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 \(Funneliformis\) mossae) 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 N2-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 N2-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 N2, 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 semi-arid 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
A block with four replications, and each experimental plot was 10 m². 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 P₂O₅ in the form of ammonium nitrate (33.5% N) and monoammonium phosphate (61% P₂O₅, 12% N), as well as 40 kg ha⁻¹ of K₂O in the form of potassium sulphate (50% K₂O, 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 homogenized 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 BaCl₂ 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 K₂HPO₄, 0.2 g MgSO₄·7H₂O, 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 10⁹ 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 × 10⁹ 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 HNO₃ 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]:

\[
\text{Protein content} \% = \text{N content} \% \times 6.25
\]

2.6. Efficiency of Biological Nitrogen Fixation

The ¹⁵N natural abundance method was used to determine the efficiency of biological nitrogen fixation (BNF). The ¹⁵N 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 ¹⁵N in the soil is higher than in atmospheric N₂ (0.3663%). The differences (Δ¹⁵N) between the ¹⁵N abundance in each sample and in the atmospheric N were calculated using Equation (2) [28]:

\[
\Delta^{15}N = \left( \frac{\text{Sample atom}\% \text{ N} - 0.3663}{0.3663} \right) \times 1000
\]

To calculate the proportion of N derived from air (% Ndfa), it is necessary to know the Δ¹⁵N of the N₂-fixing legume and the Δ¹⁵N of the non-fixing reference plant (broccoli) grown in the same soil (Equation (3)) [29]:

\[
\% \text{ Ndfa} = \left( \frac{\Delta^{15}N \text{ of reference plant} - \Delta^{15}N \text{ of legume}}{\Delta^{15}N \text{ of reference plant} - B} \right) \times 100
\]

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® 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 Žiříčková 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-N₂-fixing reference plant. Values are mean ± standard deviation (n = 4).

| Treatment (T) | 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

Between subjects
Inoculation (IT) 0.81 ns

Within subjects
Season (S) 7.37 *
S × T 1.56 ns

* Treatment: RL (*Rhizobium leguminosarum*), BC (*Burkholderia cenocepacia*), BV (*Burkholderia vietnamiensis*) and AMF (arbuscular mycorrhizal fungi). *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).

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) | Mg (mg kg⁻¹) | K (mg kg⁻¹) | P (mg kg⁻¹) | Treatment (T) | N (g kg⁻¹) | Ca (mg kg⁻¹) | Na (mg kg⁻¹) |
|---------------|--------------|-------------|-------------|---------------|------------|--------------|-------------|
| Season 1      |              |             |             | Season 1      |            |              |             |
| RL            | 295 ± 146    | 2919 ± 1263 | 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  |
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) * | Na (mg kg⁻¹) | Treatment (T) * | N (g kg⁻¹) | Ca (mg kg⁻¹) | Mg (mg kg⁻¹) | K (mg kg⁻¹) | P (mg kg⁻¹) |
|----------------|--------------|-----------------|------------|--------------|--------------|-------------|-------------|
| RL 780 ± 296   | RL 3836 ± 1080 | RL 44.3 ± 1.5   | 443 ± 88  | 194 ± 100    |
| RL + AMF 873 ± 251 | RL + AMF 3976 ± 668 | RL + AMF 41.5 ± 2.8 | 543 ± 124 | 193 ± 15    |
| BC 511 ± 96    | BC 2906 ± 391  | BC 40.8 ± 2.5   | 343 ± 60  | 105 ± 43    |
| BC + AMF 396 ± 62 | BC + AMF 2369 ± 344 | BC + AMF 44.0 ± 5.7 | 275 ± 26  | 117 ± 11    |
| BV 567 ± 153   | BV 3206 ± 672  | BV 46.1 ± 7.7   | 372 ± 97  | 134 ± 27    |
| BV + AMF 757 ± 130 | BV + AMF 3834 ± 55 | BV + AMF 44.6 ± 8.4 | 518 ± 101 | 147 ± 54    |
| AMF 516 ± 118  | AMF 2916 ± 329 | AMF 43.2 ± 3.2  | 347 ± 73  | 155 ± 13    |
| CONTROL 660 ± 340 | CONTROL 3247 ± 1299 | CONTROL 36.5 ± 2.9 | 415 ± 241 | 213 ± 87    |

**Between subjects**

| Inoculation (IT) | F values b | χ² values b |
|------------------|------------|-------------|
| *            | 4.81 *     | 3.0 ns      |
| ns           | 4.26 *     | 2.64 ns     |
| ns           | 2.28 ns    | 16.66 ***   |
| ns           | 65.65 ***  | 20.16 ***   |

