

RESEARCH ARTICLE

Efficiency of Nitrogen Fixation by *Rhizobium* Strains Associated With White Clover in the Ecuadorian Highlands

Biological Nitrogen Fixation by White Clover in Ecuador

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Abstract: The objective of this research was to evaluate the nitrogen-fixing capacity of 30 strains of *Rhizobium* isolated from white clover nodules, collected in the provinces of Pichincha and Cotopaxi under greenhouse conditions. After isolation of the bacteria and authentication of the isolates, a greenhouse experiment was conducted, in a Completely Randomized Design (CRD), with three replications and two controls: with and without nitrogen fertilization, using sterilized pomine as substrate. Superficially disinfected white clover seeds were germinated *in vitro*, transplanted into pots, and inoculated with the bacteria. Fertilization and irrigation were provided with Jensen's solution. Plants were harvested after 90 days of growth, and data were taken on the number and diameter of the nodules, dry weight and nitrogen content of the shoot. Highly significant differences were detected between *Rhizobium* strains in the five variables evaluated, and with the controls, in dry matter and nitrogen content. A high positive correlation was evidenced between the five variables evaluated. Principal component analysis explained 89.9% of the total variation. It is concluded that seven strains from Pichincha (PI44, PI42, PI41, PI40, PI36, PI34 and PI20) and six from Cotopaxi (CO63, CO60, CO55, CO54, CO49 and CO46), are promising and merit additional studies.

Keywords: Biological Nitrogen Fixation, Nodule Number, *Trifolium repens*, Principal Component Analysis

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Introduction

Nitrogen (N) is essential for all living organisms, as it integrates vital molecules such as amino acids, proteins, nucleic acids, and chlorophyll [1]. In plants, N deficiency manifests as chlorosis, stunting, and, ultimately, reduced development and

yield. N in soil is scarce and less than 2% is found in forms assimilable by plants such as ammonium, nitrates and nitrites [2]. Nitrogen fertilization has allowed the increase of yields and the sustainability of the ever-growing population, but it has also been costly, inefficient and polluting for the environment, since only 50% of the applied N is used by plants [3]. Nitrogen fertilizer is lost by runoff, and can pollute waterways, in addition, it generates emissions of greenhouse gases such as NO and N₂O [4].

Biological Nitrogen Fixation (BNF) contributes more than 60% of N fixed on the planet and is cheaper and more environmentally friendly, therefore, it is the way to sustainable agriculture [1]. BNF by rhizobia of the genus *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, *Mesorhizobium* and *Sinorhizobium*, contributes to nearly 80% of the N required for harvest [5], and it is the major contributor of N for agriculture [1, 6]. Nitrogen fixation rates may reach 400/ha/year [5].

Rhizobia bacteria are Gram-negative, natural inhabitants of soils, and stand out for their ability to establish a mutualistic symbiosis with legumes, through which they fix atmospheric nitrogen (N₂) and convert it into an assimilable form, ammonium. (NH₄⁺) [7]. This symbiosis is facilitated by the presence of specific nodulation (nod) and nitrogen fixation (nif) genes, contained in the symbiotic plasmid (pSym), which allows them to colonize legume roots and form specialized nodules [8]. The establishment of this symbiotic relationship involves a complex molecular dialogue between the plant and the bacterium, initiated in the rhizosphere. Legume roots release phenolic compounds, mainly flavonoids, which activate the expression of nod genes in rhizobia through the NodD regulator [9, 10]. Inside the nodule, an anaerobic environment is created, where the bacteria in the form of a bacteroid fixes N by the action of nitrogenase, encoded by the Nif genes. Since nitrogenase is inactivated by oxygen the involvement of leghemoglobin, encoded in the legume genome, is required to provide oxygen to the bacterium [11].

White clover (*Trifolium repens*) is a perennial forage species of global importance which establishes a symbiotic-mutualistic relationship with *R. leguminosarum* sv. *Trifolii* [12]. When associated with grasses in pastures, white clover prefers the N fixed by symbiosis rather than the N of the soil. This characteristic, together with the transfer of the fixed N to the soil that it performs, makes it a valuable species for reducing the use of industrial fertilizers, both for itself and for other crops. In Ecuador, it is commonly used in mixtures with grasses such as ryegrass (*Lolium perenne* L.) and Kikuyu (*Pennisetum clandestinum*). White clover provides protein, vitamins and minerals to forage, it is very palatable for livestock and has a low fiber content. In addition, it tolerates severe grazing, resists drought, and adapts to different types of soil. Although it originates in Africa, Asia and Europe, it adapts well to cold and humid temperate climates, which has facilitated its adaptation to the conditions of the Ecuadorian highlands [13].

In many countries, BNF is already used in the production of both grain and forage legumes, by inoculating the seeds of these species with efficient strains of *Rhizobium*, thereby reducing production costs and avoiding negative effects of Nitrogen fertilizers on the environment [14, 15]. In Ecuador, there have not been sufficient studies to identify strains of *Rhizobium* efficient in nitrogen fixation that could be used as inoculants for forage legumes. Although rhizobia exist in almost all soils, not all of them have the same level of efficiency, or fix nitrogen; therefore, it is necessary to evaluate and select efficient strains [12, 14], under the Ecuadorian conditions. The introduction of high-efficient foreign rhizobia does not always offer satisfactory results, since their adaptation and persistence are determined by the relationships of competitiveness with rhizobia and other native microorganisms, as well as environmental and soil conditions [13, 14, 16].

