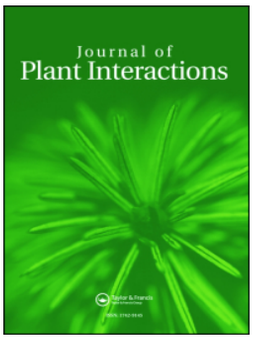

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Rhizobium etli is able to emit nitrous oxide by connecting assimilatory nitrate reduction with nitrite respiration in the bacteroids of common bean nodules

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ABSTRACT

Legumes can contribute to emissions of the potent greenhouse gas nitrous oxide (N_2O) directly by some rhizobia species that are able to denitrify under free-living conditions and in symbiotic association with the plant. In this study, the capacity of *Phaseolus vulgaris-Rhizobium etli* symbiosis to emit N_2O in response to nitrate (NO_3^-) has been demonstrated for the first time. We found that bacteroidal assimilatory nitrate reductase (NarB) as well as nitrite reductase (NirK) and nitric oxide reductase (cNor) denitrifying enzymes contribute to nitric oxide (NO) and N_2O formation in nodules. We also show that *R. etli* NarK is involved in NO_2^- extrusion and links NO_3^- reduction by NarB in the cytoplasm with NirK and cNor denitrification activities in the periplasm. The knowledge generated in this work will be instrumental for exploring strategies and sustainable practices in agricultural soil management to increase legume crop yield and mitigate greenhouse gas emissions.

HIGHLIGHTS

- Common bean nodules are able to produce N_2O in response to nitrate.
- Nitrate reduction to nitrite in *R. etli* bacteroids catalyzed by assimilatory nitrate reductase NarB is involved in N_2O emissions from common bean nodules.
- *R. etli* respiratory nitrite reductase NirK and nitric oxide reductase cNor support N_2O production in common bean nodules.
- *R. etli* NarB and cNor are involved in NO homeostasis in common bean nodules.
- *R. etli* nitrite exporter NarK links nitrate reduction in the cytoplasm with denitrification activities in the periplasm.

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Bacteroids; denitrification; nitrate reductase; nitric oxide reductase; nitrite reductase; nitrite transporter; leghaemoglobin; nodules

1. Introduction

Nitrous oxide (N_2O) is a highly stable greenhouse gas (GHG) and stands alongside carbon dioxide (CO_2), methane (CH_4) and fluorinated gases as a key driver of global warming. Moreover, the global warming potential for N_2O is around 300-fold greater than that from CO_2 and has an impact on stratospheric ozone (Müller 2021). Agriculture, Forestry and Other Land Use (AFOLU) activities accounted for around 13% of CO_2, 44% of CH_4, and 81% of N_2O emissions from human activities during 2007-2016, representing 23% of total net anthropogenic emissions of GHGs (IPCC 2019). Nitrogen (N) in the soil is converted to N_2O through two main microbial processes, nitrification and denitrification (Thomson et al. 2012; Butterbach-Bahl et al. 2013; Pilegaard 2013; Torres et al. 2016; Sánchez and Minamisawa 2019). Under low oxygen conditions some bacteria can produce energy by using nitrate (NO_3^-) or nitrite (NO_2^-) as final electron acceptors, via the denitrification pathway. Classical or 'complete' denitrification involves four enzymatic steps, which allow sequential conversion of NO_3^- to NO_2^-, nitric oxide (NO), N_2O and, finally dinitrogen (N_2). These four steps are catalysed by the periplasmic nitrate reductase (Nap) or membrane associated nitrate reductase (Nar), nitrite reductases (NirK or NirS), nitric oxide reductases (cNor, qNor or Cu_ANor) and nitrous oxide reductase (Nos) (reviewed by Zumft 1997; van Spanning et al. 2007; Torres et al. 2016; Salas et al. 2021). Anthropogenic N_2O emissions from agricultural soils are rising primarily due to the application of N fertilizers including inefficiencies such as over-application or poorly synchronization with crop demand timings (IPCC 2019; Kudeyarov 2020; Lazcano et al. 2021). Further to its contribution to N_2O emission, excessive usage of NO_3^--based fertilizers to enhance agricultural production, can also lead runoff causing soil and water contamination. Therefore, it is crucial to rationalize the use of synthetic fertilizers in agriculture (Tian et al. 2020). In this context, biological nitrogen fixation (BNF) performed by the rhizobia–legume symbiosis is proposed as an economically and environmentally friendly alternative to N fertilizers, to improve soil fertility and crop yield. Legumes are an important source of non-meat-based

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protein for humans and animals, and grain legumes such as common bean (*Phaseolus vulgaris*), account for 27% of primary crop production worldwide (Graham and Vance 2003).

These plants have the unique ability to establish a mutualist symbiosis with soil N_2-fixing bacteria, termed rhizobia, that leads to the formation of specialized organs called nodules in plant roots. Within the nodules, differentiated rhizobia i.e. bacteroids are able to convert (through the activity of the nitrogenase enzyme) an abundant atmospheric source of N_2 that is not normally available to the plant into more bioavailable forms such as ammonia (NH_4^+) that are readily plant-assimilated. In return, rhizobia obtain a safe niche and stable carbon provision in photosynthates. However, despite clear benefits, bacterial-plant endosymbiosis can be 'a double-edged sword' and have negative impacts since it may potentially contribute to N_2O emission indirectly through legumes N-rich residue deposition, or directly through some rhizobial species being able to perform denitrification inside nodules where they release free-intermediates (Inaba et al. 2009, 2012; Hirayama et al. 2011; Torres et al. 2016). In this context, several reports have demonstrated that the soybean endosymbiont *Bradyrhizobium diazoefficiens* produces N_2O in soybean nodules through the denitrification pathway in response to NO_3^- and flooding (Tortosa et al. 2015; Tortosa et al. 2020). However, information concerning the capability of common bean nodules to produce this important GHG and the proteins involved is unknown.

In order to use rhizobia as biofertilizers, it is essential to investigate the mechanisms of legume–rhizobia symbiosis from a holistic perspective including bacterial and/or plant proteins that influence N_2O emission. Although denitrification among rhizobia is rare, several of the most agronomical interesting species contain in their genomes some or all the genes required to perform the denitrification pathway (Delgado et al. 2007; Torres et al. 2016 and references herein). Complete denitrifying rhizobia should be preferred for formulation of bioinoculants, as they possess the NosZ enzyme which is currently the only known enzyme capable of reducing N_2O to the 'safe' end-product N_2 (reviewed by Hein and Simon 2019). *Rhizobium etli* CFN42 is the endosymbiont of common bean, an important globally cultivated grain legume, and does not possess the complete set of denitrification genes. The genome of this bacterium consists of one chromosome and six large plasmids (pRet42a to pRet42f) and contains the *nirK* and *norCBQD* genes on plasmid f, which encodes the NirK-type nitrite reductase and *c*-type nitric oxide reductase (cNor), respectively (Gómez-Hernández et al. 2011).

Recently, it has been reported that despite lacking genes encoding the Nap or Nar nitrate reductase enzymes, *R. etli* produces N_2O under microoxic conditions when NO_3^- is present as the only N source. This ability can be attributed to the activity of the cytoplasmic assimilatory nitrate reductase, NarB (Hidalgo-García et al. 2019). Thus, this species of rhizobia can produce N_2O from NO_3^- by coupling nitrate assimilation with the denitrification pathway. Here, NO_3^- is firstly reduced to NO_2^- in the cytosol by NarB and the reaction product NO_2^- is further reduced to NO and then N_2O by the sequential action of NirK and cNor, respectively, in the periplasmic space.

In this work, the capacity of the *Phaseolus vulgaris-R. etli*-symbiosis to emit N_2O as well as the contribution of *R. etli* NarB, NirK and cNor enzymes has been shown for the first time. *R. etli* connects nitrate reduction in the cytoplasm with nitrite respiration in the periplasm through the nitrite exporter NarK.