**Within subjects**

| Season (S) | 65.65 *** | 327.31 *** | 224.17 *** | 2.28 ns | 3.40 * |
| S x T       | 4.81 *     | 3.0 ns      | 3.2 ns     | 2.66 ns | 16.66 *** |

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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) | N (g kg⁻¹) | Na (mg kg⁻¹) | Treatment (T) | Ca (mg kg⁻¹) | Mg (mg kg⁻¹) | K (mg kg⁻¹) | P (mg kg⁻¹) |
|---------------|------------|--------------|---------------|--------------|--------------|-------------|-------------|
| Season 1      |            |              | Season 2      |              |              |             |             |
| RL            | 12.0 ± 0.3 | 1035 ± 366   | RL            | 12.39 ± 4.247| 803 ± 130    | 3967 ± 1438 | 421 ± 112   |
| RL + AMF      | 14.5 ± 1.6 | 1278 ± 777   | RL + AMF      | 3079 ± 1151  | 614 ± 314    | 5138 ± 2249 | 410 ± 197   |
| BC            | 15.5 ± 0.3 | 1932 ± 557   | BC            | 13.54 ± 619  | 1141 ± 272   | 7419 ± 2562 | 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           | 14207 ± 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      |            |              | Season 2      |              |              |             |             |
| RL            | 11.0 ± 0.7 | 2427 ± 780   | RL            | 11.223 ± 3896| 1166 ± 310   | 16856 ± 4059| 1112 ± 326  |
| RL + AMF      | 10.1 ± 1.2 | 2506 ± 600   | RL + AMF      | 23672 ± 1591 | 1795 ± 518   | 13787 ± 1829| 999 ± 259   |
| BC            | 13.2 ± 0.8 | 3047 ± 1296  | BC            | 9566 ± 2019  | 1077 ± 753   | 15491 ± 1806| 1101 ± 77   |
| BC + AMF      | 13.4 ± 0.3 | 2192 ± 319   | BC + AMF      | 17601 ± 8106 | 1357 ± 194   | 12722 ± 897 | 834 ± 119   |
| BV            | 9.8 ± 0.7  | 2649 ± 784   | BV            | 13769 ± 3957 | 1294 ± 404   | 18360 ± 8763| 1209 ± 509  |
| BV + AMF      | 11.5 ± 1.3 | 3244 ± 1136  | BV + AMF      | 19758 ± 12867| 1832 ± 248   | 18628 ± 5196| 1266 ± 494  |
| AMF           | 13.0 ± 1.3 | 2050 ± 449   | AMF           | 10705 ± 3476 | 969 ± 1044   | 14013 ± 1607| 918 ± 142   |
| CONTROL       | 11.8 ± 1.3 | 2153 ± 522   | CONTROL       | 13251 ± 4610 | 1227 ± 317   | 11744 ± 1361| 708 ± 99    |

**F values**

| Between subjects | 4.14 * | 52.79 *** | 8.16 ** | 24.00 *** |
|------------------|--------|-----------|--------|-----------|
| Within subjects  | 3.97 ***| 20.16 *** | 16.66 ***| 24.00 *** |

* Significant at * p < 0.05; ** p < 0.01; *** p < 0.001; ns: not significant (p > 0.05).
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 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 (%) |
|-----------------|-----------------------|-----------------------------|------------------------|--------------------------|-----------------|-----------------------------|
| 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 ± 738          | 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

| 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 (%) |
|-----------------|-----------------------|-----------------------------|------------------------|--------------------------|-----------------|-----------------------------|
| 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 ± 2835         | 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

| 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 |                  |        |

* 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).
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).

**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).
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.

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 Rhodococcus 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 Methylophila, 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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References

