Testing bacterial inocula isolated from the rhizosphere of *Theobroma gileri* trees as an alternative for biological fertilization of *Theobroma cacao* seedlings

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Abstract

We tested for the first time whether bacterial inocula isolated from the rhizosphere of *Theobroma gileri* trees—a wild relative of cacao—harbors diverse bacterial communities with potential to act as biofertilizers of *Theobroma cacao* seedlings, thus helping to reduce the overdrive of chemical fertilizers. Inocula enriched in N-fixing bacteria were prepared from the soil rhizosphere of *T. gileri* trees growing on primary forest soil (IPS replicates) and secondary forest soil (ISS replicates). Metagenomic analysis, based on 16S rRNA amplicon sequencing, revealed 175 genus-level clades in total, with 67 genus-level clades exclusive to IPS and 39 genus-level clades exclusive to ISS. In silico analyses designed to search for *nif* genes (known to express N-fixing function) recognized 47 genus-level clades as potential N-fixing bacteria; these included several species of *Clostridium* (anaerobic N-fixing bacteria relatively common in IPS replicates) and *Azotobacter* (aerobic N-fixing bacteria very common in ISS replicates). To assess the effect of bacterial inocula versus urea on the growth of cacao seedlings (CCN-51 variety), a 63-day greenhouse pilot experiment was set up using a two-way ANOVA design, with inoculum origin as first factor (three levels: IPS, ISS, and no-inoculum [control]) and urea fertilization as second factor (two levels: presence or absence [control] of urea). Response variables were plant survival (%), height change (mm/week), diameter change (mm/week), and chlorophyll index change (SPAD units/week). Seedlings under treatments with bacterial inocula had no mortality, while those with urea-only treatment exhibited 40% survival rate. Although no significant effects (at α=0.10) were detected for any response variable, the urea factor influenced almost significantly on height (F=2.41, P=0.14) and chlorophyll index (F=2.66, P=0.12). In conclusion, bacterial inocula isolated from the rhizosphere of *T. gileri* do not seem to have an evident effect on *T. cacao* seedlings growth, although may be beneficial to assure seedling survival.

Keywords *Theobroma cacao*, *Theobroma gileri*, biofertilizers, wild cacao, rhizosphere microbiome, N-fixing bacteria, plant growth promoters, chemical fertilization with urea, biological fertilization with bacterial inocula

1. Introduction

In recent years, the price of cocoa (*Theobroma cacao* L.) experienced unprecedented growth, tripling its value from USD 2,000 to USD 6,523 per ton between 2023 and early 2024; for 2024-2025, even record values exceeding USD 11,500 were recorded (La Hora 2025; Vásconez 2026). This encouraged many farmers to increase their production and attracted new producers, making Ecuador the third-largest cocoa producer in the world, with a +37% increase in exports for 2025. This phenomenon generated high demand for seedlings of the CCN-51 cocoa variety, which dominates the market due to its good yield (1.5–2.76 ton/ha), large pods, and moderate tolerance to moniliasis fungal disease (Quiroz et al. 2021; Vásconez 2026).

The boom in cocoa production intensified the use of nitrogen fertilizers in the early stages of cultivation. On the one hand, although nitrogen is essential for plant growth, the application of synthetic fertilizers such as urea entails significant environmental, economic, and social impacts, including soil degradation, alteration of the soil microbiota, and dependence on imported inputs (Cerruffo and Espín 2022; Troya-Medina 2024; Weinstein et al. 2025). On the other hand, the high cost and volatility of international fertilizer prices reduce the profitability of small and medium producers, undermining their economy and motivating the search for more efficient, accessible, and environmentally sustainable fertilization alternatives (Cerruffo and Espín 2022). One such fertilization alternative is the use of N-fixing bacteria, which not only can convert atmospheric nitrogen (N_2) into ammonium (NH_4^+) absorbable by plants, but also establish other beneficial interactions such as phytohormones production and root architecture improvement (Argüello-Navarro and Moreno-Rozo 2014; Reeta et al. 2022).

The application of N-fixing bacteria to cacao cultivation is carried out through the use of biofertilizers, mostly generated from commercial microbial strains or isolated from generic agricultural environments, including the cacao rhizosphere itself and its varieties (e.g., Argüello-Navarro and Moreno-Rozo 2014; Mendoza-Rodríguez and Pazmiño-Mera 2021; Jannah et al. 2022). One of the logical microhabitats where bioprospecting can be carried out in search of beneficial microorganisms for cacao cultivation is the rhizosphere associated with its wild relatives that naturally grow in wild forests. Indeed, Ecuador harbors several close relatives of cacao such as *Theobroma bicolor, T. gileri, T. subincanum*, and *T. glaucum* (Cuatrecasas 1964; Tropicos® 2026). In this study, we focus on *T. gileri*, a native species mostly distributed in the Ecuadorian Coast, and whose rhizosphere may contain an evolutionary but unexplored reservoir of microorganisms potentially adapted to local edaphoclimatic conditions. In particular, there is no experimental evidence available that evaluates the performance of the bacterial community from the rhizosphere of *T. gileri* as a biofertilizer for cacao seedlings (CCN-51 variety) under controlled nursery conditions, using inoculants from primary and secondary soils, compared to conventional nitrogen fertilization schemes (artificial) with urea, or mixed combinations (urea + biofertilizer).

Given the context presented above, we hereby propose that: (1) primary forest soils (relatively undisturbed) are reservoirs of more diverse bacterial communities than secondary (disturbed) forest soils; and, (2) bacterial communities associated with the rhizosphere of *T. gileri*—assumed to be adapted to acidic soils, high humidity, and the nutritional limitations inherent to their soils—may offer functional nutritional advantages compared to the benefits of using conventional chemical fertilizers. The precise statistical hypotheses related to these two postulates are presented in the Materials and Methods section. The ultimate goal of this study is to generate scientific evidence that supports the use of native bioinputs for more efficient and resilient fertilization, contributing to the transition towards sustainable cocoa systems, i.e. with reduced dependence on synthetic fertilizers and greater alignment with the principles of agroecology and bioeconomy.

2. Materials and methods

2.1 Sampling of rhizosphere associated with *Theobroma gileri* trees

Four adult *Theobroma gileri* trees, 15–20 m tall, were located in primary forest (three trees) and secondary forest (one tree) in the locality of Piedra Blanca, San Luis de Pambil parish, Guaranda county, Bolívar province, in the subtropical coastal zone of Ecuador (Table 1). The forest is formally classified as “Northwestern Andean Piedmont Evergreen Forest” (MAE 2013). Around the trunk of each tree, approximately 200 g of rhizospheric soil was collected at an approximate depth of 20 cm, using a spatula previously sterilized with 70% alcohol and 3% chlorine solution. Each soil sample was stored in a sterile plastic bag. The samples were transported to the laboratory in a cooler with ice and subsequently preserved in a freezer at −80 °C at the Pontificia Universidad Católica del Ecuador-Ibarra (PUCE-I). After a few days, 25 g of each of the three collected soil samples from primary forest soil were mixed to obtain a composite soil stock of 75 g, which was then thoroughly homogenized. Likewise, for the single sample collected in secondary forest soil, 75 g of soil were also weighed and homogenized.

Table 1. Geographical location of the soil samples collected from the rhizosphere of *Theobroma gileri* trees.

