Chapter 19
Rhizobium Diversity Is the Key to Efficient Interplay with *Phaseolus vulgaris*. Case of Study of Southern Ecuador

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Abstract Plants acquire different combined forms of nitrogen by addition of ammonia and/or nitrate fertilizer or manure to the soil, during organic matter decomposition, by the conversion of nitrogen into different compounds, or by biological nitrogen fixation (BNF). Diverse soil bacteria collectively called rhizobia are capable to fix N_2 from the atmosphere through symbiosis with legume plants. The N_2 fixed by the legume crops represents a renewable source of nitrogen for agricultural soils, turning symbiotic nitrogen fixation (SNF) in a natural process of significant importance in world agriculture. Within the legumes carrying out this

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process, common bean (*Phaseolus vulgaris* L.) constitutes a staple, being the most important grain legume worldwide, especially for developing countries. However, *P. vulgaris* is a low nitrogen fixer compared with other legumes, mainly attributed to the presence of high but inefficient diversity of indigenous rhizobia in soil, increasing the promiscuity of bean genotypes and lack of response under field conditions. Rhizobia diversity has been extensively studied. Polyphasic taxonomy and recently the average nucleotide identity approach have allowed to discover about 117 so-called *Rhizobium* species and the real genetic differences of microsymbionts in ecosystems around the world. Nevertheless, phylogenomic, ecological, and population genetic criteria to delineate biologically meaningful species in interplay with their host are still needed. Therefore, understanding genotypic variabilities between bean genotypes and *Rhizobium* strains contributes to achieve an efficient interaction, increase plant parameters, nitrogen fixation, and yields of common bean. Here, we discuss about the most recent studies on *Rhizobium* diversity linked to *P. vulgaris* in the American continent as the center of origin/diversification and outside this continent. The abiotic and biotic factors mediate the efficiency of the interaction, with special emphasis in the promiscuity of common bean as a constraint to achieve high nitrogen fixation rates and we show a case of study at southern Ecuador where genotypic variability among local bean genotypes and native *Rhizobium* strains was assessed to seek the efficiency of symbiosis based on its diversity.

19.1   Introduction

Legumes are one of the most widespread plants worldwide. These provide a wide range of nutritional factors, being an important source of protein-rich food and feed, oil, fiber, minerals, and vitamins (Pandey et al. 2016). The contributions of these plants to ecosystem services, especially in agroecosystems, are also of particular relevance, such as soil fertility by contributing nitrogen (N) through atmospheric N_2 fixation (Martínez-Romero 2003); improve the structure and increase soil organic carbon status (Wobeng et al. 2020); reduce the incidence of pest and diseases in cultivations (Daryanto et al. 2020), and increase the overall productivity and economic benefits of the production systems (Preissel et al. 2015).

Because N is the most necessary element for plant development, legumes become essential crops for its incorporation into agroecosystems (Misra et al. 2020). The N_2 fixed by the legume crops represents a renewable source of N for agricultural soils, turning symbiotic nitrogen fixation (SNF) in a natural process of significant importance in world agriculture. Globally, legumes in symbiosis with soil rhizobia are reported to fix 20–22 Tg of N each year in agricultural production systems (Herridge et al. 2008). Table 19.1 shows the biological systems capable of fixing N, the rates of

Table 19.1 A comparative study of the different N_2 fixers

N_2 fixers	Type	Family	Important species	N_2 fixed (kg N ha^{-1})	Recommended crops
Rhizobium	Symbiotic	Rhizobiaceae	*R. meliloti* *R. leguminosarum* *R. ciceri* *R. etli* *R. tropici* *R. fredii*	50–100	Pulse legumes like chickpea, red gram, pea, lentil, black gram, etc., oil-seed legumes like soybean, groundnut and forage legumes like berseem, and Lucerne
Azotobacter	Free living	Azotobacteriaceae	*A. chroococcum,* *A. vinelandii,* *A. beijerinckii,* *A. insignis* *A. macrocytogenes*	20	Rice, wheat, barley, oat, sunflowers, maize, line, beetroot, tobacco, tea, coffee, and coconuts
Cyanobacteria	Symbiotic, Free living and Symbiotic-Associative		*Nostoc sp.,* *Anabaena sp.,* *Gloeotrichia sp.,* *Tolypothrix sp.,* *Aulosira sp.,* *Aphanotheca sp.*	20–30	Submerged rice, barley, oats, tomato, radish, cotton, chilli, sugarcane, maize, lettuce
Azolla	Symbiotic-Associative	Salviniaceae	*A. caroliniana,* *A. microphylla,* *A. filiculoides* *A. Mexicana*	30–50	Submerged rice with maximum temperature
Azospirillum	Symbiotic-Associative	Spirilaceae	*A. lipoferum,* *A. brasilense,* *A. amazonense,* *A. halopraeferens,* *A. brasilense,* *A. trakense*	20–40	Maize, sugarcane, sorghum, pearl millet

Source: Kumar et al. (2018), cited by Misra et al. (2020)

Fig. 19.1 Comparison of dry bean harvest area and production in the last decade. FAO (2020, data 2018). http://www.faostat. fao.org/
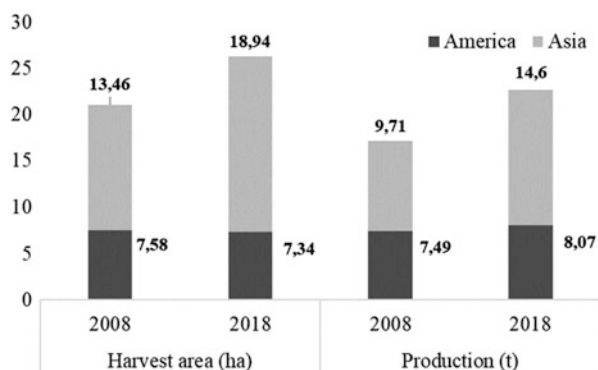
fixation, and the crops in which this important process can be carried out, highlighting legumes with an average fixation rate from 50 to 100 kg N ha^{-1}.

Among legumes, common bean (*Phaseolus vulgaris* L.) is a staple for human nutrition and one of the main sources of protein and calories in the world, mainly for developing countries (Los et al. 2018). Bean harvest areas and productions have been increasing significantly during the last decade by 22.7% and 28% respectively globally. These indicators have special relevance in Asia and America (Fig. 19.1), which are the largest producers worldwide. The total harvest area in Asia raised 28.9%, with a production of 33.5%. While in America, the total harvest area decreased in 3%, but production grew in 7.1% (FAO 2020. http://www.faostat.fao. org/, assessed on June 18, 2020). Despite its importance, the bean crop is characterized by low productivity (882.3 kg ha^{-1}) especially because of poor cropping practices, such as the inadequate supply of fertilizers and pests and diseases control (Vieira et al. 2010).

Surprisingly in America, the center of origin of this pulse and with a wide tradition of cultivation, although production has increased, the lands destined to this crop are reducing. Therefore, stimulating crop production and yields is crucial for many countries in this region. However, sustainable agricultural practices should be addressed to reduce partial or total dependence of N fertilizers and its ecological and economical costs.

