Journal of Tropical Forest Science 37(2): 238–253 (2025)
https://doi.org/10.26525/jtfs2025.37.2.238
ISSN: 0128-1283, eISSN: 2521-9847

MOLECULAR IDENTIFICATION OF NITROGEN-FIXING BACTERIA AND THE EFFECT OF INOCULATION ON TROPICAL FOREST LEGUME SPECIES

Zuñiga-Orozco A[1, 2], Solis-Ramos L[3], Hernández-Gómez L[4] & Rojas-Jiménez K[5,*]

[1] *School of Biology, University of Costa Rica, San Pedro, Montes de Oca, P.O Box 11501-2060, San José, Costa Rica*
[2] *Distance State University, San Pedro, Montes Oca, P.O Box 474-2050, San José, Costa Rica*
[3] *School of Biology and Biotechnology of Plants and Arbuscular Mycorrhizal Fungi (Biotec-PYHMA), University of Costa Rica, San Pedro, Montes de Oca, P.O Box 11501-2060, San José, Costa Rica*
[4] *Natural Products Research Center (CIPRONA), University of Costa Rica, San Pedro, Montes de Oca, P.O Box 11501-2060, San José, Costa Rica*
[5] *School of Biology and Laboratory of Genetics and Ecology of Microorganisms (LEGMi), University of Costa Rica, San Pedro, Montes de Oca, P.O Box 11501-2060, San José, Costa Rica*

**keilor.rojas@ucr.ac.cr*

Submitted August 2024; accepted November 2024

Studies on the diversity of nitrogen-fixing bacteria, particularly in tropical forest species, has been relatively less compared to other ecosystems. The aims of this study were to isolate bacteria from nodules of eight tropical forest leguminous species belonging to two subfamilies of Fabaceae (Caesalpinioideae and Papilionoideae), carry out molecular identification based on 16S rRNA gene sequencing, and evaluate their plant growth-promoting effects in the same hosts. Growth-promoting responses were determined by evaluating plant seedlings with and without inoculation of the bacteria in their respective hosts. Five-month-old seedlings were measured for the following variables: height, stem thickness, number of leaves, vigor, root length, root weight, number of nodules, leaf area, microbial respiration, and nitrogenase activity. We also quantified nutrient content in leaves and roots. The bacterial isolates were identified as *Rhizobium miluonense*, *Rhizobium multihospitium*, *Bradyrhizobium japonicum*, and *Bradyrhizobium* sp. All morphological variables were higher in inoculated seedlings compared to the uninoculated seedlings ($p < 0.05$), except for microbial respiration and root length. The Caesalpinioideae subfamily responded better to the inoculant than the Papilionoideae subfamily ($p < 0.05$). *Enterolobium cyclocarpum*, *Cojoba arborea* and *Dalbergia retusa* had higher nutrient levels at foliar and root levels when inoculated. This study provided insights into the potential of developing inoculants based on nitrogen-fixing bacteria, many of which are yet to be explored, for agriculture and reforestation with leguminous species.

Keywords: Tropical leguminous trees, biological nitrogen fixation, *Rhizobium*, *Bradyrhizobium*, biofertilisers, bacterial inoculants, nitrogenase

INTRODUCTION

Within the soil ecosystem, a vast and undervalued array of microorganisms engage in diverse interactions with plants. Among these intricate relationships are the symbiotic bacteria that colonise the roots of leguminous plants (Fabaceae). These bacteria thrive through nutrient acquisition directly from the host plant, providing a significant enhancement in the plant's capacity to absorb essential elements, with a particular emphasis on nitrogen (Moreira 2008). The incorporation of nitrogen into the soil via leguminous plants yields substantial economic benefits for agriculture, as reported by studies documenting contributions ranging from 60 to 300 kg per hectare of nitrogen (Céspedes et al. 2019).

Advances in molecular biology and genetics have facilitated a comprehensive exploration of genes related to infection and symbiotic interaction between soil bacteria and legumes. Researchers have delved deeper into the intricate molecular mechanisms that govern bacterial infections of plants, particularly rhizobia (Bulgarelli et al. 2013, Roy et al. 2020).

Mounting evidence suggests that the identity of the symbiotic bacterium present at a given time can determine whether it exhibits a generalist or specialist role, contingent upon the host plant species. Such variability in bacterial specificity has demonstrable impacts on the growth and productivity of the host plant. Notably, research has implicated nodule-specific cysteine-rich (NCR) peptides in mediating this discriminatory process (Downie & Kondorosi 2021). In a tropical country such as Costa Rica, symbiotic bacterial species have been identified in relation to agronomical species of legumes (Acuḥa 1996, Acuḥa & Uribe 1996). However, there is comparatively less knowledge about species diversity in the agroforestry area (de Bedout et al. 2022). To better characterise nitrogen-fixing symbionts, it is necessary to study soil microbiome and use techniques such as 16S rRNA gene sequencing.

In microbial diversity studies, gas chromatography is commonly used to quantify the response to inoculation and nitrogenase activity. Nitrogenase activity is based on the ability of the enzyme to reduce acetylene (C_2H_2) to ethylene (C_2H_4) (Bonaldi et al. 2011, Lamel & Cruz 2013, Ladestam et al. 2020, Bünger et al. 2021, Senthilkumar et al. 2021, Ma et al. 2022). Studies have reported that bacterial inoculation with rhizobia can improve morphometric variables and/or nitrogenase activity in different plant species (Acuḥa & Uribe 1996, Bonaldi et al. al. 2011, Lamel & Cruz 2013, Ladestam et al. 2020, Bünger et al. 2021, de Castilho et al. 2021, Simbine et al. 2021, Wyse et al. 2021, Ma et al. 2022, Nguyen et al. 2022).

The main objective of this work was to identify symbionts of eight forest species commonly used in Costa Rica. Additionally, we aimed to evaluate the effect of inoculation on plant growth and nitrogen fixation. The results obtained from this study will contribute to the identification of nitrogen-fixing rhizobia that can potentially be used as biofertilisers for the agroforestry sector.

MATERIALS AND METHODS

Isolation of bacteria from nodules

For the isolation of bacteria from root nodules, we used nursery plants of 30–70 cm height, aged 1–3 years, from two sources: CODEFORSA forest nursery (Commission for Forest Development of San Carlos) and the nursery of the School of Environmental Sciences from the National University in Costa Rica. Eight important forest species were selected, all belonging to two subfamilies of Fabaceae. The Caesalpinioideae subfamily included *Cojoba arboreum*, *Enterolobium cyclocarpum* and *Samanea saman*. The Papilionoideae subfamily included *Erythrina poepiggiana*, *Erythrina fusca*, *Platymiscium pinnatum*, *Dyphisa americana* and *Dalbergia retusa*.

To examine the presence of root nodules, plant roots were manually examined and extracted along with a part of the attached root. These nodules were washed with water, placed on paper napkins and immediately brought to the laboratory in an ice box. Once in the laboratory, the nodules were placed in 2 mL eppendorf tubes and washed twice with autoclaved distilled water. They were then superficially disinfected with 70% ethanol for 1 min and washed twice with distilled water. Finally, they were disinfected again with 2% sodium hypochlorite (NaClO) for 5 min and washed seven times with distilled water.

After disinfection, the nodules were transferred to eppendorf tubes containing 500 uL PY medium (5 g L^{-1} peptone, 1 g L^{-1} anhydrous dextrose, 0.5 g L^{-1} dipotassium phosphate, 0.2 g L^{-1} magnesium sulfate, 0.1 g L^{-1} sodium chloride) according to Zhurbenko et al. (2006). The nodules were macerated with autoclaved plastic pestles. Subsequently, the supernatant was serially diluted in 10^{-1} and 10^{-2} concentrations. From each dilution, 50 uL was plated on petri dishes of PY-agar medium with antifungal (5 g L^{-1} peptone, 1 g L^{-1} anhydrous dextrose, 0.5 g L^{-1} dipotassium phosphate, 0.2 g L^{-1} magnesium sulfate, 0.1 g L^{-1} sodium chloride, 15 g L^{-1} agar, cyclohexamide 40 mg L^{-1}). The plates were incubated for up to three weeks until colonies appeared. Once colonies appeared, they were replicated on new plates of the same medium. The isolated strains were preserved in PY+glycerol 20% medium and stored at -80 °C in the collection of the LEGMi at the School of Biology of the University of Costa Rica.

DNA extraction and molecular characterisation

The genomic DNA of the rhizobia strains was isolated using the DNEasy Powersoil kit® from

QIAGEN (Carlsbad, CA, United States) following the manufacturer's instructions. The 16S rRNA gene was amplified by PCR in a T100 thermocycler (BioRad®) from total DNA extracts using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′). The reactions were carried out with the following temperature program: 98 °C for 2 min followed by 35 cycles of 96 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s with 10 min for extension at 72 °C (James 2010). The amplification of the products was verified by electrophoresis in a 1% agarose gel for 30 min at 90 v and 200 mA. Sanger-type sequencing was performed by Macrogen (South Korea).

