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Inoculation with Different Nitrogen-Fixing Bacteria and Arbuscular Mycorrhiza Affects Grain Protein Content and Nodule Bacterial Communities of a Fava Bean Crop

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Abstract: The introduction of nitrogen fixing bacteria (NFB) and arbuscular mycorrhizal fungi (AMF) into the soil is an advisable agricultural practice for the crop, since it enhances nutrient and water uptake and tolerance to biotic and abiotic stresses. The aim of this work was to study plant nutrition, biological nitrogen fixation (BNF) and crop yield and quality, after inoculating seeds with NFBs ((*Rhizobium leguminosarum*, *Burkholderia cenocepacia*, *Burkholderia vietnamiensis*)) and/or AMFs (*Rhizophagus irregularis*, *Claroideoglomus etunicatum*, *Claroideoglomus claroideum* and *Funneliformis mosseae*) in a fava bean crop in two seasons. The composition of the nodule bacterial community was evaluated by the high-throughput sequencing analysis of bacterial 16 S rRNA genes. It was found that microbial inoculation accompanied by a 20% decrease in mineral fertilization had no significant effect on crop yield or the nutritional characteristics compared with a non-inoculated crop, except for an increase in the grain protein content in inoculated plants. None of the inoculation treatments increased biological nitrogen fixation over a non-inoculated level. The bacterial rRNA analysis demonstrated that the genus *Rhizobium* predominated in all nodules, both in inoculated and non-inoculated treatments, suggesting the previous presence of these bacteria in the soil. In our study, inoculation with *Rhizobium leguminosarum* was the most effective treatment for increasing protein content in seeds, while *Burkholderia* sp. was not able to colonise the plant nodules. Inoculation techniques used in fava beans can be considered an environmentally friendly alternative, reducing the input of fertilizers, while maintaining crop yield and quality, with the additional benefit of increasing the grain protein content. However, further research is required on the selection and detection of efficient rhizobial strains under local field conditions, above all those related to pH and soil type, in order to achieve superior nitrogen-fixing bacteria.

Keywords: *Vicia faba*; *Rhizobium* sp.; *Burkholderia* sp.; biological nitrogen fixation; bacterial community

1. Introduction

The alarming increase in the world's population means that the need for fertilizers has also grown in an attempt to reach required food production levels. In this context, the application of nitrogen-fixing bacteria (NFB) for plant cultivation is considered one of the most promising methods for improving

the management of nitrogen fertilizers and for increasing agricultural productivity [1], because it may help in achieving (i) a reduction in the external input of fertilizers; (ii) greater efficiency in the way a plant uses N; and (iii) a reduction of N leaching through the soil profile, thus preventing water pollution [2,3].

The N_2-fixing symbiosis between most legumes and bacteria is a well-studied example of nodule formation and their subsequent invasion by specific *Rhizobia* [4]. In this process, *Rhizobia* fix atmospheric N and, in exchange, bacteria receive carbon compounds derived from photosynthesis [5]. In the case of Fabaceae species, phylogenetic studies have demonstrated that most rhizobial species that form nodules belong to alpha-proteobacteria (*Bradyrhizobium, Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*), and beta- and gamma- proteobacteria (*Burkholderia* and *Enterobacter*, respectively) [4,6–10]. In various legumes, besides the rhizobia that are responsible for nodulation and N_2-fixation, other endophytic bacteria, called non-rhizobial bacteria, are also found in the nodules [11]. Some of these have proven beneficial to their legume hosts, as they enhance plant growth by producing plant hormones, fixing atmospheric N_2, and solubilizing phosphate [12]. Currently, the 16 S rRNA gene sequencing technique is commonly used for the identification, classification and quantification of microbes and can be used to shed light on both rhizobial and non-rhizobial bacteria within the complex biological mixture found in nodules.

In addition to rhizobial species, arbuscular mycorrhizal fungi (AMF) also play an important role in uptake of soil nutrients, since they act as phosphate solubilizing microorganisms that are able to mineralize organic P and increase the availability of inorganic P [13], which is considered the least accessible nutrient for plants. This nutrient is related to nodule formation and functioning, and is essential for the action of the nitrogenase enzyme in the biological nitrogen fixation (BNF) process [14,15].

Legumes often need inoculation when they are being grown in areas where they have not been traditionally grown or have not been grown for a long time [16]. In this case, or in the absence of symbiotically linked microorganisms, the inclusion of NFB in the soil is recommended [17]. In general, the use of rhizobial inoculants based on autochthonous strains is advisable, because they have superior characteristics of competitiveness in nodule infection and occupancy and, therefore, superior BNF performance in the field, due to their genetic adaptations to the local environment [18,19]. In turn, plants subjected to tripartite symbiosis (NFB-AMF-legumes), compared with non-inoculated plants or those inoculated with AMF or NFB alone, show benefits that include enhanced growth and crop yield, and an increased phosphorus and nitrogen content [20,21]. In this respect, dual inoculation has been seen to improve nodulation, nitrogenase activity, mycorrhization and nutrient content (N and P) compared with individual inoculation [22,23].

Based on these approaches, we studied the inoculation of fava bean seeds with a combination of NFBs (*Rhizobium leguminosarum*, *Burkholderia cenocepacia*, *Burkholderia vietnamiensis)* and a pool of AMFs to assess their effect on crop yield and quality and the biological quality of soils. We hypothesized that dual inoculation with NFB and AMF would increase crop yield and the protein content of the edible grain through enhancing biological N fixation more than NFB inoculation alone.

2. Materials and Methods

2.1. Cultivation Conditions and Experimental Design

This study was carried out in Cartagena, south-eastern Spain (37°41′ N 0°57′ E). The climate of the area is semiarid Mediterranean with a mean annual temperature of 18 °C and annual precipitation of 290 mm. Potential evapotranspiration surpasses 900 mm. The soil of the study site is a Haplic Calcisol (IUSS, 2014) with clay loam texture. The study was conducted in a field where cowpea had been previously grown for three months. The field experiment consisted of a complete randomized block with four replications, and each experimental plot was 10 m^2. A cultivar of fava bean (*Vicia faba* L.) 'Muchamiel' was grown under drip irrigation and following conventional management practices

for two consecutive seasons. The crop was subjected to eight treatments: 1. inoculation with *Rhizobium leguminosarum* (RL); 2. inoculation with *Burkholderia cenocepacia* (BC); 3. inoculation with *Burkholderia vietnamiensis* (BV); 4. inoculation with arbuscular mycorrhizal fungi (*Rhizophagus irregularis*, *Claroideoglomus etunicatum*, *Claroideoglomus claroideum* and *Funneliformis mosseae*) (AMF), 5. dual inoculation with *Rhizobium leguminosarum* and AMF (RL + AMF); 6. dual inoculation with *Burkholderia cenocepacia* and AMF (BC + AMF); 7. dual inoculation with *Burkholderia vietnamiensis* and AMF (BV + AMF); and 8. non-inoculated plants (control). A non-inoculated crop of broccoli (*Brassica oleracea* var. *italica* L.), also grown under drip irrigation, was included as the reference non-nitrogen fixing species to assess the BNF.