As a legume, beans can carry out biological nitrogen fixation (BNF) through symbiosis with root nodule inducing soil bacteria collectively called rhizobia, allowing plants to grow in N-deficient soils (López-Guerrero et al. 2012; Sánchez et al. 2014). *Rhizobium* is a gram-negative symbiotic bacterium that colonizes the roots of leguminous plants forming root nodules, which helps in ammonia production (Mahdi et al. 2010). SNF by root nodules of legumes contributes far more to the N economy of natural communities and to the fertility of soils than the asymbiotic systems (Table 19.1) (Gunnabo et al. 2019). N-fixing root nodules can fix 100–200 times more N$_2$ than free-living bacteria, because of the capability of nodules to continue to fix N$_2$ for long periods of time (Tanveer et al. 2019).

They utilize the photosynthetic products of plants as a carbon source and, in return, fix atmospheric N$_2$ for their host (Misra et al. 2020). BNF becomes the

cheapest and the most environmentally correct form to provide N to plants and the most intensively studied model of beneficial plant–microbe interaction (Vieira et al. 2010). They are considered to be the most competent biofertilizer for legumes in terms of the amount of N fixed (Jehangir et al. 2017). For instance, 75% of the total N in plant was derived from SNF by faba bean; 62–94% by soybean, groundnut, pea, and lentil; 54–58% by cowpea, chickpea, and pigeonpea; and 39% by common bean (Dwivedi et al. 2015). However, in some legumes like *P. vulgaris*, poor nodulation or lack of response to inoculation in field conditions has been attributed to the (1) presence of a high but inefficient population of indigenous common bean rhizobia in soil and in seeds (Andrade et al. 2002), (2) genetic instability of selected strains (Satyanarayana et al. 2018), and (3) sensitivity of the symbiosis to environmental stresses, such as high temperatures, soil dryness, and low soil fertility (Graham and Ranalli 1997; Hungria and Vargas 2000).

Rhizobia biodiversity nodulating *P. vulgaris* is one of the most important constraints to achieve a proper interplay between bacteria and legume. This wide diversity of microsymbiont leads to the promiscuity of common bean and to reduce nodulation and N fixation performance in plants (Peix et al. 2015). Only in the American continent, a total of 11 new *Rhizobium* species have been isolated from *P. vulgaris* so far (Shamseldin and Velázquez 2020). The wide rhizobia biodiversity has been classified by polyphasic taxonomy, including 16S ribosomal RNA gene sequencing, multilocus sequence analysis (MLS), biochemical properties, and phenotypic features (Shamseldin et al. 2017). Currently, the average nucleotide identity (ANI) based on nodulation host range concerning reference strains (Ormeño-Orrillo et al. 2015) is used as an alternative approach (González et al. 2019). However, although ANI is indicative of genomic clusters, phylogenomic, ecological, and population genetic criteria to delineate biologically meaningful species are still needed (Vinuesa et al. 2018).

Although is a fact that the ability of *P. vulgaris* to be nodulated by different symbiovars linked to species primarily isolated from nodules of this legume and also by symbiovars linked to species initially isolated from nodules of other legumes allows *P. vulgaris* to establish N-fixing symbiosis in very different ecosystems around the world (Shamseldin and Velázquez 2020), a few studies about the diversity of rhizobia establishing symbiosis with *P. vulgaris* have been published. To date, only two reviews have been published in the present century (Martínez-Romero 2003; Shamseldin and Velázquez 2020). Therefore, understanding rhizobia biodiversity can trigger the efficiency of the symbiotic process. Here we discuss the genotypic variability among bean genotypes and Rhizobium species, as an important role to enhance nodulation, plant parameters, N fixation, and grain yields. We show a case study in southern Ecuador that ranges from microsymbionts molecular analysis, through the search for efficiency under controlled conditions, to determine the response under field conditions.

19.2 Understanding *Rhizobium* Diversity and Distribution to Improve Interplay with *Phaseolus vulgaris*

The family Rhizobiaceae gather the seven genera *Rhizobium*, *Neorhizobium*, *Allorhizobium*, *Agrobacterium*, *Ensifer* (syn. *Sinorhizobium*), *Shinella*, and *Ciceribacter*. However, several so-called *Rhizobium* species do not exhibit robust phylogenetic positions (Mousavi et al. 2015). The current classification of *Rhizobium* species is mostly based on phenotypic features, 16S ribosomal RNA gene sequencing, multilocus sequence analysis (MLS) of housekeeping genes, DNA: DNA hybridization (DDH), and average nucleotide identity (ANI) values (González et al. 2019). To date, this genus consists of 117 described species, of which 18 had been isolated from common bean root nodules almost worldwide (Tong et al. 2018), but particularly in the Mesoamerican and Andean centers of common bean origin/diversification (Fig. 19.2).

Common bean forms N-fixing symbioses promiscuously with bacteria belonging to different genera of alpha and beta Proteobacteria (Michiels et al. 1998; Peix et al. 2015). Within alpha-Proteobacteria, the species and symbiovars nodulating *P. vulgaris* belong to the genera *Rhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Pararhizobium* (formerly *Rhizobium*), and *Bradyrhizobium* (Mousavi et al. 2015), while from the beta-Proteobacteria can be nodulated by species from genera *Burkholderia* (currently *Paraburkholderia*) (Talbi et al. 2010; Martínez-Aguilar et al. 2013; Dall'Agnol et al. 2017) and *Cupriavidus* (da Silva et al. 2012).

19.2.1 *Rhizobia Strains Identification Linked to* P. vulgaris *in the American Continent*

