

## Genetic and phenotypic diversity of *Rhizobium* isolates from Southern Ecuador

### Diversidade genética e fenotípica de isolados de *Rhizobium* do sul do Equador

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#### ABSTRACT

*Rhizobium*-legume symbioses play relevant roles in agriculture but have not been well studied in Ecuador. The aim of this study was to characterize the genetic and phenotypic diversity of *Rhizobium* isolates associated with *Phaseolus vulgaris* from southern Ecuador. Morpho-cultural characterization, biochemical tests and physiological analyses were conducted to authenticate and determine the diversity of bacteria *Rhizobium*-like isolates. The genetic diversity of the isolates was determined by molecular techniques, which consisted of bacteria DNA extraction and amplification and sequencing of the 16S rRNA gene. The nodulation parameters and nitrogen fixation for *P. vulgaris* under greenhouse conditions were also assessed to determine the phenotypic diversity among isolates. Furthermore, bacteria indole-acetic-acid production was evaluated by the colorimetric method. Morpho-cultural and biochemical characteristic assessments demonstrated that *Rhizobium*-like bacteria was associated with the *P. vulgaris* nodules. The diversity among the isolates, as determined by physiological analyses, revealed the potential of several isolates to grow at different pH values, salinity conditions and temperatures. Partial sequencing of the 16S rRNA gene identified the *Rhizobium* genus in every sampling site. From a total of 20 aligned sequences, nine species of *Rhizobium* were identified. Nodule formation and biomass, as well as nitrogen fixation, showed an increase in plant phenotypic parameters, which could be influenced by IAA production, especially for the strains *R. mesoamericanum* NAM1 and *R. leguminosarum* bv. *viciae* COL6. These results demonstrated the efficiency of native symbiotic diazotrophic strains inoculants for legume production. This work can serve as the basis for additional studies of native *Rhizobium* strains and to help spread the use of biofertilizers in Ecuadorian fields.

**Index terms:** *Phaseolus vulgaris*; diazotrophic bacteria; 16S rRNA gene; nodulation; indole acetic acid.

#### RESUMO

A simbiose *Rhizobium*-leguminosa desempenha um relevante papel na agricultura, entretanto não tem recebido suficiente atenção de estudos científicos no Equador. O objetivo deste artigo foi caracterizar a diversidade genética e fenotípica de isolados de *Rhizobium* associados com *Phaseolus vulgaris* do sul do Equador. A caracterização morfo-cultural, testes bioquímicos e análises fisiológicas foram realizados para autenticar e determinar a diversidade de isolados de bactérias *Rhizobium*. A diversidade genética foi determinada por técnicas moleculares consistindo na extração de DNA genômico bacteriano, amplificação e sequenciamento parcial do gene 16SrRNA; e parâmetros de nodulação e fixação de nitrogênio de *P. vulgaris* sobre condições de estufa foram testados para determinar a diversidade fenotípica entre os isolados. Além disso, a produção de ácido indolacético foi avaliada por um método colorimétrico. A análise fisiológica da diversidade entre os isolados revelou o potencial de crescimento de diversos isolados em diferentes níveis de pH, salinidade e temperatura. O sequenciamento parcial do gene 16S rRNA mostrou o gênero *Rhizobium* em todas os locais de amostragem. De um total de 20 sequências alinhadas, 9 espécies de *Rhizobium* foram identificadas. A formação de nódulos e biomassa, bem como a fixação de nitrogênio mostraram um aumento nos parâmetros fenotípicos das plantas, os quais devem ser influenciados pela produção de IAA, especialmente pelas cepas *R. mesoamericanum* NAM1 e *R. leguminosarum* bv. *viciae* COL6. Estes resultados demonstram a eficiência de cepas diazotróficas simbióticas nativas para produção de inoculantes para leguminosas e fornece informações valiosas e úteis para a agricultura sustentável equatoriana. Neste sentido, este trabalho deve ser um elemento essencial para a realização de futuras pesquisas aplicadas relacionadas a cepas *Rhizobium* nativas e espalhar o uso de biofertilizantes em campos equatorianos.

**Termos para indexação:** *Phaseolus vulgaris*; bactéria diazotrófica; 16S rRNA gene; nodulação; ácido indolacético.

#### INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) is the most important grain legume worldwide and plays a

significant role in human nutrition, being a major source of dietary protein and representing a rich source of minerals and vitamins (Zaccardelli; Pentagelo; Tripodi, 2013). As a

legume, beans can carry out biological nitrogen fixation (BNF) through symbiosis with root nodule-associated soil bacteria collectively called rhizobia, which contribute to plant nutrition and allow plants to grow in nitrogen deficient soils (López-Guerrero et al., 2012; Sánchez et al., 2014).

The amount of nitrogen fixed by the common bean in association with rhizobia under field conditions is often low compared to that of other legumes (Remans et al., 2008a). The success of the symbiotic process depends on the competitiveness, specificity, compatibility, and effectiveness of the rhizobia with its host legume under variable soil and environmental conditions (Naveed et al., 2015). One of the major reasons for the low response of this grain legume is that it is a highly promiscuous host (Fuentes et al., 2002; Baginsky et al., 2015). The presence of rhizobia, which are comprised of different genera and species in the same host nodule, may be a result of genetic diversification and adaptation of the bacteria to their environment. However, the efficiency of nodulation and nitrogen fixation could be influenced by the infection of several rhizobia in the same host (Wei et al., 2008; Ormeño-Orilla; Martínez-Romero, 2013).

