

Diversity of Rhizobia and Importance of Their Interactions with Legume Trees for Feasibility and Sustainability of the Tropical Agrosystems

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Abstract: Symbiotic biological nitrogen fixation (BNF) is a complex process that involves rhizobia, a diverse group of α and β -proteobacteria bacteria, and legume species. Benefits provided by BNF associated with legume trees in tropical environments include improvements to efficiency of nitrogen (N) use, increase of soil carbon sequestration, stabilization of soil organic matter, decrease of soil penetration resistance, and improvement of soil fertility. All these benefits make BNF a crucial ecosystem service to the sustainability of tropical agriculture. Due to the importance of this ecological process and the high diversity of rhizobia, these bacteria have been extensively characterized worldwide. Currently, over 400 species of rhizobia are known, distributed into seven families. In the humid tropics, *Leucaena leucocephala*, *Acacia mangium*, *Gliricidia sepium*, and *Clitoria fairchildiana* are four of the most common species used by family farmers to create sustainable agricultural systems. These four legumes perform symbiosis with different groups of rhizobia. Exploring BNF could help to enable sustainable intensification of agriculture in the humid tropics, mainly because it can increase N use efficiency in an environment where N is a limiting factor to plant growth.

Keywords: biological nitrogen fixation; nitrogen; *Leucaena leucocephala*; *Acacia mangium*; *Gliricidia sepium*; *Clitoria fairchildiana*

1. Rhizobia Strains and Legume Tree Interactions: Importance and Use in Tropical Agricultural Systems

In the humid tropics, the edaphoclimatic conditions, which involve soils with fragile structure subjected to high temperatures, rainfall, and insolation, are unfavorable for organic matter accumulation and thus N availability and nitrogen use efficiency. The adoption of inappropriate

agricultural practices for local conditions reduces nutrient availability and results in the depletion of soil fertility. Small family farmers in the humid tropics, especially in the pre-Amazon region, practice itinerant agriculture that is associated with the slashing and burning of natural vegetation. These small farmers mainly cultivate food crops such as rice, maize, cassava, and beans, using low technology. According to Moura et al. [1], this system has negative effects on the local and global environment and no longer provides social benefits to rural communities. The environmental impact of agriculture in tropical regions can include deforestation and loss of wildlife habitat, soil nutrient depletion, increased greenhouse gas emissions, and loss of biodiversity [2]. In contrast, there are excellent opportunities to avoid environmental damage through the use of ecosystem services to achieve ecological and economic benefits [3].

No-tillage alley cropping can be an alternative for the family farmers for maintaining productivity in the low-fertility soils of humid tropical regions. In this system, legume trees or shrubs are planted in two or more sets of single or multiple rows. Before and during the cropping period, the leguminous branches are periodically pruned and laid down on the soil surface between the leguminous tree sets, where other crops are planted. The use of legume trees can provide ecosystem services such as biomass production, recycling of nutrients, biological nitrogen fixation, and carbon sequestration [4].

Following the work of Fischer et al. [5], we define ecosystem services as the ecological processes of ecosystems that are used to produce human well-being. In this context, nitrogen (N) fixation by legume trees, in which bacteria living in root nodules convert atmospheric dinitrogen (N_2) gas to a plant-available form of N, is crucial to the sustainability of tropical agriculture [6]. Fixation of N in this manner can provide much of the N needed to drive crop growth and biomass creation [7]. In addition, proper management of legume tree biomass can increase soil organic matter, sequestered carbon, and recycled nutrients, which increases soil fertility and agricultural productivity [8]. In the tropics, the high biodiversity in rhizobia and legume trees, combined with long growing seasons and adequate soil moisture, provides the ideal conditions for making use of the ecosystem services provided by legume trees [9].

These phenomena offer a potential solution to agricultural sustainability challenges in the Amazon region, which are mainly associated with depletion of soil fertility [10]. The region's low-activity clay soils present difficulties for agriculture due to their reduced capacity to retain nutrients under high rainfall intensity, making agriculture an unfeasible activity [11]. Nutrient retention in the root zone can be enhanced by adding nutrients in slow-release forms and through biologically mediated processes like N-fixing symbiosis [12]. In the agroecosystems of the humid tropics, these approaches may be more sustainable than saturating the soil with soluble nutrients [13].