In both seasons, fava beans were sowed in the first week of November, flowering at the end of February, and harvested during April. Broccoli was planted in both seasons in the first week of December. The weather conditions during the first season were: a minimum air temperature of 8.3 °C, mean temperature of 13.2 °C, maximum temperature of 19.3 °C and rainfall of 9.3 mm; and for the second season, a minimum air temperature of 6.5 °C, mean temperature of 12.7 °C, maximum temperature of 17.1 °C and rainfall of 63.9 mm.

Fava bean seeds were inoculated by adding 2 g of the different NFB and 4 g of the pool of AMFs at sowing time. In the control treatment, autoclaved (121 °C for 20 min) inoculants were applied at the same rate. The inoculated and non-inoculated seeds were sown with a spacing of 100 cm between rows and 40 cm between plants (2.5 plants m^{-2}). The seeding rate was 2 seeds per sowing hole, leaving only a plant after seedling emergence. Fertilizer application in the fava bean plots started three weeks after sowing, and involved adding 20 kg ha^{-1} of N and 20 kg ha^{-1} of P_2O_5 in the form of ammonium nitrate (33.5% N) and monoammonium phosphate (61% P_2O_5, 12% N), as well as 40 kg ha^{-1} of K_2O in the form of potassium sulphate (50% K_2O, 18% S) as fertigation throughout the crop cycles. Broccoli plants were transplanted two weeks after fava bean sowing, with a planting pattern of 20 cm between plants and 100 cm between rows (5 plants m^{-2}) and fertilized similarly to the fava bean crop by fertigation. In the case of inoculated crops, the fertilizer application rate was reduced by 20% compared with the non-inoculated control, to check the efficacy of the inoculations to reduce the use of external fertilizers. No herbicide treatment was carried out, and the crop was kept free of weeds by hand-hoeing when necessary.

2.2. Soil Sampling and Analyses

The soil was sampled before the establishment of the trial and at the end of the experiment after harvesting the fava bean crop. All plots were sampled at 0–20 cm (Ap horizon). Three random soil samples per plot were collected, homogenized and sieved <2 mm to obtain a composite sample. The composite soil sample was homogeneous and divided into two sub-samples. One of the sub-samples was air-dried for 7 days, and stored at room temperature until chemical analysis, and the other sub-samples was stored at −20 °C for molecular analysis. The following chemical parameters were measured: total nitrogen (Nt) by the Kjeldahl method [24] and exchangeable Ca, Mg, Na and K, which were determined in the $BaCl_2$ extract for cation exchange capacity and measured using ICP-MS (Agilent 7500CE) [25].

2.3. Inoculum Preparation

The three strains of nitrogen-fixing bacteria (provided by the Universidade de Trás-os-Montes e Alto Douro, UTAD, Portugal) were isolated from the active root nodules of fava bean plants grown in Portugal, because of their growth-promoting effect in fava bean plants demonstrated in previous greenhouse studies. The bacterium was isolated [26], cultivated and maintained on yeast extract-mannitol (YEM) agar medium consisting of 0.4 g yeast extract, 10 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 8 g agar and 0.25% Congo Red, dissolved in 1000 mL distilled water and autoclaved at 121 °C for 20 min. For inoculation, each bacterium was cultured in 250 mL Erlenmeyer flasks containing 100 mL of YEM broth medium for 3 days at 28 °C. The contents of each flask were

diluted to 300 mL with sterilized distilled water in order to obtain 10^9 CFU per mL, estimated from the absorbance at 600 nm, and mixed with 1 kg of the sterilized carrier (compost soil:vermiculite 1:1 *v*/*v*), to give approximately 40% moisture in the inoculant (3×10^9 cells per gram of inoculum). The AMF were provided by Symbiom (Lanškroun, Czech Republic), and 1 g contained approximately 160 spores (40 spores of each fungal strain per gram).

2.4. Plant Sampling

Plants were sampled during fava bean flowering. Three plants per plot were carefully uprooted to obtain unharmed roots, and separated into root, shoot, nodules and seeds in the case of the legumes, and into root and shoot for broccoli to assess biological N fixation. An assessment of the bacterial community was only performed on nodules from plants uprooted during the second season. For this, the nodule surface was sterilized by immersing in a 95% ethanol solution for 45 s, washed with sterile water, crushed in liquid nitrogen and then stored at −80 °C until DNA extraction.

Fava bean yield (kg ha^{-1}) was determined by continuously harvesting and weighing all the fresh pods in each plot. Nodules were dried in an oven at 70 °C to reach a constant weight and their dry weight per plant was determined. The weight of fresh fava bean pods was used to measure yield since in some Mediterranean countries, such as Spain, fava bean is typically consumed as a green bean. In addition, the following quality parameters were recorded: protein content in grain (%), number of pods per plant, weight of 100 seeds and pod length.

2.5. Plant Analyses

Plant samples were oven dried and ground (A11 Basic, IKA) before incinerating at 500 °C; the ashes were dissolved in 0.6 N HNO_3 and analysed for P, Ca, Mg, Na and K by ICP-MS (7500 CE, Agilent). Nitrogen (N) was determined by the Kjeldahl method [24]. The protein content in grain was derived from the estimated N content by the following formula [27]:

$$\text{Protein content (\%)} = \text{N content (\%)} \times 6.25 \quad (1)$$

2.6. Efficiency of Biological Nitrogen Fixation

The ^{15}N natural abundance method was used to determine the efficiency of biological nitrogen fixation (BNF). The ^{15}N content of the plant samples was determined in the Stable Isotope Facility of UC-Davis, Davis, CA, USA, by CF-IRMS (Europa Scientific, Crewe, UK). This method is useful when the abundance of ^{15}N in the soil is higher than in atmospheric N_2 (0.3663%). The differences ($\delta^{15}N$) between the ^{15}N abundance in each sample and in the atmospheric N were calculated using Equation (2) [28]:

$$\delta^{15}N = \left(\frac{\text{Sample atom\% N} - 0.3663}{0.3663} \right) \times 1000 \quad (2)$$

To calculate the proportion of N derived from air (% Ndfa), it is necessary to know the $\delta^{15}N$ of the N_2-fixing legume and the $\delta^{15}N$ of the non-fixing reference plant (broccoli) grown in the same soil (Equation (3)) [29]:

$$\text{\% Ndfa} = (\delta^{15}N \text{ of reference plant} - \delta^{15}N \text{ of legume}) / (\delta^{15}N \text{ of reference plant} - B) \times 100 \quad (3)$$

As 'B' value we used −0.50, based on 'B' values for fava bean shoot taken from the literature [30].