Sequences were assembled and edited using BioEdit® and MEGA®. Taxonomic assignments were made by sequence comparisons in several databases including SILVA, NCBI, BacDive, GBIF, and PANGAEA. Once the strains were identified, a phylogenetic tree was generated using MAFFT v7 tool (https://mafft.cbrc.jp/alignment/server/) to align the sequences. A maximum likelihood phylogenetic tree was generated using Archeopteryx software with 1000 Bootstrap replicates under the Hasegawa–Kishino–Yano (HKY) model of evolution.

Selection and inoculation of bacteria

After sequencing and molecular identification, the prevalent bacterium identified from each forest species was chosen for inoculation and quantification of the responses. Before inoculation, seeds of each forest species were sown between February and March 2022. The seeds were treated with 1% NaClO for 1 min, followed by five washes of distilled water. Subsequently, they were submerged in water for 8 hours and put them to germinate in semi-hermetic plastic containers with moistened filter paper in the dark. Humidity was checked every 3 days.

Fifteen days after sowing the seeds, a germination count was carried out and the seeds were placed in previously autoclaved sterile soil of the Usteps Andisol type in 0.5 L pots with a perforated black plastic bag. Soil was sterilised for 12 hours at 100 °C in a 250 L steriliser (LPV ®). In these pots, 10 embryos were grown for each forest species to be inoculated with the bacteria and 10 embryos without inoculation. A total of 160 plants were used. The average agroclimatic conditions of the experimental site (Dulce Nombre, Cartago, Costa Rica), according to the nearest meteorological station (Technological Institute of Costa Rica, TEC) for February–September 2022 were temperature range of 18.1–20.5 °C (19.3 °C on average), accumulated precipitation range of 36.7–211.7 mm (959 mm on average), and relative humidity range of 86–90% (87.5% on average).

Seven days before inoculating, each bacterium was cultured in a 0.5 L erlenmeyer containing 0.2 L of PY medium (without agar). The bacteria were grown under shaking at 100 rpm for 3–7 days at 23 °C in a multiple orbital shaker until reaching an OD of 1 at 600 nm (Osei et al. 2018). At the time of inoculating the embryos, they were planted in black plastic bags measuring 25 x 25 cm (1 L) in a shade house with double-layer saran that allowed 70% green light penetration. Once the bacterial suspension was obtained, a volume of 1 mL was applied to each seeded embryo using a syringe. As the plants grew, the percentage of shade was reduced to full sunlight. The treatments carried out were shown in Table 1, following strain codes given during bacterial isolation.

Morphometric variables

Once the plants began to grow, the germination percentage was measured by counting the number of seedlings that emerged versus the number of seedlings planted (15 days after germination). At five months of age, the following morphometric variables were quantified: height (cm), stem thickness (mm), number of leaves (units), vigor (1–5 scale: 1 being the smallest and 5 being the largest), length of root (cm), root weight (g), number of nodules (units) and leaf area (cm^2). The leaf area was determined by analysing photographic images using Bioleaf® and LeafArea® software.

Foliar nutrient analysis

To determine the contents of chemical elements in the composition of each species and treatment, three replicates of the foliar tissue and three replicates of the root tissue were taken. Fresh tissue samples were sent to the CIA Lab at University of Costa Rica for atomic absorption spectroscopy analysis.

Table 1 Description of the treatments employed using symbiotic nitrogen-fixing bacteria in the inoculation of leguminous forest species

Treatment	Forest species	Subfamily	Bacterial strain	No. of replicates
1	*Erythrina poepiggiana*	Papilionoideae	With inoculation of 163b* strain	10
2			Without inoculation (control)	10
3	*Erythrina fusca*	Papilionoideae	With inoculation of 163b* strain	10
4			Without inoculation (control)	10
5	*Cojoba arborea*	Caesalpinioideae	With inoculation of 12c* strain	10
6			Without inoculation (control)	10
7	*Platymiscium pinnatum*	Papilionoideae	With inoculation of 119d* strain	10
8			Without inoculation (control)	10
9	*Enterolobium cyclocarpum*	Caesalpinioideae	With inoculation of 20j* strain	10
10			Without inoculation (control)	10
11	*Samanea saman*	Caesalpinioideae	With inoculation of 24b* strain	10
12			Without inoculation (control)	10
13	*Dyphisa americana*	Papilionoideae	With inoculation of 118c* strain	10
14			Without inoculation (control)	10
15	*Dalbergia retusa*	Papilionoideae	With inoculation of 15a* strain	10
16			Without inoculation (control)	10

*strains were encoded at the beginning of the experiment

Microbial respiration

Microbial respiration was recorded 5 months after sowing by quantifying CO_2 levels using an IRGA chamber (IRGA, LI-COR® Biosciences) with a range between 0–10000 ppm. The CO_2 concentration was measured every 4 s for a period of 15 min to obtain an average data per plant. The measurements were made 24 hours at an average temperature of 20 °C. A shade was used over the chamber to prevent light interference with the measurement.

Nitrogenase activity

Each root was washed, weighed and placed into 60 mL vials. A septum type screw cap was used to seal the vials and allow the entry of a 20 mL 22–24-gauge syringe. To measure the reduction of acetylene to ethylene by means of the nitrogenase enzyme, 10% air was replaced with acetylene according to the methods of Hardy et al. (1973) and Senthilkumar et al. (2020). A gas chromatograph (GC) coupled to a mass spectrometer (Shimadzu GC-2014) was used.

To identify the response time to form ethylene and produce a calibration curve, *Lupinus costaricensis* D.B Bunn (Fabaceae) plants of about 3 months old nodulated in the field were used. Nine plants with different degrees of nodulation were taken, and three replicates were quantified at 1, 24, 48, 72, and 96 hours after injection. From the above data, it was determined that the optimum period for measurement was 72 hours.

Statistical analysis

Evaluation of the inoculation experiment was carried out in an unrestricted randomised design with 10 replicates per treatment. To evaluate the normality of the data and its distribution, the Shapiro-Wilk and Kolmogorov-Smirnov tests were applied to determine if the data followed a normal distribution. Due to non-normality of the data, a Wilcoxon-Man-Whitney test was performed with $\alpha = 0.05$ (95% reliability) for the morphometric variables, microbial respiration by CO_2 (ppm), and nitrogenase activity (µmol N_2 h^{-1}). Rstudio® version 4.2.2 and Infostat® version 2020 software were used for data analysis. Finally, a Pearson correlation test was performed to determine if there was a correlation between variables.

RESULTS

Identification of bacterial strains

The bacterial isolates were assigned to three species and two genera from the phylum Pseudomonata, class Alphaprotobacteria, order Hyphomicrobiales, families Rhizobiaceae (*Rhizobium miluonense*, *Rhizobium multihospitium*) and Bradyrhizobiaceae (*Bradyrhizobium japonicum* and *Bradyrhizobium* sp.). It was not possible to identify three bacterial strains at species level. However, it was possible to verify that they belong to the genus *Bradyrhizobium* (Table 2). In Figure 1 we show the taxonomy of the isolates according to their phylogenetic relationship.

Response to inoculation

All morphometric measurements determined for the seedlings of the eight tropical forest tree species, except for root length and microbial respiration, showed significant differences ($p < 0.05$) in the response to inoculation (Figure 2). The morphometric measurements, microbial respiration and nitrogenase activity grouped by species are shown in Table 3. Each species showed different response to inoculation. In general, *E. cyclocarpum*, *S. saman* and *E. fusca* presented higher values when they were inoculated in six out of ten significant variables measured ($p < 0.05$). *Cojoba arborea* and *D. retusa* presented four variables with higher values when the bacterial inoculant was applied. *Erythrina poepiggiana*, *D. americana* and *P. pinnatum* obtained three, two and one variables with significantly different values, respectively. When grouping of the species was done by subfamilies, it was observed that both subfamilies responded to the bacterial inoculation. However, the Caesalpinioideae subfamily presented a better response to inoculation in most of the variables compared to the Papilionaceae subfamily ($p < 0.05$) (Figure 3).