This study sought to evaluate, under greenhouse conditions, the nitrogen-fixing capacity of thirty strains of *Rhizobium* isolated from white clover nodules collected in grasslands of the provinces of Pichincha and Cotopaxi. The ultimate goal was to identify the most promising strains, which, after being subjected to subsequent complementary investigations, could eventually be included in inoculant formulations for white clover in Ecuadorian highlands.

Materials and Methods

Sampling Sites

Nodules of white clover of variable ages in the field, were collected in five geo-referenced localities in the province of Pichincha, and in three localities in the province of Cotopaxi (Table 1). White clover plants, showing healthy, and abundant growth were found in commercial pastures, usually in mixtures with ryegrass (*Lolium perenne*) and/or kikuyu (*Pennisetum clandestinum*). Roots of the plants were extracted to a depth of approximately 30 cm, preserving the block of soil that was

attached to them, in a radius of 15 cm around it. Samples with nodules were placed in sacks and assigned an identification code consisting of the two initial letters of the province and an ascending two-digit numbering. Subsequently, the samples were transported to the Plant Biotechnology laboratory of the Faculty of Agricultural Sciences of the Central University of Ecuador, in Quito, for the isolation of the bacteria.

Table 1: Collection sites of white clover nodules from which nitrogen-fixing *Rhizobium* bacteria were isolated for greenhouse evaluation, provinces of Pichincha and Cotopaxi - Ecuador

Province	Canton	Parish	Location	Georeference	No. of samples
Pichincha	Quito	Tumbaco	La Morita	LS0°13'49"; LO78°22'4"	2
	Mejía	Machachi	La Bomba	LS0°29'47.1"; LO78°34'23.3"	6
	Mejía	Machachi	Pedregal	LS0°30'33.6"; LO78°32'49.6"	6
	Cayambe	Cayambe	San José de Ayora	LS0°04'28.1"; LO78°09'08.1"	5
	Cayambe	Cayambe	Tupigachi	LS0°5'34.2"; LO78°10'04.1"	6
Cotopaxi	Salcedo	San Miguel	Quevedo	LS1°03'10.0"; LO78°33'46.7"	1
	Salcedo	San Miguel	Rumipamba	LS1°01'82"; LO78°59'55"	2
	Salcedo	San Miguel	Rumipamba	LS1°01'02.2"; LO78°36'04.4"	1
	Salcedo	San Miguel	Pastocalle	LS0°40'58.8"; LO78°38'02.4"	1

Rhizobia Isolation

After removing the soil from the roots and washing them with tap water, two or three large nodules were extracted, whose interior was pink, indicative of the presence of leghemoglobin and therefore active in nitrogen fixation. Immediately, nodules were superficially disinfected by soaking them in 95% ethanol for 60 seconds, then in 2.5% sodium hypochlorite for five minutes with agitation and five rinses with sterile distilled water for one minute each. The nodules were then macerated with a previously flamed metal rod, in an Eppendorf tube, until a milky liquid was obtained, which was striated on the surface of the culture medium Yeast Mannitol Agar + Congo Red (YMA + CR) with a flamed and cooled bacterial loop. Petri dishes were then sealed with parafilm and incubated in an oven at 28 °C for five days.

Purification, Purity Testing, and Preservation of Isolates

The purification of the isolates was carried out by the striated exhaustion method with a bacterial loop. After incubation at 28°C for five days, an individual colony with typical characteristics of *Rhizobium* was chosen and striated in a new Petri dish with YMA+CR medium.

To verify the absence of contaminants, the isolates were subjected to the following purity tests, following the [17] with minor variations.

Gram stain: from an individual colony, a small portion was taken with a bacterial loop and smeared on a slide containing a drop of sterile distilled water. The bacterium was fixed by heat and then a drop of violet crystal was added for one minute, then a drop of iodine solution for one minute, then washed with 75% ethanol and counterstained with safranin for one minute. The bacterium was washed with sterile distilled water. It was left to dry and observed under a microscope with the immersion lens (100X). Gram-negative bacilli were observed.

Growth in culture medium Peptone Glucose Agar (PGA): An aliquot of an individual colony was striated with the bacterial loop in PGA medium (peptone: 10 g/L, glucose: 5 g/L, agar: 15 g/L), 10.0 ml/L of bromocresol purple 1 % in ethanol, pH 6.8, and incubated at 28°C for five days. No bacterial growth or color change of the culture medium was observed, indicating the absence of contaminants, as *Rhizobium* does not grow in this culture medium [18].

Production of 3-ketoactose in Yeast Lactose Agar (YLA) medium: A portion of a bacterial colony was striated in the YLA medium, pH 6.8, and incubated at 28°C for five days with a bacterial loop. Then, the culture was covered with 10 ml of Benedict's reagent and observed for 10 minutes. The non-appearance of yellow color confirmed the absence of contaminants with *Agrobacterium*, a soil bacterium that also belongs to the Rhizobiaceae family, which produces nodules, but does not fix nitrogen [18]. The isolates that passed the purity and authentication tests continued with the conservation and greenhouse experiment.