2.7. DNA Extraction, PCR Amplification and Processing of Sequencing Data

Total genomic DNA was extracted from 0.5 g of nodule from three replicates using Genomic DNA for plant (Nucleo Spin^R Plant II, Macherey-Nagel, Düren, Germany) following the manufacturer´s protocol. The DNA concentration was determined using spectrophotometer (Nanodrop, 2000, Thermo Fisher Scientific The Meern, The Netherlands). The DNA was PCR amplified using the barcoded

primers 515 F and 806 R [31] in three PCR reactions per sample as previously described by Žifčáková et al. [32]. The PCR reactions contained 2.5 µL of 10 × buffer for DyNAzyme DNA Polymerase; 1.5 µL of BSA (20 mg mL^{-1}); 1 µL of each primer (0.01 mM); 0.5 µL of PCR Nucleotide Mix (10 mM each); 0.75 µL of polymerase (DyNAzyme II DNA polymerase 2 U/µL + PFU 200 µL D + 6.6 µL PFU); 1 µL of template DNA; and 17.75 µL of water. The cycling conditions were as follows: 94 °C for 4 min, 35 cycles of 94 °C for 45 s, 50 °C for 1 min, 72 °C for 75 s, and a final extension at 72 °C for 10 min. The sequencing of bacterial amplicons was performed on Illumina MiSeq, and the sequences were generated with a MiSeq Reagent Kit v2 in paired-end mode with sizes of 251 base pairs in the C4SYS facility at the Institute of Microbiology of the CAS, Prague, Czech Republic.

The amplicon sequencing data were processed using the pipeline SEED 2 [33]. Briefly, pair-end reads were merged using fastq-join [34], and whole amplicons were processed. Chimeric sequences were deleted using Usearch 7.0.1090 [35]. UPARSE implemented in Usearch [36] was used for clustering at a 97% similarity level. Consensus sequences were constructed for each cluster, and the closest hits at genus or species level were identified using BLASTn against the Genbank databases. Sequences identified as non-bacterial and singletons were excluded from further analyses. Sequences were upload to NCBI repository.

Diversity indexes (Shannon and Simpson indexes) were assessed using Paleontological Statistics (PAST) [37].

2.8. Statistical Analyses

Data were checked to ensure normal distribution using the Kolmogorov–Smirnov test and ln-transformed when necessary to ensure normal distribution. A one-way repeated measures ANOVA, with season (1, 2) as within-subject factor, and inoculation treatment (RL, RL + AMF, BC, BC + AMF, BV, BV + AMF, AMF and CONTROL) as between-subject factors was developed. Data without normal distribution were submitted to a non-parametric ANOVA (Kruskal–Wallis test) for the factor inoculation treatment. To assess the effect of season, the paired-data Friedman non-parametric test was used. Relationships among properties were studied using Pearson's correlations. Statistical analyses were performed with the IBM software SPSS for Windows, Version 22.

3. Results

3.1. Soil Fertility

The soil before the establishment of the trial showed the following average values of nutrients: Nt (g kg^{-1}) = 0.94 ± 0.07; exchangeable Ca (mg kg^{-1}) = 2726 ± 126; exchangeable Mg (mg kg^{-1}) = 606 ± 24; exchangeable Na (mg kg^{-1}) = 301 ± 31 and exchangeable K (mg kg^{-1}) = 369 ± 63. The soil at the end of the experiment showed the following ranges of nutrients: 1.2–1.3 g kg^{-1} for Nt; 1946–2482 mg kg^{-1} for exchangeable Ca; 354–506 mg kg^{-1} for exchangeable Mg; 154–209 mg kg^{-1} for exchangeable Na; and 268–386 mg kg^{-1} for exchangeable K. Inoculation treatments did not affect nutrient content in soil at the end of the experiment.

3.2. Biological Nitrogen Fixation

The inoculation treatment did not significantly affect BNF in roots (Table 1), while the season did so, with significantly lower values in the second season. In addition, BNF was not significantly correlated with plant nutrients, nodule weight, crop yield or crop quality parameters (data not shown).

Table 1. Biological nitrogen fixation (% Ndfa) in fava bean roots, using broccoli as a non-N_2-fixing reference plant. Values are mean ± standard deviation (n = 4).

Treatment (T) [a]	Season 1	Season 2
RL	62.8 ± 6.2	53.3 ± 5.9
RL + AMF	61.2 ± 6.5	63.8 ± 6.7
BC	63.0 ± 2.5	56.7 ± 10.7
BC + AMF	61.3 ± 7.8	48.3 ± 2.9
BV	57.8 ± 6.2	55.0 ± 12.4
BV + AMF	59.6 ± 2.7	55.1 ± 10.1
AMF	59.1 ± 0.8	58.3 ± 1.2
CONTROL	61.8 ± 10.3	56.6 ± 8.2
F values [b]		
Between subjects		
Inoculation (IT)	0.81 ns	
Within subjects		
Season (S)	7.37 *	
S × T	1.56 ns	

[a] Treatment: RL (*Rhizobium leguminosarum*), BC (*Burkholderia cenocepacia*), BV (*Burkholderia vietnamiensis*) and AMF (arbuscular mycorrhizal fungi). [b] Significant at * $p < 0.05$; ns: not significant ($p > 0.05$).

