UNIVERSIDAD AUTÓNOMA CHAPINGO
UNIDAD REGIONAL UNIVERSITARIA DE ZONAS ÁRIDAS
Posgrado En Recursos Naturales Y Medio Ambiente
En Zonas Áridas

Study of the Bacteriome of Nodules from Native Mexican Beans: Characterization and Selection of Plant Growth–Promoting Bacteria

Estudio del Bacterioma de Nódulos de Frijol Nativos de México, Caracterización y Selección de Bacterias Promotoras del Crecimiento Vegetal

T H E S I S

Submitted as a partial requirement to obtain the degree of:

MAESTRA EN CIENCIAS EN RECURSOS NATURALES Y MEDIO AMBIENTE EN ZONAS ÁRIDAS

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ACKNOWLEDGEMENTS

My sincere appreciation to the Secretaría de Ciencia, Humanidades, Tecnología e Innovación (**SECIHTI**) for the financial support that strengthened my scientific training and enabled the completion of this research project.

To **UACh–URUZA**, for receiving me into the Chapingo community and allowing me to continue my academic training and giving me the opportunity to meet remarkable colleagues and academics.

To **Dr. Ricardo Trejo Calzada**, I express my gratitude for his guidance, teachings, and constant support. His influence and encouragement at the beginning of my academic path inspired me to move forward with this project.

To **Dr. Erika Nava Reyna**, I am especially grateful to you for your close mentorship throughout this process. Your patience and confidence were essential to my academic and professional development. You have been part of my training since my undergraduate studies, and your guidance has been key to my growth.

To **Dr. Ma. del Rosario Jacobo Salcedo**, I am deeply thankful for accepting me as her thesis student. Her involvement meant a great deal to me. Her character, professionalism, and commitment have motivated me to approach my work with greater discipline and responsibility. Her support was decisive in helping me move forward at this stage.

To **Dr. Oscar Martín Antúnez Ocampo** and **Dr. Aurelio Pedroza Sandoval**, I express my sincere appreciation for their support and the clarity with which they helped me understand topics that were new to me. Their guidance was crucial for the progress of this project.

To my friends, **Diana** and **José Antonio**, thank you. Your companionship, support, and camaraderie made this journey more manageable, more human, and deeply meaningful.

DEDICATED TO

To **Francisco**, my partner through adventures and conflicts, through sadness and joy. Thank you for your unconditional support.

To our blessing, **Noah**, and to the two new additions to our family, my **sisters**. I love you, even when you drive me crazy.

Finally, to my **mother** and **grandparents**, who would be happy and proud to see the achievements I have reached and eager for the ones still to come.

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RESUMEN GENERAL

Estudio del Bacterioma de Nódulos de Frijol Nativos de México, Caracterización y Selección de Bacterias Promotoras del Crecimiento Vegetal

En México, el frijol común (*Phaseolus vulgaris* L.) es un cultivo estratégico por su relevancia nutricional, económica y sociocultural. Como otras leguminosas, posee la capacidad de establecer simbiosis con bacterias fijadoras de nitrógeno; sin embargo, el papel de otras bacterias asociadas a este proceso ha sido poco explorado, a pesar de su potencial para mejorar la productividad agrícola y la sanidad vegetal. El objetivo de este estudio fue caracterizar dichas poblaciones y su metabolismo mediante técnicas de metabarcoding y ensayos *in vitro*, con el fin de identificar cepas con potencial como biofertilizantes. Se compararon muestras de dos regiones ecológicas contrastantes: una zona semiárida (Durango) y una subtropical húmeda (Guerrero). Los resultados funcionales revelaron que el origen ambiental influye significativamente en las capacidades metabólicas de los aislados: aquellos provenientes de la región húmeda presentaron una mayor actividad enzimática (proteasas y amilasas), mientras que la producción de sideróforos fue más prevalente en la región semiárida. Se seleccionaron cepas sobresalientes, como G9 y D7, por su notable capacidad de producción de amonio, ácido indolacético y sideróforos. Por otro lado, los análisis moleculares confirmaron que, si bien el género *Rhizobium* es el más abundante, los nódulos albergan un consorcio diverso de bacterias no rizobiales, donde predominan los phyla Proteobacteria y Bacteroidota. En las variedades mejoradas de la zona norte, se identificó un "núcleo microbiano conservado", dado que no se observaron diferencias significativas en la diversidad bacteriana entre los genotipos evaluados. Este estudio demuestra que tanto el ambiente como la selección del hospedero modelan la arquitectura del microbioma nodular, hallazgos que permiten avanzar hacia la reducción de fertilizantes sintéticos mediante el aprovechamiento estratégico de la diversidad bacteriana nativa.

Palabras clave: *Phaseolus vulgaris, nódulos, metabarcoding, rizobios.*

Tesis de Maestría en Ciencias en Recursos Naturales y Medio Ambiente de Zonas Áridas
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GENERAL ABSTRACT

Study of the Bacteriome of Nodules from Native Mexican Beans, Characterization and Selection of Plant Growth–Promoting Bacteria

In Mexico, the common bean (*Phaseolus vulgaris* L.) is a strategic crop due to its nutritional, economic, and sociocultural significance. Like other legumes, it can establish symbiotic relationships with nitrogen-fixing bacteria; however, the role of other bacteria associated with this process remains underexplored, despite their potential to enhance agricultural productivity and plant health. This study aimed to characterize these populations and their metabolism using metabarcoding techniques and in vitro assays to identify strains with potential as biofertilizers. Samples from two contrasting ecological regions were compared: a semi-arid zone (Durango) and a humid subtropical zone (Guerrero). Functional results revealed that environmental origin significantly influences the metabolic capabilities of the isolates: those from the humid region exhibited higher enzymatic activity (proteases and amylases), while siderophore production was more prevalent in the semi-arid region. Outstanding strains, such as G9 and D7, were selected for their notable capacity to produce ammonium, indole-acetic acid, and siderophores. Furthermore, molecular analyses confirmed that while the genus Rhizobium is the most abundant, the nodules harbor a diverse consortium of non-rhizobial bacteria, predominantly within the phyla Proteobacteria and Bacteroidota. In improved varieties from the northern region, a "conserved microbial core" was identified, as no significant differences in bacterial diversity were observed among the evaluated genotypes. This study demonstrates that both the environment and host selection model the architecture of the nodule microbiome. These findings contribute to the transition from synthetic fertilizer dependence toward the strategic utilization of native bacterial diversity.

Keywords: *Phaseolus vulgaris, nodules, metabarcoding, rhizobia.*

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CHAPTER 1

General Introduction

Legumes (Fabaceae or Leguminosae) are among the most important botanical families from an agricultural perspective, second only to cereal crops in the grass family (Poaceae or Gramineae). They occupy between 12% and 15% of cultivated land, provide 33% of dietary proteins, and account for 27% of major crops worldwide, making legumes key components of sustainable agriculture and global food security (Etesami & Adl, 2020).

Within the legume family, the common bean (*Phaseolus spp*.) is one of the oldest domesticated crops and one of the most dominant in the Americas. Bean crops exhibit remarkable diversity in cultivation methods, adapting to different environments and modifying their morphological variability. They can be found from sea level up to 3,000 m.a.s. and are cultivated in monoculture, associations, or rotations. Beans are currently consumed as mature grain, immature seeds, and vegetables (both leaves and pods), owing to their versatility and ease of adaptation (Broughton et al., 2003). For these reasons, common bean represents one of the most important protein sources for populations in Latin America and worldwide (Rocha et al., 2020).

Mexico contributes 4% of the world's bean production and is recognized as a primary center of domestication and genetic diversity. Beans are primarily cultivated in Zacatecas, Sinaloa, Durango, Chihuahua, and Nayarit, although they are grown in nearly all states of Mexico, with varietal preferences varying according to regional consumption patterns. Bean cultivation generates approximately 382,000 permanent jobs and involves more than 476,000 producers (SADER, 2021). Mexico also harbors diverse wild populations and landraces that have adapted locally through genetic variation, resulting in differences in floral development, pod morphology, and seed traits (Delgado & Gama-López, 2015). Furthermore, native bean species possess high levels of

nutraceutical compounds and may improve yield and quality under various agronomic practices, including biofertilization.

Bean plants can partially obtain their nitrogen through symbiosis with soil bacteria (rhizobia), which convert atmospheric nitrogen into ammonium, later used to synthesize nitrogenous compounds such as amino acids and amino sugars (Mus et al., 2016).

The beneficial association between rhizobia and legumes has been known for more than a century. In 1888, Beijerink obtained the first pure bacterial culture from a legume nodule responsible for nitrogen fixation. One year later, Frank (1889) named this bacterium *Rhizobium leguminosarum*, and since then, bacteria forming legume nodules have been referred to as “rhizobia” (Peix et al., 2015). These bacteria receive carbon sources from the plant and induce cell division in the root cortex, forming the nodule. The nodule is later invaded by the bacteria, where nitrogen fixation occurs (Gage, 2004; Quinto & Cárdenas, 2007).

This “infection” by rhizobacteria requires that they migrate toward regions enriched in chemical compounds such as flavonoids, organic acids, and amino acids released by the host roots. The resulting bacterial migration and complex biochemical interactions between bacteria and the host roots constitute biological nitrogen fixation—an essential process that reduces production costs and increases the sustainability of agricultural systems across various soil types (De Bedout-Mora et al., 2022).

In addition, some endophytes can coexist with rhizobia inside legume nodules and are referred to as nodule-associated bacteria (NAB) or non-rhizobial bacteria (Majeed et al., 2023; Youseif et al., 2021). These organisms may also promote plant growth, especially under stress conditions (Muresu et al., 2019). Both rhizobia and non-rhizobial endophytes contribute to nitrogen and other nutrients (such as phosphorus, potassium, and iron), produce plant growth hormones, and even protect against diseases. Thus, the presence of rhizobia in root nodules and free-living microbial associations in the rhizosphere plays a decisive role in the

development and normal functioning of both legume and non-legume crops (Hassen et al., 2023).

According to the Food and Agriculture Organization (FAO), future agriculture faces a major challenge: by 2050, it must produce 50% more food, feed, and biofuel than in previous years due to global population growth (FAO, 2017). Similarly, increased variability in temperature, altered precipitation patterns, droughts, floods, pests, and diseases—aggravated by resistance to indiscriminate chemical use—are expected to affect crop yields (Rodríguez-Sahagún et al., 2020).

These challenges require new strategies and systems to improve food production efficiency, reduce costs, and preserve natural resources in the long term (Rodríguez-Sahagún et al., 2020). Soil degradation remains the main limitation to achieving higher yields in developing regions, particularly among low-resource farmers. The intensive and unbalanced use of pesticides and chemical fertilizers has produced significant social, environmental, and economic concerns (Riaz et al., 2020).

Ecological disturbances in soil and water bodies—including biomagnification of toxic agrochemicals, development of pest resistance, and negative effects on human and animal health—continue to increase (Mondal et al., 2022). Excessive fertilizer use has made soil ecology inhospitable for microflora and microfauna (Yadav et al., 2023), causing environmental pollution and health risks such as the accumulation of heavy metals and inorganic contaminants. High applications of nitrogen fertilizers are known to increase N_2O emissions, nitrogen runoff, and soil acidification. Toxic substances may also accumulate in plant tissues, posing risks to human and animal consumers (Yang et al., 2022).

Leveraging soil–plant–microbe interactions is essential for sustainable agriculture and reducing the use of toxic agrochemicals. Plants and microorganisms are fundamental components of the biosphere, and their interactions sustain healthy biogeochemical cycles. Therefore, the efficiency of beneficial soil microbes must

be considered to improve both the quantity and quality of agricultural production (Mondal et al., 2022).

Studying bacterial populations within legume nodules, their metabolism, plant-growth-promoting traits, nodulation capacity, and their physiological effects on the plant is therefore crucial. Recent studies indicate that rhizobia and non-rhizobial endophytes are important components of nodule microbiota (Bender et al., 2022; Leite et al., 2017; Majeed et al., 2023), yet their effects on plants remain understudied. Since many microorganisms inside nodules are not cultivable, their presence may go unnoticed in culture-dependent studies, highlighting the need for advanced molecular tools such as metabarcoding (Hakim et al., 2018).

Biological fertilization relies on organic inputs such as fungi, bacteria, manure, organic waste, and wastewater. These bioinputs improve the rhizosphere's capacity to fix nutrients, stimulate plant growth, enhance soil stability, provide biological control, decompose organic matter, recycle nutrients, promote mycorrhizal symbiosis, and contribute to bioremediation in contaminated soils. Moreover, biofertilizers increase soil fertility, reduce water and soil pollution, improve productivity, require less energy, and support antagonistic interactions against phytopathogens (Yadav et al., 2023).

The use of native rhizobacteria has been shown to improve plant growth and establish more effectively than commercial strains because they are better adapted to local environmental conditions (Banjare et al., 2022). However, to use bacteria as biofertilizers, they must be isolated from nodules or soil, confirmed as non-pathogenic, evaluated for plant-growth-promoting benefits, and produced in a suitable formulation that ensures their delivery and persistence in the plant environment (Trivedi et al., 2005).

Therefore, the objective of this research is to study the bacteriome within nodules of native and improved beans using metabarcoding to characterize the bacterial populations and their metabolism in two different agroecological regions: semiarid and dry tropical regions. Subsequently, rhizobial and nodule-associated bacteria from studied bean roots will be isolated, evaluated for plant-growth-promotion

efficiency, and assessed for nodulation capacity and physiological effects in *Phaseolus vulgaris*. This will serve as an initial step toward developing a biofertilizer to sustainably increase yield and crop quality.

1.1 Hypothesis

Bacterial communities identified via metabarcoding in native bean nodules harbor strains with significant biotechnological potential. These isolates possess traits that not only promote plant growth but also offer a basis for the biocontrol of pathogens and pests in agricultural systems.

1.2 General Objective

Characterizing the bacterial communities within the nodules of native and improved beans across semi-arid and dry tropic agroecological regions using metabarcoding, and to evaluate their potential as multi-functional agents for plant growth promotion and the biocontrol of pests and pathogens.

1.2.1 Specific objectives

- Identifying and comparing the taxonomic composition of bacterial communities within the nodules of native and improved beans across the target agroecological regions using high-throughput sequencing (metabarcoding).
- Isolating and establishing a collection of culturable rhizobial and non-rhizobial strains from bean root nodules to serve as a resource for functional screening.
- Evaluating the multi-functional potential of the selected isolates by assessing their efficiency in promoting plant growth and their mechanisms against key agricultural pests and pathogens.

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CHAPTER 2

2.1 Common bean: importance and domestication

The common bean (*Phaseolus vulgaris* L.) is widely recognized as one of the most important legume crops globally due to its nutritional value, adaptability to diverse environments, and socioeconomic relevance, particularly in Latin America. In many regions, it constitutes an affordable dietary component for vulnerable populations while providing fiber, minerals, proteins, and bioactive compounds that contribute to food security (Broughton et al., 2003).

The common bean is a grain legume belonging to the family Fabaceae (Leguminosae). It was first described by Carl Linnaeus (1753) in his botanical compendium *Species Plantarum* and is commonly included among crops referred to as “pulses” or “legumes.” Taxonomically, it corresponds to a species of the genus *Phaseolus*, within the tribe Phaseoleae, subfamily Papilionoideae, family Fabaceae, and order Fabales (Table 1) (Ulloa et al., 2011).

Table 1. Taxonomic classification of common bean (Ospina et al., 1980; Ulloa et al., 2011).