*P. vulgaris* nodule rhizobia have been extensively investigated (Kaschuk et al., 2006; Rahmani et al., 2011; Zhang et al., 2014), and it has been shown that, at its sites of origin, there are preferred symbionts, but in introduced areas it is promiscuous and may function as a less functional host plant (Michiels et al., 1998), forming nodules with diverse indigenous bacteria (Martínez-Romero, 2003). In Ecuador, an Andean region from which common bean originates (Rodiño et al., 2010), few rhizobia identification studies have been conducted, despite potentially being an important source of rhizobial diversity, which is a key determinant of common bean productivity (Baginsky et al., 2015). Recently, Ribeiro et al. (2015) determined the taxonomic affiliations of isolated strains from Ecuadorian soil previously reported by Bernal and Graham (2001) and Mexican strains, 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). This was one of the first studies in this Andean region, and additional research must be conducted to increase our understanding of the distribution patterns of rhizobial species in Ecuador.

Although a great deal of knowledge has been amassed concerning the diversity and genetics of

common bean symbionts, the basis of a successful inoculation and efficient nitrogen fixation remain elusive (Martínez-Romero, 2003). The few previous studies from the Ecuadorian Andean region have focused only on determining the phylogenetic potential of symbionts (Bernal; 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 tolerance to pH, temperature and salinity (Marquina; González; Castro, 2011), the stimulatory effect of indole-3-acetic acid (Remans et al., 2008b), as well as nodule formation and biomass production when they are inoculated in the host plant (Wei et al., 2008; Mehboob et al., 2013). 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.

The aim of this study was to characterize the genetic and phenotypic diversity of *Rhizobium* isolates associated with *Phaseolus vulgaris* from southern Ecuador. The ability of isolated strains to stimulate plant growth was assessed to evaluate the diversity and phenotypic variation of *Rhizobium* isolates from the southern region of Ecuador, taking into account molecular analysis (16S rRNA gene), cultural parameters, indole acetic acid-produced by bacteria and the effect of isolates on nodulation and biomass in the common bean in a greenhouse assay. Encouraging results will help change agricultural practices and revitalize the worldwide interest in BNF, particularly in low input systems (Naveed et al., 2015).

## MATERIAL AND METHODS

### Sampling

Sampling was performed in nine municipalities of Loja province in the southern region of Ecuador. In each of the sampling areas, roots of *Phaseolus vulgaris* plants with nodules were taken randomly and stored in moisturized Ziploc® bags to prevent drying. Global positioning system data were recorded at each collection point and altitudinal levels (meters over sea level, m.o.s.l.) were determined. Table 1 shows the sampling sites, geolocation position, altitudinal levels and soil types at each sampling site.

**Table 1:** Sampling sites, altitudinal levels and geolocation.

Municipality	Samples	Geolocation			Soil type	Altitudinal levels**
		S	W	Height* (m.o.s.l.)		
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	3
Loja	8	03°56'86"	79°12'48"	2120	Entisols	
Celica	2	04°05'95"	79°57'78"	2029	Inceptisols	
Saraguro	5	03°36'56"	79°15'26"	2691	Inceptisols	

\*Average height of the sampling sites in meters over sea level (m.o.s.l.).

\*\*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.

### Sample preparation and bacterial isolation

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 minutes, followed by immersion in 0.1% HgCl<sub>2</sub> for 2-5 minutes. Finally, the nodules were washed profusely with sterile distilled water.

The surface-disinfected root nodules were crushed with the flattened end of a flamed glass rod in a sterile plate containing 1 ml of sterile Milli-Q water. Afterwards, the bacterial suspension was streaked on solid YMA medium (Vincent, 1970) and the plates were incubated at 28 °C for ±7 days. Single colonies were purified by repeatedly streaking on YMA plates. After 2 days of purification, single colonies were individually analyzed by Gram-staining, morpho-cultural (growth rate, color, elevation, edges and slime production), and biochemical (growth in YMA+Congo Red, MacConkey and Kligler medium; catalase production; and production of acid or base) characterization (Somasegaran; Hoben, 1994). Cultural analyses were performed by testing growth at different pH values (4, 5, and 9), NaCl concentrations (0.1, 1 and 2%) and temperature (10, 35, 40 °C) in YMA medium at 30 °C (Bécquer; Prévost; Prieto, 2000; Kulkarni; Nautiyal, 2000). For pH assays, the YMA medium was adjusted to the desired pH with HCl or NaOH before sterilization with an

autoclave. All pure cultures were maintained on YMA and preserved at 4 °C for temporary storage or cryopreserved at -80 °C with 50% (v/v) glycerol for long-term storage.

### DNA extraction

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 was performed using a ChargeSwitch® gDNA Mini Bacteria Kit (Invitrogen™, USA), according to the manufacturer's instructions. DNA quality was checked by quantification in Nanodrop (Nanodrop 2000, Thermo Scientific, USA) and electrophoresis in a 1% agarose gel (1 g agarose in 100 ml TBE buffer).

### 16S rRNA gene amplification, purification and sequencing

The 16S rRNA gene of isolates was amplified with the conserved primers: ARI C/T (5'CTGGCTCAGGAC/TGAACGCTG3') and pH (5'AAGGAGGTGATCCAGCCGCA3') (Clermont et al., 2009), which amplify almost the full length of the 16S rRNA gene (1500 bp). Each 50 µl amplification reaction contained: 5 µl dNTPs (2 mM of each), 5 µl GeneAmp 10X-PCR buffer (100 mM Tris-HCl (pH 8.3)), 500 mM KCl, 15 mM MgCl<sub>2</sub>, 0.01% (w/v) gelatin, 0.5 µl of each primer (50 ng µl<sup>-1</sup>), 1 µl AmpliTaq DNA polymerase (1 U µl<sup>-1</sup>), 34.5 µl Milli-Q water and 5 µl of template (DNA extracted from isolated colonies).