3.3. Plant Nutritional Characteristics

Inoculation treatment significantly affected the N content of seeds, shoots and roots. Inoculation with *Rhizobium leguminosarum* (RL) led to a significant increase of N in seeds compared to the control (Table 2). In turn, dual inoculation with RL and AMF significantly increased the N concentration in shoots in both seasons compared with inoculation with RL alone (Table 3). However, the N content of roots was significantly higher after inoculation with *Burkholderia cenocepacia* (BC) and BC + AMF than in the control (Table 4). N content in different plant parts (seed, shoot and root) was also significantly higher after inoculation with AMF than in the control in Season 2. As a general pattern, most nutrients measured in the different plant parts (seeds, shoot and root) were higher in the second than in the first growing season. Seed Mg, Na, K and P concentrations were positively correlated with each other ($R > 0.75$; $P < 0.01$). In shoots, Ca, Mg, Na and P concentrations were positively correlated with each other ($R > 0.73$; $P < 0.01$), as were Mg, K and P concentrations in roots ($R > 0.69$; $P < 0.01$).

3.4. Crop Yield and Quality

Inoculation did not significantly influence the dry weight of nodules, crop yield, weight of 100 seeds and number of pods per plant (Table 5). However, it affected the protein content of the grain, and higher values were recorded after inoculation with NFB, AMF and the combined inoculations than in the control. Season influenced crop yield and the number of pods per plant. Crop yield and number of pods per plant were higher in the first season than in the second.

Table 2. Nutrient content of fava bean seeds in two seasons (nitrogen content, calcium, magnesium, sodium, potassium and phosphorus). Values are mean ± standard deviation (n = 4).

	Treatment (T) [a]	Mg (mg kg^{-1})	K (mg kg^{-1})	P (mg kg^{-1})	Treatment (T) [a]	N (g kg^{-1})	Ca (mg kg^{-1})	Na (mg kg^{-1})
Season 1	RL	295 ± 146	2919 ± 1283	1090 ± 474	RL	48.2 ± 0.6	457 ± 320	91.7 ± 52.6
	RL + AMF	195 ± 75	2333 ± 838	762 ± 249	RL + AMF	42.4 ± 1.0	172 ± 46	81.6 ± 27.2
	BC	184 ± 65	1992 ± 687	760 ± 263	BC	41.6 ± 1.3	166 ± 75	52.2 ± 6.4
	BC + AMF	236 ± 59	2899 ± 598	988 ± 232	BC + AMF	44.4 ± 5.1	217 ± 58	84.5 ± 5.7
	BV	208 ± 167	2120 ± 1726	825 ± 658	BV	45.8 ± 1.8	186 ± 151	53.6 ± 28.6
	BV + AMF	114 ± 45	1441 ± 544	501 ± 148	BV + AMF	44.6 ± 1.9	117 ± 45	49.8 ± 9.6
	AMF	316 ± 96	2910 ± 727	454 ± 283	AMF	46.0 ± 0.8	237 ± 56	79.0 ± 23.4
	CONTROL	217 ± 44	2482 ± 530	883 ± 181	CONTROL	47.1 ± 1.0	176 ± 55	72.5 ± 9.0
Season 2	RL	780 ± 296	13,820 ± 3735	3836 ± 1060	RL	44.3 ± 1.5	443 ± 88	194 ± 100
	RL + AMF	873 ± 251	14,764 ± 2411	3976 ± 668	RL + AMF	41.5 ± 2.8	543 ± 124	193 ± 15
	BC	511 ± 96	10,954 ± 1090	2906 ± 391	BC	40.8 ± 2.5	343 ± 60	105 ± 43
	BC + AMF	396 ± 62	10,101 ± 931	2369 ± 344	BC + AMF	44.0 ± 5.7	275 ± 26	117 ± 11
	BV	567 ± 153	11,609 ± 1961	3206 ± 672	BV	46.1 ± 7.7	372 ± 97	134 ± 27
	BV + AMF	757 ± 130	14,656 ± 2942	3834 ± 55	BV + AMF	44.6 ± 5.4	518 ± 101	147 ± 54
	AMF	516 ± 118	10,777 ± 1249	2916 ± 329	AMF	43.2 ± 3.2	347 ± 73	155 ± 13
	CONTROL	660 ± 340	12,510 ± 3505	3247 ± 1299	CONTROL	36.5 ± 2.9	415 ± 241	213 ± 87
	F values [b]				χ^2 values [b]			
Between subjects								
	Inoculation (IT)	4.26 *	2.64 ns	2.80 ns	Inoculation (IT)	4.81 *	3.0 ns	3.2 ns
Within subjects								
	Season (S)	65.65 ***	327.31 ***	224.17 ***	Season (S)	2.66 ns	16.66 ***	20.16 ***
	S × T	2.28 ns	2.55 ns	3.40 *				

[a] Treatment: RL (*Rhizobium leguminosarum*), BC (*Burkholderia cenocepacia*), BV (*Burkholderia vietnamiensis*) and AMF (arbuscular mycorrhizal fungi). [b] Significant at * $p < 0.05$; *** $p < 0.001$; ns: not significant ($p > 0.05$).

Table 3. Nutrient content of fava bean shoots for two consecutive seasons (nitrogen content, calcium, magnesium, sodium, potassium and phosphorus). Values are mean ± standard deviation (n = 4).