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Fabales
Family	Fabaceae
Subfamily	Papilionoideae
Tribe	Phaseoleae
Genus	*Phaseolus*
Species	*Phaseolus vulgaris* L.

Globally, approximately 150 bean species have been identified, 65 of which are found in Mexico. These species are distributed across a wide range of ecological environments, altitudes, and climatic conditions. Notably, 31 of these species are endemic to the country, highlighting Mexico's role as a key center of Phaseolus diversity (Delgado & Gama-López, 2015).

Mesoamerica serves as the primary center of domestication for *Phaseolus vulgaris* L. Kaplan (1973) posited that this process likely originated in the Tehuacán Valley (Puebla, Mexico) approximately 7,000 years ago, occurring in co-evolutionary association with maize (*Zea mays*). Domestication emerges from the interplay between natural evolutionary mechanisms and empirical human selection. This transformative process has yielded crops tailored to specific human requirements, favoring traits such as increased fruit size, enhanced biomass, prolific inflorescence, and robust tolerance to abiotic stressors like salinity and drought (Hernández-López et al., 2013).

As a consequence of the domestication process, the common bean exhibits remarkable diversity in seed morphology, including variations in size, shape, and color, which reflect the genetic diversity preserved through traditional cultivation practices (Espinoza- García et al., 2016).

he common bean is an annual herbaceous legume characterized by four distinct growth habits (Table 2). As a thermophilic species, it exhibits high sensitivity to frost (Barrios-Gómez & López-Castañeda, 2009). Cultivars are traditionally classified based on their intended use (dry grain or green pods), maturity group (early or late-maturing), and photoperiod sensitivity (sensitive, insensitive, or neutral). Furthermore, varieties are categorized by their response to biotic and abiotic constraints (resistant or susceptible) and, for commercial purposes, by grain attributes such as size, pigmentation, and nutritional profile (Ulloa et al., 2011).

Table 2. Main growth habits of common bean according to Ospina et al. (1980).

Growth habit	Main characteristics
Type I	Stem and branches end in a developed inflorescence and growth stops.
Determinate bush	Strong stem with a low number of short internodes (5–10).
	Height of 30–50 cm.
	Short flowering stage and generally synchronous pod maturation.
Type II	Erect stems without climbing characteristics; branches do not produce vines.
Indeterminate bush	Higher number of branches and nodes (>12) than Type I.
	Growth continues during flowering, although at a slow rate.
Type III	Prostrate or semi-prostrate plant with well-developed branching.

Indeterminate prostrate	Height greater than 80 cm. Greater number of nodes and branches than Types I and II. Branches end in vines.
Type IV *Indeterminate climbing*	Stems develop a double twisting capacity from the first trifoliate leaf. Branches are poorly developed due to apical dominance. The stem can have between 20 and 30 nodes and may reach heights greater than 2 m with adequate support. Longer flowering stage, with simultaneous flowering, pod formation, and pod filling.

2.2 Nutritional contribution and socioeconomic role of common bean

The nutritional composition of the common bean makes it a vital component of the human diet, particularly in developing regions such as Mexico, where it serves as a readily and sufficient source of protein, fiber, and micronutrients. Protein content ranges from 14 to 26% depending on the bean variety, and it is rich in amino acids such as phenylalanine (1.34 ± 0.10 g/100 g protein) and lysine (1.6 ± 0.15 g/100 g protein), although it has low levels of sulfur-containing amino acids such as cysteine and methionine (OECD, 2019).

Table 1. Nutritional values corresponding to 100 g of raw and dry beans (Food and Agriculture Organization of the United Nations, 2016).

Common name	protein (g)	Fat (g)	Dietary fiber (g)	Available carbs (g)	Fe (mg)	Mg (mg)	P (mg)	K (mg)	Zn (mg)	Cu (mg)
Pinto bean	21.4	1.23	15.5	47.1	5.07	176	411	1393	2.28	0.893
Black bean	21.3	1.2	21.8	37	6.5	188	471	1416	2.9	0.83
White bean	22.3	1.5	15.3	45.5	5.49	175	407	1185	3.65	0.834

In addition to its nutritional functionality, its high fiber content contributes to reducing the risk of cardiovascular diseases and may even contribute to lowering the risk of chronic diseases such as cancer. This is due to the high amount of polyphenols present in the grain, which capture free radicals and prevent cellular damage (Bernardi et al., 2024). Furthermore, according to biological evaluations,

the protein of cooked beans can reach up to 70% quality compared to a reference animal protein that is assigned a quality value of 100% (Guzmán et al., 2002).

The carbohydrate profile of raw beans varies by cultivar, typically providing between 25 and 65 g per 100 g (OECD, 2019), with starch serving as the primary energy fraction. Although a portion of this starch is rendered indigestible during cooking, transforming into resistant starch, it remains the dominant caloric component. Additionally, beans are an excellent source of dietary fiber (14 to 29 g per 100 g), with soluble fiber accounting for up to half of this content (Food and Agriculture Organization of the United Nations, 2016). This fibrous fraction is primarily composed of pectins, pentosans, cellulose, lignin, and hemicellulose. Furthermore, beans provide essential micronutrients, including significant concentrations of zinc, phosphorus, magnesium, iron, and calcium, alongside key vitamins such as niacin, thiamine, and folic acid (Guzmán et al., 2002).

Economically, bean cultivation generates significant productive activity and employment in Mexico. Moreover, its cultivation is distributed across various states with different edaphoclimatic conditions, making it suitable for studies that relate genotype, environment, and microbiome. Recent data and reviews on the contribution of beans to sustainable agriculture and food security provide context on their multifunctional role in agroecological systems (Foyer et al., 2016).

With approximately 570,000 producers in Mexico, the common bean is cultivated for its high nutritional value and relevance in the Mexican basic food basket. This crop generates approximately 382,000 direct jobs within the national agricultural sector and can produce nearly 17 billion Mexican pesos. The most prominent producing states are Zacatecas, Nayarit, and Sinaloa, which together generate almost 50% of the country’s total production (Del Rosario-Arellano et al., 2025).

2.3 Ecological importance of common beans in agroecosystems

In agricultural ecosystems, legumes represent a major biological source of nitrogen because they form symbiotic relationships with nitrogen-fixing bacteria.

This process allows atmospheric nitrogen to be incorporated into the agricultural system in forms that are assimilable by plants, thereby reducing the dependence on synthetic nitrogen fertilizers, since the industrial production of nitrogen fertilizers through the Haber–Bosch process requires large amounts of energy and contributes substantially to greenhouse gas emissions. In this context, crops such as common bean contribute to improving soil fertility, increasing nutrient use efficiency, and maintaining productivity in low-input agricultural systems (Foyer et al., 2016; Uebersax et al., 2023).

The common bean has been considered an important model for the study of food legumes due to its agronomic relevance, genetic diversity, and wide adaptation to different edaphoclimatic environments. In addition to its nutritional value for human consumption, its cultivation has relevant ecological implications in agroecosystems, as it contributes to nitrogen cycling, improves soil structure, and promotes microbial diversity associated with the rhizosphere and root nodules (Broughton et al., 2003; Graham et al., 2006).

Among the ecological functions attributed to legumes, biological nitrogen fixation (BNF) represents one of the key ecological processes operating in agroecosystems, a process through which symbiotic bacteria convert atmospheric nitrogen (N_2) into ammonium (NH_4^+), a form that can be utilized by plants. This interaction occurs within specialized structures known as root nodules, where bacteria differentiate into bacteroids capable of carrying out nitrogenase activity under microaerobic conditions (Mus et al., 2016).

BNF not only directly benefits the host plant but also contributes to the enrichment of soil with available nitrogen, generating positive effects on subsequent crops within crop rotation or polyculture systems. This phenomenon has been widely documented in agricultural systems where legumes are part of sustainable management strategies, as they can improve the overall productivity of the agricultural system and reduce the costs associated with chemical fertilization (Uebersax et al., 2023).

Additionally, common bean cultivation may influence the structure and diversity of soil microbial communities. The interaction between the plant and microorganisms associated with the rhizosphere promotes the formation of complex microbial communities that participate in processes such as nutrient mineralization, organic matter degradation, and the biological suppression of soil-borne pathogens (Compant et al., 2019).

2.3.1 Role in the biological structure of soil

Bean roots release a variety of organic compounds into the soil through processes collectively known as rhizodeposition. These compounds include sugars, amino acids, organic acids, phenolic compounds, and mucilage that act as sources of carbon and energy for soil microorganisms (Bulgarelli et al., 2013).

The release of these compounds favors the establishment of specialized microbial communities in the rhizosphere, a soil zone characterized by intense biological and biochemical activity. Within this region, bacteria, fungi, and other microorganisms interact with the host plant through a complex network of symbiotic, mutualistic, commensal, or competitive relationships (Trivedi et al., 2020).

In the case of legumes, these interactions acquire particular relevance due to the presence of nitrogen-fixing bacteria and the formation of root nodules. These structures function not only as sites of nitrogen fixation but also as microhabitats where diverse microbial communities coexist and may perform complementary functions to the main symbiotic process (Martínez-Hidalgo & Hirsch, 2017).

Likewise, the incorporation of plant residues derived from bean cultivation contributes to the accumulation of organic matter in the soil and promotes the formation of stable aggregates. This process improves soil physical properties such as porosity, water retention, and structural stability, which are fundamental factors for the sustainability of agricultural systems (Foyer et al., 2016). Within this ecological framework, the interaction between legumes and nitrogen-fixing

bacteria represents one of the most important biological processes influencing nutrient cycling in agroecosystems.

2.4 Legume–rhizobium symbiosis: signaling, nodule formation, and modulating factors

The association between legumes and nitrogen-fixing bacteria is one of the best-characterized examples of plant–microorganism symbiosis. This process begins with an exchange of chemical signals between the host plant and soil bacteria, triggering a series of molecular and morphological events that culminate in the formation of functional root nodules (**Figure 1**) (Gage, 2004).

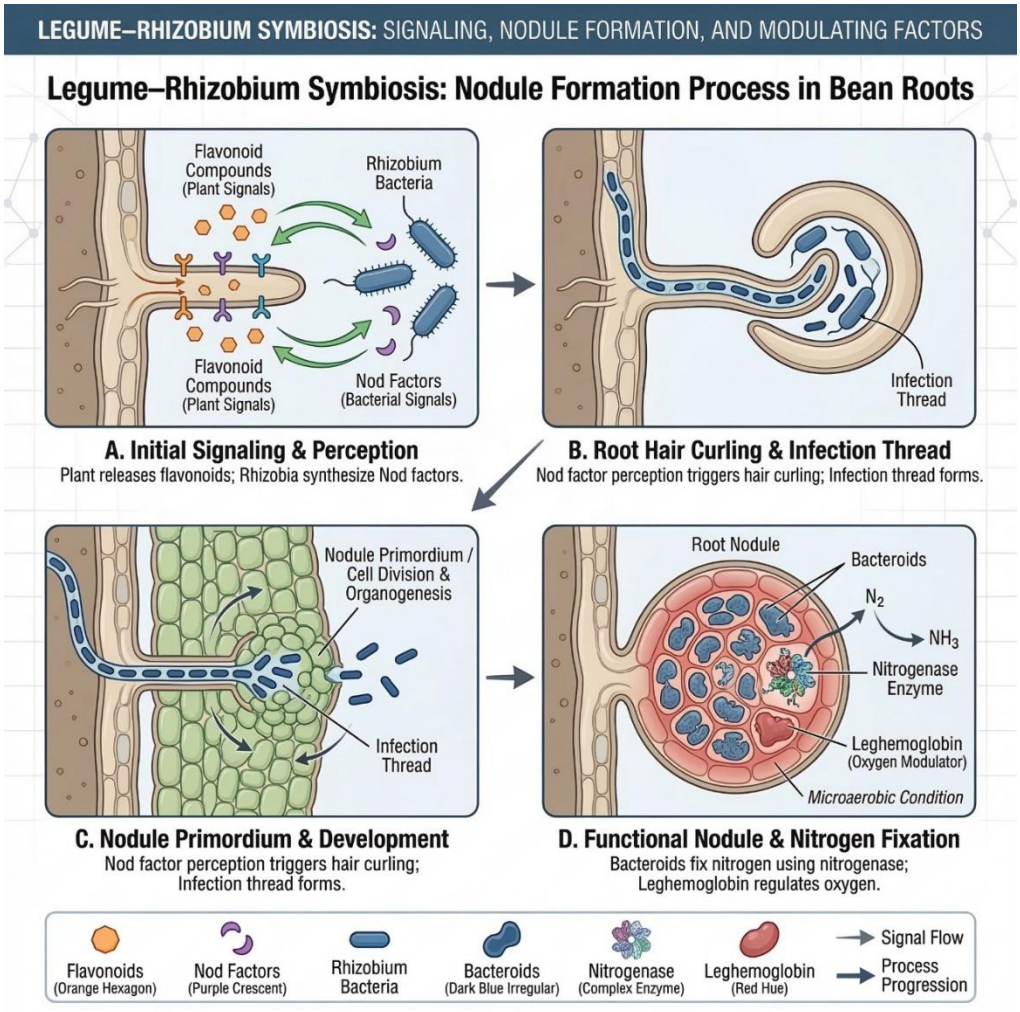

Figure 1. Nodulation process of root legumes (Nano Banana IA).

Nodulation is initiated when legume roots exude flavonoid compounds into the rhizosphere. These compounds act as chemical signals that induce the expression of nod genes in rhizobial bacteria. In response, rhizobia synthesize Nod factors, which are lipochitooligosaccharides capable of triggering morphogenic responses in plant root hairs (Oldroyd et al., 2011).

The perception of these factors by the plant causes root hair curling and the formation of structures known as infection threads. Through these channels, bacteria penetrate the cortical tissues of the root, where nodule development begins through processes of cell division and organogenesis (Gage, 2004).

Within the nodule, bacteria differentiate into bacteroids, a specialized form capable of carrying out biological nitrogen fixation through the enzyme nitrogenase. This process requires microaerobic conditions that are regulated by the presence of leghemoglobin, a protein that modulates oxygen availability within the nodule (Mus et al., 2016).

2.4.1 Environmental factors affecting symbiosis

The efficiency of nodulation and biological nitrogen fixation depends not only on the genetic compatibility between the host plant and the symbiotic bacteria but also on multiple environmental factors that influence the establishment and functioning of the symbiosis (**Figure 2**). Among the most important factors are soil pH, the availability of essential nutrients such as phosphorus and molybdenum, soil temperature, salinity, and water stress. These factors can affect both bacterial survival in the soil and the expression of genes involved in nodulation and nitrogen fixation (de Bruijn & Hungria, 2022).

In the case of phosphorus, its availability is particularly important for the establishment of symbiosis, since this nutrient participates in key energetic processes related to nitrogenase activity and bacterial metabolism. Its deficiency can significantly limit nodule formation and the efficiency of biological nitrogen fixation (Graham et al., 2006).