The following temperature cycling program was used: 5 min at 95 °C to denature DNA, three amplification

cycles (45 sec at 94 °C, 2 min at 55 °C, and 1 min at 72 °C), 30 amplification cycles (20 sec at 94 °C, 1 min at 55 °C, and 1 min at 72 °C) and 5 min at 72 °C for a final primer extension. All PCR products were analyzed by electrophoresis in a 1% agarose gel (1 g agarose in 100 ml TAE buffer) at 80 V for 45 minutes.

The PCR-amplified 16S rDNA fragments were purified using a PureLink® PCR Purification Kit (Invitrogen™, USA) according to the manufacturer's instructions and evaluated afterwards by electrophoresis in a 1% agarose gel.

Table 2 shows the primers used for each sequencing reaction (Clermont et al., 2009). For each sequencing reaction, a mixture was made of 1 µl of purified PCR product, 0.5 µl of the Big Dye™ Termination Ready Reaction Mix (Applied Biosystems), 3.75 µl sterile Milli-Q water and 3 µl (20 ng µl<sup>-1</sup>) of one of the eight sequencing primers used.

The sequence analysis was performed using an Applied Biosystems 3100 DNA Sequencer following the manufacturer's protocols (Perkin-Elmer). 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 (Pearson, 1990). Sequences were also compared with those available in the GenBank database.

### 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. This work is a more detailed study of the *Rhizobium*-common bean (*Mantequilla*) interaction, which was published previously (Granda-Mora et al., 2016). 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. The pots contained a substrate that was a mixture of soil, sand and organic matter (2:1:1) sterilized at 120 °C for 2 hours. Seeds were surface disinfected as described previously by Vlassak et al. (1998) and pre-germinated for two days on moist filter paper in the dark at 28 °C. One pre-germinated seedling was planted per pot.

The inocula preparation consisted of pure bacteria cultures grown overnight in liquid medium (YM) at 30 °C with shaking at 250 rpm in an incubated shaker (Techine TS1500, USA). The inocula were prepared as reported by Remans et al. (2007). Cells were washed twice with 10 mM MgSO<sub>4</sub> and resuspended in 10 mM MgSO<sub>4</sub> at a density of 10<sup>7</sup> colony forming units (CFU) ml<sup>-1</sup>. Finally, common bean seedlings were inoculated with 0.2 ml of an inoculum containing 10<sup>7</sup> CFU.

A complete randomized experimental design was performed with ten pots as replicates for every treatment. Plants were grown under greenhouse conditions to control moisture, pests and weeds during the entire assay.

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) according to the Kjeldahl method (Page; Miller; Keeney, 1982).

### Quantification of indole acetic acid (IAA) production by isolates

IAA quantification was carried out by a colorimetric method using Salkowski reagent, consisting of 4.5 g FeCl<sub>3</sub> per liter in 10.8 M H<sub>2</sub>SO<sub>4</sub> (Glickmann; Dessaux, 1995) in the supernatant of cultures.

**Table 2:** Primers used for sequencing reaction.

Name	Direction	Primer sequence	Amplification region
Gamma	Forward	5'CTCCTACGGGAGGCAGCAGT3'	339-358
	Reverse	5'ACTGCTGCCTCCCGTAGGAG3'	358-339
*PD	Forward	5'CAGCAGCCGCGGTAATAC3'	519-536
	Reverse	5'GTATTACCGCGGCTGCTG3'	536-519
*O	Forward	5'AACTCAAAGGAATTGACGG3'	908-926
	Reverse	5'GTTGCGCTCGTTGCGGGACT3	1112-1093
*3	Forward	5'AGTCCCGCAACGAGCGCAAC3'	1093-1112
	Reverse	5'GCTACACACGTGCTACAATG3'	1241-1222

A previous experiment that was done to test the ability of strains to produce IAA without any IAA precursor in the media did not generate a result after measuring the optical density (OD) in a spectrophotometer. Therefore, all strains, including the wild-type strain as a positive control, were grown overnight in YM media with the addition of L-tryptophan (Sigma, 2.5 mg ml<sup>-1</sup>) and were incubated in a shaker (Techine TS1500, USA) at 30 °C, 250 rpm. From each culture, 1 ml was transferred to a sterile Eppendorf tube and centrifuged for 5 min at 6000 rpm. For the reaction, the supernatant was transferred to a fresh Eppendorf tube and 1 ml of the supernatant was added to 1 ml of Salkowski reagent and left in the dark for 30 min at room temperature. From the mixed solution, 1 ml was transferred to a micro-cuvette and the absorbance was measured at 530 nm (Jen Way 6505 UV/VIS, UK). The IAA concentration at the exponential phase of growth was estimated using a standard curve. For this purpose, a serial dilution of IAA (Sigma; 5, 10, 20, 50 and 100 µg ml<sup>-1</sup>) was made and incubated with Salkowski reagent using the same procedure described previously. The (OD) absorbance was measured at a wavelength of 530 nm and a standard curve was obtained by plotting the ODs against the concentrations. Three replicates were taken for statistical analysis and curve standardization.

### Statistical analysis

Data from each assay were tested for normality and homogeneity of variances for each variable and then assessed by analysis of variance (ANOVA), using a significance level of  $p < 0.05$ . SPSS Statistics 21.0 for Windows (IBM) was used and the means for each treatment were compared using Tukey's HSD as a post hoc significance test.