Preservation of Isolates

From the previously purified isolates, an individual colony with a flamed and cooled bacterial loop was taken, with which 5 ml of Mannitol Yeast broth pH 6.8 culture medium was inoculated in 12x2 cm test tubes. The tubes were sealed with Parafilm and incubated in an orbital shaker at 200 rpm at 28°C, for three days. In labeled vials or cryotubes, 500 µl of 1x10⁸ CFU of bacterial broth was mixed with 500 µl of sterilized glycerol and stored in the ultra-freezer at -80°C, for long-term preservation.

Evaluation of the Nitrogen-Fixing Capacity of Isolates in the Greenhouse

To evaluate the nodulation and nitrogen fixation ability of the 30 *Rhizobium* isolates, a greenhouse experiment was carried out on white clover plants. Black polyethylene bags of 2 kg were used and were filled with sterile pomine substrate. Pomine is a porous volcanic material composed of silica and alumina, it is completely inert and therefore provides no nutrients, including nitrogen. This material was chosen to create a controlled environment that allows observation of the strains' performance without external interference. It also promotes drainage that favors root development. The pomine was obtained from a local supplier who extracts this material from the igneous sediments of the volcanic foothills of the mountain range. It was then sieved through a 4x4 mesh screen, washed with running water, and encased and sealed in polyfan bags and sterilized in an autoclave at 121°C for 4 hours. Inside the laminar flow cabinet, white clover seeds were superficially disinfected, successively immersed in 95% ethyl alcohol for 60 seconds, then in 2.5% sodium hypochlorite for five minutes with stirring, and five rinses with sterile distilled water for one minute each. The seeds were then placed inside Petri dishes containing moistened and previously sterilized napkins. The Petri dishes were sealed with parafilm and incubated in a Memmert oven at 24°C, until germination.

The greenhouse experiment was arranged in a completely randomized design with three replications. A total of 96 experimental units (black plastic bags with pomine substrate) were used: 30 strains + 2 controls (with and without nitrogen fertilization) with three replications.

Prior to transplanting, 30 g of sterilized humus was added to give initial support to the seeds. The substrate was then moistened with Jensen's nutrient solution [17]. The transplant was carried out using a sterilized forceps, placing four pre-germinated seeds of white clover in each bag, forming a cross. Inoculation was performed when the seedlings were 7 days old transplanted [17]. With a micropipette, 1000 µl of bacterial broth 1x10⁸ CFU mL⁻¹ was placed at the base of each seedling, according to the McFarland curve, determined with a spectrophotometer (Jasco, model V-630). The uninoculated controls were given 1 ml of sterile distilled water. Fertilization of the nitrogen control was carried out by adding 50 ml of potassium nitrate solution (KNO₃), 0.75 g/L to each plant [17]. The experiment remained for three months in the greenhouse, at 24 °C and a photoperiod of 12/12 hours light dark, and irrigation was applied every day, 100 ml of distilled water + Jensen solution (10 L of distilled water + 50 ml of Jensen solution), all sterilized.

At the time of harvest, three months after transplanting, the number, diameter, internal color and distribution of the nodules formed on the roots of the white clover plants were determined (the diameter and internal color were determined in 10 nodules taken at random, the diameter was measured with a Vernier). In addition, the dry weight of the shoot (dried in an oven at 65 °C for 24 hours [19] and the N content of the aerial part of the plants were evaluated by the Kjeldahl method [20, 21] in a steam distiller Selecta, Pro-Nitro M model, Barcelona, Spain), using 0.2 g of dry and ground tissue.

Statistical Analysis

The statistical analysis consisted of an analysis of variance, Tukey mean separation tests at 5%, correlation analysis and a multivariate principal component analysis. The normal distribution of data was analyzed using the Shapiro Wilk test and the homogeneity of variances with the Levene test. Additionally, a non-parametric Kruskal Wallis test with Dwass Steel Critchlow Fligner multiple comparisons was performed to address the unequal group sizes between the Cotopaxi and Pichincha strains. Statistical analyses were done in [22], and principal components analysis in InfoStat version 2008 [23].

Results

Highly significant differences ($p < 0.01$) were detected between *Rhizobium* strains in the five quantified variables (Table 2). When comparing the strains from the two localities, Cotopaxi and Pichincha, from which the nodules were collected, significant differences ($p < 0.05$) were observed in the number and diameter of nodules, highly significant differences ($p < 0.01$) in shoot nitrogen content (%) and amount of nitrogen per plant (mg) and No Significant differences (NS) in shoot dry weight. The non-

parametric Kruskal Wallis test with Dwass Steel Critchlow Fligner multiple comparisons, performed to address the unequal group sizes between the Cotopaxi and Pichincha strains, did not detect statistically significant differences among strains from the two localities.

Table 2: Analysis of variance of the five quantitative variables evaluated

Source	DF	Nitrogen Percentage		Nitrogen mg		Dry shoot weight		Number of Nodules		Diameter of Nodules	
		F Value	Pr > F	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F	F Value	Pr > F
Strain	31	13.06	<.0001	549.13	<0.0001	37.03	<.0001	63.11	<.0001	1315.81	<.0001
Error	64						Pr >F	60			
Total	95							89			

Number of Nodules

All the strains inoculated in white clover formed nodules in the roots of the legume. The average number of nodules per plant (Figure 1) ranged from 6.33 in the PI22 strain, as the least nodulating, to 297.00 in the PI38 strain, as the highest nodulating strain. Except for the PI08 (87.67 nodules) and PI22 strains, all other strains had more than 100 nodules per plant. The number of nodules of strains collected in Cotopaxi varied from 242.33 (CO55 strain) to 276.33 (CO46 strain). On average, the Cotopaxi strains with 254.05 nodules outperformed the Pichincha strains with 215.90 nodules ($P<0.05$). The absolute and N-fertilized controls did not form nodules.