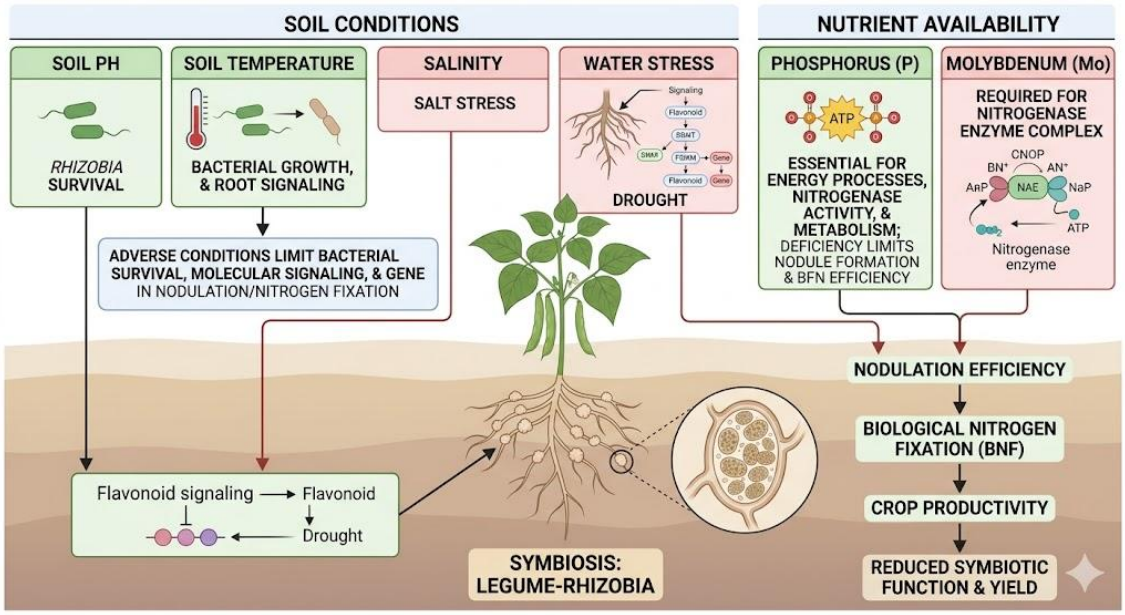

Figure 2. Environmental drivers of symbiotic efficiency in legumes. Impact of soil conditions, nutrient status, and abiotic stressors on nodulation and biological nitrogen fixation (BNF) (Nano Banana IA).

Furthermore, abiotic stress conditions such as drought or salinity can alter molecular signaling between plant and bacteria, reducing nodulation efficiency and affecting crop productivity (Trivedi et al., 2020).

2.5 Nodules as microecosystems: non-rhizobial bacteria and the nodular community

For many years, root nodules in legumes were believed to contain exclusively rhizobial bacteria responsible for nitrogen fixation. However, recent studies based on high-throughput sequencing techniques have demonstrated that nodules harbor much more complex microbial communities than previously thought (Martínez-Hidalgo & Hirsch, 2017)

These communities include non-rhizobial bacteria. They are commonly referred to as NRE (non-rhizobial endophytes) or NAB (nodule-associated bacteria). Several studies have reported the recurrent presence of bacterial genera such as *Pseudomonas*, *Bacillus*, *Microbacterium*, *Enterobacter*, *Paenibacillus*, and

Stenotrophomonas within legume nodules (Muresu et al., 2019; Tapia-García et al., 2020).

The detection of these bacteria suggests that root nodules may function as complex microbial habitats rather than simple binary symbiotic structures where multiple microorganisms interact with each other and the host plant. In this context, the nodule has been proposed as part of a plant holobiont, in which the plant and its associated microbiome function as an integrated ecological unit (Compant et al., 2019).

2.5.1 Functional implications of microbial coexistence

The coexistence of rhizobial and non-rhizobial bacteria within nodules may have important functional implications for plant growth and health. Some nodular endophytic bacteria possess characteristics typical of plant growth-promoting bacteria (PGPB), including the production of phytohormones, the solubilization of mineral nutrients, and the production of siderophores capable of improving iron acquisition by the plant (Trivedi et al., 2020).

Additionally, some of these bacteria may exert indirect effects through biocontrol mechanisms against soil pathogens, via the production of antimicrobial compounds or hydrolytic enzymes capable of degrading cellular structures of pathogenic microorganisms (Compant et al., 2019).

However, interactions among microorganisms within the nodule may also include competitive relationships. In some cases, non-symbiotic bacteria may interfere with nodulation or compete with rhizobia for available resources within the nodule. For this reason, understanding the structure and dynamics of nodular microbial communities is essential for identifying microorganisms with potential agronomic applications (Martínez-Hidalgo & Hirsch, 2017).

2.6 Diversity of rhizobia associated with *Phaseolus vulgaris*

Phaseolus vulgaris is characterized by remarkable symbiotic promiscuity, enabling it to establish associations with a diverse array of nitrogen-fixing bacterial genera and species. Unlike legumes with high host specificity, the common bean can form nodules with various rhizobial lineages (Shamseldin & Velázquez, 2020), most notably *Rhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Pararhizobium*, *Bradyrhizobium*, and *Mesorhizobium*. The assembly of these rhizobial communities is highly dynamic, influenced by soil physicochemical properties, geographic location, agricultural management, and host genotype (Andrews & Andrews, 2017).

In Mexico, researches on both wild and cultivated beans has revealed a high level of microbial diversity. These findings suggest that Mexican soils serve as critical reservoirs for legume-associated bacteria, driving the adoption of advanced molecular tools to further characterize these complex microbial ecosystems (Tapia-García et al., 2020).

2.7 Modern methodologies for studying the nodular microbiome: metabarcoding and ASV analysis

In recent decades, the development of high-throughput sequencing technologies has greatly improved the study of plant-associated microbial communities (Next Generation Sequencing, NGS). These approaches now allow researchers to explore microbial diversity across different plant compartments, including the rhizosphere, endosphere, and root nodules, with a much higher resolution than that achieved with traditional culture-based methods (Trivedi et al., 2020).

In legumes, the use of sequencing techniques has demonstrated that root nodules harbor more complex microbial communities than previously assumed. While classical microbiological approaches focused primarily on the isolation of nitrogen-fixing rhizobia, sequencing-based studies have revealed the presence of

multiple non-rhizobial endophytic bacteria that form part of the nodular microbiome (Martínez-Hidalgo & Hirsch, 2017).

One of the most widely used strategies to study these microbial communities is metabarcoding, an approach that consists of amplifying and sequencing conserved regions of bacterial ribosomal DNA, particularly the 16S rRNA gene. This molecular marker contains highly conserved regions interspersed with variable regions that allow discrimination among different bacterial taxonomic groups (Klindworth et al., 2013).

Among the most commonly used regions of the 16S rRNA gene are the V3–V4 segments, as they provide an appropriate balance between taxonomic resolution and sequence length compatible with sequencing platforms such as Illumina MiSeq (Figure 3). The metabarcoding process generally includes several stages, including total DNA extraction, PCR amplification of the target region, amplicon purification, incorporation of sequencing indices, and subsequent bioinformatic analysis of the obtained sequences (Bolyen et al., 2019; Callahan et al., 2016).

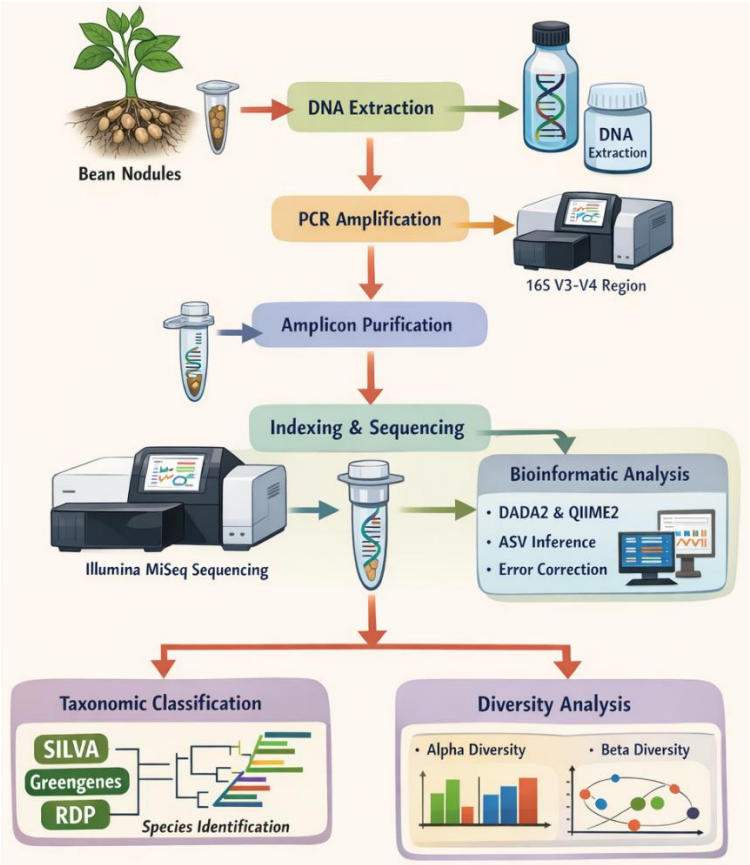

Figure 3. Metabarcoding process of gen 16S rRNA of been nodule samples (Nano Banana IA).

In the bioinformatic analysis of sequencing data, OTUs (Operational Taxonomic Units) have traditionally been used, which group sequences based on a defined similarity threshold, commonly 97%. However, more recent approaches have introduced the concept of ASVs (Amplicon Sequence Variants), which allow the distinction of individual sequences with higher resolution through statistical models that correct sequencing errors (Callahan et al., 2016).

The use of ASVs presents several advantages compared to traditional OTUs. First, it allows a more precise taxonomic resolution, since it does not depend on arbitrary similarity thresholds. Additionally, it facilitates direct comparison among different studies, as sequence variants can be real biological units that are reproducible across different analyses (Callahan et al., 2016).

Several bioinformatic tools have been developed for processing metabarcoding data. Among the most widely used is QIIME2, an open-source platform for

microbiome analysis that integrates multiple modules for data processing, statistical analysis, and visualization of microbial data (Bolyen et al., 2019). Within this framework, the DADA2 algorithm is widely used for ASV inference through the removal of sequencing errors and reconstruction of real sequences present in the samples (Callahan et al., 2016).

Subsequently, the obtained sequences are compared against reference databases such as SILVA, Greengenes, or RDP, which allows taxonomic assignment of the different identified ASVs (McDonald et al., 2024; Quast et al., 2013). This taxonomic classification constitutes the basis for analyzing the structure and composition of microbial communities present in the samples.

Once ASV abundance matrices are obtained, microbial diversity can be evaluated using different ecological metrics. Alpha diversity describes diversity within an individual sample and can be evaluated using indices such as Shannon, Simpson, observed richness (observed features), or Faith's phylogenetic diversity. On the other hand, beta diversity allows comparison of microbial community composition among samples using metrics such as Bray–Curtis, Jaccard, or UniFrac (Bolyen et al., 2019).

The combined use of these tools makes it possible to identify patterns of variation in microbial community structure as a function of factors such as plant genotype, soil conditions, agricultural practices, or edaphoclimatic environment. In the case of the nodular microbiome, these approaches have allowed evaluation of whether different bean varieties select specific microbial communities or whether microbiome composition is mainly determined by the microbial reservoir present in the soil (Shamseldin & Velázquez, 2020).

In this context, ASV-based metabarcoding denotes a powerful tool for studying bacterial diversity associated with *Phaseolus vulgaris* nodules, as it allows the characterization of complex microbial communities and the detection of both dominant taxa and rare microorganisms that may be relevant roles within the symbiotic system.

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CHAPTER 3

Diversity and functional potential of bacteria isolated from bean nodules (*Phaseolus vulgaris*) in two contrasting regions of Mexico.

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Summary

Introduction: The common bean (*Phaseolus vulgaris* L.) is a key legume for food security and agricultural sustainability, particularly in regions with contrasting environmental conditions. Symbiotic interaction with root nodule-associated bacteria plays a fundamental role in plant nutrition, stress tolerance, and nutrient recycling. However, there is limited knowledge about how environmental conditions influence the functional traits of these bacterial communities.

Objective/**Hypothesis**: This study aimed to characterize bacteria associated with bean nodules from contrasting agroecosystems (semi-arid and subhumid) and evaluate their potential as plant growth promoters. The hypothesis was that environmental origin differentially influences the metabolic and enzymatic profiles of nodular bacteria.

Methodology: Bacteria were isolated from bean root nodules collected in Durango (semi-arid) and Guerrero (subhumid), Mexico. The isolates were characterized morphologically and microscopically and evaluated qualitatively and quantitatively for their ability to produce hydrolytic enzymes (proteases, amylases, cellulases), siderophores, indoleacetic acid (IAA), ammonium, and hydrocyanic acid (HCN).

Results: Seventeen bacterial isolates with wide morphological and functional diversity were obtained. The isolates from Guerrero showed greater proteolytic and amylolytic activity, while those from Durango showed a higher frequency of siderophore production. Some isolates exhibited multiple plant growth-promoting traits, including IAA production (D3 and G8), Amylases (D1, D8 and G4), Proteases (D2, D1 and G9) and Cellulases (D7, D5 and G9, G8), and compounds with biocontrol potential like HCN production (D1, D3 and G8) and Siderophore production (D7 and G8).

Implications: Environmental conditions influence the functional expression of nodular bacteria, which has implications for the selection of bioinoculants adapted to specific regions. However, greenhouse and field evaluations are required to validate their agronomic performance.

Conclusion: Bean nodule-associated bacteria exhibit high functional diversity depending on the environment of origin, supporting their potential as biotechnological tools for sustainable agricultural systems.

Keywords: *Phaseolus vulgaris*; nodule bacteria; hydrolytic enzymes; plant-growth promotion.

This chapter is derived from the manuscript entitled "Diversity and functional potential of bacteria isolated from bean nodules (Phaseolus vulgaris) in two contrasting regions of Mexico"
Submitted to the journal Tropical and Subtropical Agroecosystems.
Status: *Under Review.*

3.1 Introduction

The common bean *(Phaseolus vulgaris* L.) is one of the most important legumes worldwide, with a production of 27.2 million tons (t) in 2024 (FIRA, 2022). Along with soybeans (*Glycine max* L.) and peanuts (*Arachis hypogaea* L.), this specie show a great adaptability remarkable adaptability to diverse climatic and edaphic conditions and comprises numerous varieties differing in growth cycle, size, and seed color (De Ron, 2015). In 2023, the leading producers India, Brazil, and Myanmar with 6.4, 2.8, and 2.6 million tons, respectively, while, while in Latin America, Brazil, Argentina, and Mexico held the highest production levels. Mexico alone contributed 723 thousand tin 2023 (FAOSTAT, 2025).

In Mexico, beans are a staple food, particularly for low-income populations, due to their high nutritional value and affordability (Sánchez *et al*., 2022). Beyond their economic and dietary relevance, common beans represent an important source of plant-based protein and dietary fiber, providing approximately 21-23 g of protein and 15-25 g of fiber per 100 g of dry seed. In addition, beans contribute essential micronutrients to the human diet, including iron (≈ 6-7 mg), folate (≈ 390-440 µg), and magnesium (≈ 170-180 mg per 100 g), reinforcing their role in food an nutritional security in developing regions (Singh *et al*., 2020; USDA, 2023). Ecologically, *P*. *vulgaris* contributes to agricultural sustainability through symbiotic associations with soil microorganisms, especially nitrogen-fixing bacteria (NFB), which increase soil nitrogen and reduce reliance on synthetic fertilizers (Islam *et al*., 2024; Shumi, 2018).

Nodulating bacteria are considered a valuable biotechnological tool for sustainable agriculture (Tabande & Naseri, 2020), promoting plant growth using mechanisms such as nitrogen fixing, phosphate solubilization, phytohormone production, and synthesis of hydrolytic enzymes (Santoyo *et al.*, 2021; Vanegas & Uribe-Vélez, 2014). Moreover, microbial consortia composed of compatible strains can enhance these effects synergistically, improving nutrient uptake and soil fertility. For instance, combinations of *Azotobacter* and *Azospirillum* species decreased nitrogen fertilizer requirements by up to 30 kg ha^{-1} due to their

efficiency in biological nitrogen fixation and nutrient availability (Sharma *et al*., 2021).