1. Lugtenberg, B.J.J.; Chin-A-Woeng, T.F.C.;Blobom, G.V. Microbe-plant interactions: Principles and mechanisms. Antonie Van Leeuwenhoek 2002, 81, 373–383.
2. Peoples, M.B.; Herridge, D.E.; Ladh, J.K. Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production? Plant Soil 1995, 174, 3–28.
3. Giller, K.E. Nitrogen Fixation in Tropical Cropping Systems, 2nd ed.; CABI Publishing: Wallingford, UK, 2001.
4. Lu, J.; Yang, F.; Wang, S.; Ma, H.; Liang, J.; Chen, Y. Co-existence of rhizobia and diverse non-rhizobial bacteria in the rhizosphere and nodules of Dalbergia odorifera seedlings inoculated with Bradyrhizobium elkanii, Rhizobium multilotubisite like and Burkholderia pyrrophytica like strains. Front. Microbiol. 2017, 8, 2255, doi: 10.3389/fmicb.2017.02255.
5. Laranjo, M.; Alexandre, A.; Oliveira, S. Legume growth-promoting rhizobia: An overview on the Mesorhizobium genus. Microbiol. Res. 2014, 169, 2–17.
6. Vandamme, P.; Goris, J.; Chen, W.M.; De Vos, P.; Willems, A. Burkholderia tuberum sp. nov. and Burkholderia phymatum sp. nov., nodulate the roots of tropical legumes. Syst. Appl. Microbiol. 2002, 25, 507–512.
7. Benhizia, Y.; Benhizia, H.; Bonguedouar, A.; Muresu, R.; Giacomini, A.; Squartini, A. Gamma proteobacteria can nodulate legumes of the genus Hedysarum. Syst. Appl. Microbiol. 2004, 27, 462–468.
8. Glick, B.R. Plant Growth-Promoting Bacteria: Mechanisms and Applications; Hindawi Publishing Corporation: Scientific Waterford, ON, Canada, 2012.
9. Zaidi, A.; Ahmad, E.; Khan, M.S.; Saif, S.; Rizvi, A. Role of plant growth promoting rhizobacteria in sustainable production of vegetables: Current perspective. Sci. Hortic. 2015, 193, 231–239.
10. Nadeem, S.M.; Ahmad, M.; Zahir, Z.A.; Javaid, A.; Ashral, M. The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. Biotechnol. Adv. 2014, 32, 429–448.
11. Busby, R.R.; Rodriguez, G.; Gebhart, D.L.; Yannarell, A.C. Native Lespedeza species harbour greater non-rhizobial bacterial diversity in root nodules compared to the coexisting invader, L. cuneata. Plant Soil 2016, 401, 427–436.
12. Peix, A.; Ramirez-Bahena, M.H.; Velazquez, E.; Bedmar, E.J. Bacterial associations with legumes. Front. Microbiol. 2011, 2, 429–448.
13. Sharda, J.N.; Koide, R.T. Exploring the role of root anatomy in P-mediated control of colonization by arbuscular mycorrhizal fungi. Botany 2010, 88, 165–173.
14. Al-Niemi, T.S.; Kahn, M.L.; Mc Dermott, T.R. P metabolism in the bean-Rhizobium tropic symbiosis. Plant Physiol. 1997, 113, 1233–1242.
15. Shen, J.; Yuan, L.; Zhang, J.; Li, H.; Bai, Z.; Chen, X.; Zhang, W.; Zhang, F. Phosphorus dynamics: From soil to plant. Plant Physiol. 2011, 156, 997–1005.
16. Brockwell, J.; Bottomley, P.J. Recent advances in inoculant technology and prospects for the future. Soil Biol. Biochem. 1995, 27, 683–697.
17. Catroux, G.; Hartmann, A.; Revellin, C. Trends in rhizobial inoculant production and use. Plant Soil 2001, 230, 21–30.
18. Meghvansi, M.K.; Prasad, K.; Mahna, S.K. Symbiotic potential, competitiveness and compatibility of indigenous Bradyrhizobium japonicum isolates to three soybean genotypes of two distinct agro-climatic regions of Rajasthan. India Saudis J. Biol. Sci. 2010, 17, 303–310.
19. Pastor-Bueis, R.; Sánchez-Cañizares, C.; James, E.K.; González-Andrés, F. Formulation of a highly effective inoculant for common bean based on an autochthonous elite strain of Rhizobium leguminosarum bv. phaseoli, and genomic-based insights into its agronomic performance. Front. Microbiol. 2019, 10, 2724, doi:10.3389/fmicb.2019.02724.
20. Pacovsky, R.S.; Fuller, G.; Stafford, A.E. Nutrient and growth interactions in soybeans colonized with Glomus fasciculatum and Rhizobium japonicum. Plant Soil 1986, 92, 37–45.
21. Azcon, R.; Rubio, R.; Barea, J.M. Selective interactions between different species of mycorrhizal fungi and Rhizobium melliloti strains and their effects on growth, N2 fixation (15N) and nutrition of Medicago sativa L. New Phytol. 1991, 117, 399–404.
22. Tajini, F.; Trabelsi, M.; Dreven, J.J. Combined inoculation with Glomus intraradices and Rhizobium tropici CIAT899 increases phosphorus use efficiency for symbiotic nitrogen fixation in common bean (Phaseolus vulgaris L.). Saudi J. Biol. Sci. 2012, 19, 157–163.
23. Abd-Alla, M.H.; El-Enany, A.W.E.; Nafady, N.A.; Khalaf, D.M.; Morsy, F.M. Synergistic interaction of Rhizobium leguminosarum bv. viciae and arbuscular mycorrhizal fungi as a plant growth promoting biofertilizers for faba bean (Vicia faba L.) in alkaline soil. Microbiol. Res. 2014, 169, 49–58.
