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► **To cite this version:**

Catherine Hénault, Elodie Barbier, Alain Hartmann, Cécile Revellin. New Insights into the Use of Rhizobia to Mitigate Soil N<sub>2</sub>O Emissions. *Agriculture*, 2022, 12 (2), 10.3390/agriculture12020271 . hal-03604574

**HAL Id: hal-03604574**

**<https://hal.inrae.fr/hal-03604574>**

Submitted on 29 Aug 2023

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## Article

# New Insights into the Use of Rhizobia to Mitigate Soil N<sub>2</sub>O Emissions

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**Abstract:** Agriculture is a major anthropogenic source of the greenhouse gas N<sub>2</sub>O, 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<sub>2</sub>O emissions, this study presents an in situ abatement of 70% of soil N<sub>2</sub>O emission using the strain *nosZ*<sup>+</sup> G49 vs. *nosZ*<sup>-</sup> 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*<sup>+</sup> LL200 strain. The clear demonstration of the N<sub>2</sub>O reduction capacity of clover symbiotic strains suggests that opportunities for mitigation might also occur on grassland.

**Keywords:** N<sub>2</sub>O mitigation; rhizobia; legumes; *nosZ* gene; phenotypes; multiscale approach



**Citation:** Hénault, C.; Barbier, E.; Hartmann, A.; Revellin, C. New Insights into the Use of Rhizobia to Mitigate Soil N<sub>2</sub>O 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

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## 1. Introduction

Agriculture, through soil emissions, is a major anthropogenic source of the greenhouse gas N<sub>2</sub>O, which has a Global Warming Potential about 300 times higher than CO<sub>2</sub> on a molar basis, and which is also involved in stratospheric ozone depletion [1]. In soils, N<sub>2</sub>O is mainly produced through the microbial processes of denitrification and nitrification [2]. The last step of the denitrification process, N<sub>2</sub>O reduction into N<sub>2</sub>, is currently the only known pathway for the terrestrial removal of N<sub>2</sub>O. N<sub>2</sub>O reduction is catalyzed by the N<sub>2</sub>O reductase enzyme (Nos) encoded by the *nosZ* gene. Strategies to mitigate N<sub>2</sub>O emissions from agricultural soil could be based on promoting the reduction of N<sub>2</sub>O into N<sub>2</sub> in soils [3]. Recently, Bakken and Frostegård [4] reviewed emerging options for mitigating N<sub>2</sub>O emissions from food production by manipulating the soil microbiota. Amongst these options, the use of rhizobial inoculants to mitigate N<sub>2</sub>O emissions was underlined. These authors also specified that Bradyrhizobia may be stronger N<sub>2</sub>O sinks than many other fully fledged denitrifying bacteria, because their nitrate reductase (Nap) competes poorly with Nos, resulting in the preferential reduction of N<sub>2</sub>O over that of nitrate [5]. Moreover, the N<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>O emissions; and (ii) to analyze the genotypic and phenotypic characteristics of the N<sub>2</sub>O 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 *nosZ* 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 *nosZ* gene [16,17].