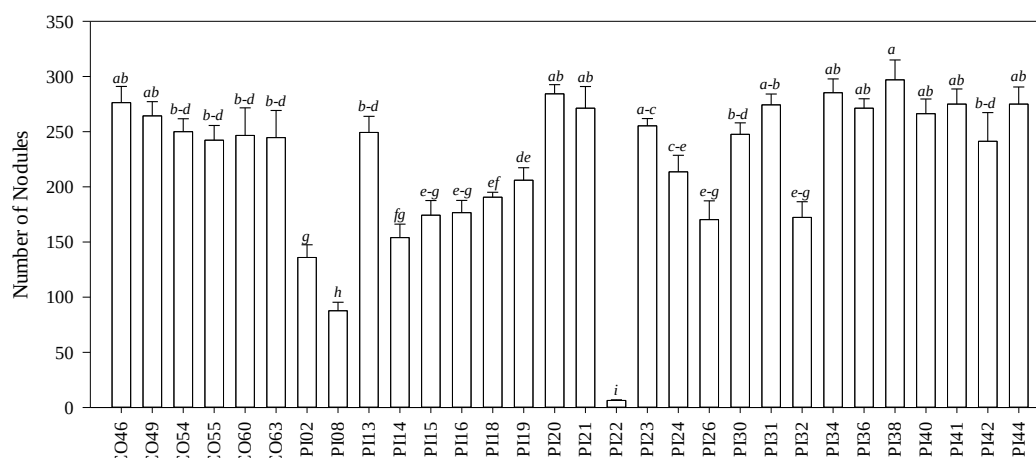


Fig. 1: Average number of nodules of 90-day-old white clover plants grown in greenhouses and inoculated with *Rhizobium* strains collected in grasslands of Pichincha and Cotopaxi. The results are presented as mean + SD, significant differences at $p<0.01$ % (*) (ANOVA, Tukey's test)

Nodule Diameter

The diameter of the nodules (Figure 2) ranged from 1.27 mm in the PI22 strain to 3.94 mm in the PI26 strain. The Cotopaxi strains had nodule diameter values between 3.27 mm (CO63 strain) and 3.15 mm (CO60 strain). On average, the Cotopaxi strains with 3.22 mm outnumbered those from Pichincha with 2.93 mm, in the diameter of the nodules ($P<0.05$).

Dry Shoot Weight

The dry weight of the shoot (Figure 3) ranged from 4.00 g in the PI24 strain to 0.30 g in the PI22 strain. Except for the PI22 strain, all other strains outperformed absolute (0.4 g) and N-fertilized (1.7 g) controls in terms of shoot dry weight. Among the Cotopaxi strains, the CO54 strain had the highest dry weight of the shoot with 3.57 g, while the lowest weight corresponded to the CO46 strain with 3.33 g. On average, the Cotopaxi and Pichincha strains did not exhibit significant differences ($P>0.05$) in terms of the dry weight of the shoot.

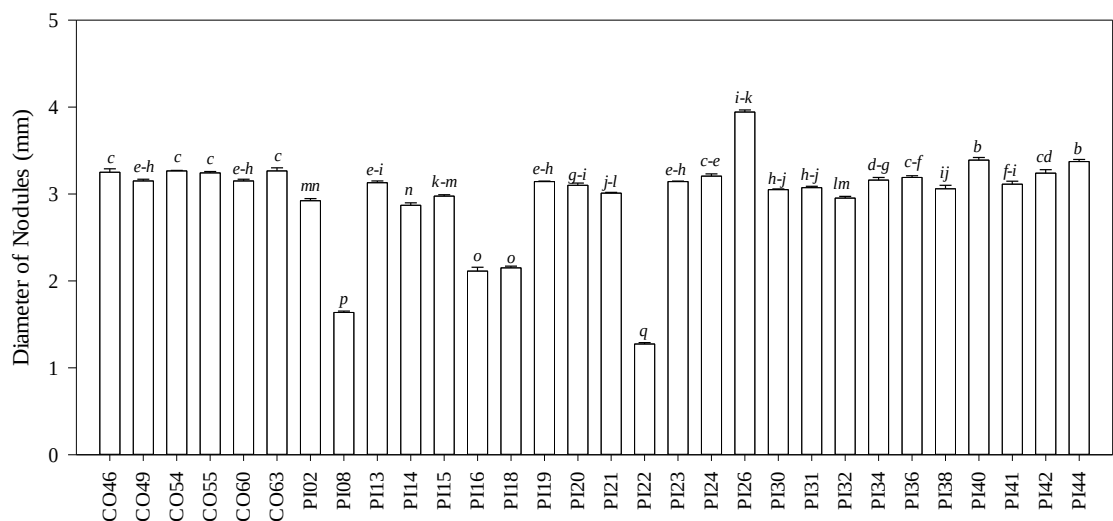


Fig. 2: Average diameter of nodules of 90-day-old white clover plants grown in greenhouses and inoculated with *Rhizobium* strains collected in grasslands of Pichincha and Cotopaxi. The results are presented as mean + SD, significant differences at $p < 0.01$ % (*) (ANOVA, Tukey's test)