2. Material and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 1. *R. etli* strains were grown at 30°C in TY rich

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Abbreviated name	Reference
Rhizobium etli			
CE3	A streptomycin-resistant derivative of CFN42, wild-type, Nal^rSm^r	WT	Noel et al. 1984
DR4004	CE3 derivative, Δ*narK,* Nal^rSm^r	*narK*	This work
DR4004-pDR4005	DR4004 with pDR4005 plasmid. Complemented *narK* strain, $Nal^rSm^rKm^r$	*narK*+NarK	This work
DR4000	CE3 derivative, Δ*narB*::ΩSpSm, $Nal^rSm^rSp^r$	*narB*	Hidalgo-García et al. 2019
CFNX702	CE3 derivative, *nirK*::*lox*P, Nal^rSm^r	*nirK*	Gómez-Hernández et al. 2011
CFNX701	CE3 derivative, *norC*::*lox*Sp, $Nal^rSm^rSp^r$	*norC*	Gómez-Hernández et al. 2011
WT-pTR102	CE3 with the empty pTR102 plasmid, $Nal^rSm^rTc^r$	WT/pTR102	This work
WTpTR102::*narB*	CE3 with the pTR102 plasmid carrying *R. etli narB*, $Nal^rSm^rTc^r$	WT+NarB$^+$	This work
DR4004-pTR102::*narB*	CE3 Δ*nark,* Nal^rSm^r with the pTR102 plasmid carrying *R. etli narB*, $Nal^rSm^rTc^r$	*narK*+NarB$^+$	This work
DR4000-pTR102::*narB*	CE3 Δ*narB*::ΩSpSm with the pTR102 plasmid carrying *R. etli narB*, $Nal^rSm^rSp^rTc^r$	*narB*+ NarB$^+$	This work
CFNX702-pTR102::*narB*	CE3 *nirK*::*lox*P with the pTR102 plasmid carrying *R. etli narB*, $Nal^rSm^rTc^r$	*nirK*+NarB$^+$	This work
CFNX701-pTR102::*narB*	CE3 *norC*::*lox*Sp with the pTR102 plasmid carrying *R. etli narB*, $Nal^rSm^rSp^rTc^r$	*norC*+NarB$^+$	This work
DR4004-pDR4005- pTR102::*narB*	CE3 Δ*narK* with DR4005 plasmid and the pTR102 plasmid carrying *R. etli narB*, $Nal^rSm^rKm^rTc^r$	*narK*+NarK +NarB$^+$	This work
Escherichia coli			
DH5 α	*supE*44Δ*lacU*169(φ80*lacZ*ΔM15) *hsd*R17*rec*A1*end*A1*gyrA*96 *thi*-1*rel*A1		Sambrook et al. 1989
S17.1	*thi, pro*, recA, *hsdR, hsdM*, RP4Tc::Mu, Km::Tn7, Tp^r Sm^r Spc^r		Simon et al. 1983
Plasmids			
pBluescript KS	Cloning vector, Ap^r		Invitrogen
pK18*mobsacB*	Suicide cloning vector, Km^r		Schäfer et al. 1994
pBBR1-MCS2	Broad host range cloning vector, Km^r		Kovach et al. 1994
pTR102	Broad host range plasmid, Tc^r		Weinstein et al. 1992
pDR4004	pK18*mobsac*B carrying *narK* with a 1083 bp deletion, Km^r		This work
pDR4000	pBBR1MCS-2 derivative carrying *R. etli narK,* Km^r		This work
pDR4002	pBBR1MCS-2 carrying *narB*, Km^r		Hidalgo-García et al. 2019
pTR102::*narB*	pTR102 derivative carrying *R. etli narB*, Tc^r		This work

medium (Tryptone Yeast, Beringer 1974) or in Y minimal medium (MMY) with succinate (10 mM) and ammonium chloride (10 mM) as carbon and N sources, respectively (Bravo and Mora 1988). To study the growth and N_2O production capacity of *R. etli* from different N sources, 10 mM NH_4Cl from MMY medium was replaced by 10 mM KNO_3 (this medium was named 'MMN') or 1 mM $NaNO_2$. Antibiotics were added to *R. etli* cultures at the following concentrations (μg/mL): nalidixic acid (Nal) 20, kanamycin (Km) 30, spectinomycin (Sp) 100, streptomycin (Str) 100, tetracycline (Tc) 10. *Escherichia coli* strains were grown at 37°C in LB medium (Sambrook and Russell 2001) with antibiotics added at the following concentrations (μg/mL): Sp 25, Str 25, Km 20, Tc 25 and ampicillin (Ap) 200. For growth under microoxic conditions, 17 mL tubes containing 3 mL of cell cultures were sealed with rubber septa and flushed for 5 min at the start of the incubation with 2% (v/v) O_2 and 98% N_2 (v/v).

For determination of growth rates, extracellular NO_2^- concentration and N_2O emission, cells were firstly grown aerobically in TY medium for 24 h, harvested by centrifugation at 8,000 g for 10 min, and washed twice with MMN. Cells were then incubated in minimal medium for another 24 h under the desired oxygen conditions. Initial optical density for cultures (measured at 600 nm, OD_{600}) was around 0.05.

Inoculum for plants was prepared by growing *R. etli* aerobically in TY rich medium for 24 h, then cells were harvested by centrifugation at 8,000 g for 10 min, washed twice and grown aerobically for another 24 h in MMN. Initial and final OD_{600} of culture were around 0.3 and 0.6, respectively. The final inoculum was prepared by harvesting cells by centrifugation, washing them with MMN and resuspending them in fresh MMN to a final OD_{600} of 0.8, corresponding to $\sim 10^8$ cells/mL.

2.2. Construction and complementation of a R. etli narK mutant

The oligonucleotide primers used in this work were purchased from Sigma (Sigma-Aldrich, Saint Louis, MO, USA) and are listed in Supplementary Table S1. Genomic and plasmid DNA isolation was carried out using the REALPURE Genomic DNA purification Kit (Real) and Qiagen Plasmid Kit (Qiagen), respectively. PCR was performed using the High-Fidelity DNA polymerase Phusion® enzyme (Thermo Fisher Scientific, MA, USA) and DNA digestions were carried out using Fast Digest restriction enzymes (Thermo Fisher Scientific, MA, USA).

To generate a *narK* mutant, two regions flanking the *narK* gene (fragments F1 and F2) were amplified by PCR using narK_up_For/narK_up_Rev and narK_down_For/narK_down_Rev primer pairs. Then, fragments F1 and F2 were cloned into the pBluescript KS (pBSKS) vector (Thermo Fisher Scientific, MA, USA) as *Xba*I/*Bam*HI and *Bam*HI/*Eco*RI fragments, respectively, to generate plasmid pBSKS_F1F2. The *Xba*I/*Eco*RI fragment from pBSKS_F1F2 was cloned into the pK18*mobsacB* suicide vector (Schäfer et al. 1994) yielding plasmid pDR4004 and integrity was confirmed by sequencing. Replacement of the *R. etli* wild-type *narK* allele with the truncated mutant allele in plasmid pDR4004 was carried out by double recombination using the *sacB* sucrose-selectable marker present in plasmid pK18*mobsacB*. Double recombinants were selected as resistant to 12.5% sucrose and Km sensitive. The derivatives were verified by PCR using primers narK_up-For and narK_down-Rev (Supplementary Table S1), and the *narK* mutant strain obtained was named DR4004.

The *R. etli narK* gene was constitutively expressed under control of the *lacZ* promoter to generate a complemented *narK* strain. The *narK* gene was amplified by PCR using narK_compl_For/narK_compl_Rev primers par (Supplementary Table S1) and cloned as a *Hind*III/*Bam*HI fragment into the broad-host range cloning vector pBBR1MCS-2 (Kovach et al. 1994). The plasmid obtained (pDR4005) was sequenced using primers M13_For, M13_Rev and narK_compl_IN (Supplementary Table S1), and then transferred to the *R. etli narK* + $NarB^+$ strain by bi-parental conjugation using *E. coli* S17.1. The resulting strain containing pDR4005 (*narK* + $NarB^+$ + NarK) was checked by plasmid isolation and PCR.

2.3. Construction of R. etli strains overexpressing NarB

In previous work (Hidalgo-García et al. 2019), it was reported that overexpression of *narB* led to induction of NR activity and N_2O production by free-living *R. etli* cells. Here, overexpression was performed by introducing plasmid pDR4002 (pBBR1MCS-2 carrying the *narB* gene) into *R. etli* WT and *narB* mutant strains. In this current study we were interested in exploring the role of NarB in N_2O metabolism during endosymbiosis, by using the *R. etli narB* deletion mutant and NarB overexpressing strains. The pTR102 plasmid has been reported to be highly stable in cells of *Sinorhizobium meliloti* during symbiosis with alfalfa (Weinstein et al. 1992) and so, this platform was used to carry the gene to ensure the stability and persistence of the overexpression system over the course of plant experiments. To generate this overexpression platform, the pDR4002 plasmid (pBBR1MCS-2 carrying *narB* gene) was digested with *Xba*I/*Hind*III restriction enzymes, and the resultant fragment carrying *narB* gene was cloned as an *Xba*I/*Hind*III fragment into plasmid pTR102, yielding the pTR102::*narB* plasmid, where *narB* gene is under the control of the *lacZ* promoter.

Once pTR102::*narB* was constructed, it was introduced into *R. etli* WT, and *narB*, *nirK*, *norC* and *narK* mutant strains by conjugation with *E. coli* S17.1. The derivatives strains containing pTR102::*narB* ($NarB^+$) were verified by plasmid isolation and PCR. Concurrently, a WT strain containing pTR102 empty vector was obtained (WT-pTR102).