Environmental factors significantly influence the composition and functional potential of nodule-associated microbiota. Arid and semiarid soils—characterized by low organic matter content (< 1-2%), high soil temperatures (>25-30 °C), and restricted nutrient availability (particularly low available nitrogen and phosphorus) (Ayala-Niño *et al,* 2018), tend to select for stress-tolerant and metabolically specialized bacteria, often linked to the synthesis of siderophores, extracellular enzymes, and other adaptive metabolites (osmoprotectants like, trehalose) (Taoufiq *et al*., 2024; Vurukonda, *et al. 2016*). In contrast, subtropical environments, generally higher in matter content (> 3-6%) and exposed to higher soil moisture availability, can support more diverse and metabolically flexible microbial communities. These contrasting environmental conditions provide a meaningful context for comparing the functional attributes of nodule-associated bacteria across regions (Shanko & Andargie, 2025). However, despite the extensive research on rhizobia, little is known about how such environmental variation shapes the diversity and functional traits of nodule-associated bacteria in common beans. Comparative analyses between arid and subtropical regions remain scarce, and no studies have examined whether isolates from these environments differ in enzymatic activity or plant-growth–promoting traits.

Based on this, we hypothesize that contrasting environmental conditions will differentially influence the metabolic traits of the nodule-associated bacteria of beans. Therefore, this study aimed to evaluate plant growth-promoting mechanisms of nodule-associated bacteria isolated from diverse common beans from arid and subtropical agroecosystems.

3.2 Material and Methods

3.2.1 Geographic location and sampling

Root nodules samples of common bean were collected from two different agroecosystems in Mexico: one located in the state of Guerrero (a subhumid agroecosystem), and the other in Durango (a semiarid agroecosystem) (Figure 4). The Guerrero site represented native bean populations growing under traditional management in southern Mexico. The soil in this area exhibits a clay–silt texture, with an average pH of 7.8, relatively high nitrogen and phosphorus availability (12 ppm), but low potassium content (0.93 meq/100 g), along with a very high organic matter content (6.78%). The climate is classified as warm subhumid [A(w1)], with a mean annual temperature of 26.7 °C and a mean annual precipitation of 965.4 mm (CONAGUA, 2010). In contrast, the Durango site comprised improved bean cultivars grown under experimental field conditions in northern Mexico. The soil is predominantly loamy in texture, with an average pH of 8.48, and is characterized by low nitrogen (3.51 ppm) and phosphorus (7.45 ppm) contents, but high potassium availability (2.18 meq/100 g), as well as low organic matter content (1.02%). The climate is classified as temperate semiarid [BS1kw(w)(e)], with a mean annual temperature of 18.3 °C and a mean annual precipitation of 566.9 mm (CONAGUA, 2020).

In Guerrero, samples were obtained from the experimental area of the Colegio Superior Agropecuario del Estado de Guerrero (18° 15' 58“N; 99° 38' 49” W, at 635 m a. s. l.) during February. In Durango, samples were collected in April from the “Valle del Guadiana” Experimental field, belonging to the Centro de Investigación Regional Norte Centro (CIRNOC) of the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) (23°59'25.0“N 104°37'22.3” W, at 1,880 m a. s. l.).

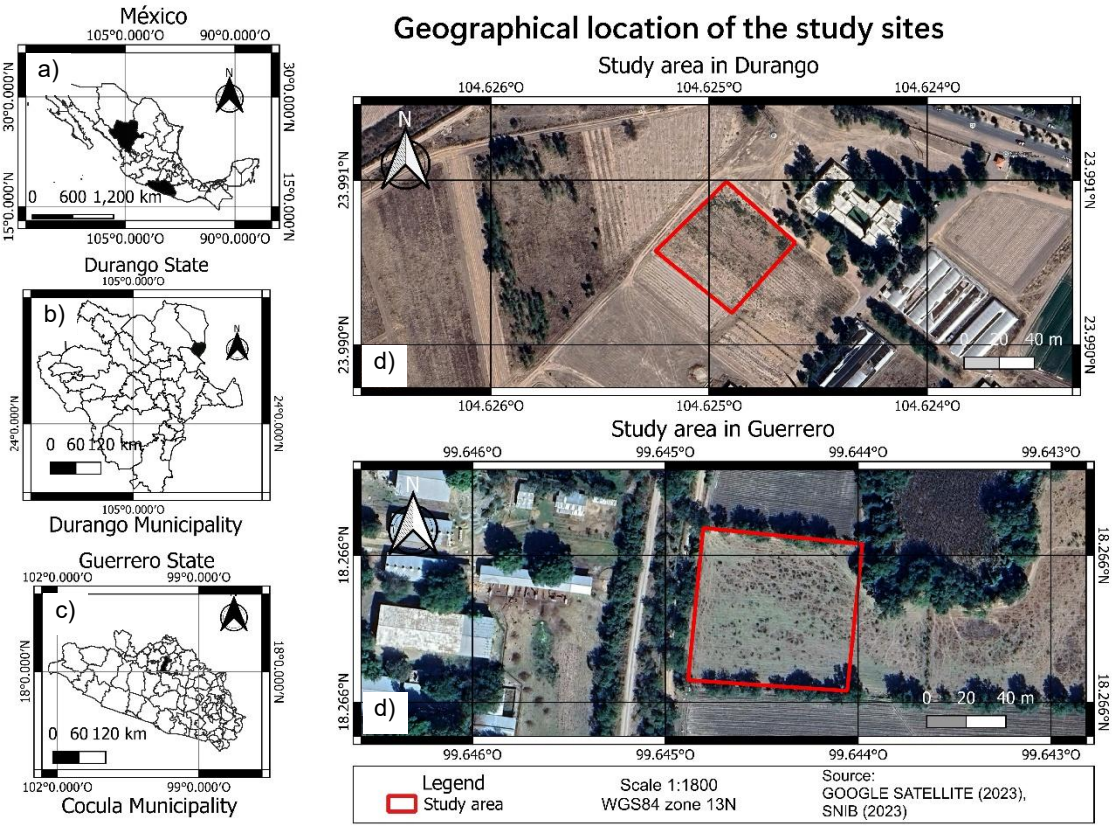

Figure 4. Geographical location of the study area: (a) map of Mexico, (b) map of the state of Durango, (c) map of the state of Guerrero, (d) location of the experimental area in Durango, and (e) location of the experimental area in Guerrero.

In both locations, nodules were collected six weeks after seedling emergence, corresponding to the flowering and early pod development stages. Five plants from different bean varieties were randomly selected (Table 3). Plants were carefully uprooted to preserve the root system, and adhering soil was gently removed. All visible nodules were detached and placed in sterile transport vials for subsequent laboratory analysis.

Nodule samples were transported and analyzed at the Centro Nacional de Investigación Disciplinaria en Relación Agua, Suelo, Planta, Atmósfera (CENID-RASPA) of INIFAP, where a set of microbiological and physiological assays were performed to compare enzymatic activity profiles and plant growth–promoting traits of nodule-associated bacteria isolated from arid and subtropical bean agroecosystems.

Table 3. Bean varieties and ecotypes selected for the analysis. Materials from Durango correspond to improved varieties with documented agronomic descriptions.

Region	Type of bean	ID	Description	Origin Isolation	Reference
Durango	Pinto Bravo	PIN	Growth habit II (indeterminate bush type), growth cycle of approximately 90–95 days, large pinto seeds with slow darkening, tolerant to anthracnose and rust.	D1, D2, D3, D4	(Rosales-Serna & Flores-Gallardo, 2022)
	NOD1	NGO	Growth habit II (indeterminate bush type), growth cycle of approximately 95–100 days, small to medium dull black seeds, tolerant to anthracnose, rust, and common bacterial blight.	D5, D6, D7	(Rosales-Serna & Flores-Gallardo, 2023)
	Jamapa	JAM	Growth habit III (indeterminate prostrate type), growth cycle of approximately 90–100 days, small black seeds, widely adapted traditional variety.	D8	(Rosales-Serna et al., 2004)
Guerrero	White Bean	ACA-4	Type III growth habit (indeterminate prostrate type), growth cycle of 110–120 days, dull bone-colored seeds	G1, G2, G3	
	Chinese Bean	CU-8	Type III growth habit (indeterminate prostrate type), growth cycle of 110–120 days, dull light-brown seeds.	G4, G5, G6, G7	
	White Bean	ACA-16	Type III growth habit (indeterminate prostrate type), growth cycle of 110–120 days, dull bone-colored seeds.	G8	
	Caña Black Bean	AP-2	Type IV growth habit (indeterminate climbing type), growth cycle of 100–120 days, dull black seeds.	G9	

Materials from Guerrero correspond to locally collected native ecotypes; therefore, the type of bean reported for this region refers only to seed color and not to a formally registered variety.

3.2.2 Nodules preparation and bacteria isolation

A total of 10 undamaged root nodules were selected from each plant for bacterial isolation. The nodules were surface sterilized by immersion in 70% (v/v) ethanol for one minute, followed by soaking in a 6% (v/v) sodium hypochlorite solution for three minutes. Subsequently, the nodules were rinsed six times with sterile distilled water to ensure complete removal of residual disinfectant (Somasegaran & Hoben, 1985).

Once the process was complete, the nodules were crushed with sterile tweezers on a Petri dish and suspended in 2.5 ml of sterile distilled water. 100 µl of the suspension was inoculated into yeast-mannitol agar (15 g L^{-1} agar, 10 g L^{-1} mannitol, 0.5 g L^{-1} yeast extract, 0.2 g L^{-1} NaCl, 0.5 g L^{-1} K_2HPO_4, and 0.1 g L^{-1} $MgSO_4$), modified to contain 0.00125% Congo red (w/v), and plates were

incubated for 24 h at 28 °C. The presumptive selection criterion for the strains was their ability to fix N, observed in the medium by the pale pink coloration of the colonies. Seventeen different strains were selected, eight strains from the bean nodules of Durango and nine from the bean nodules of Guerrero (Table 4). The selected strains were described considering shape, edge, surface, texture, elevation, and color as macroscopic characteristics. Similarly, a smear of the described colonies was prepared and stained using the Gram method to observe shape and coloration.

3.2.3 Evaluation of plant growth-promotion mechanisms

Hydrolytic enzyme activities

Lipase, protease, amylase, and cellulase production of the isolated bacteria were assessed qualitatively using agar diffusion assays. The production of extracellular enzymes was evaluated because these enzymes facilitate root infection and contribute to nutrient degradation, such as polysaccharides and proteins (Dutta *et al.*, 2022).

Lipase activity was assessed on tributyrin agar (12 g L^{-1} agar, 3 g L^{-1} yeast extract, 10 mL tributyrin) incubated at 28 °C for 72 h. The formation of transparent halos around the colonies indicated lipase production (Braun *et al.*, 2001).

Protease activity was determined on skim milk agar (15 g L^{-1} agar, 3 g L^{-1} meat extract, 5 g L^{-1} skim milk, and 5 g L^{-1} peptone) incubated at 28 °C for 24 h. The appearance of clear halos indicated proteolytic activity (Panicker & Sayyed, 2022).

Amylase activity was determined on starch agar (12 g L^{-1} agar, 10 g L^{-1} starch, and 3 g L^{-1} meat extract) plates at 28 °C for 24 h. After incubation, plates were flooded with Lugol's iodine solution; transparent halos around the colonies demonstrated amylase activity (Chaiharn *et al.*, 2008).

Cellulase activity was evaluated on carboxymethylcellulose (CMC) agar (15 g L^{-1} agar, 2.5 g L^{-1} yeast extract, 10 g L^{-1} sodium carboxymethylcellulose, 2.5 g L^{-1}

peptone, 0.5 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $CaCl_2$, 0.1 g L^{-1} K_2HPO_4, and 0.1 g L^{-1} KH_2PO_4) incubated at 28 °C for five days. Plates were flooded with a 1% (w/v) Congo red solution, and cellulose-degrading colonies were measured using a Vernier caliper (Stewart *et al.*, 1982).

Siderophore production

Siderophores have an important role in iron solubilization, and some of them exhibit biocontrol activity (Wang *et al.*, 2014). Thus, their production was determined following the CAS assay of Schwyn and Neilands (1987) method. The isolates were incubated at 28 °C for one week; transparent or yellowish halos around the colonies indicated siderophore production. All materials used for medium preparation were cleaned with 6 M HCl. The CAS medium was prepared from four separately sterilized components: (i) the CAS–Fe(III)–HDTMA indicator solution; (ii) a low-iron MM9 salt solution; (iii) a piperazine–HCl buffer (pH 5.6); and (iv) casamino acids (10%) and glucose (20%) as supplements. The components were combined at ~50 °C before pouring plates, following the original formulation.

Hydrocyanic Acid (HCN) production

HCN production was evaluated following the colorimetric method described by Lorck (1948). Bacterial cultures previously grown in YM broth, were inoculated into nutrient broth supplemented with glycine (4.4 g L^{-1}). A picrate-impregnated paper strip (2% sodium carbonate in 0.5% picric acid w/v) was placed on the inner side of the tube cap, ensuring it did not contact the medium, and the tubes were sealed with parafilm. Cultures were incubated at 28 °C for four days. A color shift of the picrate paper from yellow to orange or light brown was considered indicative of HCN production.

Indole-3-Acetic Acid (IAA) production

IAA was determined by the method described by Gordon and Weber (1951). Fresh cultures were grown on yeast-mannitol broth (10 g L^{-1} mannitol, 0.5 g L^{-1} yeast extract, 0.2 g L^{-1} NaCl, 0.5 g L^{-1} K_2HPO_4, 0.1 g L^{-1} $MgSO_4$) supplemented with 0.2% L-tryptophan at 28 °C for 24 h. After incubation, the medium was

centrifuged at 8,000 g for 10 min, and equal volumes of the supernatant and Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% $HClO_4$ solution) were mixed in a microplate and incubated for 30 min in the dark. Absorbance was measured at 530 nm, and IAA production was calculated using a standard IAA curve (0-250 mg L^{-1}).

Ammonium production

Qualitative ammonium production was determined by inoculating bacterial isolates into peptone water (10 g L^{-1} peptone and 5 g L^{-1} NaCl) and incubating for 72 h at 28 °C. Five milliliters of Nessler's reagent were then added to each culture tube. The appearance of yellow to orange/brown coloration indicated ammonium production. Sterile peptone water served as the negative control (Ahmad et al., 2008; Cappuccino & Sherman, 1992).

Data analysis

Datasets were previously evaluated for normality and homogeneity of variances. Then, data were analyzed by one-way ANOVA, using bacterial isolates as the source of variation, and analyses were performed separately for each region. Tukey's *post hoc* test was used to identify significant differences among means ($P < 0.05$). Additionally, an independent samples *t*-test was used to compare overall plant growth-promoting mechanisms between regions. Statistical analyses were conducted using IBM SPSS Statistics 27, and figures were able using Microsoft Excel.