When analyzing the biodiversity of *Rhizobium* species nodulating common bean, the American continent and specifically Center and South America play a preponderant role. A total of 11 new *Rhizobium* species have been isolated from *P. vulgaris* in this continent: *Rhizobium etli* (sv. *phaseoli*), *Rhizobium acidisoli* (sv. *phaseoli*), *Rhizobium hidalgonense* (sv. *phaseoli*), *Rhizobium esperanzae* (sv. *phaseoli*), and *Rhizobium mesoamericanum* (sv. *phaseoli*) in Mexico; *Rhizobium phaseoli* (sv. *phaseoli*) in the USA, *Rhizobium freirei* (sv. *tropici*), *Rhizobium leucaenae* (sv. *tropici*), and *Rhizobium paranaense* (sv. unnamed) in Brazil; *Rhizobium tropici* (sv. *tropici*) in Colombia, and *Rhizobium ecuadorense* (sv. *phaseoli*) in Ecuador. Interestingly, only *R. phaseoli*, *R. etli*, and *R. tropici* are considered indigenous of the American continent, just like their symbiovars: *phaseoli* and *tropici* (Shamseldin and Velázquez 2020). Other species, on the other hand, have not been reported in other nearby American countries such as *R. acidisoli, Rhizobium anhuiense, Rhizobium mesoamericanum, R. hidalgonense,* and *Rhizobium ecuadorenses*. Finally, *R. hidalgonense* and *R. ecuadorense* have been found outside the American continent, in Croatia, Southeast Europe (Rajnovic et al. 2019).
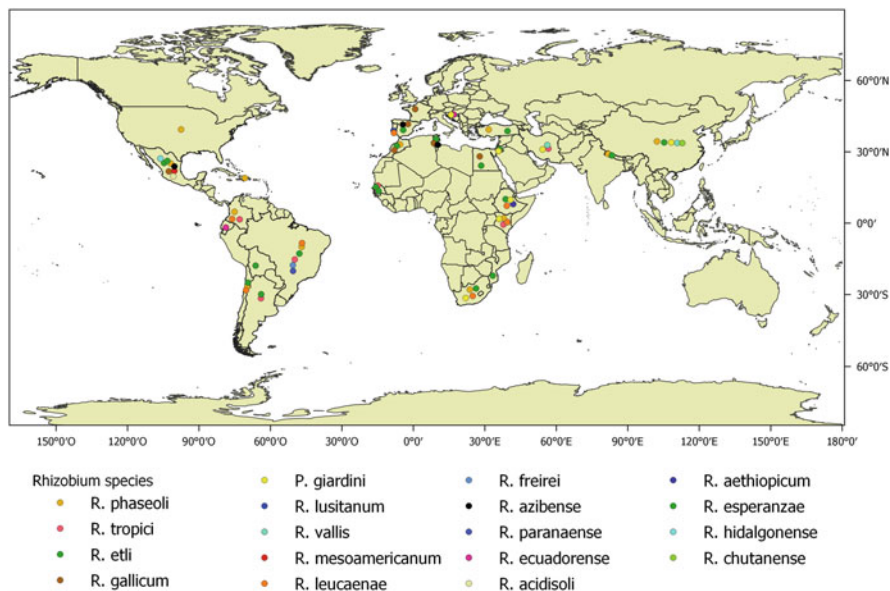
Fig. 19.2 Species distribution of *Rhizobium* originally isolated from *P. vulgaris*

Rhizobium etli and *Rhizobium phaseoli* were some of the first isolates from common beans in the continent, together with *Rhizobium tropici*. However, *R. phaseoli* was reclassified as biovar (currently symbiovar) of *R. leguminosarum* (Jordan 1982) and then of *Rhizobium etli* (formerly *R. leguminosarum* type I strains) (Eardly et al. 1992; Segovia et al. 1991). Currently, *R. phaseoli* is recognized as an independent species from *R. leguminosarum* and *R. etli* mainly because of the presence of divergent core genes (Ramírez-Bahena et al. 2008). *Rhizobium tropici*, similarly to *R. etli*, was previously classified as *Rhizobium leguminosarum* (type II) (Martínez-Romero et al. 1991). It was the first description of a rhizobial species to include sequences from 16 s rRNA gene and also the first species to carry the symbiovar *tropici*. In contrast to *R. leguminosarum* sv. *phaseoli* type II, *R. tropici* strains tolerate high levels of acidity and high temperatures in culture, and are also symbiotically more stable (Ormeño-Orrillo et al. 2012). Interest in the evolution of *R. tropici* results from its close genetic resemblance with Agrobacteria. *R. tropici* and some *Agrobacterium* strains resemble one another in morphology, growth rate, tolerance to pH, DNA-DNA hybridization, and in the 16S and 23S rRNA sequences (Martínez-Romero 1994), which indicates the possibility of discovering the linkage between symbiosis and pathogenicity (Gomes et al. 2012).

Formerly, the species *R. tropici* was designated as having two subgroups, A and B. Nevertheless, Ribeiro et al. (2013) proposed the reclassification of *R. tropici* type A strains as a novel species: *Rhizobium leucaenae* sv. *tropici*. The species *R. tropici* and *R. leucaenae* found in Colombia and Brazil were also found in soils of other American countries such as Argentina (Aguilar et al. 2001) and Chile (Baginsky

et al. 2015), supporting the possibility of co-evolution between *P. vulgaris* and its microsymbionts in the centers of host genetic diversification. Similarly, the distribution of species *R. etli* and *R. phaseoli* supports this hypothesis (Shamseldin and Velázquez 2020). In addition to the distribution of these bacteria, *R. phaseoli, R. etli, R. tropici*, and *R. leucaenae* were also found outside the American continent in Europe (García-Fraile et al. 2010; Valverde et al. 2011), Africa (Diouf et al. 2000; Mhamdi et al. 2002; Shamseldin and Werner 2005; Aserse et al. 2012; Zinga et al. 2017), and Asia (Adhikari et al. 2013; Cao et al. 2014; Chen 2019).

During the last decade, reports of new *Rhizobium* species isolated from common bean in America were linked to symbiovar *phaseoli* such as *Rhizobium acidisoli* (Román-Ponce et al. 2016), *Rhizobium hidalgonense* (Yan et al. 2017), *Rhizobium esperanzae* (Cordeiro et al. 2017) in Mexico, and *Rhizobium ecuadorenses* in Ecuador (Ribeiro et al. 2015). These last two species have been reclassified from the clade containing *R. phaseoli/R. etli/R. leguminosarum*, using mainly the approach based on nodulation host range and genomic ANI. *R. esperanzae* and *R. ecuadorense* have been reported to have higher similarity with *R. etli* on the 16S rRNA gene sequence phylogeny and phylogeny of nifH analysis (Ribeiro et al. 2015; Cordeiro et al. 2017). Similarly, *R. acidisoli* and *R. hidalgonense* have identical 16S rRNA genes to other *Rhizobium* species. For instance, the phylogeny of 16S rRNA gene sequences of *R. acidisoli* suggests it is most closely related to *Rhizobium anhuiense* (99.7% similarity) (Román-Ponce et al. 2016), and *R. hidalgonense* was found to be similar to *R. acidisoli* using 16S rRNA and nifH gene sequences (Yan et al. 2017).

19.2.2 Microsymbionts Beyond America

The first described nodulating common beans outside of America were *Rhizobium gallicum* (sv. *gallicum* and *phaseoli*), *Rhizobium giardinii* (sv. *giardinii* and *phaseoli*), and *Rhizobium lusitanum* (sv. *tropici* and *phaseoli*) in Europe. However, only *R. lusitanum*, isolate in Portugal, is considered indigenous of this continent (Valverde et al. 2011). Since then, *R. gallicum* has been found in American countries (Amarger et al. 1997; Mhamdi et al. 1999; Sessitsch et al. 1997), and *R. giardinii* has also been found in Asian and African soils (Herrera-Cervera et al. 1999; Mhamdi et al. 2002; Aserse et al. 2012; Wang et al. 2016; Rouhrazi et al. 2016). The phylogenetic analysis of these bacteria shows that *R. gallicum* has similarities with *R. etli* (98%) and recent evidence suggests that the NodC of the pSym of *R. etli* is distributed in some strains of *R. gallicum* (Verástegui-Valdés et al. 2014). On the other hand, 16 s rRNA gene sequence analysis places *R. giardinii* on a lineage independent of *Rhizobium* (Amarger et al. 1997), prompting Mousavi et al. (2015) to place *Rhizobium giardinii* in the new genus *Pararhizobium*.