### 2.3. In Situ N<sub>2</sub>O Fluxes Measurements

An experimental block trial involving the N<sub>2</sub>O-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<sub>2</sub>O [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<sub>2</sub>O 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
<i>Ensifer meliloti</i> MIAE00427 (MSDJ848, RCR2011, <b>2011</b> )	<i>Medicago sativa</i> (alfalfa)	[20]/RCR
<i>Rhizobium leguminosarum</i> <i>bv.</i> <i>trifolii</i> MIAE03027 (MSDJ134, <b>T117</b> )	<i>Trifolium pratense</i> (clover)	[21]
MIAE03034 (MSDJ141, <b>T132</b> )	<i>Trifolium pratense</i> (clover)	[21]
<i>Bradyrhizobium lupini</i> MIAE00428 (MSDJ718, <b>LL13</b> )	<i>Lupinus luteus</i> (lupine)	[22]
<i>Bradyrhizobium</i> sp. ( <i>Lupinus</i> ) MIAE00428 (MSDJ718, <b>LL200</b> )	<i>Lupinus luteus</i> (lupine)	[20]
<i>Rhizobium leguminosarum</i> <i>bv.</i> <i>viciae</i> MIAE01211 (MSDJ822, <b>FH34</b> )	<i>Vicia faba</i> (faba bean)	[20]
MIAE01211 (MSDJ822, <b>FS16</b> )	<i>Vicia faba</i> (faba bean)	[20]
<i>Bradyrhizobium diazoefficiens</i> MIAE00426 (MSDJ1996, <b>G49</b> )	<i>Glycine max</i> (soybean)	[23]
<b>USDA 110</b>	<i>Glycine max</i> (soybean)	USDA
<i>Bradyrhizobium japonicum</i> <b>USDA138</b>	<i>Glycine max</i> (soybean)	USDA
<i>Ensifer</i> sp. ( <i>Trigonella</i> ) MIAE06325 (MSDJ3523, <b>3523</b> )	<i>Trigonella foenum-graecum</i> (fenugreek)	MIAE
MIAE06333 (MSDJ3531, <b>3531</b> )	<i>Trigonella foenum-graecum</i> (fenugreek)	MIAE
MIAE01335 (MSDJ3538, <b>3538</b> )	<i>Trigonella foenum-graecum</i> (fenugreek)	MIAE
MIAE06345 (MSDJ3546, <b>3546</b> )	<i>Trigonella foenum-graecum</i> (fenugreek)	MIAE
<i>Bradyrhizobium</i> sp. <i>Arachis</i> MIAE06284 (MSDJ3482, <b>IC7017</b> )	<i>Arachis hypogaea</i> (peanut)	ICRISAT
MIAE04980 (MSDJ2177, <b>ST2</b> )	<i>Arachis hypogaea</i> (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.,  $5 \times 10^6$  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 ( $0.5 \times 0.5$  m<sup>2</sup> 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<sub>2</sub>O 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<sub>2</sub>O was linearly distributed within the chamber, and that the N<sub>2</sub>O concentration of a sample taken at mid-height was representative of the mean concentration within the chamber. N<sub>2</sub>O emission measurements were performed during the evening–night period to avoid any possible (but currently unknown) interactions with photosynthesis, and because the N<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>O at the atmospheric concentration (i.e.,  $0.34 \mu\text{L L}^{-1}$ ) with a precision better than  $0.1 \mu\text{L L}^{-1}$ , as verified with 4 certified bottles between 0.4 and  $1.5 \mu\text{L L}^{-1}$ . These nocturnal fluxes were calculated by the difference (when significant  $p < 0.05$ ) of concentration of N<sub>2</sub>O in each chamber over time between the initial and the final sampling.

While not classical [24], this N<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>OR 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<sup>-1</sup> of KNO<sub>3</sub>. The atmosphere of the tubes sealed with rubber stoppers was removed and replaced with N<sub>2</sub>. After 7 days of incubation at 28 °C with agitation at 150 rpm, we determined the NO<sub>3</sub><sup>-</sup> consumed [26] and the N<sub>2</sub>O produced. N<sub>2</sub>O content was determined on GC3000, (SRA Instruments, Marcy l'Etoile, France), equipped with a TCD detector (detection limit 10 µL.L<sup>-1</sup>) as follows: (i) very low when no production was clearly determined, (ii) low when N<sub>2</sub>O represents between 0.1% and 10% of the consumed NO<sub>3</sub><sup>-</sup>, (iii) high when N<sub>2</sub>O represents between 10% and 50% of the consumed NO<sub>3</sub><sup>-</sup>, and (iv) very high when N<sub>2</sub>O represents between 50% and 100% of the consumed NO<sub>3</sub><sup>-</sup>. Isolates that had consumed NO<sub>3</sub><sup>-</sup> with a low or very low N<sub>2</sub>O content in flasks were then incubated in the same conditions, except with the addition of C<sub>2</sub>H<sub>2</sub> (7%) used to inhibit the N<sub>2</sub>O reductase activity [27]. Strains were identified as C-N<sub>2</sub>OR<sup>+</sup> if they accumulated N<sub>2</sub>O in the high or very high range, only in the presence of C<sub>2</sub>H<sub>2</sub>.