2.4. Extracellular NO_2^- determination.

Cultures were centrifuged at 8,000 g for 10 min and NO_2^- concentration was estimated in supernatants by using the Griess-Yallosway diazotization protocol (Nicholas and Nason 1957). Briefly, equal volumes of 0.1% (w/v) NNEDA [n-(1-naphthyl)ethylenediamine dihydrochloride] and 1% (w/v) sulfanilamide were added to one volume of supernantant. NO_2^- was measured colorimetrically at $\lambda = 540$ nm after 20 min incubation with the reagents and extrapolating from a standard curve constructed with increasing concentrations of $NaNO_2$ (0, 20, 40, 60, 80 and 100 μM) from a stock solution of 100 μM.

2.5. N_2O production by free-living cells

Cells were cultured as indicated above in section 2.1. After 24 h, 1 ml from the headspace was taken using a Hamilton® Gastight syringe, and manually injected into an HP 4890D gas chromatography instrument equipped with an electron capture detector (ECD) (Hewlett Packard, San Jose, CA, USA) as described by Torres et al. (2014).

2.6. Protein concentration measurement

Protein concentration was measured using the Protein Assay Dye Reagent Concentrate (Bio-Rad, CA, USA). Bovine serum albumin (BSA) was used as standard protein for calibration curves.

2.7. Plant inoculation and growth conditions

Seeds of common bean (*P. vulgaris*, var. Negro jamapa) were surface sterilized as performed by Delgado et al. (1994) with little modifications. Briefly, seeds were immersed in 96% (v/v) ethanol for 30 s, in 30% (v/v) H_2O_2 for 10 min and then thoroughly washed five times with sterilized distilled water. After incubation for 2 h in sterile distilled water in darkness, the seeds were placed on plates containing 1% water-agar and were allowed to germinate at 30°C for 72 h. Three days after sowing, one germinated seed was transplanted into a sterilized Leonard jar (Trung and Yoshida 1983) with a 0.25 L pot filled with vermiculite (N° 3) as growth substrate. Each seedling was inoculated with 1 mL of a single bacterial strain (10^8 cells/mL). Plants were grown in controlled environmental chambers (16/8 light/dark cycle, 26/22°C, photosynthetic photon flux density of 180 µmol photons/$m^2 \times s$, and relative humidity 60–70%). Different sets of plants were watered throughout the experiment with mineral solution (Rigaud and Puppo 1975) containing 0, 1, 2 or 4 mM KNO_3 or treated with 10 mM KNO_3 for one or four days at the end of the experiment. The plants were grown for a total of 25 days following inoculation with *R. etli*.

2.8. N_2O measurement in detached nodules

Plants (10 from each treatment) were harvested from Leonard jars, vermiculite carefully removed from roots and two nodules from each plant transferred into 20 mL headspace vials containing a circle of absorbent paper on its base (20 nodules/vial). Subsequently, 100 µL of mineral solution was added to each vial which were hermetically sealed and incubated at 30°C. After 5, 8 and 24 h of incubation, gas samples were taken from the headspace using luer-lock gastight syringes with Mininert® valves and immediately transported to the gas chromatograph for analysis following the protocol described by Tortosa et al. (2015). The emission flux rate [nmol N_2O/h x g NFW] was calculated in the interval between 5 and 24 h: Δ N_2O molar concentration (24h – 5 h) / Δ incubation time increase (19 h).

2.9. Leghaemoglobin content

Leghaemoglobin (Lb) content was measured as previously described by LaRue and Child (1979). Essentially, nodules (0.3 g) were ground with 6 mL Lb extraction buffer (50 mM $Na_2HPO_4{\cdot}2H_2O/NaH_2PO_4{\cdot}H_2O$, pH 7.4; 0.02% w/v $K_3Fe(CN)_6$; 0.1% w/v $NaHCO_3$) supplemented with 0.1 g polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 12,000 g at 4°C for 20 min to retain the supernatant. 50 µL of clear supernatant and 3.15 mL of saturated oxalic acid (66 g/L) were mixed in screw-capped glass tubes, which were sealed and autoclaved for 30 min at 120°C and then allowed to cool to room temperature. The fluorescence of the solutions was measured using a Shimadzu spectrofluorometer (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with a mercury-xenon lamp and a RF-549 red-sensitive photomultiplier. The excitation and the emission wavelengths were 405 and 600 nm, respectively. The difference in fluorescence between heated and unheated samples was proportional to haem protein content.

2.10. Bacteroidal nitrate, nitrite and nitric oxide reductase activities

Bacteroids from detached nodules were extracted according to Mesa et al. (2004). In brief, 1–2 g of fresh nodules were ground with a ceramic mortar and pestle with 7.5 mL of extraction buffer (50 mM Tris-HCl, pH 7.5, 250 mM mannitol). The homogenate was filtered through cheeseclothe and centrifuged at 250 *g* at 4°C for 5 min to remove nodule debris. The supernatant was recentrifuged at 12,000 *g* at 4°C for 10 min to pellet the bacteroids, which were washed twice with 50 mM Tris-HCl pH 7.5 for nitrate reductase (NR) and nitrite reductase (NiR) activities or 25 mM sodium phosphate pH 7.4 for nitric oxide reductase (Nor) activity.

For NR activity, bacteroids were disrupted using a French pressure cell (SLM Aminco, Jessup, MD, USA) at about 120 MPa. The resultant cell extract was used to measure methyl viologen-dependent (MV)-NR activity as described by Delgado et al. (2003). Briefly, equal volumes of reaction mixture (10 mM KNO_3 and 200 µM MV) were added to bacteroid cell extract aliquots containing 0.2–0.4 mg of protein and mixed gently. Enzymatic reactions started by addition of freshly prepared sodium dithionite (144 mM) dissolved in $NaHCO_3$ (300 mM). After incubation for 30–60 min at 30°C, the reaction was stopped by vigorous shaking until the samples lost their blue color. Controls were done in parallel by oxidizing sodium dithionite at the start of the reaction (by shaking). Nitrite production was estimated after diazotization as described previously in section 2.4. Results are expressed as nmol NO_2^- produced/h x mg protein.

For MV-dependent NiR activity measurements, equal volumes of reaction mixture (0.1 mM $NaNO_2$ and 200 µM MV) were added to bacteroid aliquots containing 0.1–0.3 mg of protein and thereafter the procedure was followed as previously described above for NR activity in reactions carried out for 15–30 min. Results were expressed as nmol NO_2^- consumed/h x mg protein.

Bacteroidal NO consumption rate (Nor activity) was determined by using an ISONOP NO electrode APOLLO 4000® (World Precision Instruments, Friedberg, Germany). The reaction chamber (2 mL) was temperature-controlled, magnetically stirred, and contained 760 µL of 25 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7.4) and 900 µL of cell suspension (1–2 mg protein). To generate an anoxic atmosphere, the method described in Cabrera et al. (2016) was used. Once a steady base line was obtained, 15 µL of 2 mM NO were added to the chamber and NO consumption was recorded. Results were expressed as nmol NO consumed/h x mg protein.

2.11. Bacteroidal N_2O emission

Bacteroids from detached nodules were extracted as described above in section 2.10. Once bacteroids were isolated, they were resuspended in a sterile solution of 50 mM Tris-HCl (pH 7.5), 10 mM KNO_3, 10 mM sodium succinate dibasic hexa-hydrate. Then, 3mL of the suspension was placed in 17mL tubes and treated as free-living cells to obtain microoxic conditions (see section 2.1.). These were then incubated at 30°C at 170 rpm for 24 h. The initial and final OD_{600} of the suspensions was 2.5. N_2O concentration in the headspace was measured as specified in section 2.5.

2.12. Detection of LbNO complexes in whole nodules by Electron Paramagnetic Resonance (EPR) spectroscopy

Continuous wave X-band EPR spectroscopy was performed on whole-nodule samples as described previously with minor modifications (Sánchez et al. 2010). Individual frozen and intact root nodules with a size between 2.5- and 2.8-mm diameter were selected and transferred under liquid nitrogen to pre-cooled 20-cm Wilmad® quartz (CFQ) EPR sample tubes with 3 mm internal diameter. Each sample contained intact nodules closely packed to a sample depth of 25 mm in the tubes (sample biomass was 79 ± 8 mg wet wt.). To improve signal to noise during data collection, a 10-cm length of ethylene-tetrafluoroethylene tubing (GE Healthcare, Piscataway, NJ, USA) was inserted into the tube and was secured with parafilm at the tube entrance to prevent nodule movement and allow liquid gas boil-off. Control experiments confirmed that neither the tubes nor tubing gave rise to any EPR signals in the absence of nodules.

EPR spectra were recorded using a Bruker ELEXSYS 500 spectrometer with an ER049X SuperX microwave bridge and a super high Q cavity (Bruker Analytische Messtechnik GmBH, Karlsruhe, Germany). Low temperature experiments were performed using an Oxford Instruments ESR-900 helium flow cryostat and an ITC3 temperature controller (Oxford Instruments, Oxfordshire, UK). Microwave frequency was 9.45 GHz with 2 mW power and 0.3 mT (3 Gauss) modulation amplitude applied. Spectra were recorded at 40 ± 1 K and are the average of three scans.

