New Insights into the Use of Rhizobia to Mitigate Soil N2O Emissions

Catherine Hénault, Elodie Barbier, Alain Hartmann, Cécile Revellin

agriculture

Article

New Insights into the Use of Rhizobia to Mitigate Soil N_2O Emissions

Catherine Hénault *, Elodie Barbier, Alain Hartmann and Cécile Revellin

Agroécologie, INRAE, Institut Agro, Université Bourgogne, Université Bourgogne Franche-Comté, F-21000 Dijon, France; elodie.barbier@inrae.fr (E.B.); alain.hartmann@inrae.fr (A.H.); cecile.revellin@inrae.fr (C.R.)

* Correspondence: catherine.henault@inrae.fr

Abstract: Agriculture is a major anthropogenic source of the greenhouse gas N_2O, which is also involved in stratospheric ozone depletion. While the use of rhizobial inoculants has already been reported as an emerging option for mitigating soil N_2O emissions, this study presents an in situ abatement of 70% of soil N_2O emission using the strain *nosZ*$^+$ G49 vs. *nosZ*$^-$ USDA138 in association with soybean. Therefore, we consider that the choice of the inoculant strain of a leguminous crop should take into account the capacity of strains to reduce nitrous oxide in addition to their N fixation capacity. This study also clearly suggests that this mitigation option could be considered not only for soybean but also for different leguminous crops, with emphasis currently placed on lupin because of the potential of its association with the *nosZ*$^+$ LL200 strain. The clear demonstration of the N_2O reduction capacity of clover symbiotic strains suggests that opportunities for mitigation might also occur on grassland.

Keywords: N_2O mitigation; rhizobia; legumes; *nosZ* gene; phenotypes; multiscale approach

check for updates

Citation: Hénault, C.; Barbier, E.; Hartmann, A.; Revellin, C. New Insights into the Use of Rhizobia to Mitigate Soil N_2O Emissions. *Agriculture* **2022**, *12*, 271. https://doi.org/10.3390/agriculture12020271

Academic Editor: Paolo Ruisi

Received: 3 January 2022
Accepted: 12 February 2022
Published: 15 February 2022

1. Introduction

Agriculture, through soil emissions, is a major anthropogenic source of the greenhouse gas N_2O, which has a Global Warming Potential about 300 times higher than CO_2 on a molar basis, and which is also involved in stratospheric ozone depletion [1]. In soils, N_2O is mainly produced through the microbial processes of denitrification and nitrification [2]. The last step of the denitrification process, N_2O reduction into N_2, is currently the only known pathway for the terrestrial removal of N_2O. N_2O reduction is catalyzed by the N_2O reductase enzyme (Nos) encoded by the *nosZ* gene. Strategies to mitigate N_2O emissions from agricultural soil could be based on promoting the reduction of N_2O into N_2 in soils [3]. Recently, Bakken and Frostegård [4] reviewed emerging options for mitigating N_2O emissions from food production by manipulating the soil microbiota. Amongst these options, the use of rhizobial inoculants to mitigate N_2O emissions was underlined. These authors also specified that Bradyrhizobia may be stronger N_2O sinks than many other fully fledged denitrifying bacteria, because their nitrate reductase (Nap) competes poorly with Nos, resulting in the preferential reduction of N_2O over that of nitrate [5]. Moreover, the N_2O reduction activity of inoculated rhizobia possibly persists in the soil for a long time [6]. While this approach was mainly conducted with the association of soybean–*Bradyrhizobium*, other leguminous crops are of great interest for food and other uses and currently cover substantial surface areas that have been predicted to increase in the future [7]. As far as the use of the soybean–*Bradyrhizobium* association for mitigating soil N_2O emissions is concerned, experiments have demonstrated mitigation on the laboratory and greenhouse scales [8,9]; however, currently, very few field-scale results have been reported [6,10,11]. Field experiments testing mitigation options remain scarce because they are difficult to conduct. They are nevertheless essential to carry out the TRL (Technology Readiness Level scale) approach. Therefore, each field experiment requires painstaking preparation. The

aims of this paper are (i) to present results obtained during a field experiment with the soybean–*Bradyrhizobium diazoefficiens* G49 association, previously well characterized during laboratory and greenhouse experiments [9], to obtain in situ proof of concept of the use of rhizobial inoculants to mitigate N_2O emissions; and (ii) to analyze the genotypic and phenotypic characteristics of the N_2O reduction capacity of different leguminous crop–rhizobia associations not yet investigated regarding this property, prior to testing them in field conditions.

2. Materials and Methods

2.1. Bacterial Strains Used in the Study and Culture Conditions

The strains used in this study are described in Table 1. They were obtained from the collection "Microorganisms of Interest for Agriculture and Environment" hosted by INRAE, UMR Agroecologie at Dijon (France). Strains were grown from cryo-conserved stocks kept at −80 °C on modified Bergersen medium plates. From these plates, liquid cultures were obtained on either YEM medium [12] or TY medium [13], for plant inoculation in greenhouse trials or molecular detection of the *nosZ* gene, respectively.

2.2. Molecular Detection of nosZ Gene Sequences

Primers nosZ1F (5′ CSY TGT TCM TCG ACA GCC AG 3′) and nosZ2R (5′ CAK GTG CAK BGC RTG GCA GAA 3′) described by Henry et al. [14] were used to detect the presence of *nosZ* genes in bacterial strains described in Table 1. These primers (with degenerated sequences in order to take into account sequence variability) proved to be efficient in detecting *nosZ* genes among a wide diversity of bacterial genera, especially in all the proteobacteria tested, including rhizobia. Subsequent sequencing of the PCR products demonstrated the specificity of these primers [14]. Amplification was performed, using proteinase K treated cells as templates [14]. Briefly, PCR Thermal cycling conditions for the nosZ1F and nosZ2R primers were as follows: an initial cycle of 95 °C for 4 min; 95 °C for 30 s; touch down on the annealing temperature 65 °C (2 cycles), 59 °C (2 cycles), 56 °C (3 cycles), and 53 °C (4 cycles) for 30 s; 72 °C for 40 s; 34 cycles of 95 °C for 30 s and 50 °C for 30 s; 72 °C for 40 s. The size of the PCR product was 699 bp. The *nos*Z gene was also detected using primers Nos661F* (5′ CGG YTG GGG CCW RAC SAA 3′) and Nos1773R* (5′ ATR TCG ATC ARC TGB TCG TT 3′) modified from Scala and Kerkhof [15]. The PCR conditions used were those described by the authors, and the expected PCR product size was 1112 bp. Some PCR products obtained in our study were also sequenced to confirm the identity with known *nosZ* sequences (Sanger Sequencing, Genewiz, France). The sequences obtained were deposited in Genbank. Reference strains, namely *B. diazoefficiens* strain USDA110 and *Ensifer meliloti* strain 2011, were used as positive controls of PCR since they are known to carry a *nos*Z gene [16,17].