Habitat and tree	UTM Coordinates (zone 17M)	Geographical coordinates	Altitude (m)
Primary forest: tree 1	696633 – 9859451	1°16'13.7"S 79°13'57.9"W	524
Primary forest: tree 2	697017 – 9860214	1°15'48.9"S 79°13'45.5"W	430
Primary forest: tree 3	697049 – 9860251	1°15'47.7"S 79°13'44.5"W	425
Secondary forest: tree 1	697064 – 9859567	1°16'09.9"S 79°13'44.0"W	439

2.2. Preparation of bacterial inocula from *Theobroma gileri* rhizosphere

In order to obtain the bacterial inocula, all soil samples were first cultured in Jensen culture medium in order to promote the growth of N-fixing bacteria. The Jensen culture medium was manually prepared using the recipe reported by HiMedia (2026). First, a 1-liter stock of base medium was prepared by mixing 20 g×l^{-1} sucrose ($C_{12}H_{22}O_{11}$), 1 g×l^{-1} dipotassium dihydrogen phosphate (KH_2PO_4), 0.5 g×l^{-1} magnesium sulfate ($MgSO_4$), 0.5 g×l^{-1} sodium chloride (NaCl), and 2 g×l^{-1} calcium carbonate ($CaCO_3$); this base medium was then autoclaved. Second, because of the thermal instability of some micronutrients (which prevents them from being autoclaved), stock solutions of 0.1 g×l^{-1} ferrous sulfate ($FeSO_4$) and 0.005 g×l^{-1} sodium molybdate (Na_2MoO_4) had to be prepared separately and filtered using a 0.22 µm membrane. This filtrate was then added to the autoclaved base media. The $FeSO_4$ solution was prepared at a concentration of 10% w/v (100 mg×ml^{-1}) by weighing 1.0 g of $FeSO_4 \cdot 7H_2O$ in 10 ml of distilled water; while the Na_2MoO_4 solution was prepared at a concentration of 0.1% w/v (1 mg.ml^{-1}) by dissolving 10 mg of $Na_2MoO_4 \cdot 2H_2O$ in 10 ml of distilled H_2O.

Once the stock of Jensen culture medium was ready, it was mixed with two "replicates" of each rhizosphere soil type, in the proportions shown in Table 2. Note that the two "replicates" obtained for each soil type were really not statistically independent, as the soil stock from which they came was the same (see section 2.1). The four cultures (2 soil types × 2 replicates of each) were incubated at 30 °C and 180 rpm in an orbital shaker for seven days. At the end of this period, the liquid cultures obtained were used as bacterial inocula for the greenhouse experiment with cacao seedlings (see further below).

Table 2. Amounts of soil and Jensen culture media used in the production of bacterial inocula originated from the rhizosphere of *Theobroma gileri* trees. IPS: Inoculum from primary soil rhizosphere. ISS: Inoculum from secondary soil rhizosphere.

Inoculum*	Fresh soil weight (g)	Volume of Jensen liquid medium (ml)	Origin of the soil
IPS1	1	250	Rhizosphere soil from three *T. gileri* trees growing in primary forest; mixed and homogenized
IPS2	1	250	
ISS1	1	250	Rhizosphere soil from one *T. gileri* tree growing in secondary forest; homogenized
ISS2	1	250	

* Note that the two “replicates” obtained for each soil type were really not statistically independent, as the soil stock from which they came was the same. Yet, to facilitate reading of this paper, we anyhow use the term “replicate” to refer to them.

2.3. DNA analyses of bacterial inocula from *Theobroma gileri* rhizosphere

With the aim of characterizing the bacterial diversity present in the culture media, the total DNA of each replicate (see Table 2) was extracted, sequenced and subjected to a metagenomic bioinformatic analysis. In November 2025, 100 ml of each culture was sent to the company IDgen (Ecuador) for DNA extraction and subsequent high-throughput sequencing. Total DNA was extracted using FastDNA™ Spin Kit for Soil DNA Extraction (MP Biomedicals), following the manufacturer's instructions. Quality and concentration of extracted DNA were evaluated by spectrophotometry (Nanodrop) and 1% agarose gel electrophoresis. To detect bacterial taxa, the hypervariable V3-V4 region of the 16S rRNA gene was amplified by PCR using the following universal primers:

- 341F (5′-CCTACGGGNGGCWGCAG-3′)
- 805R (5′-GACTACHVGGGTATCTAATCC-3′).

Library preparation and amplicon sequencing were performed on the Illumina NovaSeq platform using paired-end reads (2 × 250 bp), following the manufacturer's standard protocols. Bioinformatic analysis of the obtained sequences was conducted in QIIME2 (v2023.9) (Bolyen et al. 2019). Raw demultiplexed reads were imported as a “Sample Data [Paired End Sequences With Quality]” artifact. Quality filtering, denoising, and chimera removal were performed using the DADA2 plugin for QIIME2 (Callahan et al. 2016) with the denoise-paired method. Forward and reverse reads were truncated to 247 bp and 245 bp, respectively by trimming 17 bp (forward) and 21 bp (reverse) to remove primer sequences (341F: 17 bp; 805R: 21 bp), based on an expected amplicon length of approximately 468 bp and a minimum overlap of 24 bp. This process generated amplicon sequence variants (ASVs), a feature table, and denoising statistics. Taxonomic assignment of representative ASV sequences was carried out using a pre-trained Naive Bayes classifier against the SILVA v138 reference database (Anwesh 2021). Subsequently, ASVs classified as mitochondria or chloroplasts were filtered from the feature table.

In order to define those genera that are probably N-fixing bacteria, the list of taxa was subjected to an *in silico* detection of sequences from the structural genes *nifH, nifD*, and *nifK*, given that these three genes are associated with the N-fixing function. This search used the *rentrez* package of the R software (Winter 2017; R Core Team 2025), which accesses the NCBI nucleotide database—including the PubMed, GenBank, and SNP databases—and searches there for taxa containing gene sequences specified according to the user's needs.

2.4. Community structure analyses of bacterial inocula from *Theobroma gileri* rhizosphere

Basic community structure analyses—i.e., abundance and diversity patterns—were mostly conducted at the taxonomic level of genus (or genus-level clade) in order to have high confidence on taxonomic identification during data analyses and interpretation. For each bacterial inoculum (replicate), we first calculated the relative abundance of each genus-level clade, defined as the percentage (%) of sequences of each genus with respect to the total sequences obtained in the replicate (according to the bioinformatic analyses). In each replicate, the topmost abundant genus-level clades (quaquantile range ≥0.95) were then selected, and their potential functional role identified based on published bibliography. For the diversity analysis, the following alternative statistical hypothesis was proposed in the context of our first postulate (see Introduction): "Alpha (α) diversity of bacterial inocula isolated from the rhizosphere of primary forest soil (IPS) is higher than that from secondary forest soil (ISS)". We used Hill´s effective number of species (Hill 1973) because this diversity estimate effectively reduces any interpretational bias on the observed community diversity patterns (see e.g., Jost 2006).

2.5. Pilot agricultural experiment on *Theobroma cacao* seedlings growth: effect of bacterial inocula isolated from *T. gileri* rhizosphere vs. urea fertilization

In order to evaluate the effect of bacterial inoculation versus urea application on the growth and chlorophyll content of *Theobroma cacao* seedlings (CCN-51 variety), a pilot agricultural experiment was established with commercially produced cocoa seedlings. The seedlings were produced approximately one year before the experiment, following standard commercial propagation techniques by grafting. The propagation was carried out at the "***Vivero Pamelita***" located in San Luis de Pambil parish, Guaranda county, Bolívar province, in the coastal region of Ecuador (1°13'21.0"S 79°16'38.7"W). The final substrate in which seedlings were transplanted was constituted by well-homogenized local soil, which is routinely used for cocoa seedling production by farmers in the area. The initial height of the experimental seedlings at the beginning of the experiment ranged from approximately 30 to 50 cm.