2.13. Statistical analyzes

For each assay, the total number of replicates is shown in each figure and table. Descriptive statistical analyzes (mean and standard deviation) for each parameter were calculated and data were checked for normal distribution and homoscedasticity. The analyzes of variance (ANOVA) within treatments were performed using the *post hoc* HSD Tukey test ($P \leq 0.05$). Statistical software used for these analyzes was IBM SPSS Statistics v.26.

3. Results

3.1. Rhizobium etli *RHE_CH01783 encodes a NarK-type nitrate/nitrite transporter*

The gene RHE_CH01783 from *R. etli* CFN42 lies upstream of *nirB* (RHE_CH01782), *nirD* (RHE_CH01781) and *narB* (RHE_CH01780) genes (Figure 1). According to bioinformatics predictions, *nirB* and *nirD* have been proposed to encode the large and small subunits of the sirohaem-ferredoxin nitrite reductase (NirB, NirD), respectively. The *narB* gene encodes the large subunit of the $Mo[MGD]_2$ and iron-sulfur-binding nitrate reductase (NarB) that has been demonstrated in previous work to be involved in assimilatory nitrate reduction (Hidalgo-García et al. 2019). Upstream of RHE_CH01783, two other genes are located, RHE_CH01784 and RHE_CH01785, which have been annotated as a NO_3^--ABC transporter, ATP-binding protein (NrtCch), and a two-component response regulator protein (NasT), respectively (Figure 1). Although RHE_CH01784 is annotated as *nrtCch*, this gene is more likely to be *nasS* given its proximity to *nasT* (RHE_CH01785). The alignment of the *NrtCch* amino acids sequence revealed between 60 and 50% similarity to NasS proteins from *B. diazoefficiens* USDA 110 and *Paracoccus denitrificans* PD1222, respectively (Source: NCBI Blast, data not shown). Consequently, these two genes are likely to encode the NasS-NasT two component regulatory system for NO_3^-/NO_2^- perception and control of genes involved in NO_3^-/NO_2^- assimilation that has been previously characterized in the α-proteobacteria *P. denitrificans* (Luque-Almagro et al. 2013) and *B. diazoefficiens* (Cabrera et al. 2016).

Concerning RHE_CH01783, this gene has been predicted to encode a probable NO_3^--transporter belonging to the major facilitator superfamily (MFS)-type transporter (Source: https://www.genome.jp/kegg/genes.html). A sequence alignment (Supplementary Figure S1A) showed that RHE_CH01783 protein shares from 79% to 38% amino acid similarity with NarK-like transporters (a subgroup of MFS proteins) from *P. denitrificans*, *Thermus thermophilus*, *E. coli* and *B. diazoefficiens* (Supplementary Figure S1B). Interestingly, among rhizobia, *B. diazoefficiens* NarK, whose translated sequence has 40% similarity with the predicted primary sequence of *R. etli* NarK, has been reported to be involved in NO_2^- export (Cabrera et al. 2016).

Since proline residues usually have a crucial role in dynamic processes involving transmembrane α-helices, we looked at the *R. etli* NarK sequence (Supplementary Figure S1A) and we found six prolines located within the residues that conform the α-helices (P58, P157, P240, P302, P364 and P375; Supplementary Figure S1A). Among them, four are highly conserved within NarK1 homologous (P58, P157, P302 and P375), and three are highly conserved within NarK2 homologous (P58, P157 and P375). Particularly, P302 has been reported to be essential for NarK1 function, meanwhile P58, P157 and P375 were shown to be essential for NarK2 function in *P. denitrificans* (Goddard et al. 2017).

As shown in Supplementary Table S2, NarK homologous are present in many rhizobia species, however is only in *B. diazoefficiens* (Cabrera et al. 2016) and *Sinorhizobium meliloti* (Ruiz et al. 2022) where a connection between assimilatory and denitrification pathways has been reported.

3.2. Involvement of R. etli *NarK in nitrite extrusion and nitrous oxide production by free-living cells*

To investigate the role of *R. etli* NarK in NO_2^- export and N_2O production, a *narK* knockout mutant was constructed. The *narK* mutant strain was able to grow with NO_3^- as the sole N source at the same rate as the WT cells under oxic, as well as microoxic, conditions (Figure 2A,C, respectively). This demonstrated that NO_3^- assimilation was not impaired

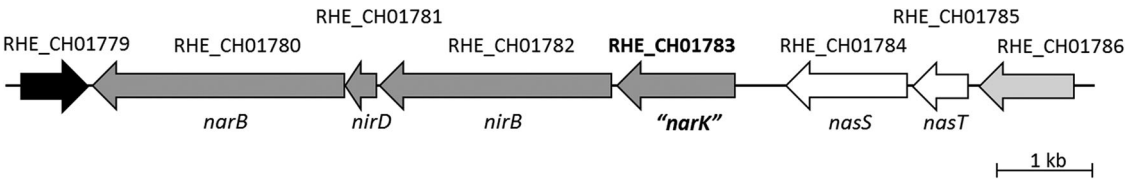

Figure 1. Genomic context of RHE_CH01783 gene. Source: https://www.ncbi.nlm.nih.gov/gene.

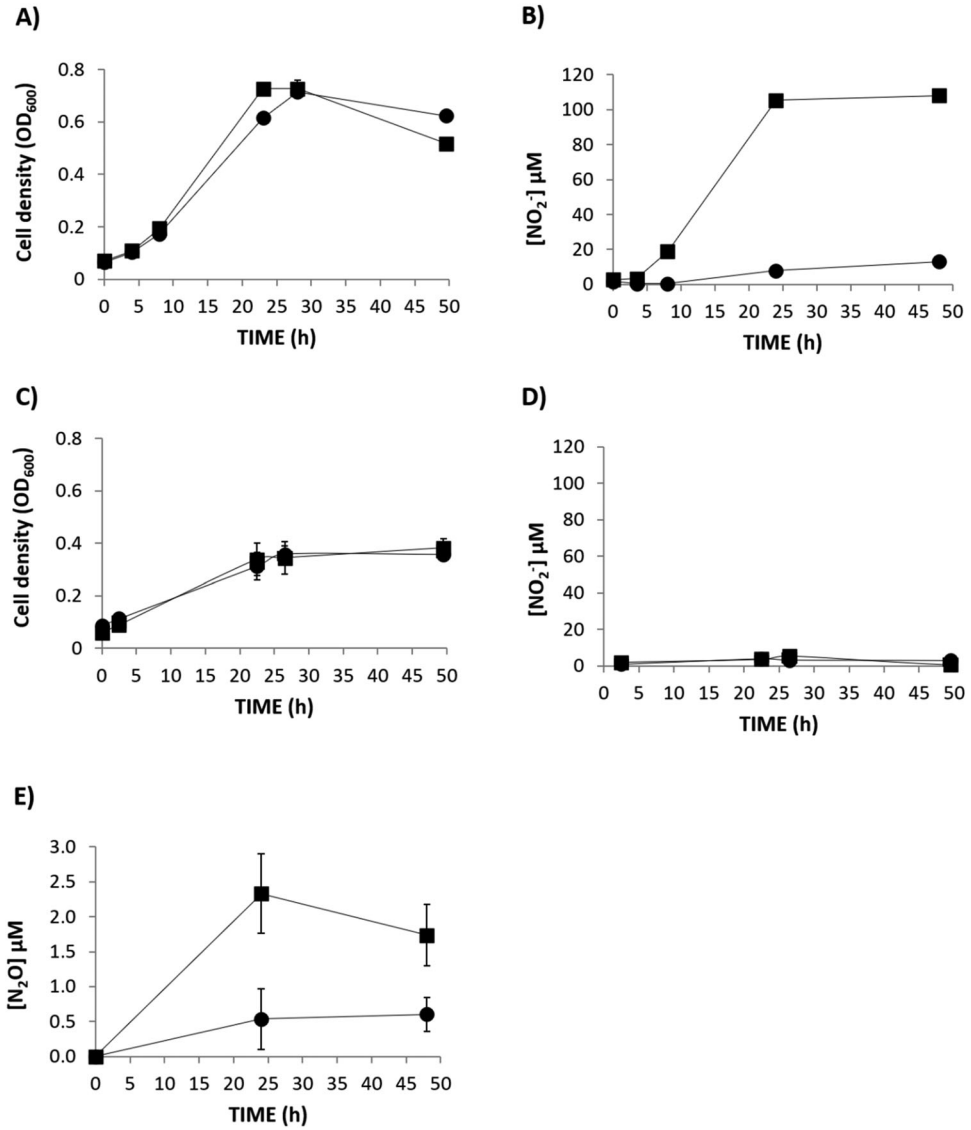

Figure 2. Nitrate-dependent growth (A, C), extracellular nitrite (NO_2^-) concentration (B, D) and N_2O accumulation (E) of *R. etli* CE3 (WT) (■) and *narK* (●). Cells were cultured with NO_3^- as the sole N source under oxic (A, B) or microoxic (C, D, E) conditions. Data are means with standard error bars from at least two independent cultures assayed in triplicate.