#### 2.5. Characterization of Greenhouse N<sub>2</sub>O Reduction by the Different Legume–Rhizobia Associations (A)

The phenotype determined at this scale is denoted A-N<sub>2</sub>OR. 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<sup>2</sup> with 4 plants per pot. The varieties used were Huia, Lublanc, Espresso, 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  $5 \times 10^6$  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<sup>-1</sup> to verify the air tightness of the chambers by the absence of change in the Kr concentration during incubation. N<sub>2</sub>O was added to set the initial atmosphere chamber at an N<sub>2</sub>O concentration of around 3 µL L<sup>-1</sup> as a trade-off between (i) a screening approach, (ii) the expression of the N<sub>2</sub>O reductase activity increasing with ambient N<sub>2</sub>O concentration [9], and (iii) the N<sub>2</sub>O analyzer detection limit. During the 48 h period, the Kr and N<sub>2</sub>O concentrations in the chambers were periodically determined by gas chromatography, as described previously, with N<sub>2</sub>O concentrations first determined on the GC equipped with the ECD detector (0.3 µL L<sup>-1</sup> precision verified at 3 µL L<sup>-1</sup>) and Kr concentrations then determined on the GC equipped with the TCD detector (detection limit of 100 µL L<sup>-1</sup>). 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<sub>2</sub>O concentrations per leguminous plant at the beginning of incubation, a rhizobia–leguminous couple was considered as an N<sub>2</sub>O reducer (A-N<sub>2</sub>OR<sup>+</sup>) when the

N<sub>2</sub>O concentration at the end of incubation was statistically ( $p < 0.05$ ) and quantitatively (decrease of 0.3  $\mu\text{L L}^{-1}$ ) significantly lower than that of the N<sub>2</sub>O 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.

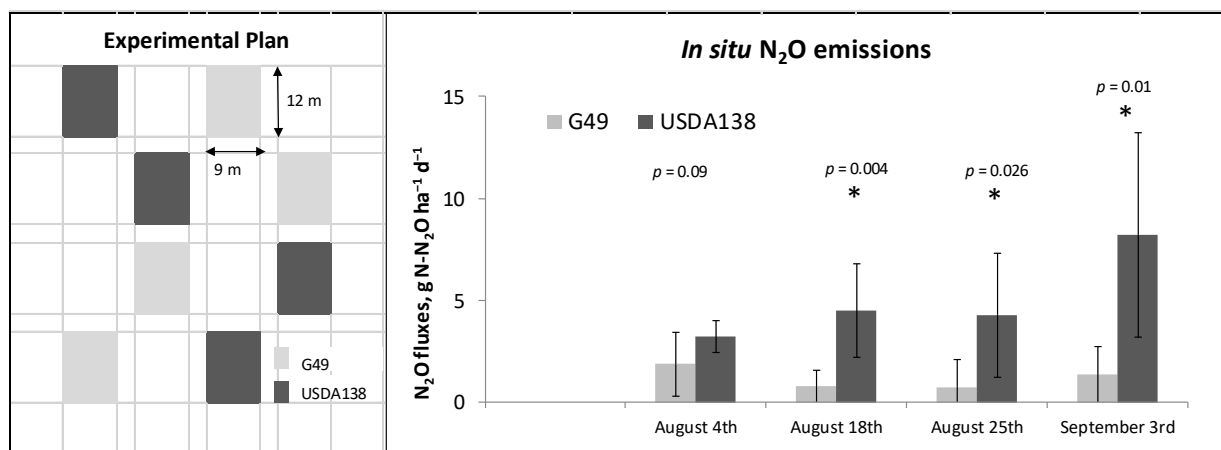
#### 3.2. In Situ Proof of Concept of N<sub>2</sub>O Emission Mitigation by the Soybean–*B. diazoefficiens* G49 Association

During the summer of 2015, the soils' N<sub>2</sub>O emissions were very low on all the measurement dates, systematically lower than 10 g N ha<sup>-1</sup> d<sup>-1</sup> (Figure 1). Nevertheless, N<sub>2</sub>O emissions on plots cultivated with the soybean inoculated with the G49 strain never exceeded 2 g N-N<sub>2</sub>O ha<sup>-1</sup> d<sup>-1</sup>, 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<sub>2</sub>O emission abatement was then estimated as 70% on average, which corresponds exactly to the abatement of soil N<sub>2</sub>O assessed using the soybean–G49 association, as calculated in [9].