The experiment was carried out in one of the greenhouses at the Pontificia Universidad Católica del Ecuador in Ibarra city, province of Imbabura (0°20'55.34"N 78°06'20.02"W) following a two-way ANOVA experimental design. The first factor (independent variable), with two levels, was related to the use of conventional chemical fertilization: treatment with urea (U+) and control without urea (U-). The second factor (independent variable), with three levels, was related to the use of biological fertilization with native bacterial inocula originated from the rhizosphere of *Theobroma gileri* trees, previously cultured to promote growth of N-fixing bacteria: treatment with inoculum from primary soil (IPS+), treatment with inoculum from secondary soil (ISS+), and control without inoculum (IPS-/ISS-). This design resulted in six possible different combinations (Figure 1). Each combination had a sample size of n=5 seedlings, for a total of N=30 seedlings for the entire experiment. The concentration of bacteria in the inoculum was estimated to be 10^8 CFU (Colony-Forming Unit) and the weight of the urea applied to each treatment was 1.5 g (Figure 1). The dose of 1.5 g of urea applied to each seedling represents an intermediate value of what is usually recommended for urea fertilization doses (see e.g., Sudarma and Masdar 2022; Acuña 2025). The urea granules were applied directly to the soil, buried at an approximate depth of 10 cm. During the experimental period, the seedlings were maintained under the following controlled greenhouse conditions: average temperature of 30 °C, relative humidity between 40 and 60%, and automated sprinkler irrigation with filtered drinking water for 30 seconds every 30 minutes.

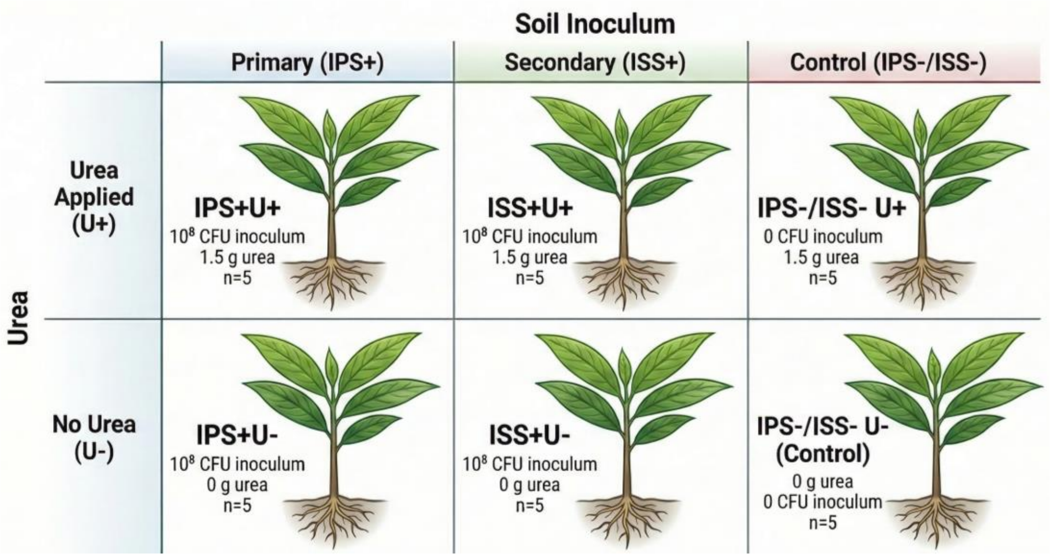

Figure 1. Two-way ANOVA experimental design used to evaluate the effect of bacterial inocula isolated from *Theobroma gileri* rhizosphere versus urea application on the growth and chlorophyll content of *Theobroma cacao* seedlings (CCN-51 variety). The first factor had two levels: urea treatment (U+) and control without urea (U-). The second factor had three levels: treatment with primary soil inoculum (IPS+), treatment with secondary soil inoculum (ISS+), and control without inoculum (IPS-/ISS-). *n* = number of replicates in each possible combination. Image generated by NotebookLM online app.

An independent ANOVA was performed for each of the following dependent variables: height, diameter, and chlorophyll index. These variables were chosen because they tend to relate well to the growth and physiological state of the seedlings. Height was defined as the distance (in mm) from where the stem emerges from the soil to the highest apical bud; stem diameter (in mm) was measured using a Vernier caliper near the base of the stem (for subsequent measurements, the point of diameter measurement was marked); finally, chlorophyll index was measured using a SPAD 502DL instrument, taking readings (in SPAD units) on three leaves per plant (at high, medium, and low heights) and three readings per leaf (upper, middle, and lower parts of a leaf blade). All these variables were monitored over a period of 63 days, with data recorded approximately every 15 days (day 0, day 16, day 30, day 44, and day 63).

For the statistical analyses, the dependent variables of height and diameter were expressed as growth rates over time (mm/week), calculated using a linear regression (slope of the linear equation). For the chlorophyll index variable, all nine values per plant, for each observation day, were first averaged, and then its rate of change over time (SPAD units/week) was calculated, using also a linear regression. The normality of each of these three dependent variables (height, diameter, and chlorophyll) was evaluated with the Shapiro-Wilk test; in case of lack of normality, a logarithmic transformation was applied prior to ANOVA. We decided to use a value of $\alpha=0.10$ (not $\alpha=0.05$) as the significance level, considering that this was not an experiment under laboratory conditions (highly controlled) and that there was notable morphological heterogeneity of the experimental seedlings used. Data were organized and curated in Excel® and statistically analyzed in Past® 4.03.

In the context of the second postulate (see Introduction), the formal alternative statistical hypotheses proposed for the two-way ANOVA of the three independent variables of the pilot agricultural experiment (height growth rate, diameter growth rate, and chlorophyll index change rate) were: (1) “There are significant differences on the average values of the independent variable among the treatments of the first factor: absence vs. presence of urea”; (2) “There are significant differences on the average values of the independent variable among the treatments of the second factor: type of bacterial inoculum”; and (3) “There is a significant interaction between the two experimental factors”.

3. Results and discussion

3.1. Community structure of bacterial inocula isolated from the rhizosphere of *Theobroma gileri*

Taxonomic information and absolute abundances of the 175 genus-level clades detected are shown in Supplementary Table 1 (available online). In the bacterial samples cultured in Jensen medium, according to the metagenomic analysis, there were 36,786 sequences for IPS1, 32,998 sequences for IPS2, 44,962 sequences for ISS1, and 31,016 sequences for ISS2. Across all replicates, the DADA2 software recognized, at the species-level, 264 clades; at the genus-level, 175 clades; and at the family-level, 84 clades. Many unidentified genus-level clades were assigned a known taxonomic code by the DADA2 software, and many had at least their family identified. Indeed, 115 (65.7%) of the genus-level clades and 60 (71.4%) of the family-level clades were assigned a formal scientific latinized name. Note that this does not necessarily mean that there are new families or genera to be discovered—it simply means that a number of genetic sequences could not be readily assigned to a known and named genus or family clade by the software used. For the present study, in order to simplify data interpretation and avoid uncertainties in the taxonomical identification, we focus on reporting diversity and abundance patterns at the genus-level only.