In Asia, the microsymbionts of *P. vulgaris* were directly introduced from American countries to the China region through *P. vulgaris* seeds. This hypothesis is supported by the high similarities in the symbiotic genes (nodC and nifH) between

the Chinese and American *R. etli* populations (Cao et al. 2014). Indeed, two new species isolated in this continent carry the symbiovar *phaseoli*: *Rhizobium vallis* (Wang et al. 2011), reported also in Iran (Rouhrazi et al. 2016), and *Rhizobium chutanense* (Huo et al. 2019). According to 16S rRNA sequence analyses, *R. vallis* showed the most similarity with *Rhizobium lusitanum* (99.1% sequence similarity) and *Rhizobium rhizogenes* (99.0%) strains, although DNA–DNA relatedness values were very lower (Wang et al. 2011). Also using 16S rRNA analyses, *Rhizobium chutanense* was found to be most similar to *R. ecuadorense, R. fabae, R. pisi,* and *R. esperanzae* (Huo et al. 2019).

African diversity of nodulating rhizobia *of P. vulgaris* has their origins with the introduction of American and European strains (Mhamdi et al. 1999; Aserse et al. 2012; Zinga et al. 2017; Kawaka et al. 2018). In the last decade, two new species within the genus *Rhizobium* have been isolated in this continent: *Rhizobium azibense* sv. *gallicum* (Mnasri et al. 2014) and *Rhizobium aethiopicum* sv. *phaseoli* (Aserse et al. 2017), although the first specie has strains belonging to North Tunisia, Spain, and Mexico (Silva et al. 2005). *R. azibense* was previously unsigned as *Rhizobium gallicum*, based on 16S rRNA, *recA*, and *atpD* gene sequences (Mnasri et al. 2014), but phylogenetic analysis based on *recA, atpD, dnaK*, and *thrC* sequences showed that it is distinguished from a group closely related to *Rhizobium gallicum*. On the other hand, the closest phylogenetic reference (combined *recA* and *glnII* sequences) of *Rhizobium aethiopicum* were strains of *R. etli* (94% similarity) and *Rhizobium bangladeshense* (93%) (Aserse et al. 2017). Lastly, the distribution of *R. azibense* and *R. aethiopicum* is still unknown, given that these bacteria have not been reported in other countries, though recently *R. azibense* together with *R. bangladeshense* were found nodulating in legumes in Bangladesh-South Asia (Tanim et al. 2019).

19.3   The Efficiency of *Rhizobium*–Bean Interaction Mediated by Biotic and Abiotic Factors

Several factors directly influence the efficiency of symbiotic plant–*Rhizobium* interactions, particularly in field conditions. Among them, (1) compatible strain and host selection, (2) saprophytic competence, (3) root hair attachment and competition for infection, (4) growth characteristics of bacteria within infection threads as well as (5) effects of plant physiology, and (6) the abiotic environment (Terpolilli et al. 2012). The first of the factors, related to the strains type and host selection, is one of the most significant to achieve the desired effect of N_2 fixer bacteria application.

Rhizobia are soilborne bacteria with a key role in the SNF process as symbiotic partners of legumes. *Rhizobium* spp. populations reside in soils and are in constant evolution, being able to adapt to different habitats (Martínez-Romero 2003). The colonization success and symbiotic N_2 fixation depends on their attachment abilities to biotic and abiotic substrates (Wielbo et al. 2015). Wang and Chen (2004) described how *Rhizobium* colonize new environments, facing two different

problems. In the first place, *Rhizobium* needs to establish a population in the local community among groups of indigenous bacteria, and there exists a competition between space and nutrients. In the second place, is the adaptation of *bacteria* to the soil environmental factors, such as humidity, salt content, pH, temperature, and symbiotic partners, which are determinant for the establishment of rhizobia in a specific environment (Misra et al. 2020).

Bean cultivation is considerably varied by soil type, land use pattern, and genotype. It is demonstrated that root exudates act as substrates in soils and signaling molecules, which are required for establishing plant–bacteria interactions (Kour et al. 2019). The successful root colonization is an important and initial step in the interaction of beneficial bacteria with plants (Hungria and Mendes 2015). In order to acquire beneficial effects from the microbial communities, the effective colonization of the plant root area is crucial (Yadav et al. 2015).

Successful root colonization by a bacterium is the result of interactions with physical, chemical, and biological characters of the environment as well as properties of the bacterium itself. A clear understanding of the bacterial colonization process toward the plant is required (Dutta et al. 2014). Between these bacterial colonizers, some genera of rhizobia are cataloged as effective colonizers. They synthesize plant growth hormones and can make organic and inorganic phosphates soluble (Avis et al. 2008).

Many rhizobacteria like rhizobia possessing the enzyme ACC deaminase catalyze the conversion of ACC to ammonia and α-ketobutyrate, which indirectly decrease the ethylene concentration in plants under drought stress (Glick 2012). By facilitating the development of longer roots, these rhizobacteria may enhance the survival of seedlings, which help in combating the effect of stress ethylene (Zahir et al. 2009). The root elongation plants under drought stress can allow a better access to water and uptake of nutrients (Misra et al. 2020). Ethylene is also known to compromise the nodule formation and N fixation in legume (Sapre et al. 2019). *Rhizobium* with ACC deaminase activity can diminish the deleterious effect of ethylene under drought stress by increasing the nodulation and N fixation in its symbiotic legume partner (Belimov et al. 2009).

Salinity of the agriculture soil is a significant issue all over the world and it is also a determinant environmental factor for reduction of growth and yield of agricultural crops (Misra et al. 2020). The use of plant growth-promoting rhizobacteria (PGPR), as well as those described for the genus *Rhizobium*, can stimulates mechanisms of action for the amelioration of salt stress, and to reduce the application of chemical fertilizers and pesticides in the agricultural fields and improve soil health (Yadav and Saxena 2018). ACC deaminase producing *Rhizobium* strains can improve the growth and quality of mung beans under salinity stress (Ahmad et al. 2012). Bacteria that are tolerant to stress have better nodulation ability and greater ability for N fixation of legumes to grow and survive under stressed conditions. Rhizobial populations vary in their tolerance to major environmental factors. Together, the expression of all these features can improve the efficiency of the rhizobia–legume symbiosis (Naveed et al. 2015).

19.3.1 Promiscuity as a Biotic Constraint for Achieving a High Rate of N Fixation in Common Bean

Common bean is nodulated by different *Rhizobium* species and symbiovars, allowing this pulse to establish N-fixing symbiosis in very different ecosystems around the world (Shamseldin and Velázquez 2020). However, among the biotic factors restricting the *Rhizobium*–bean interplay, the microsymbionts biodiversity in the soil and in nodules is crucial. As reported previously, *P. vulgaris* is a promiscuous legume in its interaction with *Rhizobium* species and symbiovars (Michiels et al. 1998; Pérez-Ramírez et al. 1998). Indeed, *P. vulgaris* has been considered as a promiscuous host because it can be nodulated by several divergent nodC symbiovars (Peix et al. 2015). Also, in the laboratory under axenic conditions, *P. vulgaris* is nodulated by many more *Rhizobium* species than in agricultural fields. In addition, most of these species belong to the genus *Rhizobium* such as *R. calliandrae, R. grahamii, R. jaguaris, R. leguminosarum, R. mayense, R. mongolense, R. miluonense, R. multihospitium, R. rhizogenes, R. yanglingense, R. sophorae, R. sophoriradicis,* and *R. laguerreae sv. viciae* (Jiao et al. 2015; Flores-Félix et al. 2019).