## RESULTS AND DISCUSSION

It is generally accepted that *P. vulgaris* is native to the Americas. The analysis of natural bean populations has led to the proposal of two major centers of diversification: the Mesoamerican center (Mexico, Central America and Colombia) and the Andean center (Ecuador, Peru and Bolivia) (Beebe et al., 2001; Junier et al., 2014). However, the genetic diversity of rhizobium-nodulating beans in Ecuador is, for the most part, unknown (Ribeiro et al., 2015). In previous studies, Bernal and Graham (2001) and Ribeiro et al. (2013) reported on the diazotrophic rhizobial communities in soils of northern and central Ecuador. Recently, a new species, *Rhizobium ecuadorensis* sp. nov., which was obtained from a common bean nodule, has been

described as a lineage from Ecuador (Ribeiro et al., 2015). This suggests that Ecuador is the center of genetic diversification for the common bean (Debouck et al., 1993), and symbionts of the several species of *Rhizobium* should be isolated from this region (Ribeiro et al., 2013).

The first results of this work, involving the isolation of bacteria from bean nodules, identified 34 isolates, from which 20 corresponded to *Rhizobium*-like bacterial strains (found at all sampling sites), taking into account their morphological and biochemical characterization. All 20 isolates were fast growers, short Gram-negative rods, produced bacterial colonies that ranged from white to opaque color, were elevated with regular edges and produced slime (Kuykendall; Family, 2005). The biochemical analysis showed that all strains grew on YMA+Congo Red with colonies ranging from pink to transparent in color. Furthermore, none of the strains fermented glucose in MacConkey medium, fermented neither lactose nor glucose and was a hydrogen sulfide producer in Kligler medium, and tested catalase positive and were acid producers, reinforcing that the isolated strains were *Rhizobium* (Somasegaran; Hoben, 1994).

The cultural analysis showed the diversity in the ability isolates to grow at different pH values, NaCl concentrations and temperatures (Table 3). These assessments are crucial to determine the ability of isolates to grow under different environmental stress conditions. Table 3 shows that most of the strains (70%) were able to grow at an alkaline pH (9), while at pH 4 only four strains (25%) grew. From them, isolates SOS1 and SOS4 grew moderately well and CB1, LP1 and COL6 strains exhibited only slight growth. The isolates VP2, Z3 and PIN1 did not grow at any of the pH values. The rest of the isolates (85%) were able to grow at pH 5. Finally, only five isolates (COL6, CB1, LP1, SOS1 and SOS4) were capable of growing at the three pH values tested.

The assessment of growth at different NaCl concentrations showed that nine isolates (45%) grew in 0.01% NaCl, five isolates (25%) grew in 1% NaCl and three isolates (15%) grew in 2% NaCl. A total of 11 isolates (55%) were susceptible at the three NaCl concentrations assessed, while only three isolates (COL6, CB1 and SOS1) were able to grow in all NaCl conditions.

Eight isolates (40%) were characterized as psychrophiles, growing at 10 °C, all the isolates (100%) were able to grow at 28 °C, and 15 grew at 35 °C (75%). Of the latter group, eight exhibited slight growth, four exhibited moderate growth and three exhibited abundant growth. At 40 °C, eight isolates (40%) exhibited slight growth.

**Table 3:** Cultural characteristics of isolates by sampling sites and altitudinal levels.

Strain codes	Sampled municipality	pH			NaCl (%)			Temperature (°C)			
		4	5	9	0.01	1	2	10	28	35	40
NAR1	Paltas	-	++	+	+	-	-	+	+	-	+
PIN1	Pindal	-	-	-	-	-	-	-	+	-	-
PIN3	Pindal	-	+	-	+	-	-	-	+	+	-
TAB1	Calvas	-	+	+	-	-	-	+	++	++	+
COL1	Calvas	-	+	-	-	-	-	-	+	+	-
COL6	Calvas	+	++	++	+	++	++	+	+++	+++	+
TAM1	Catamayo	-	+	+	-	-	-	-	+	+	-
CB1	Catamayo	+	+	+	+	+	+	+	+	+	+
NAM1	Gonzanamá	-	+	+	-	-	-	-	+	-	-
SOS1	Sosoranga	++	++	++	++	++	+	+	+	+	+
SOS4	Sosoranga	++	++	++	++	+	-	-	+++	+++	-
LP1	Célica	+	+	+	+	+	-	+	+	-	+
VP1	Loja	-	+	+	-	-	-	-	++	++	-
VP2	Loja	-	-	-	-	-	-	-	++	++	-
RC2	Loja	-	++	+	-	-	-	-	+	-	-
TUR1	Loja	-	+	+	+	-	-	+	+	+	+
RAI1	Loja	-	+	+	-	-	-	-	++	++	-
Q2	Saraguro	-	++	+	+	-	-	-	+++	+++	-
Z1	Saraguro	-	+	-	-	-	-	-	+	+	-
Z3	Saraguro	-	-	-	-	-	-	+	+	+	+

Legend: - Non growth, + slight growth, ++ moderated growth, +++ abundant growth.

By comparing the cultural parameters of isolates with the average of altitudinal level by sampling site, it was evident that the strains isolated at level 2 (from 1078 to 1680 m.o.s.l.) were the most adapted to growth at different pH values, NaCl concentrations and temperatures. The strains CB1 (from Catamayo, 1078 m.o.s.l.), COL6 (from Calvas, 1193 m.o.s.l.) and SOS1 (from Sosoranga, 1549 m.o.s.l.) grew under all the conditions tested.