Biologically mediated processes are one of the most important targets in modifying and improving N uptake efficiency by roots [14]. This assertion is particularly true in humid tropical conditions, where N use efficiency is usually very low. High temperatures increase the volatilization rates of fertilizers [15], while high rainfall rates increase nitrate (NO_3^-) concentration and leaching [16]. Inefficient use of N, beyond its negative economic impact, contributes to agriculture disservices through greenhouse gas emissions and groundwater pollution [17]. Indeed, in the humid tropics, N uptake is impaired by N leaching due to high rainfalls, and by reduced root growth in hard-setting soil when rainfall is low [18]. The poor agronomic efficiency of N, reaching as low as 14 kg/kg (maize grain/applied N) has been the main reason why many family farmers have resisted changing from the traditional slash-and-burn systems to conventional tillage systems, despite recommendations to switch.

Some authors have reported the positive effect of the symbiosis between rhizobia and legume trees on agroecosystem services, one of the main results of which is an increase in tropical agriculture sustainability. Sena et al. [10] showed that the total N content of maize was increased by around 50% when adding biomass of gliricidia (*Gliricidia sepium* (Jacq.)) on its own and with urea. Aguiar et al. [13] reported that a combination of high- and low-quality residues from legume trees increased N concentrations in maize. A combination of gliricidia with sombreiro (*Clitoria fairchildiana* (R. A. Howard)) resulted in a nearly threefold increase in N accumulation compared to bare soil with urea

(148.4 vs 54.1 kg ha⁻¹). Treatments involving legume residues plus urea led to higher N accumulation in comparison to the treatments with residues but without urea [13].

N fixation by legume trees also has a positive effect on soil carbon sequestration. N-fixing trees sequester CO₂ directly through their growth or indirectly via the turnover of their N-rich tissues, whose decomposition increases plant growth due to higher soil N [7]. However, some authors have highlighted the influence of quality of the applied residue (i.e., residues with high N contents and low to medium cellulose contents) on the stabilization of the soil organic matter [19,20]. According to Bradford et al. [21], high-quality leaf litter, which increases microbial activity, results in more organic matter that can be physicochemically stabilized. Therefore, the higher the symbiotic efficiency between rhizobia and legume trees, the higher the quality and amount of stabilized organic matter or sequestered carbon. According to Sena et al. [10], total carbon stock was 30% higher in treatments with high-quality legume tree residue than in treatments with bare soil. Thus, compared with the control treatment, the treatment with gliricidia, when added to the accumulated organic content, resulted in the carbon stock increasing by 5.0 g kg⁻¹.

Improvement in soil fertility from legume tree biomass can be physical (decreased soil penetration resistance), or chemical (increased base cations due to recycled nutrients) [18]. After four rainless days, the effect of biomass application on penetration resistance in a hard-setting soil extended to a depth of 17.5 cm, due less to soil moisture conservation than to the increase in soil organic matter [22]. This biomass may also capture a considerable amount of recycled base cations that were previously out of the reach of crops, improving the environment of the root zone [9,23].

Since the Forest Code in Brazil specifies that only 20% of a farmer's land can be used for agriculture in the Amazon, local research and policy agendas must consider N-fixing symbiosis between rhizobia and legume trees. This would allow sustainable intensification of agriculture in this region, for which increased N availability and use efficiency is crucial [24]. However, strategy to meet the challenges of the systematic use of ecosystem services provided by interactions of leguminous trees and rhizobia will require the mastery of a vast knowledge base [25]. First, we need to know how symbiotic relationships between different species could work through the ecological gradient, like the one that makes up the Amazon and its periphery. Such a process can determine the efficiency of N input in tropical family farm systems [26], increasing the ease of adoption by farmers. Second, it will be necessary to identify and select rhizobia communities which have greater efficiency to produce high quality biomass if the aim is to take advantage of the large tropical biodiversity [24].