Out of the 175 total genus-level clades detected, 136 occurred in primary forest soil inocula IPS1 and IPS2 (67 exclusive), and 108 occurred in secondary forest soil inocula ISS1 and ISS2 (39 exclusive). The 69 shared genera (39.4% of the total) reflect a stable rhizosphere-associated microbiome that persists across successional stages, likely due to conserved plant host selection mechanisms (see Bai et al. 2022; Lundberg et al. 2012). This core microbiome may play a key role in maintaining essential plant-microbe interactions irrespective of forest age.

Alpha diversity, evaluated through Hill numbers (Table 3), was consistently higher in the IPS replicates than in the ISS replicates across all three orders of diversity (q=0, q=1, and q=2). The difference was particularly pronounced for q=1 (Shannon-based) and q=2 (Simpson-based), which weigh taxa by their relative abundances, indicating that primary forests harbor not only higher species richness but also a more even distribution of abundances across taxa. The higher diversity and number of genus-level clades observed in primary forests, compared to secondary forests, suggests that mature, undisturbed ecosystems may support greater habitat specialization and niche partitioning, as proposed by classical ecological theory (see Barberán et al. 2014). However, the relationship between forest succession and microbial diversity remains debated in the literature. While our findings show higher diversity in primary forests, contrasting patterns have been reported, such as the recent work in karst ecosystems in southwest China where bacterial diversity was higher, not lower, in secondary forests (Jiang et al. 2024). This discrepancy likely reflects the strong influence of local abiotic factors—such as climate, soil parent material, nutrient availability, and disturbance history—in modulating microbial community assembly during succession (Pan et al. 2025; Jiang et al. 2024). Indeed, in secondary forests, early successional dominant taxa may temporarily outcompete other taxa, causing reduced abundance evenness across taxa, and thus lower diversity values (Fierer et al. 2012). Recent work by Romero (2025) on native potato rhizospheres in the Peruvian Andes similarly showed that management intensity (analogous to disturbance) significantly decreased microbial diversity.

Table 3. Diversity in Hill numbers of the bacterial communities found in each inoculum replicate isolated from the rhizosphere of *Theobroma gileri* trees growing in primary soil (IPS1 and IPS2 replicates) and secondary soil (ISS1 and ISS2).

Inoculum code (in Jensen media)	Species richness (q=0)	Effective number of species based on Shannon exp (q=1)	Effective number of species based on Simpson inv (q=2)
IPS1	87	9.98	5.57
IPS2	114	23.78	14.24
ISS1	64	4.43	2.86
ISS2	84	6.44	3.06

Regarding relative abundance patterns, around four to six very abundant genus-level clades fell within the ≥0.95 quantile threshold of the abundance distribution in each replicate (IPS1, IPS2, ISS1, ISS2) (Figure 2). On the other hand, the rarest genus-level clades falling within the ≤0.05 quantile range summed 9 taxa for IPS1 (5 not identified to the genus-level), 13 taxa for IPS2 (8 not identified to the genus-level), 4 taxa for ISS1 (1 not identified to the genus-level), and 7 taxa for ISS2 (4 not identified to the genus-level). In general, most clades unidentified at the genus level occurred at very low relative abundances, suggesting they represent rare components of the rhizosphere, with minimal contribution to the overall community structure (see Lynch and Neufeld 2015).

As expected after culturing in Jensen medium, potential N-fixing bacteria were commonly detected in bacterial inocula across all replicates (81.4% of the total bacterial abundance in IPS1, 39.7% in IPS2, 95.1% in ISS1, and 86.4% in ISS2). Indeed, out of the total 175 genus-level clades found across all replicates, 32 contained the “complete” set of *nif* genes (*nifH, nifD*, and *nifK*), while an additional 15 contained one or two of these genes. Furthermore, among the topmost abundant genera (Figure 2), most contained at least one *nif* gene, demonstrating a widespread genetic potential for biological N-fixation in the soil from both forest types (primary and secondary forests). Note that *Clostridium* clades had high relative abundance in bacterial inocula from primary forest soil (IPS), while *Azotobacter* was very common in bacterial inocula from secondary forest soil (Figure 2). Both are well known anaerobic (*Clostridium*) and aerobic (*Azotobacter*) N-fixing bacteria (Becking 2006; Dos Santos et al. 2012). The high abundance of *Clostridium* in primary forest soil suggests that anaerobic or microaerophilic microsites may be prevalent in this habitat, whereas the dominance of *Azotobacter* in secondary forest soil points to a shift in soil conditions, possibly related to higher oxygen availability, differences in organic matter quality, or disturbance history associated with forest succession. The observed compositional patterns depicted in Figure 2 align with observations by Eaton et al. (2020) in Costa Rican forests, where distinct assemblages of N-fixing bacteria were found between primary and secondary forest soils under leguminous trees. While the genetic potential for N-fixation seems widespread, the successful establishment of specific diazotrophic genera is probably influenced by many abiotic and biotic factors.

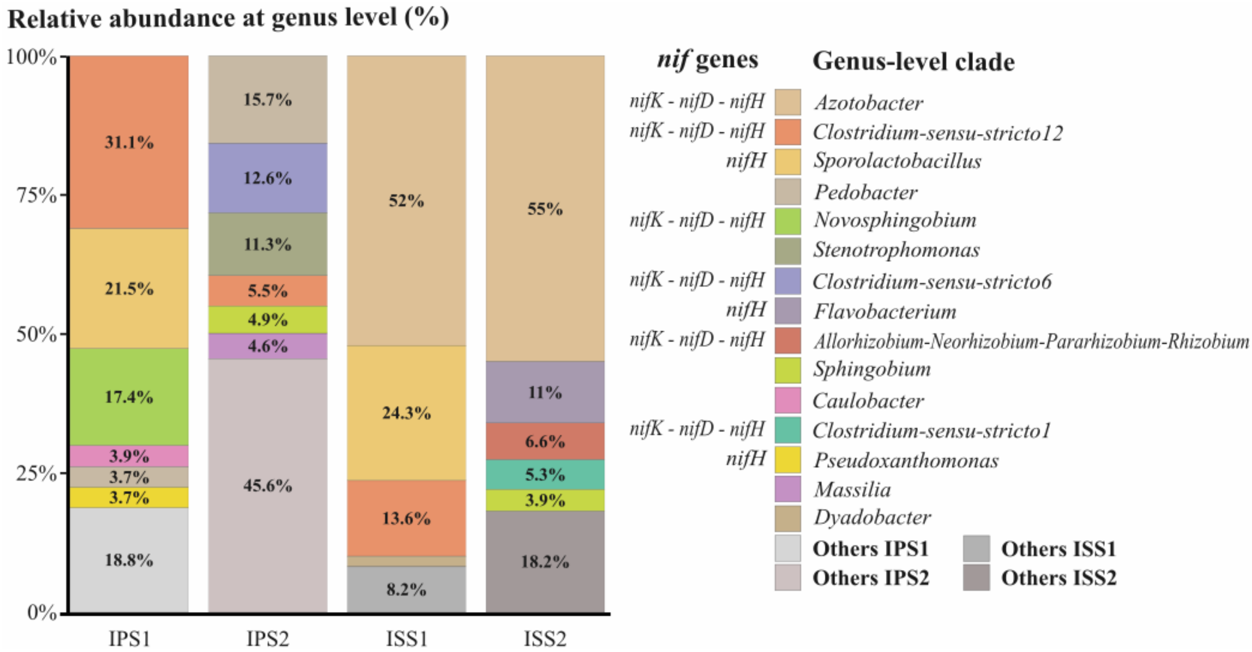

Figure 2. Relative abundance (%) of the most abundant genera (quantile ≥0.95 of the abundance distribution) found in each replicate of bacterial inocula isolated from the rhizosphere of *Theobroma gileri* trees growing in primary soil (IPS1 and IPS2 replicates) and secondary soil (ISS1 and ISS2). The presence of different *nif* genes in each genus-level clade is also indicated. Taxonomic names were those given by the DADA2 software.

