Analysis of the denitrification pathway and greenhouse gases emissions in Bradyrhizobium sp. strains used as biofertilizers in South America

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Abstract

Aims: Greenhouse gases are considered as potential atmospheric pollutants, with agriculture being one of the main emission sources. The practice of inoculating soybean seeds with Bradyrhizobium sp. might contribute to nitrous oxide (N₂O) emissions. We analysed this capacity in five of the most used strains of Bradyrhizobium sp. in South America.

Methods and Results: We analysed the denitrification pathway and N₂O production by Bradyrhizobium japonicum E109 and CPAC15, Bradyrhizobium diazoefficiens CPAC7 and B. elkanii SEMIA 587 and SEMIA 5019, both in free-living conditions and in symbiosis with soybean. The in silico analysis indicated the absence of nosZ genes in B. japonicum and the presence of all denitrification genes in B. diazoefficiens strains, as well as the absence of nirK, norC and nosZ genes in B. elkanii. The in planta analysis confirmed N₂O production under saprophytic conditions or symbiosis with soybean root nodules. In the case of symbiosis, up to 26.1 and 18.4 times higher in plants inoculated with SEMIA5019 and E109, respectively, than in those inoculated with USDA110.

Conclusions: The strains E109, SEMIA 5019, CPAC15 and SEMIA 587 showed the highest N₂O production both as free-living cells and in symbiotic conditions in comparison with USDA110 and CPAC7, which do have the nosZ gene. Although norC and nosZ could not be identified in silico or in vitro in SEMIA 587 and SEMIA 5019, these strains showed the capacity to produce N₂O in our experimental conditions.

Significance and Impact of the Study: This is the first report to analyse and confirm the incomplete denitrification capacity and N₂O production in four of the five most used strains of Bradyrhizobium sp. for soybean inoculation in South America.
reduction, and this causes the release of N gases involved in global climate change (Hellin et al. 2012). Legumes, a large group of plants capable of growing under different edaphic and climatic conditions, form a broad family named Fabaceae (Leguminosae) that comprises around 20,000 species and 750 genera with representatives in nearly every terrestrial biome on Earth (Lewis et al. 2005; Peix et al. 2015). Together with actinorhizal plants, legumes are unique among higher plants because of their ability to establish N2-fixing symbiotic associations with soil bacteria, collectively referred to as rhizobia. Soybean (Glycine max L.) is a crop legume grown all over the world, and Brazil and Argentina are the second and third largest soybean producers worldwide (Meade et al. 2016). In 2017, both countries were responsible for around 47% of the total global soybean production, with a planted area in the 2016/2017 season of 33.9 and 19.6 million hectares respectively (United States Department of Agriculture, 2017). However, a gradual increase in these figures is expected in future seasons (Bolsa de Cereales de Buenos Aires, 2018). Soybean establishes symbiotic N2-fixing associations with members of the family Nitrobacteriaceae (Bradyrhizobiaceae), which belongs to the Rhizobial order of Alphaproteobacteria. These associations often occur mainly with species of the genus Bradyrhizobium, although Ensifer and Mesorhizobium have also been shown to be microsymbionts of Glycine (Shamseldin et al. 2017). Many legume-nodulating rhizobacteria do not perform complete denitrification. So far, only B. diazoefficiens (formerly Bradyrhizobium japonicum) has been shown to contain and to express the complete set of denitrification genes leading to the reduction of NO3− to N2 (Bedmar et al. 2005; Bueno et al. 2012). Inoculation of soybean has been a common, extensive agricultural practice in South America for over 40 years, mainly in Argentina and Brazil. Along with the cultivation of soybean, Bradyrhizobium species were introduced into the soil (Campos et al. 2001). Currently, the most commonly found strains used for inoculant production and use in South America are B. japonicum E109 and CPAC15 (formerly SEMIA 5079), B. diazoefficiens CPAC7 (formerly SEMIA 5080) and B. elkanii SEMIA 587 and SEMIA 5019. While our research was underway, the sequenced genomes of strains E109 (Torres et al. 2015), CPAC 15 and CPAC 7 (Siqueira et al. 2014) were published. Therefore, there is now available knowledge on the presence or the absence of the denitrification genes in those strains at the in silico level. On the other hand, the genomes of strains SEMIA 587 and SEMIA 5019 have not been reported, and their denitrifying activity is unknown.