3.3 Results and Discussion

3.3.1 Morphological and microscopic characterization

Seventeen bacterial isolates displayed diversity in colony morphology (Table 4), differing in color, shape, surface texture, and elevation. Most isolates were Gram-negative cocci or bacilli, consistent with previously reports describing rhizobia and other associative bacteria commonly related to legumes (Vasileva *et al*., 2019). Bacterial colony characteristics ranged from circular to rhizoid shapes, with

smooth, rough, or mucoid textures, and colors varying from white to pink, and red. This variability aligns with the broad spectrum of genera commonly associated with legumes, including *Rhizobium*, *Bradyrhizobium*, and *Bacillus* (Kumar *et al*., 2016). In addition, most isolates showed little to no absorption of Congo red when incubated in the dark, a classical trait of rhizobia (Niste *et al.*, 2015).

Table 4. Morphological characterization of different strains obtained from two different ecological regions: Durango and Guerrero States, Mexico

ID	Origin	Shape	Edge	Surface	Texture	Elevation	Color	Gram Stain	
D1	Durango	Circular	Round	Dry	Rough	Convex	Soft Pink	Coccus	Positive
D2		Circular	Round	Soft	Shiny	Raised	Colorless	Coccoid	Negative
D3		Circular	Round	Soft	Creamy	Raised	Light Pink	Coccoid	Negative
D4		Circular	Round	Dry	Powdery	Raised	Red	Filamentous, coccobacilli	Positive
D5		Circular	Round	Soft	Creamy	Raised	Light Pink	Coccoid	Negative
D6		Irregular	Irregular	Mucoid	Mucoid	Convex	Light Pink	Coccoid	Negative
D7		Circular	Round	Soft	Creamy	Convex	Light Pink	Coccoid	Negative
D8		Rhizoid	Irregular	Dry	Rough	Pulvinate	Light Pink	Filamentous, coccobacilli	Variable
G1	Guerrero	Circular	Entire	Smooth	Soft	Pulvinate	Pink	Bacilli	Negative
G2		Rhizoid	Rhizoid	Yeast-Like	Yeast-Like	Umbonate	Red	Filamentous, coccobacilli	Negative
G3		Circular	Entire	Creamy	Soft	Convex	Pink	Bacilli	Negative
G4		Circular	Entire	Smooth	Rough	Umbonate	Pink	Coccus	Positive
G5		Circular	Entire	Smooth	Soft	Convex	Pink	Coccoid	Negative
G6		Circular	Entire	Smooth	Soft	Convex	Red	Bacilli	Negative
G7		Circular	Entire	Smooth	Shiny	Convex	Pink	Bacilli	Negative
G8		Circular	Entire	Smooth	Soft	Pulvinate	White	Coccus	Positive
G9		Rhizoid	Lobulated	Dry	Rough	Umbonate	Pink	Bacilli	Negative

Bacteria D1-D8 represent isolates obtained from the state of Durango, and G1-G9 represents bacteria isolated from the state of Guerrero.

Microscopically, both coccoid and bacillary morphotypes were observed, with a predominance of Gram-negative bacteria, an outcome consistent with previous findings reporting that bean nodules are typically dominated by Gram-negative taxa (Santoyo *et al.*, 2021). Gram-positive bacteria isolates, although less frequent, may correspond to endophytic Actinobacteria or Firmicutes commonly reported in nodules—often displaying coccoid or cocco-bacillary morphologies rather than classical rod-shaped forms—and are known for their stress tolerance and production of extracellular enzymes (Soni & Keharia, 2021; Trujillo *et al.*,

2015).It is likely that non rhizobial bacteria were isolated in this study such as other researchers have found in legume nodules (Etesami, 2022). On the other hand, three of the isolated bacteria exhibited filamentous and rod-shaped morphology, with typical fungi growing characteristics, which suggested the presence of Rhizobium species (Ferras-Negrin et al., 2025).

Interestingly, isolates from Durango, a semi-arid region characterized by high solar radiation and low soil organic matter, tended to produce dry or powdery colonies, whereas isolates from the more humid Guerrero environment displayed predominantly creamy or mucoid textures. These differences suggests that environmental gradients influence bacterial phenotypes, as previously described in *Rhizobium* species under contrasting ecological regimes (Patel *et al.*, 2023). The diversity of morphotypes observed reinforces the notion that bean nodules harbor complex microbial communities beyond classical rhizobia, including plant growth-promoting bacteria (PGPB) with multiple ecological functions mainly related with nutrients biogeochemical cycles, biological control and stress resistance in plants (Hnini & Aurag, 2024).

3.3.2 Evaluation of plant growth-promotion mechanisms

Lipase Activity

The results of qualitative analysis of isolated microorganisms reported that in all evaluated cases, lipase activity was not detected in both regions, Durango and Guerrero. These results are consistent with reports indicating that lipase secretion is often low or absent under in vitro laboratory conditions (Kouker & Jaeger, 1987). Although lipase can contribute to pathogen degradation, their absence does not limit the potential functional importance of the isolates.

Protease Activity

Protease activity was detected in most isolates, although halo size varied significantly among strains (Figure 5). The greatest proteolytic activity was observed in strains G9 (42.6 mm), and D2 (32.8 mm), indicating strong

extracellular protein degradation capacity. This trait is frequently associated with antagonism activity against phytopathogens via hydrolysis of fungal or bacterial cell walls (Dutta *et al.*, 2022).

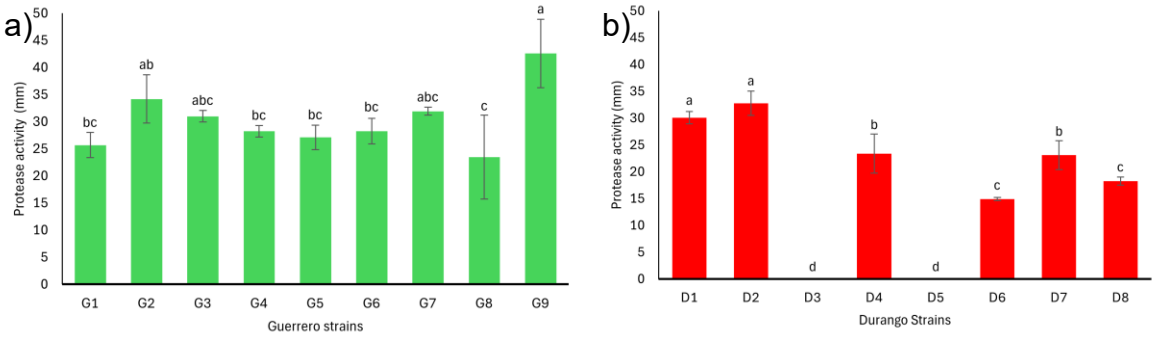

Figure 5. Protease activity of bacterial strains. The X-axis represents bacterial strains, and the Y-axis represents enzymatic activity expressed as hydrolysis halo diameter (mm). Panel (a) (green) corresponds to bacterial isolates from Guerrero, and panel (b) (red) corresponds to bacterial isolates from Durango. Each bar represents the mean of four independent replicates, and error bars indicate the standard deviation. Different letters above the bars indicate statistically significant differences among strains within each region, according to one-way ANOVA followed by Tukey's *post hoc* test ($P < 0.05$).

In contrast, isolates D3 and D5 (Durango) showed no detectable protease activity. This lack of expression may reflect metabolic strategies for conservation under poor nutrient arid soils. Significant differences in protease activity between regions (Table 5) suggest that isolates from the more humid Guerrero environment express higher enzymatic activity, potentially due to greater soil organic matter availability and increased microbial competition (Cestari-Abreu *et al.*, 2024).

Table 5. Comparison of plant growth–promoting traits between the two study regions based on independent samples *t*-tests.

Test	Semi-arid	Subhumid	F	Sig.
Amylases	12,66 ± 5,1	14,51 ± 3.89	6,676	**< 0,05***
Proteases	17,82 ± 11,9	30,28 ± 6,41	67,598	**< 0,05***
Cellulases	21,17 ± 17,37	20,74 ± 15,91	0,967	0,329
Siderophores	7,67 ± 4,53	1,57 ± 0,52	47,769	**< 0,05***
IAA	1,15 ± 0,49	5,73 ± 2,37	0,060	0,808

Semi-arid region corresponds to Durango and subhumid region corresponds to Guerrero. Each value represents the mean and standard deviation of four independent replicates. Values marked with an asterisk indicate statistically significant differences among traits.

Protease-producing bacteria such as *Bacillus*, *Pseudomonas*, and *Aeromonas* are well-known for their biocontrol capacity, often degrading fungal cell walls or insect cuticles, and producing volatile secondary metabolites (Mishra *et al.*, 2020). Protease activity also contributes to soil ecosystem functioning by accelerating organic matter decomposition, it breaks large proteins into amino acids (Greenfield *et al.* 2021). The strong proteolytic activity observed in strains such as G9 suggests potential dual functions as biofertilizers and biocontrol agents.

Amylase Activity

All isolates exhibited amylase activity, confirming their capacity to hydrolyze starch and use complex carbohydrates as an energy source. Among them, strains D1 (21.47 mm) and G4 (21.1 mm) produced the largest halos (Figure 6). Amylase secretion is associated with nutrient release, rhizosphere colonization and improve microbial competitiveness (Arora *et al*., 2007).

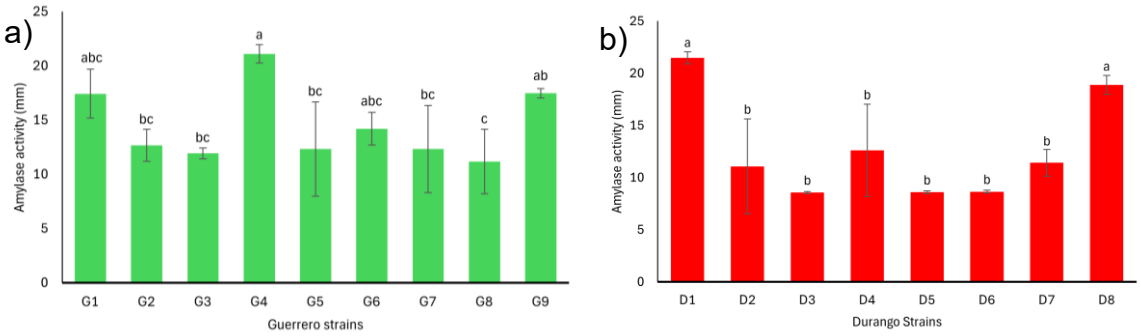

Figure 6. Amylase activity of bacterial strains. The X-axis represents bacterial strains, and the Y-axis represents enzymatic activity expressed as hydrolysis halo diameter (mm). Panel (a) (green) corresponds to bacterial isolates from Guerrero, and panel (b) (red) corresponds to bacterial isolates from Durango. Each bar represents the mean of four independent replicates, and error bars indicate the standard deviation. Different letters above the bars indicate statistically significant differences among strains within each region, according to one-way ANOVA followed by Tukey's *post hoc* test ($P < 0.05$).

Higher amylase activity in Guerrero isolates (Table 5) parallels the trend observed for protease activity, reinforcing the influence of environmental factors on metabolic expression. Amylase-secreting bacteria may also contribute to pathogen inhibition, as hydrolysis of starch-like compounds in pathogen exudates

can suppress spore germination and hyphal development like *Fusarium* and *Phytophthora* (Sayed *et al.*, 2022). The concurrent production of amylase and protease supports the notion of multifunctional PGPB, highly desirable for integrated nutrient cycling and disease suppression (Saberi et al., 2024).

Cellulase Activity

Cellulase activity was detected in 11 of the 17 isolates, with D7 (37.4 mm) and G9 (36.02 mm) showing the greatest halos (Figure 7). No significant regional differences were detected (Table 5). Cellulase enzymes are critical for cellulose decomposition and organic residue turnover, indicating that these isolates have strong potential for enhance nutrient cycling (Mgbechidinma *et al.*, 2024).

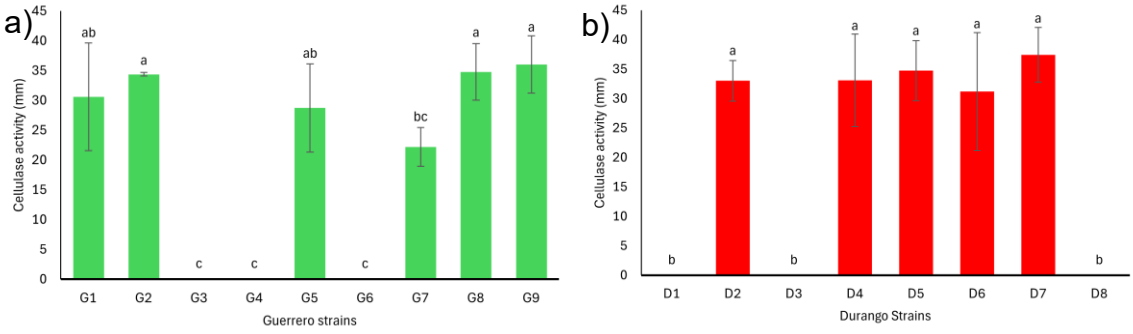

Figure 7. Cellulase activity of bacterial strains. The X-axis represents bacterial strains, and the Y-axis represents enzymatic activity expressed as hydrolysis halo diameter (mm). Panel (a) (green) corresponds to bacterial isolates from Guerrero, and panel (b) (red) corresponds to bacterial isolates from Durango. Each bar represents the mean of four independent replicates, and error bars indicate the standard deviation. Different letters above the bars indicate statistically significant differences among strains within each region, according to one-way ANOVA followed by Tukey's *post hoc* test ($P < 0.05$).

In legumes, cellulase activity is also implicated in early symbiotic processes such as root hair curling and nodule invasion (Shanmugam *et al.*, 2002). Thus, high cellulase activity in strains like G9 and D7 may reflect enhanced potential for root tissues colonization. Similar roles have been documented for *Bacillus subtilis* and *Pseudomonas fluorescens*, which rely on cellulases and other hydrolases to facilitate endophytic interactions (Arora et al., 2007; Saberi et al., 2024).

The absence of cellulase activity in six isolates does not preclude their value as symbionts, as non-cellulolytic bacteria can still participate in nutrient solubilization or hormone modulation through complementary mechanisms (Chen *et al.*, 2015). Nevertheless, cellulolytic activity remains a useful indicator of rhizosphere competitiveness.

Collectively, the presence of hydrolytic enzymes such as cellulases, amylases, and proteases indicates strong potential for biological control, as these enzymes degrade structural polymers in phytopathogens and compromise their cell wall integrity (Mir *et al.*, 2024). Moreover, hydrolytic enzymes are important during nodule formation in legumes and, as a consequence, for nitrogen fixation (Kumari et al., 2010).

Siderophore production

Siderophores production was detected in a minority of isolates, with D7 showing the largest halo (18.7 mm) (Figure 8). Moreover, only one isolation from Guerrero exhibited siderophore activity, whereas multiple isolates from Durango did so. Those patterns suggest that siderophore production may be advantageous under the iron limited condition typical of arid soils, such as those of Durango (Rocha *et al.*, 2023).

Siderophores play a crucial role in Fe^{3+} acquisition and contribute to essential plant processes including chlorophyll biosynthesis, photosynthesis, and oxygen metabolism (Sultana *et al.*, 2021).