Finally, there were various genera with variable, or unexpected, abundance among replicates. For example, *Sporolactobacillus* was relatively abundant in the IPS1 and ISS1 replicates (i.e., in different forest types); *Novosphingobium* exhibited high abundance only in IPS1; and *Pedobacter* was much more abundant in IPS2 than in IPS1 (Figure 2). *Sporolactobacillus* and *Novosphingobium* are potentially N-fixing bacteria (containing at least on *nif* gene sequence), but not *Pedobacter*. The relatively inconsistent variation among replicates, even from the same forest type, suggests that stochastic processes or fine-scale spatial heterogeneity certainly influence microbial community assembly.

3.2. Effect of biological fertilization (bacterial inocula) vs. chemical fertilization (urea) on the growth of *Theobroma cacao* CCN-51 seedlings

3.2.1. Seedlings survival during the experimental period

All except one of the treatments recorded a survival rate of 100% during the experimental period (63 days). The exception was the IPS-/ISS-_U+ treatment (i.e., no bacterial inocula added, but only 1.5 g of urea added) which only had a survival rate of 40% (3 out of the 5 plants died by day 44). The 100% survival rate in all inoculated treatments, regardless of urea addition, underscores the potential of native bacteria associated to *Theobroma gileri* rhizosphere as effective bioprotective agents during early establishment. In other words, the presence of microbial inoculants in the rhizosphere of *Theobroma cacao* seedlings may buffer against the potential stress induced by chemical fertilization. Indeed, recent studies have shown that microbial inoculants can enhance plant resilience to abiotic stressors by modulating phytohormone levels and improving nutrient imbalance or uptake efficiency (Trivedi et al. 2020; Liu et al. 2023). Further, the 100% survival rate in all inoculated seedlings is consistent with a growing body of evidence supporting the use of native microbial consortia over single-strain inoculants for improving seedling performance in tropical reforestation programs (Eaton et al. 2020).

3.2.2. Height growth rate

To avoid interpretative biases, statistical analyses (ANOVA) in this and the following sections only considered those plants that completed the experimental period (i.e., n=2 seedlings for the IPS-/ISS-_U+ treatment, and n=5 for the control and rest of the treatments; see Figure 1). Total sample size (N) analyzed was therefore reduced from N=30 to N=27 seedlings.

Raw height data collected during the monitoring period is shown in Supplementary Table 2 (available online). The variable of height growth rate (mm/week) did not show a normal distribution (Shapiro Wilk test: W=0.8504, P=0.0012). However, by log-transforming the data, the distribution became relatively normal (W=0.9525, P=0.2467). Using these transformed data, a two-way ANOVA analysis was conducted (see Figure 2), which did not detect any significant effect at an α=0.10 (Table 4, Figure 3). In other words, neither of the two factors—absence/presence of urea vs. bacterial inocula—had a significant effect on height growth rate.

However, note that the P-value associated to the urea factor was somewhat close to the predefined α=0.10 (F=2.41, P=0.14) meaning that urea addition *tended* to influence height growth rate. This almost significant result aligns with the well-documented role of nitrogen as a limiting nutrient in tropical soils (Vitousek and Howarth 1991), but the lack of statistical significance suggests that the 63-day experimental period or the sample size used may have been insufficient to capture full treatment effects on aboveground growth. Woody perennials often exhibit delayed growth responses to fertilization and inoculation, as initial resource allocation may prioritize root development over shoot elongation (Poorter et al. 2012). In this context, it would be appropriate to measure radicular biomass in future experiments, although that would imply sacrificing the plants. In addition, future studies should also extend the observational experimental period and increase sample size.

Table 4. Two-way ANOVA table corresponding to the response variable of height change (mm/week; logarithmically transformed) in *Theobroma cacao* seedlings subjected to biological fertilization (bacterial inoculants) vs. chemical fertilization (urea). Experimental design is shown in Figure 1.

	Sum of sqrs	**df**	**Mean square**	***F***	***P* (same)**
Inoculum (I)	0.15	2	0.08	0.80	0.46

Urea (U)	0.23	1	0.23	2.41	0.14
Interaction I x U	0.03	2	0.02	0.17	0.84
Within:	1.97	21	0.09		
Total:	2.42	26			

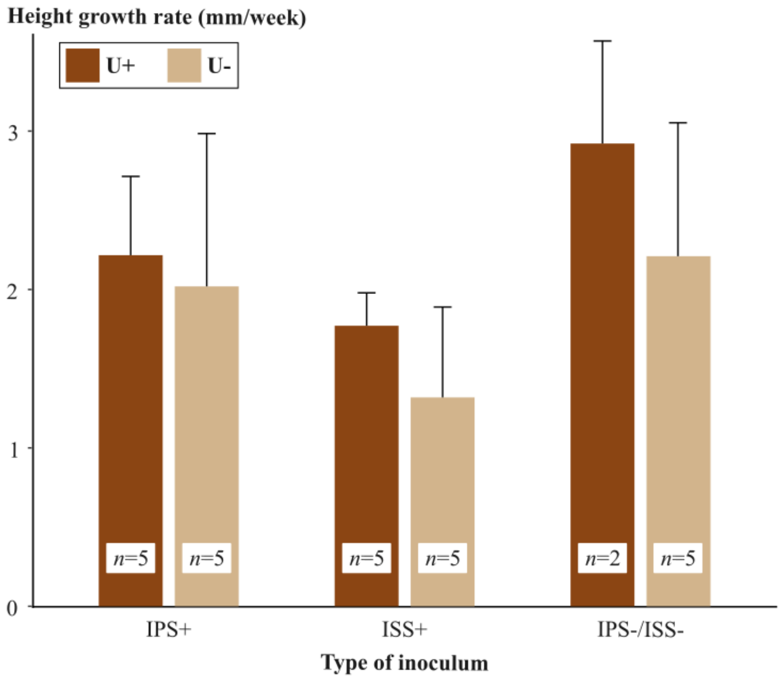

Figure 3. Height change (mm/week) in *Theobroma cacao* seedlings subjected to different bacterial inocula and urea treatments. Each bar and its corresponding error represent the mean + 1SEM (one standard error of the mean). The experimental design was a two-way ANOVA (Figure 1) where the first factor had two levels: urea treatment (U+) and control without urea (U-); while the second factor had three levels: treatment with primary soil inoculum (IPS+), treatment with secondary soil inoculum (ISS+), and control without inoculum (IPS-/ISS-). *n* = number of replicates in each possible combination. The corresponding ANOVA table is shown as Table 4.