by mutation of *narK* gene. However, extracellular NO_2^- accumulation was significantly lower in the growth medium of the *narK* mutant compared to the WT strain (Figure 2B) under oxic conditions indicating a NarK role in nitrite extrusion. When cultured microoxically, extracellular NO_2^- was not detected in the growth medium of any strains (Figure 2D). This observation suggests that, as previously reported (Hidalgo-García et al. 2019), under microoxic conditions, NO_2^- exported from the cytoplasm might be subsequently reduced to NO and N_2O in the periplasm by NirK and cNor denitrification enzymes, respectively. However, we can assume that NO_2^- export is also affected under microoxic conditions since N_2O production is notably decreased in the headspace of the *narK* cultures compared to that from the WT cultures (Figure 2E). In order to confirm that the decrease of N_2O production in the *narK* mutant was due to an impaired NO_2^- transport from cytoplasm to periplasm, we performed the same experiment but using NO_2^- as the sole N source (Figure 3). Under these conditions the WT and *narK* mutant grew (Figure 3A) and emit N_2O (Figure 3B) at similar rates. Hidalgo-García et al. (2019) reported that *R. etli* CE3 overexpressing NarB shows increased levels of extracellular NO_2^- and headspace N_2O under microoxic conditions with NO_3^- as the sole N source. To further confirm the NarK involvement in NO_2^- extrusion and N_2O production, WT and *narK* strains overexpressing NarB were used (Figure 4). As previously reported by Hidalgo-García et al. (2019), extracellular NO_2^- accumulation in growth medium (80 µM

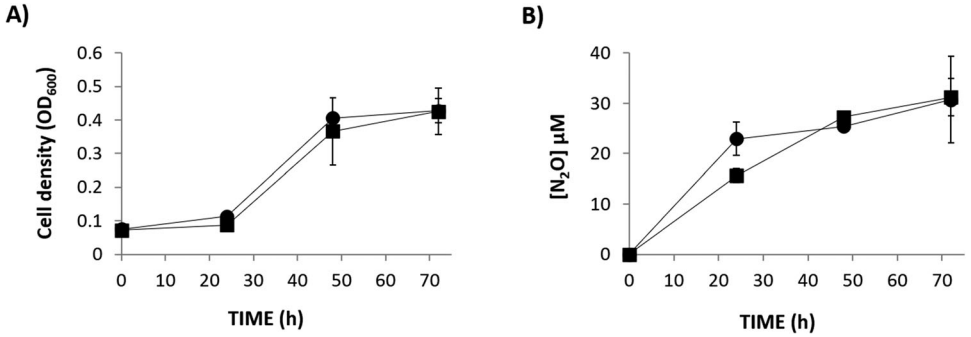

Figure 3. Growth (A) and N_2O accumulation (B) by *R.etli* CE3 (WT) (■) and *narK*(●), cultured microoxically with NO_2^- as the sole N source. Data are means with standard error bars from at least two independent cultures assayed in triplicate.

NO_2^- after 25 h incubation) for a WT + $NarB^+$ strain was observed during microoxic culture with NO_3^-. However, when *narB* was overexpressed in the *narK* mutant, NO_2^- was practically undetectable in the medium (Figure 4B). The WT + $NarB^+$ and *narK* + $NarB^+$ strains showed similar growth (Figure 4A), however, a noticeable difference was N_2O production that was much lower in the *narK* mutant compared to that observed in the WT, both overexpressing NarB (Figure 4C). Collectively, these *in vivo* results suggest that NarK is involved in NO_2^- transport from the cytoplasm to the periplasm and, therefore in N_2O emission, since the NO_2^- supplied to the periplasm by NarK is the substrate for NirK and cNor denitrification enzymes that reduce it to NO and N_2O, respectively. Complementation of the *narK* + $NarB^+$ mutant with an expression plasmid carrying the *narK* gene under the control of a constitutive promotor, restored the extracellular NO_2^- and N_2O production to levels comparable with those observed for the WT + $NarB^+$ strain (Figure 4B,C, respectively).

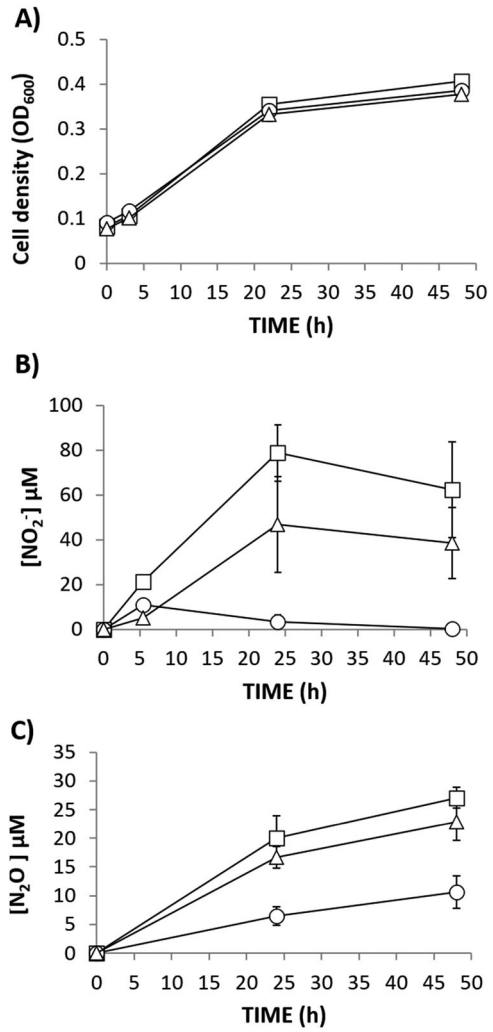

Figure 4. Nitrate-dependent growth (A) extracellular NO_2^- concentration (B) and N_2O accumulation (C) by *R.etli* WT + $NarB^+$ (□), *narK* + $NarB^+$ (○) and *narK* + $NarB^+$+$NarK^+$ (Δ), cultured microoxically with NO_3^- as the sole N source. Data are means with standard error bars from at least two independent cultures assayed in triplicate.

3.3. Nitrate induces N_2O emission by common bean nodules

The capacity of common bean nodules to emit N_2O was explored in plants inoculated with *R. etli* CE3 and N_2O emission was measured in detached nodules by gas chromatography. Several treatments were tested to determine which plant growth conditions could support rhizobia denitrification and, therefore, N_2O production by common bean nodules. Figure 5 shows a positive correlation between 1, 2, and 4 mM KNO_3 concentration in the mineral solution applied to irrigate the plants and N_2O emission. Treatment of 10 mM NO_3^- for one day before nodules harvesting slightly increases N_2O production by nodules compared to 1, 2 or 4 mM treatment during the entire plant growth period. However, the highest N_2O emission rate was reached after the application of 10 mM KNO_3 four days before plant harvesting (Figure 5). Although this treatment significantly induced N_2O production by the nodules, it had an adverse effect in nodule biomass and fitness since decreased nodules fresh weight as well as leghaemoglobin content of nodules was observed in 10 mM KNO_3 treated plants compared to plants watered with a N-free mineral solution throughout the experiment (Supplementary Figure S2).

3.4. R. etli *NarB, NirK, NorC and NarK proteins are involved in N_2O emission from common bean nodules*

Common bean plants were inoculated with *R. etli* CE3 (WT) and a set of mutant strains individually lacking the *narB*, *nirK*, *norC* or *narK* genes. Plants were watered with N-free mineral solution and 4 days before plant harvesting, they were treated with 10 mM KNO_3. N_2O production was detected in nodules from common bean plants regardless of the *R. etli* strain used as inoculum (Figure 6). We found that only nodules formed in plants inoculated with the *nirK* mutant emitted 41% less N_2O than those from plants inoculated with the WT strain (Figure 6). A parallel

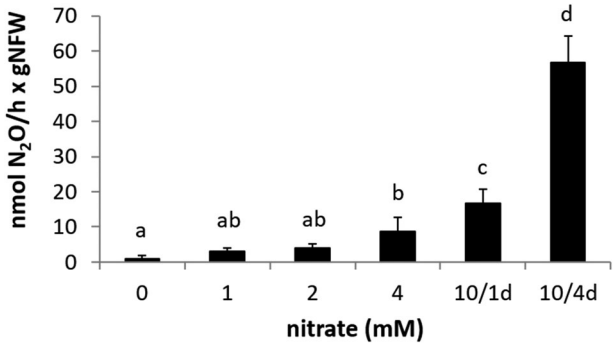

Figure 5. N_2O emission by detached nodules from plants inoculated with *R. etli* CE3 and watered with mineral solution containing 0, 1, 2 or 4 mM KNO_3 throughout the growth period or with 10 mM KNO_3 for one day (10/1d) or four days (10/4d) before plant harvesting. Data are expressed as the mean value and standard deviation error bars of two independent experiments. In each experiment, 5 replicates collected from ten plants were assayed. Lower-case letters indicate comparisons between nitrate treatments. Same lower-case letters are not statistically significant according to HSD Tukey test at $p \leq 0.05$. NFW, nodule fresh weight.

experiment was performed using the WT and the same set of mutants overexpressing NarB (WT + $NarB^+$, *narB* + $NarB^+$, *nirK* + $NarB^+$, *norC* + $NarB^+$ and *narK* + $NarB^+$). Overexpression of the *narB* gene in WT and *narB* strains resulted in about 2-fold increase of N_2O emission. Interestingly, nodules formed by *nirK* + $NarB^+$, *norC* + $NarB^+$ and *narK* + $NarB^+$ mutants emitted significantly less N_2O than the WT + $NarB^+$ (Figure 6). In fact, *nirK* + $NarB^+$ nodules produced about 77% less N_2O than WT + $NarB^+$ nodules. In the case of *norC* + $NarB^+$ nodules, they emitted around 56% less N_2O than WT + $NarB^+$ nodules (Figure 6). Finally, N_2O production capacity of the nodules induced by *narK* + $NarB^+$ mutant was only decreased by about 25% compared to WT + $NarB^+$ nodules (Figure 6).