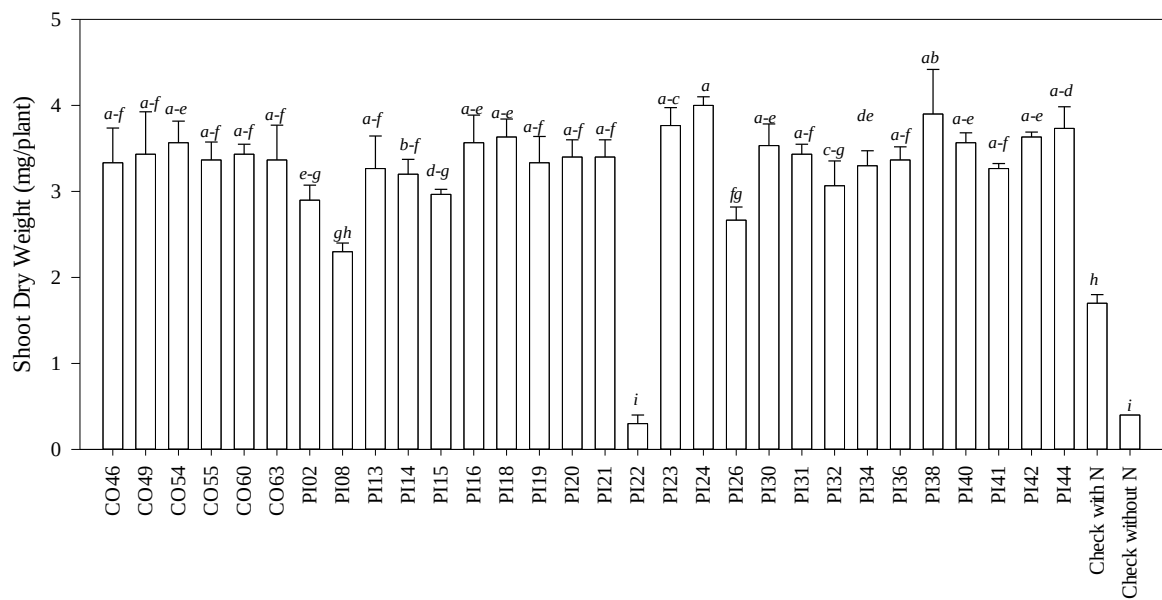


Fig. 3: Dry weight of the shoot of 90-day-old white clover plants grown in a greenhouse and inoculated with *Rhizobium* strains collected in grasslands of Pichincha and Cotopaxi. Results are presented as mean+SD, significant differences at $p < 0.01$ % (*) (ANOVA, Tukey's test)

Nitrogen Percentage

The percentage of nitrogen (%) (Figure 4) ranged from 3.9% in the PI40 strain to 0.97% in the PI22 strain. All strains, except the PI22 strain, exceeded the absolute control (2.7%), while the nitrogenous control (2.9%) exceeded the PI38 (2.8%), PI08 (2.77%) and PI14 (2.73%) strains. The Cotopaxi strains had a range of values from 3.80% for the CO63 strain to 3.33% for the CO60 strain. On average, the Cotopaxi strains outperformed those from Pichincha ($p < 0.05$).

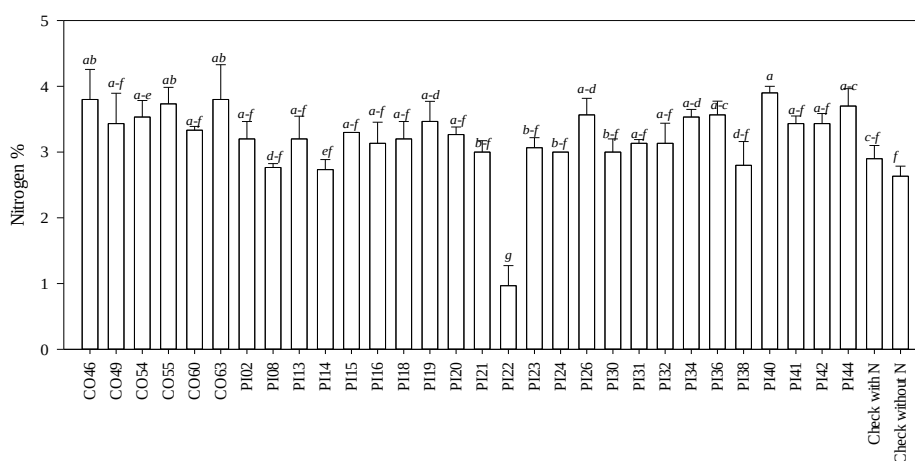


Fig. 4: Shoot nitrogen content (%) of 90-day-old white clover plants grown in a greenhouse and inoculated with *Rhizobium* strains collected in grasslands of Pichincha and Cotopaxi. The results are presented as mean + SD, significant differences at $p < 0.01$ % (*) (ANOVA, Tukey's test)

Amount of Nitrogen Per Plant

The amount of nitrogen per plant (Figure 5) ranged from 139.50 mg in the PI40 strain to 2.73 mg in the PI22 strain. All strains, except PI22, outperformed absolute (10.87 mg) and N-fertilized (49.13 mg) controls. Cotopaxi strains ranged in the amount of nitrogen per plant from 127.23 mg to 115.20 mg. On average, the Cotopaxi strains (122.46 mg) were superior ($p < 0.01$) to those from Pichincha (104.08 mg), in terms of the amount of nitrogen per plant.