In previous reports, out of 250 strains of Bradyrhizobium sp. isolated from the nodules of soybean grown in Argentina, only 41 reduced NO3− to N2 (Fernández et al. 2008). Considering the vast extension of soil cultivated with soybean in this country, together with Brazil, and that NO3− is usually added during its growth, the cultivation of soybean is one of the main sources for N2O released into the atmosphere. Our research aimed to determine the denitrification capacity and N2O production in B. japonicum strains E109 and CPAC15, B. diazoefficiens strain CPAC7, and B. elkanii strains SEMIA 587 and SEMIA 5019, the most used strains for soybean inoculation in South America, both under free-living and symbiotic conditions.

**Materials and methods**

**Bacterial strains and growth conditions**

*Bradyrhizobium elkanii* SEMIA 587, SEMIA 5019; *B. japonicum* CPAC15 and E109 and *B. diazoefficiens* CPAC7 used in this study were provided by the Instituto de Microbiología y Zoología Agrícola, INTA-IMyZA, Castellar, Buenos Aires, Argentina. We also used *B. diazoefficiens* USDA 110 (formerly *B. japonicum* USDA) and its mutant derivatives GRC 131, lacking the norC gene (Mesa et al. 2002), and GRZ 3035, lacking the nosZ gene (Velasco et al. 2004). Bacteria were routinely grown in peptone–salts–yeast extract (PSY) medium (Regensburger and Hennecke 1983) supplemented with arabinose (0.1% w/v) for 5 days at 30°C. Growth under microaerophilic conditions was performed in 17-ml tubes containing 5 ml Bergersen’s minimal medium (Bergersen 1977) supplemented with 10 mmol l−1 KNO3, independently inoculated with 1 ml (~106 CFU per ml) of a suspension of each strain and sealed with rubber septa stoppers. Then, the headspace atmosphere of the tubes was replaced by a gas mixture (2% oxygen, 98% argon) before the cultures were incubated at 30°C. The gas was replaced every 12 h by flushing the tubes with the same gas mixture. The culture medium composition was adapted for the individual strains by addition of the following antibiotics (µg ml−1): chloramphenicol 20 (USDA110), spectinomycin 200 (GRC131), streptomycin 100 (GRZ3035) and tetracycline 100 (GRZ3035).

**DNA extraction and PCR amplifications for SEMIA 587 and SEMIA 5019**

Genomic DNA was isolated from bacterial cells using the Real Pure Genomic DNA Extraction kit (l Durviz, Valencia, Spain), following the manufacturer’s instructions. The quantity of DNA was determined using a Nanodrop spectrophotometer (NanoDrop ND1000; Thermo Fisher Scientific, Waltham, MA). The denitrification genes *napA*, *nirK*, *norB* and *nosZ* were amplified using the specific

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primers described by Fernández et al. (2008) for Bradyrhizobium. The reaction conditions were the same for all genes analysed in all the strains of interest. Briefly, the reaction mixture contained: genomic DNA (80–100 ng), 2 mmol l−1 of each dNTP, 15 pmol of each oligonucleotide primer, 1 U of Taq DNA polymerase (Promega, Madison, WI) and 6% of DMSO. PCR was performed under the following conditions: (i) initial denaturation at 95°C for 5 min; (ii) five cycles at 95°C for 1 min, starting with an annealing temperature of 58°C for 1 min, which decreased by 1°C every cycle, 72°C for 2 min; (iii) 30 cycles of denaturation at 95°C for 1 min, primer annealing at 53°C for 1 min and primer extension at 72°C for 2 min; (iv) a final extension at 72°C for 10 min.