Table 2 Relationship between bacteria-host found in eight legume forest species and previously reported hosts

Bacteria	Host/s	Observation	Hosts reported
R. miluonense	*S. saman*	With a percentage of similarity of 99.57% with *R. miluonense* Sr28bT (MW958076.1). Its genome size is approximately 6.81 Mbp (Hḍrdt et al. 2020).	*Lespedeza* spp. (Gu et al. 2018), *Inga laurina* (Salles et al. 2015), *Phaseolus vulgaris* (Oliveira et al. 2017), *Milletia pinnata* (Arpiwi et al. 2012), *Clitoria brachystegia* (Soto-Valenzuela et al. 2020) and *Arachis hypogaea* (Nguyen et al. 2022).
R. multihospitium	*C. arborea*	With a percentage of similarity of 99.89% with *R. multihospitium* J5R5RIT (MT409548.1). Its genome size is approximately 7.32 Mbp (Hḍrdt et al. 2020).	*Lotus frondosus, L. tenuis, Alhagi toum, Astragalus aksuensis, A. betetovii, Halimodendron halodendron, Oxytropis meinshausenii, O. glabra, Robinia pseudoacacia, Sophora alopecuriodes, Caragana jubata, Lathyrus odoratus, Vicia hirsuta* (Han et al. 2008), *Aeschynomene aspera* (Mir et al. 2021), *Clitoria brachystegia* (Soto-Valenzuela et al. 2020) and *Arachis hypogaea* (Nguyen et al. 2022).
B. japonicum	*E. fusca*, *E. poepiggiana* & *D. americana*	Isolates obtained a similarity percentage of 100% with *B. japonicum* YC8T (MN173253.1) and 99.91% with *B. japonicum* GI-4T (LC386869.1). Its genome size is approximately 9.21 Mbp (Hḍrdt et al. 2020).	*Glycine max*, *Glycine soja*, *Vigna radiata*, *Macroptilium atropurpureum* (Chun et al. 1994), *Vigna unguiculata* (Gḍttferd et al., 1990), *Clitoria brachystegia* (Soto-Valenzuela et al. 2020) and *Acacia albida* (Dupuy et al. 1994)
Bradyrhizobium sp.	*E. cyclocarpum*, *D. retusa* & *P. pinnatum*	Isolates obtained between 99.78–100% similarity with *Bradyrhizobium* sp. (accessions AB933533.1, AY603956.1 and AB933529.1).	This genus has been recorded in herbaceous and woody species, in temperate and tropical environments, as well as in various ecological niches (Vinuesa et al., 2003). They have a diverse metabolism in which they can carry out denitrification processes and growth promoters in legumes and even in non-legumes (Bedmar et al. 2005).

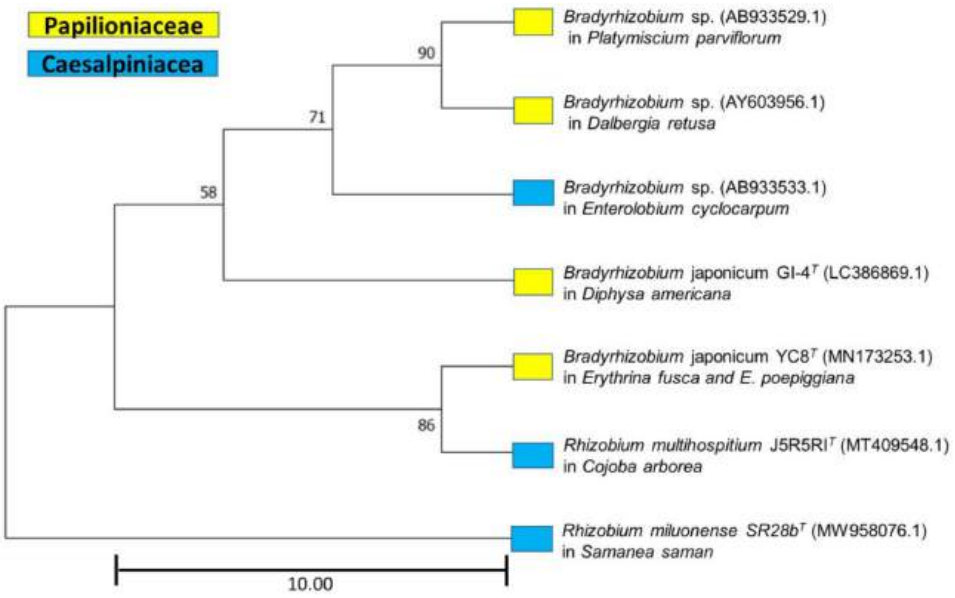

Figure 1 Phylogenetic relationship of bacterial strains isolated from different leguminous forest plant species of Costa Rica and identified with the 16S rRNA marker gene

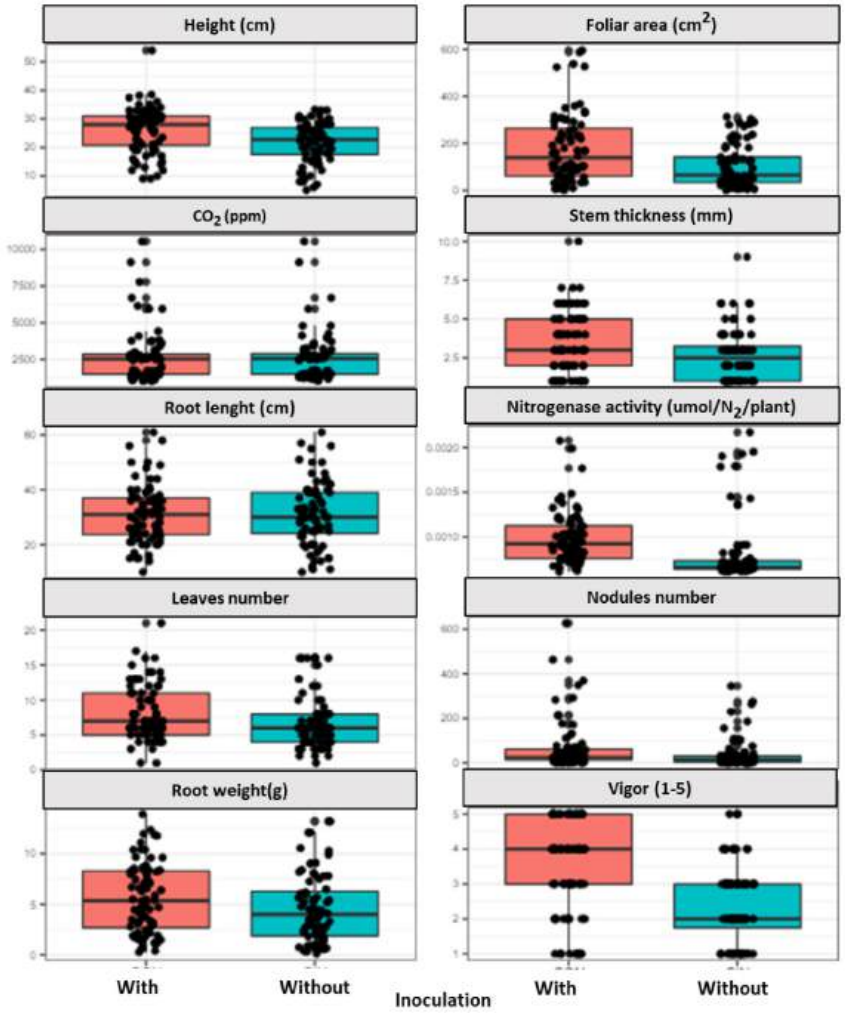

Figure 2 Boxplots of morphometric measurements by treatment, determined for seedlings of eight tropical forest legume species with and without application of bacterial inoculants (differences at $p < 0.05$ Wilcoxon-Man-Whitney)

Table 3 Values of means of germination percentage, survival percentage, morphometric measurements, nitrogenase activity and microbial respiration evaluated at five months after sowing in the leguminous forest species studied in Costa Rica, under inoculation and no inoculation (different letters indicate $p < 0.05$ Wilcoxon-Man-Whitney)