To better understand the involvement of NarB, NirK and cNor enzymes in N_2O emission from common bean nodules, NR, NiR and Nor activities, as well as N_2O production, were analyzed in isolated bacteroids from nodules of plants inoculated with the WT strain or the *narB*, *nirK* and *norC* mutants. With respect to NR, very low levels of activity were observed in bacteroids of the WT and *narB* mutant (Figure 7A). However, the overexpression of NarB protein in the WT and *narB*

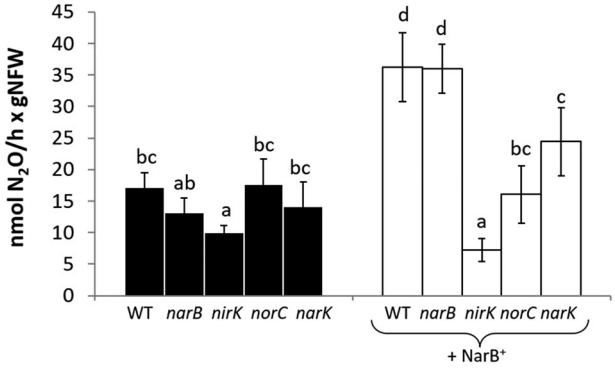

Figure 6. N_2O emission by detached nodules from plants inoculated with *R. etli* CE3 (WT), *narB, nirK, norC* and *narK* (black bars) or *R. etli* WT + $NarB^+$, *narB* + $NarB^+$, *nirK* + $NarB^+$, *norC* + $NarB^+$ and *narK* + $NarB^+$ (white bars). Plants were treated with 10 mM KNO_3 for four days before plant harvesting. Data are expressed as the mean value and standard deviation error bars of two independent experiments. In each experiment, 5 replicates collected from ten plants were assayed. Lower-case letters indicate comparisons between nitrate treatments. Same lower-case letters are not statistically significant according to HSD Tukey test at $p \leq 0.05$. NFW, nodule fresh weight.

strains resulted in a significant induction of NR activity by more than double compared to the strains that did not overexpress *narB* gene (Figure 7A). NiR activity was clearly impaired in bacteroids from nodules recovered from plants inoculated with the *nirK* mutant strain compared to those of WT strain in both scenarios: with or without overexpression of NarB protein (Figure 7B). Bacteroids produced by the *norC* mutant consumed around 25% less NO than WT bacteroids. This difference was more notable between WT and *norC* strains overexpressing NarB protein, where the rate of NO consumption of *norC* + $NarB^+$ bacteroids decreased around 58% compared to WT + $NarB^+$ bacteroids (Figure 7C). Finally, we analyzed N_2O production by bacteroids induced by WT, *norC*, WT + $NarB^+$ or *norC* + $NarB^+$ strains (Figure 7D). Interestingly, NarB overexpression in the WT strain increased the bacteroid N_2O emission by around 10-fold (Figure 7D). By contrast, very low levels of N_2O were produced by *norC* and *norC* + $NarB^+$ bacteroids compared to those produced by WT + $NarB^+$ bacteroids (Figure 7D).

3.5. R. etli *NarB and cNor proteins are involved in NO homeostasis in common bean nodules*

Common bean plants were inoculated with WT, *norC*, WT + $NarB^+$ and *norC* + $NarB^+$ strains of *R. etli* and treated with 10 mM KNO_3 four days before harvesting. Electron paramagnetic resonance (EPR) spectroscopy was used to detect NO bound to Lb (LbNO complexes) within intact frozen nodules recovered from the plant root systems. It has been previously reported that soybean nodules from plants inoculated with *B. diazoefficiens*, watered with 4 mM KNO_3 and subjected to hypoxia produced an EPR signal specific for LbNO complexes (Meakin et al. 2007; Sánchez et al. 2010). Therefore, in this current study, nodules from soybean plants treated with nitrate and flooding were used as a positive control for detection of EPR signals from LbNO complexes (Figure 8). Interestingly, overexpression of the *narB* gene in the WT strain gave rise to clear formation of these complexes in common bean nodules (Figure 8). Furthermore, the magnitude of the specific EPR signal was greater in nodules from plants inoculated with the *R. etli norC* or *norC* + $NarB^+$ strains compared to nodules recovered from plants inoculated with either WT or WT + $NarB^+$ strains, indicating a higher accumulation of LbNO complexes in nodules deficient in the bacteroidal NO reductase. Notably, nodules from plants inoculated with the *norC* + $NarB^+$ strain gave the largest EPR signal for LbNO (Figure 8).

4. Discussion

4.1. Rhizobium etli *NarK is involved in nitrite extrusion and N_2O emission by free-living cells*

R. etli is a N_2-fixing bacterium able to live freely in soil or symbiotically associated with common bean plants. It is considered an incomplete denitrifier since its genome only contains the *nirK* and *nor* denitrification genes, which code for the nitrite and nitric oxide reductases, respectively, but lacks genes that encode for the respiratory nitrate reductases (Nar or Nap) and nitrous oxide reductase (Nos). In a previous work (Hidalgo-García et al. 2019), it was demonstrated that *R. etli* is able to produce NO and N_2O when cultivated under microoxic conditions with NO_3^- as the sole N source,

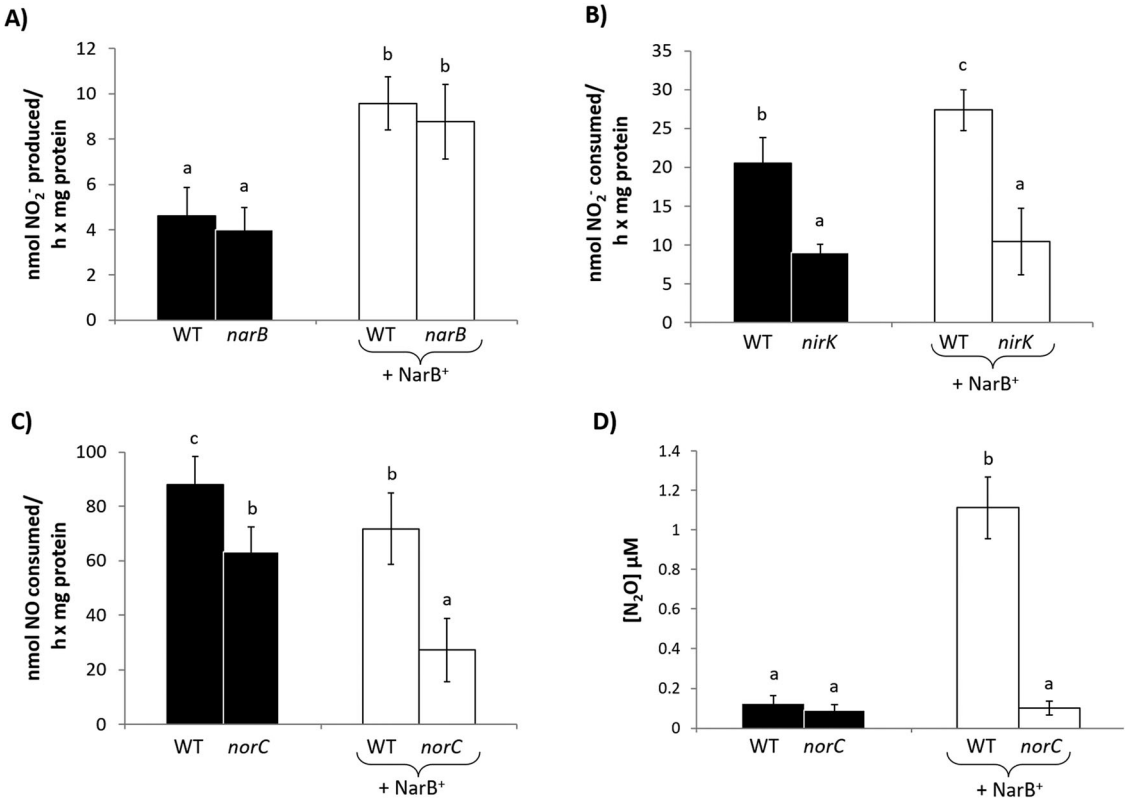

Figure 7. Nitrate reductase (A), nitrite reductase (B), nitric oxide reductase (C) activities and N_2O production (D) of bacteroids from nodules of common bean plants inoculated with *R. etli* CE3 (WT), *narB*, *nirK* or *norC* (black bars) or WT + NarB$^+$, *narB* + NarB$^+$, *nirK* + NarB$^+$, or *norC* + NarB$^+$ (white bars). Data are expressed as the mean value and standard deviation error bars of two independent experiments. In each experiment, bacteroids from 3 replicates collected from ten plants were assayed. Lower-case letters indicate comparisons between strains. Same lower-case letters are not statistically significant according to HSD Tukey test at $p \leq 0.05$.