Denitrification activity and N2O detection in free-living cells

To determine methyl viologen (MV)-dependent NO3− and NO2− reductase activities, aliquots of microaerophilic bacterial cultures, from 0.2 to 0.4 mg of protein, were added to a reaction mixture (Sánchez et al. 2010). Reactions were started with the addition of sodium dithionite, run at 30°C for 15 min and stopped through the oxidation of sodium dithionite by vortexing. Controls were run in parallel, but in these reactions sodium dithionite was oxidized at the start. Nitrite was estimated after diazotation by adding the sulphanilamide/naphthylethylene diamine dyhydrochloride reagent (Nicholas and Nason 1954). To determine NO reductase activity, a kinetic MV-dependent assay was used (Sánchez et al. 2010).

Essentially, for each assay, a 3-ml cuvette was filled with 1 phosphate buffer (pH 7.0), 10 mmol l−1 sodium dithionite solution was freshly made and sparged before its addition with a gas-tight Hamilton syringe to the cuvette. Enough sodium dithionite was added to turn the solution blue, with an absorbance of approximately 2 at 600 nm in the spectrophotometer (DW-2000 (SLM-Aminco Instruments Inc., Rochester, NY) or U-3310 (Hitachi High-Technologies, Tokyo, Japan)). Once a steady base line was observed, 100 µl of a saturated NO solution was added to the cuvette to begin the reaction. Each assay was run until the absorbance had dropped to zero, that is, when all MV was oxidized. To assay N2O accumulation, cells were grown in Bergersen minimal medium under microoxic conditions with NO3− for 7 days. Gas samples (100 µl) were withdrawn every 12 h from the headspace with gas-tight syringes and injected into a gas chromatograph (Hewlet-Packard HP 4890D) equipped with an electron capture detector and a Porapak Q 80/100 mesh (8 ft) packed column. N2 was the carrier gas at 30 ml/min flow rate and the injector, column and detector temperatures were 125, 60 and 375°C respectively. N2O concentrations were calculated using 2% (v/v) N2O standard (Air Liquid). The N2O production in liquid cultures was corrected using the N2O Bunsen solubility coefficient (47.2% at 30°C).

Protein determination

The protein concentration of cell suspensions was estimated by using the Bio-Rad assay, with a standard curve of varying bovine serum albumin concentrations.

Inoculation of soybean plants and growth conditions

Soybean (Glycine max L. Merr. cv. Williams) seeds were surface sterilized with 96% ethanol (v/v) for 30 s, further immersed in H2O2 (15%, v/v) for 8 min, washed thoroughly with sterile water and, finally, germinated in darkness at 30°C. Selected seedlings were planted in autoclaved 1.5-l Leonard jar assemblies filled with a sand–vermiculite mixture (1 : 1). Plants (two per pot) were inoculated at sowing with 1 ml of a single bacterial strain (~108 CFU per ml), provided with a mineral solution (Rigaud and Puppo 1975) supplemented with or without a minimal concentration of NO3− (4 mmol l−1 NO3K) to induce the denitrification process without repressing the nodule formation (Dogra and Dudeja 1993). Plants were grown in a greenhouse with a 16 h per day photoperiod at 28°C. After 35-day-old plants were harvested. Dry weight was obtained by drying the fresh samples in an oven at 65°C. When a constant weight was reached, this was considered an indicator of complete drying.

NO detection in nodules

Plant growth conditions

Soybean seeds were disinfected, planted, inoculated and cultured as described above. The NO indicator dye DAF-2 DA (CalbiochemTM, Sigma-Aldrich, St. Louis, MO, USA) was used to detect NO in nodule sections according to (Zafra et al. 2010). Nodules were detached from the roots of soybean plants, cut into halves by hand using a scalpel, immersed in MES/KCl pH 6.15 for 10 min, and transferred to 10 µmol l−1 DAF-2 DA for 10 min. They were then washed with MES/KCl buffer for 15 min and observed under the microscope. Parallel sets of samples were treated the same, although they were previously incubated for 1 h with the NO-scavenger cPTIO (Sigma-
Aldrich, San Luis, Mo, USA) in a concentration of 400 μmol l⁻¹ in Tris-HCl 10 mmol l⁻¹, pH 7.4. Negative controls were treated with MES-KCl buffer only, instead of with DAF-2 DA solution. Observations were carried out in a Nikon C1 confocal microscope using an Ar-488 laser source and different levels of magnification (20× to 60×). Multiple optical sections were captured and processed to generate 3-D reconstructions of the whole nodule surface. The fluorescent signal was obtained exclusively in the range of the 515-560 nm emission wavelengths, and recorded in green. Auto-fluorescence, mainly due to the presence of chlorophyll and other pigments and secondary metabolites, was isolated and displayed in red. Identical settings were used for image capture in both control/test experiments in order to ensure reproducibility and accurate quantification. Reconstructions were performed by using the Nikon EZ-C1 3.90 FreeViewer software. Quantification of fluorescence intensity in the infection area of nodules was made by using Image J free software.