A large number of texts have recently appeared in the scientific literature on these different issues, but often with an orientation less applied to the tropical environment and its agrosystems. This paper provides an overview of recent developments in the diversity of rhizobia for N fixation but is mostly concerned with contributing to meet the challenges of feasibility and sustainability of the agrosystems in tropical family farms.

2. Biological N Fixation and Indigenous Rhizobia Communities in Tropical Environments

N is the most limiting nutrient for plants, especially in the tropics, where it is particularly important in secondary forest succession [7], which is common in the humid tropics due to the practice of shifting cultivation. The largest source of N is the atmosphere, where it is found as N₂, a form that is unavailable to most living beings. Most plant-available N is extracted from the atmosphere using biological nitrogen fixation (BNF).

N can also be fixed by the Haber–Bosch process, an industrial procedure used to produce N fertilizers, or by electrical discharges in the atmosphere. However, the Haber–Bosch process has a high economic and environmental cost, since it requires a high amount of energy, requires fossil fuel inputs, and generates harmful by-products [27]. BNF is important in agriculture due to its low/no cost and non-polluting process. This environmental service contributes to the production of high protein content seeds for food and forage. In addition, it considerably reduces the use of fertilizers, favoring the productivity of ecosystems and reducing losses from N leaching [28]. BNF consists of the conversion of atmospheric N to ammonia and can be carried out by free-living, associative, or symbiotic bacteria. This symbiosis represents an inexpensive and sustainable approach to crop

production [27]. BNF is mediated by nitrogenase, a complex metalloenzyme, with well-preserved structural and mechanical characteristics.

Nodulation in legumes evolves a highly specific interaction between these plants and rhizobia, which are gram-negative soil bacteria. This symbiosis includes several processes, including the recognition of symbionts, infection and colonization of plants by bacteria, the formation of root nodules by the plant, and biological N fixation. Modern genetic and molecular methods are effective in identifying N-fixing organisms [29]. Indigenous or native rhizobia are those naturally found in the soils of a given location. Studies have shown that tropical soils have native rhizobia communities with high diversity and varied efficiency and are a source of genetic variability in the search for strains capable of being used as inoculants [25,30,31]. Inoculants are products composed of living microorganisms capable of benefiting the development of different plant species. The rhizobia were the first microorganisms used as inoculants. Currently, the use of inoculants is widespread and indicated in agriculture, mainly for legumes such as soybeans, common beans, faba beans, and cowpea, but the production of inoculants for other legumes and non-legumes has increased in order to obtain greater yield [32].

The high variability among strains of indigenous communities is largely the result of the numerous stresses and natural interactions to which these communities are subject due to edaphoclimatic factors. However, it must be emphasized that the genes that regulate BNF are highly conserved in the organisms that express it. Therefore, they are not subject to significant variations [29,33]. The efficiency of strains in the native community has a direct impact on BNF with legumes that are native or introduced in certain areas. BNF is determined by the efficiency and number of rhizobia present in the soil and influenced by the host and environmental conditions [34,35]. The characteristics of the native community of rhizobia, especially features related to competitiveness and efficiency, are critical for the management of legumes of agricultural importance or those that want to explore the BNF. Inoculation with efficient rhizobia is usually recommended for locations where there are no compatible rhizobia, reduced soil rhizobia populations, or where the native community of rhizobia has low efficiency [36].

Currently, the benefits of using inoculants are well known and the target of frequent studies. However, potential environmental impacts related to this practice are generally overlooked. The use of microbial inoculants can influence the indigenous microbial communities. In addition, there is a major concern about how the impact on taxonomic groups can be related to effects on functional capabilities of the soil microbial communities. The use of microbial inoculants may cause major changes in microbial soil communities. This change is capable of ultimately inducing unpredictable feedback reactions. This is because the responses in vegetative development (plant growth), contrary to expectations, may be related to indirect effects of using the inoculant such as induction or repression of resident microbial populations which can influence beneficial soil functions such as biological nitrogen fixation [37].

