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

GEI emissions from *Bradyrhizobium* sp.

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Abstract

Aims: Greenhouse gases are considered 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 (N_2O) emissions. We analyzed this capacity in five of the most used strains of *Bradyrhizobium* sp. in South America.

Methods and Results: We analyzed the denitrification pathway and N_2O production by *B. japonicum* E109 and CPAC15, *B. diazoefficiens* CPAC7, and *B. elkanii* SEMIA 587 and SEMIA 5019, both in free-living conditions and 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 *nir*K, *nor*C and *nos*Z genes in *B. elkanii*. The *in planta* analysis confirmed the N_2O production under saprophytic conditions or symbiosis with soybean roots nodules. In the last case 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 N_2O production both as free-living cells and in symbiotic conditions in comparison with USDA110 and CPAC7, which do have the *nos*Z gene. Although *nor*C and *nos*Z could not be identified *in silico* or *in vitro* in SEMIA 587 and SEMIA 5019, these strains showed capacity to produce N_2O in our experimental conditions.

Significance and Impact of Study: This is the first report to analyze and confirm the incomplete denitrification capacity and N_2O production in four of the five most used strains of *Bradyrhizobium* sp. for soybean inoculation in South America.

Keywords: greenhouse gases; denitrification; soybean; *Bradyrhizobium*; biofertilizers.

Introduction

Denitrification is an alternative respiration pathway in which microorganisms under oxygen-limiting conditions reduces nitrate (NO_3^-) to nitrite (NO_2^-) through nitrate reductase enzymes encoded by the *narG*/*napA* genes. Nitrite reductase enzymes encoded by the *nirS*/*nirK* genes then reduce NO_2^- to nitric oxide (NO) and nitric oxide reductase encoded by the *norB*/*norC* genes reduce NO to nitrous oxide (N_2O). Finally, N_2O is reduced by nitrous oxide reductase, which is encoded by the *nosZ* gene. This leads to the formation of molecular nitrogen (N_2) as an end product (Bueno *et al.* 2012). However, not all denitrifying organisms have the genetic capacity for NO and N_2O reduction, and this causes the release of N-gases involved in global climate change (Hallin *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 N_2-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 N_2-fixing associations with members of the family Nitrobacteriaceae (Bradyrhizobiaceae), which belongs to the Rhizobial order of Alphaproteobacteria. These associations 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 *B. japonicum*) has been shown to contain and to express the complete set of denitrification genes leading to the reduction of nitrate to N_2 (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 inoculants 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 nitrate to N_2 (Fernández *et al.* 2008). Considering the vast extension of soil cultivated with soybean in this country, together with Brazil, and that nitrate is usually added during its growth, the cultivation of soybean is one of the main sources for N_2O released into the atmosphere. Our research aimed to determine the denitrification capacity and N_2O 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

B. 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, Castelar, Buenos Aires, Argentina. We also used *B. diazoefficiens* USDA 110 (formerly *B. japonicum* USDA) and its mutant derivatives GRC 131, lacking the *nor*C gene (Mesa *et al.* 2002), and GRZ 3035, lacking the *nos*Z 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 d at 30 ºC. Growth under microaerophilic conditions was performed in 17 ml tubes containing 5 ml Bergersen's minimal medium (Bergersen, 1977) supplemented with 10 mM KNO_3, independently inoculated with 1 ml (~10^8 CFU.ml^{-1}) 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 ºC. 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 (Durviz, Spain), following the manufacturer's instructions. The quantity of DNA was determined using a Nanodrop spectrophotometer (NanoDrop ND1000, Thermo Fisher Scientific, USA). The denitrification genes *nap*A, *nir*K, *nor*B and *nos*Z were amplified using the specific primers described by Fernández *et al.* (2008) for *Bradyrhizobium.* The

reaction conditions were the same for all genes analyzed in all the interest strains. Briefly, the reaction mixture contained: genomic DNA (80–100 ng), 2 mM of each dNTP, 15 pmol of each oligonucleotide primer, 1 U of Taq DNA polymerase (Promega, WI) and 6 % of DMSO. PCR was performed under the following conditions: (1) initial denaturation at 95 °C for 5 min; (2) 5 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; (3) 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; (4) a final extension at 72 °C for 10 min.