3.2.3. Diameter growth rate

Raw diameter data collected during the monitoring period is shown in Supplementary Table 2 (available online). The diameter growth rate variable (mm/week) showed an almost normal distribution (Shapiro Wilk test: W=0.8172, P=0.06). The two-way ANOVA analysis (see Figure 1) did not detect any significant effect at an α=0.10 (Table 5, Figure 4). In other words, neither of the two factors—absence/presence of urea vs. bacterial inocula—had a significant effect on the stem diameter growth rate. This lack of any effect whatsoever is not in accord to the recognized potential of native N-fixing bacteria as growth-promoting agents, independent of chemical fertilization (Eaton et al. 2020; Trivedi et al. 2020). Indeed, recent meta-analyses have shown that microbial inoculants can increase stem diameter in tree seedlings, with effects becoming more pronounced after the initial establishment phase (Rafiq & Reshi 2025)—unfortunately, this pattern was not apparent in this study.

Table 5. Two-way ANOVA table corresponding to the response variable of diameter change (mm/week) in *Theobroma cacao* seedlings subjected to biological fertilization (bacterial inoculants) vs. chemical fertilization (urea). Experimental design is shown in Figure 1.

	Sum of sqrs	**df**	**Mean square**	***F***	***P* (same)**
Inoculum (I)	0.04	2	0.02	1.22	0.32
Urea (U)	0.02	1	0.02	0.88	0.36
Interaction I x U	0.05	2	0.02	1.31	0.29
Within:	0.36	21	0.02		
Total:	0.46	26			

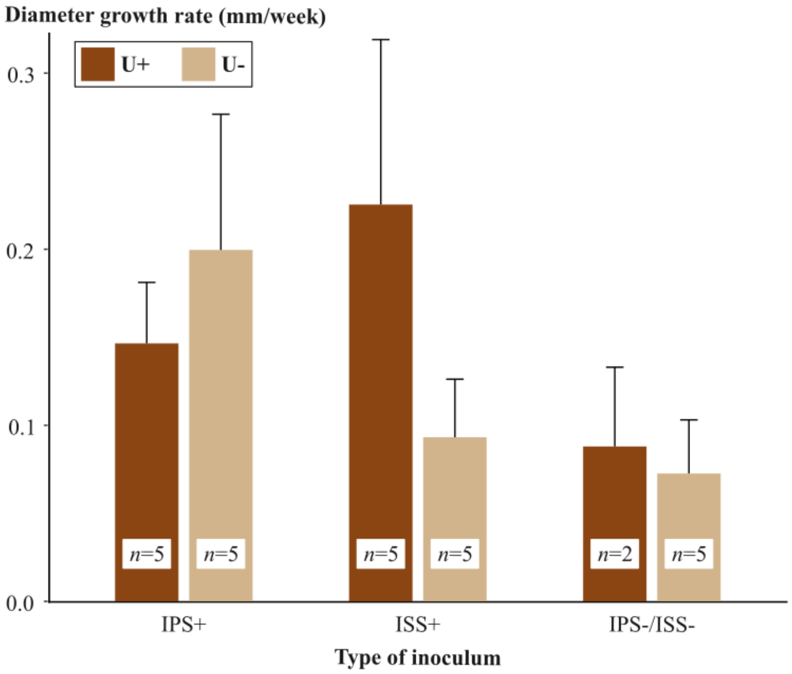

Figure 4. Diameter change (mm/week) in *Theobroma cacao* seedlings subjected to different bacterial inocula and urea treatments. Each bar and its corresponding error represent the mean + 1SEM (one standard error of the mean). The experimental design was a two-way ANOVA (Figure 1) where the first factor had two levels: urea treatment (U+) and control without urea (U-); while the second factor had three levels: treatment with primary soil inoculum (IPS+), treatment with secondary soil inoculum (ISS+), and control without inoculum (IPS-/ISS-). *n* = number of replicates in each possible combination. The corresponding ANOVA table is shown as Table 5.

3.2.4. Chlorophyll index (SPAD) rate

Raw chlorophyll data collected during the monitoring period is shown in Supplementary Table 2 (available online). The change of the chlorophyll index over time (SPAD units/week) showed an almost normal distribution (Shapiro Wilk test: W=0.9273, P=0.06). The two-way ANOVA analysis (see Figure 1) did not detect any significant effect at an α=0.10 (Table 6, Figure 5). In other words, neither of the two factors—absence/presence of urea vs. bacterial inocula—had any effect on the chlorophyll index.

However, note that the *P*-value associated to the urea factor was somewhat close to the predefined α=0.10 (F=2.66, P=0.12) meaning that urea addition *tended* to influence the change in chlorophyll content. Given that nitrogen is a key component of chlorophyll molecules and photosynthetic machinery (Evans 1989; Mu and Chen 2021), this almost significant urea effect on chlorophyll change over time suggests that chemical nitrogen is more immediately available for chlorophyll synthesis, while microbially-fixed nitrogen may be preferentially allocated to root development or other tissues during early establishment (Poorter et al. 2012), thus somewhat delaying the presence of biologically-fixed nitrogen in leaves (Liu et al. 2023; Koskey et al. 2021).

Table 6. Two-way ANOVA table corresponding to the response variable of chlorophyll index change (SPAD units/week) in *Theobroma cacao* seedlings subjected to biological fertilization (bacterial inoculants) vs. chemical fertilization (urea). Experimental design is shown in Figure 1.

	Sum of sqrs	**df**	**Mean square**	***F***	***P* (same)**
Inoculum (I)	0.40	2	0.20	0.63	0.54
Urea (U)	0.85	1	0.85	2.66	0.12
Interaction I x U	0.35	2	0.18	0.55	0.59
Within:	6.74	21	0.32		
Total:	8.29	26			

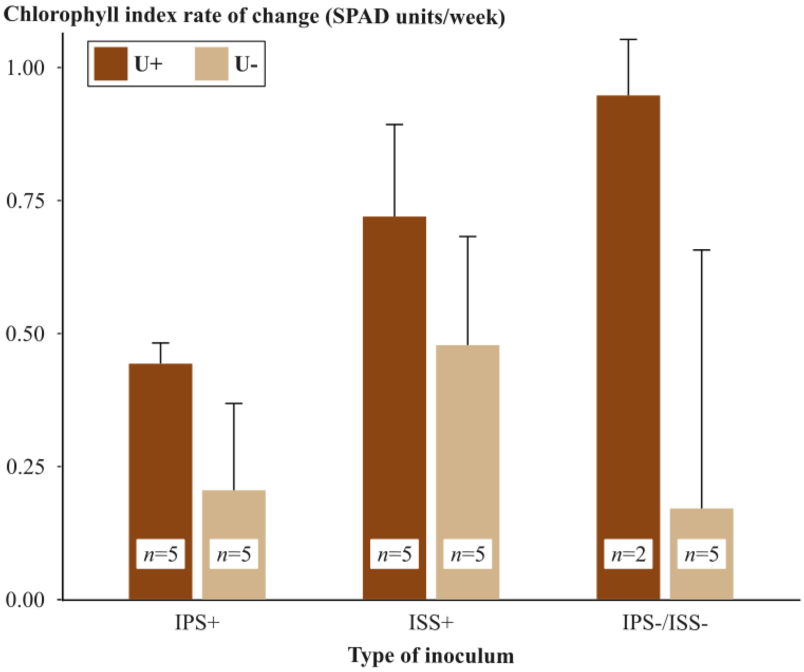

Figure 5. Chlorophyll index change (SPAD units/week) in *Theobroma cacao* seedlings subjected to different bacterial inocula and urea treatments. Each bar and its corresponding error represent the mean + 1SEM (one standard error of the mean). The experimental design was a two-way ANOVA (Figure 1) where the first factor had two levels: urea treatment (U+) and control without urea (U-); while the second factor had three levels: treatment with primary soil inoculum (IPS+), treatment with secondary soil inoculum (ISS+), and control without inoculum (IPS-/ISS-). *n* = number of replicates in each possible combination. The corresponding ANOVA table is shown as Table 6.