24. Hoeger, R. Training Papers Nitrogen Determination According to Kjeldahl; BUCHI Labortechnik AG: Flawil, Switzerland, 1998; pp. 4–10.
25. Roig, A.; Romero, M.; Lax, A.; Fernández, F.G. Estudio comparativo de métodos de determinación de capacidad de cambio catiónica en suelos calizos. Anal. Edaf. Agrobiol. 1980, 39, 2021–2032.
26. Vincent, J.M. A Manual for the Practical Study of Root Nodule Bacteria; Blackwell: Oxford-Edimburg, UK, 1970.
27. Cunniff, P. Official Methods of Analysis of AOAC International, 16th ed.; Association of Official Analytical Chemists: Washington, DC, USA, 1995.
28. Keeney, D.R.; Nelson, D.W. Nitrogen-inorganic forms. In Methods of Soil Analysis, Part 2 Chemical and Microbiological Properties; Page, A.L., Miller, R.H., Keeney D.R., Eds.; Madison, WI, USA, 1982; pp. 643–693.
29. Bedard-Haughn, A.; Van Groenigen, J.W.; Van Kessel, C. Tracing 15N through landscapes: Potential uses and precautions. J. Hydrol. 2003, 272, 175–190.
30. Unkovich, M.; Herridge, D.; Peoples, M.; Cadish, G.; Boddey, R.; Giller, K.; Alves, B.; Chalk, P. Measuring Plant Associated Nitrogen Fixation in Agricultural Systems; Clarus Design Pty Ltd.: Canberra, Australia, 2008.
31. Caporaso, J.G.; Lauber, C.L.; Walters, W.A.; Berg-Lyons, D.; Huntley, J.; Fierer, N.; Owens, S.M.; Betley, J.; Fraser, L.; Bauer, M.; et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 2012, 6, 1621–1624.
32. Žifčáková, L.; Vetrovsky, T.; Howe, A.; Baldrian, P. Microbial activity in forest soil reflects the changes in ecosystem properties between summer and winter. Environ. Microbiol. 2016, 18, 288–301.
33. Vetrovsky, T.; Baldrian, P.; Morais, D. SEED 2: A user-friendly platform for amplicon high-throughput sequencing data analyses. Bioinformatics 2018, 34, 2292–2294.
34. Aronesty, E. Comparison of sequencing utility programs. Open Bioinform. J. 2013, 7, 1–8.
35. Edgar, R.C. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010, 1, 2460–2461.
36. Edgar, R.C. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 2013, 10, 996–998.
37. Hammer, O.; Harper, D.; Ryan, P. PAST: Paleontological statistics software package for education and data analysis. Palaeontol. Electron. 2001, 4, 1–9.
38. Zahir, Z.A.; Arshad, M.; Frankenberger, W.T., Jr. Plant growth promoting rhizobacteria application and perspectives in agriculture. Adv. Agron. 2004, 81, 96–168.
39. Philippot, L.; Raaijmakers, J.M.; Lemanceau, P.; van der Putten, W.H. Going back to the roots: The microbial ecology of the rhizosphere. Nat. Rev. Microbiol. 2013, 11, 789–799.
40. Nardi, S.; Concheri, G.; Pizzeghello, D.; Sturano, A.; Rella, R.; Parvoli, G. Soil organic matter mobilization by root exudates. Chemosphere 2000, 5, 653–658.
41. Walker, T.S.; Bais, H.P.; Grootweld, E.; Vivanco, J.M. Root exudation and rhizosphere biology. Plant Physiol. 2003, 132, 44–51.
42. Alsinia, I.; Dubova, L.; Karlovskva, A., Steinberga, V.; Strauta, L. Evaluation of effectiveness of Rhizobium leguminosarum strains on broad beans. Acta Hortic. 2016, 1142, 417–422.
43. Sistani, N.R.; Kaul, H.P.; Desalegn, G.; Wienkhoop, S. Rhizobium Impacts on Seed Productivity, Quality, and Protection of Pisum sativum upon Disease Stress Caused by Didymellapinodes: Phenotypic, Proteomic, and Metabolomic Traits. Front. Plant Sci. 2017, 8, 1961.
44. Elsheikh, E.A.; Elzidany, A.A. Effects of Rhizobium inoculation, organic and chemical fertilizers on yield and physical properties of faba bean seeds. Plant Foods Hum. Nutr. 1997, 51, 137–144.
45. Massa, N.; Cesaro, P.; Todeschini, V.; Capraro, J.; Scarafoni, A.; Cantamessa, S.; Copetta, A.; Anastasia, F.; Gamalero, E.; Lingua, G.; et al. Selected autochthonous rhizobia, applied in combination with AM fungi, improve seed quality of common bean cultivated in reduced fertilization condition. Appl. Soil Ecol. 2020, 148, 103507.
46. Zhang, B.; Chang, S.X.; Angya, A.O. Mycorrhizal inoculation and nitrogen fertilization affect the physiology and growth of spring wheat under two contrasting water regimes. Plant Soil 2016, 398, 47–57.
47. Requena, N.; Perez-Solis, E.; Azcon-Aguilar, C.; Jeffries, P.; Barea, J. Management of indigenous plant-microbe symbioses aids restoration of desertified ecosystems. Appl. Environ. Microbiol. 2001, 67, 495–498.
48. Lodwig, E.M.; Hosie, A.H.F.; Bourdes, A.; Findlay, K.; Allaway, D.; Karunakaran, R.; Dowie, J.A.; Poole, P.S. Amino-acid cycling drives nitrogen fixation in the Legume-Rhizobium symbiosis. Nature 2003, 422, 722–726.
49. Scheublin, T.R.; Ridgway, P.; Van Der Heijden, M.G. Nonlegumes, legumes, and root nodules harbor different arbuscular mycorhizal fungal communities. *Appl. Environ. Microbiol.* 2004, 70, 6240–6246.