**N₂O production by nodulated soybean roots**

To measure N₂O produced by nodulated roots, plants were harvested from the Leonard jars and vermiculite was carefully removed. Then, roots were placed into 100-ml bottles and closed with rubber septa and kept at room temperature. Gas samples were taken from the headspace using gas-tight syringes and injected into the gas chromatograph mentioned above. The N₂O flux was recorded chronologically to check for linearity of N₂O emissions, which were calculated within the linear interval between sampling times (Parkin and Kaspar 2006).

**Statistical analysis**

Statistical analyses were carried out by using the software package SPSS ver. 17.0. Data were analyzed using analysis of variance and the HSD Tukey pairwise comparisons. All tests were subjected to a 95% confidence limit.

**Results**

**In silico analysis, denitrification activity and N₂O detection in free-living cells**

We analysed the whole-genome sequences of strains CPAC7, CPAC15, E109 and USDA110 and the draft genomes of strains SEMIA 5019 and SEMIA 587, and we found that only in CPAC7 all genes described in the canonical denitrification pathway are present (napA, nirK and norC and nosZ); while CPAC15 and E109 have an incomplete pathway in which nosZ is absent. In the case of SEMIA 5019 and SEMIA 587, no sequences of any denitrification pathway genes were identified in their draft genomes (Table 3). With regard to denitrification activity, all strains were cultured from an initial optical density of (OD₆⁰₀) 0.01 and after 7 days all of them reached OD₆⁰₀ ~0.80. This means *Bradyrhizobium* strains grow under microaerophilic conditions (i.e. 2% oxygen) with NO₃⁻ as the sole nitrogen source (Table S1). After incubation, all strains showed NO₃⁻ reductase (NO₂⁻ production) activity with values ranging from 2.52 ± 0.5 to 7.05 ± 0.4 nmol l⁻¹ NO₂⁻ produced h⁻¹ mg protein⁻¹, corresponding to strains E109 and SEMIA 587 respectively (Table 1). They also displayed nitrite reductase (NO₂⁻ consumption) activity, with values ranging from 0.92 ± 0.1 to 1.42 ± 0.2 nmol l⁻¹ NO₂⁻ consumed h⁻¹ mg protein⁻¹, corresponding to strains SEMIA 587 and CPAC15 respectively (Table 1). Nitrate and NO₂⁻ reductase activity evidenced the capability of the respective strains to metabolize NO₃⁻ and NO₂⁻ under microaerophilic conditions. All the bradyrhizobial strains consumed NO at rates ranging from 7.85 ± 0.17 to 61.94 ± 2.9 nmol l⁻¹ NO consumed h⁻¹ mg protein⁻¹, shown by strains SEMIA 587 and E109 respectively. On the other hand, CPAC 7 and USDA 110 showed a lower NO production than the other strains, while E109 tripled the nm NO consumption by USDA 110. In all cases, NO reduction activity was detected. N₂O accumulation was also detected after incubation for 7 days. The lowest value was 0.15 ± 0.01 and the highest was 41.42 ± nmol l⁻¹ N₂O h⁻¹ mg protein⁻¹, by strains USDA 110 and E109 respectively. As expected, the amount of N₂O accumulated by the wild-type strain USDA 110 was very scarce (Table 1). Summarizing, in those strains belonging to *B. diazoefficiens* (USDA110 and CPAC 7) and containing nosZ gene, the values for N₂O emissions were minimal; while the production by *B. elkanii* (SEMEA 587 and SEMIA 5019) was higher than the first ones but up to six times lower than that by *B. japonicum* (E109 and CPAC 15) (Table 1).