**Table 2.** Genotypic characterization of the strains.

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

NT: not tested.



**Figure 1.** *In situ* experiment. Experimental plan and measured N<sub>2</sub>O 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<sub>2</sub>O in flask during incubation and were therefore considered as being unable to reduce N<sub>2</sub>O (C-N<sub>2</sub>OR<sup>-</sup>). Moreover, amongst the strains first observed to consume NO<sub>3</sub><sup>-</sup> 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<sub>2</sub>O only in the presence of C<sub>2</sub>H<sub>2</sub> were clearly shown to be able to reduce N<sub>2</sub>O in pure culture and present the phenotype C-N<sub>2</sub>OR<sup>+</sup>.

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

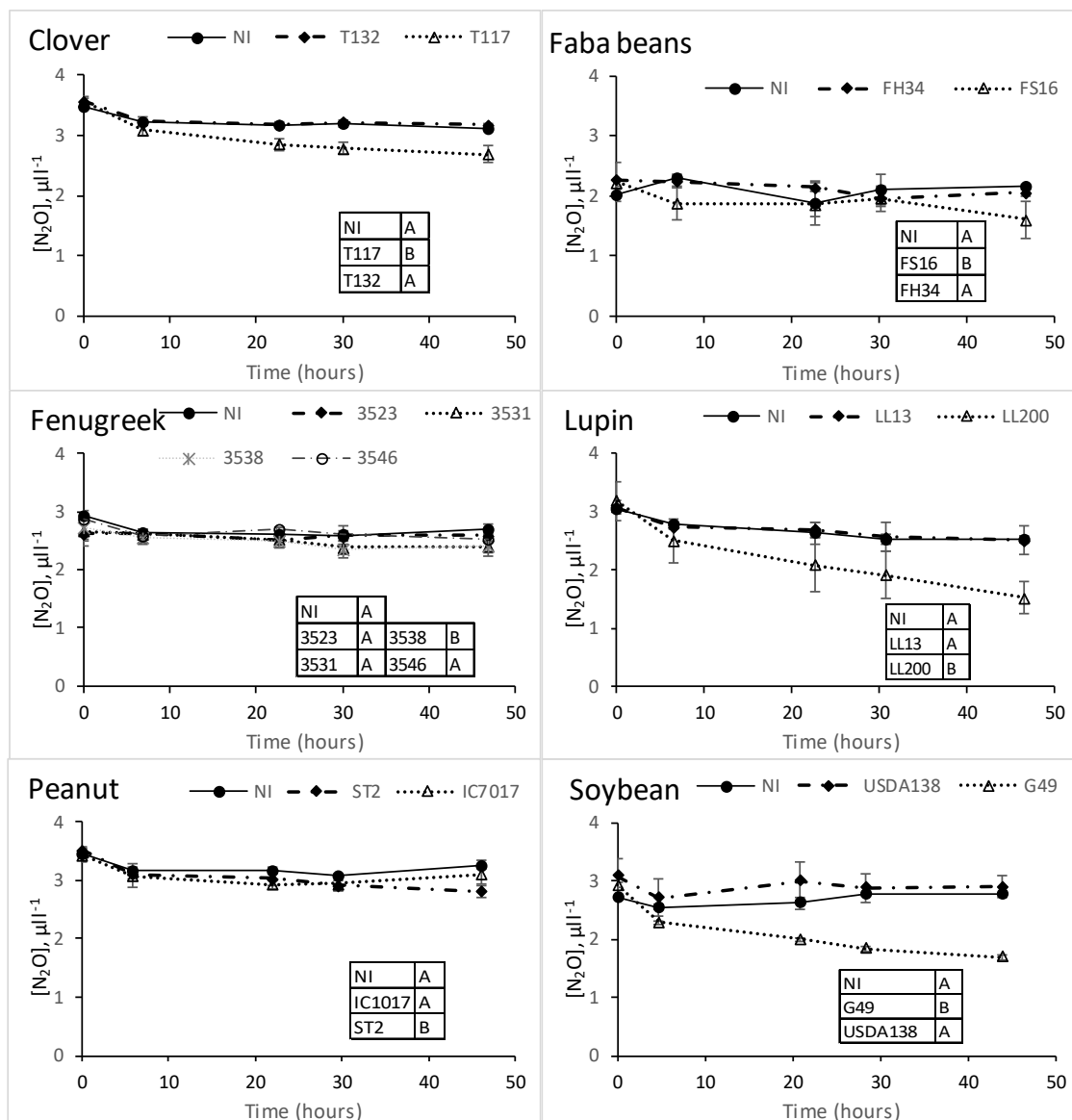
Species	Collection Name	Without Acetylene		With Acetylene	
		Nitrate Consumption % of Added Nitrate	N <sub>2</sub> O Accumulation % of Consumed Nitrate	Nitrate Consumption % of Added Nitrate	N <sub>2</sub> O Accumulation % of Consumed Nitrate
<i>E. meliloti</i>	2011	62	0.4 (low)	14	34 (high)
<i>R. legumin. bv. trifolii</i>	T117	100	0 (very low)	0	0 (very low)
<i>B. lupini</i>	LL13	100	100 (very high)	NT	NT
<i>R. legumin. bv. viciae</i>	FH34	0	0 (very low)	NT	NT
<i>R. legumin. bv. viciae</i>	FS16	32	0 (very low)	2	0 (very low)
<i>B. diazoefficiens</i>	G49	100	0 (very low)	100	100 (very high)
<i>B. diazoefficiens</i>	USDA 110	94	0 (very low)	26	100 (very high)
<i>B. japonicum</i>	USDA 138	96	84 (very high)	NT	NT
<i>B. sp. arachis</i>	IC7017	0	0 (very low)	NT	NT
<i>B. sp. arachis</i>	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<sub>2</sub>O 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<sub>2</sub>O, and soybean–USDA138, which was unable to reduce N<sub>2</sub>O, were confirmed during this experiment, the level of N<sub>2</sub>O 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<sub>2</sub>O 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  $\mu\text{L}\cdot\text{L}^{-1}$ , 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<sup>+</sup> phenotype, with significant differences appearing also from the second sampling with low but clearly detectable activity in our experimental conditions, reaching 0.45  $\mu\text{L}\cdot\text{L}^{-1}$  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 ( $0.3 \mu L L^{-1}$ ). 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.