2.3. In Situ N_2O Fluxes Measurements

An experimental block trial involving the N_2O-reducing strain G49 [9] and the strain USDA 138 depleted of the *nosZ* gene (this study) was performed in 2015 at the INRAE Center in Centre Val de Loire Region (Ardon) in France. The experimental soil, with a pH between 5.5 and 6.0, had been tested previously as being scarcely able to reduce N_2O [3]. Before this experiment, this soil had never been cultivated with soybean and therefore was free of soybean symbionts [18]. The trial used 8 sub-plots each with 2 manual chambers for N_2O flux measurements [19]. The day of sowing (22 May), soja seeds were divided into two groups, each receiving one of our two microbial inoculants. First, the commercial liquid Rhizoflo (BASF), lot G490314D, was used for the inoculation of G49.

Table 1. Bacterial strains used in this study.

Species/ **Strain Number (Synonyms)**	**Host Plant of Origin**	**Reference/Source/Origin**
Ensifer meliloti		
MIAE00427 (MSDJ848, RCR2011, **2011**)	*Medicago sativa* (alfalfa)	[20]/RCR
Rhizobium leguminosarum bv. trifolii		
MIAE03027(MSDJ134, **T117**)	*Trifolium pratense* (clover)	[21]
MIAE03034 (MSDJ141, **T132**)	*Trifolium pratense* (clover)	[21]
Bradyrhizobium lupini		
MIAE00428 (MSDJ718, **LL13**)	*Lupinus luteus* (lupine)	[22]
Bradyrhizobium sp. *(Lupinus)*		
MIAE00428 (MSDJ718, **LL200**)	*Lupinus luteus* (lupine)	[20]
Rhizobium leguminosarum bv. viciae		
MIAE01211 (MSDJ822, **FH34**)	*Vicia faba* (faba bean)	[20]
MIAE01211 (MSDJ822, **FS16**)	*Vicia faba* (faba bean)	[20]
Bradyrhizobium diazoefficiens		
MIAE00426 (MSDJ1996, **G49**)	*Glycine max* (soybean)	[23]
USDA 110	*Glycine max* (soybean)	USDA
Bradyrhizobium japonicum		
USDA138	*Glycine max* (soybean)	USDA
Ensifer sp. *(Trigonella)*		
MIAE06325 (MSDJ3523, **3523**)	*Trigonella foenum-graecum* (fenugreek)	MIAE
MIAE06333 (MSDJ3531, **3531**)	*Trigonella foenum-graecum* (fenugreek)	MIAE
MIAE01335 (MSDJ3538, **3538**)	*Trigonella foenum-graecum* (fenugreek)	MIAE
MIAE06345 (MSDJ3546, **3546**)	*Trigonella foenum-graecum* (fenugreek)	MIAE
Bradyrhizobium sp. *Arachis*		
MIAE06284 (MSDJ3482, **IC7017**)	*Arachis hypogaea* (peanut)	ICRISAT
MIAE04980 (MSDJ2177, **ST2**)	*Arachis hypogaea* (peanut)	USA

In bold: name used in this paper; RCR: Rhizobium Collection Rothamsted; USDA: U.S. Department of Agriculture, Beltsville, MD; MIAE: Microbes d'Intérêt Agro-Environnemental; ICRISAT: International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Hyderabad, India.

The second was a USDA 138 inoculant produced in our laboratory on Burton culture media. The inoculation rate was higher than the minimum generally used for soybean seed inoculation, i.e., 5×10^6 CFU per seed, for both inoculants. After carefully mixing the seeds with the appropriate inoculant, they were sown in the soil at the following density: one seed every 10 cm in rows 30 cm apart. Obviously, no N fertilizers were added during the culture period. The chambers (0.5×0.5 m^2 enclosing 10 plants; 0.15 m height) were inserted into the soil when the plants reached about 5 cm height (mid-June) to a depth of approximately 8 cm. N_2O flux measurements started at the beginning of August just before flowering. During the 4 measurements performed before culture destruction, 1.2 m props without any gas circulation system were placed above the chambers. This system was designed to limit damage to the plants. The production of an upward concentration gradient in these large chambers showed that N_2O was linearly distributed within the chamber, and that the N_2O concentration of a sample taken at mid-height was representative of the mean concentration within the chamber. N_2O emission measurements were performed during the evening–night period to avoid any possible (but currently unknown) interactions with photosynthesis, and because the N_2O fluxes were suspected to be low since the legume crops were unfertilized. The gas samples were collected in 20 mL tubes that had been thoroughly purged, by way of 3 replicates per chamber in the evening just after the chambers were closed, and early in the morning. The N_2O analyses were carried out by GC equipped with an electron capture detector (Thermo Scientific™—TRACE 1310) coupled to an automatic sampler (TriPlus RSH™), (Thermo Fisher Scientific, Courtaboeuf, France) able to detect N_2O at the atmospheric concentration (i.e., 0.34 μL L^{-1}) with a precision better than 0.1 μL L^{-1}, as verified with 4 certified bottles between 0.4 and 1.5 μL L^{-1}. These nocturnal fluxes were calculated by the difference (when significant $p < 0.05$) of concentration of N_2O in each chamber over time between the initial and the final sampling.

While not classical [24], this N_2O flux measurement methodology was defined as a trade-off between ensuring the physical integrity of the plants, our physiological knowledge of symbiotic legumes, the N_2O flux detection limit in an unfertilized system, compliance with the working conditions of the personnel involved, and the comparative aim of the experiment. The mean flux of N_2O for each treatment was calculated as the arithmetic mean of the eight replicates. The hypothesis of homogeneity of means between treatments was tested using the ANOVA procedure followed by a Newman and Keuls test ($p < 0.05$) on Microsoft Excel Stat.