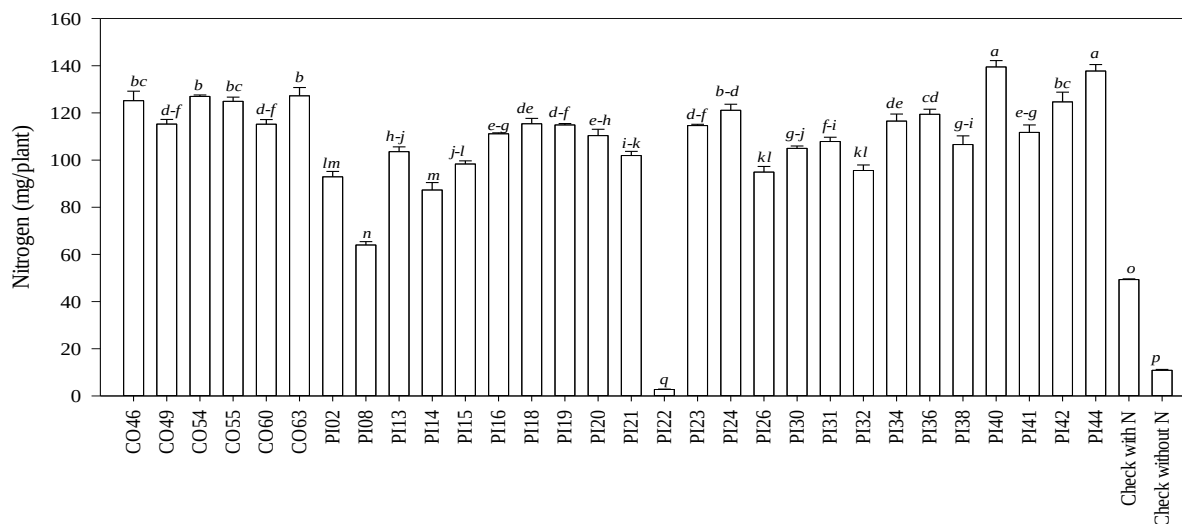


Fig. 5: Amount of nitrogen in the shoot in mg of 90-day-old white clover plants grown in a greenhouse and inoculated with *Rhizobium* strains collected in grasslands of Pichincha and Cotopaxi. The results are presented as mean + SD, significant differences at $p < 0.01$ % (*) (ANOVA, Tukey's test)

Internal Color and Distribution of Nodules in the Root System of White Clover

In all the *Rhizobium* strains evaluated in this research, pink to red nodules were present, except for the PI22 strain whose nodules were white. Nodules were mainly distributed in the secondary roots in all strains.

Correlations Between Quantitative Variables

The five quantitative variables evaluated were positively correlated with each other, with the highest index among the variables shoot dry weight vs. amount of nitrogen per plant ($r = 0.86$, $p < 0.0001$) and the lowest index between shoot dry weight vs. total nitrogen % ($r = 0.52$, $p < 0.0001$) (Table 3).

Table 3: Correlation indices between quantitative variables in the evaluation of *Rhizobium* strains collected in the provinces of Pichincha and Cotopaxi

Variable	Nodule number	Nodule diameter	Shoot dry weight	Nitrogen mg	Nitrogen %
Nodule number	1				
Nodule diameter	0.70498 <.0001	1			
Shoot dry weight	0.75018 <.0001	0.59306 <.0001	1		
Nitrogen mg	0.81210 <.0001	0.72993 <.0001	0.86223 <.0001	1	
Nitrogen %	0.63025 <.0001	0.69943 <.0001	0.52382 <.0001	0.84085 <.0001	1

Principal Component Analysis

The analysis of the principal components (Figure 6) of the 30 *Rhizobium* strains and the five variables manages to explain 89.9% of the total variability, where CP1 contributes 81.3% and CP2 contributes 8.6%. When observing the CP1 values, the strains were separated into two groups. On the left side and with the lowest values were located eight strains from Pichincha and on the right side and with the highest values were located the rest of the strains from Pichincha and the six strains from Cotopaxi. The variables with the highest contribution to CP1 were amount of nitrogen per plant (mg) and dry weight of the shoot, while the variables that contributed the most to the CP2 were diameter of nodules and dry weight of the shoot. It is also observed that on the right side of the graph are located the four vector-variables, and by the angle they form it can be assumed that they are positively correlated, agreeing with the correlation analysis.