4. Conclusions

We tested for the first time whether native bacterial inocula isolated from the rhizosphere of *Theobroma gileri* trees—a wild relative of cacao—harbors diverse bacterial communities with potential to act as biofertilizers of *Theobroma cacao* seedlings, thus helping to reduce the overuse of chemical fertilizers, particularly urea. We postulated that (1) the rhizosphere of *T. gileri* trees growing in primary forest will host more diverse bacterial communities than the rhizosphere of *T. gileri* trees growing in secondary (disturbed) forest soils; and that (2) bacterial communities isolated from the rhizosphere of *T. gileri* may offer functional nutritional advantages compared to the benefits of using conventional chemical fertilizers. The first postulate was supported by our data, given that the rhizosphere of *T. gileri* trees growing in primary forest soils harbored more diverse bacterial communities than that of secondary forest soils. However, we could not find strong support for the second postulate, even though genetic potential for biological N-fixation was detected in many of the genera, and that N-fixing bacteria genera were much more abundant than other types of bacteria in all but one of the replicates (in primary forest). Given that chemical fertilization with urea almost significantly affected chlorophyll index and height, but biological fertilization with bacterial inocula did not show a clear effect on the growth of *T. cacao* seedlings, we conclude—at least for our pilot experiment—that bacterial inocula isolated from the rhizosphere of *T. gileri* do not seem to have an evident effect on *T. cacao* seedlings growth, although may be beneficial to assure seedling survival (because the urea-only treatment had 60% mortality rate among seedlings).

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Declarations

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Conflicts of interest/Competing interests The authors declare no conflicts of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Data Availability The original datasets (Supplementary Tables 1 and 2) generated and analyzed in the current study are available online as electronic complementary material:

- **Supplementary Table 1.** Taxonomical information and absolute abundances of all genus-level clades detected via a metagenomic analysis of bacterial inocula isolated from the rhizosphere of *Theobroma gileri* trees, growing in primary forest soil (IPS replicates) and secondary forest soil (ISS replicates).

- **Supplementary Table 2.** Raw monitoring data of *Theobroma cacao* seedlings growth (height and diameter) and their chlorophyll index (SPAD units) throughout the duration of the pilot agricultural experiment designed to test the effect of biological fertilization (bacterial inocula) vs. chemical fertilization (urea).

Code availability Not applicable.

Authors'contributions Conceptualization: HRS, NHB, SMA; literature review: NHB, HRS, SMA; data collection (field and lab work): NHB; data analyses: NHB, HRS; writing original draft: NHB; writing review and editing: HRS; funding acquisition: SMA, NHB; supervision: SMA.

References

Acuña, C. (2025) Efecto del compost en la producción de plantones de cacao (T*heobroma cacao* L.) [Thesis], Universidad Nacional de Educación Enrique Guzmán y Valle, Perú. https://repositorio.une.edu.pe/server/api/core/bitstreams/947c93c6-a779-46ab-8f47-b7cb2b93864b/content

Anwesh, M. (2021) Silva 138 classifiers. https://github.com/anw-sh/silva-138_classifiers Accessed 5 January 2026

Argüello-Navarro, A. Z., & Moreno-Rozo, L. Y. (2014) Evaluación del potencial biofertilizante de bacterias diazótrofas aisladas del cultivo de cacao (*Theobroma cacao* L.). Acta Agronómica 63(3):38–245. https://doi.org/10.15446/acag.v63n3.41033

Bai, B., Liu, W., Qiu, X., Zhang, J., Zhang, J., & Bai, Y. (2022) The root microbiome: community assembly and its contributions to plant fitness. Journal of Integrative Plant Biology 64(2):230–243. https://doi.org/10.1111/jipb.13226

Barberán, A., Ramirez, K. S., Leff, J. W., Bradford, M. A., Wall, D. H., & Fierer, N. (2014) Why are some microbes more ubiquitous than others? Predicting the habitat breadth of soil bacteria. Ecology Letters 17(7):794–802. https://doi.org/10.1111/ele.12282

Becking, J. H. (2006) The family Azotobacteraceae. The Prokaryotes 6:759–783. https://doi.org/10.1007/0-387-30746-x_26

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., Cope, E. K., Da Silva, R., Diener, C., Dorrestein, P. C., Douglas, G. M., Durall, D. M., Duvallet, C., Edwardson, C. F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J. M., Gibbons, S. M., Gibson, D. L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G. A., Janssen, S., Jarmusch, A. K., Jiang, L., Kaehler, B. D., Kang, K. B., Keefe, C. R., Keim, P., Kelley, S. T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M., Lee, J., Ley, R., Liu, Y. X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B. D., McDonald, D., McIver, L. J., Melnik, A. V., Metcalf, J. L., Morgan, S. C., Morton, J. T., Naimey, A. T., Navas-Molina, J. A., Nothias, L. F., Orchanian, S. B., Pearson, T., Peoples, S. L., Petras, D., Preuss, M. L., Pruesse, E., Rasmussen, L. B., Rivers, A., Robeson, M. S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S. J., Spear, J. R., Swafford, A. D., Thompson, L. R., Torres, P. J., Trinh, P., Tripathi, A., Turnbaugh, P. J., Ul-Hasan, S., van der Hooft, J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K. C., Williamson, C., Willis, A. D., Xu, Z. Z., Zaneveld, J. R., Zhang, Y., Zhu, Q., Knight, R., & Caporaso, J. G. (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology 37:852–857. https://doi.org/10.1038/s41587-019-0209-9

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016) DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods 13(7):581–583. https://doi.org/10.1038/nmeth.3869

Cerruffo , H. A., & Espín , W. S. (2022) Análisis económico de la aplicación de compost orgánico en cultivos de cacao. Un enfoque a pequeños productores de cacao CCN-51 del litoral ecuatoriano. [Thesis], Escuela Superior Politécnica del Litoral, Ecuador. http://www.dspace.espol.edu.ec/handle/123456789/57466

Cuatrecasas, J. (1964) Cacao and its allies: A taxonomic revision of the genus *Theobroma*. Contributions from the United States National Herbarium 35(6):379–614.