	Treatment (T) [a]	Na (mg kg^{-1})	Treatment (T) [a]	N (g kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	K (mg kg^{-1})	P (mg kg^{-1})
Season 1	RL	1032 ± 487	RL	30.5 ± 6.4	2158 ± 1242	584 ± 290	9055 ± 3097	726 ± 192
	RL + AMF	986 ± 139	RL + AMF	36.7 ± 4.3	3270 ± 779	939 ± 164	10,229 ± 1548	851 ± 145
	BC	935 ± 195	BC	27.5 ± 2.3	2457 ± 1144	732 ± 242	9197 ± 2141	961 ± 233
	BC + AMF	1250 ± 176	BC + AMF	29.7 ± 5.8	2308 ± 1159	670 ± 267	8254 ± 2192	599 ± 303
	BV	1094 ± 561	BV	33.3 ± 3.3	2308 ± 720	688 ± 256	9028 ± 4288	842 ± 251
	BV + AMF	1372 ± 147	BV + AMF	20.7 ± 7.4	3143 ± 429	961 ± 146	6536 ± 1176	1057 ± 136
	AMF	795 ± 33	AMF	30.1 ± 2.3	1347 ± 239	403 ± 83	4375 ± 1085	556 ± 130
	CONTROL	2182 ± 287	CONTROL	27.4 ± 1.8	4283 ± 743	1407 ± 91	16,445 ± 2527	1442 ± 166
Season 2	RL	1550 ± 159	RL	25.3 ± 3.2	6633 ± 1373	1250 ± 291	20,623 ± 3343	1538 ± 369
	RL + AMF	1680 ± 180	RL + AMF	35.1 ± 4.2	4552 ± 1389	931 ± 219	19,849 ± 177	1479 ± 631
	BC	2670 ± 674	BC	29.8 ± 0.7	23,068 ± 6704	3576 ± 288	34,786 ± 7097	3877 ± 730
	BC + AMF	2410 ± 780	BC + AMF	34.0 ± 1.7	42,355 ± 3941	3588 ± 852	20,314 ± 4004	4046 ± 377
	BV	1422 ± 293	BV	26.7 ± 2.0	7630 ± 822	1520 ± 130	23,080 ± 5230	2247 ± 1065
	BV + AMF	2018 ± 756	BV + AMF	37.1 ± 7.2	8728 ± 4136	1810 ± 899	29,378 ± 12020	2348 ± 143
	AMF	2436 ± 319	AMF	28.4 ± 1.7	46,638 ± 7428	4413 ± 860	26,915 ± 4053	4327 ± 133
	CONTROL	2326 ± 340	CONTROL	25.9 ± 1.7	47,820 ± 2898	3810 ± 484	21,441 ± 3150	3666 ± 122
	F values [b]		χ^2 values [b]					
Between subjects								
	Inoculation (IT)	4.14 *	Inoculation (IT)	11.60 ***	0.96 ns	1.33 ns	0.03 ns	0.85 ns
Within subjects								
	Season (S)	39.75 ***	Season (S)	4.16 *	20.16 ***	16.66 ***	24.00 ***	24.00 ***
	S × T	2.47 ns						

[a] Treatment: RL (*Rhizobium leguminosarum*), BC (*Burkholderia cenocepacia*), BV (*Burkholderia vietnamiensis*) and AMF (arbuscular mycorrhizal fungi). [b] Significant at * $p < 0.05$; *** $p < 0.001$; ns: not significant ($p > 0.05$).

Table 4. Nutrient content of fava bean roots for two consecutive seasons (nitrogen content, calcium, magnesium, sodium, potassium and phosphorus). Values are mean ± standard deviation (n = 4).

	Treatment (T) [a]	N (g kg^{-1})	Na (mg kg^{-1})	Treatment (T) [a]	Ca (mg kg^{-1})	Mg (mg kg^{-1})	K (mg kg^{-1})	P (mg kg^{-1})
Season 1	RL	12.8 ± 0.9	1035 ± 366	RL	12,338 ± 4247	803 ± 103	3967 ± 1838	421 ± 112
	RL + AMF	14.5 ± 1.6	1278 ± 773	RL + AMF	3079 ± 1151	614 ± 314	5138 ± 2249	410 ± 197
	BC	15.5 ± 0.3	1932 ± 557	BC	13,544 ± 6189	1141 ± 272	7419 ± 3562	740 ± 193
	BC + AMF	14.6 ± 3.7	967 ± 536	BC + AMF	2570 ± 532	433 ± 134	3622 ± 1503	280 ± 62
	BV	11.8 ± 0.9	903 ± 118	BV	8198 ± 790	610 ± 57	4096 ± 1218	380 ± 67
	BV + AMF	14.3 ± 2.7	1054 ± 391	BV + AMF	2585 ± 448	517 ± 139	4411 ± 2084	374 ± 130
	AMF	12.6 ± 2.5	1364 ± 972	AMF	14,207 ± 9458	1012 ± 618	4678 ± 2389	557 ± 343
	CONTROL	14.0 ± 0.5	814 ± 636	CONTROL	2665 ± 1409	396 ± 250	3755 ± 2614	283 ± 186
Season 2	RL	11.0 ± 0.7	2427 ± 780	RL	11,223 ± 3896	1166 ± 310	16,856 ± 4059	1112 ± 326
	RL + AMF	10.1 ± 1.2	2506 ± 600	RL + AMF	23,672 ± 15915	1795 ± 518	13,787 ± 1829	999 ± 259
	BC	13.2 ± 0.8	3047 ± 1296	BC	9566 ± 2019	1077 ± 753	15,491 ± 1806	1101 ± 77
	BC + AMF	13.4 ± 0.3	2192 ± 319	BC + AMF	17,601 ± 8106	1357 ± 194	12,722 ± 897	834 ± 119
	BV	9.8 ± 0.7	2649 ± 784	BV	13,769 ± 3957	1294 ± 404	18,360 ± 8763	1209 ± 509
	BV + AMF	11.5 ± 1.3	3244 ± 1136	BV + AMF	19,758 ± 12867	1832 ± 248	18,628 ± 5196	1266 ± 494
	AMF	13.0 ± 1.3	2050 ± 449	AMF	10,705 ± 3476	969 ± 1044	14,013 ± 1607	918 ± 142
	CONTROL	11.8 ± 1.3	2153 ± 522	CONTROL	13,251 ± 4610	1227 ± 317	11,744 ± 1361	708 ± 99
		F values [b]			χ^2 values [b]			
Between subjects								
	Inoculation (IT)	4.58 *	0.81 ns	Inoculation (IT)	0.08 ns	0.97 ns	0.003 ns	0.003 ns
Within subjects								
	Season (S)	20.70 ***	52.79 ***	Season (S)	8.16 **	10.66 **	24.00 ***	24.00 ***
	S × T	1.11 ns	1.25 ns					

[a] Treatment: RL (*Rhizobium leguminosarum*), BC (*Burkholderia cenocepacia*), BV (*Burkholderia vietnamiensis*) and AMF (arbuscular mycorrhizal fungi). [b] Significant at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: not significant ($p > 0.05$).

Table 5. Crop yield, dry weight of nodules and crop quality parameters (weight of 100 seeds, number of pods per plant and protein content of grain). Values shown are mean ± standard deviation (n = 4).