Species	Treatment	% germ.	% surv.	Height (cm)	Stem thickness (mm)	Leaves number	Vigor (1-5)	Nodules number	CO_2 (ppm)	Root length (cm)	Root weight (g)	Foliar area (cm^2)	Nitrogenase activity (µmol N_2 hr^{-1} $plant^{-1}$)
Eryhtrina fusca	WITH	100	100	32.47 a	6.2 a	5.1 a	4.5 a	19.4 a	2329 a	25.9 a	9.8 a	267.48 a	0.97 a
	WITHOUT	100	100	25.27 b	5.4 b	3.7 b	2.7 b	4.7 b	2221 a	23.0 a	9.7 a	209 a	0.81 b
Enterolobium cyclocarpum	WITH	100	100	35.45 a	3.5 a	8.8 a	3.6 a	82.0 a	2300 a	25.6 a	6.6 a	66.6 a	1.31 a
	WITHOUT	100	100	25.51 b	3.0 a	6.6 b	2.2 b	21.3 b	2286 a	22.3 a	3.9 b	41.5 a	0.7 b
Cojoba arborea	WITH	100	100	29.7 a	3.1 a	5.7 a	3.8 a	307.8 a	2373 a	37.7 a	6.1 a	381.9 a	1.17 b
	WITHOUT	96	100	26.5 a	2.6 a	4.7 a	2.5 b	177.0 b	2318 a	35.5 a	4.9 a	171.6 b	1.58 a
Erythrina poepiggiana	WITH	98	100	25.5 a	5.9 a	4.9 a	3.7 a	24.5 a	2676 a	39.8 a	7.7 a	193.5 a	0.86 a
	WITHOUT	98	100	23.2 a	3.7 b	4.1 a	2.9 a	18.0 a	2569 a	35.6 a	7.14 a	121.8 b	0.68 b
Diphysa americana	WITH	95	100	28.0 a	1.9 a	14.7 a	3.2 a	25.6 a	2365 a	37.6 a	2.32 a	81.1 a	0.83 a
	WITHOUT	95	100	25.1 a	1.6 a	14.3 a	2.8 a	20.8 a	2326 a	35.0 a	1.69 a	46.6 b	0.69 a
Dalbergia retusa	WITH	100	100	18.8 a	1.7 a	7.8 a	3.1 a	48.8 a	4187 a	40.2 a	2.6 a	114.4 a	0.93 a
	WITHOUT	96	100	13.4 b	1.4 a	6.1 b	2.2 a	25.0 b	2429 a	32.5 a	1.78 a	72.9 a	0.64 b
Samanea saman	WITH	100	100	26.4 a	3.8 a	12.1 a	3.8 a	15.2 a	3346 a	31.3 a	7.2 a	256.2 a	0.98 a
	WITHOUT	100	90	20.9 b	2.4 b	8.3 b	2.3 b	13.3 a	4187 a	34.6 a	5.0 a	120.2 b	0.67 b
Platymiscium pinnatum	WITH	65	70	12.9 a	1.7 a	4.9 a	2.4 a	1.4 a	2212 a	19.6 a	1.2 a	28.2 a	0.77 a
	WITHOUT	55	60	7.2 b	1.0 a	4.0 a	1.3 a	0.0 b	2588 a	14.8 a	0.5 a	7.4 a	0.62 b
		Standard error		0.6	0.1	0.3	0.1	7.6	139.4	0.03	0.9	0.3	10.2

Results from the chemical analysis of tissues (foliar and root) showed that the chemical elements presented significant differences in some species when they were inoculated compared to those not inoculated, and this occurred both in leaves and roots (Wilcoxon-Mann-Whitney, $p < 0.05$). *Cojoba arborea* presented higher content of B in the leaf and of P, K, Mg, S, B, Cu and Fe in the root. *Enterolobium cyclocarpum* obtained higher amounts of N and S in the leaf, as well as N, S, Mn, Zn, B, Cu and Fe in the root. *Dalbergia retusa* obtained a greater amount of Mg in leaf, while in the root the majority of B, Cu and Fe were present (Figures 4 and 5). Four species (*S. saman, E. fusca, D. americana* and *E. poepiggiana*) did not present significant differences in terms of nutrient content, both in leaf and foliage, when they were inoculated. Samples from *P. pinnatum* could not be statistically analysed due to the small amount of tissue that was obtained due to its very slow growth. However, both in leaf and root, a higher content of N, P, K, Mg, Ca, S, B, Zn and Mn was observed in inoculated plants than in those

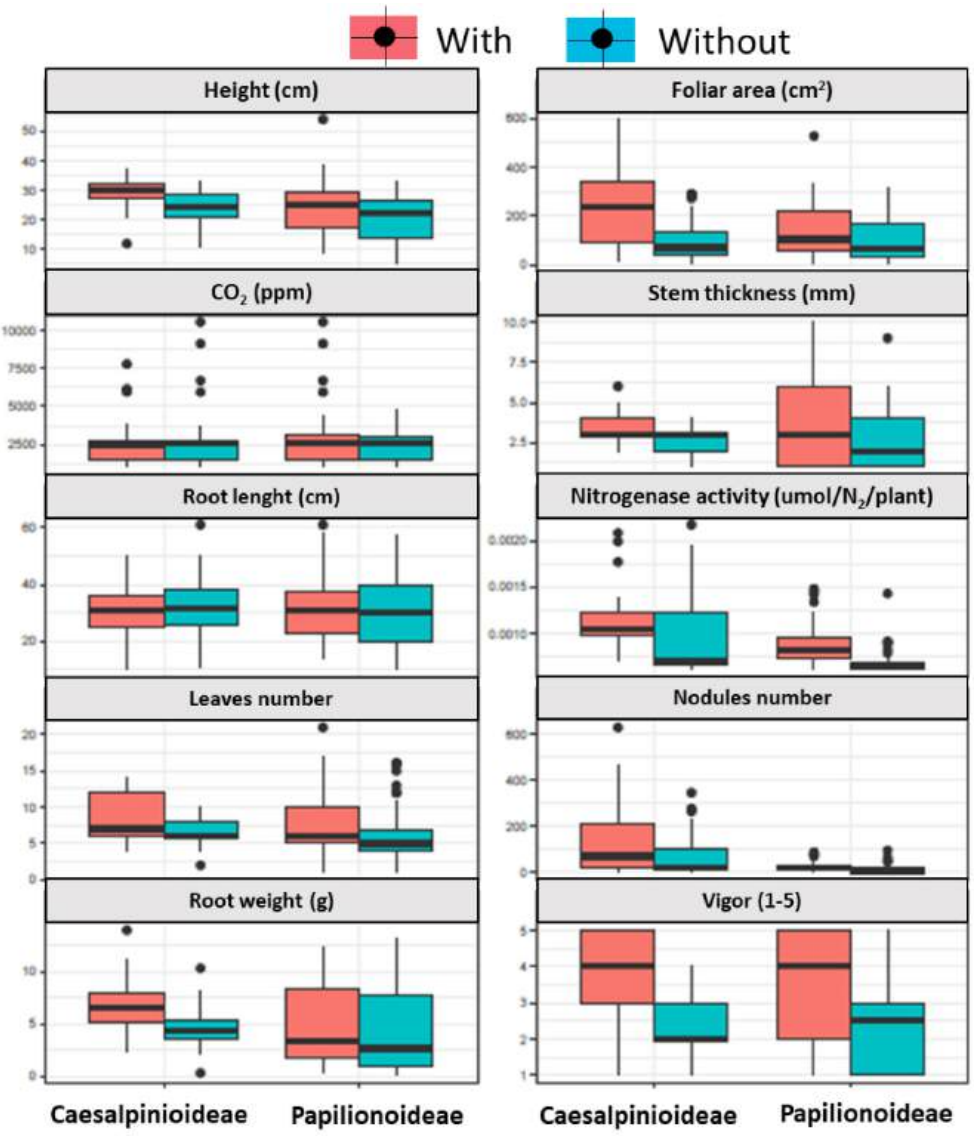

Figure 3 Boxplot of morphometric measurements, nitrogenase activity and microbial respiration by subfamily evaluated at five months after sowing in the leguminous forest species studied in Costa Rica, under inoculation and no inoculation. Grouped by subfamily (differences at $p < 0.05$ Wilcoxon-Man-Whitney

that were not inoculated (Figures 4 and 5). Additionally, high levels of Fe were found in the root of inoculated plants (Figure 5).

When grouping of the species was done by subfamily, Caesalpinioideae presented higher nutrient content both in leaf and root compared to Papilionoideae. A greater amount of P, S and Fe was obtained in leaf, as well as N, S, Mg, B, Zn, Fe and Mn in root. In Papilionoideae, only the Mg level was higher than in the Caesalpinioideae subfamily (Figure 6).

We identified some correlations between the variables (Table 4). Among those variables, the highest correlation values (> 0.7) were between height with vigor, and root weight with stem thickness. Among those of intermediate correlation values (0.4–0.7) were between leaf area with multiple variables, including height, stem thickness, vigor, number of nodules and root weight. Intermediate correlation was also seen between stem thickness with vigor and height. The rest showed smaller correlation values (<0.4).