due to the action of an assimilatory nitrate reductase, NarB. In the cytosol, NO_3^- is reduced to NO_2^- via NarB, then NO_2^- can be further reduced to NH_4^+ or exported outside the cell since this molecule can be toxic at certain concentrations. However, once NO_2^- passes into the periplasmic space, it can be subsequently reduced to NO and N_2O by NirK and cNor denitrification enzymes, respectively. Until now, it was unknown which transporter(s) were involved in NO_2^- export to the periplasmic space in *R. etli*, acting as a nexus between the nitrate assimilatory and denitrification pathways. Bioinformatic analyses showed that, in the *R. etli* genome, upstream *nirB*, *nirD,* and *narB* genes, a gene (RHE_CH01783) was predicted to encode a putative NarK-like NO_3^--transporter belonging to the MFS-type transporters superfamily. Using a *R. etli narK* knockout mutant, we have demonstrated that NarK performs a role in NO_2^- export to the periplasm and supports N_2O production under microoxic conditions. Consistent with a NO_2^- extrusion role for NarK, when the *narK* mutant was cultured with NO_2^- as the sole N source under microoxic conditions, N_2O emission was similar to that observed for WT cultures. In this case, NO_2^- is externally provided and its export from cytosol to periplasm is not necessary for N_2O formation in the periplasmic space.

In bacteria, there are two classes of MFS transporters involved in NO_3^- transport: NarK1 are predicted to be NO_3^-/proton symporters (Moir and Wood 2001), whereas NarK2 are believed to be NO_3^-/ NO_2^- antiporters (Moir and Wood 2001; Goddard et al. 2008). In this context, it has been also reported that in *P. denitrificans*, NarK1 and NarK2 are fuzed into a single protein where the NarK1-like domain functions primarily as a NO_3^- transporter, while the NarK2-like domain is more specialized in NO_3^-/ NO_2^- antiport (Goddard et al. 2017). *R. etli* NarK shows higher amino acid sequence similarity with NarK1 protein from *P. denitrificans*, however our results suggest a NO_2^- export role for this protein. Accordingly, among rhizobia, *B. diazoefficiens* NarK has also been previously reported to be involved in NO_2^- export (Cabrera et al. 2016). The capacity of the *narK* mutant to grow on NO_3^- or NO_2^- suggests that NarK is not implicated, or at least is not essential, for the import of such substrates to the cytoplasm in *R. etli.* This suggests that other unknown importers that are encoded by genes remote to the NO_3^- or NO_2^- assimilatory genes are involved in the transport of these molecules to the cytosol. In this context, it has been reported that free nitrous acid (HNO_2) diffusion might be a way of NO_2^- transport across the inner membrane (Gates et al. 2011).

4.2. N_2O is emitted by common bean nodules by the coupling, through NarK transporter, of R. etli nitrate assimilation and denitrification routes

Our next goal was to explore the role of *R. etli* NarB, NarK, NirK and cNor in N_2O emission by common bean root nodules. It is well known that soybean nodules produce N_2O in response to nitrate and flooding (Tortosa et al. 2015; Tortosa et al. 2020). However, due to the high sensitivity of common bean to flooding (Hidalgo-García et al. 2016), this standard laboratory experimental conditions could not be used to study N_2O emissions by common bean root nodules. Instead, plants were exposed to different NO_3^- treatments to investigate the capacity of the nodules to produce N_2O. The application of 10 mM KNO_3 for four days

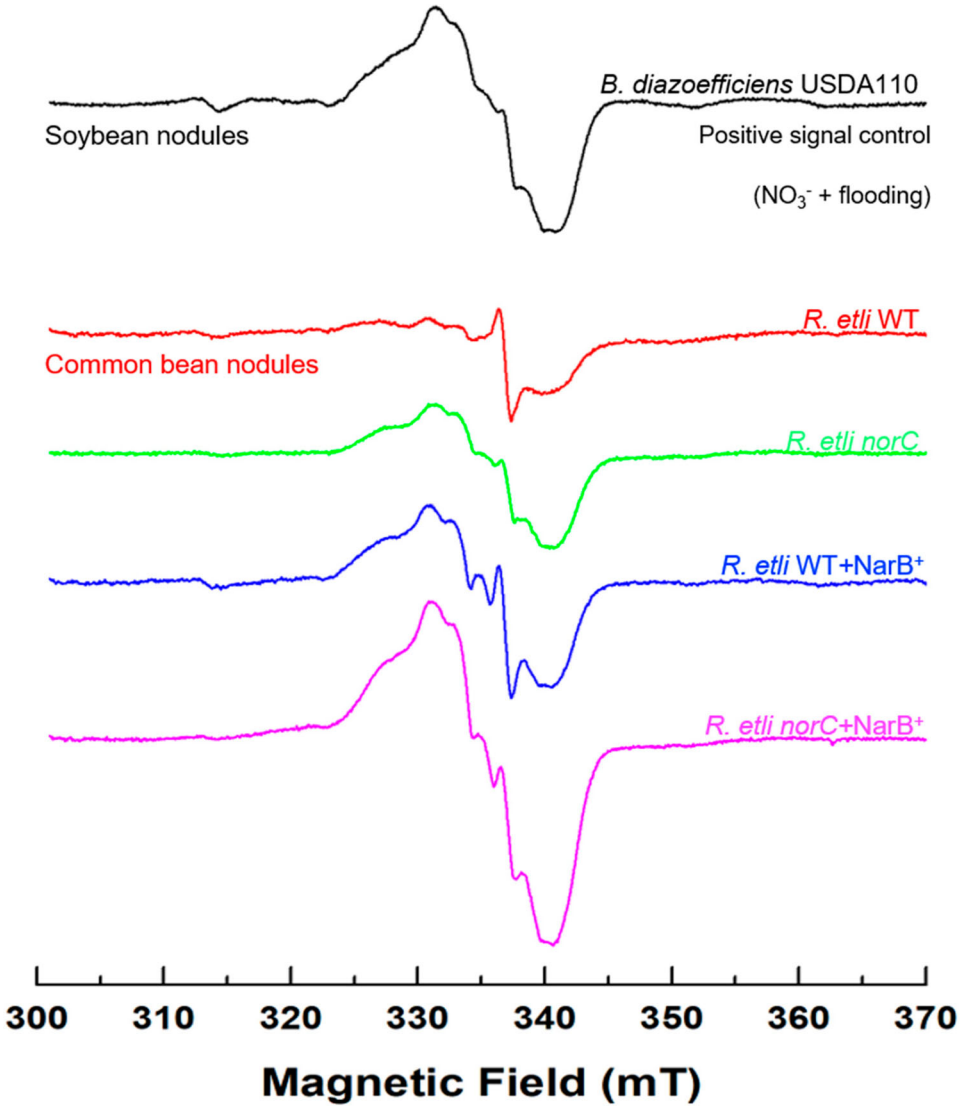

Figure 8. Leghaemoglobin-NO (LbNO) complexes detected by electron paramagnetic resonance (EPR) from whole intact nodules of common bean plants inoculated with *R. etli* CE3 (WT), *norC*, WT + NarB$^+$ and *norC* + NarB$^+$. Nodule EPR signal from soybean plants subjected to NO_3^- and flooding was used as positive control. For each strain, a representative EPR spectrum observed from triplicate nodule samples from three different nodule harvests is presented.

resulted in the highest N_2O production in nodules. This treatment also reduced the fitness of the nodules as determined by the analysis of Lb content. As observed in our work, it has been previously reported that 10 mM KNO_3 treatment of common bean or soybean for 4 or 6 days, respectively, affected nodules functionality by inducing their premature senescence (Calvo-Begueria et al. 2018). The nitrate treatment (10 mM KNO_3 four days before plant harvesting) used in this work to induce N_2O emissions is quite improbable under natural conditions and it is detrimental to symbiosis and nitrogen fixation. However, those conditions allowed us to demonstrate N_2O emissions from common bean nodules as well as the involvement of *R. etli* NarB, NarK, NirK and cNor proteins under high nitrate fertilization when nodulation is established.