Bakhoun et al. [38] demonstrated that, in addition to the inoculant, soil origin and the plant provenance influenced the plant growth, bacterial structure, and diversity and soil functioning in plant rhizosphere. This means that inoculation with selected rhizobial strains is a suitable tool for increasing plant growth; however, for each soil origin, plant species, or provenance, a rhizobial strain must be objectively selected. In this scenario, an inoculum mixture is an alternative to be considered. Thilakarathna and Raizada [39], through a meta-analysis that encompassed several studies on soybean inoculation, demonstrated that inoculants consisting of indigenous rhizobia, sometimes, result in greater yields than non-local, improved rhizobia under field conditions and that these native rhizobia are generally more competitive than the improved rhizobia in terms of nodule occupancy. This indicates that native rhizobia may have potential for commercial inoculant production at a local level. Additionally, the review pointed out that locally adapted rhizobia strains are capable of performing better under environmental stress conditions than introduced rhizobia. This characteristic is especially important for the choice of inoculant strains for some regions with limiting characteristics such as semi-arid regions.

The rhizobia host specificity refers to the ability to interact between symbionts. It is a concept that can be applied to both bacteria and plants. Legume root exudates contain phenolic compounds called flavonoids that may or may not activate nod gene expression in the bacterial partner. If activated, nod genes expression results in the production and secretion of a set of molecular signals known as nod factors, that may or may not elicit the appropriate responses in the plant required for rhizobial root infection and nodule development [40]. Strains of rhizobia and legumes that can establish symbiotic relationships with several species are considered promiscuous. Usually, a particular species of rhizobia can nodulate a limited rate of legumes [36,41,42]. Therefore, the harnessing of N fixation as a tool for agricultural development is dependent on immersing oneself in the vast diversity of species and characteristics to identify and select the agronomically more efficient ones. The symbiotic relationships between two species and the diversification of rhizobia in functional, adaptive characteristics through the ecological gradient can determine the N input in several ecological niches [36,43].

Biological nitrogen fixation includes several processes, such as the recognition of symbionts, infection and colonization of plants by bacteria, the formation of root nodules by the plant, and biological N fixation. The plants regulate the formation of root nodules that house nitrogen-fixing rhizobia and adjust investment into nodule development and growth [44]. Legumes occupy a variety of habitat types, and they can also take on a range of growth forms (trees, vines, shrubs, and herbs) and substantially differ in their promiscuity with rhizobial partners (generalists vs. specialists) and their ability to regulate symbiotic nitrogen fixation (obligate vs. facultative) [45].

For nitrogen-fixing rhizobia, assemblages are mostly determined by filtering by the host as well as abiotic soil conditions [46]. Therefore, legume–rhizobia association is a powerful model of the limits of host control over microbes [44].

An important consideration when assessing the effects of mutualisms such as legumes and rhizobia is the specificity/promiscuity of each partner in the symbiosis. If a species in the mutualism is a generalist (will engage in symbiosis with multiple partner species), then increasing partner diversity can increase its contribution to ecosystem functioning [45]. However, for legumes adapted to highly variable environments and nutrient-poor soils, filtering out rhizobial partners may not be an effective strategy to ensure symbiotic benefits [46]. Strain-specific legume rhizobia symbioses can develop in particular habitats and lateral gene transfer of specific symbiosis genes within rhizobial genera is an important mechanism allowing legumes to form symbioses with rhizobia adapted to particular soils [47]. The generalists (i.e., promiscuous legumes) obtain fewer average benefits from rhizobia than specialists [45]. However, the “Jack-of-all-trades is a master of none” hypothesis asserts that specialists persist because the fitness of a generalist utilizing a particular habitat is lower than that of a specialist adapted to that habitat [48].

Specialized interactions help structure communities, but persistence of specialized organisms is puzzling because a generalist can occupy more environments and partake in more beneficial interactions [48]. A specialist–generalist trade-off would suggest that hosts often benefit from blocking many rhizobia strains, which conflicts with the fitness interest of rhizobia to increase nodulation [44]. Therefore, species-specific legume–rhizobial interactions can directly influence niche and fitness differences among legume species and between legumes and non-legumes, with important consequences for the richness–productivity relationship [45].