DISCUSSION

Bacterial candidates with suitable characteristics were identified for the development of a multispecies biofertiliser. These bacterial species have been reported in other hosts different from those studied in this work, which broadens the spectrum of hosts in which they are present and produce beneficial growth effects in forest tree species commonly used in Costa Rica. *Rhizobium miluonense, R. multihospitium* and *B. japonicum* had been recorded in association with many plant species, indicating these bacterial species as being a generalist. *Bradyrhizobium japonicum* is widely used for soybean production and its complete genome has been sequenced (Hḍrdt et al. 2020; Kaneko et al. 2002). The identified bacterial species are possibly the first records of presence in the plant species used in this study. For the unidentified *Bradyrhizobium* species, it is recommended to use other molecular markers such as recA, atpD, rpoB, gyrB and dnaK or the

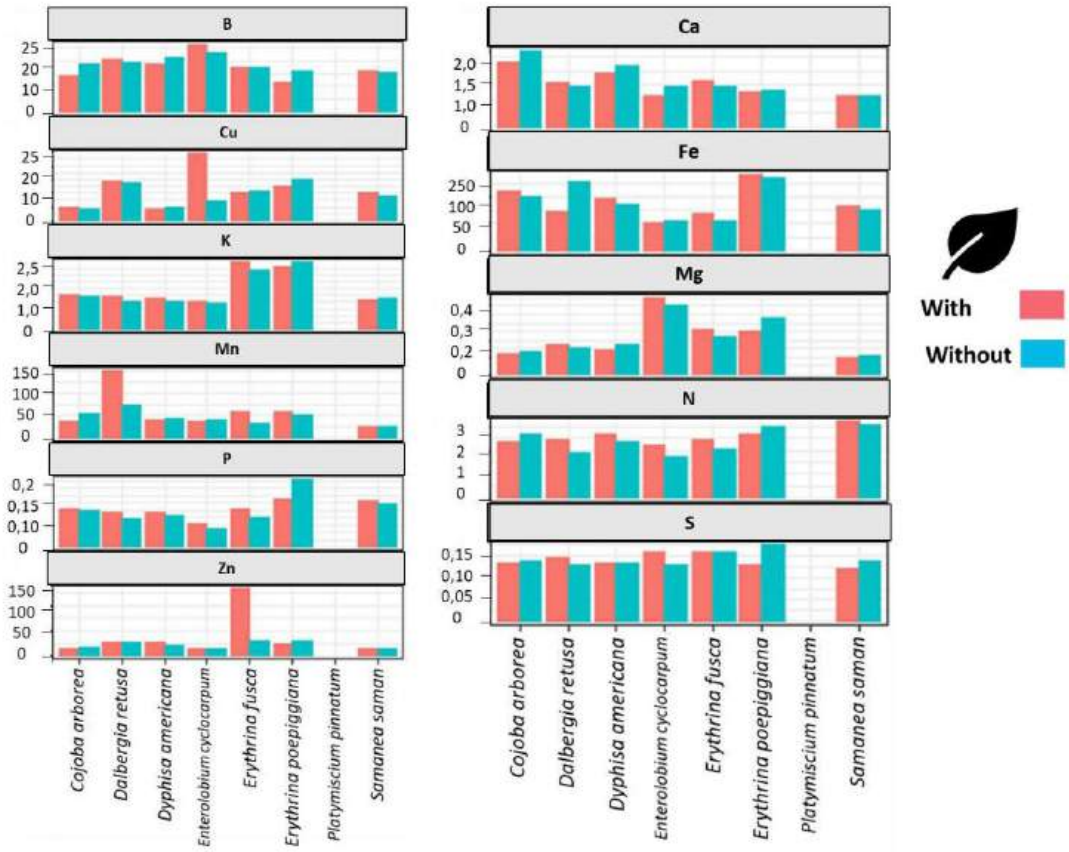

Figure 4 Nutrient content in leaf ( ) evaluated in leguminous forest species studied in Costa Rica with inoculation (red) and no inoculation (blue) at five months after sowing (differences at $p < 0.05$ Wilcoxon-Man-Whitney

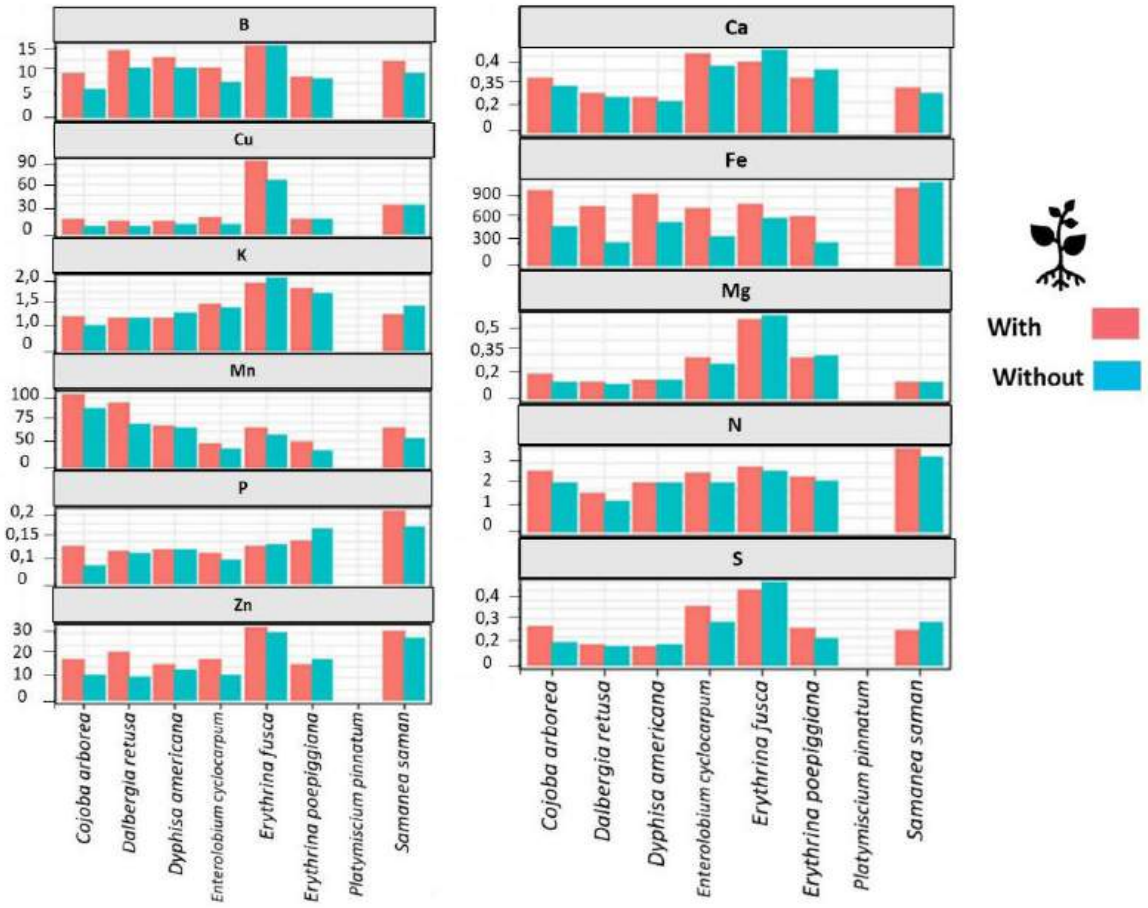

Figure 5 Nutrient content in root ( ) evaluated in leguminous forest species studied in Costa Rica with inoculation (red) and no inoculation (blue) at five months after sowing (differences at $p < 0.05$ Wilcoxon-Man-Whitney)

whole genome sequence to determine their identity (Rivas et al. 2009).

Analysis on nitrogenase activity showed that there was a correlation between the number of nodules and the activity of the enzyme, with the inoculated treatments generally having the highest activity. The above evidence indicated of a cause-effect relationship where the inoculation increased the number of nodules, and this in turn, increased the magnitude of nitrogen fixation. An enzyme activity of 0.62–1.58 µmol N_2 hr^{-1} $plant^{-1}$ was observed in this study, while Lamel & Cruz (2013), Bünger et al. (2021) and Bonaldi et al. (2011) reported lower activity of 0.1–0.4 (*Phaseolus vulgaris*), 0.02–0.18 (*Mimosa scabrella* and *P. vulgaris*) and 0.36–0.64 (*Aeschynomene afraspera* and *A. indica*) µmol N_2 hr^{-1} $plant^{-1}$, respectively. Ladestam et al. (2020) and Ma et al. (2022) reported higher activity of 0.4–8.7 (*Medicago sativa, Pisum sativum, P. vulgaris, Glycine max, Arachis hypogeal* and *M. atropurpureum*) and 2.9–9 (*G. max*) µmol N_2 hr^{-1} $plant^{-1}$, respectively. This finding shows the variability in nitrogen fixation between the symbiotic species.