Dos Santos, P. C., Fang, Z., Mason, S. W., Setubal, J. C., & Dixon, R. (2012) Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. BMC Genomics 13(1):162. https://doi.org/10.1186/1471-2164-13-162

Eaton, W. D., McGee, K. M., Hoke, E., Lemenze, A., & Hajibabaei, M. (2020) Influence of two important leguminous trees on their soil microbiomes and nitrogen cycle activities in a primary and recovering secondary forest in the northern zone of Costa Rica. Soil Systems 4(4): 65. https://doi.org/10.3390/soilsystems4040065

Evans, J. R. (1989) Photosynthesis and nitrogen relationships in leaves of C3 plants. Oecologia 78:9–19. https://doi.org/10.1007/bf00377192

Fierer, N., Leff, J. W., Adams, B. J., Nielsen, U. N., Bates, S. T., Lauber, C. L., Owens, S., Gilbert, J. A., Wall, D. H., & Caporaso, J. G. (2012) Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. Proceedings of the National Academy of Sciences 109(52):21390–21395. https://doi.org/10.1073/pnas.1215210110

Hill, M. O. (1973) Diversity and evenness: A unifying notation and its consequences. Ecology 54(2):427–432. https://doi.org/10.2307/1934352

HiMedia. (2026). Jensen’s Medium (Agar). Himedialabs.com. https://www.himedialabs.com/us/m710-jensens-medium-agar.html Accessed 5 January 2026

Jannah, N. A., Mustafa, I., & Jatmiko, Y. D. (2022) Isolation and identification of Nitrogen-fixing rhizobacteria associated with cocoa plantation (*Theobroma cacao* L.) as biofertilizer agent. The Journal of Experimental Life Sciences 12(3):75–80. https://doi.org/10.21776/ub.jels.2022.012.03.01

Jiang, S., Song, M., Du, H., Wang, F., Song, T., Chen, H., Zeng, F., & Peng, W. (2024) Soil properties regulate soil microbial communities during forest succession in a karst region of southwest China. Microorganisms 12(11):2136. https://doi.org/10.3390/microorganisms12112136

Jost, L. (2006) Entropy and diversity. Oikos 113(2):363-375. https://doi.org/10.1111/j.2006.0030-1299.14714.x

Koskey, G., Mburu, S. W., Njeru, E. M., Kimiti, J. M., Ombori, O., & Maingi, J. M. (2017) Potential of native rhizobia in enhancing nitrogen fixation and yields of climbing beans (*Phaseolus vulgaris* L.) in contrasting environments of eastern Kenya. Frontiers in Plant Science 8:443. https://doi.org/10.3389/fpls.2017.00443

La Hora. (2025) Precio del cacao alcanza sus mayores niveles en 47 años y Ecuador tiene las condiciones para aprovechar ese boom. "La Hora" newspaper. https://www.lahora.com.ec/archivo/Precio-del-cacao-alcanza-sus-mayores-niveles-en-47-anos-y-Ecuador-tiene-las-condiciones-para-aprovechar-ese-boom-20240308-0029.html Accessed 5 January 2026

Liu, Q., Cheng, L., Nian, H., Jin, J., & Lian, T. (2023) Linking plant functional genes to rhizosphere microbes: a review. Plant Biotechnology Journal 21:902–917. https://doi.org/10.1111/pbi.13950

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., Tremblay, J., Engelbrektson, A., Kunin, V., Rio, T. G. del, Edgar, R. C., Eickhorst, T., Ley, R. E., Hugenholtz, P., Tringe, S. G., & Dangl, J. L. (2012) Defining the core *Arabidopsis thaliana* root microbiome. Nature 488(7409):86–90. https://doi.org/10.1038/nature11237

Lynch, M. D. J., & Neufeld, J. D. (2015) Ecology and exploration of the rare biosphere. Nature Reviews Microbiology 13(4):217–229. https://doi.org/10.1038/nrmicro3400

MAE - Ministerio del Ambiente del Ecuador. (2013) Sistema de Clasificación de los Ecosistemas del Ecuador Continental. Subsecretaría de Patrimonio Natural. Quito.

Mendoza-Rodrigez, M. D., & Pazmiño-Mera, A. J. (2021) Microorganismos (Hongos y bacterias "género *Bacillus*") asociados a la rizósfera del cacao en diferentes sistemas de producción en el cantón Portoviejo. [Thesis], Escuela Superior Politécnica Agropecuaria de Manabí, Ecuador.

Mu, X., & Chen, Y. (2021) The physiological response of photosynthesis to nitrogen deficiency. Plant Physiology and Biochemistry 158:76–82. https://doi.org/10.1016/j.plaphy.2020.11.019

Pan, M., Xiao, R., & Ni, H. (2025) Changes in soil microbial diversity across different forest successional stages: A meta-analysis of Chinese forest ecosystems. Forests 16(8):1319–1319. https://doi.org/10.3390/f16081319

Poorter, H., Niklas, K. J., Reich, P. B., Oleksyn, J., Poot, P., & Mommer, L. (2012) Biomass allocation to leaves, stems and roots: meta-analyses of interspecific variation and environmental control. New Phytologist 193(1):30–50. https://doi.org/10.1111/j.1469-8137.2011.03952.x

Quiroz, J., Mestanza, S., Parada, N., Morillo, L., Samaniego, I., & Garzón, I. (2021) Catálogo de cultivares de cacao en Ecuador. Iniap.gob.ec; Yaguachi, EC: INIAP, Estación Experimental Litoral Sur, Programa de Cacao. https://repositorio.iniap.gob.ec/items/8825b24f-b2f6-42a1-b949-966921431073 Accessed 20 March 2026

R Core Team (2025) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/ Accessed 20 March 2026

Rafiq, I., & Reshi, Z. A. (2025) Global meta-analysis of endophytic inoculation effects on seed germination, seedling growth, and nutrient uptake of trees. Rhizosphere 34:101079. https://doi.org/10.1016/j.rhisph.2025.101079

Reeta, K., Sonal , B., & Charu , K. (2022) Nitrogen assimilation in plants. In Advances in Plant Nitrogen Metabolism. CRC Press, pp 17. https://doi.org/10.1201/9781003248361 (ISBN: 9781032163949)

Romero, Y. (2025) Diversidad microbiana en el suelo rizosférico de papa nativa bajo manejo orgánico y convencional en Huancayo [Thesis], Universidad Nacional Federico Villarreal, Perú. https://hdl.handle.net/20.500.13084/11449

Sudarma, J. A., & Masdar, H. (2022) Effects of manure and urea fertilizer on cocoa growth (*Theobroma cacao* L.). Journal of Social Science 3(5):1122–1130. https://doi.org/10.46799/jss.v3i5.432

Trivedi, P., Leach, J. E., Tringe, S. G., Sa, T., & Singh, B. K. (2020) Plant-microbiome interactions: from community assembly to plant health. Nature Reviews. Microbiology 18(11):607–621. https://doi.org/10.1038/s41579-020-0412-1

Troya-Medina, N. L. (2024) Análisis del impacto ambiental de los agroquímicos usados en el cultivo de cacao CCN-51 (*Theobroma cacao* L.) en la comunidad El Encanto del Cantón San Lorenzo, Ecuador. Reincisol 3(6):2962–2982. https://doi.org/10.59282/reincisol.v3(6)2962-2982

Tropicos.org. (2026) Missouri Botanical Garden. https://www.tropicos.org Accessed 20 March 2026

Vásconez, L. (2026) El precio del cacao vuelve a niveles de 2023 tras una fuerte caída y pone en alerta a Ecuador. “El Comercio” newspaper.. https://www.elcomercio.com/actualidad/negocios/precio-cacao-vuelve-niveles-2023-tras-fuerte-caida-pone-alerta-ecuador/ Accessed 20 March 2026

Vitousek, P. M., & Howarth, R. W. (1991) Nitrogen limitation on land and in the sea: How can it occur? Biogeochemistry 13:87–115. https://doi.org/10.1007/bf00002772

Weinstein, M., Graber, E. R., Yermiyahu, U., Sperling, O., Tsehansky, L., Elmakias, A., Grinshpun, J., Rosenfeld, L., & Baram, S. (2025) Nitrogen affects cacao (*Theobroma cacao* L.) tree Growth, macronutrient demand, and water use. Journal of the American Society for Horticultural Science 150(5):290–299. https://doi.org/10.21273/jashs05518-25

Winter, D. J. (2017) rentrez: an R package for the NCBI eUtils API The R Journal 9(2):520-526