Denitrification activity and N_2O detection in free-living cells

To determine methyl viologen (MV)-dependent nitrate and nitrite 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 sulfanilamide/naphthylethylene diamine dyhydrochloride reagent (Nicholas and Nason 1954). To determine nitric oxide reductase activity, a kinetic MV-dependent assay was used (Sánchez *et al.* 2010). Essentially, for each assay, a 3 ml cuvette was filled with 2.5 ml of 10 mM phosphate buffer (pH 7.5), 100 µl of cell solution (0.2-0.4 mg protein), and 25 µl of 100 mM MV solution, then sealed with a mini suba-seal and made anaerobic by sparging with oxygen-free nitrogen gas for 10 min. A 100 mM 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, N

Y, U.S.A.] or U-3310 [Hitachi High-Technologies, Tokyo]). 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, i.e. when all MV was oxidized. To assay nitrous oxide accumulation, cells were grown in Bergersen minimal medium under microoxic conditions with nitrate for 7 d. 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. N_2 was the carrier gas at 30 ml/min flow rate and the injector, column and detector temperatures were 125 °C, 60 °C and 375 °C, respectively. N_2O concentrations were calculated using 2 % (v/v) N_2O standard (Air Liquid). The N_2O production in liquid cultures was corrected using the N_2O Bunsen solubility coefficient (47.2 % at 30 ºC).

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 (BSA) 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 H_2O_2 (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 (2 per pot) were inoculated at sowing with 1 ml of a single bacterial strain (~10^8 $CFU.ml^{-1}$), provided with a mineral solution (Rigaud and Puppo, 1975) supplemented or not with a minimal concentration of nitrate (4 mM NO_3K) to induce denitrification process without repress the nodule formation (Dogra and Dudeja, 1993). Plants were grown in a greenhouse with

16 h 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 65ºC. When 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 (Calbiochem) 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 μM 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) in a concentration of 400 μM in Tris-HCl 10 mM, 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. Autofluorescence, 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.

N_2O production by nodulated soybean roots

To measure N_2O 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 N_2O flux was recorded chronologically to check for linearity of N_2O 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 version17.0. Data were analyzed using Analysis of Variance (ANOVA) and the HSD Tukey pairwise comparisons. All tests were subjected to a 95 % confidence limit.

Results

In silico analysis, Denitrification activity and N_2O detection in free-living cells

We analyzed 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 (*nap*A, *nir*K and *nor*C and *nos*Z); while CPAC15 and E109 have an incomplete pathway in which *nos*Z 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 strain were cultured from an initial optical density of (OD_{600}):0,01 and after 7 days all of them reached OD_{600} ~ 0,80. This means *Bradyrhizobium* strains grow under microaerophilic conditions (i.e. 2 % oxygen) with nitrate as the sole nitrogen source (Table S1). After incubation, all strains showed nitrate reductase (NO_2^- production) activity with

values varying from 2.52 ± 0.5 to 7.05 ± 0.4 nM NO_2^- produced. h^{-1}. mg $protein^{-1}$, corresponding to strains E109 and SEMIA 587, respectively (Table 1). They also displayed nitrite reductase (NO_2^- consumption) activity, with values ranging from 0.92 ± 0.1 to 1.42 ± 0.2 nM NO_2^- consumed.h^{-1}. mg $protein^{-1}$, corresponding to strains SEMIA 587 and CPAC15, respectively (Table 1). Nitrate and nitrite reductase activity evidenced the capability of the respective strains to metabolize nitrate and nitrite under microaerophilic conditions. All the bradyrhizobial strains consumed NO at rates ranging from 7.85 ± 0.17 to 61. 94 ± 2.9 nM 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. N_2O accumulation was also detected after incubation for 7 d. The lowest value was 0.15 ± 0.01 and the highest was 41.42 ± nM $N_2O.h^{-1}$.mg $protein^{-1}$, by strains USDA 110 and E109, respectively. As expected, the amount of N_2O 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 *nos*Z gene, the values for N_2O emissions were minimal; while the production by *B. elkanii* (SEMIA 587 and SEMIA 5019) was higher than the first ones but up to 6 times lower than that by *B. japonicum* (E109 and CPAC 15) (Table1).