Next, the contribution of *R. etli* NarB, NirK, cNor, and NarK proteins in N_2O emissions by common bean nodules was assessed. In contrast to free-living conditions where NarB is required for N_2O production (Hidalgo-García et al. 2019), mutation of *narB* did not significantly affect N_2O emission by common bean nodules. Similar to our observations, it has been shown in *Medicago truncatula* nodules that *S. meliloti* nitrate assimilation pathway encoded by *nirBDnarB* did not play any detectable role in the production of NO, the N_2O precursor (Ruiz et al. 2022). Despite this, we have demonstrated the involvement of NarB in bacteroidal NR activity as well as in nodule and bacteroidal N_2O emissions by overexpressing *narB*. Our results also reveal an important contribution of bacteroidal NirK to N_2O emissions by common bean nodules. This was supported with *nirK* and *nirK* + NarB$^+$ nodules emitting significantly less N_2O than WT and WT + NarB$^+$ nodules, respectively, presumably due to the impaired bacteroidal Nir activity. The involvement of cNor in N_2O production was only observed in nodules produced by a *norC* mutant additionally overexpressing *narB*. However, *norC* + NarB$^+$ still produced significant levels of N_2O (about 44%). These results suggest that, in addition to cNor, other plant or bacterium proteins may be involved in N_2O formation. Since, *norC* or *norC* + NarB$^+$ bacteroids were defective in N_2O production, a plant protein may provide an additional source of N_2O in common bean nodules. Supporting this hypothesis, it has been reported that N_2O can be produced from NO in plant mitochondria (Timilsina et al. 2020 and references herein).

Although the *norC* mutation resulted in a lower capacity of bacteroids to consume NO compared to WT, the NO

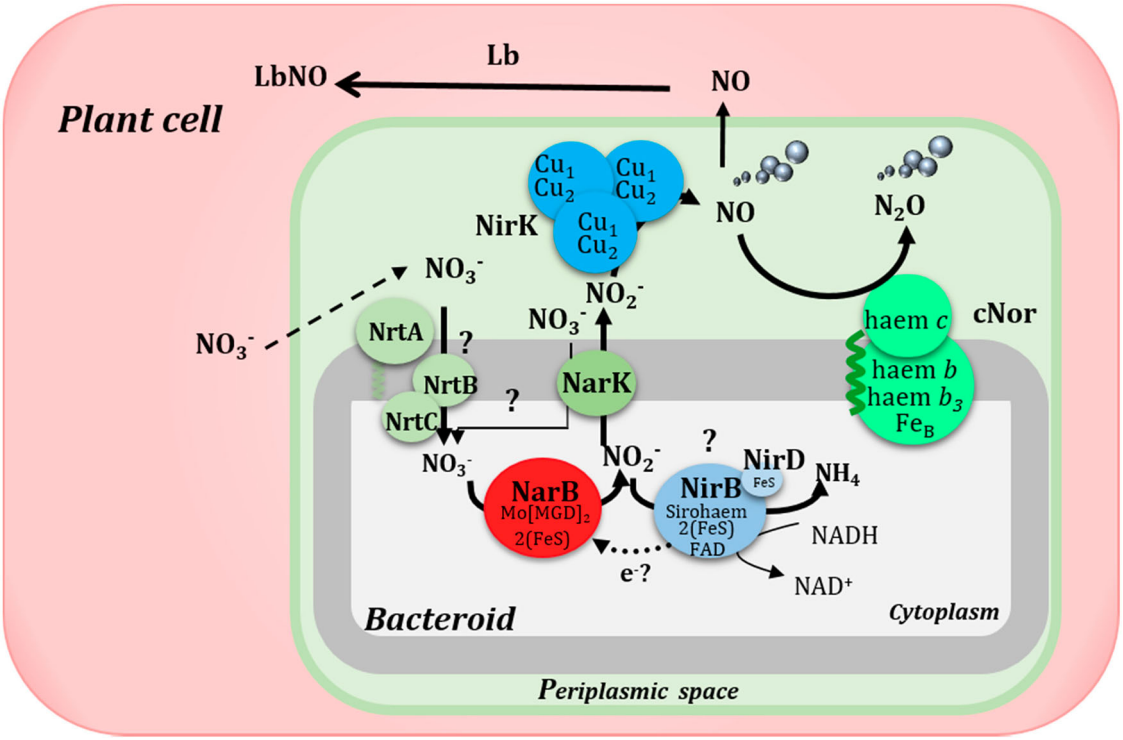

Figure 9. Schematic representation of the contribution of NarB, NarK, NirK and cNor to NO and N_2O production in common bean nodules. In this model, NO_3^- provided in the nutrient solution enters the nodule and can be transported to the cytoplasm of the bacteroid where, by the action of NarB it would be reduced to NO_2^-. Then, NO_2^- can be exported to the periplasmic space through NarK and there the denitrifying enzymes NirK and cNor would reduce it to NO and N_2O, respectively. In the plant cell, NO produced by the bacteroids would be bound to Lb to form LbNO complexes. Question marks denote mechanisms still unknown. Adapted from Salas et al. 2021.

consumption activity remained higher than that observed under free-living conditions, where NO consumption by the *norC* mutant was almost abolished compared to WT cells (Hidalgo-García et al. 2019). This discrepancy between free-living and symbiotic lifestyles might indicate the existence of other systems to detoxify NO that are induced specifically under symbiotic conditions in *R. etli* bacteroids. In this context, in addition to cNor, a combined role for *S. meliloti* flavohaemoglobin (Hmp), and NnrS1 and NnrS2 proteins in NO degradation has been reported in *Medicago truncatula* nodules (Blanquet et al. 2015). Furthermore, in soybean nodules, the *B. diazoefficiens* hemoglobin, Bjgb, indirectly contributes to NO levels modulation in the nodules (Salas et al. 2020). Finally, *R. etli* NarK also plays an important role in N_2O formation by contributing to NO_2^- export from the bacteroidal cytosol to the periplasmic space. However, in contrast to free-living conditions, *narK* nodules still produce significant levels of N_2O. These results suggest the involvement of additional specific NO_2^- transport systems in the bacteroids or alternatively the participation of plant proteins in N_2O formation.

4.3. R. etli *NarB and cNor proteins modulate NO levels in common bean nodules*

In addition to being a precursor to N_2O, NO is a very important signaling molecule in plants and is involved in many processes including vegetative development, defence against pathogens, abiotic stress signalling among others. Interestingly, NO also plays a role at different stages of the rhizobia-legume symbiotic relationship, from the early steps of infection to nodule senescence (reviewed by Berger et al. 2019). Moreover, NO inhibits nitrogenase activity (Sánchez et al. 2010). Consequently, NO concentration has to be finely controlled inside nodules to maintain viability. It has been previously reported that denitrification performed by *B. diazoefficiens* or *S. meliloti* is an important source of NO in soybean or *M. truncatula* nodules (Horchani et al. 2010; Sánchez et al. 2010; Tortosa et al. 2015). This molecule can bind to leghaemoglobin yielding LbNO complexes which can be detected readily by EPR (Sánchez et al. 2010). In order to investigate the contribution of *R. etli* NarB and cNor to NO levels in common bean nodules, LbNO complexes were analysed by EPR in intact nodules. Overexpression of *R. etli narB* in the WT strain gave rise to clear LbNO complex signals in intact nodules suggesting that NarB contributes to NO formation in common bean nodules. Similar to what has been reported for soybean nodules by using a *B. diazoefficiens norC* mutant (Sánchez et al. 2010; Calvo-Begueria et al. 2018), mutation of *R. etli norC* gene also resulted in higher levels of LbNO complexes demonstrating the involvement of *R. etli* NarB and NorC in NO modulation in common bean nodules.

5. Conclusions

Legumes contribute to N_2O emissions by providing N-rich residues for decomposition or directly by some rhizobia that can denitrify under free-living and symbiotic conditions. In the case of common beans, its endosymbiont, *Rhizobium etli* CFN42, is an incomplete denitrifier since it lacks the *nar* or *nap* genes encoding the respiratory nitrate reductases (Nap or Nar) as well as the *nos* genes encoding the nitrous oxide reductase enzyme. In this work, we have demonstrated the capacity of common bean nodules to produce N_2O by coupling NO_3^- assimilation and denitrification in the

bacteroids (Figure 9). In this pathway, NO_2^- formed by the *R. etli* assimilatory nitrate reductase NarB is exported by NarK from the cytosol to the periplasm. Then, the denitrifying enzymes NirK and NorC reduce NO_2^- to NO and N_2O, respectively (Figure 9). In conclusion, this work highlights the importance of rhizobia NO_3^- assimilation and denitrification in emission of NO and N_2O by common bean nodules under high nitrate fertilization when the nodulation is established. The knowledge generated in this work including the identification of proteins involved in NO and N_2O production by legume root nodules will contribute to development of new solutions to improve legume crop production while mitigating greenhouse gas emissions.

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Disclosure statement

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