Although promiscuity could be an important feature for plant breeding, in *P. vulgaris* is one of the most important constraints to achieve high rates of N fixation by this pulse (Dwivedi et al. 2015). Symbiotic interactions between common bean and its microsymbionts are not always equally effective in the N fixation (Peix et al. 2015). For example, compared with other *Rhizobium* species, the strains of *R. tropici, R. freirei*, and *R. paranaense* show higher tolerance to environmental stress and high efficiency in N_2 fixation, so are thus considered for use in commercial inoculants, especially in countries where they were discovered (Martínez-Romero et al. 1991; Zurdo-Piñeiro et al. 2004; Gomes et al. 2015; Mwenda et al. 2018; Ipsilantis et al. 2019; Elizalde-Díaz et al. 2019). Moreover, *R. etli* and *R. tropici* strains showed different efficiency on different plant genotypes, which affect the stability of the performance of inoculants (Gunnabo et al. 2019).

19.4 Seeking Efficiency of *Rhizobium* Species Based on Its Biodiversity

Since bean is a poor N fixer compared to other grain legumes (de Sá et al. 1993; Naveed et al. 2015; Yadav et al. 2020), inoculation of bean lines or genotypes with *Rhizobium* strains well suited to different agroecological regions with high capacities to fix atmospheric N is required. However, the response of the crop and inoculation may not be satisfactory in cases in which highly competitive native *Rhizobium* populations are present in the soil, restricting root colonization by the inoculant strain (Hungria and Vargas 2000; Brito et al. 2015; Hungria and Mendes 2015; da Conceição et al. 2018). Therefore, to recommend a *Rhizobium* strain with high

Table 19.2 Summary of results on the relationships among nodule number, nodule weight, % N_2 in shoot, total N_2 fixed, nitrogenase activity, root weight, shoot weight, harvest index, seed yield, and 100-seed weight in common bean

Number and type of germplasm	Trait combination	Correlation coefficient	References
50 Iranian germplasm	Grain yield, 100-seed weight, and harvest index with nodule number, N% in shoot, and total N_2 fixed	0.208^a–0.584^a	Reza Golparvar (2012)
	Nodule number with total N% in shoot and total N_2 fixed	0.466^a–0.517^a	
	Total N% in shoot and total N_2 fixed	0.671^a	
47 Andean, Meso-American gene pool	Root and nodule weight linearly correlated with mg N fixed per plant	0.71^a–0.74^a	Vadez et al. (1999)
8 cultivars	Shoot weight and mg N fixed per plant	0.46^a	Westermann and Kolar (1978)
	Acetylene reduction activity (mmol C_2H_4 per plant) and mg N fixed per plant	0.38^a–0.54^a	
	Nodule weight with mg N_2 fixed per plant	0.84^a	
	Plant weight and seed yield with mg N_2 fixed per plant	0.55^a–0.74^a	

Source: Dwivedi et al. (2015)
[a]Weight, refers to dry weight of the sample

agronomic performance, previous several tests are necessary to confirm its competitiveness (Leite et al. 2018).

Although the competitive effect associated with rhizobia promiscuity in common bean is well known, it would be important to take this feature into account when searching effective strains in symbiosis with bean genotypes. This is a fact that makes the *Rhizobium* biodiversity in this crop should not only be approached as a negative aspect but also as an opportunity to obtain adequate genotypic variability among *Rhizobium* strain × bean genotypes.

To test the effectiveness of *Rhizobium* inoculation in common bean, several types of researches have been developed under laboratory, greenhouse, and field conditions, where the strain–bean genotype interactions play an important role to seek the proper genotypic variability to enhance plant parameters and yields. In this regard, Dwivedi et al. (2015) reported that genotype × environment and genotype × *Rhizobium* strain interactions are a prerequisite to identifying germplasm and *Rhizobium* strains for effective symbiosis in legumes. Correlations between these factors can enhance plant parameters related to an effective symbiosis, such as nodule number, nodule weight, root and shoot weight, N_2 fixation, and yields in bean plants (Table 19.2).

The infectivity of *Rhizobium* species nodulating common bean has been assessed in different studies. Mhamdi et al. (2002) have reported nine groups of rhizobia isolated from *P. vulgaris* in Tunisia. They delineated: *Rhizobium gallicum biovar* (bv.) *gallicum, Rhizobium leguminosarum bv. phaseoli* and *bv. viciae, Rhizobium*

etli bv. *phaseoli, Rhizobium giardinii* bv. *giardinii,* and four groups related to species of the genus *Sinorhizobium, Sinorhizobium meliloti, Sinorhizobium medicae*, and *Sinorhizobium fredii*. Among the isolates assigned to *R. leguminosarum*, two-thirds were ineffective in nitrogen fixation with *P. vulgaris* and harbored a symbiotic gene typical of the biovar *viciae*. The *S. fredii*-like isolates did not nodulate soybean plants but formed numerous effective nodules on *P. vulgaris.*

In Africa, the increasing interest in the use of rhizobia as biofertilizers in smallholder agricultural farming systems has prompted the identification of a large number of tropical rhizobia strains and led to studies on their diversity. Besides in Tunisia, Koskey et al. (2018) obtained 41 *Rhizobium* isolated from the root nodules of MAC 13 and MAC 64 climbing beans in agro-ecological zones of Eastern Kenya. The analysis of molecular variance based on restriction digestion of 16S rRNA genes showed that the largest proportion of significant ($p < 0.05$) genetic variation was distributed within the rhizobia population (97.5%) than among rhizobia populations (1.5%). The high degree of morphological and genotypic diversity of rhizobia within Eastern Kenya shows that the region harbors novel rhizobia strains worth exploiting to obtain strains efficient in biological N fixation with *P. vulgaris*.

Near this continent, in Spain, Mulas et al. (2011) analyzed the rhizobia present in nodules of the variety "Riñón," in order to select native rhizobial strains to be used as biofertilizers. The analysis of *rrs* and housekeeping genes of the strains showed that they belong to two phylogenetic groups within *Rhizobium leguminosarum*. Strains LCS0306 from group I and LBM1123 from group II were the best N fixers among all strains isolated and were selected for field experiments. The field research showed that the biofertilization of common bean with native and selected rhizobial strains can completely replace the fertilization with chemical N fertilizers.

Approaching the American continent, Díaz-Alcántara et al. (2014) analyzed phylogenetic relationships with rhizobia isolated from the American and European countries linked by trade routes since the discovery of America. In this study, effective rhizobial strains nodulating common bean were isolated in the Dominican Republic. A total of 25 isolates were obtained from nodules, concluding that the strains of *R. phaseoli* sv. phaseoli, as well as those from *R. etli* sv. *phaseoli*, are indigenous to mainland America from where they were introduced along with *P. vulgaris* seeds. The results of this study showed that the analysis of *P. vulgaris* endosymbionts present in the islands located between America and Spain is important for biogeographical studies of these rhizobia, as well as for increasing the knowledge of the coevolution of *Rhizobium–Phaseolus vulgaris* symbiosis.