**NO detection in soybean nodules**

In plants treated with minimal concentration of NO₃⁻ in culture medium, fluorometric NO detection detected varying fluorescence emissions in nodules formed by bradyrhizobial strains, with values ranging from 70 ± 5.0 to 787 ± 69.2 relative fluorescence intensity units in the wild-type USDA110 and the norC mutant GRC131 respectively (Table 2). Except for SEMIA 587, fluorescence intensity in the nodules formed by the remaining strains had higher values than those found in nodules formed by the wild type USDA110 and lower than those belonging to the norC mutant (Table 2). Fluorescence due to NO emission was seen in nodules formed by GRC131 in plants treated with NO₃⁻, but not in nodules by the same strain in plants watered with
Nodulated roots of SEMIA 5019 and E109 produced N$_2$O, albeit at different concentrations (Table 2). Nodules from soybean roots inoculated with strain USDA 110, E109, CPAC15, CPAC7, SEMIA 587 and SEMIA 5019 strains emitted fluorescence, albeit with different intensities. As expected, the mutant norC strain GRC131 showed maximum DAF-2DA staining (Fig. 3).

N$_2$O emissions from inoculated soybean roots

Nodules from soybean roots inoculated with strains USDA 110, GRZ 3035, E109, CPAC15, CPAC7, SEMIA 587 and SEMIA 5019 produced N$_2$O, albeit at different concentrations (Table 2). Nodulated roots of SEMIA 5019 and E109 were the highest N$_2$O producers, with values that were, respectively, 26.2 times and 18.4 times that of the N$_2$O emitted by the wild-type strain USDA 110 (83 ± 6.9 nmol l$^{-1}$ N$_2$O h$^{-1}$ g nodule fresh weight$^{-1}$). Moreover, the amount of N$_2$O produced by either E109 or SEMIA 5019 was higher than that produced by the nosZ 3035 mutant, which lacks N$_2$O reductase activity (Table 2).

**Discussion**

*In silico* and molecular analysis of denitrification genes

Molecular analyses were performed to identify the presence of denitrification genes in *Bradyrhizobium* strains.
B. japonicum species have particular denitrification genes and pathways, were B. diazoefficiens has all the canonical denitrification pathways and consequently all the related genes, B. japonicum has an incomplete pathway including napA, nirK and norC, but excluding nosZ, and 4 of 12 strains of B. elkanii have napA as the unique denitrification gene of the canonical pathway, while only one has an incomplete pathway including napA, nirK and norC.