Legumes use nitrogen fixation to rapidly colonise ecological niches and outcompete other species, as occurred in neotropical forests (Gei et al. 2018). By stimulating nitrogenase activity through bacterial inoculants, this effect is potentiated, both in the studied species and in majority of species in the Fabaceae family. Thus, the application of rhizobia can reduce the application of nitrogen fertilisers and environmental pollution, as well as increase the yield and quality of plants. With regards to the morphometric variables, it was generally observed that there was a response when bacterial inoculant was applied to the eight leguminous forest species. The same effect was seen when the forest species were grouped by subfamily. When the values were observed based on species, the response obtained were dependent

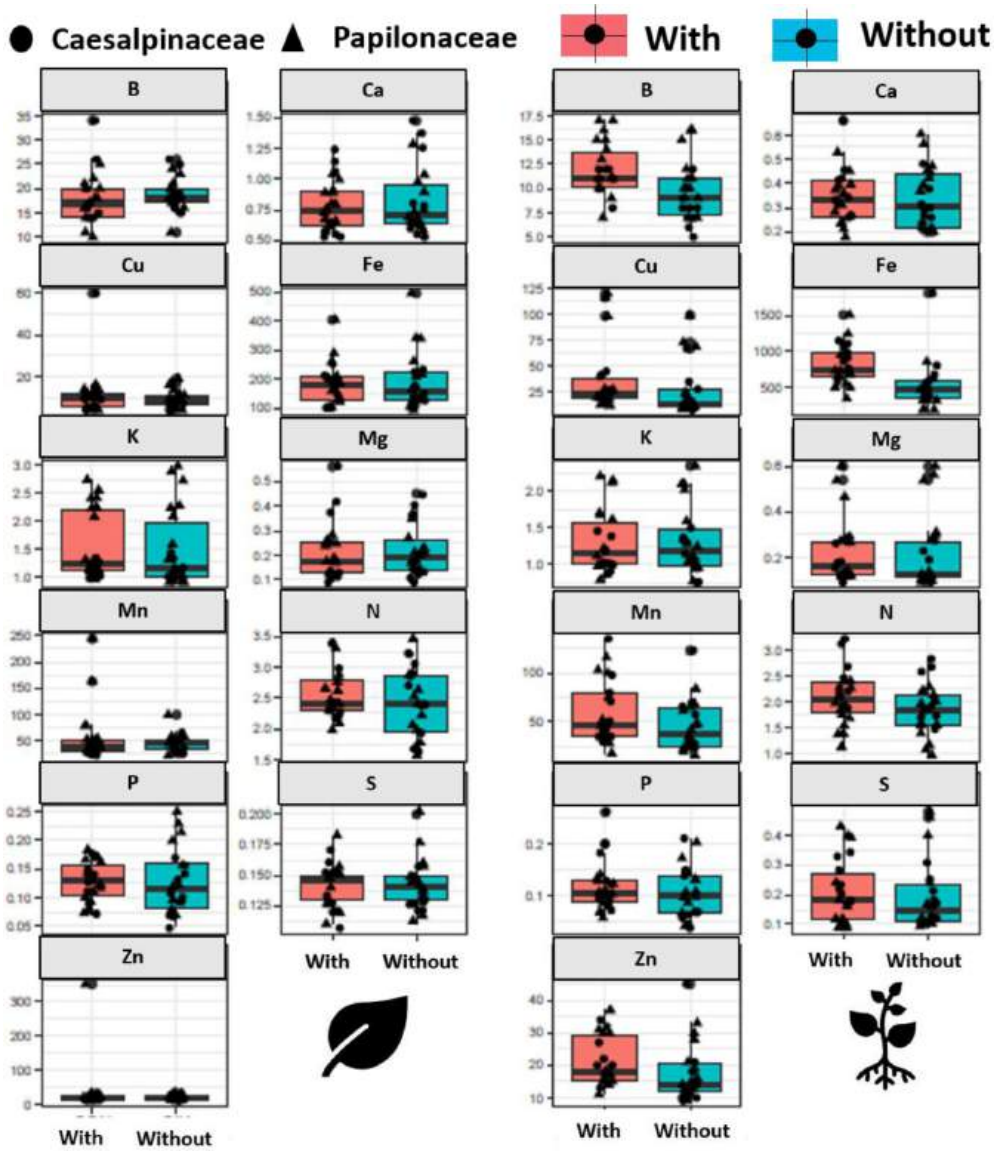

Figure 6 Nutrient content in leaf (left) and root (right) evaluated at five months after sowing for the leguminous forest species studied in Costa Rica with inoculation (red) and no inoculation (blue). Grouped by subfamily (differences at $p<0.05$ Wilcoxon-Man-Whitney)

on the particularities of each species. However, a positive response to the bacterial inoculation was always manifested in all species studied. The genera *Rhizobium* and *Bradyrhizobium* have been widely studied and known to consist of a large number of species that fix nitrogen in plant hosts from the Fabaceae and other families (Fahde et al. 2023).

Inoculation of *R. miluonense* showed that the nutrient levels were not significantly increased in *S. saman*. However, six out of 10 quantified variables were improved. Therefore, it is considered that the bacterium had a positive effect on growth. Wyse et al. (2021) applied *Bradyrhizobium elkanii* BR 6205 and *B. elkanii* BR 6212 to *S. saman*, increasing nodulation and morphometric variables by 70%. In the present study, nodulation with *R. miluonense* was increased by 13%. Accordingly, *S. saman* could also be a species that accepts various species of symbiotic bacteria.

In the case of *R. multihospitium*, there was an increase in the levels of some nutrients in *C. arborea*, both at the foliar and root levels. This in turn, improved four growth variables. Therefore, it is considered that the bacterium showed a positive effect on *C. arborea* where 43% more nodulation was obtained compared to the control without inoculation. Nguyen et al. (2022) isolated this species along with others

Table 4 Correlations between variables used to evaluate the efficacy of inoculation with symbiotic bacteria from forest legumes

	Height (cm)	Stem thickness (mm)	Leaf number	Vigor (1–5)	Nodules number	CO_2 (ppm)	N. activity (µmol N_2 hr^{-1} $plant^{-1}$)	Root length (cm)	Root weight (g)	Leaf area (cm^2)
Height (cm)	1									
Stem thickness (mm)	**0.58***	1								
Leaf number	0.29	-0.22	1							
Vigor (1–5)	**0.71****	**0.50***	0.26	1						
Nodules number	0.32	-0.01	-0.08	0.22	1					
CO_2 (ppm)	-0.15	-0.11	-0.03	-0.13	-0.05	1				
N. activity (µmol N_2 hr^{-1} $plant^{-1}$)	0.39	0.17	0.02	0.26	**0.47***	-0.07	1			
Root length (cm)	0.14	-0.07	0.23	0.18	0.21	0.00	0.11	1		
Root weight (g)	**0.63***	**0.80****	-0.17	**0.57***	0.12	-0.09	0.24	0.05	1	
Leaf area (cm^2)	**0.52***	**0.48***	-0.07	**0.57***	**0.45***	-0.05	0.32	0.20	**0.64***	1

* = 0.4–0.7; ** = > 0.7

such as *R. miluonense* from *Arachis hypogaea*. Subsequently, these species were inoculated in peanuts and rice, obtaining an improvement in germination. In that study, the *R. multihospitium* and *R. miluonense* bacteria grew at temperatures of 15–44 °C and could tolerate up to 2% NaCl, indicating them as candidates for studies in plantations in dry and high salinity areas.

Inoculation of *B. japonicum* in the three plant species studied, indicated that the levels of nutrients in plant tissues did not improve. However, the morphometric measurements improved in six variables for *E. fusca*, three in *E. poepiggiana* and one in *D. americana*. Based on these findings, it is considered that the bacterium was not a good inoculant for *E. poepiggiana* and *D. americana*, but it was for *E. fusca*. In addition to the widely documented effect of *B. japonicum* on soybean, it was also shown that the bacterium can associate with other plant species such as rice, corn, wheat and sorghum (Fahde et al. 2023). In *Avena sativa* and *Lolium multiflorum*, root volume was improved compared to non-inoculated plants (de Castilho et al. 2021). In Costa Rica, this bacterium should be given special attention due to the fact that *E. fusca* and *E. poepiggiana* are species commonly associated with coffee agroforestry systems.

Inoculation of *Bradyrhizobium* sp. in the three plant species studied, showed increased nutrient values. In *D. retusa* and *E. cyclocarpum*, levels of N, Mg and S increased in foliage. While levels of N, P, K, Mg, S, Fe, Mn, Cu, B and Zn increased in roots. In *P. pinnatum*, although it was only possible to obtain a replica in this species due to its slow-growth, practically all the nutrients were increased both in the foliar and root. In addition, it improved six, four and two variables for *E. cyclocarpum*, *D. retusa* and *P. pinnatum*, respectively. Due to the above, where a tendency to improve different variables and nutrient content was observed, it is advisable to identify the related species. In other studies, different *Bradyrhizobium* species have been evaluated on *V. radiata*, finding relative effectiveness greater than 80% in 72–83% of the strains collected in various agro-ecological zones (Simbine et al. 2021).

In general, the improvement in morphometric variables by inoculation recorded in this study is consistent with what was reported by Acuḥa & Uribe (1996), Wyse et al. (2021) and de Castilho et al. (2021), where at least one variable was improved. Under the conditions of this experiment, they were improved from one to six, depending on the plant species. This is important given that the quality of forest seedlings is a factor of great importance, especially in the first months after planting when they are subjected to adverse environmental conditions. With bacterial inoculants, greater seedling survival and growth can be obtained (Salles et al. 2015) by disease suppression, growth promotion and nitrogen fixation (Bulgarelli et al. 2013).

