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Efficient Rhizobium Spp. Strains in Nitrogen Fixation Enhance the Yield of Phaseolus Vulgaris Var. “Panamito” in Acidic Soils

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Rhizobia, Gram-negative soil bacteria, play a fundamental role in inducing the formation of nitrogen-fixing nodules in the roots of legumes such as *Phaseolus vulgaris*, an important plant due to its agricultural, economic, and ecological characteristics. This study evaluated the effect of *Rhizobium* spp. strains on biological nitrogen fixation in *P. vulgaris* var. “Panamito”. In a field design, seven treatments were compared: five strains of *Rhizobium* (*Rhizobium tropici* CIAT899, *Rhizobium freirei* PRF81, *Rhizobium* sp. E10, *Rhizobium* sp. Colombia, and *Rhizobium* sp. Hg), a nitrogen control (120 kg N/ha), and a non-inoculated control, with five replications each. Vegetative and production parameters were measured. Inoculation with *Rhizobium* spp. strains was effective in biological nitrogen fixation, with strain E10 achieving a yield of 1,257.50 ± 143.10 kg/ha. This yield was superior to that of the other strains and treatments, exceeding the nitrogen treatment (1,104.83 ± 84.84 kg/ha) by 13.81% and the non-inoculated control (822.31 ± 41.27 kg/ha) by 52.92%. This efficiency is attributed to the origin of the strain and its adaptability to various ecosystems, showing greater infectivity and effectiveness in nitrogen fixation in symbiosis with *P. vulgaris* var. “Panamito”. Strain E10 is recommended as an inoculant to improve common bean productivity in acidic soils in the San Martín region. This study provides a novel contribution by evaluating specific strains of *Rhizobium* spp. in acidic and low-fertility soils of Aucaloma, Peru, which could enhance agricultural production and environmental sustainability in legume crops.

1. Introduction

Biological nitrogen fixation (BNF) transforms atmospheric N_2 into NH_3, essential for the synthesis of plant biomolecules, with *Rhizobium* spp. strains being a key group that establishes symbiosis with legumes (Soumare et al., 2020). The inclusion of nitrogen-fixing organisms in crops reduces the need for fertilizers, promoting agricultural sustainability and improving crop yield and quality, especially in systems with legumes (Abd-Alla et al., 2023). More than 70 % of legumes develop symbiosis with rhizobia, with some fixing up to 200 kg of N/ha/year (Shah et al., 2021), while *Phaseolus vulgaris* can fix between 16.3 and 71.9 kg of N/ha (Kebede, 2021). However, the efficiency of this process varies due to the diversity of rhizobia species and legumes, as well as environmental conditions (Horácio et al., 2024). The cultivation of common beans in Peru encompasses the coast, highlands, and jungle regions, with an annual production of 90,497.30 t and an average yield of 1.39 t/ha. However, in the San Martín region a low yield of 0.98 t/ha was recorded in 2023 (MIDAGRI-SIEA, 2023). Improving this yield through BNF techniques, including the implementation of effective rhizobial inoculants, is important for advancing agricultural sustainability in the region. The soils of Aucaloma, where this study was conducted, have acidic pH and low fertility (Ríos-Ruiz et al., 2019), leading bean producers to use chemical products to improve fertility. The use of rhizobia is proposed as a sustainable alternative to facilitate nitrogen assimilation without adverse environmental impacts. This study evaluates specific strains of *Rhizobium* spp. in acidic and low-fertility soils in Aucaloma, Peru, highlighting their potential to improve the yield of *Phaseolus vulgaris* var. 'Panamito' and offering a sustainable solution to enhance productivity in challenging soil conditions, representing an innovation in local agricultural management.