Denitrification activities in free-living cells

The denitrification process consists of the reduction of \(\text{NO}_3^-/\text{NO}_2^-\) to \(\text{N}_2\) (Zumft 1997). Previous reports have noted that not all rhizobial strains are able to grow in denitrifying conditions (Monza et al. 2006; Zhong et al. 2009). In our study, the best adaptation to the microaerophilic conditions was observed for USDA110. However, all the strains were able to grow under denitrifying conditions. They survive and assimilate either \(\text{NO}_3^-\) or \(\text{NO}_2^-\) as a final electron acceptor, which implies the induction of denitrifying genes under these conditions to carry out an assimilation process (Bedmar et al. 2005). Under our experimental conditions, \(\text{NO}_3^-\) reduction activity was observed to be further induced than that of \(\text{NO}_2^-\) reduction activity (Table 1). Sánchez et al. (2010) reported that MV-dependent \(\text{NO}_3^-\) and \(\text{NO}_2^-\) reductase activities in free-living denitrifying conditions were about 10 times higher than those observed under aerobic conditions. In this report, the 88 and 93% of the \(\text{NO}_3^-\) reductase and \(\text{NO}_2^-\) reductase activities detected in anaerobically grown cells correspond to Nap and NirK enzymes respectively. Nevertheless, all the strains were able to produce and consume \(\text{NO}_2^-\), expressed as an induction and activation of Nar and Nir. The determination of \(\text{NO}_3^-\) and \(\text{NO}_2^-\) reductase activity allows us to infer the presence of napA and nirK genes in B. diazoefficiens CPAC 7 and B. japonicum E109 and CPAC 7, but not in B. elkanii SEMIA 587 and 5019 in which according to the \textit{in silico} and \textit{in vitro} analysis other mechanism not yet described should be operating for denitrification process. The reduction of \(\text{NO}_3^-\) to \(\text{NO}_2^-\) is catalysed by \(\text{NO}_3^-\) reductase and encoded by the napA gene in B. japonicum USDA110, while free-living cells of the napA mutant are unable to reduce \(\text{NO}_3^-\) (Delgado et al. 2003). According to Velasco et al. (2001), the nirK gene in B. diazoefficiens USDA110 strain is required for breathing in anaerobic conditions with \(\text{NO}_3^-\) as a single source of nitrogen. As well as the \textit{in silico} analysis for E109, CPAC15 and CPAC7, the presence of nirK can be deduced for these strains. Zumft (1997) and Sánchez et al. (2010) reported that the denitrification process is the main pathway for oxide nitric production in free-living bacteria. Under our experimental conditions, all the strains accumulated NO after the addition of \(\text{NO}_2^-\) inside the chamber. Velasco et al. (2001) reported that cells of B. diazoefficiens USDA 110, which are mutants for the nirK or norC genes, accumulated \(\text{NO}_2^-\) and NO, respectively, when they were grown.

**Figure 2** Nitric oxide production in nodules of soybean (Glycine max L.) inoculated with Bradyrhizobium diazoefficiens GRC 131 lacking norC. Plants were watered with mineral nutrient solution containing 5 mmol \(l^{-1}\) NO\(_3\)K. NO production was imaged by DAF-2DA staining (green fluorescence). Nodules were treated (a) or not (b) with cPTIO. a' and b', images under bright field. Bars represent 500 \(\mu m\). [Colour figure can be viewed at wileyonlinelibrary.com]
in anaerobic conditions with NO$_3^-$ as a nitrogen source. In all cases in our results, there was an activation of the NO$_2^-$ reductase enzyme (Nir) and the nirK gene codified this activity. Although they just provide anaerobic conditions in measuring, this information indicates that the capacity of rhizobia to produce large amounts of N$_2$O in denitrifying conditions could be very variable but a flux trend is continuing.

Fernández et al. (2008) described the denitrifying ability of *Bradyrhizobium* isolates from five Argentinian soybean-cultivated soils. This study showed that 41 of the 250 isolates exhibited behaviour typical of true respiratory denitrifiers. This pattern has been correlated with complete denitrification and is not common in rhizobial strains, which lack N$_2$O reductase (Hallin et al. 2017). As reported by Sameshima-Saito et al. (2006a), who evaluated the conversion of $^{15}$N-N$_2$O to $^{15}$N-N$_2$ and N$_2$O reductase activity in USDA 110 and USDA110 n$^{o}$Z-lacking strains, the wild-type strain showed N$_2$O reductase activity, whereas the n$^{o}$Z mutant did not. Of the strains analysed in this study, only CPAC7 can be considered a complete denitrifier and N$_2$O lower producer, which confirms the low abundance of rhizobia capable of emitting molecular nitrogen as a final product.

**Figure 3** Nitric oxide production in nodules of soybean (*Glycine max* L.) inoculated with *Bradyrhizobium* strains USDA 110 (a), E109 (b); SEMIA 5019 (c), CPAC15 (d), CPAC7 (e), SEMIA 587 (f) and GRC131 (g) lacking norC. a’, b’, c’, d’, e’, f’ and g’, images under bright field. Plants were watered with mineral nutrient solution containing 5 mmol l$^{-1}$ NO$_3$K. NO production was imaged by DAF-ZDA staining (green fluorescence). Bars represent 500 µm. [Colour figure can be viewed at wileyonlinelibrary.com]