2.4. Phenotypic Characterization of Strains Grown as Pure Culture (C) on Growing Medium

The phenotype determined at this scale is denoted C-N_2OR and was determined according to [25]. Strains 2011, T117, LL13, FH34, FS16, G49, USDA100, USDA138, IC7017, and ST2 were tested by inoculation of 1 mL of a preculture (optical density of 0.2 at 620 nm in YEM medium) added to 40 mL tubes containing 9 mL of the same medium added with 1 g L^{-1} of KNO_3. The atmosphere of the tubes sealed with rubber stoppers was removed and replaced with N_2. After 7 days of incubation at 28 °C with agitation at 150 rpm, we determined the NO_3^- consumed [26] and the N_2O produced. N_2O content was determined on GC3000, (SRA Instruments, Marcy l'Etoile, France), equipped with a TCD detector (detection limit 10 µL.L^{-1}) as follows: (i) very low when no production was clearly determined, (ii) low when N_2O represents between 0.1% and 10% of the consumed NO_3^-, (iii) high when N_2O represents between 10% and 50% of the consumed NO_3^-, and (iv) very high when N_2O represents between 50% and 100% of the consumed NO_3^-. Isolates that had consumed NO_3^- with a low or very low N_2O content in flasks were then incubated in the same conditions, except with the addition of C_2H_2 (7%) used to inhibit the N_2O reductase activity [27]. Strains were identified as C-N2OR$^+$ if they accumulated N_2O in the high or very high range, only in the presence of C_2H_2.

2.5. Characterization of Greenhouse N_2O Reduction by the Different Legume–Rhizobia Associations (A)

The phenotype determined at this scale is denoted A-N2OR. Plants were cultivated on the inert "perlite" substrate with calcined clay beads at the bottom for drainage, for about 2 months in a greenhouse in pots, each with a surface area of 0.031 m^2 with 4 plants per pot. The varieties used were Huia, Lublanc, Expresso, Navigator, Hanka for clover, lupin, faba bean, soybean, and fenugreek, respectively. For peanuts, the variety used was unknown, as the grains were sold as food for parrots. The inoculated plants had received the inoculant at sowing with corresponding specific rhizobial strains (Table 1) at a rate higher than 5×10^6 CFU per seed. Both inoculated and non-inoculated plants were watered daily with a nitrogen-free solution. Nodulation was observed for all inoculated plant species at the end of the experiment. After about 60 days of growth, each of the cultivated pots was placed in an airtight chamber (24 cm diameter, 64 cm height) in dark conditions for 48 h in the presence of the inert gas krypton (Kr) at a concentration of 2000 µL L^{-1} to verify the air tightness of the chambers by the absence of change in the Kr concentration during incubation. N_2O was added to set the initial atmosphere chamber at an N_2O concentration of around 3 µL L^{-1} as a trade-off between (i) a screening approach, (ii) the expression of the N_2O reductase activity increasing with ambient N_2O concentration [9], and (iii) the N_2O analyzer detection limit. During the 48 h period, the Kr and N_2O concentrations in the chambers were periodically determined by gas chromatography, as described previously, with N_2O concentrations first determined on the GC equipped with the ECD detector (0.3 µL L^{-1} precision verified at 3 µL L^{-1}) and Kr concentrations then determined on the GC equipped with the TCD detector (detection limit of 100 µL L^{-1}). Statistical analyses of these data were performed with Microsoft Excel Stat using the ANOVA procedure, followed by a Newman and Keuls test with a 5% significance level. After having verified the homogeneity of the means of the N_2O concentrations per leguminous plant at the beginning of incubation, a rhizobia–leguminous couple was considered as an N_2O reducer (A-N2OR$^+$) when the

N_2O concentration at the end of incubation was statistically ($p < 0.05$) and quantitatively (decrease of 0.3 μL L^{-1}) significantly lower than that of the N_2O concentration of the same non-inoculated leguminous crop, also at the end of incubation.

3. Results

3.1. Detection of the nosZ Gene Amongst the Strains Tested

The two sets of primers used gave fully correlated results concerning the detection or the absence of detection of the *nosZ* gene, and the results obtained with the reference strains, *B. diazoefficiens* strain USDA110 and *Ensifer meliloti* strain 2011, were consistently positive. All the results are summarized in Table 2. Sequencing of several PCR products confirmed that amplification products corresponded to *nosZ* sequences. The sequences obtained in this study were aligned with known *nosZ* sequences, analyzed with ORFfinder (NCBI) and deposited in Genbank under the accession numbers given in Table 2.

3.2. In Situ Proof of Concept of N_2O Emission Mitigation by the Soybean–B. diazoefficiens G49 Association

During the summer of 2015, the soils' N_2O emissions were very low on all the measurement dates, systematically lower than 10 g N ha^{-1} d^{-1} (Figure 1). Nevertheless, N_2O emissions on plots cultivated with the soybean inoculated with the G49 strain never exceeded 2 g N-N_2O ha^{-1} d^{-1}, and thus were significantly lower than those observed on plots cultivated with the soybean inoculated with the USDA138 strain ($p < 0.05$), all data considered together. More specifically, daily fluxes were significantly lower on plots cultivated with the soybean inoculated with the G49 strain than those on plots cultivated with the soybean inoculated with the USDA 138 strain on 18 and 25 August and 3 September. N_2O emission abatement was then estimated as 70% on average, which corresponds exactly to the abatement of soil N_2O assessed using the soybean–G49 association, as calculated in [9].

Table 2. Genotypic characterization of the strains.

Species	Collection Name	Detection of the nosZ Gene		Genbank Accession Numbers of Sequences
		Primers nosZ1F/nosZ2R	*Primers nos661F*/nos1773R**	
E. meliloti	2011	+	NT	
B. diazoefficiens	G49	+	+	OL741415
B. diazoefficiens	USDA 110	+	+	
B. japonicum	USDA 138	–	–	
R. leguminosarum bv. trifolii	T132	–	–	
R. leguminosarum bv. trifolii	T117	+	+	OL691940
B. lupini	LL13	–	–	
Bradyrhizobium sp.	LL200	+	+	
R. leguminosarum bv. viciae	FH34	–	–	
R. leguminosarum bv. viciae	FS16	+	+	OL691939
Ensifer sp.	3523	+	+	
Ensifer sp.	3531	+	+	
Ensifer sp.	3538	+	+	
Ensifer sp.	3546	+	+	
Bradyrhizobium sp. *arachis*	IC7017	–	–	
Bradyrhizobium sp. *arachis*	ST2	+	+	OL741416

NT: not tested.