NO detection in soybean nodules

In plants treated with minimal concentration of nitrate in culture medium, fluorometric NO detection detected varying fluorescence emission in nodules formed by bradyrhizobial strains, with values ranging from 70 ± 5.02 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 nitrate, but not in nodules by the same strain in plants watered with N-free mineral nutrient solution (Figure 1). Treatment of the nodules with c-PTIO prior to the imaging of NO production resulted in the suppression of fluorescence emission, which indicates that the response was representative of bacterial NO production (Figure 2). Nodules from nitrate-treated plants formed by 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 (Figure 3).

N_2O 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_2O, albeit at different concentrations (Table 2). Nodulated roots of SEMIA 5019 and E109 were the highest N_2O producers, with values that were, respectively, 26.2 times and 18.4 times that of the N_2O emitted by the wild type strain USDA 110 (83 ± 6.9 nM N_2O h^{-1}.nodules fresh $weight^{-1}$). Moreover, the amount of N_2O produced by either E109 or SEMIA 5019 was higher than that produced by the *nosZ* 3035 mutant, which lacks nitrous oxide 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. In this manuscript we found that the reference strain *B. diazoefficiens*

USDA 110 has the full set of genes for the denitrification pathway, as it was previously reported by (Bedmar *et al.*2005). In our experimental conditions, no amplification of any denitrification pathway gen was obtained for *B. elkanii* SEMIA 587 and SEMIA 5019 (Figure 1S), but the presence of such genes was confirmed *in silico* and *in vitro* (Table S2) for *B. diazoefficiens* CPAC7 and *B. japonicum* CPAC15 and E109. We broaden the scope of our study and analyzed the genome sequences of *B. elkanii* strains USDA 3259, 3254, 94 and 76; WSM1741 and 2783;

BLY6-1 and 3-8; CCBAU 43297 and 05737; UASWS1015 and WSM1741 by the use of MicroScope (http://www.genoscope.cns.fr/agc/microscope/home/index.php); KEEG (https://www.genome.jp/kegg/) and BLAST (http://ncbi.nlm.nih.gov/BLAST) databases. In the case of USDA 3259, 3254 and 94 we found homologous sequences for the canonical *nap*A, but we did not found any sequence for *nir*K, *nor*C and *nos*Z in none of such genomes. In the case of WSM 2783 we found homologous sequences for the canonical *nap*A, *nirK* and *norC* but we did not found any sequence for *nos*Z in the genome. These results, allows us to infer that different *Bradyrhizobium* species have particular denitrification genes and pathways, were *B. diazoefficiens* has all the canonical denitrification pathway and consequently all the related genes; *B. japonicum* has an incomplete pathway including *nap*A, *nir*K and *nor*C, but excluding *nos*Z and four of twelve strains of *B. elkanii* have *nap*A as the unique denitrification gene of the canonical pathway; while only one has an incomplete pathway including *nap*A, *nir*K and *nor*C.

Denitrification activities in free-living cells

The denitrification process consists in the reduction of nitrate/nitrite to 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 nitrate or nitrite 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, nitrate reduction activity was observed to be further induced than that of nitrite reduction activity (Table 1). Sánchez *et al.* 2010, reported that MV-dependent nitrate and nitrite 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 nitrate reductase and nitrite reductase activities detected in anaerobically grown cells correspond to Nap and NirK enzymes, respectively. Nevertheless, all the strains were able to produce and consume nitrite, expressed as an induction and activation of Nar and Nir. The determination of nitrate and nitrite reductase activity, allows us to infer the presence of *nap*A and *nir*K 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 *in silico* and *in vitro* analysis other unravel mechanism should be operating for denitrification process. The reduction of nitrate to nitrite is catalyzed by nitrate reductase and encoded by the *nap*A gene in *B. japonicum* USDA110, while free-living cells of the *nap*A mutant are unable to reduce nitrate (Delgado *et al.* 2003). According to Velasco *et al.* 2001, the *nir*K gene in *B. diazoefficiens* USDA110 strain is required for breathing in anaerobic conditions with nitrate as a single source of nitrogen. As well as the *in silico* analysis for E109, CPAC15 and CPAC7, the presence of *nir*K 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 nitrite inside the chamber. Velasco *et al.* 2001 reported that cells of *B. diazoefficiens* USDA 110, which are mutants for the *nir*K o *nor*C genes, accumulated nitrite and nitric oxide,