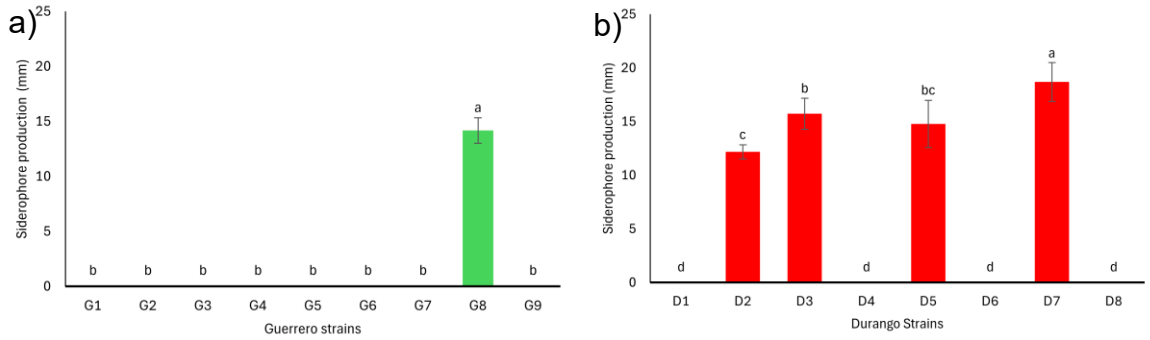

Figure 8. Siderophore production of bacterial strains. The X-axis represents bacterial strains, and the Y-axis represents enzymatic activity expressed as hydrolysis halo diameter (mm). Panel (a) (green) corresponds to bacterial isolates from Guerrero, and panel (b) (red) corresponds to bacterial isolates from Durango. Each bar represents the mean of four independent replicates, and error bars indicate the standard deviation. Different letters above the bars indicate statistically significant differences among strains within each region, according to one-way ANOVA followed by Tukey's *post hoc* test ($P < 0.05$).

This production also contributes indirectly to biocontrol by reducing the availability of iron to competing or pathogenic microorganisms (Mir *et al.*, 2024). The observed activity is consistent with well-known siderophore producers such as *Pseudomonas* and *Bacillus* (Sultana *et al.* 2021; Sayed *et al.* 2022).

HCN production

Four isolates were positive for HCN production (Table 6), two from Guerrero and Two from Durango. The HCN is traditionally considered a biocontrol compound, capable of inhibiting the growth of fungi and pathogenic bacteria, although its effectiveness strongly depends on microenvironment factors such as gas diffusion, soil oxygenation, and other competitors that displace its beneficial activity (Knowles & Bunch, 1986). However, Rijavec & Lapanje (2016) proposed a second ecological role: iron chelation. HCN can form metal complexes with soil metals like iron, releasing phosphates and increasing nutrients availability. Thus, HCN production may contribute both to pathogen suppression and to nutrient mobilization.

Table 6. Qualitative evaluations of Ammonia and Hydrocyanic Acid (HCN) production of each strain.

ID	Origin	Ammonia	HCN
D1		+	+
D2		-	-
D3		-	+
D4	Durango	-	-
D5		-	-
D6		+	-
D7		-	-
D8		+	-
G1		-	-
G2		-	-
G3		-	-
G4		+	+
G5	Guerrero	-	+
G6		-	-
G7		-	-
G8		-	-
G9		+	-

Ammonium production

Only five bacterial isolates showed positive ammonium production (Table 6). The nitrogen-fixing biological capacity of bacteria is a critical characteristic for obtaining plant nutrients, because it facilitates the acquisition of an assimilable nitrogen source; they convert dinitrogen (N_2) into ammonia (NH_3) (Rocha *et al.*, 2023), Genera such as *Rhizobium*, *Frankia*, and *Pseudomonas* have been well documented as growth promoters because they facilitate nitrogen assimilation in plants (Priyadarshini *et al.*, 2021). In addition, the accumulation of ammonium can partially increase the soil pH to 9-9.5, which can limit the growth of certain fungi and inhibit the germination of potentially pathogenic spores (Swamy *et al.*, 2016).

IAA production

In terms of IAA production, isolate G1 (32.2 mg L^{-1}) had the highest production of this auxin compared to the rest and the Guerrero region, while D3 (5.14 mg L^{-1}) had the highest production in the Durango region (Figure 9). No significant regional differences were observed (Table 5). The production of phytohormones is another desirable quality of bacteria, as it promotes plant growth. IAA is a growth regulator that stimulates seed germination and modulates growth in the vegetative stage (AlAli *et al.*, 2021). Low IAA production can stimulate primary root elongation, while high levels promote secondary root formation and reduce primary root growth, as well as increasing root hair formation (Vacheron *et al.*, 2013). Moreover, IAA also improve plant resistance to biotic and abiotic stress, included drought and infections (Uzma *et al.* 2022), Remans *et al.* (2008) found that the levels of bacterial IAA production and the host can determine whether the amount produced is positive or negative. They used two genotypes of *P. vulgaris,* BAT477 and DOR364; the first was more sensitive to low concentrations (≈10 mg L^{-1}) and the second only showed effects at higher levels (≈100 mg L^{-1}).

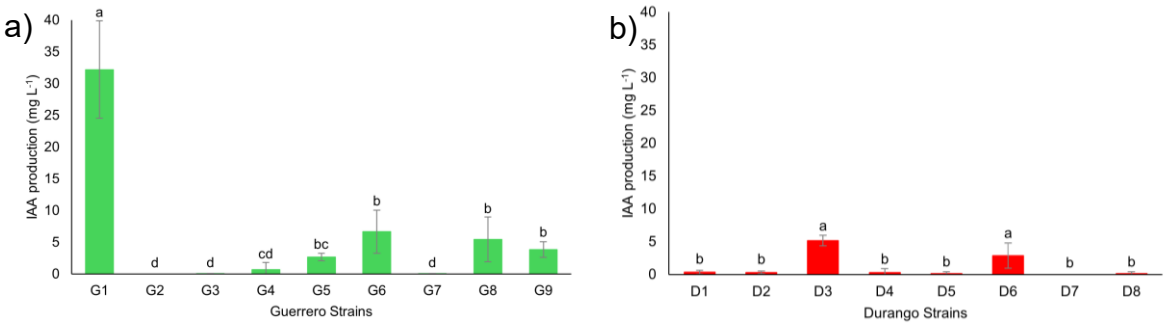

Figure 9. IAA production of bacterial strains. The X-axis represents bacterial strains, and the Y-axis represents IAA production (mg L^{-1}). Panel (a) (green) corresponds to bacterial isolates from Guerrero, and panel (b) (red) corresponds to bacterial isolates from Durango. Each bar represents the mean of four independent replicates, and error bars indicate the standard deviation. Different letters above the bars indicate statistically significant differences among strains within each region, according to one-way ANOVA followed by Tukey's *post hoc* test ($P < 0.05$).

3.4 Conclusions

The findings of the study show a high bacterial diversity and functionality that varies depending on the ecological region. Enzyme profiles and growth-promoting

characteristics showed substantial variation. Strains such as G9 and D7 showed strong cellulolytic and proteolytic capacity, while others, such as G4 and D1, excelled in the production of siderophores, HCN, IAA, or ammonium. Thus, environmental origin influenced metabolic expression; isolates from the humid subtropical region of Guerrero showed higher protease and amylase activity, while siderophore production was more frequent in isolates from the semi-arid region of Durango. These patterns suggest that ecological conditions highly determine the functional traits relevant to nutrient utilization, colonization capacity, and biological control.

Multifunctional properties showed in several isolates identify a great potential as growth-promoting of the bean plant. However, greenhouse and field trials are necessary to validate their agronomic performance and evaluate possible synergistic effects.

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CHAPTER 4

Functional Stability of the Common Bean (*Phaseolus vulgaris*) Nodule Microbiome in Semi-Arid Regions

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Summary

Phaseolus vulgaris L. is a strategic crop whose sustainable productivity depends on symbiosis with nitrogen-fixing bacteria. However, the composition and functional potential of the nodule microbiome in varieties adapted to semi-arid regions remain poorly characterized. Therefore, this research aimed to evaluate the influence of host genotype on nodule-associated bacterial communities in three improved common bean varieties (Pinto Bravo, NOD1, and Jamapa) using high-throughput sequencing of the V3–V4 regions of the 16S rRNA gene. Alpha and beta diversity analyses revealed no significant differences among varieties, indicating a highly similar nodular microbiome regardless of genotype. Moreover, at the phylum level, Proteobacteria and Bacteroidota dominated all samples, suggesting a conserved microbial core. At the genus level, *Rhizobium* was the most abundant taxon, consistent with its central role in biological nitrogen fixation. Non-rhizobial genera, including *Acinetobacter* and JC017 lineage, were also detected, indicating a diverse nodule-associated consortium with potential plant growth–promoting traits. Furthermore, functional prediction with PICRUSt2 showed highly conserved metabolic profiles among varieties. Dominant pathways were associated with amino acid biosynthesis, central carbon metabolism, aerobic respiration, and fatty acid biosynthesis, reflecting strong functional redundancy and potential adaptation to osmotic, thermal, and oxidative stress typical of semi-arid environments. Thus, results indicate that under semi-arid conditions, the common bean nodule microbiome is shaped primarily by host species–level filtering and environmental pressures rather than by varietal differences.

Keywords: metabarcoding, bean nodules, improved varieties

This chapter is derived from the manuscript entitled "Functional Stability of the Common Bean (Phaseolus vulgaris) Nodule Microbiome in Semi-Arid Regions "
Submitted to the journal Diversity.
Status: *Under Review.*

4.1 Introduction

The common bean (*Phaseolus vulgaris* L.) is one of the most important grain legumes for human consumption and plays a central role in global food security due to its high protein, mineral content, and low cost, making it an accessible source of nutrients, especially in developing countries (De Ron et al., 2016). According to data from the Food and Agriculture Organization (FAO), in 2023 the global bean production reached approximately 28.5 million tons (FAOSTAT, 2025), where Mexico is one of the world's leading bean producers and is also recognized as the center of origin and domestication of this crop (Bitocchi et al., 2012). Moreover, in Mexico, beans are grown in virtually all regions under a wide variety of climatic and soil conditions, mainly in the states of Zacatecas, Sinaloa, and Durango, and they are an essential part of the Mexican diet as a staple food and nutritional supplement (García-Urióstegui et al., 2025).

Despite its wide geographical distribution, a significant proportion of bean production in Mexico occurs under arid and semi-arid conditions, where limited water availability, high temperatures, and low soil fertility are limiting factors for crop growth and productivity (Karavidas et al., 2022). Several studies conducted in semi-arid regions of the country have shown that climatic and edaphic variables have a decisive influence on the physiological and productive performance of beans, highlighting the vulnerability of the crop to environmental stress and the need for strategies to promote its adaptation (Cid-Ríos et al., 2025; Servin-Palestina et al., 2022). In this restrictive environmental context, the symbiotic interactions between beans and associated microorganisms take on special relevance.

Similarly to other legumes, common beans establish symbiotic relationships with various soil microorganisms, forming a specialized microbiome that contributes to plant growth, improves nutrient absorption, increases stress tolerance, and participates in pathogen resistance (Bender et al., 2022). Among them, ecological services offered by this symbiotic interaction include biological nitrogen fixation (BNF), which is one of the most important functions that microorganisms provide

to plants, that could reduce the need of synthetic nitrogen fertilizers and helps make farming more sustainable (Moura et al., 2022). In addition, numerous bacteria associated with plants have mechanisms that promote their growth, such as the synthesis of phytohormones (indole-3-acetic acid, cytokinins, and gibberellins), production of siderophores with antagonistic activity against phytopathogens, and solubilization of phosphates and other mineral nutrients (Dutta et al., 2022; Santoyo et al., 2021). Additionally, the symbiosis between bacteria and legumes constitutes an intensely studied biological model, where plant growth-promoting bacteria have been widely documented, as *Rhizobium*, *Bacillus*, *Azospirillum*, *Pseudomonas,* and *Serratia* (El-Esawi et al., 2024; Okon et al., 2015; Velasco-Jiménez et al., 2020). For example, it has been demonstrated that genera such as *Rhizobium* and *Azospirillum* increase the nitrogen supply to the host plant through root colonization and the exchange of nitrogenous compounds for photosynthates within root nodules (Banjare et al., 2023).

Even though the symbiosis between bacteria and legumes has been widely studied, recent evidence suggests that the composition and structure of microbial communities associated with plants do not depend exclusively on soil microbiota, instead being strongly influenced by the plant host (Barraza et al., 2020). Studies on the assembly of the microbiome in crops have shown that the selection made by the host, determined by its genetic identity, acts as a filter that influences the diversity, structure, and complexity of the associated microbial communities (Xiong et al., 2021). Hence, elucidating the mechanisms underlying specific symbiotic interactions between host plants and their associated microbiomes remains a major challenge, yet it is essential for the development of strategies aimed at enhancing crop productivity (Bender et al., 2022). In this context, next-generation sequencing (NGS)–based analyses of nodule-associated microbiomes offer valuable insights into the microbial factors that may account for differences in symbiotic efficiency and performance. Moreover, the use of this technology revealed that microbial diversity in legume root nodules is greater than previously documented (Favero et al., 2021; Somasundaram et al., 2025).

Nevertheless, information on the specificity of nodular bacterial communities associated with improved common bean varieties remains limited, particularly under restrictive environmental conditions (Tapia-García et al., 2020).

Therefore, the objective of this study was to characterize the bacterial communities associated with root nodules of improved common bean varieties, with the aim of assessing their specificity across genotypes and identifying the main metabolic functions that define the nodular microbiome.

4.2 Material and Methods

4.2.1 Seed material and field management description

Three improved varieties of common bean; pinto (PIN), dull-black (NGO), and Jamapa (JAM), derived from double and multiple biparental crosses involving genetically diverse parental genotypes representing the Durango (pinto) (Rosales-Serna & Flores-Gallardo, 2022), Mesoamérica (dull black) (Rosales-Serna & Flores-Gallardo, 2023), and Antigua bean races (Jamapa) (Rosales-Serna et al., 2004) (Table 7).

Table 7. Improved bean varieties selected for analysis.

Region	Type of bean	ID	Description	Reference
Durango	Pinto Bravo	PIN	Growth habit II, cycle ≈90–95 days, large pinto grain with slow darkening, tolerant to anthracnose and rust.	(Rosales-Serna & Flores-Gallardo, 2022)
	NOD1	NGO	Growth habit II, cycle ≈95–100 days, small to medium black opaque grain, tolerant to anthracnose, rust and common bacterial blight.	(Rosales-Serna & Flores-Gallardo, 2023)
	Jamapa	JAM	Growth habit III, cycle ≈90–100 days, small black grain, widely adapted traditional variety.	(Rosales-Serna et al., 2004)

The three bean varieties were established in a completely randomized design in a plot consisting of two rows 5 m in length, with a spacing of 0.81 m between rows. Manual hoeing and hand weeding were performed for weed control. During the first weeding, the crop was fertilized with a 35-50-00 ($N–P_2O_5–K_2O$) fertilizer rate. At the onset of flowering, a foliar fertilizer application of UAN 32 (6 L ha^{-1})

and MaxiGrow Excel (60 mL ha^{-1}) was carried out. Dimethoate was applied on three occasions for the control of insect pests (*Diabrotica undecimpunctata*, *Peridroma saucia*, and *Agrotis ipsilon*). Prior to sowing, the crop received an initial irrigation, followed by four supplemental irrigations to prevent severe water stress in the plants.

4.2.2 Sampling and Nodule Conservation

Samples were collected from improved common bean plants cultivated in the main bean-producing region of Durango, located in semiarid northern Mexico. To evaluate the effect of genotype on microbial community composition, all seed materials were grown under uniform field conditions at the *Valle del Guadiana* experimental station of the North Central Regional Research Center (CIRNOC), National Institute of Forestry, Agricultural and Livestock Research (INIFAP) (23°59′25.0″ N, 104°37′22.3″ W; 1,880 m a.s.l.) (Figure 10). Nodules were collected six weeks after emergence, during the flowering-fruiting stage.