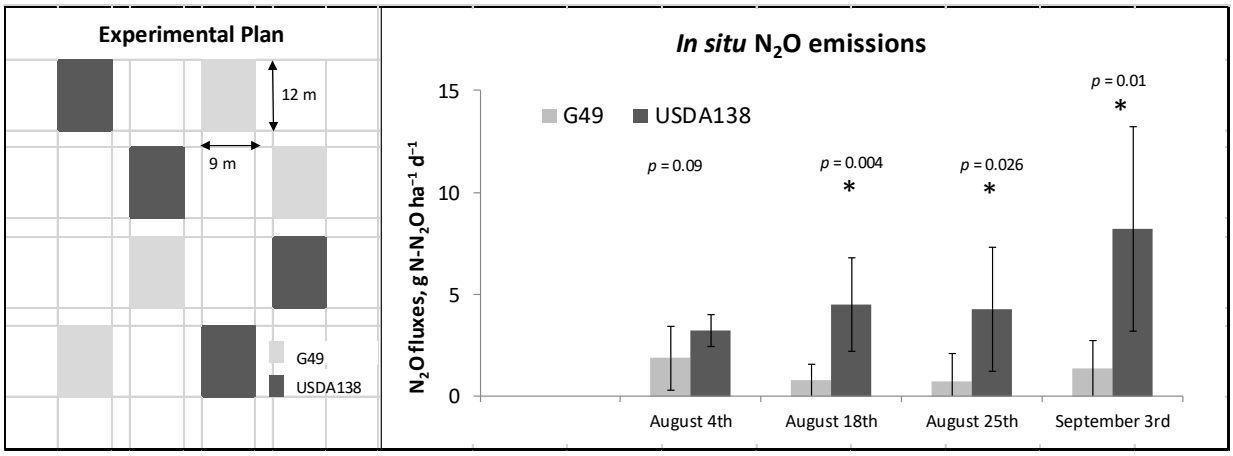

Figure 1. *In situ* experiment. Experimental plan and measured N_2O fluxes including means and standard deviation are represented as error bars, and results of statistical analysis performed on daily fluxes. * Significant difference between fluxes measured on each treatment.

3.3. Phenotypes in Pure Culture

About two-thirds of the tested strains had consumed all the added nitrate during the 7 days of anaerobic incubations (T117, LL13, G49, USDA110, USDA138, and ST2), while strains 2011 and FS16 exhibited a partial consumption of nitrate and FH34 and IC7017 did not consume any nitrate (Table 3), suggesting that they were not able to grow in the incubation conditions [28]. LL13 and USDA 138 accumulated very high levels of N_2O in flask during incubation and were therefore considered as being unable to reduce N_2O (C-N2OR$^-$). Moreover, amongst the strains first observed to consume NO_3^- without acetylene, some showed a lower nitrate consumption (i.e., 2011, USDA110) in the presence of acetylene while some strains (T117, FS16) did not consume any nitrate in this condition, making the pure culture phenotype impossible to determine. On the other hand, strains G49, USDA110, ST2, and 2011 that accumulated N_2O only in the presence of C_2H_2 were clearly shown to be able to reduce N_2O in pure culture and present the phenotype C-N2OR$^+$.

Table 3. Changes in N forms in incubation flasks during pure culture growth.

Species	Collection Name	Without Acetylene		With Acetylene	
		Nitrate Consumption % of Added Nitrate	N_2O Accumulation % of Consumed Nitrate	Nitrate Consumption% of Added Nitrate	N_2O Accumulation% of Consumed Nitrate
E. meliloti	2011	62	0.4 (low)	14	34 (high)
R. legumin. bv. trifolii	T117	100	0 (very low)	0	0 (very low)
B. lupini	LL13	100	100 (very high)	NT	NT
R. legumin. bv. viciae	FH34	0	0 (very low)	NT	NT
R. legumin. bv. viciae	FS16	32	0 (very low)	2	0 (very low)
B. diazoefficiens	G49	100	0 (very low)	100	100 (very high)
B. diazoefficiens	USDA 110	94	0 (very low)	26	100 (very high)
B. japonicum	USDA 138	96	84 (very high)	NT	NT
B. sp. *arachis*	IC7017	0	0 (very low)	NT	NT
B. sp. *arachis*	ST2	100	0 (very low)	71	100 (very high)

NT: not tested.

3.4. Greenhouse Phenotypic Characterization on Living Rhizobia-Legume Associations

The change over time of N_2O concentration in airtight systems was variable amongst the different legume–rhizobia associations tested (Figure 2). Although the results for the couples soybean–G49, which was able to reduce N_2O, and soybean–USDA138, which was unable to reduce N_2O, were confirmed during this experiment, the level of N_2O reduction was very variable amongst the six legume–rhizobia associations that were tested. For four associations, at least one strain was observed to be clearly able to reduce N_2O when growing

with the plant: (i) the already known soybean–G49 association and, as novelties, (ii) the clover–T117 association, (iii) the faba bean–FS16 association, and (iv) the lupin–LL200 association. Indeed, N_2O reduction was rapid for the G49–soybean and T117–clover associations since the N_2O concentration in the airtight system of these associations was significantly lower ($p < 0.05$) than that of their corresponding non-inoculated legume directly from the second gas sampling, i.e., 6 to 7 h after the beginning of incubation. The quantitative difference rapidly exceeded our precision limit. For FS16–faba bean and LL200–lupin associations, the N_2O concentration in the airtight system was significantly lower than that of their non-inoculated legume ($p = 0.003$) only for the last sampling, while the difference in N_2O concentration appeared large, reaching 0.55 and 1 $\mu L.L^{-1}$, respectively. Although the N_2O reduction rate appeared low for ST2 (Figure 2), statistical tests applied on N_2O concentration revealed that this strain has an A-N2OR$^+$ phenotype, with significant differences appearing also from the second sampling with low but clearly detectable activity in our experimental conditions, reaching 0.45 $\mu L\ L^{-1}$ for the last sampling. This is in accordance with the detection of the *nosZ* gene in this strain.