respectively, when they were grown in anaerobic conditions with nitrate as a nitrogen source. In all cases in our results, there was an activation of the nitrite reductase enzyme (Nir) and the *nir*K 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_2O in denitrifying conditions could be very variable but a fluxes 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 out of the 250 isolates exhibited behavior typical of true respiratory denitrifiers. This pattern has been correlated with complete denitrification and is not common in rhizobial strains, which lack N_2O reductase (Hallin et al. 2017). As reported by Sameshima-Saito *et al.* 2006a, who evaluated the conversion of $^{15}N\text{-}N_2O$ to $^{15}N\text{-}N_2$ and N_2O reductase activity in USDA 110 and USDA110 *noz*Z-lacking strains, the wild type strain showed N_2O reductase activity, whereas the *nos*Z mutant did not. Out of the strains analyzed in this study, only CPAC7 can be considered a complete denitrifier and N_2O lower producer, which confirms the low abundance of rhizobia capable of emitting molecular nitrogen as a final product.

Nitric oxide and nitrous oxide production in Bradyrhizobium–soybean interaction

The correlation between NO production and its reduction to nitrous oxide has not been clarified, while for NO_2^- to NO reduction numerous alternative pathways through enzymatic or non-enzymatic 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 nitrous oxide (Sánchez *et al.* 2010; Cabrera *et al.* 2016). As can be observed in Figure 3, under our conditions fluorometric NO detection showed some endogenous NO accumulation in *nor*C nodules from plants that were not treated with nitrate- Moreover, the presence of nitrate 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. diazoefficiens* USDA110 or GRC131 in the presence of nitrate. They found that application of flooding allowed the detection of NO accumulation, mostly in GRC131 nodules, and that it stimulated NO production by USDA 110.

Nitrous oxide (N_2O) is one of the three main biogenic greenhouse gases (GHGs), and agriculture represents close to 30 % of the total N_2O emissions (Tortosa *et al.* 2015).Various authors have reported that leguminous plants associated to *Bradyrhizobium* sp. can fix nitrogen from the atmosphere but also emit N_2O (Duxbury *et al.* 1982; Inaba *et al.* 2009). In the present study, treatments inoculated with USDA110 and CPAC7 strains had very low N_2O production values in comparison with all the other strains used, which are considered incomplete denitrifiers that lack the *nos*Z gene (Table 3). In this sense, Itakura *et al.* 2013 reported that in pure culture and pot experiments N_2O emission was lower in $nosZ^{+}$ strains and $nosZ^{++}$ strains (mutants with increased nitrous oxide reductase activity) of *Bradyrhizobium japonicum* than in $nosZ^{-}$ strains (mutants lacking nitrous oxide reductase activity).

According to our results, (summarized in Table 3), *Bradyrhizobium*-soybean interaction N_2O production was higher for CPAC15, E109 and SEMIA 587 treatments. All these groups are commercially used as the active ingredient in the formulation of bioproducts 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 *nos*Z produce nitrous oxide as a final denitrification product, and this kind of bacteria are found in agricultural soils (Sameshima *et al.* 2004; Fernández *et al.* 2008). Natural *Bradyrhizobium* populations which lack the *nos*Z gene and N_2O reductase activity are often dominant in the soils of soybean fields (Chèneby *et al.* 1998; Sameshima-Saito *et al.* 2006a). Considering the vast extension of soil cultivated with soybean in South America, nitrous oxide may be emitted from the nodulated soybean roots, and the legume-*Bradyrhizobium* symbiosis could play an important role in nitrous oxide 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 nitrate and nitrite reduction activity capacity and produce nitric oxide and nitrous oxide in our experimental conditions (Table 1 and Figure S1). Sameshima-Saito *et al.* 2006b reported absence of *nir*, *nor* and *nos* genes in *B. elkanii* and NO_2^- and NO_3^- as denitrification end products for these strains, while our results evidenced N_2O as denitrification end products. Also, they found *nap*A gen through Southern hybridization in *B. elkanii* USDA94, which coincides 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 N_2O production *Bradyrhizobium* sp. and *B. elkanii* strains found that SEMIA 587 strain produced lower N_2O values than USDA110 and CPAC7 (Nascimento 2011, cited by Alves *et al.* 2016). *B. elkanii* SEMIA 587 and SEMIA 5019 have no denitrification genes codified in their genomes, but they have capacity to reduce nitrate and produce nitrous oxide. In this sense, nitrate, nitrite

and nitric oxide reduction activity, as well as N_2O 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 N_2O. 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 (Chèneby *et al.* 1998; Fernández *et al.* 2008). An alternative explanation for the inability of amplify *nap*A, *nir*K, *nor*C and *nor*C 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 Fernandez et al., (2008). Even when *in silico* results did not indicate 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 N_2O 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 nitrous oxide emissions in global climate change.