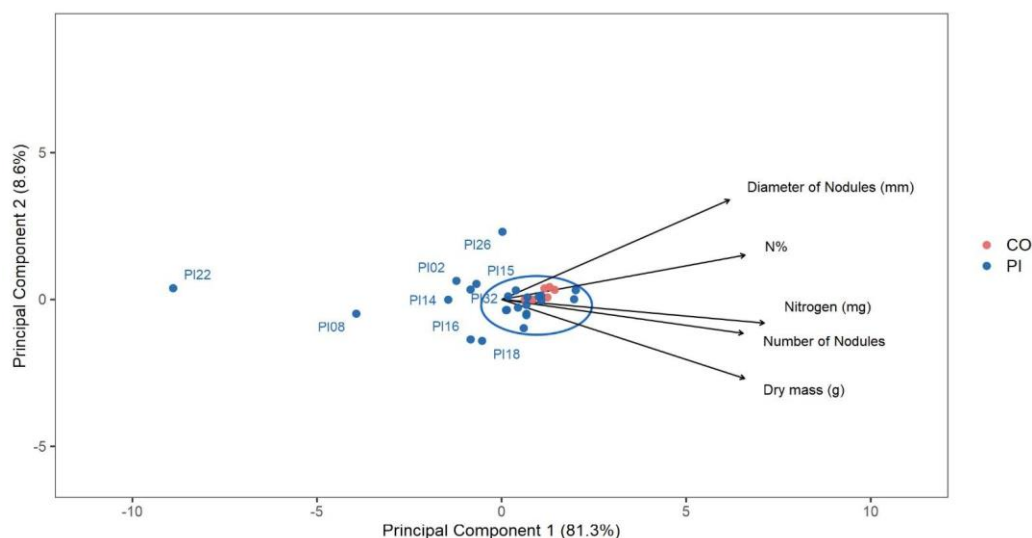


Fig. 6: Principal component analysis of the five quantitative variables analyzed: number of nodules, diameter of nodules, dry shoot biomass and nitrogen content in percentage and in milligrams of white clover plants inoculated with *Rhizobium* strains collected in Pichincha and Cotopaxi

Discussion

Ability of *Rhizobium* Strains to form Nodules on White Clover Roots

In this research, the nodulation capacity of *Rhizobium* strains in the roots of white clover plants was assessed by counting the nodules, measuring their diameter and determining the N-fixing ability. Results showed a considerable variability in

effectiveness of symbiosis and significant differences in all variables tested. Our results indicated that the PI22 strain was ineffective, and overall the study demonstrated considerable heterogeneity when selecting strains for BNF.

The formation of functional nodules depends on genetic-molecular factors of the plant and bacteria; therefore, this process only occurs between compatible and specific organisms [24, 25]. Consequently, the number and size of the nodules are important indicators to evaluate the nodulating ability of the bacterium in interaction with the legume [26]. Several studies characterizing the nitrogen-fixing efficiency of *Rhizobium*-legume systems have used these evaluation parameters [12, 26, 27].

The average number of nodules varied widely among the different strains (6.33 to 297.00), while the diameter varied somewhat less (1.27 to 3.94 mm); the nodules were mainly distributed in the secondary roots. As for the internal color, they were all pink or red, which indicated that they were effective in nitrogen fixation [28], except for PI22. The two controls, with and without nitrogen fertilization, did not present nodules.

The highly significant differences (ANOVA and Tukey, $p < 0.01$), detected between the strains in relation to the number and diameter of the nodules, suggest the existence of a level of variability in the nodulating capacity of the bacteria under study and the importance of selecting strains with greater nodulating efficiency [12].

Three strains from Pichincha [PI38 (297 nodules), PI34 (285 nodules) and PI20 (284 nodules)] and one from Cotopaxi [CO46 (276 nodules)] were the ones with the highest number of nodules, while the PI22 strain (6.33 nodules) was the one that formed the lowest number of nodules. Regarding the diameter, the PI26 strain (3.94 mm) and the PI22 strain (1.27 mm) had the largest and smallest diameter nodules, respectively.

[29, 30] indicate that the number of nodules by itself does not necessarily have a direct relationship with the nitrogen fixation capacity of bacteria, since it may happen that strains that are inefficient in nitrogen fixation are highly nodulating, with a predominance of small and white nodules. [31] consider that an optimal number of nodules, which would depend on the species and the nutritional condition of the plant, is associated with efficient nitrogen fixation. Since this is a highly energy-demanding process, legumes would regulate the number and size of the nodules that they could support without negatively affecting their growth and development [32]. In our case, a positive correlation was found between the number of nodules and nitrogen content, both in percentage ($r = 0.63$) and in amount per plant ($r = 0.81$), and, as indicated above, the nodules were pink or red. In addition, in principal component analysis, the number of nodules contributes significantly to explaining the observed variability. [27] found that in one *Rhizobium* strain evaluated, no correlation was seen between nodulation and nitrogen fixation efficiency in a trial similar to this one but in beans and concluded that additional data is required to select efficient strains.

Additionally, there are reports that legumes have developed the ability to defend themselves against infection by inefficient rhizobia; however, this property would be relaxed in modern cultivars, because of the genetic improvement, to which the species have been subjected without considering this characteristic of nodulation [33].

In relation to the size of the nodules, [31], consider that the regular ones have a higher capacity to fix atmospheric nitrogen. It is important to note that excessive size can present problems, as overly large nodules may have less efficient areas due to an uneven distribution of rhizobia, reducing the overall efficiency of the nodule. In addition, the larger size implies greater demands on plant resources, such as carbohydrates and nutrients, to maintain the metabolic activity of the rhizobia [32]. In this research, a positive correlation was found between the diameter of the nodules and the nitrogen content of the shoot ($r = 0.71$) and per plant ($r = 0.73$). In principal component analysis, nodule diameter contributes to observed variability.

[16, 34] mention the existence of mechanisms in leguminous plants to privilege efficient nodules in BNF and impose "sanctions" on inefficient ones, through the asymmetric distribution of photosynthates, favoring the development of the most efficient.

Plant genes such as Too Much Love (TML) and peptides such as CLE play crucial roles in the regulation of nodulation, ensuring an optimal number and size of nodules to maximize nitrogen fixation efficiency without compromising plant health [35].