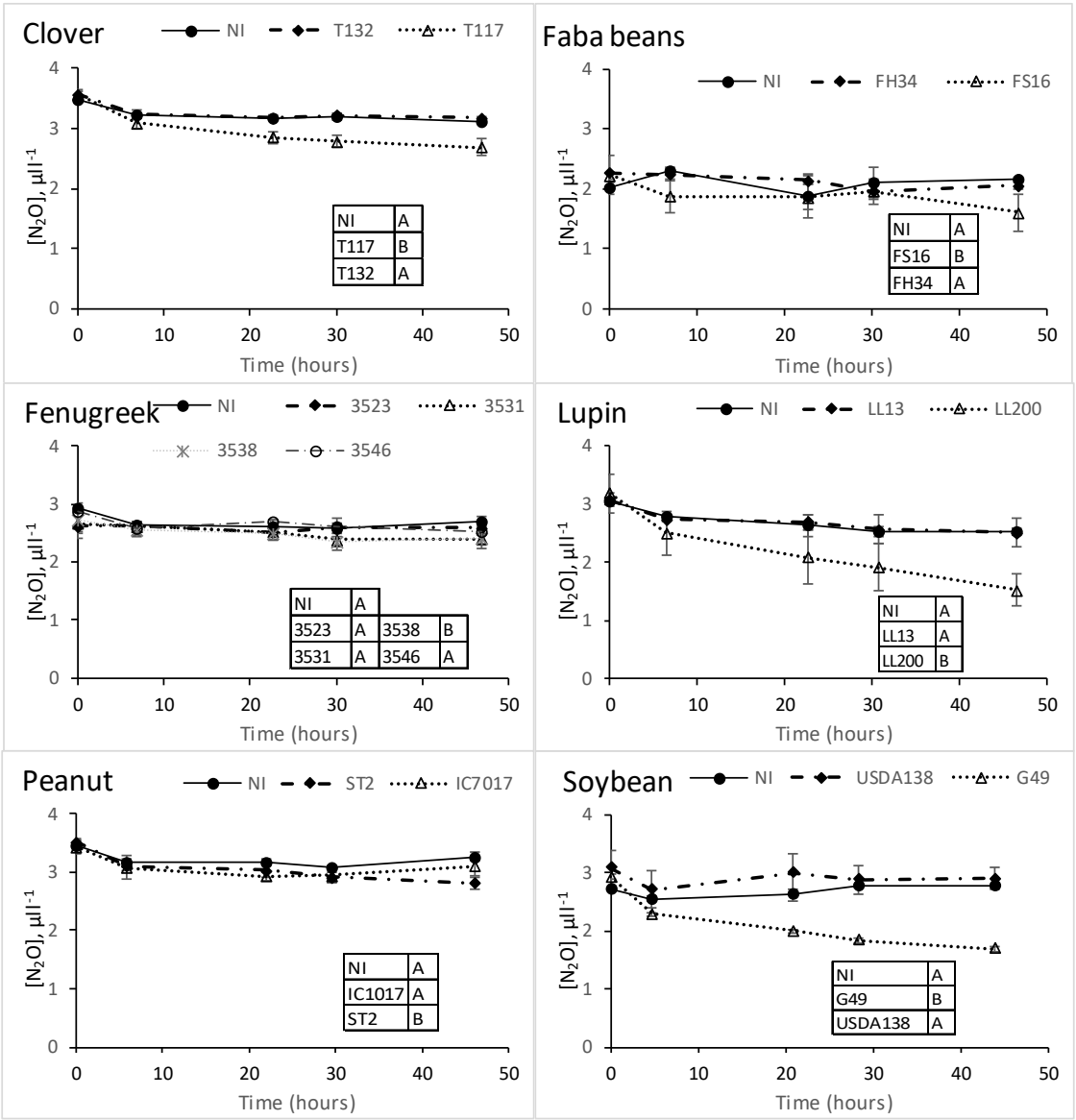

Figure 2. Changes in N_2O concentration over time for different intact leguminous–strain couples incubated in an airtight system enriched with N_2O and statistical differences (Newman and Keuls test $p < 0.05$) between inoculation treatments at the last incubation time.

As far as the fenugreek legume crop is concerned, despite the fact that four strains were tested, we did not clearly obtain any clear positive answer for any of them. A significant signal was obtained with 3538 (p = 0.023) at the last sampling, but the final N_2O concentration difference between chambers with 3538 and those from non-inoculated fenugreek corresponded to our precision limit (0.3 µL L^{-1}). We then considered its phenotype as A-$N_2OR^{+/-}$ and that more investigations will be required on this strain before envisaging its use on the field scale. All the results are summarized in Table 4.

Table 4. Genotypic and phenotypic determination of the strains.

Genotype			Phenotypic Characterization			
				Strain in Symbiosis with Its Host Plant		
Species (Abbreviated)	**Collection Name**		**Strain in Growth Medium with Anaerobic Conditions**	**Host Plant**	**Varieties**	**Efficient Reduction of N_2O Observed on Living Inoculated Plants**
E. meliloti	2011	*nosZ*$^+$	C-N2OR$^+$			NT
R. legumin. trif.	T132	*nosZ*$^-$	NT	Clover	HUIA	A-N2OR$^-$
R. legumin. trif.	T117	*nosZ*$^+$	NNC*	Clover	HUIA	A-N2OR$^+$
B. lupini	LL13	*nosZ*$^-$	CN2OR$^-$	Lupin	LUBLANC	A-N2OR$^-$
B. sp. (*Lup.*)	LL200	*nosZ*$^+$	NT	Lupin	LUBLANC	A-N2OR$^+$
R. legumin. vic.	FH34	*nosZ*$^-$	NNC	Faba beans	EXPRESSO	A-N2OR$^-$
R. legumin. vic.	FS16	*nosZ*$^+$	NNC*	Faba beans	EXPRESSO	A-N2OR$^-$
B. diaz.	G49	*nosZ*$^+$	C-N2OR$^+$	Soybean	NAVIGATOR	A-N2OR$^+$
B. diaz.	USDA 110	*nosZ*$^+$	C-N2OR$^+$	NT		
B. japon.	USDA 138	*nosZ*$^-$	C-N2OR$^-$	Soybean	NAVIGATOR	A-N2OR$^-$
E. sp. (*Trigon.*)	3523	*nosZ*$^+$	NT	Fenugreek	HANKA	A-N2OR$^-$
E. sp. (*Trigon.*)	3531	*nosZ*$^+$	NT	Fenugreek	HANKA	A-N2OR$^-$
E. sp. (*Trigon.*)	3538	*nosZ*$^+$	NT	Fenugreek	HANKA	A-N2OR+/$^-$
E. sp. (*Trigon.*)	3546	*nosZ*$^+$	NT	Fenugreek	HANKA	A-N2OR$^-$
B. sp. (*Arachis*)	IC7017	*nosZ*$^-$	NNC	Peanut	Unknown	A-N2OR$^-$
B. sp. (*Arachis*)	ST2	*nosZ*$^+$	C-N2OR$^+$	Peanut	Unknown	A-N2OR$^+$

NT: not tested; NNC: no nitrate consumption was observed; NNC*: no nitrate consumption was observed in the presence of C_2H_2.