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Conflicts of Interest

The authors declare no competing interests.

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8. Tables headers

Table 1. Nitrate reductase (Nap) or NO_2^- production; Nitrite reductase (Nir) or NO_2^- consumption; nitric oxide reductase (Nor) or NO consumption activity and NO accumulation in *Bradyrhizobium* strains. Cells were grown in Bergersen's minimal medium with 10 mM NO_3K as the sole nitrogen source under 2 % O_2 for 7 d. Values represent the mean ± standard error of 4 replicates.

Table 2. Nitric oxide (NO) and nitrous oxide (N_2O) production by nodules of soybean (*G. max* L.) inoculated with *Bradyrhizobium* strains. The wild type strain USDA 110 and its mutant derivatives *norC* GRC131 and *nosZ* GRZ 3035 were used as control. Values represent the mean ± standard error of 4 replicates.

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

Supplementary Tables

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. The genes *napA*, *nirK*, *norB* and *nosZ* were amplified using the specific primers described by Fernández *et al.* (2008).

9. Legend to Figures

Figure 1. Nitric oxide production in nodules of soybean (*G. max* L.) inoculated with *B. diazoefficiens* GRC131 lacking *norC*. Plants were watered with mineral nutrient solution containing (A) or not (B) 5 mM NO_3K. NO production was imaged by DAF-2DA staining (green fluorescence). A' and B', images under bright field. Bars represent 500 μm.

Figure 2. Nitric oxide production in nodules of soybean (*G. max* L.) inoculated with *B. diazoefficiens* GRC 131 lacking *norC*. Plants were watered with mineral nutrient solution containing 5 mM NO_3K. 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 μm.

Figure 3. Nitric oxide production in nodules of soybean (*G. 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 mM NO_3K. NO production was imaged by DAF-2DA staining (green fluorescence). Bars represent 500 μm.

Supplementary Figure

Figure 1S. Agarose gel showing PCR amplification of *nap*A, *nir*K, *nor*C and *nos*Z genes from *B. elkanii* strains SEMIA 587 and SEMIA 5019 using the primers proposed by Fernández et al. (2008). Lanes: DNA marker (1Kb); *B. dizoefficiens* USDA110 (positive control); SEMIA 5019 and SEMIA 587.

Table 1.

strain	(*) Nap activity or (**) NO_2^- production(1)	(*) Nir activity or (**) NO_2^- consumption(2)	(*) Nor activity or (**) NO consumption(3)	N_2O accumulation(4)
USDA 110	$5.52^{b} \pm 0.4$	$1.34^{ab} \pm 0.2$	$22.20^{c} \pm 1.4$	$0.15^{e} \pm 0.01$
E109	$2.52^{d} \pm 0.5$	$1.23^{b} \pm 0.3$	$61.94^{a} \pm 2.9$	$41.42^{a} \pm 2.4$
CPAC 15	$7.04^{a} \pm 0.4$	$1.42^{a} \pm 0.2$	$11.28^{c} \pm 1.1$	$12.59^{b} \pm 0.7$
CPAC 7	$4.05^{c} \pm 0.3$	$1.10^{bc} \pm 0.1$	$33.35^{b} \pm 2.2$	$0.19^{e} \pm 0.01$
SEMIA 587	$7.05^{a} \pm 0.4$	$0.92^{c} \pm 0.1$	$7.85^{d} \pm 0.17$	$5.99^{c} \pm 0.06$
SEMIA 5019	$4.05^{c} \pm 0.6$	$0.93^{c} \pm 0.1$	$66.61^{a} \pm 161$	$4.36^{d} \pm 0.01$

Units: (1) nM NO_2^- produced. h^{-1}. mg $protein^{-1}$; (2) nM NO_2^- consumed.h^{-1}. mg $protein^{-1}$, (3) nM NO_2^- consumed.h^{-1}. mg $protein^{-1}$; (4) nM $N_2O.h^{-1}$.mg $protein^{-1}$.