In Mesoamerican and Andean region, the center of origin and diversification of *P. vulgaris*, various studies based on microsymbiont diversity have been conducted (Segovia et al. 1991; Souza et al. 1994; Hungria and Vargas 2000; Martínez-Romero 2003; Aguilar et al. 2004; López-López et al. 2010; Santos et al. 2011; Servín-Garcidueñas et al. 2012; Ribeiro et al. 2013; Verástegui-Valdés et al. 2014; Torres-Gutiérrez et al. 2017; Tong et al. 2018; Ramírez-Puebla et al. 2019). In Ecuador, an Andean region from which common bean originates (Rodiño et al. 2010), few rhizobia identification studies have been carried out, despite potentially being an important source of rhizobial diversity, which is a key determinant of common bean

productivity (Baginsky et al. 2015). Ribeiro et al. (2015) determined the taxonomic affiliations of isolated strains from Ecuadorian soil (*Rhizobium ecuadorense*) previously reported by Bernal and Graham (2001), showing that all the Ecuadorian isolates corresponded to three novel lineages from the *Rhizobium etli* group that fall into the *R. phaseoli/R. etli/R. leguminosarum* clade. One of these lineages, with representatives isolated mostly from Ecuador, seems to be a dominant lineage associated with beans from that northern and central region (Ribeiro et al. 2013).

The few previous studies from the Ecuadorian Andean region have focused only on determining the phylogenetic potential of symbionts (Bernal and Graham 2001; Ribeiro et al. 2013). However, the effect of rhizobia on plant phenotypic parameters and the variability among isolated strains are rarely studied under laboratory, greenhouse, or field conditions. Therefore, it is important to assess the phenotypic parameters of isolated strains, such as nodule formation and plant biomass production. The determination of these parameters is not only necessary to elucidate the capability of isolated strains to grow under different environmental conditions but also to understand how bacterial inoculation enhances plant growth and yields (Torres-Gutiérrez et al. 2017).

19.4.1 Genotypic Variability Among Local Bean Genotypes and Native Rhizobium Strains. Case of Study of Southern Ecuador

19.4.1.1 Rhizobium Biodiversity at Southern Ecuador

Serial experiments were performed to unravel *Rhizobium* biodiversity and its genotypic variability with local bean genotypes. Here we describe the first study published by Torres-Gutiérrez et al. (2017), in which they show the genetic diversity of native *Rhizobium* strains from the southern region of Ecuador.

For *Rhizobium* isolation, firstly, sampling was performed in nine municipalities of Loja province in the southern region of Ecuador. Global positioning system data were recorded at each collection point and altitudinal levels (meters over sea level) were determined (Table 19.3).

In each of the sampling areas, roots nodules of *P. vulgaris* were taken randomly and stored in moisturized Ziploc® bags to prevent drying. The isolation methodology proposed by Sánchez et al. (2014) was used with minimal modification. Briefly, individual nodules were dissected from the roots using a flame sterilized scalpel and tweezers and were washed thoroughly in distilled water using a sieve to remove all traces of soil. Subsequently, nodules were transferred to a sterile Petri dish and surface disinfected by immersion in 10 mL of 3% NaClO for 3 min, followed by immersion in 0.1% $HgCl_2$ for 2–5 min. Finally, the nodules were washed profusely with sterile distilled water. For bacteria molecular identification, isolated colonies were grown overnight in Yeast Extract-Mannitol (YM) medium at 30 °C with shaking at 250 rpm in a shaker incubator (Techine TS1500, USA). DNA extraction

Table 19.3 Georeferentiation of sampling sites at southern Ecuador

| Municipality | Samples | Georeferentiation | | Height[a] (m.o.s.l) | Soil type | Altitudinal levels[b] |
		S	W			
Pindal	4	04°07′06″	80° 06′32″	800	Inceptisols	1
Paltas	3	04°02′46″	79°46′78″	940	Entisols	
Catamayo	4	04°05′99″	79°18′29″	1078	Inceptisols	2
Calvas	2	04°26′56″	79°35′52″	1193	Inceptisols	
Sosoranga	3	04°19′50‴	79°47′ 35″	1549	Entisols	
Gonzanamá	3	04°07′87″	79°25′50″	1680	Inceptisols	
Loja	8	03°56′86″	79°12′48″	2120	Entisols	3
Celica	2	04°05′95″	79°57′78″	2029	Inceptisols	
Saraguro	5	03°36′56″	79°15′26″	2691	Inceptisols	

[a]Average height of the sampling sites in meters over sea level (m.o.s.l.)
[b]Altitudinal level 1: from 800 to 940 m.o.s.l., altitudinal level 2: from 1078 to 1680 m.o.s.l., altitudinal level 3: from 2120 to 2691 m.o.s.l

was performed using a ChargeSwitch® gDNA Mini Bacteria Kit (InvitrogenTM, USA), according to the manufacturer's instructions. DNA quality was checked by&spi2;quantification in NanoDrop (NanoDrop 2000, Thermo Scientific, USA) and electrophoresis in a 1% agarose gel (1 g agarose in 100 mL TBE buffer). The&spi2;16S rRNA gene of isolates was amplified with the conserved primers: ARI C/T (5'CTGGCTCAGGAC/TGAACGCTG3') and pH (5'AAGGAGGTGA-TCCAGCCGCA3') (Clermont et al. 2009), which amplify almost the full length of the 16S rRNA gene (1500 bp). The PCR-amplified 16S rDNA fragments were purified using a PureLink® PCR Purification Kit (InvitrogenTM, USA) and the sequence analysis was performed using an Applied Biosystems 3100 DNA Sequencer. Sequence assembly was performed with BioNumerics version 4.5 (Applied Maths, Sint-Martens-Latem, Belgium). The closest related sequences were identified using the FASTA program and compared with those available in the GenBank database.

The 16S rDNA assay (Table 19.4) demonstrated the presence of nine species of *Rhizobium* among the 20 isolated strains, including *Rhizobium tropici, R. etli, R. etli* bv. *mimosae*, *R. leguminosarum*, *R. leguminosarum* bv. *viciae, R. mesoamericanum, R. undicola* and two unclassified species, *Rhizobium* sp. and uncultured *Rhizobium* sp. These results show the wide *Rhizobium* diversity at southern Ecuador, as well as the prevalence of *R. tropici*, which nodulates *P. vulgaris* in this region.