More than 90% of arable land has some type of stress, which causes more than 50% of crop losses worldwide (Yamal et al., 2016). For example, fluctuations in pH, nutrient availability, temperature, and salinity, among other environmental stress factors, greatly influences the growth, survival, and metabolic activity of soil microorganisms and plants and their ability to enter into symbiotic interactions. In most cases, the microsymbiont is the more affected partner (Miransari et al., 2013). Therefore, the selection of stress-tolerant

strains of *Rhizobium* are important under stressful environmental conditions (Zahran, 1999). In the present study, the physiological characterization showed a high tolerance of the strains COL6, CB1 and SOS1 to grow at different pH values, salinity conditions and temperatures. Similar results reported by Andrade, Murphy and Giller (2002) and Shamseldin and Werner (2005) suggest that strains of *Rhizobium* can tolerate acidic pH values and high concentrations of NaCl. Thus, these native strains constitute a sustainable alternative to use as biofertilizer in sites with problems of environmental stress.

The 16S rDNA assay demonstrated the presence of nine species of *Rhizobium*, 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., supporting the idea that there is a wide diversity of species of this genera in Ecuador,

as well as the predominant *R. tropici*, which nodulates *P. vulgaris* in southern Ecuador (Table 4). Although few studies have reported on the genetic diversity of *Rhizobium* in Ecuador (Ribeiro et al., 2013), this is the first report of species diversity in the southern region of the country. Similar to this study, *R. etli*, *R. leguminosarum* and *R. tropici* have been previously isolated from common bean nodules (Martínez-Romero, 2003; Aguilar; Riva; Peltzer,

2004; Oliveira et al., 2011) and are considered to be the predominant occupant species of bean nodules in the Andean region (Junier et al., 2014; Ribeiro et al., 2015).

Ecuador, north of Peru, Colombia and the Andean Region are currently considered to be the centers of domestication/diversity of the common bean (Oliveira et al., 2011). Thus, it is important to isolate native strains from this region to determine their ability to fix nitrogen,

**Table 4:** Genetic diversity of *Rhizobium* isolates by altitudinal levels.

Strain Code	Sampled municipality	Altitudinal levels*	Accession N°	Closest FASTA hit	% Sequence identity
NAR1	Paltas	1	KP027690.1	<i>Rhizobium tropici</i> str. MMUST-006	100
PIN1	Pindal	1	JQ797311.1	<i>Rhizobium etli</i> str. ECRI 15	100
PIN3	Pindal	1	KP027691.1	<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> str. MMUST-003	100
TAB1	Calvas	2	EF555479.1	<i>Rhizobium</i> sp. rf033	98
COL1	Calvas	2	KP027691.1	<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> str. MMUST-003	100
COL6	Calvas	2	KP027691.1	<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> str. MMUST-003	100
TAM1	Catamayo	2	KP027690.1	<i>Rhizobium tropici</i> str. MMUST-006	100
CB1	Catamayo	2	KP027690.1	<i>Rhizobium tropici</i> str. MMUST-006	100
NAM1	Gonzanamá	2	JX122134.1	<i>Rhizobium mesoamericanum</i> str.	100
SOS1	Sosoranga	2	KM672515.1	<i>Rhizobium undicola</i> str. MR68	100
SOS4	Sosoranga	2	KC172298.1	<i>Rhizobium uncultured</i> . clone DM6-85	100
LP1	Célica	3	KP027690.1	<i>Rhizobium tropici</i> str. MMUST-006	100
VP1	Loja	3	CP006986.1	<i>Rhizobium etli</i> bv. <i>mimosae</i> str. IE4771	99
VP2	Loja	3	KP027690.1	<i>Rhizobium tropici</i> str. MMUST-006	100
RC2	Loja	3	KP027690.1	<i>Rhizobium tropici</i> str. MMUST-006	100
TUR1	Loja	3	KP027679.1	<i>Rhizobium leguminosarum</i> str. KSM-004	100
RAI1	Loja	3	KP027690.1	<i>Rhizobium tropici</i> str. MMUST-006	100
Q2	Saraguro	3	KP027691.1	<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> str. MMUST-003	100
Z1	Saraguro	3	KP027691.1	<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> str. MMUST-003	100
Z3	Saraguro	3	KP027691.1	<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> str. MMUST-003	100

\*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 20120 to 2691 m.o.s.l.

and stimulate bean plants to obtain inoculants that provide benefits to farmers. The genetic diversity of the strains was remarkable at the three altitudinal levels. However, with respect to the cultural characterization, altitudinal level 2 (1078 to 1680 m.o.s.l.) was had the most variation in *Rhizobium* isolates, with the presence of six species: *R. tropici*, *R. leguminosarum* bv. *viciae*, *R. mesoamericanum*, *R. undicola*, *Rhizobium* sp. and an uncultured *Rhizobium* sp. These results reinforce the promiscuity of *Phaseolus vulgaris* to nodulate with several *Rhizobium* species, including Ecuadorian cultivars. A total of 35% of the strains under study were positioned in the *R. tropici* clade, which was dominant in the region regardless the sampling site or altitudinal levels. For altitudinal level 1, one strain was identified as *R. tropici*, while for altitudinal level 2, two strains matched this species and at altitudinal level three, four strains belonged to this species.

Effective nodulation is essential for a functioning legume/*Rhizobium* symbiosis. In a previous study, we demonstrated that plants most susceptible to infection and capable of producing effective nodules should have greater potential to fix more atmospheric nitrogen (Sánchez et al., 2014). At 21 days after inoculation, all isolates were able to nodulate the host plant in the pot experiment under greenhouse conditions. The nodule number and nodule biomass were variable among the isolates (Figure 1), 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.