Both the rhizobia–host specificity and the diversity of the native rhizobia community are fundamental for the efficient use of inoculants. Poor inoculation responses may be related to large and diverse indigenous populations of well adapted nodulating bacteria. This occurs with the inoculation of beans in Latin American agricultural fields [49].

3. Identification of Bacterial Strains and Determination of Rhizobia Diversity

Rhizobia have been extensively characterized in global studies due to their high diversity and importance for agriculture [50]. These studies of diversity generally involve sampling of nodules in the fields (soils and seeds), rhizobia isolation from nodules, identification of the isolates at the genus and species levels, molecular characterization through genotyping of isolates, and characterization

of symbiosis-related genes, as well as nodulation tests, phenotyping, and description of new rhizobium species [51].

Currently, several molecular tools are available to study and identify bacterial diversity. Molecular methods have been developed to reveal the rhizobia diversity at the genetic, strain, species, genus, or higher levels [51]. Genetic analysis includes DNA–DNA hybridization, G + C contents, and PCR analysis using a large number of genes, including housekeeping genes [52]. For molecular characterization of the rhizobial strains, different PCR methods can be used [50]. To further improve the identification process, the analysis of conserved housekeeping genes has been used successfully to detect rhizobia diversity with precision [52]. The housekeeping genes used in PCR and sequence analyses for further characterization of representative strains of each *recA* genotype include the 16S rRNA, *atpD*, *glnII*, *dnaK*, *gap*, *glnA*, *gltA*, *gyrB*, *pnp*, *rpoB*, and *thrC*. Recommended strategies for studies on rhizobia diversity include 1, screening by *recA* phylogeny; 2, phylogenetic analyses of housekeeping genes; 3, BOX-PCR; 4, phenotypic characterization; 5, chemical taxonomy; 6, phylogenetic analyses of symbiosis genes and symbiotic specificity; 7, genome analysis; 8, description of new species and genera [51].

Sequencing the 16S rRNA gene, one of the most important tools in current studies on microbiology, has been used mainly to identify and classify isolates from pure cultures and estimate bacterial diversity in the environment without culture samples, through metagenomic approaches [53]. The 16S rRNA gene is a component of the 30S subunit of the prokaryote ribosomes, whose other components are 23S and 5S in the RNA molecules. The 16S gene is considered a good phylogenetic marker because it displays some conserved regions that are useful for unveiling phylogenetic relationships between distant species and also more variable regions, which are used to differentiate closely related species. For example, Salvina et al. [54] carried out preliminary tests to select the best procedure for genetic identification of rhizobia species and selected the 16S rRNA gene due to its reliable genetic identification of bacteria. Moreover, Hakim et al. [55] used the sequencing of the 16S rRNA by the illumina® system (Illumina, Inc, San Diego, USA) to obtain an overview of microbial diversity using a metagenomic approach and the relative distribution of the endophytic population (rhizobial and non-rhizobial) in mung bean nodules grown in different areas.

Although important, molecular methods should not be limited to the 16S rRNA gene alone, since a taxonomic consensus is best achieved when different types of data are combined. Such an integrated model, which includes phenotypic, genotypic, and phylogenetic information, is referred to as polyphasic taxonomy [56]. To identify rhizobia at the species and subspecies levels, identification and classification of bacteria and particularly rhizobia using polyphasic approaches is becoming the most accepted method [52].

Currently, several methodologies and other housekeeping genes have been used in phylogenetic studies of bacteria, mainly because the 16S rRNA has limitations in resolving the taxonomy and phylogeny of some genera, such as *Bradyrhizobium*, where high conservation of this gene hampers diversity analysis and species identification [56,57]. Other methods that have been used include characterization and screening of nodulating bacteria and sequencing of repetitive DNA-like BOX, ERIC sequences, and the 16S-23S rRNA intergenic spacer. However, so far, limited databases are available to compare results from different studies [52]. Moreover, screening by *recA* is advantageous because such phylogenetic analyses can simultaneously determine the genus and species of the rhizobial strains. On the other hand, many rhizobial species share highly similar (>97%) or even identical sequence of 16S rRNA.