Table 19.4 Genetic diversity of *Rhizobium* strains at southern Ecuador

Strain code	Sampled municipality	Altitudinal levels[a]	Accession no.	Closets to FASTA hit	Sequence identity (%)
NAR1	Paltas	1	KP027690.1	*Rhizobium tropici* str. MMUST-006	100
PIN1	Pindal	1	JQ797311.1	*Rhizobium etli* str. ECRI 15	100
PIN3	Pindal	1	KP027691.1	*Rhizobium leguminosarum* bv. *viciae* str. MMUST-003	100
TAB1	Calvas	2	EF555479.1	*Rhizobium* sp. rf033	98
COL1	Calvas	2	KP027691.1	*Rhizobium leguminosarum* bv. *viciae* str. MMUST-003	100
COL6	Calvas	2	KP027691.1	*Rhizobium leguminosarum* bv. *viciae* str. MMUST-003	100
TAM1	Catamayo	2	KP027690.1	*Rhizobium tropici* str. MMUST-006	100
CB1	Catamayo	2	KP027690.1	*Rhizobium tropici* str. MMUST-006	100
NAM1	Gonzanamá	2	JX122134.1	*Rhizobium mesoamericanum* str.	100
SOS1	Sosoranga	2	KM672515.1	*Rhizobium undicola* str. MR68	100
SOS4	Sosoranga	2	KC172298.1	*Rhizobium uncultured.* Clone DM6-85	100
LP1	Célica	3	KP027690.1	*Rhizobium tropici* str. MMUST-006	100
VP1	Loja	3	CP006986.1	*Rhizobium etli* bv. *mimosae* str. IE4771	99
VP2	Loja	3	KP027690.1	*Rhizobium tropici* str. MMUST-006	100
RC2	Loja	3	KP027690.1	*Rhizobium tropici* str. MMUST-006	100
TUR1	Loja	3	KP027679.1	*Rhizobium leguminosarum* str. KSM-004	100
RAI1	Loja	3	KP027690.1	*Rhizobium tropici* str. MMUST-006	100
Q2	Saraguro	3	KP027691.1	*Rhizobium leguminosarum* bv. *viciae* str. MMUST-003	100
Z1	Saraguro	3	KP027691.1	*Rhizobium leguminosarum* bv. *viciae* str. MMUST-003	100
Z3	Saraguro	3	KP027691.1	*Rhizobium leguminosarum* bv. *viciae* str. MMUST-003	100

[a]Altitudinal level 1: from 800 to 940 m.o.s.l., altitudinal level 2: from 1078 to 1680 m.o.s.l., altitudinal level 3: from 2120 to 2691 m.o.s.l

19.4.1.2 Authentication of *Rhizobium* Isolates and N Fixation under Greenhouse Assay

All isolates were assessed to determine their capability to promote nodule number, biomass production, and N content of *P. vulgaris* in pot experiments under greenhouse conditions. For the experiment, a complete randomized experimental design with ten replicates was performed. The inoculation with the wild-type strain *Rhizobium etli* CNPAF512 (obtained from the culture collection of the Centre for Microorganism and Plant Genetic of Catholic University of Leuven, Belgium) and a treatment without inoculation were the controls.

Certified *P. vulgaris* cv. Mantequilla seeds were obtained at Loja Market. Seeds were surface disinfected as described previously by Vlassak et al. (1998) and pre-germinated for 2 days on moist filter paper in the dark at 28 °C. One pre-germinated seedling was planted per pot. Bean plants were harvested at 21 days after inoculation (DAI) to determine nodule number, nodule dry weight (mg), and total nitrogen content in the shoots (% total N). The best response strains were grouped by Ward's method, using Euclidean distance, taking into account the results of nodulation, dry weight of nodules, and N fixation.

Figure 19.3 (panel A) shows that at 21 days after inoculation, all isolates were able to nodulate the host plant. The nodule number and nodule biomass were variable among the isolates, but most of them yielded significantly higher values than the controls, primarily for nodule number, highlighting the potential of native strains to nodulate a local beans cultivar. The inoculation with *R. leguminosarum* bv. *viciae* COL6, *R. etli* bv. *mimosae* VP1, and *R. mesoamericanum* NAM1 was statistically significant among the treatments. However, no significant difference was observed among them and with *R. tropici* (NAR1), *R. undicola* (SOS1), *R. tropici* (LP1), *R. tropici* (VP2), *R. leguminosarum* bv. *viciae* (Q2), and *R. leguminosarum* bv. *viciae* (Z1). These nine bacterial isolates belonged to the group with the best nodule formation in bean plants (group A) forming an average of 75 nodules per plant. Following this group, a total of ten isolates (*R. etli* PIN1, *R. leguminosarum* PIN3, *R.* sp. TAB1, *R. leguminosarum* bv. *viciae* COL1, *R. tropici* TAM1, *R. tropici* CB1, *R.* uncultured SOS4, *R. tropici* RC2, *R. tropici* RAI1, and *R. leguminosarum* bv. *viciae* Z3) were clustered in the second most important group (B) for nodulation, and only one isolate (*R. leguminosarum* TUR1) and wild type strain CNPAF512 were included in group C, having the lowest nodules number. Although no significant differences were shown among several native strains and *R. etli* wild type strain CNPAF512, the low responses by the reference strain could be associated with the fact of adaptation to a new environment. Thies et al. (1991) state that native rhizobia are generally more competitive than introduced strains. Finally, as expected, the control treatment was unable to nodulate the host plant.

In panel B, the nodular biomass showed significant differences among the isolates. A group A, with a total of seven isolates (R. tropici RC2, *Rhizobium mesoamericanum* NAM1, *R. tropici* LP1, *R. leguminosarum* bv. *viciae* Z1, R. sp. TAB1, *R. leguminosarum* PIN3, and *R. leguminosarum* bv. *viciae* COL6) exhibited

a

b
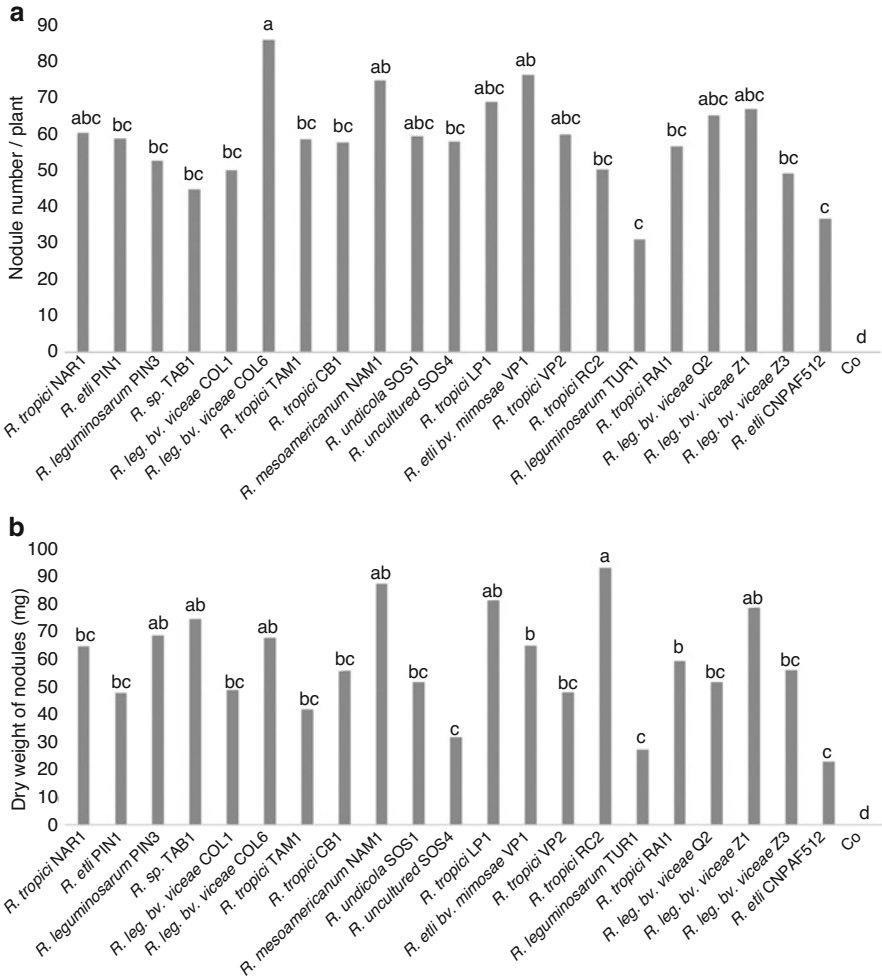
Fig. 19.3 Nodule formation (**a**) and nodular biomass (**b**) by strain. Plants were inoculated with the isolates showed in each column. Co: plant no inoculated. Letters on bars indicate significant differences between the mean ranges according to the Kruskal–Wallis/Mann–Whitney test for $p \leq 0.05$ $n = 10$