When analysing the growth response by species, there were some that were more sensitive to inoculation than others. One of the reason to explain this observation is due to the relationship between host plant and bacteria. In the species with the best development, it is possible that a very specific symbio-variety relationship existed as proposed by Rogel et al. (2011). An example of the above is the case of *E. fusca* and *E. poepiggiana* where *B. japonicum* was isolated from both species, but the response to inoculation was better in one species than the other. This could be because the isolated strain possibly has a greater adaptation and symbiotic evolution with *E. fusca*.

Although a positive response to the inoculation was obtained in most of the species, competition could have been involved in the experiment. This is because the experiment was carried out under open environmental conditions and the measurement of microbial respiration was the same in both inoculated and non-inoculated treatments. Competition between bacteria has been reported by Denton et al. (2002). Therefore, re-inoculation and increased frequency must be considered to keep the bacterium of interest in contact with the roots and colonise the infection sites before other species and/or strains. This is particularly important since hosts tend to choose strains of higher infective quality (Zanetti et al. 2010).

Another aspect to consider is the behavior of so-called 'promiscuous' legumes. While it can be advantageous to inoculate them with various bacterial species, it also opens up the possibility of competition between these bacteria. This has been widely reported in beans. Talbi et al. (2010) reported of five species of *Rhizobium* capable of nodulating beans, besides *Burkholderia phymatum*. In the present study, *S. saman* was inoculated with *R. miluonense*. However, there are also reports that its growth improves with inoculation of *B. elkanii* strains BR 6205 and BR 6212 (Wyse et al. 2021). On the other hand, there are other legumes that have a narrow host specificity such as *Pterocarpus indicus* (Lok et al. 2006).

Considering the possibility of competition, the promiscuity of some legumes, symbiovar specificity, and the potential of nitrogen-fixing bacteria to reduce the application of synthetic fertilisers, it is recommended that a potential biofertiliser should be formulated specifically for a forest crop. Additionally, the possibility of formulating a bacterial consortium for promiscuous plant species should be assessed. The quality of each bacterium to infect in the presence of other bacterial species should also be assessed. The bacterial species identified in this study could also have the potential to be applied to other plant species, since symbiotic nitrogen-fixing bacteria perform bio stimulation in legume and even non-legume species. In addition, it is possible that these are new records in hosts that have not been reported to date. Although small experiments located under controlled conditions in laboratories and protected environments are useful, they may not account for all the factors that can intervene under field conditions. Therefore, studies of this nature offer a perspective of the response that can be obtained in a multifactorial dynamic like the conditions that occur in a forest plantation.

CONCLUSIONS

Three species of nitrogen-fixing symbiotic bacteria were isolated, as well as a genus where there are no species records to date. It is possible that with subsequent analysis, a new species of the genus *Bradyrhizobium* associated with tropical leguminous forests may be identified. No record of the identified bacteria present in leguminous forest trees was found; therefore, it is possible that they are new records for tropical forests.

When carrying out bacterial inoculation in their respective hosts, improvement in growth was observed in all forest species. The magnitude depended on the species and the association

with the bacterium. At subfamily level, the Caesalpinioideae presented a better response to the bacterial inoculant than the Papilionoideae. Some chemical elements are absorbed by plants to a greater extent when symbiotic nitrogen-fixing bacteria are inoculated. The magnitude depended on the species, the tissue and the bacterial association. The use of bacterial inoculants showed an improvement in leguminous forest trees. This verifies their potential for development as biofertilisers. Future studies to investigate the effects of these bacteria on other plant species and explore the potential for developing bacterial consortia should be carried out. Considering the microbiological dynamism of soil and the changing environmental conditions, we do not rule out re-inoculation as a highly recommended practice.

ACKNOWLEDGEMENTS

This study was financed by Project C0-524 and B9-204 and carried out at LEGMI of the School of Biology of the University of Costa Rica. We thank CIPRONA of the University of Costa Rica for providing the facilities and for the accompanying by the specialised personnel.

REFERENCES

Acuña O. 1996. Manejo y tecnología de la fijación biológica de nitrógeno en leguminosas de importancia agrícola. *Proc X Congreso Nacional Agronómico y de Recursos Naturales*. 8–12 July 1996, San José.

Acuña O & Uribe L. 1996. Inoculación del frijol común con tres cepas seleccionadas de Rhizobium leguminosarum bv. phaseoli. *Agronomía Mesoamericana* 7: 35–40.

Arpiwi NL, Yan G, Barbour EL, Plummer JA & Watkin E. 2012. Phenotypic and genotypic characterization of root nodule bacteria nodulating *Millettia pinnata* (L.) Panigrahi, a biodiesel tree. *Plant Soil* 367: 363–377. https://doi.org/10.1007/s11104-012-1472-4.

Bedmar EJ, Robles EF & Delgado MJ. 2005. The complete denitrification pathway of the symbiotic nitrogen-fixing bacterium *Bradyrhizobium japonicum*. *Biochemichal Society Transactions* 33: 141–144. https://doi.org/10.1042/BST0330141.

Bonaldi K, Gargani D, Prin Y et al. 2011. Nodulation of *Aeschynomene afraspera* and *A. indica* by photosynthetic *Bradyrhizobium sp.* strain ORS285: The Nod-Dependent versus the Nod-Independent Symbiotic Interaction. *MPMI* 24: 1359–1371. https://doi.org/10.1094/MPMI-04-11-0093.

Bulgarelli D, Schlaeppi K, Spaepen S, Van Themaat E & Schulze-Lefert P. 2013. Structure and functions of the bacterial microbiota of plants. *Annual Review of Plant Biology* 64: 807–838.

Bünger W, Sarkar A, Grönemeyer J et al. 2021. Root nodule rhizobia from undomesticated shrubs of the dry woodlands of Southern Africa can nodulate Angolan teak *Pterocarpus angolensis*, an important source of timber. *Frontiers in Microbiology* 12: 611704. https://doi.org/10.3389/fmicb.2021.611704.

Céspedes S, Zuñiga A, Mendoza A, Montero K & Chaves A. 2019. Evaluación de la incorporación de *Mucuna pruriens* L. (Fabaceae) y *Crotalaria spectabilis* Roth (Fabaceae), sobre el aporte y absorción de nutrientes en el cultivo de arroz. *Repertorio Científico* 22: 29–37. https://doi.org/10.22458/rc.v23i1.2941.

Chen G & Weil RR. 2009. Penetration of cover crops roots through compacted soils. *Plant and Soil* 331: 31–43. https://doi.org/10.1007/s11104-009-0223-7.

Chun JY, Sexton G, Evans R & Stacey G. 1994. Identification and characterization of a novel *Bradyrhizobium japonicum* gene involved in host-specific nitrogen fixation. *Journal of Bacteriology* 176: 6717–6729.

De Castilho CL, Volpiano CG, Ambrosini A et al. 2021. Growth-promoting effects of *Bradyrhizobium soybean* symbionts in black oats, white oats, and ryegrass. *Brazilian Journal of Microbiol* 52: 1451–1460. https://doi.org/10.1007/s42770-021-00523-1.

De Bedout M, Solis-Ramos L, Valverde-Barrantes O & Rojas-Jiménez K. 2022. Capacidad de nodulación en especies forestales leguminosas (Fabaceae) según su filogenia y características morfológicas. *Revista Forestal Mesoamericana Kurú* 19: 1–8. https://doi.org/10.18845/rfmk.v19i45.6315.

Denton MD, Coventry DR, Murphy PJ, Howieson Jg & Bellotti WD. 2002. Competition between inoculant and naturalised *Rhizobium leguminosarum* bv. trifolii for nodulation of annual clovers in alkaline soils. *Australian Journal of Agriculture Research* 53: 1019–1026. https://doi.org/10.1071/AR01138.

Downie JA & Kondorsi E. 2021. Why should nodule cysteine-rich (ncr) peptides be absent from nodules of some groups of legumes but essential for symbiotic N-fixation in others? *Frontiers in Agronomy* 3: 654576. https://doi.org/10.3389/fagro.2021.654576.

Dupuy N, Willems A, Pot B et al. 1994. Phenotypic and genotypic characterization of *Bradyrhizobia* nodulating the leguminous tree *Acacia albida*. *International Journal of Systematic and Evolutionary Microbiology* 44: 461–473. https://doi.org/10.1099/00207713-44-3-461.

Fahde S, Boughribil S, Sijilmassi B & Amri A. (2023). Rhizobia: a promising source of plant growth-promoting molecules and their non-legume interactions: Examining applications and mechanisms. *Agriculture* 13: 1279. https://doi.org/10.3390/agriculture13071279.