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2. Methodology

2.1 Biological material

The *Rhizobium* spp. strains used were provided by the Microbial Ecology and Biotechnology Laboratory "Marino Tabusso" at the Universidad Nacional Agraria La Molina, Lima, and preserved in 30 % glycerol at -20 °C in the Agricultural Microbiology Laboratory "Raúl Ríos Reátegui" at the Universidad Nacional de San Martín (UNSM), Tarapoto. These strains were: 1) *Rhizobium tropici* strain CIAT899 (Colombia), widely distributed worldwide (Martínez-Romero et al., 1991); 2) *Rhizobium freirei* strain PRF81 (Brazil), effective and widely used as an inoculant for bean cultivation (Dall'Agnol et al., 2013); 3) *Rhizobium* sp. strain Colombia; 4) *Rhizobium* sp. strain E10 (Peru); and 5) *Rhizobium* sp. strain Hg (Cuba), strains that form nodules with a large number of *P. vulgaris* (Schmeisser et al., 2009). The *P. vulgaris* variety used was the "Panamito" variety, a bushy plant reaching a height of 40 to 45 cm. It is characterized by terminal inflorescences, flowers ranging in color from white to purple, and green pods with purple stripes, each containing 4 to 6 red-colored grains.

2.2 Reactivation of strains and production of the inoculum

The strains were reactivated from glycerol at -20 °C using streaking and purification on Petri dishes with Yeast Extract Mannitol Agar (YEMA). The agar comprised K_2HPO_4, yeast extract, NaCl, $MgSO_4.7H_2O$, agar, and mannitol, with pH adjusted to 6.8-7 and incubated at 28 °C for 72 hours until colonies appeared. Pre-inoculum was prepared by seeding colonies into YEMA broth and incubating at 170 rpm for 48 hours. The contents were then transferred to a larger flask to constitute the inoculum, which was incubated at 170 rpm until maximum turbidity was reached. Cell counting confirmed populations of > 5 x 10^9 CFU/mL, with 100 µL spread on each Petri dish.

2.3 Determining the most probable number of native rhizobia in experimental soil

To assess the number of native rhizobia capable of nodulating common bean, an initial decimal dilution (1:10) was performed. This involved suspending 100 g of soil in 900 mL of physiological saline solution (PSS) (0.85 %) and continuing dilutions to the fourth (1:4) by diluting 2 mL of the previous solution in 6 mL of PSS to obtain the 4^{-8} dilution. Polypropylene bags with nitrogen-free Jensen nutrient solution were utilized for seedling growth, following Somasegaran and Hoben's (1994) method. Disinfected and pre-germinated common bean seeds of the variety "Panamito" were planted in these bags and inoculated at a rate of 1 mL of seed/dilution. After 21 days in a photoperiod chamber, nodulation assessment was conducted, counting units with at least one nodule. The most probable number was calculated using nodulation evaluation tables suggested by Somasegaran and Hoben (1994).

2.4 Experiment setup

The experiment was conducted in the Aucaloma area, San Martín province, at the Academic and Agroforestry Research Center of Aucaloma of the UNSM, located at coordinates -6°26'17.55" south latitude and -76°25'28.30" west longitude, at an altitude of 333 meters above sea level. The experiment was carried out under field conditions using a completely randomized block design with seven treatments, including: five strains of *Rhizobium* (*Rhizobium tropici* strain CIAT899 from Colombia; *Rhizobium freirei* strain PRF81 from Brazil; *Rhizobium* sp. strain Colombia; *Rhizobium* sp. strain E10 from Peru; and *Rhizobium* sp. strain Hg from Cuba), a nitrogen control (120 kg N/ha), and a non-inoculated control, each treatment with five replications. The experimental area covered 822.5 m^2, with 35 experimental units (plots) measuring 4.7 m x 5 m (23.5 m^2). For data collection, an area of 1.5 m x 1.3 m (1.95 m^2) (approximately 10 plants) was designated for each treatment and replication. Chemical fertilization was carried out based on the soil analysis of the experimental area, the results of which are detailed in Table 1.

Table 1: Physical and chemical attributes of the soil of the experimental area before planting

pH	EC µs/cm	N %	OM %	P ppm	K ppm	Mechanical analysis (%) Sand	Silt	Clay	Textural class
5.86	65.5	0.1	2.71	4.2	58.1	53	19	28	Sandy loam

The physical and chemical analyses of the substrate were conducted at the Soil and Water Laboratory of the Faculty of Agricultural Sciences, Universidad Nacional de San Martín, following the methodologies described in Ríos-Ruiz et al. (2019). The parameters evaluated included electrical conductivity (EC), nitrogen (N), organic matter (OM), phosphorus (P), and potassium (K).