4. Discussion

While the use of rhizobial inoculants appears to be a potentially interesting option to mitigate N_2O emissions, further demonstrations and quantifications of their benefits at the field scale remain necessary for their deployment. Nevertheless, this kind of demonstration, which (i) requires multidisciplinary skills (inoculation of strains, field N_2O measurements), (ii) requires access to fields with a naturally low N_2O reduction rate, and (iii) is subject to in situ hazards (climate, pest attacks, etc.), is difficult to complete. Despite such in situ hazards during our field experiment, we nevertheless obtained clear evidence that the use of the *nosZ*$^+$ strain G49 mitigates N_2O emission during the growing period of soybean crops. Although emissions were obviously not very high in the unfertilized crop conditions, the abatement obtained was as high as 70%, in accordance with previous assessments [9]. As the G49 strain is already known to be efficient for N fixation [29], this study further demonstrated the interest of using it to mitigate soil N_2O emissions on the field scale. The agreement between the observed and calculated abatement validated our understanding of the process on the field scale, while the calculated abatement was based on specific N_2O reduction rates and the mass of nodules determined during laboratory, greenhouse, and field experiments [9]. The other field trials dealing with the use of rhizobial strains to reduce soil N_2O emissions were performed in Japan and covered 10] both the growing and the post-growth period. Itakura et al. [6] observed field N_2O reduction rates exceeding 60% even during the post-harvest period with the Tsu125 (*nosZ*$^+$) strain, while a reduction rate close to 55% was observed both during the growing and the post-harvest periods with the mutant 5M09 (*nosZ*$^{++}$) strain. Akiyama et al. [10] obtained a significant reduction rate during the post-harvesting period of around 30%, by inoculation with a mixed culture of indigenous *nosZ*$^+$ strains of the *B. diazoefficiens* USDA110 group isolated from Japanese fields. While these authors finally concluded that isolating *nosZ*$^+$ strains from local soybean

fields would be more applicable and feasible than generating mutants, both the isolated *nosZ*$^+$ strains from the local field approach and the screening of efficient strains for N fixation and N_2O reduction among culture collections, such as the G49 strain, remains of great interest.

After having obtained the in situ proof of concept that the inoculation of a *nosZ*$^+$ soybean nodulating *Bradyrhizobium* allows reducing N_2O emissions, we developed a multi-scale approach (genotypic and phenotypic) to screen strains from culture collection and then detected legume–rhizobia associations capable of reducing N_2O on the field scale. Figure 3 summarizes (i) the conceptual scheme for selecting efficient strains for both nitrogen fixation and N_2O reduction, including the final step of in situ agronomic mitigation of N_2O emission, and (ii) the outcome of this process for the different legume species and rhizobial strains tested in this study.

Soybean - G49 - USDA 138	Clear signal of a positive strain (G49) and of a negative one (USDA138)	Clear signal of a positive strain (G49) and of a negative one (USDA138)	Clear signal of a positive strain (G49) and of a negative one (USDA138)	Clear signal of in situ mitigation by the use of G49
Clover - T117 - T132	Clear signal of a positive strain (T117) and of a negative one (T132)	Difficulty to grow in pure culture	Clear signal of a positive strain (T117) and of a negative one (T132)	Ready to field experiment
Lupin - LL13 - LL200	Clear signal of a positive strain (LL200) and of a negative one (LL13)	LL200 not tested LL13 exhibiting a negative signal	Clear signal of a positive strain (LL200) and of a negative one (LL13)	Ready to field experiment
Faba bean - FH34 - FS16	Clear signal of a positive strain (FS16) and of a negative one (FH34)	Difficulty to grow in pure culture	Weak signal of a positive strain and of a negative one	Not ready for field experiment : - To test some other strains inside the collection - To test under some other greenhouse conditions
Fenugreek - 3523 - 3531 - 3538 - 3546	Clear signal of 4 positive strains	Not tested	No clear signal of any positive strains	Not ready for field experiment : - To test some other strains inside the collection - To test under some other greenhouse conditions
Peanut - IC7017 - ST2	Clear signal of a positive strain (ST2) and of a negative one (IC7017)	Clear signal of a positive result (ST2) Difficulty to grow in pure culture	Clear signal of a positive strain and of a negative one	Ready to field experiment

Figure 3. Chart of the process of selecting rhizobial strains that could reduce N_2O in the framework of in situ field mitigation of N_2O emissions (concept and application to our study).

The genotypic characterization of strains, now based on robust tools, as revealed by the consistency of the results obtained, is essential in the process proposed, making it possible to choose the strains that will be tested in the greenhouse. To date, we have detected several *nosZ*$^+$ strains in all the rhizobial species tested. This might be explained by the use of "universal" degenerated *nosZ* primers targeting potentially most Proteobacteria. While *nosZ* genes were already described in *Bradyrhizobium* and *Ensifer* species, we described for the first time, to our knowledge, the occurrence of functional *nosZ* genes in *Rhizobium leguminosarum* bv. *trifolii* and bv. *viciae*. Indeed, to date, the only evidence of *nosZ* occurrence is reported from the complete genome of one strain of *R. leguminosarum* bv. *trifoli*i, strain WSM597 (UniProt database). Since *R. leguminosarum* bv. *viciae* is the symbiont of pea, which is a major crop worldwide, further selection of strains of this species that are efficient for biological nitrogen fixation and N_2O reduction is of particular interest for inoculant production. More generally, rhizobial strains used in inoculants should be selected for their nitrogen fixation efficiency, but also, if possible, for their capacity to reduce nitrous oxide. The detection of *nosZ*$^-$ strains is also important since they can be used as controls during phenotypic assessment. However, sequence variability might hamper in certain cases the detection of *nosZ* sequences in some bacterial species or strains, in which case, only phenotypic methods can be used. The phenotypic step performed on pure culture

grown anaerobically gave further information on the physiology of the strains. This step allowed rapid confirmation that *nosZ* genes detected by the genotypic approach might be functional—pure culture tests are obviously more rapid when compared to plant tests. For the strains growing in these conditions, the results obtained were always consistent with the *nosZ* gene status (namely strains 2011, LL13, G49, USDA110, USDA138, ST2). For other strains (strains FH34, IC1017), we suspected poor growth [28] even with a preadaptation period [16] and, surprisingly, we observed that the growth of some strains was affected by acetylene (T117, FS16). However, we are not able to explain these observations at present. In line with the study of Mahne and Tiedje, [30], dedicated to the identification of denitrifiers, we have proposed a priori ranges of N_2O production for defining the N_2O reduction phenotype of strains grown as pure culture (PC) on growing medium. These ranges appeared relevant and could be used as a general guideline for further studies. Finally, the essential phenotypic step performed in planta in the greenhouse on the legume–rhizobia associations validated the activity of N_2O reductase before carrying out the in situ step. While all the associations exhibiting the phenotype A-N2OR$^+$ (i.e., T117, LL200, G49, ST2) were consistently composed with a *nosZ*$^+$ strain, none of the couples composed with fenugreek were clearly observed to reduce N_2O, although strains harbor a *nosZ*$^+$ genotype. Our experimental conditions were probably not adapted for the expression of N_2O reduction for these associations, and this point must be understood before organizing a field test for fenugreek associations. Currently, we do not know the extent to which the plant variety can affect the N_2O reduction phenotype of the association. The question is also raised if research should seek new *nosZ*$^+$ strains of *Ensifer* sp., which are also A-N2OR$^+$, with fenugreek plants. Therefore, we consider that currently, clover–T117, lupin–LL200, and peanut–ST2 associations are ready to be tested in field conditions. However, further investigations are required for associations with faba bean (FS16) and fenugreek (3523, 3531, 3546, and even 3538) since the gene *nosZ* was detected in these strains. The association cowpea–Ac70c tested by Woliy et al. [31] could probably join the first list.