Gei M, Rozendaal DMA, Poorter L et al. 2018. Legume abundance along successional and rainfall gradients in Neotropical forests. *Nature Ecology and Evolution* 2: 1104–1111. https://doi.org/10.1038/s41559-018-0559-6.

Göttferd M, Grob P & Hennecke G. 1990. Proposed regulatory pathway encoded by the nodV and nodW genes, determinants of host specificity in *Bradyrhizobium japonicum*. *Proceedings of Natural Academy of Science USA* 87: 2680–2684.

Gu CT, Wang ET, Tian CF et al. 2008. *Rhizobium miluonense* sp. nov., a symbiotic bacterium isolated from *Lespedeza* root nodules. *International Journal of Systematic and Evolutionary Microbiology* 58: 1364–1368. https://doi.org/10.1099/ijs.0.65661-0.

Han TX, Wang ET, Wu LJ et al. 2008. *Rhizobium multihospitium* sp. nov., isolated from multiple legume species native of Xinjiang, China. *International Journal of System Evolutionary Microbiology* 58: 1693–1699. https://doi.org/10.1099/ijs.0.65568-0.

Hardy RWF, Burns RC & Holsten RD. 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biology and Biochemistry* 5: 47–81. https://doi.org/10.1016/0038-0717(73)90093-X.

Hördt A, García M, Meier-Koltholff J et al. 2020. Analysis of 1,000+ type-strain genomes substantially improves taxonomic classification of Alphaproteobacteria. *Frontiers in Microbiology* 11: 468. https://doi.org/10.3389/fmicb.2020.00468.

James G. 2010. Universal bacterial identification by PCR and DNA sequencing of 16S rRNA gene. Pp 209–214 in Schuller M et al. (eds) *PCR for Clinical Microbiology*. Springer Nature, Dordrecht.

Kaneko T, Nakamura Y, Sato S et al. 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. *DNA Research* 9: 189–197. https://doi.org/10.1093/dnares/9.6.189.

Kaye JP & Quemada M. 2017. Using cover crops to mitigate and adapt to climate change. *Agronomy for Sustainable Development* 37: 1–17. https://doi.org/10.1007/s13593-016-0410-x.

Kitagawa W, Takami S, Miyauchi K et al. 2002. Novel 2,4-dichlorophenoxyacetic acid degradation genes from oligotrophic *Bradyrhizobium sp.* strain HW13 isolated from a pristine environment. *Journal of Bacteriology* 184: 509–518. https://doi.org/10.1128/JB.184.2.509-518.2002.

Ladestam Z, Retnaningrum E & Dwidya I. 2020. Symbiotic performance of several leguminous plants with legume nodule bacteria isolated from Siratro (*Macroptilium atropurpureum*) at Mount Merapi eruption, Indonesia. Pp 14–27 in Trisakti et al. (eds) *The 6th International Conference on Biological Science (ICBS): Biodiversity as a Cornerstone for Embracing Future Humanity*. 10–11 October 2019, Yogyakarta. https://doi.org/10.1063/5.0015969.

Lamel D & Cruz L. 2013. Diversity and symbiotic effectiveness of beta-rhizobia isolated from subtropical legumes of a Brazilian Araucaria Forest. *World Journal of Microbiology and Biotechnology* 29: 2335–2342. https://doi.org/10.1007/s11274-013-1400-7.

Lane DJ. 1991. 16S/23S rRNA sequencing. Pp 115–175 in Stackebrant et al. (eds) *Nucleic acid techniques in bacterial systematics*. John Wiley and Sons, New York.

Lok EH, O'hara G & Dell B. 2006. Nodulation of the legume *Pterocarpus indicus* by diverse strains of rhizobia. *Journal of Tropical Forest Science* 18: 188–194.

Ma J, Zhou Y, Li J & Song Z. 2022. Novel approach to enhance *Bradyrhizobium diazoefficiens* nodulation through continuous induction of ROS by manganese ferrite nanomaterials in soybean. *Journal of Nanobiotechnology* 20: 168. https://doi.org/10.1186/s12951-022-01372-2.

Mir MI, Kumar BK, Gopalakrishnan S, Vadlamudi S & Hameeda B. 2021. Characterization of rhizobia isolated from leguminous plants and their impact on the growth of ICCV 2 variety of chickpea (*Cicer arietinum* L.). *Heliyon* 7: e08321. https://doi.org/10.1016/j.heliyon.2021.e08321.

Moreira FMS, Huisin EJ & Bignel DE. 2008. Nitrogen-fixing Leguminosae-nodulating bacteria. Pp 107–129 in Moreira et al. (eds) *Handbook of tropical soil biology, sampling & characterization of belowground biodiversity*. Routledge, Oxfordshire.

Nguyen TH, Vo TM, Nguyen MK et al. 2022. Isolation and characterization of *Rhizobium* spp. and *Bradyrhizobium* spp. from legume nodules. *Ho Chi Minh City Open University Journal of Science, Engineering and Technology* 12: 70–98. https://doi.org/10.46223/HCMCOUJS.tech.en.12.2.2116.2022.

Oliveira DP, Figueiredo MA De, Soares BL et al. 2017. Acid tolerant *Rhizobium* strains contribute to increasing the yield and profitability of common bean in tropical soils. *Journal of Soil Science and Plant Nutrition* 17: 922–934. https://doi.org/10.4067/S0718-95162017000400007.

Rogel Ma, Ormeño-Orrillo E & Martinez-Romero E. 2011. Symbiovars in rhizobia reflect bacterial adaptation to legumes. *Systematic and Applied Microbiology* 34: 96–104. https://doi.org/10.1016/j.syapm.2010.11.015.

Roy S, Liu W, Nandety RS et al. 2020. Celebrating 20 years of genetic discoveries in legume nodulation and symbiotic nitrogen fixation. *Plant Cell* 32: 15–41. https://doi.org/10.1105/tpc.19.00279.

Rivas R, Martens M, De Lajudie P & Willems A. 2009. Multilocus sequence analysis of the genus *Bradyrhizobium*. *Systematic and Applied Microbiology* 32: 101–110. https://doi.org/10.1016/j.syapm.2008.12.005.

Salles G, Gross E, Brito-Rocha E & Schramm M. 2015. Efeitos da inoculação com bactérias diazotróficas e da adubação nitrogenada no crescimento e na qualidade de mudas de Inga laurina (sw.) willd. (fabaceae). *Revista Árvore* 39: 1031–1038. https://doi.org/10.1590/0100-67622015000600005.

Senthilkumar M, Amaresan N & Sankaranarayanan A. 2021. *Plant-Microbe Interactions: Laboratory Techniques. Chapter 5: Quantitative Estimation of Nitrogenase Activity: Acetylene Reduction Assay*. Springer Protocols Handbook, New York.

Simbine MG, Mohammed M, Jaiswal SK & Dakora F. 2021. Functional and genetic diversity of native rhizobial isolates nodulating cowpea (*Vigna unguiculata* L. Walp.) in Mozambican soils. *Scientific Reports* 11: 12747. https://doi.org/10.1038/s41598-021-91889-7.

Soto-Valenzuela J, Ormeño-Orrillo E & Zúḥiga-Dávila D. 2021. Diversity and biological nitrogen fixation of rhizobia isolated from *Clitoria brachystegia*, in tropical dry forest remnants of Ecuador and Peru. *Revista Mexicana de Biodiversidad* 92: e923426. https://doi.org/10.22201/ib.20078706e.2021.92.3426.

Truscott S, Lewis RS & Watt GD. 2021. Positive cooperativity during *Azotobacter vinelandii* nitrogenase-catalyzed acetylene reduction. *Biophysical Chemistry* 277: 106650. https://doi.org/10.1016/j.bpc.2021.106650.

Vinuesa P, Silva C, Werner D & Romero-Martínez E. 2003. Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in *Bradyrhizobium* species cohesion and delineation. *Molecular Phylogenetics and Evolution* 34: 29–54. https://doi.org/10.1016/j.ympev.2004.08.020.

Wyse G, Saggin OJ & Miana S. 2021. Interaction of substrates and inoculants for *Samanea Saman* (Jacq.) Merr. seedling production. *Floresta e Ambiente* 28: e20210046. https://doi.org/10.1590/2179-8087-FLORAM-2021-0046.

Zanetti ME, Blanco FA, Beker MP, Battaglia M & Aguilar OM. 2010. A C subunit of the plant nuclear factor NF-Y required for rhizobial infection and nodule development affects partner selection in the common bean-*Rhizobium etli* symbiosis. *Plant Cell* 22: 4142–4157. https://doi.org/10.1105/tpc.110.079137.

Zhurbenko R, Rodríguez C, Díaz M, Durán A, López OD & Viera DA. 2006. Caracterización de la peptona de soya para el cultivo de microorganismo. *Revista Cubana de Medicina Tropical* 58: 109–118.