Nodule sampling was performed during the flowering/fruiting stage at the sixth week after emergence. Five plants of three improved bean varieties (Table 7) were selected completely at random. They were extracted from the soil taking special care not to damage the roots; excess soil was removed, and all visible nodules were collected in transport vials that contains moisture absorber drying silica beads. The samples were then sent to the National Center for Disciplinary Research in Water, Soil, Plant, and Atmosphere Relations (CENID-RASPA) of INIFAP for subsequent analysis.

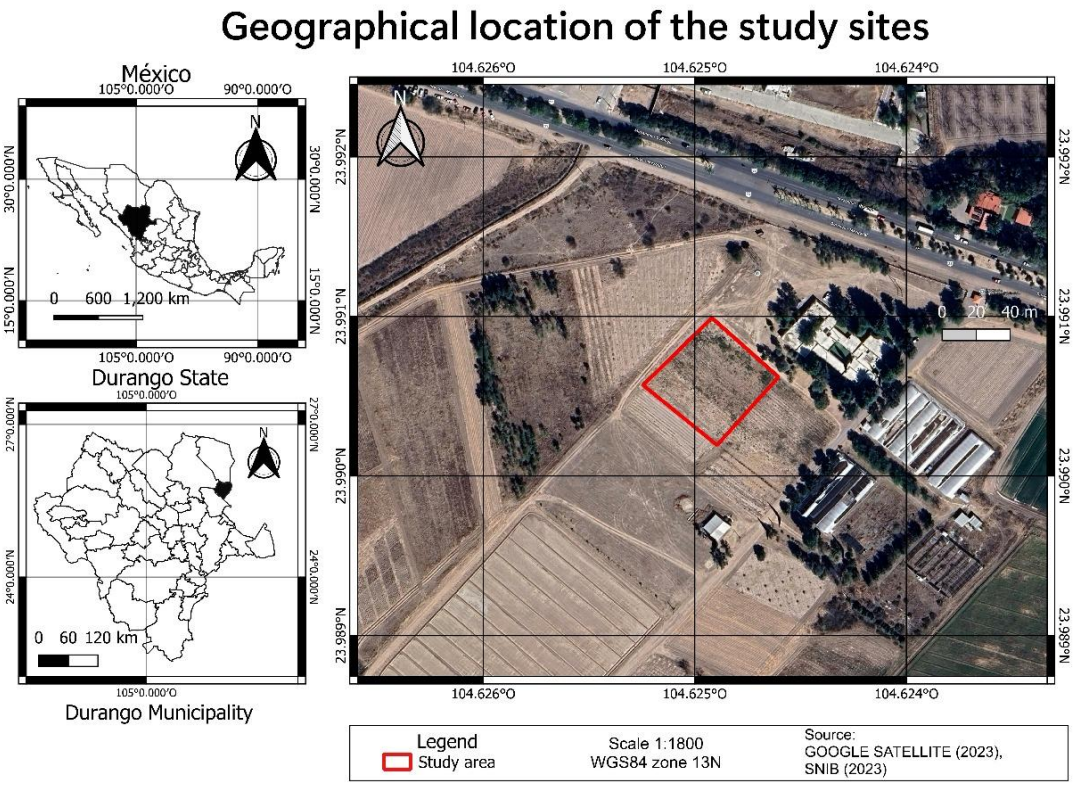


Figure 10. Geographical location of the study area.

4.2.3 Nodules washing and Selection of Viable Nodules for DNA Extraction

Six undamaged nodules were randomly selected from each sampled plant for washing and bacterial isolation. The nodules were immersed in 70% ethanol (v/v) for 1 min, rinsed, and soaked in a 6% sodium hypochlorite solution (v/v) for 3 min. Following the soak, they were rinsed six times with sterile distilled water to completely eliminate the sodium hypochlorite.

Once sterile, the nodules were crushed with sterile forceps on a Petri dish, and 2.5 ml of distilled water was added to macerate them. From the maceration product, 100 µl were taken and resuspended in 750 µl of BashingBead™ lysis buffer in a ZR BashingBead™ lysis tube (Zymo Research™).

4.2.4 DNA Extraction, Amplification, and Sequencing of the Nodule Bacteriome

Genomic DNA was extracted from the samples using the Xpedition™ ZymoBIOMICS DNA MiniPrep kit (Zymo Research™). Extractions were carried out inside a UV-sterilized laminar flow cabinet under stringent aseptic conditions. Sample processing was conducted at Novogene Corporation, Inc. (Davis, CA, USA), where the V3–V4 hypervariable region of the 16S rRNA gene was amplified with primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGTATCTAAT).

PCR amplification was performed in reactions containing 15 µL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 2 µM of each primer, and approximately 10 ng of template DNA. The cycling conditions included an initial denaturation step at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s.

PCR products were mixed with an equal volume of 1× loading buffer containing SYBR Green and visualized by electrophoresis on a 2% agarose gel. Amplicons were pooled in equimolar proportions (1:1) and purified using a Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were prepared using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) according to the manufacturer's instructions, incorporating index adapters. Library integrity and concentration were evaluated using a Qubit® 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system. Finally, libraries were sequenced on an Illumina NovaSeq platform to generate 250 bp paired-end reads.

4.2.5 Bioinformatics Analysis

Bioinformatic processing was carried out in QIIME2 (Quantitative Insights Into Microbial Ecology) within a Linux–Ubuntu operating system (Bolyen et al., 2019). Sequence quality control, chimera removal, and inference of amplicon sequence variants (ASVs) were performed using the DADA2 pipeline (Callahan et al., 2016, 2017). Taxonomic classification was assigned against the Greengenes2 reference database (McDonald et al., 2024). ASVs identified at the species level were

subsequently validated through comparison with the National Center for Biotechnology Information (NCBI) database using BLAST, applying strict criteria of 100% query coverage, E-value ≤ 0.0, and ≥ 98% sequence identity.

The relative abundance of predominant bacterial taxa was visualized through heatmaps constructed with Morpheus (Broad Institute). To enable comparison of nodule-associated microbiota among the three bean species, sequencing depth was normalized by rarefaction across samples. Based on the rarefied dataset, four alpha diversity indices were estimated: observed ASVs, Shannon diversity, Pielou's evenness, and Faith's phylogenetic diversity. Differences among species were evaluated using the Kruskal–Wallis test ($p < 0.05$).

For beta diversity analyses, four dissimilarity metrics were calculated: Jaccard distance (presence/absence-based), Bray–Curtis dissimilarity (abundance-based), unweighted UniFrac, and weighted UniFrac. Jaccard and Bray–Curtis values range from 0 (identical communities) to 1 (completely distinct communities), whereas UniFrac metrics incorporate phylogenetic information, reflecting differences in lineage composition (unweighted) or both phylogeny and relative abundance (weighted). Statistical differences among species were assessed for each beta diversity matrix using PERMANOVA ($p < 0.05$).

Functional prediction of the bacterial communities was conducted with PICRUSt2 (version 2.5.2) (Douglas et al., 2020), which infers metagenomic functional profiles from 16S rRNA gene data based on phylogenetic relationships. Analyses were executed on a Linux–Ubuntu system following established workflows (Douglas et al., 2020; Langille et al., 2013). ASVs generated in QIIME2 were aligned to reference genomes from the IMG/M and KEGG Orthology (KO) databases using HMMER for hidden Markov model searches and EPA-NG for phylogenetic placement. Phylogenetic placements were processed with GAPPA to reconstruct a reference tree.

Predicted gene family abundances were normalized by 16S rRNA gene copy number to reduce phylogenetic bias. The Nearest Sequenced Taxon Index (NSTI) was calculated to evaluate prediction accuracy; NSTI values closer to zero

indicate greater reliability of functional inference based on 16S rRNA sequences. Functional pathway abundances were obtained from the MetaCyc database (Caspi et al., 2020) using the file *path_abun_unstrat.tsv.gz*. Relative pathway abundances were computed across samples, and functional profiles were visualized in RStudio.

4.3 Results and Discussion

4.3.1 Influence of genotype in nodule microbial communities

The mean of number of reads was 191,983.5, 172,982.25, 193,458.25 for the Pinto Bravo, dull black (NOD1), and Jamapa lines, respectively (Table 8).

Table 8. Denoising statistics and weighted Nearest Taxon Index (NSTI) values for bacterial communities across improved *Phaseolus vulgaris* varieties.

Sample id	Input numeric	Filtered numeric	Percentage of input passed filter numeric	Denoised numeric	Merged numeric	Percentage of input merged numeric	Non-chimeric numeric	Percentage of input non-chimeric numeric	Weighted NSTI
JAM1	204659	195902	95.72	195850	195373	95.46	192406	94.01	0.0232
JAM2	203402	194379	95.56	194317	193855	95.31	191694	94.24	0.0175
JAM3	203709	194520	95.49	194460	194312	95.39	191650	94.08	0.0176
JAM4	209566	200348	95.6	200304	199942	95.41	198092	94.52	0.0112
NGO1	203373	196413	96.58	196347	195802	96.28	193631	95.21	0.0186
NGO2	205671	196312	95.45	196228	195834	95.22	194507	94.57	0.0172
NGO3	209357	199849	95.46	199759	199294	95.19	198527	94.83	0.0422
PIN1	205884	197391	95.87	197363	196963	95.67	195735	95.07	0.0225
PIN2	202300	193439	95.62	193399	193051	95.43	190570	94.2	0.0288
PIN3	184678	177465	96.09	177325	176870	95.77	176087	95.35	0.0327
PIN4	216228	207179	95.82	207119	206543	95.52	205543	95.06	0.0223

Denoising metrics include the number of inputs, filtered, denoised, and non-chimeric sequences obtained through the DADA2 pipeline. The weighted NSTI values represent the average phylogenetic distance between the ASVs in the samples and the available sequenced genomes, serving as a measure of the accuracy of functional predictions.

The total number of reads obtained from the three varieties was 2,233,696 (767,934 for Pinto Bravo, 773,833 for Jamapa, and 691,929 for NOD1). According

to the results, the taxonomic richness described in Table 9 was identified. All sequences were uploaded to the National Center for Biotechnology Information (NCBI) for public knowledge (BioProject: PRJNA1437770).

Table 9. Taxonomic richness identified in the bacterial communities of nodules from improved bean varieties.

Variety	Phylum	Class	Order	Family	Genus	Specie	Latinized
Pinto Bravo	4	6	8	9	9	18	6
Jamapa	4	5	7	7	7	12	3
NOD1	8	10	18	20	22	34	9

With respect to both alpha and beta diversity, no significant differences were detected among the analyzed varieties (

Table **10**), indicating a high degree of similarity in the microbial communities inhabiting root nodules.

Table 10. Alpha and beta diversity metrics of bacterial communities in root nodules across improved Phaseolus vulgaris varieties.

Index	H	p
Alpha diversity		
Observed features	2.355	0.307
Shannon	1.416	0.492
Evenness	2.227	0.328
Faith	0.575	0.749
Beta diversity		
Bray-Curtis	1.28	0.346
Jaccard	1.215	0.121
Unweighted UniFrac	0.943	0.626
Weighted UniFrac	1.35	0.31

Statistical significance for alpha diversity was determined using Kruskal-Wallis tests, while beta diversity was assessed via PERMANOVA. The H value corresponds to the test statistics in each case, and p values indicate statistical significance ($p < 0.05$).

At the phylum level, Proteobacteria (74,94%) and Bacteroidota (21,22%) predominated across the three analyzed varieties, indicating a conserved and

potentially essential role of these taxa in plant–associated microbial interactions within improved legume varieties, as well as a high degree of community interconnectivity. Proteobacteria was consistently the most abundant phylum in all samples (Figure 11), a pattern widely reported in studies of soil-associated microbial niches, including the endosphere and rhizosphere (Mataranyika et al., 2024; Pang et al., 2022). Moreover, this phylum is especially dominant in soils near roots with nodules and nodule samples from different legumes (Klūga et al., 2023; Tian et al., 2021).

Proteobacteria represents one of the largest and most phylogenetically diverse bacterial phyla, encompassing Gram-negative taxa such as rhizobial species and numerous diazotrophic bacteria, including *Rhizobium*, *Bradyrhizobium*, and *Pseudomonas* (Etesami, 2022). In leguminous plants, root nodules harbor not only nitrogen-fixing rhizobia but also a diverse assemblage of non-rhizobial nodule-associated bacteria (NAB). These microorganisms have been documented in multiple legume species and are associated with plant growth–promoting traits, including siderophore production, phytohormone (auxin) synthesis, and hydrolytic enzyme activity (Martínez-Hidalgo et al., 2022).

In addition to Proteobacteria, Bacteroidota is among one of the most prevalent phyla in soil ecosystems and includes several plant growth–promoting bacteria (PGPB), particularly within the legume rhizosphere (Pang et al., 2022). In the present study, Bacteroidota represented the second most abundant phylum in common bean nodule bacteriome. Members of this phylum have been linked to carbon cycling processes, notably organic matter mineralization and nitrite oxidation in legume-based cropping systems (Adnan et al., 2023; Cai & Zhao, 2024).