Nitric oxide and nitrous oxide production in *Bradyrhizobium*–soybean interaction

The correlation between NO production and its reduction to N$_2$O has not been clarified, while for NO$_2^-$ to NO reduction numerous alternative pathways through enzymatic or nonenzymatic conversion have been found...
in plants (Gupta et al. 2011; Mur et al. 2013) and bacteria (Zumft 1997). The bacterial pathway has been reported as the main route for NO production and it occurs both in free-living bacteria under microaerophilic conditions and soybean nodules (Meilhoc et al. 2011). Thus, it has been proven that there are several NO detoxification sources (in free-living cells and symbiotic conditions) and that NO is not only reduced to N₂O (Sánchez et al. 2010; Cabrera et al. 2016). As can be observed in Fig. 3, under our conditions, fluorometric NO detection showed some endogenous NO accumulation in nodules from plants that were not treated with NO₃⁻. Moreover, the presence of NO₃⁻ in the mineral solution increased NO production. However, our results stand in contrast with the report by Sánchez et al. (2010), who could not detect differences in nodular NO accumulation between plants inoculated either with B. diaeoefficiens USDA110 or GRC131 in the presence of NO₃⁻. They found that the application of flooding allowed the detection of NO accumulation, mostly in GRC131 nodules, and that it stimulated NO production by USDA 110.

Nitrous oxide is one of the three main biogenic greenhouse gases, and agriculture represents close to 30% of the total N₂O emissions (Tortosa et al. 2015). Various authors have reported that leguminous plants associated with Bradyrhizobium sp. can not only fix nitrogen from the atmosphere but also emit N₂O (Duxbury et al. 1982; Inaba et al. 2009). In the present study, treatments inoculated with USDA110 and CPAC7 strains had very low N₂O production values in comparison with all the other strains used, which are considered incomplete denitrifiers that lack the nosZ gene (Table 3). In this sense, Itakura et al. (2013) reported that in pure culture and pot experiments, N₂O emission was lower in nosZ⁺ strains and nosZ⁺⁺ strains (mutants with increased N₂O reductase activity) of B. japonicum than in nosZ⁻ strains (mutants lacking N₂O reductase activity).

According to the results obtained in this research, (summarized in Table 3), Bradyrhizobium–soybean interaction N₂O production was higher for CPAC15, E109 and SEMIA 587 treatments. All these groups are commercially used as the active ingredient in the formulation of bio-products for soybean (Glycine max L. (Merr.)) in Argentina and Brazil. Because of this, the genomes of CPAC15 and E109 have been sequenced, and our results can confirm that they are incomplete denitrifying rhizobia. In this respect, several authors have reported that various strains of Bradyrhizobium that lack nosZ produce N₂O as a final denitrification product, and these kind of bacteria are found in agricultural soils (Sameshima-Saito et al. 2004; Fernández et al. 2008). Natural Bradyrhizobium populations which lack the nos gene and N₂O reductase activity are often dominant in the soils of soybean fields (Chêneby et al. 1998; Sameshima-Saito et al. 2006a). Considering the vast extension of soil cultivated with soybean in South America, N₂O may be emitted from the nodulated soybean roots, and the legume–Bradyrhizobium symbiosis could play an important role in N₂O emissions, thus contributing to global warming (IPCC 2006).

### Denitrification activity for SEMIA 5019 and SEMIA 587

Even though in SEMIA 5019 and SEMIA 587 we did not amplify the canonical denitrification genes by PCR methodology, these strains do have NO₃⁻ and NO₂⁻ reduction activity capacity and produce NO and N₂O in our experimental conditions (Table 1 and Fig. S1). Sameshima-Saito et al. (2006b) reported the absence of nir, nor and nos genes in B. elkanii and NO₂⁻ and NO₃⁻ as denitrification end products for these strains, while our results evidenced N₂O as denitrification end products. Also, they found napA gene through Southern hybridization in B. elkanii USDA94, which coincides