In panel A, the inoculation with *R. leguminosarum* bv. *viciae* COL6, *R. etli* bv. *mimosae* VP1 and *Rhizobium mesoamericanum* NAM1, were 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 surprisingly wild type strain CNPAF512 were included in group C, having the lowest nodule number. 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 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 *R. 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 (Remans et al., 2008b), 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 Hungría et al. (2003) demonstrated the low activity of the reference strain as CIAT899 when used to inoculate *P. vulgaris*.

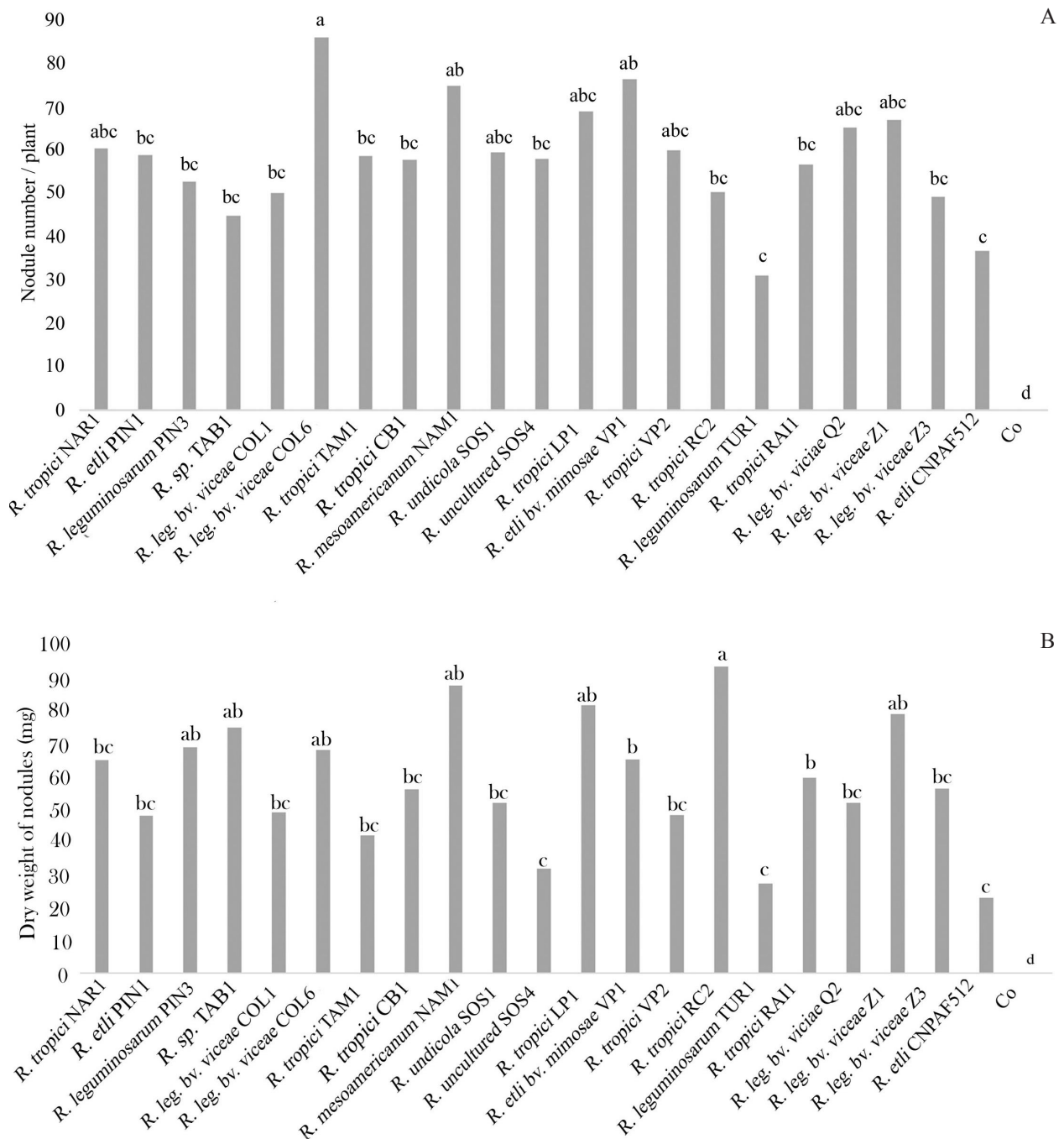
Despite the high diversity of morphological, genetic and nodulation parameters, nitrogen fixation was rather homogenous for most of the treatments assessed (Figure 2). The native strains, including uncultured *Rhizobium* sp. SOS4, *R. tropici* VP2, *R. leguminosarum* bv. *viciae* Z3, *R. leguminosarum* 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 respect to the amount of N<sub>2</sub> symbiotically fixed by leguminous plants, such as common beans inoculated with efficient *Rhizobium* strains, increased N fixation is strongly correlated with the number of nodules and nodular biomass. However, the results displayed with the inoculation of *Rhizobium* sp. SOS4, which did not induce higher nodule numbers and had one of the lower nodule biomass production values, is rather interesting. The results suggest that this strain had a negative interaction with the tested bean genotype for nodulation parameters, but the nodules formed can fix enough N to contribute to plant nutrition. Surprisingly, 11 isolated strains did not affect the N content in plants, having no significant difference with the control, demonstrating that only some *Rhizobium*-Mantequilla interactions can produce an efficient symbiosis. Similar results were observed by Cardoso et al. (2017), who identified several native *Rhizobium* strains that stimulated nodule dry weight in common beans, but