A phenotypic or morphocultural characterization is a culture-dependent, fast, and low-cost method that provides information on groups of viable and cultivable microorganisms in a soil sample [58]. The phenotypic data involves biochemical and physiological traits, and analysis includes Gram staining; cell morphology and motility; oxidase and catalase activity; Biolog tests; NaCl tolerance, antibiotic, pH and temperature profiling; fatty acid composition; nodulation; and N fixation [52]. In this method, evaluating the morphological characteristics is the first step to identify new taxonomic groups of microorganisms. Due to its low cost, the phenotypic characterization can be useful in laboratories that do not have access to sophisticated technologies [59]. Currently, despite the high

value of molecular methods to bacteria taxonomy, phenotypic characterization still plays an important role in classification [60]. The isolation and characterization of rhizobia from nodules collected in the field or from bait plants require few materials and are carried out, according to Vincent [61], in the following stages: 1, rehydration of nodules, if desiccated, in sterile water; 2, superficial disinfection of nodules; 3, crushing and streaking the internal content of the nodule in a petri dish containing Yeast-extract-mannitol (YEM) broth. The most important characteristics observed are the time of emergence of isolated colonies and pH reaction in YEM broth with bromothymol blue; however, exopolysaccharide production, shape, size, color, texture, consistency, and optical details of the colony can also be observed [62]. Thomas-Oates et al. [63] also point out physiological and symbiotic characteristics as important for the characterization of strains of rhizobia.

Analyses of the chemical composition of *Rhizobium* cells include the content of cellular fatty acids, protein, respiratory quinones, polar lipids, and G+C mol% of the genomic DNA [53]. Although the data from these analyses are not so valuable for species differentiation, they can be used as descriptive characteristics for the species that are currently known [52].

Genome analysis complements studies on bacterial diversity. With the development of genome sequence analysis, the DNA–DNA hybridization (DDH) methods were replaced by average nucleotide identity (ANI) and digital hybridization of genome sequences in the description of novel species and genera [64,65]. Several analyses mentioned here are used to describe new species and genera. The International Committee on Systematics of Prokaryotes hosts the Subcommittee for the Taxonomy of Rhizobia and Agrobacteria, which holds regular meetings to discuss relevant issues and keep track of newly published species and genera. Importantly, the subcommittee publishes recommendations for the description of new species and genera of rhizobia and agrobacteria, and authors are expected to follow these guidelines [51].

Using these described methods, we identified different types of nodular bacteria and verified that nitrogen-fixing Leguminosae-nodulating bacteria (NFLNB) or rhizobia are included in a diverse phylogenetic classification list with species distributed in alphaproteobacteria and betaproteobacteria of the following genera (Table 1):

Table 1. Classification and number of species of bacteria identified as rhizobia.

Family	Genus/Type Species	Species of Rhizobia within the Genus, According to List of Prokaryotic Names with Standing in Nomenclature (2018)	References
<i>Rhizobiaceae</i>	<i>Rhizobium</i> . Type species: <i>R. leguminosarum</i>	133 species	[66]
	<i>Ensifer</i> / <i>Sinorhizobium</i> . Type species: <i>E. adhaerens</i> ; <i>S. fredii</i> ; <i>S. xinjiangensis</i> .	The genus <i>Ensifer</i> / <i>Sinorhizobium</i> covers about 24 species	[67,68]
	<i>Allorhizobium</i> . Type species: <i>A. undicola</i>	9 species	[69]
	<i>Pararhizobium</i> . Type species: <i>P. giardinii</i> .	<i>P. giardinii</i> ; <i>P. herbae</i>	[70]
	<i>Shinella</i> . Type species: <i>S. granuli</i>	<i>S. kummerowiae</i>	[71,72]
	<i>Neorhizobium</i> . Type species: <i>N. galegae</i>	<i>N. alkalisoli</i> , <i>N. galegae</i> and <i>N. huaatlense</i>	[73]
<i>Hyphomicrobiaceae</i>	<i>Azorhizobium</i> . Type species: <i>A. caulinodans</i>	<i>A. caulinodans</i> , <i>A. doebereineriae</i> and <i>A. oxalatophilum</i>	[74–76]
	<i>Devosia</i> . Type species: <i>D. riboflavina</i>	<i>D. neptuniae</i>	[77,78]
<i>Bradyrhizobiaceae</i>	<i>Bradyrhizobium</i> . Type species: <i>B. japonicum</i>	60 species	[79–81]
	<i>Blastobacter</i> . Type species: <i>B. tienricii</i>	<i>B. denitrificans</i>	[82]