	Treatment (T) [a]	Crop Yield (kg ha^{-1})	Nodule Dry Weight (g/Plant)	Weight of 100 Seeds (g)	Number of Pods Per Plant	Treatment (T) [a]	Protein Content of Grain (%)
Season 1	RL	32,541 ± 3295	3.4 ± 0.5	152.4 ± 13.9	33 ± 2	RL	30 ± 0
	RL + AMF	30,133 ± 6311	4.9 ± 2.4	178.3 ± 14.9	33 ± 5	RL + AMF	26 ± 1
	BC	33,333 ± 2480	4.0 ± 1.4	178.7 ± 17.5	34 ± 2	BC	26 ± 1
	BC + AMF	28,583 ± 4026	2.6 ± 1.1	165.0 ± 28.8	31 ± 1	BC + AMF	28 ± 3
	BV	28,166 ± 7381	3.8 ± 0.9	179.9 ± 8.4	30 ± 4	BV	29 ± 1
	BV + AMF	30,250 ± 4073	3.5 ± 0.8	158.5 ± 21.7	32 ± 7	BV + AMF	28 ± 1
	AMF	27,100 ± 7258	4.9 ± 2.1	176.6 ± 28.8	29 ± 10	AMF	29 ± 0
	CONTROL	27,016 ± 2759	4.8 ± 0.5	137.0 ± 7.3	29 ± 6	CONTROL	29 ± 1
Season 2	RL	23,672 ± 2797	3.1 ± 0.5	170.7 ± 15.2	30 ± 1	RL	28 ± 1
	RL + AMF	18,805 ± 6022	4.8 ± 1.6	183.5 ± 13.1	20 ± 7	RL + AMF	26 ± 2
	BC	19,857 ± 4828	5.3 ± 2.7	199.6 ± 46.3	22 ± 6	BC	25 ± 2
	BC + AMF	14,753 ± 2033	5.8 ± 3.4	169.8 ± 24.4	16 ± 2	BC + AMF	27 ± 4
	BV	20,379 ± 7372	4.7 ± 1.7	158.8 ± 27.7	24 ± 10	BV	29 ± 5
	BV + AMF	19,056 ± 2855	3.7 ± 0.7	154.0 ± 21.2	23 ± 2	BV + AMF	28 ± 3
	AMF	24,137 ± 2599	6.2 ± 5.3	162.7 ± 15.8	26 ± 4	AMF	27 ± 2
	CONTROL	19,506 ± 3791	4.3 ± 2.3	176.1 ± 8.0	22 ± 5	CONTROL	23 ± 2
		F values [b]				χ^2 values [b]	
	Between subjects						
	Inoculation (IT)	0.42 ns	0.56 ns	0.87 ns	1.07 ns	Inoculation (IT)	4.81 *
	Within subjects						
	Season (S)	68.55 ***	1.94 ns	1.35 ns	23.73 ***	Season (S)	2.66 ns
	S × T	2.20 ns	1.03 ns	1.45 ns	1.02 ns		

[a] Treatment: RL (*Rhizobium leguminosarum*), BC (*Burkholderia cenocepacia*), BV (*Burkholderia vietnamiensis*) and AMF (*arbuscular mycorrhizal fungi*). [b] Significant at * $p < 0.05$; *** $p < 0.001$; ns: not significant ($p > 0.05$).

3.5. Bacterial Diversity and Community Structure

The Shannon and Simpson diversity index pointed to no significant differences among inoculation treatments (F = 261 and F = 254 respectively; P > 0.05) (Figure 1).

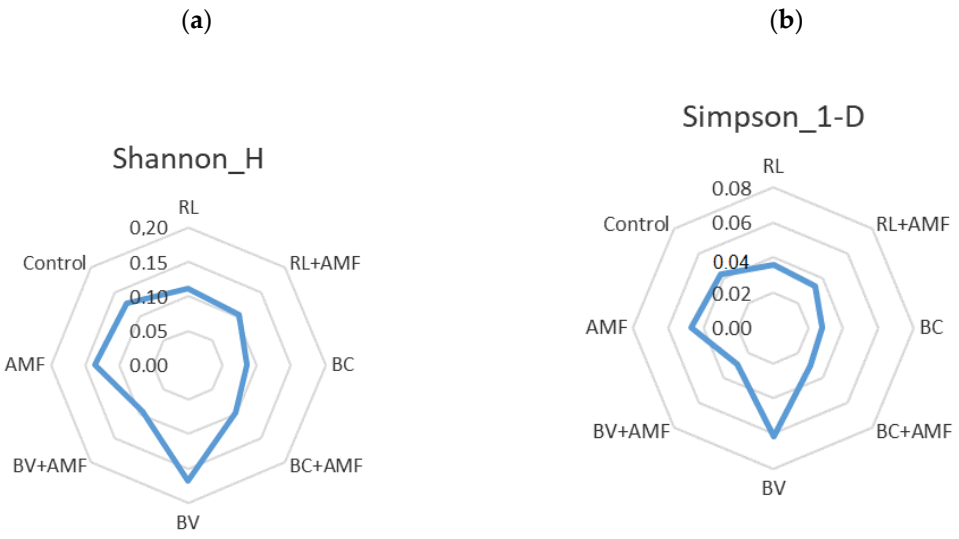

Figure 1. Diversity indices (**a**) Shannon_H and (**b**) Simpson in nodules of fava bean for the different inoculation treatments. Treatment: RL (*Rhizobium leguminosarum*), BC (*Burkholderia cenocepacia*), BV (*Burkholderia vietnamiensis*) and AMF (arbuscular mycorrhizal fungi).

A total of 353,442 high-quality bacterial sequences were obtained after removing low-quality sequences and chimeras. Based on 97% sequence similarity, bacterial sequences were clustered into 382 OTUs. The major bacterial phyla for all samples were Proteobacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia and Firmicutes. As shown in Figure 2, Proteobacteria formed the most abundant phylum, accounting for 98.03% of all OTUs, followed by Bacteroidetes (0.65%) and Actinobacteria (0.39%). Within Proteobacteria, the most abundant class were alphaproteobacteria, genera *Rhizobium* (97.23%), *Methylophilus* (0.16%) and *Novosphingobium* (0.05%), and within betaproteobacteria, genera *Methylotenera* (0.08%), *Acidovorax* (0.05%) and *Variovorax* (0.05%). However, the genus *Burkholderia* was not detected. In the case of Bacteroidetes, the most abundant genera were *Flavobacterium* (0.31%), *Dyadobacter* (0.15%) and *Chryseobacterium* (0.05%). The most abundant Actinobacteria genera were *Nocardioides* (0.13%), *Aeromicrobium* and *Mycobacterium* (0.05%). No identified bacterial genus showed a relative abundance lower than 0.05%.

Among the different NFB inoculation treatments, Proteobacteria were the least abundant and Verrucomicrobia the most abundant in the BV treatment, while, in the AMF treatment, Bacteroidetes were the most abundant compared to the other treatments (Figure 2).