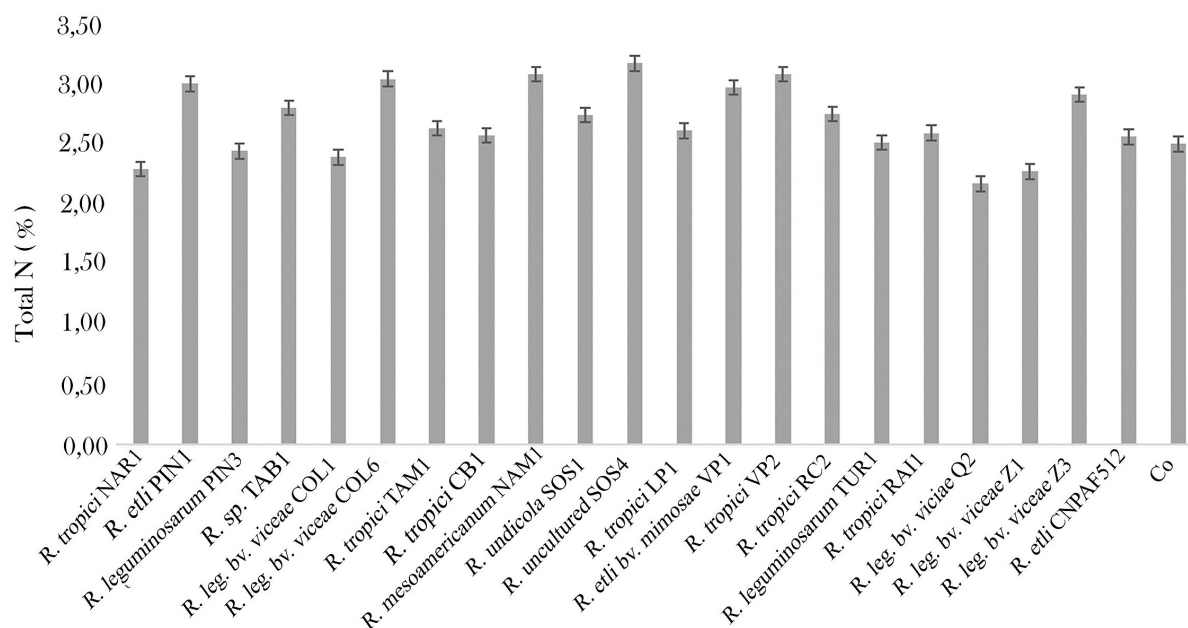


most of them were unable to produce a positive effect on total N content. In this study, more than half of the strains fixed less N than the control treatment without inoculation,

and approximately 20% of isolates showed a symbiotic efficiency similar to or better than the best Rhizobium reference strain CIAT899.



**Figure 1:** Nodule number (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 ANOVA and Tukey's test for  $p \leq 0.05$ ,  $n = 10$ .



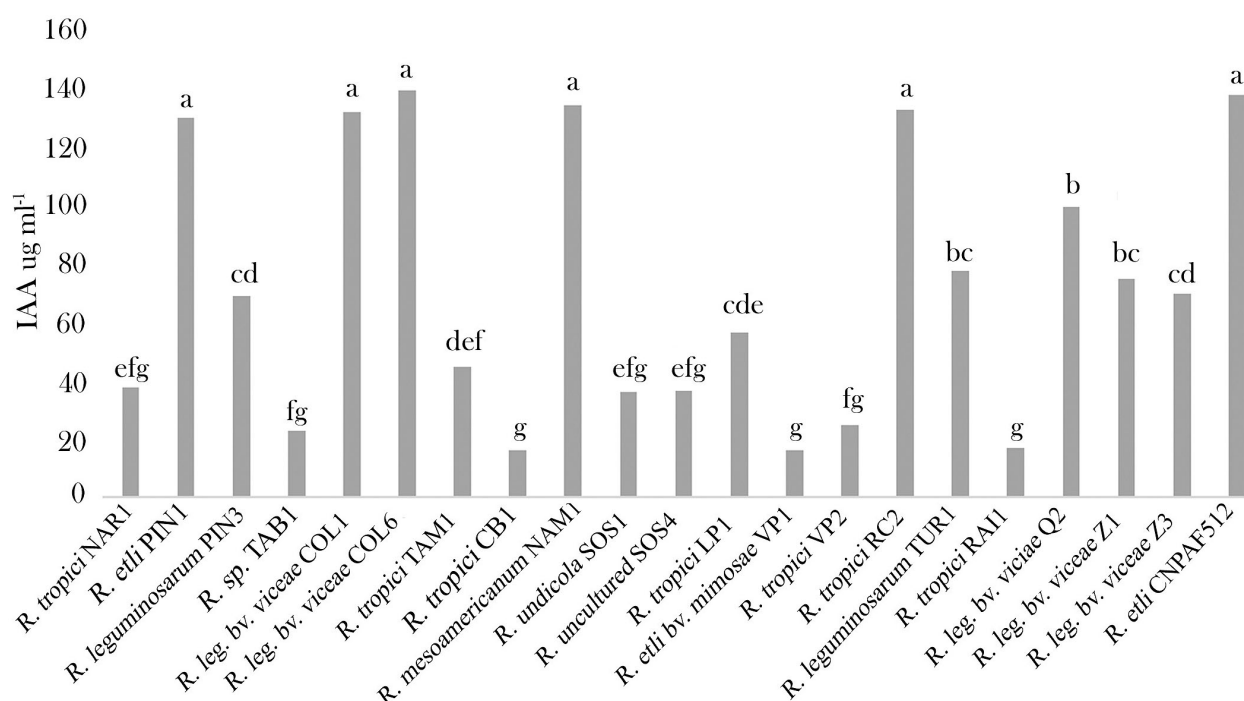
**Figure 2:** Nitrogen fixation by *Phaseolus vulgaris* cv. Mantequilla inoculated with *Rhizobium* strains and non-inoculated treatments. For the analysis, the shoot from each treatment was dried and processed by the Kjeldahl method. Letters on bars indicate significant differences between the mean ranges according to the ANOVA and Tukey's test for  $p \leq 0.05$ ,  $n = 10$ . In addition to the ability of bacteria to survive under different salt concentrations, pH values or temperatures, the ability to produce plant growth promoting substance are important characteristics that contribute to a proper interaction with host plants and helps increase plant growth parameters and yields. In the study, the quantification of IAA production by bacteria showed that all isolates, and wild-type strain *R. etli* CNPAF512 as positive control, had the ability to release the phytohormone (Figure 3).