the most significant results, with nodule dry weight values ranging from 70 to 92 mg. The largest group (group B) of strains (11 isolates) had moderate nodule dry weights, with values from 38 to 65 mg, and the low values (group C) belonged to *Rhizobium* uncultured SOS4, *R. leguminosarum* TUR 1, and the wild-type strain CNPAF512. The results for these two strains were consistent with the nodule number. The results obtained with wild-type strain, as well as for TUR1, could be related to their erratic interaction with *P. vulgaris* cv. Mantequilla. *R. etli* CNPAF512 was isolated from Mesoamerican soils and has been shown to be effective with the common bean
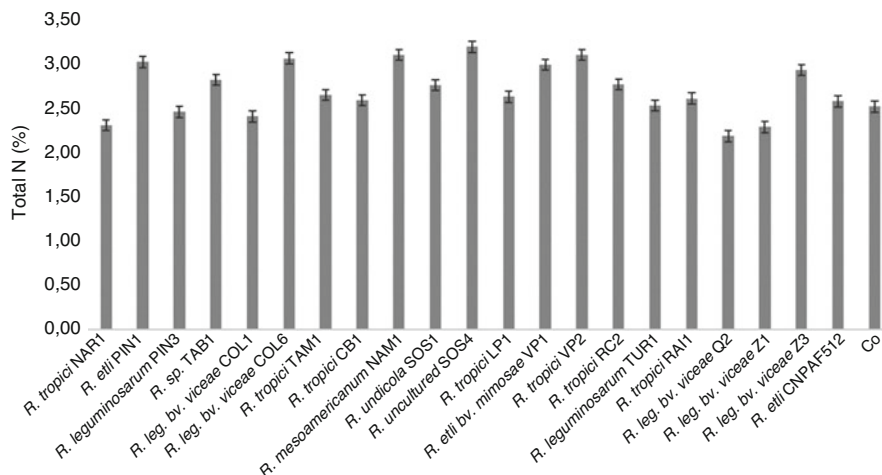
Fig. 19.4 Nitrogen fixation by *Phaseolus vulgaris* cv. Mantequilla inoculated with *Rhizobium* strain and non-inoculated treatments. Values are average of total nitrogen expressed in percentage from ten replicates. For the analysis, shoot from each treatment was dried and processed by Kjeldahl method

(Remans et al. 2007), but it may not be as efficient at promoting nodule formation and nodule biomass for Andean cultivars. Several studies have focused on the lack of response of wild-type strains in common beans. Mostasso et al. (2002) and Hungria et al. (2003) demonstrated the low activity of the reference strain as CIAT899 when used to inoculate *P. vulgaris*.

Despite the high diversity of morphological, genetic, and nodulation parameters, N fixation was rather homogenous for most of the treatments assessed (Fig. 19.4). The native strains, including uncultured *Rhizobium* sp. SOS4, *R. tropici* VP2, *R. leguminosarum* bv. *viciae* Z3, R. *leguminosarum* bv. *viciae* COL6, *R. mesoamericanum* NAM1, and *R. etli* PIN1, yielded the highest shoot N content. Results obtained using COL6 and NAM1 were expected for N fixation, due to the performance observed for the nodulation parameters. Voisin et al. (2003) and Yadegari and Rahmani (2010) reported that with the inoculation of efficient *Rhizobium* strains, the amount of N_2 symbiotically fixed by common beans is increased and is strongly correlated with the number of nodules and nodular biomass.

These results allowed the selection of the best strains according to their response to nodulation, nodular biomass, and N fixation (Fig. 19.5) to assess their efficiency in further researches under field conditions.

The results shown by native strains from southern Ecuador are in agreement with other previous studies (Slattery et al. 2004; Figueiredo et al. 2008; Peoples et al. 2009; Mulas et al. 2011; Karaca and Uyanöz 2012; Kawaka et al. 2014), which have been conducted with the purpose to select efficient *Rhizobium* strains to enhance nodulation, N fixation, and growth of common bean genotypes.

Fig. 19.5 Dendrogram constructed using Ward's method for the selection of *Rhizobium* strains
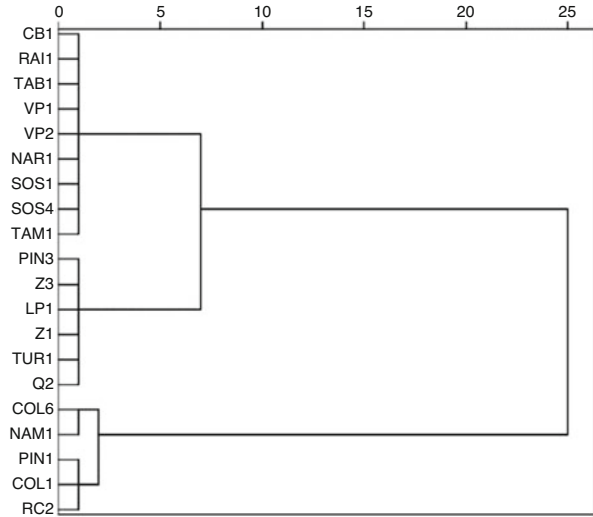
19.5 Conclusions and Perspectives

The rhizobia diversity plays an important role in establishing an effective and efficient symbiotic relationship with *Phaseolus vulgaris*. Through this exhaustive review, the biogeographic and genetic distribution of microsymbionts capable to nodulate common bean globally has been shown. Although there is a net of *Rhizobium* genetic distribution worldwide, we show that most of these strains belong to symbiovars indigenous from America, such as *phaseoli* and *tropici*, where this legume has its origin center. It is evident that biotic factors like the promiscuity can effectively limit beneficial interactions, being a constraint for stimulation of nodule formation and nitrogen fixation on *P. vulgaris*. Compatible interactions, as shown in the case of study in southern Ecuador, help to understand the genotypic variability between *Rhizobium* strains and local bean genotypes. Despite the amount of scientific information regarding the diversity of diazotrophic bacteria, more studies are needed focusing on the application of effective interplay under different agroclimatic conditions. The goal should be directed to achieve plant growth stimulation and to increase yields under field conditions with biofertilizers application and thus the reduction and/or elimination of nitrogenous fertilizers to carry out sustainable agricultural processes.

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