Overall, two α-rhizobia (*Rhizobium* and *Bradyrhizobium*) were detected, the most abundant being *Rhizobium* in all the treatments. However, *Bradyrhizobium* was only detected in the RL treatment. Among the non-rhizobial bacteria detected were the genera *Pseudomonas*, *Devosia*, *Agrobacterium* and *Rhodococcus* (Figure 3). *Pseudomonas* was more abundant in the AMF treatment, followed by RL + AMF, BC and BVAMF. *Agrobacterium* was only detected in RL, RL + AMF; BC, BV and; BV + AMF. *Devosia* was only detected in BC + AMF, BV + AMF, AMF and the Control, while *Rhodococcus* was detected in all the treatments except for RL and BV + AMF (Figure 3).

Among the non-nodulating bacterial endophites detected were the genera *Variovorax*, *Arthrobacter*, *Bacillus*, *Streptomyces* and *Ensifer*. Of these, *Variovorax* were most abundant in AMF followed by RL + AMF, RL, BV and BV + AMF. *Arthrobacter* was only detected in BV, RL + AMF and BC + AMF, and *Bacillus* only in RL + AMF, BV, AMF and the Control. *Streptomyces* was highest in RL and BV and *Ensifer* was lower than control values in all the treatments.

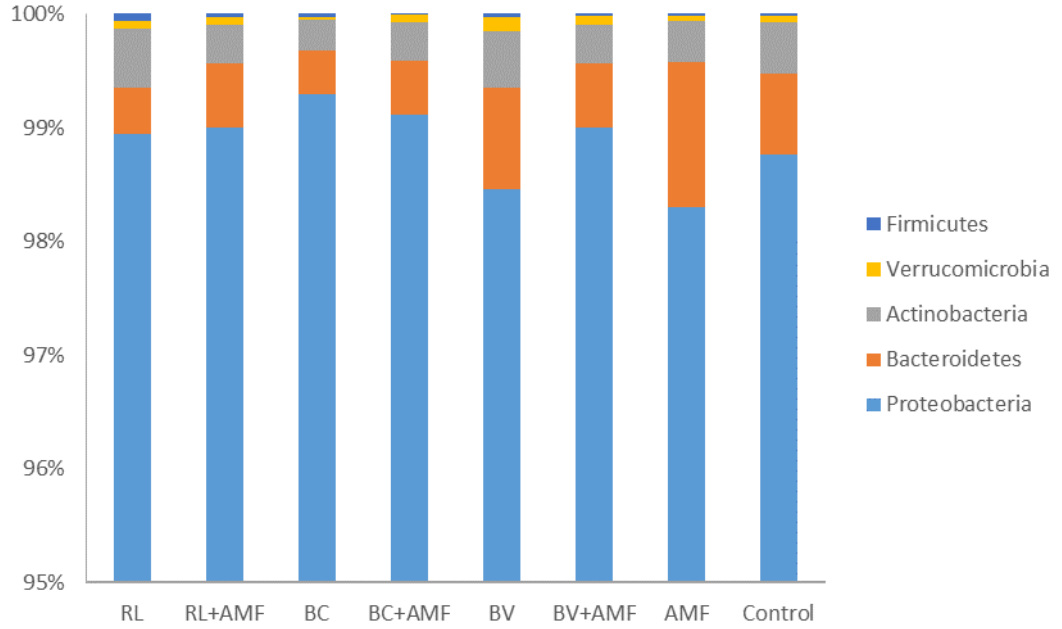

Figure 2. Relative abundance (%) of the predominant phyla in fava bean nodules of the different inoculation treatments. Others phyla and bacteria were not shown. Treatment: RL (*Rhizobium leguminosarum*), BC (*Burkholderia cenocepacia*), BV (*Burkholderia vietnamiensis*) and AMF (arbuscular mycorrhizal fungi).

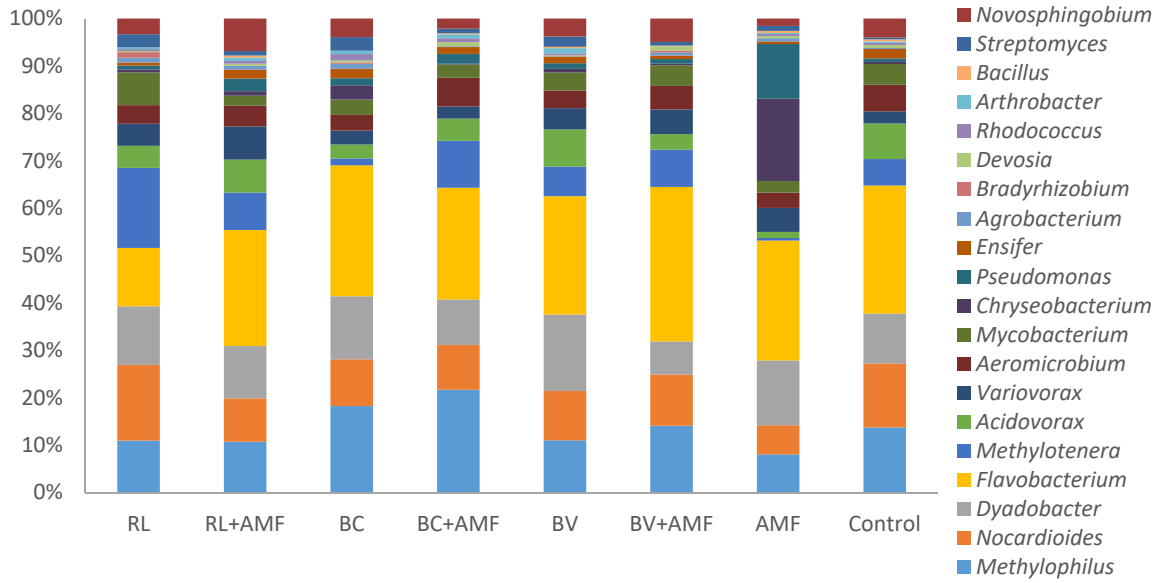

Figure 3. Relative abundance of different genera in fava bean nodules of the different inoculation treatments. The genus *Rhizobium* is not shown due to its high relative abundance, and the rest of the genera were relativized. Treatment: RL (*Rhizobium leguminosarum*), BC (*Burkholderia cenocepacia*), BV (*Burkholderia vietnamiensis*) and AMF (Arbuscular mycorrhizal fungi).

As regards the other genera found (Figure 3), *Flavobacteria* and *Dyadobacter* showed the highest abundance in BV and AMF; *Methylotenera* and *Acidovorax* in BV and *Chrysebacterium* in the AMF treatment.