50. Pennanen, T.; Liski, J.; Baath, E.; Kitunen, V.V.; Uotila, J.; Westman, C.J. Structure of the microbial communities in coniferous forest soils in relation to site fertility and stand development stage. *Microb. Ecol.* 1999, 38, 168–179.

51. Menge, D.; Wolfl, A.; Funk, J. Diversity of nitrogen fixation strategies in Mediterranean legumes. *Nat. Plants* 2015, 1, 15064, doi:10.1038/nplants.2015.64.

52. Bottomley, D.J. Ecology of *Bradyrhizobium* and *Rhizobium*. In *Biological Nitrogen Fixation*, Stacey, G., Burris, R.H., Evans, H.J., Eds.; Chapman and Hall: New York, NY, USA, 1992; pp. 293–348.

53. Zgadzaj, R.; James, E.K.; Kelly, S.; Kawaharada, Y.; de Jonge, N.; Jensen, D.B.; Madsen, L.H.; Radutoiu, S. A legume genetic framework controls infection of nodules by symbiotic and endophytic bacteria. *PLoS Genet.* 2015, 11, e1005280, doi:10.1371/journal.pgen.1005280.

54. Martínez, J.; Negrete-Yankelevich, S.; Godinez, L.G.; Reyes, J.; Esposti, M.D.; Martínez Romero, E. Short-term evolution of rhizobial strains towards sustainability in agriculture. In *Microbial Models: From Environmental to Industrial Sustainability*; Springer Singapore, 2016.

55. Hoque, M.S.; Broadhurst, L.M., Thrall, P.H. Genetic characterization of root-nodule bacteria associated with *Acacia salicina* and *A. stenophylla* (Mimosaceae) across south-eastern Australia. *Int. J. Syst. Evol. Microbiol.* 2011, 61, 299–309.

56. Xu, L.; Zhang, Y.; Wang, L.; Chen, W.; Wei, G. Diversity of endophytic bacteria associated with nodules of two indigenous legumes at different altitudes of the Qilian Mountains in China. *Syst. Appl. Microbiol.* 2014, 37, 457–465.

57. Leite, J.; Fischer, D.; Rouws, L.F.; Fernandes-J´unior, P.I.; Hofmann, A.; Kublki, S.; Schloter, M.; Xavier, G.R.; Radl, V. Cowpea nodules harbor non-rhizobial bacterial communities that are shaped by soil type rather than plant genotype. *Front. Plant Sci.* 2017, 7, 2064.

58. Samac, D.A.; Willert, A.M.; McBride, M.J.; Kinkel, L.L. Effects of antibiotic producing *Streptomyces* on nodulation and leaf spot in Alfalfa. *Appl. Soil Ecol.* 2003, 22, 55–66.