(*) *B. diazoefficiens* USDA 110 and CPAC 7; *B. japonicum* E109 and CPAC 15

(**) *B. elkanii* SEMIA 587 and 5019

Table 2.

Strain	NO production[(1)]	N_2O production[(2)]
USDA 110	$70^{d} \pm 5.02$	$83^{e} \pm 6.9$
E109	$148^{c} \pm 9.5$	$1528^{b} \pm 131$
CPAC 15	$114^{c} \pm 5.4$	$628^{d} \pm 15$
CPAC 7	$298^{c} \pm 12.1$	$91^{e} \pm 5.2$
SEMIA 587	$76^{d} \pm 4.3$	$687^{d} \pm 17.3$
SEMIA 5019	$447^{b} \pm 16.12$	$2174^{a} \pm 165$
GRC 131	$787^{a} \pm 69.2$	nd
GRZ 3035	nd	$1002^{c} \pm 85.4$

nd: not determined

Units: [(1)] relative fluorescence intensity units (RFIU); [(2)] nM N_2O h^{-1}.g nodule fresh weight^{-1}

Table 3.

Strain	Denitrification genes[1]				Cited in	NO Production[2]		Cited in
	*nap*A	*nir*K	*nor*C	*nos*Z		*free living*	Nodules	
B. diazoefficiens USDA 110	+	+	+	+	Bedmar *et al*. (2005)	Low	Low	Meakin *et al.* (2006)
B. diazoefficiens CPAC7	+	+	+	+	This study	Low	Low	This study
B. japonicum E109	+	+	+	-	This study	High	High	This study
B. japonicum CPAC15	+	+	+	-	This study	High	High	This study
B. elkanii SEMIA 5019 [3]	-	-	-	-	This study	Medium	High	This study
B. elkanii SEMIA 587 [3]	-	-	-	-	This study	Medium	Medium	This study

References: (+) Presence; (-) Absence

[1] *in silico* and *in vitro* analysis

[2] N_2O levels in comparison with reference strain (USDA 110)

[3] Genome sequence is not complete for SEMIA 587 or not available for SEMIA 5019

Figure 1

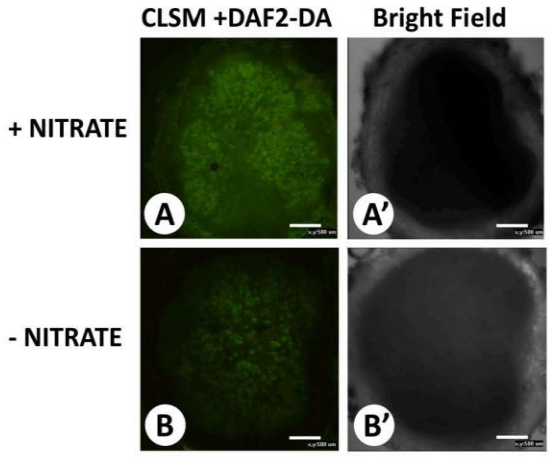

Figure 2

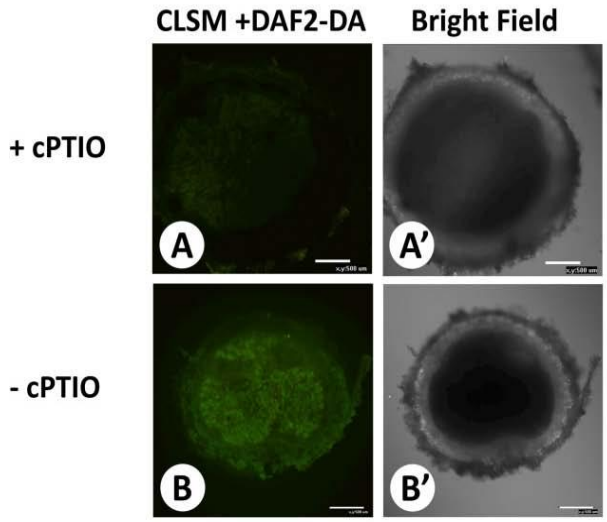

Figure 3