Nitrogen fertilization was exclusively implemented in the nitrogen treatment, applying two doses of 60 kg/ha in the form of urea ($CO(NH_2)_2$) (total of N = 120 kg/ha). The first application was carried out at 12 days after sowing

(DAS), and the second at 25 DAS. Regarding phosphorus fertilization, it was done by incorporating 260 kg/ha in the form of single superphosphate ($Ca(H_2PO_4)_2$). For potassium, 90 kg/ha were applied in two doses, at 12 and 25 DAS (total of K = 180 kg/ha), using potassium chloride (KCl) as the source.

2.5 Seed inoculation and planting

Seed inoculation involved using 200 mL of inoculum per 50 kg of seed, with both the inoculant and seeds placed in a polypropylene bag for 3 hours to enhance adherence. Seeds were then manually planted at a density of 3 seeds every 0.3 m and buried at a depth of 2 to 4 cm, with continuous manual weed control. Fungus control was achieved using Azoxyistrobin + Difenoconazole (2 mL/L), and insect control with Lambda-cyhalothrin + Thiamethoxam (1 mL/L), applied twice at 12 and 25 days after sowing (DAS). The following variables were evaluated: chlorophyll content (measured using a Konica Minolta SPAD 502 plus meter), fresh weight per plant (assessed from 10 plants), number of nodules per plant (counted from the main and secondary roots of 10 plants), number of pods per plant (evaluated from 10 selected plants), weight of 100 seeds (measured for each treatment), and yield (assessed at the end of the crop's phenological cycle at 131 days, extrapolated to kg/ha).

2.6 Statistical analysis

All data obtained for each variable were transformed to $\sqrt{x}$ and subjected to analysis of variance. Once homogeneity of variances and normal distribution of data were verified, means were compared using the Fisher's test. A significance level of $p < 0.05$ with n = 5 was used for all cases. Data were analyzed using the InfoStat 2019 software.

3. Results and discussion

3.1 Most probable number of native rhizobia

The Most Probable Number (MPN) plant infection test was used to quantify the presence of native rhizobia in the soils of Aucaloma, capable of forming nodules on the roots of common beans. The soil in the study area, classified as sandy loam with acidic pH 5.86 (Table 1), showed a native population of rhizobia capable of nodulating *P. vulgaris* var. “Panamito” at 1.6×10^3 rhizobia/g soil. Extreme soil acidity conditions can affect the formation of symbiosis between legumes and rhizobia. Legumes adapted to acidic soils have developed tolerance mechanisms to these conditions, such as the production of nod factors that differ from those produced in neutral soils (Goyal and Habtewold, 2023). Results showing a high population of rhizobia, as found by Bender et al. (2022), suggest the importance of identifying efficient strains for nitrogen fixation in legumes such as common bean (*P. vulgaris*). Although this legume can interact with a variety of rhizobia to form nodules, not all strains are equally efficient in BNF (Moura et al., 2022), highlighting the need to research and select suitable strains to improve plant yield under different environmental conditions.

3.2 Variables evaluated

3.2.1 Chorophyll content

Regarding chlorophyll content, evaluated during crop development (Figure 1), Fisher's mean comparison analysis ($p < 0.05$) (Table 2) shows that all treatments had statistically equal means, although numerically different, with treatment E10 having the highest value with an average of 39.97 SPAD. Were found non-significant results, similar to previous studies (López-Alcocer et al., 2020), but we also observed significant variations in other research (Horácio et al., 2024), indicating the influence of specific environmental factors on BNF. The chlorophyll content, which was significantly higher in the E10 strain, is a crucial indicator of plant health and productivity. Chlorophyll plays a fundamental role in photosynthesis, directly influencing biomass production (Wang and Shi, 2024).