It should be noted that the amount of IAA produced by most of the isolated strains was high compared with other studies, such as those conducted by Asghar et al. (2002) and Waheed et al. (2014), where the average concentrations of IAA produced by *Rhizobium* strains reached 10.5 and 14.25  $\mu\text{g ml}^{-1}$ , respectively. In this study, we obtained an average of IAA production of 138  $\mu\text{g ml}^{-1}$  and the lowest values ranged between 16 and 22  $\mu\text{g ml}^{-1}$  (strains CB1, VP1, RAI1 and TAB1).

The statistical analysis revealed that several strains, including *R. leguminosarum* bv. *viciae* COL6, *R. mesoamericanum* NAM1, *R. tropici* RC2, *R. etli* CNPAF512, *R. leguminosarum* bv. *viciae* COL1 and *R. etli* PIN1 produced the highest amounts of IAA. Interestingly strains such as *R. leguminosarum* bv. *viciae* COL6, *R. mesoamericanum* NAM1 also produced high amounts of IAA.

Most *Rhizobium* species have been shown to produce IAA via different pathways (Theunis et al., 2004), and many studies indicate that changes in the

auxin balance in the host plant are a prerequisite for nodule organogenesis (Mathesius et al., 1998). Although the exact role of IAA in the different stages of the *Rhizobium*/plant symbiosis remains unclear (Janczarek et al., 2014), auxins are involved in multiple processes, including cell division, differentiation and vascular bundle formation (Theunis et al., 2004). These three events are also necessary for nodule formation. Thus, it seems likely that auxins play a role in nodulation, as well as in the regulation of root system development in plants (Crespi; Frugier, 2009). Therefore, the production of IAA by *Rhizobium* species isolated from southern Ecuador, and in particular the isolates *R. leguminosarum* bv. *viciae* COL6, *R. mesoamericanum* NAM1 and *R. tropici* RC2 strains, indicate that their plant/growth promoting activity may include not only symbiotic nitrogen fixation but also an adequate ability to produce phytohormones that could be related to the increase in nodulation after inoculation with the common bean cv. Mantequilla.



**Figure 3:** Quantification of IAA production by bacteria. Letters on bars indicate significant differences between the mean ranges according to the ANOVA and Tukey test for  $p \leq 0.05$ ,  $n = 3$ .

Taking into account the holistic results of this research, the strain *R. leguminosarum* bv. *viciae* COL6 is a promising candidate for use in inoculants (biofertilizers) and for future studies under greenhouse and field conditions to further study the *Rhizobium*-legume symbiosis and the genotypic diversity among *Rhizobium* species and local bean cultivars.

## CONCLUSIONS

This study presented the first report of the isolation, genetic identification and phenotypic diversity of *Rhizobium* species associated with *Phaseolus vulgaris* cv. Mantequilla in southern Ecuador. Several strains (*R. leguminosarum* bv. *viciae* COL6, *R. tropici* CB1 and *R. undicola* SOS1) exhibited an ability to grow at different pH values, salinity conditions, temperatures and altitudes (from 800 to 2691 m.o.s.l). In fact, only these strains were able to grow under all the evaluated parameters, while the molecular analysis identified a high diversity of *Rhizobium* species (*R. tropici*, *R. etli*, *R. leguminosarum* bv. *viciae*, *R. mesoamericanum*, *R. undicola*, *R. etli* bv. *mimosae* *R. uncultured* and *R. sp.*) at the sampling sites. However, the predominance of *R. tropici* was remarkable. The presence of a non-cultured

*Rhizobium* isolated suggest the needs to perform more molecular analyses to elucidate the potential presence of novel *Rhizobium* species in the south of Ecuador. The results under greenhouse conditions showed the promiscuity of a local *Phaseolus vulgaris* cultivar to form nodules with several species of *Rhizobium*. The stimulation of nodulation by *Rhizobium* species and the legume *P. vulgaris* cv. Mantequilla was evident. However, all the tested strains produced different results in terms of numbers of nodules, nodular dry biomass and percentage nitrogen fixation, with *R. leguminosarum* bv. *viciae* COL6, *R. mesoamericanum* NAM1 producing the most significant results for most of the parameters analyzed. IAA-producing *Rhizobium* species, in particular by *Rhizobium leguminosarum* COL6 and *R. mesoamericanum* NAM1 indicate that the promoter activity of plant/growth may include not only symbiotic nitrogen fixation but also a high production of phytohormone. These results suggest that these promising strains could to be used as biofertilizers for field applications. However, more trials need to be conducted to determine the effectiveness of the stimulation on nodulation, biomass, and nitrogen fixation related to yields of several beans cultivars.

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