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**Table 3** Correlation between the presence/absence of denitrification pathway genes and nitrous oxide production in both, free-living cells of Bradyrhizobium and nodulated soybean roots

| Strain                  | Denitrification genes* | NO production† | Cited in                  | Strain                  | Denitrification genes* | NO production† | Cited in                  |
|------------------------|------------------------|----------------|---------------------------|------------------------|------------------------|-------------------|---------------------------|
| B. diaeoefficiens USDA110 | + + + +                | Low + Low      | Meakin et al. (2006)      |            | + + + +                | Low + Low       | Meakin et al. (2006)      |
| B. diaeoefficiens CPAC7 | + + + +                | Low + Low      | This study                |            | + + + +                | High + High     | This study                |
| B. japonicum E109      | + + + +                | High + High    | This study                |            | + + + +                | High + High     | This study                |
| B. japonicum CPAC15    | + + + +                | High + High    | This study                |            | + + + +                | High + High     | This study                |
| B. elkanii SEMIA 5019† | – – – –                | Medium + High  | This study                |            | – – – –                | Medium + High   | This study                |
| B. elkanii SEMIA 587‡  | – – – –                | Medium + Medium| This study                |            | – – – –                | Medium + Medium | This study                |

(†) presence; (—) absence.

*in silico and in vitro analysis.

†N₂O levels in comparison with reference strain (USDA 110).

‡Genome sequence is not complete for SEMIA 587 or not available for SEMIA 5019.
with in silico results of *B. elkanii* USDA 3259, 3254 and WSM2783, but contrasting with our in silico and in vitro results for SEMIA 5019 and SEMIA 587. An unique report related to N₂O production *Bradyrhizobium* sp. and *B. elkanii* strains found that the SEMIA 587 strain produced lower N₂O values than USDA110 and CPAC7 (Nascimento 2011, cited by Alves et al. 2016). *Bradyrhizobium elkanii* SEMIA 587 and SEMIA 5019 have no denitrification genes codified in their genomes, but they have the capacity to reduce NO₃⁻ and produce N₂O. In this sense, NO₃⁻, NO₂⁻ and NO reduction activity, as well as N₂O accumulation was demonstrated in this manuscript in two strains of *B. elkanii*. Fernández et al. (2008) mentioned that not all denitrification genes were detected in *Bradyrhizobium* isolates, but gas chromatography revealed that these strains produced N₂O. Inefficient amplification reactions might have occurred due to differences between the nucleotide sequences of the primers and genetic sequences for the enzymes among denitrifying bacteria (Chènèbe et al. 1998; Fernández et al. 2008). An alternative explanation for the inability of amplify napA, nirK, norC and norC in SEMIA 5019 and SEMIA 587 should be attributed to the genes’ polymorphism (Jaton et al. 2010), which might inhibit the target sequences’ amplification proposed by Fernández et al. (2008). Even when in silico results did not indicate the presence of denitrification genes of the canonical pathway for almost all strains of *B. elkanii*, we shall not exclude an inherent limitation of the PCR methodology affecting the amplification of those genes under our experimental conditions. Future research could reveal the presence of these genes or an alternative pathway involved in the denitrification and N₂O emissions for *B. elkanii*.

This is the first study to describe and confirm the incomplete denitrification pathway in most strains used in the production of inoculants for soybean in South America in particular, and it constitutes one step in the process of establishing the role of *Bradyrhizobium* strains and N₂O emissions in global climate change.

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**Conflict of Interest**

The authors declare no competing interests.

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**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Agarose gel showing PCR amplification of *napA*, *nirK*, *norC* and *nosZ* genes from *B. elkanii* strains SEMIA 587 and SEMIA 5019 using the primers proposed by Fernández et al. (2008).

**Table S1.** Maximum growth of *B. japonicum* E109 and CPAC 15; *B. elkanii* SEMIA 5019 and SEMIA 587 and *B. diazoefficiens* CPAC 7 cultured under microaerophilic conditions in Bergersen’s minimal medium (Bergersen 1977)

**Table S2.** Results of the molecular analysis for denitrification pathway genes in *B. japonicum* E109 and CPAC 15; *B. elkanii* SEMIA 5019 and SEMIA 587 and *B. diazoefficiens* CPAC 7.