Figure 1: Experimental area with plots of different bean crop treatments at the flowering stage

Table 2: Mean values and statistical parameters of the variables measured during the assessment of five strains of Rhizobium spp. in symbiosis with P. vulgaris, under field conditions

Treatment	Chorophyll content (SPAD)	Fresh weight/plant (g)	N° de nodules/plant	N° of pods/plant	Weight of 100 seeds (g)	Yield (kg/ha)
E10	39.97 ± 1.74a	54.70 ± 7.02a	50.50 ± 3.85a	13.16 ± 1.37a	108.65 ± 10.45a	1,257.50 ± 143.10a
Hg	38.27 ± 2.27a	49.13 ± 6.24ab	24.50 ± 2.77b	11.93 ± 1.55ab	102.91 ± 10.20a	1,225.68 ± 178.57ab
CIAT899	38.87 ± 1.29a	47.95 ± 8.27ab	25.30 ± 5.81b	10.64 ± 0.85bc	101.01 ± 9.06a	1,127.58 ± 90.75ab
Colombia	38.73 ± 2.69a	42.72 ± 6.98bc	16.60 ± 4.91c	10.31 ± 1.04bc	103.73 ± 17.45a	1,115.28 ± 101.12ab
PRF81	37.97 ± 1.04a	40.62 ± 4.41bc	15.90 ± 3.79c	10.09 ± 1.05bc	106.03 ± 6.12a	1,080.85 ± 131.27b
Nitrogen	38.47 ± 1.78a	47.56 ± 3.14ab	8.90 ± 3.02d	9.82 ± 0.89c	107.15 ± 6.59a	1,104.83 ± 84.84ab
Control	38.17 ± 1.74a	38.27 ± 8.21c	8.10 ± 2.18d	9.27 ± 1.98c	98.38 ± 17.76a	822.31 ± 41.27c
CV (%)	2.17	8.20	12.34	6.85	6.15	5.69

E10, Hg, CIAT899, Colombia and PRF81 = *Rhizobium* spp. strains; Nitrogen = 120 kg N ha $^{-1}$; Control = No inoculation; SPAD = Soil Plant Analysis Development; CV = Coefficient of variability. The data corresponds to the average of 5 repetitions. Different letters within each column indicate significant statistical variances according to Fisher's test ($p < 0.05$) (± Standard deviation).

3.2.1 Fresh plant weight

Regarding the fresh weight/plant, Fisher's mean comparison analysis ($p < 0.05$) (Table 2) reveals that treatment E10 presented the highest value with an average of 54.70 g, followed by treatments Hg, CIAT899, and Nitrogen with 49.13 g; 47.95 g, and 47.56 g, respectively. All treatments inoculated with the strains, as well as the nitrogen treatment, showed higher averages and statistically significant differences compared to the control (without inoculation), which recorded an average value of 38.27 g. Fresh weight of the bean plant was favored by all strains, although previous studies did not find significant differences, possibly due to variations in experimental environments (Granda-Mora et al., 2017).

3.2.2 Number of nodules

Regarding the number of nodules/plant, Fisher's mean comparison analysis ($p < 0.05$), detailed in Table 2, highlights that treatment E10 exhibited the highest value with a significant average of 50.50 nodules/plant. Following in order are treatments CIAT899 and Hg with 25.30 and 24.50 nodules/plant, respectively. All inoculated treatments showed higher and statistically significant values compared to the nitrogen and control treatments (without inoculation), which had average values of 8.9 and 8.1 nodules/plant, respectively.

A remarkable infective capacity was observed in all strains used under field conditions, consistent with previous findings by López-Alcocer et al. (2020). The nodules formed in the nitrogen control treatments and the uninoculated controls indicate the presence of native symbiotic rhizobia of *P. vulgaris* in the studied soils. However, their effectiveness may be lower than that of the inoculated strains, underscoring the importance of overcoming nodulation competition to achieve effective BNF (Mwenda et al., 2023). According to Maitra et al. (2023), nodule formation is essential for the assimilation of atmospheric nitrogen, reducing the need for nitrogenous fertilizers and enhancing agricultural sustainability.