The surface areas dedicated to soybean cropping are considerable worldwide, based either on natural or inoculated symbiosis. In accordance with the conclusions of Bakken and Frostegård [4], who suggested favoring the use of N_2O-reducing strains for the production of legume inoculants, we strongly recommend using *nosZ*$^+$ strains since N_2O reduction does not affect N fixation [26] and allows N_2O emission mitigation. While previous studies have shown variable efficiency of N_2O reduction rates for *nosZ*$^+$ strains hosted by soybean, strains must be tested for this property before application. To our knowledge, the G49 currently appears to be the most efficient amongst the *Bradyrhizobia* tested [9]. Therefore, commercial inoculants containing G49 belonging to *B. diazoefficiens* species (like those used in France) are likely to reduce N_2O emission from soils cropped with soybean. On the contrary, inoculants based on *nosZ*$^-$ *B. japonicum* such as USDA138 would not be able to do so. This fact was also reported for soybean inoculation in South America [11,32], where the inoculant strains used are *nosZ*$^-$ and are unable to reduce N_2O. Nevertheless, in situations with natural nodulating populations, more investigations are required on the competitiveness of selected strains in order to obtain a successful inoculation (i.e., the inoculant strain occupying a significant proportion of nodules formed on the roots). To date, the association lupin–LL200, as efficient as the soybean–G49 association during the greenhouse experiment, also presents genuine potential for further application. The association clover–T117 with its specific application for grassland could also be of great interest.

5. Conclusions

While the use of rhizobial inoculants to mitigate N_2O emissions has already been presented as an emerging option for mitigating N_2O emissions from food production, this study presents an in situ proof of concept of this phenomenon in a soybean crop, by demonstrating an N_2O emission abatement of 70% when using the *nosZ*$^+$ G49 strain compared to the *nosZ*$^-$ USDA138 strain, in association with soybean. This study also clearly

demonstrated that this mitigation option might be considered not only for soybean but also for other leguminous crops, with priority currently given to lupin due to the potential of the lupin–LL200 association. The clear demonstration of the capacity of N_2O reduction by the clover–T117 association suggests that the opportunity for mitigation might also be possible for grassland. Further field studies should be carried out to confirm the mitigation potential of these legumes associated with nitrogen fixing and N_2O-reducing strains.

Author Contributions: All authors contributed to the conceptualization, methodology, investigation, and writing of this manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Conseil Regional Centre Val de Loire through the PUIGES project, the Conseil Régional de Bourgogne through the SYMGES project, and the programme "Investissement d'Avenir", ISITE-BFC (contract ANR-15-IDEX-0003) through the NatAdGES project.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank A.S. Ferré, V. Bienvenue, H. Gaillard, A. Ayzac, D. Laloua, and C. Le Lay for their help during the experiments.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. UNEP. *Drawing Down N2O to Protect Climate and the Ozone Layer*; A UNEP Synthesis Report; United Nations Environment Programme (UNEP): Nairobi, Kenya, 2013.
2. Butterbach-Bahl, K.; Baggs, E.; Dannenmann, M.; Kiese, R.; Zechmeister-Boltenstern, S. Nitrous oxide emissions from soils: How well do we understand the processes and their controls. *Philos. Trans. R. Br. Soc.* **2013**, *368*, 20130122. [CrossRef] [PubMed]
3. Hénault, C.; Bourennane, H.; Ayzac, A.; Ratié, C.; Saby, N.; Cohan, J.P.; Eglin, T.; Gall, C.L. Management of soil pH promotes nitrous oxide reduction and thus mitigates soil emissions of this greenhouse gas. *Sci. Rep.* **2019**, *9*, 20182. [CrossRef]
4. Bakken, L.R.; Frostegård, Å. Emerging options for mitigating N_2O emissions from food production by manipulating the soil microbiota. *Curr. Opin. Environ. Sustain.* **2020**, *47*, 89–94. [CrossRef]
5. Mania, D.; Woliy, K.; Degefu, T.; Frostegård, Å. A common mechanism for efficient N_2O reduction in diverse isolates of nodule-forming bradyrhizobia. *Environ. Microbiol.* **2020**, *22*, 17–31. [CrossRef]
6. Itakura, M.; Uchida, Y.; Akiyama, H.; Hoshino, Y.T.; Shimomura, Y.; Morimoto, S.; Tago, K.; Wang, Y.; Hayakawa, C.; Uetake, Y.; et al. Mitigation of nitrous oxide emissions from soils by *Bradyrhizobium japonicum* inoculation. *Nat. Clim. Chang.* **2013**, *3*, 208–212. [CrossRef]
7. Stagnari, F.; Maggio, A.; Galieni, A.; Pisante, M. Multiple benefits of legumes for agriculture sustainability: An overview. *Chem. Biol. Technol. Agric.* **2017**, *4*, 2. [CrossRef]
8. Sameshima-Saito, R.; Chiba, K.; Hirayama, J.; Itakura, M.; Mitsui, H.; Eda, S.; Minamisawa, K. Symbiotic Bradyrhizobium japonicum Reduces N_2O Surrounding the Soybean Root System via Nitrous Oxide Reductase. *Appl. Environ. Microbiol.* **2006**, *72*, 2526–2532. [CrossRef] [PubMed]
9. Hénault, C.; Revellin, C. Inoculants of leguminous crops for mitigating soil emissions of the greenhouse gas nitrous oxide. *Plant Soil* **2011**, *346*, 289–296. [CrossRef]
10. Akiyama, H.; Hoshino, Y.; Itakura, M.; Shimomura, Y.; Wang, Y.; Yamamoto, A.; Tago, K.; Nakajima, Y.; Minamisawa, K.; Hayatsu, M. Mitigation of soil N_2O emission by inoculation with a mixed culture of indigenous Bradyrhizobium diazoefficiens. *Sci. Rep.* **2016**, *6*, 32869. [CrossRef]
11. Obando, M.; Antonelli, C.; Casanave, S.; Maguire, V.; Torres, D.; Pérez, G.; Bailleres, M.; Donadio, F.; Creus, C.; Videla, C.; et al. Evaluation of nitrous oxide emission by soybean inoculated with Bradyrhizobium strains commonly used as inoculants in South America. *Plant Soil.* **2022**. [CrossRef]
12. Vincent, J.M. *A Manual for the Practical Study of Root Nodule Bacteria (Vol. IBP Handbook no. 15)*; Blackell Scientific Publication: Oxford, UK, 1970.
13. Beringer, J.E.R. factor transfer in *Rhizobium leguminosarum*. *J. Gen. Microbiol.* **1974**, *84*, 188–198. [CrossRef] [PubMed]
14. Henry, S.; Bru, D.; Stres, B.; Hallet, S.; Philippot, L. Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and *nosZ* genes in soils. *Appl. Environ. Microbiol.* **2006**, *72*, 5181–5189. [CrossRef] [PubMed]
15. Scala, D.J.; Kerkhof, L.J. Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments. *FEMS Microbiol. Lett.* **1998**, *162*, 61–68. [CrossRef]
16. Velasco, L.; Mesa, S.; Xu, C.; Delgado, M.J.; Bedmar, E.J. Molecular characterization of nosRZDFYLX genes coding for denitrifying nitrous oxide reductase of *Bradyrhizobium japonicum*. *Antonie Van Leeuwenhoek* **2004**, *85*, 229–235. [CrossRef] [PubMed]