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

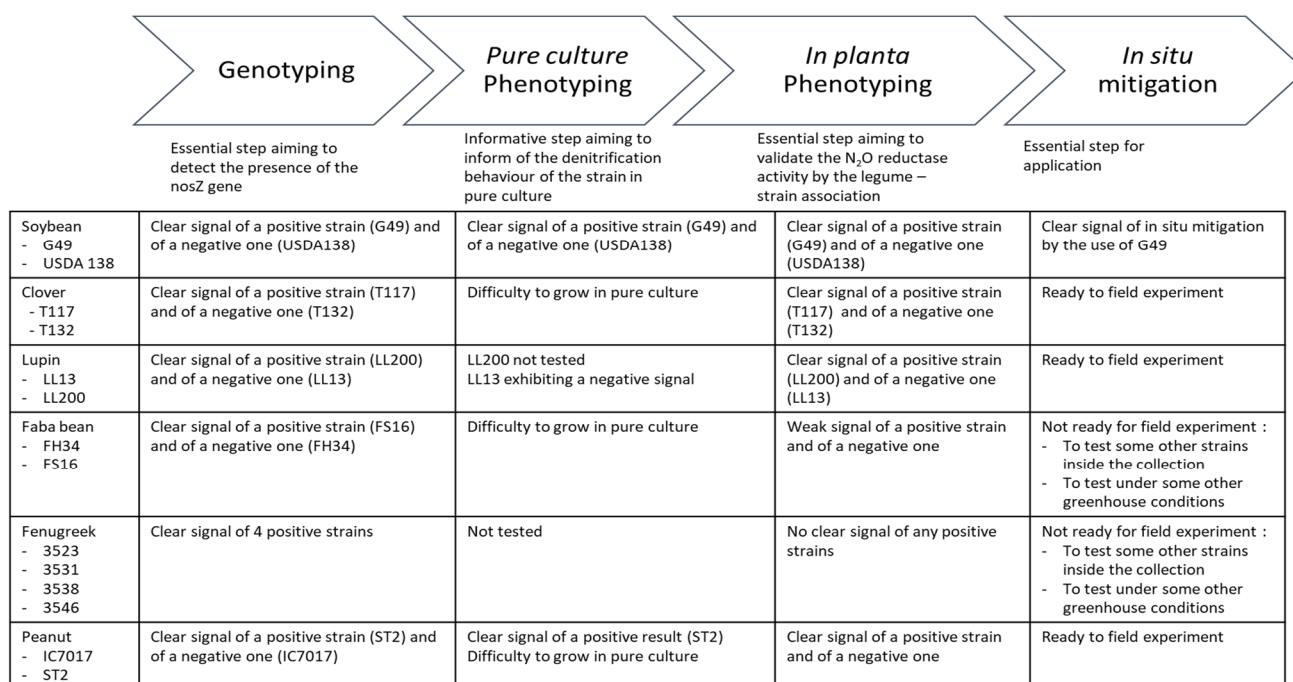
NT: not tested; NNC: no nitrate consumption was observed; NNC\*: no nitrate consumption was observed in the presence of  $C_2H_2$ .