<i>Phyllobacteriaceae</i>	<i>Mesorhizobium</i> . Type species: <i>M. loti</i>	59 species	[83,84]
	<i>Aminobacter</i> . Type species: <i>A. aminovorans</i>	<i>A. anthyllidis</i>	[85,86]
	<i>Phyllobacterium</i> Type species: <i>P. myrsinacearum</i>	8 species: <i>P. ifriqiense</i> , <i>P. leguminum</i> , <i>P. bourgognense</i> , <i>P. brassicacearum</i> , <i>P. endophyticum</i> , <i>P. loti</i> , <i>P. sophorae</i> , <i>P. trifolii</i>	[87,88]
<i>Methylobacteriaceae</i>	<i>Methylobacterium</i> . Type species: <i>M. organophilum</i>	<i>M. nodulans</i>	[89,90]
	<i>Microvirga</i> Type species: <i>M. subterranea</i>	<i>M. lupini</i> , <i>M. lotonononidis</i> , <i>M. zambiensis</i> and <i>M. vignae</i>	[91,92]
<i>Brucellaceae</i>	<i>Ochrobactrum</i> . Type species: <i>O. anthropi</i>	<i>O. lupini</i> , (synonym of <i>O. anthropic</i>); <i>O. cytisi</i>	[93–96]
<i>Burkholderiaceae</i>	<i>Burkholderia</i> . Type species: <i>B. cepacian</i> . Sawana et al. (2015) proposed the division of the genus into <i>Burkholderia</i> and <i>Paraburkholderia</i> ,	The genus <i>Burkholderia</i> has 32 species and <i>Paraburkholderia</i> has 69 species	[97–99]
	<i>Cupriavidus</i> . Type species: <i>C. necator</i>	<i>C. taiwanenses</i>	[100–102]
	<i>Ralstonia</i> . Type species: <i>R. pickettii</i> ,	<i>R. taiwanensis</i> (Sinonimous: <i>Cupriavidus taiwanensis</i> ; <i>Wautersia taiwanensis</i>)	[103,104]

4. Diversity of Rhizobia in Nodules of Legume Trees in the Humid Tropics

In the humid tropics, more specifically, in the periphery of the Amazon region, legume trees are important for their ability to colonize and supply N in environments with poor soils due to the continuous production of biomass throughout the year, even in long periods of drought, which provides better soil coverage [105].

Legume trees of economic and environmental interest are distributed throughout the world, and some non-native species are widely distributed in the tropics [106]. These legumes can be used to recover degraded areas by providing N-rich biomass for agroforestry systems, produce high-quality forage for livestock, stabilize slopes against erosion, and provide shade for planting crops, fruits, and vegetables for human consumption, among others [33,107]. It should also be noted that the success of the BNF of legume, both introduced and native, depends on the presence of compatible and efficient rhizobia in the native community of the soil [106]. Therefore, the evaluation of the presence or absence of efficient strains in the native community of the area and forecast of the need for inoculation is the first step towards the establishment of legume trees, regardless of the purpose. This review will focus on four legume trees, the leucaena (*Leucaena leucocephala* (Lam.) de Wit), acacia (*Acacia mangium* Willd.), sombreiro (*C. fairchildiana* R. A. Howard), and gliricidia (*G. sepium* (Jacq.) Kunth), common in sustainable agricultural systems in the humid tropics due to their effectiveness in the use of N, mainly by low-income family farmers [10,13].