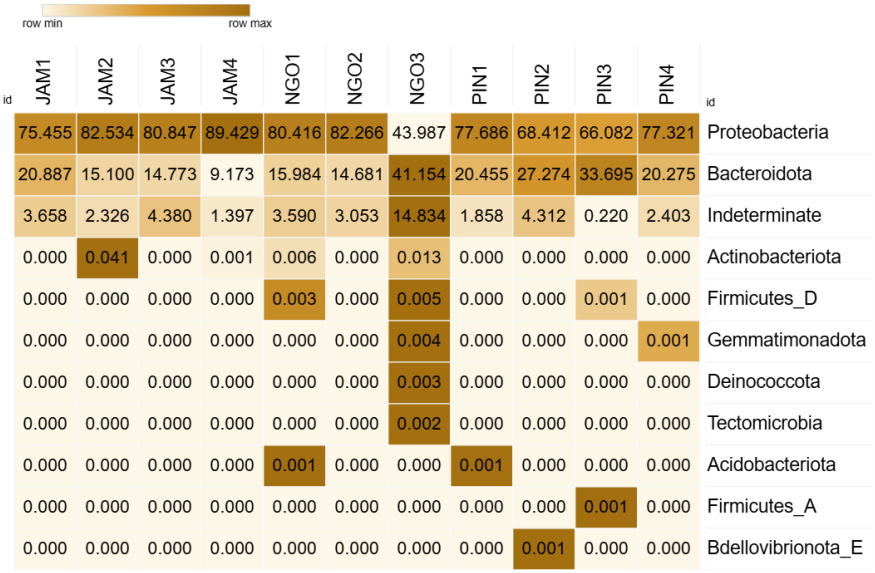
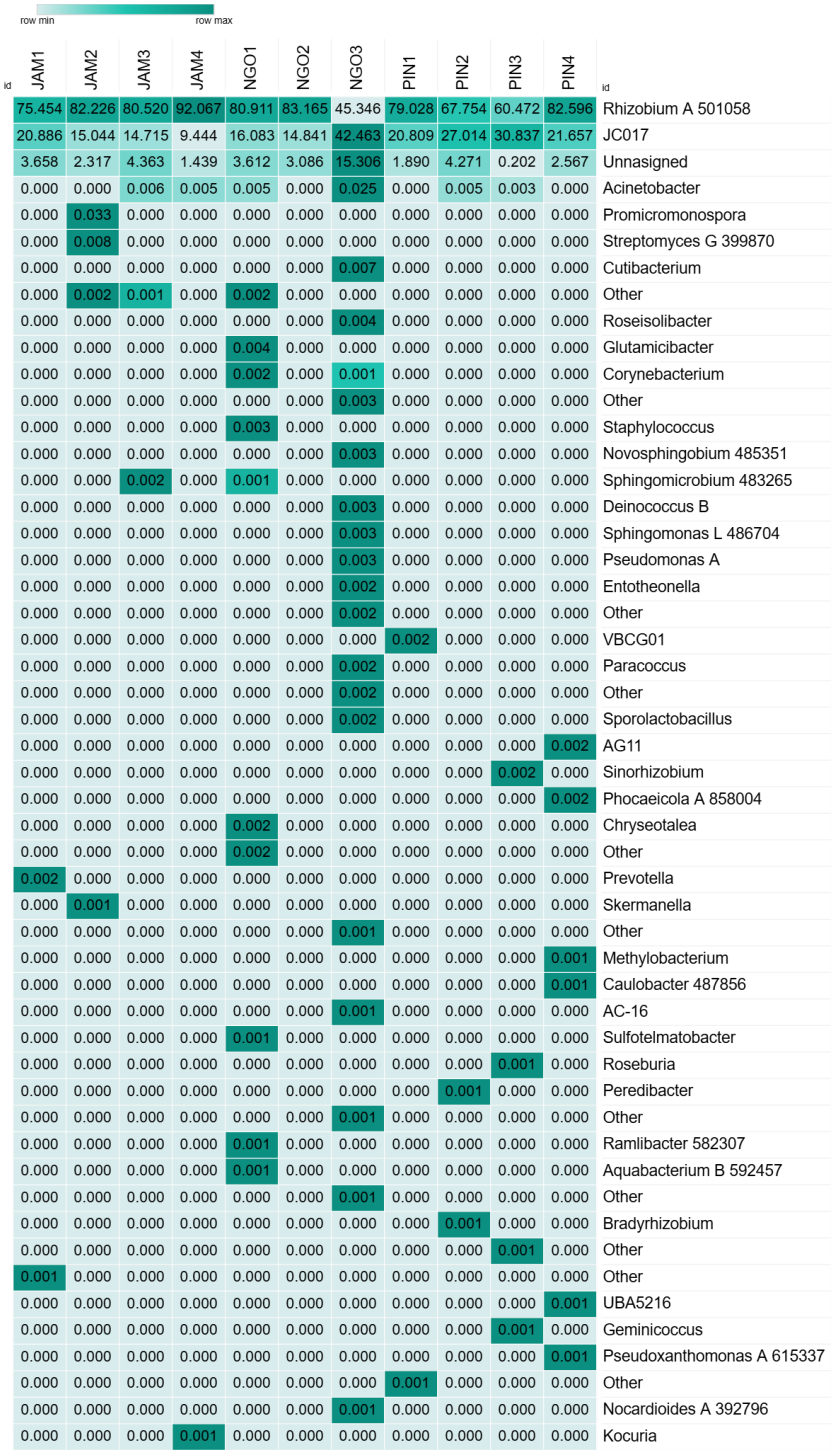


Figure 11. Relative abundance at the phylum level in the bacterial communities of nodules from improved bean varieties. PIN – Pinto bravo; NGO – NOD1; JAM – Jamapa.

At the genus level, *Rhizobium (75,41%)* was identified as the most abundant taxa across the analyzed samples (Figure 12). The genus *Rhizobium* has been extensively documented through both culture-dependent isolations and high-throughput sequencing approaches in root nodules of *Phaseolus vulgaris* and other leguminous species (El Attar et al., 2019; Hossain et al., 2023; Shamseldin & Velázquez, 2020), as was reported in this research with *Rhizobium A_501058* as the most abundant specie in all samples (Table 11). The composition and relative abundance of *Rhizobium* species are often heterogeneous, reflecting the combined influence of multiple abiotic and biotic factors, and are frequently shaped by host plant genotype (Mendoza-Suárez et al., 2021). Additionally, the non-rhizobial genus *Acinetobacter* has been reported in legume nodules, where it contributes to plant development through phosphate solubilization and indole acetic acid production (Hakim et al., 2020; Zhao et al., 2018). On the other hand, *Roseisolibacter agri* is a chemoheterotroph member of Gemmatimonadetes phylum, firstly identified in African savannah from floodplain soil for maize crops

(Pascual et al., 2018); however, its ecological role and functional potential remain largely unexplored.



id	JAM1	JAM2	JAM3	JAM4	NGO1	NGO2	NGO3	PIN1	PIN2	PIN3	PIN4	id
	75.454	82.226	80.520	92.067	80.911	83.165	45.346	79.028	67.754	60.472	82.596	Rhizobium A 501058
	20.886	15.044	14.715	9.444	16.083	14.841	42.463	20.809	27.014	30.837	21.657	JC017
	3.658	2.317	4.363	1.439	3.612	3.086	15.306	1.890	4.271	0.202	2.567	Unnasigned
	0.000	0.000	0.006	0.005	0.005	0.000	0.025	0.000	0.005	0.003	0.000	Acinetobacter
	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	Promicromonospora
	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	Streptomyces G 399870
	0.000	0.000	0.000	0.000	0.000	0.000	0.007	0.000	0.000	0.000	0.000	Cutibacterium
	0.000	0.002	0.001	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	Other
	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000	0.000	Roseisolibacter
	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.000	Glutamicibacter
	0.000	0.000	0.000	0.000	0.002	0.000	0.001	0.000	0.000	0.000	0.000	Corynebacterium
	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	Other
	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000	0.000	Staphylococcus
	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	Novosphingobium 485351
	0.000	0.000	0.002	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	Sphingomicrobium 483265
	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	Deinococcus B
	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	Sphingomonas L 486704
	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	Pseudomonas A
	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	Entotheonella
	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	Other
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	VBCG01
	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	Paracoccus
	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	Other
	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	Sporolactobacillus
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	AG11
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	Sinorhizobium
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	Phocaeicola A 858004
	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	Chryseotalea
	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	Other
	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	Prevotella
	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	Skermanella
	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	Other
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	Methylobacterium
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	Caulobacter 487856
	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	AC-16
	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	Sulfotelmatobacter
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	Roseburia
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	Peredibacter
	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	Other
	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	Ramlibacter 582307
	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	Aquabacterium B 592457
	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	Other
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	Bradyrhizobium
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	Other
	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	Other
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	UBA5216
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	Geminicoccus
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	Pseudoxanthomonas A 615337
	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	Other
	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	Nocardioides A 392796
	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	Kocuria

Figure 12. Relative abundance of bacterial genera in root nodules across improved *Phaseolus vulgaris* varieties.

Table 11. Taxonomic assignment of dominant bacterial species in root nodules of improved *Phaseolus vulgaris* varieties from Northern Mexico.

Specie	Query cover	E value	Percent of identity
Rhizobium rhizogenes	100%	0	100.00
Acinetobacter johnsonii	100%	0	100.00
Roseisolibacter agri	100%	0	99.28
Corynebacterium otitidis	100%	0	99.53
Segatella copri	100%	0	100.00
Deinococcus_B terrestris	100%	0	99.04
Sporolactobacillus sp. strain HS17_27A	100%	0	100.00
Phocaeicola dorei strain CLA-AA-H245	100%	0	99.76

Species identification was performed by aligning representative Amplicon Sequence Variants (ASVs) against the NCBI database using the BLASTn algorithm. Only the most abundant species with a sequence identity ≥99 and an E-value ≤10^{-5} are displayed.

These results suggest that the structure and composition of the nodular bacteriome in common bean are largely determined by host plant species–level factors rather than by other secondary factors, such as different soil characteristics or management practices; that is, it is suggested that the plant species decides which bacteria to "accept" in the symbiotic relationship, as previously reported (Osogo et al., 2025; Park et al., 2023). Consistently, despite the inclusion of distinct varieties in the present study, no differences were observed in the bacteriome associated with *Phaseolus vulgaris* nodules across samples. This pattern supports the notion that the assembly of the nodular bacteriome in semiarid conditions is predominantly governed by species-specific traits—such as root exudate profiles and nodulation signaling pathways—while varietal effects, if present, likely play a secondary or marginal role in shaping these microbial communities (Chen & Liu, 2024; Dollete et al., 2024). Moreover, in arid and semi-arid regions, the soil microbiome is strongly shaped by environmental stressors, particularly drought and salinity (Ben Gaied et al., 2024). Soil water availability is a key determinant of microbial survival, activity, and diversity in these ecosystems. Water deficit modifies soil physicochemical properties by increasing

solute concentration and promoting soil compaction, thereby reducing pore connectivity and limiting microbial habitat and function (Mickan et al., 2019). Zhou et al. (2020) reported that rhizospheric microbial communities associated with different legume species in arid environments are predominantly composed of members of the orders *Rhizobiales, Xanthomonadales, Burkholderiales, Sphingomonadales, Solirubrobacterales,* and *Nitrosomonadales*. Their findings indicate that drought stress is a crucial driver of bacterial community specificity in the rhizosphere, selecting for taxa capable of persisting and proliferating under low-moisture conditions across multiple host plants. Most of these orders belong to the phylum Proteobacteria, which was likewise the dominant phylum detected in the present study.

On the other hand, salinity represents an additional major selective factor affecting soil microbial communities, mainly through osmotic imbalance and ion toxicity, both of which constrain microbial growth and metabolic activity. Consequently, salinity acts as a strong filter in plant-mediated rhizosphere microbiome assembly, favoring microbial groups that contribute to improved plant tolerance to salt stress (Yang et al., 2020). In the analysis made by Ben Gaied et al. (2024), authors report that under saline conditions, legume rhizospheres are primarily dominated by members of the phyla Proteobacteria, Actinobacteriota, Chloroflexi, and Planctomycetes. Despite these advances, most studies to date have focused on natural or unmanaged ecosystems. In contrast, agricultural legumes exposed to combined drought and salinity stress remain comparatively understudied, particularly regarding the structure and function of nodulating microbial communities.

4.3.1 Metabolic profiles inference

The NSTI values for the five pools ranged from 0.011 to 0.033, with an average of 0.023, indicating high phylogenetic similarity between the ASVs and their closest reference genomes. The metabolic pathways with the highest relative abundance corresponded to the pyruvate fermentation to isobutanol ($\bar{x}$ = 27.02%),

Aerobic respiration I (cytochrome c) ($\bar{x}$ = 23.72%), cis-vaccenate biosynthesis ($\bar{x}$ = 17.29%), L-isoleucine biosynthesis ($\bar{x}$ = 16.23%), and L-valine biosynthesis ($\bar{x}$ = 16.23%) (Figure 13).

The functional prediction analysis performed with PICRUSt2 revealed that the dominant metabolic pathways in the nodule-associated microbial communities of the three improved common bean varieties were highly conserved, with no significant differences detected in either taxonomic diversity (alpha and beta) or functional profiles. The prevalence of core pathways related to amino acid biosynthesis (e.g., branched-chain amino acids), central carbon metabolism, aerobic respiration, and fatty acid biosynthesis suggests a metabolically robust and functionally redundant microbiome (Figure 13). These functions are closely linked to microbial tolerance to environmental stress, particularly osmotic and oxidative stress typical of semi-arid regions (Hu et al., 2013; Xiong et al., 2025). Amino acid biosynthesis pathways likely contribute to the production of osmoprotective compounds that help maintain cellular homeostasis under water deficit (Abuzahrah et al., 2022; Pholchan et al., 2025; Qui et al., 2024; Wu et al., 2024), while the prominence of flexible energy metabolism pathways indicates an enhanced capacity to cope with fluctuations in oxygen and nutrient availability within the nodular niche (Lee et al., 2023; Miranda-Molina et al., 2025). Additionally, the enrichment of lipid biosynthesis pathways may support membrane stability under high temperature and desiccation conditions (Lee et al., 2023; Zhao et al., 2023; Zhukov & Shumskaya, 2020). Together, these findings indicate that the bean nodule microbiome is functionally pre-adapted to semi-arid environments, and that environmental filtering and host selection may favor the maintenance of a stable core of stress-resilient metabolic functions regardless of host variety.

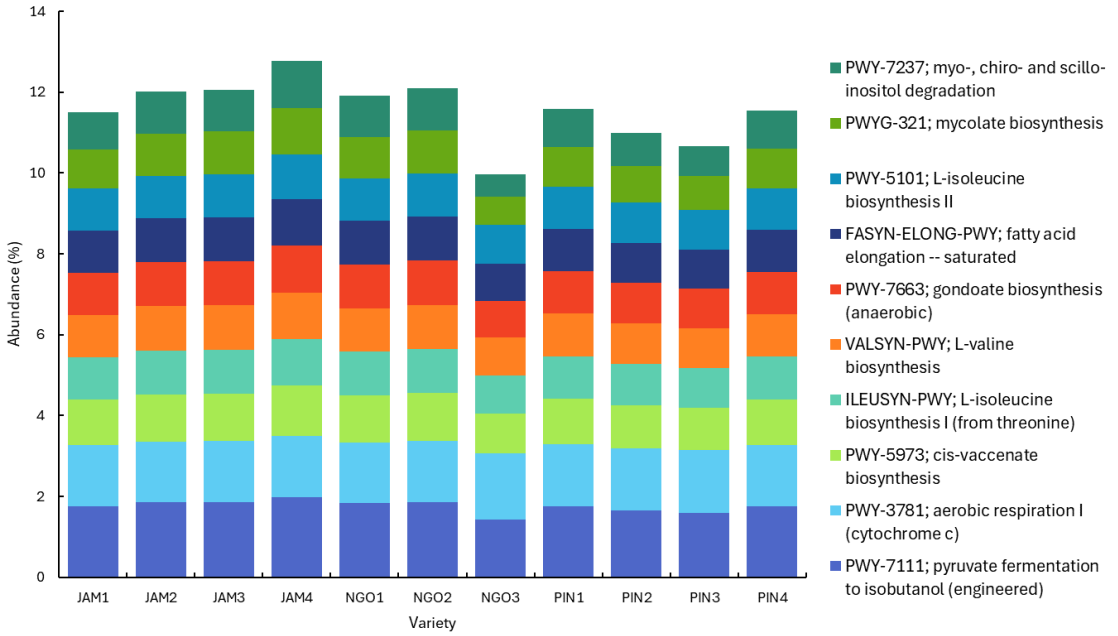


Figure 13. Profile of the ten most abundant functional metabolic pathways in root nodules of three improved *Phaseolus vulgaris* varieties. Varieties are identified as follows: PIN (Pinto Bravo), NGO (NOD1), and JAM (Jamapa). Pathway abundance was predicted based on PICRUSt2 analysis of 16S rRNA gene sequences

4.4 Conclusions

The three improved common bean varieties grown under semi-arid conditions exhibited remarkably similar nodule-associated microbial assemblages, with no significant variation in alpha or beta diversity or in the predicted metabolic functions. The bacteriome was consistently dominated by Proteobacteria and Bacteroidota, and *Rhizobium* remained the most abundant genus, highlighting the presence of a conserved symbiotic core across genotypes. Functional inference indicated a high degree of metabolic redundancy, particularly in pathways associated with osmotic adjustment, central energy metabolism, and lipid biosynthesis, which are linked to tolerance to environmental stress. Overall, these findings indicate that host species–level filtering and environmental pressures play a stronger role than varietal differences in shaping the structure and functional potential of the common bean nodule microbiome.

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