Taken together, these results suggest that microbial inoculation accompanied by a 20% decrease in mineral fertilization had no significant effect on crop yield or the nutritional characteristics compared with non-inoculated plants, except for an increase in the grain protein content in inoculated plants.

Furthermore, the genus *Rhizobium* predominated in all nodules, both in inoculated and non-inoculated plants, suggesting the previous presence of these bacteria in the soil.

4. Discussion

Despite the reduction in the amount of fertilizer applied in the inoculated treatments compared to that applied in the control treatment, crop yield, quality and nutritional characteristics were maintained. This may be attributed to the beneficial properties of the inoculated microorganisms, alone or in combination, including better mineral solubilization and organic matter mineralization as a result of phytohormones, organic compounds or enzymes being released [38,39]. These microorganisms would interact with plant root exudates in the rhizosphere [40,41], inducing an improvement in plant growth [10].

The differences with regard to season observed in most plant properties could be related to differences in atmospheric temperature. As indicated in Section 2.1, the first season was warmer than the second, which may have contributed to higher crop growth and production, and the increased nutrient content. The higher rainfall during the second season had no effect on production since the crop was irrigated, and there was no water deficit at any time during the entire cycle.

Inoculation with *Rhizobium leguminosarum* (RL) led to a higher nitrogen and protein content in fava bean seeds than in the non-inoculated seeds, suggesting that subsequent N assimilation by the plant was enhanced with RL, and that the efficiency of protein anabolism was improved. This finding agrees with that of Alsina et al. [42] who found that some RL strains promoted protein accumulation in the seeds of two fava bean cultivars, their efficacy being dependent on interaction between strain, soil conditions and cultivar. Therefore, RL could be considered as a key bacterial symbiont to improve and protect yield components and seed quality for sustainable agricultural systems [43]. The ability of rhizobia to increase the protein content in fava beans has been observed previously, particularly when supplemented with nitrogen fertilizer [44]. In a previous study with common bean cultivated in reduced fertilization, a higher protein content was found in seeds of *Rhizobium*-inoculated plants in combination or without AMF than in inoculated plants, showing that AMF-inoculated plants had higher nutrient seed accumulation [45]. In our study, inoculation with AMF led to higher nitrogen content in seeds, shoots and roots in the second season than in the non-inoculated plants, indicating the influence of AMF on plant growth, due to a higher nutrient use efficiency by the plant [46]. Contrary to our hypothesis, the dual inoculation of rhizobial bacteria and AMF did not increase nitrogen fixation or crop yield in the same way as other authors showed [47,48]. This could be due to competition between the different AMF applied and those already present in the soil, which would affect the final extent of plant colonization. Moreover, Scheublin et al. [49] found that AMF colonisation of the nodules may inhibit N fixation.

Inoculation with NFB, alone or combined with AMF, did not lead to a higher amount of biologically-fixed N, which may also be due to the competition with native microbes or a higher nutrient use efficiency [50]. In this respect, Menge et al. [51] observed greater biological nitrogen fixation when N was a determinant nutrient. The high abundance of *Rhizobium* and other rhizobia bacteria in nodules observed in the different treatments would imply that rhizobia bacteria (an integral part of the soil microbial community) can remain viable in soil for several seasons [52], and that inoculation with specific rhizobia bacteria would not be sufficient to overcome the effect of the native ones. Thus, the differences found in the ability to colonise the nodules by the rhizobia bacteria demonstrate that specific bacterial determinants contribute to their acceptance by the host [53].

Legume nodules occupy a distinctive ecological niche, with a programme adapted to the accommodation of compatible soil microbes [4]. As our results indicate, legume nodules are often occupied by a phylogenetically diverse microbial community apart from rhizobia. However, the RL treatment induced a higher nitrogen and protein content than the other treatments. It is assumed that when legume plants are exposed to complex communities, they selectively regulate the access and accommodation of bacteria occupying this specialized environmental niche—the root nodule [54].

Furthermore, *Burkholderia* was not detected in nodules of different treatments, probably due to its low ability to form nodules in the presence of other native strains, which showed superior characteristics of competitiveness, and its lack of adaptation to the local environment [55]. It has been recently demonstrated that species of the genus *Burkholderia* have higher affinity for acidic soils, while they can be replaced by alpha-rhizobia in alkaline habitats [56], as could happen in the soil where this experiment was carried out.

In addition to rhizobia, non-rhizobial bacteria are considered to be able to infect legume nodules and multiply within. The fact that we found *Pseudomonas*, *Devosia*, *Agrobacterium* and *Rhodococcus* in nodules points to the coexistence of both types of bacteria in fava bean nodules, where they might have different roles [52,57]; for example, inoculation with *Bradyrhizobium* and *Pseudomonas* was seen to play a growth promoting role in the nodule ecosystem [58], and *Agrobacterium* and *Rhodococus* induced nodule formation [59,60].

Non-nodulating bacterial endophites (*Variovorax*, *Arthobacter*, *Bacillus*, *Streptomyces* and *Ensifer*) were also found in the nodules, as other authors have reported [61,62]. According to Xu et al. [63], *Bacillus* is the most abundant non-nodulating genus found in nodules, whereas it was only detected in three treatments in our experiment.

No significant differences were observed in the Shannon diversity index in nodules, although the BV and AMF treatments showed a slight increase compared to the other treatments and the control, but no correlation was found with crop yield and quality parameters, or with nitrogen fixation. In AMF, the genera *Bacillus*, *Flavobacteria*, *Dyadobacter* and *Chrysebacterium* were more abundant than in the other treatments; however in BV, the genera *Methylotenera*, *Acidovorax*, *Bacillus*, *Streptomyces*, *Dyadobacter*, *Arthrobacter* and *Flavobacteria* were more abundant than in the other treatments, while they have also been found in the nodules of other legumes [64,65].

In conclusion, the inoculation with *Rhizobium leguminosarum* (RL) produced the highest seed N content and protein in grain. However, none of the treatments increased biological nitrogen fixation compared with the plants grown from non-inoculated seeds. *Rhizobium* predominated in all nodules, and non-rhizobial and non-nodulate bacteria were also present. The lower amount of mineral fertilizers applied to the assayed soil can be considered an environmentally friendly alternative for reducing their use. Inoculation with RL was seen to be the most effective treatment, while *Burkholderia* sp. were not able to colonise the plant nodules. However, further studies are needed on the selection and detection of efficient rhizobial strains under local field conditions in order to obtain superior nitrogen-fixing bacteria.

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