Leucaena is a fast-growing legume tree belonging to the subfamily Mimosoideae, native to Central America and widely distributed in tropical regions. It has a high BNF (average of 177–247 kg ha⁻¹), high leaf N content (about 1.3%), and high biomass production (about 5 t ha⁻¹) [108]. Leucaena is used to rehabilitate degraded areas by vegetation covering, in agroforestry systems, as forage, human food, firewood, wood, green manure, shade, support for scandent species, and to control wind and soil erosion [109]. It appears that leucaena is non-specific with respect to its symbiotic relationships with NFLNB, since it can form nodules with species of several genera of rhizobia. A survey of several studies carried out in tropical regions indicated that nodulation of leucaena occurs, preferably, with fast-growing rhizobia. Four recent studies have identified species of *Rhizobium* in leucaena nodules [105,109–111], while others have found species belonging to the genus

Mesorhizobium [109–112]. Only one study reported the occurrence of symbiosis between leucaena and *Bradyrhizobium* [111] and between leucaena and *Cupriavidus* [113].

The BNF capacity of acacia in symbiosis with rhizobia is one of its most advantageous characteristics. This legume establishes symbiosis with species of *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium*, and *Ochrobactrum* [114,115]. However, symbiosis between acacia and strains of *Bradyrhizobium* is more common than with species of other genera of rhizobia, as shown in studies on the identification of rhizobia strains in symbiosis with acacia in several regions [94,116–119]. Some authors have reported that effective nodulation of acacia occurs only with specific strains of *Bradyrhizobium* sp., such as *B. elkanii*, although acacia seedlings show high variability in their ability to fix N₂ in symbiosis with their specific strains of *Bradyrhizobium* [114,117,118,120].

Another legume of interest is gliricidia, a medium-sized tree that can reach up to 12 m in height [121]. The species is native to Mesoamerica and is considered the second most important multifunctional legume tree only behind leucaena [122]. Due to the high capacity of climate adaptation, this legume has been transported to most tropical countries, where it is widely distributed. Gliricidia can produce about 5 t ha⁻¹ of dry matter and 186 kg ha⁻¹ of N. The capacity for BNF makes the use of this species advantageous, especially for green manure purposes and for use in agroforestry systems. This legume establishes symbiosis, preferably with fast-growing rhizobia, mainly of the genus *Rhizobium* [25,123]. This fact is confirmed by the massive presence of species of the genus *Rhizobium* in a survey carried out with the main studies that addressed symbiosis of gliricidia with rhizobia, identified by a wide variety of molecular or classic methodologies [25,105,109,110,124,125].

The sombreiro is a rustic, medium to large fast-growing legume tree. The species is native to Brazil, and its phytogeographic domain includes the Amazon rainforest. Although it has a clear preference for fertile and moist soils, the sombreiro can also occur in open and altered areas [126]. The BNF capacity of this tree is known, but little studied, despite its high production of biomass (about 8 t ha⁻¹) and N (328 kg ha⁻¹) [127], with reports of symbiosis with *Rhizobium* strains [114] and *Bradyrhizobium* [25,120].

5. Final Considerations

The huge diversity of rhizobia strains and their interactions with legume trees in tropical soils, if used wisely, can contribute to the sustainability of tropical agroecosystems. According to studies carried out in these regions, tropical soils have a high diversity of rhizobia, which interact with different legumes. Furthermore, the efficiency of this symbiosis depends on both the symbionts and external factors. Indeed, BNF is a highly complex process that involves a diverse group of bacteria, currently distributed into seven families and 19 genera identified through several methods based on phenotypic/morphological, biochemical, and molecular characterizations, the latter of which is making major contributions to modern taxonomy. Fortunately, four of the most suitable legumes for use in achieving more sustainable agricultural systems for family farmers in the humid tropics, *L. leucocephala*, *A. mangium*, *G. sepium*, and *C. fairchildina*, perform symbiosis with different groups of rhizobia. Future research must be focused on efficiency of the interaction between the symbionts and external factors, which can lead to higher legume biomass, rich in N. In this scenario, the exploration of BNF as a key ecological service could bring economic, ecological, and agronomic benefits to assist in the process of sustainable intensification of agriculture in the humid tropics, mainly because BNF can increase the efficiency of N use in this environment, where it is a major limiting factor.

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