3.2.3 Number of pods

The Fisher's mean comparison analysis ($p < 0.05$) corresponding to the number of pods/plant (Table 2) reveals that treatment E10 presented the highest value with an average of 13.16 pods/plant, followed by treatments Hg, CIAT899, Colombia, and PRF81 with 11.93; 10.64; 10.31, and 10.09 pods/plant, respectively. All treatments inoculated with the strains showed higher and statistically different averages compared to the nitrogen and control treatments (without inoculation), which had average values of 9.82 and 9.27 pods/plant, respectively.

The notable increase in the number of pods per plant observed in the treatment inoculated with the E10 strain coincides with similar findings in other studies. For instance, Cantaro-Segura et al. (2019) found significant differences when evaluating four bean varieties and two rhizobial strains compared to non-inoculated treatments. Similarly, Horácio et al. (2024) examined the interaction between six common bean cultivars, specifically of the carioca variety, and rhizobial strains, observing a higher number of pods in the inoculated treatments compared to the non-inoculated ones. This increase may be attributed to better assimilate accumulation due to the plants' ability to obtain nitrogen from symbiotic bacteria (Assegid and Abera, 2023).

3.2.4 Fresh weight of 100 seeds

Regarding the fresh weight of 100 seeds, the Fisher's mean comparison analysis ($p < 0.05$) (Table 2) shows that all treatments had statistically equal averages but numerically different, with treatment E10 presenting the highest value with an average of 108.65 g compared to the control (without inoculation) which had an average value of 98.38 g. Although there were no significant differences in the fresh weight of 100 seeds among the treatments, a positive effect on the overall crop was observed.

3.2.5 Yield

Regarding the yield, the Fisher's mean comparison analysis ($p < 0.05$) (Table 2) reveals that treatment E10 exhibited the highest yield with an average of 1,257.5 kg/ha; followed by treatments Hg; CIAT899; Colombia, and Nitrogen, with 1,225.68 kg/ha; 1,127.58 kg/ha; 1,115.28 kg/ha, and 1,104.83 kg/ha, respectively. All treatments inoculated with the strains and the nitrogen treatment showed higher and statistically different averages compared to the control (without inoculation), which had an average yield of 822.31 kg/ha.

The yield results demonstrate significant improvements in the inoculated treatments and the nitrogen control, with the inoculated treatment with the E10 strain showing a remarkable 52.92 % increase in yield compared to the non-inoculated control and a 13.81 % increase compared to the nitrogen control. These findings are consistent with other studies, such as Cantaro-Segura et al. (2019), who observed that inoculated treatments reached 90 % of the yields obtained with nitrogen fertilizers. Furthermore, Wekesa et al. (2023) investigated the interaction between common beans and rhizobia, finding that *Rhizobium Phaseoli* showed the capacity to substitute nitrogen fertilizers. This suggests that inoculation with rhizobial strains can significantly enhance agricultural productivity and reduce dependency on chemical nitrogen fertilizers. The ability of the strains used in this study to tolerate soil acidity and promote a healthy symbiosis with *P. vulgaris* highlights their potential to improve soil fertility and increase agricultural productivity, especially in acidic and degraded soils like those in the study area. The results obtained by Ríos-Ruiz et al. (2024) reinforce these observations, as the authors identified rhizospheric bacteria from legumes capable of solubilizing inorganic phosphates such as aluminium phosphate and iron phosphate. The study demonstrates that these bacteria not only improve phosphorus availability in acidic soils but also contribute to crop health and productivity.

4. Conclusions

Inoculation with strains of *Rhizobium spp.* proved to be efficient in promoting biological nitrogen fixation in *Phaseolus vulgaris* var. “Panamito” under field conditions, with the E10 strain significantly standing out in the evaluated parameters. This finding underscores its potential as an inoculant to enhance the productivity of common beans in acidic and low-fertility soils, surpassing both other strains and treatments with nitrogen fertilization. The manuscript highlights the importance of carefully selecting *Rhizobium* strains that are adapted to specific soil conditions, such as acidity, to maximize the benefits of nitrogen fixation and promote more sustainable agricultural practices, thereby reducing dependence on chemical fertilizers.

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