17. Torres, M.J.; Rubia, M.I.; de la Peña, T.C.; Pueyo, J.J.; Bedmar, E.J.; Delgado, M.J. Genetic basis for denitrification in *Ensifer meliloti*. *BMC Microbiol.* **2014**, *14*, 142. [CrossRef]
18. Revellin, C.; Pinochet, X.; Beauclair, P.; Catroux, G. Influence of soil properties and soya bean cropping history on the Bradyrhizobium japonicum population in some French soils. *Eur. J. Soil Sci.* **1996**, *47*, 505–510. [CrossRef]
19. Hutchinson, G.L.; Livingston, G.P. Use of chamber systems to measure trace gas fluxes. In *Agricultural Ecosystem Effects on Trace Gases and Global Climate Change*; Rolston, D.E., Harper, L.A., Mosier, A.R., Duxbury, J.M., Eds.; ASA Spec. Publ. 55; ASA, CSSA and SSSA: Madison, WI, USA, 1993; pp. 63–78.
20. Amarger, N. Selection of *Rhizobium* strains on their competitive ability for nodulation. *Soil Biol. Biochem.* **1981**, *13*, 481–486. [CrossRef]
21. Mazurier, S.I. Diversité de Populations Naturelles Nodulantes de *Rhizobium leguminosarum*. Ph.D. Thesis, Université Claude Bernard Lyon 1, Lyon, France, 1989.
22. Laguerre, G.; Geniaux, E.; Mazurier, S.I.; Casartelli, R.R.; Amarger, N. Conformity and diversity among field isolates of *Rhizobium leguminosarum bv. viciae*, *bv. trifolii*, and *bv. phaseoli* revealed by DNA hybridization using chromosome and plasmid probes. *Can. J. Microbiol.* **1993**, *39*, 412–419. [CrossRef]
23. Lagacherie, B.; Hugot, R.; Amarger, N. Selection de souches de *Rhizobium japonicum* d'après leur compétitivité pour l'infection. *Ann. Agron.* **1977**, *28*, 379–389.
24. Rochette, P.; Eriksen-Hamel, N.S. Chamber Measurements of Soil Nitrous Oxide Flux: Are Absolute Values Reliable? *Soil. Sc. Soc. Am. J.* **2008**, *72*, 331–342. [CrossRef]
25. Cheneby, D.; Hartmann, A.; Hénault, C.; Topp, E.; Germon, J.C. Diversity of denitrifying microflora and ability to reduce N_2O in two soils. *Biol. Fert. Soils.* **1998**, *28*, 19–26. [CrossRef]
26. Castaldo, D.A.; Harroon, M.; Schrader, L.E.; Youngs, V.L. Rapid colorimetric determination of nitrate in plant-tissue by nitration of salycilic acid. *Commun. Soil Sci. Plant Anal.* **1975**, *6*, 71–80. [CrossRef]
27. Yoshinari, T.; Hynes, R.; Knowles, R. Acetylene inhibition of nitrous oxide reduction and measurement of denitrification and nitrogen fixation in soil. *Soil Biol. Biochem.* **1977**, *9*, 177–1833. [CrossRef]
28. Green, S.J.; Prakash, O.; Gihring, T.M.; Akob, D.M.; Jasrotia, P.; Jardine, P.M.; Watson, D.B.; Brown, S.D.; Palumbo, A.V.; Kostka1, J.E. Denitrifying Bacteria Isolated from Terrestrial Subsurface Sediments Exposed to Mixed-Waste Contamination. *Appl. Environ. Microbiol.* **2010**, *76*, 3244–3254. [CrossRef] [PubMed]
29. Catroux, G. Inoculant quality standards and controls in France. In *Expert Consultation on Legume Inoculant Production and Quality Control*; Thompson, V.A., Ed.; FAO: Rome, Italy, 1991; Volume 19–21, pp. 113–120.
30. Mahne, I.; Tiedje, J.M. Criteria and methodology for identifying respiratory denitrifiers. *Appl. Environ. Microbiol.* **1995**, *61*, 1110–1115. [CrossRef]
31. Woliy, K.; Degefu, T.; Frostegard, A. Host Range and Symbiotic Effectiveness of N_2O Reducing Bradyrhizobium Strains. *Front. Microbiol.* **2019**, *10*, 10. [CrossRef]
32. Obando, M.; Correa-Galeote, D.; Castellano-Hinojosa, A.; Gualp, J.L.; Hidalgo, A.; Alche, J.; Bedmar, E.J.; Cassan, F.D. Analysis of the denitrification pathway and greenhouse gases emissions in *Bradyrhizobium* sp. strains used as biofertilizers in South America. *J. Appl. Microbiol.* **2019**, *127*, 739–749. [CrossRef]