#### 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*<sup>+</sup> 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*<sup>+</sup>) strain, while a reduction rate close to 55% was observed both during the growing and the post-harvest periods with the mutant 5M09 (*nosZ*<sup>++</sup>) 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*<sup>+</sup> strains of the *B. diazoefficiens* USDA110 group isolated from Japanese fields. While these authors finally concluded that isolating *nosZ*<sup>+</sup> strains from local soybean

fields would be more applicable and feasible than generating mutants, both the isolated *nosZ*<sup>+</sup> strains from the local field approach and the screening of efficient strains for N fixation and N<sub>2</sub>O 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*<sup>+</sup> soybean nodulating *Bradyrhizobium* allows reducing N<sub>2</sub>O 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<sub>2</sub>O on the field scale. Figure 3 summarizes (i) the conceptual scheme for selecting efficient strains for both nitrogen fixation and N<sub>2</sub>O reduction, including the final step of in situ agronomic mitigation of N<sub>2</sub>O emission, and (ii) the outcome of this process for the different legume species and rhizobial strains tested in this study.



**Figure 3.** Chart of the process of selecting rhizobial strains that could reduce N<sub>2</sub>O in the framework of in situ field mitigation of N<sub>2</sub>O 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*<sup>+</sup> 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. *trifolii*, 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<sub>2</sub>O 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*<sup>−</sup> 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<sub>2</sub>O production for defining the N<sub>2</sub>O 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<sub>2</sub>O reductase before carrying out the in situ step. While all the associations exhibiting the phenotype A-N<sub>2</sub>O<sup>+</sup> (i.e., T117, LL200, G49, ST2) were consistently composed with a *nosZ*<sup>+</sup> strain, none of the couples composed with fenugreek were clearly observed to reduce N<sub>2</sub>O, although strains harbor a *nosZ*<sup>+</sup> genotype. Our experimental conditions were probably not adapted for the expression of N<sub>2</sub>O 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<sub>2</sub>O reduction phenotype of the association. The question is also raised if research should seek new *nosZ*<sup>+</sup> strains of *Ensifer* sp., which are also A-N<sub>2</sub>O<sup>+</sup>, 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<sub>2</sub>O-reducing strains for the production of legume inoculants, we strongly recommend using *nosZ*<sup>+</sup> strains since N<sub>2</sub>O reduction does not affect N fixation [26] and allows N<sub>2</sub>O emission mitigation. While previous studies have shown variable efficiency of N<sub>2</sub>O reduction rates for *nosZ*<sup>+</sup> 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<sub>2</sub>O emission from soils cropped with soybean. On the contrary, inoculants based on *nosZ*<sup>−</sup> *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*<sup>−</sup> and are unable to reduce N<sub>2</sub>O. 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<sub>2</sub>O emissions has already been presented as an emerging option for mitigating N<sub>2</sub>O emissions from food production, this study presents an in situ proof of concept of this phenomenon in a soybean crop, by demonstrating an N<sub>2</sub>O emission abatement of 70% when using the *nosZ*<sup>+</sup> G49 strain compared to the *nosZ*<sup>−</sup> 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<sub>2</sub>O 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<sub>2</sub>O-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.

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