Environmental factors, such as soil quality and nutrient availability also play a crucial role in regulating nodulation. Under phosphate deficiency conditions, genes such as PHR1 limit the number of nodules to optimize resource use. Similarly, in the presence of nitrate, peptides such as NIC1/CLE35 inhibit nodulation by signaling through specific receptors such as NARK.

This interaction between nutritional cues and self-regulation of nodulation (AON) demonstrates how plants adjust nodule formation in response to their nutritional status [32].

In addition, regulatory factors such as cytokinins and auxins, which are regulated by NOD factors, play crucial roles in the expansion of the nodule. The SUNN gene in *Medicago truncatula*, for example, regulates both the number and size of nodules, interacting with NOD factors and hormonal components to maintain a balance in nodular development [36].

Nitrogen Fixing Efficiency of the Strains Evaluated

To determine the nitrogen-fixing efficiency of the evaluated strains, the dry weight and nitrogen content of the shoot of the white clover plants inoculated with them were analyzed [12, 37], measuring nitrogen content both in percentage and quantity per plant [26]. This is justified given that the Legume-*Rhizobium* symbiosis has a direct impact on dry weight and N accumulation [36]. In addition, legumes tend to grow more vigorously and produce higher biomass, due to the constant supply of N necessary for the synthesis of proteins and enzymes, key elements in cell growth and expansion [38]. Thus, the dry weight and nitrogen content of the shoot reflect the efficiency of the symbiotic process within legume nodules [12].

Both the dry weight of the shoot, as well as the nitrogen content (%) and the amount of nitrogen per plant (mg), had highly significant differences between the strains evaluated ($p < 0.01$). In dry weight of the shoot, the PI24 strain (4.0 g/plant) and the PI38 strain (3.9 g/plant) stand out as the most efficient, while the PI22 strain appears as the least efficient with values close to absolute control (0.3 g/plant).

In relation to the amount of nitrogen per plant (mg) (Figure 5) and nitrogen content (%) (Figure 4), the PI40 strain stands out with a value of 139.50 mg/plant and 3.9% respectively, which classifies it as highly efficient compared to the PI22 strain, which only presented 2.73 mg/plant and 0.97%, and the absolute control with 10.87 mg/plant and 2.7%. Consequently, the PI40 strain has a superior ability to fix nitrogen and improve plant nutrition than the PI22 strain, which shows significantly low efficiency.

Our results indicate that, under greenhouse conditions, the plants inoculated with the strains evaluated in white clover achieved a great mean dry weight of the shoot of 3.27 g, 107.76 mg of nitrogen per plant and 3.24% of nitrogen in the shoot; the latter, higher than the average reported for white clover of 2.45 % [12]. [39, 40] estimate that the efficiency of symbiosis in N fixation is a function of host genotype, *Rhizobium* strain genotype, and environmental factors, such as soil fertility, type and intensity of fertilization, and climate. Within soil fertility, nitrogen availability is crucial to establishing the symbiosis between legumes and rhizobia. Legumes use rhizobia in soils with low or no assimilable nitrogen, underlining the importance of soil conditions in the effectiveness of nitrogen fixation and the need for strategies adapted to different environmental contexts [41].

Another aspect to highlight from this study is the positive correlation found between the dry weight of the shoot and the nitrogen fixation indices: total nitrogen (%) ($r = 0.52$) and the amount of nitrogen per plant ($r = 0.86$), which was also observed by [42] (96%) in a field study and [43] in a review of several studies on biological nitrogen fixation in perennial forage legumes, including clover, where dry matter and nitrogen fixation were positively correlated ($p < 0.001$). In the principal component analysis, it is seen that dry matter and nitrogen content are important contributors to variability in the CP1 axis.

On the other hand, the poor performance of strain PI22 is noteworthy. It exhibits small, white nodules and plant performance values close to those of absolute control, thus qualifying it as inefficient. Considering theories of host-imposed sanctions and defenses relaxation [33, 34], this could be interpreted as PI22 being capable of initiating infection, indicating that immune suppression is sufficient for nodulation. However, the nodules are unable to establish effective nitrogen fixation, as evidenced by their white color and reduced plant growth. This poor performance of the symbiosis is consistent with either colonization by ineffective *Rhizobium* strains or unsuitable environmental conditions that would impair nitrogenase activity. Given the low nitrogen delivery by the nodules, the plant would likely initiate sanctioning mechanisms, reducing the supply of carbon and oxygen to these inefficient nodules and thus limiting their symbiotic activity.

Additionally, in a study evaluating the effect of *Rhizobium* strains on the growth of *Brassica napus* under salinity stress, two promising strains significantly increased chlorophyll content (SPAD index), leaf area, relative water content, shoot biomass, and dry weight compared with other strains and the non-inoculated control. The authors attributed these improvements to the strains' 1-Aminocyclopropane-1-Carboxylate (ACC) deaminase activity and indole-3-acetic acid (IAA) production, which enhanced plant tolerance to salinity stress. These mechanisms may also explain the superior performance of the most effective strains identified in the present study [44].

Effects of locality

In the five variables evaluated, the six Cotopaxi strains exhibit a significantly higher overall average than the average of the 24 Pichincha strains ($p < 0.05$). A first reason must be related to the number of strains in each locality (6 vs 24). A second reason may be related to the extent of the area in which the strains were collected. The Cotopaxi strains all belong to the Salcedo canton, while those of Pichincha correspond to three cantons: Quito, Mejía and Cayambe, which means greater extension and therefore greater variability of climate and soil, which can modulate the strains that adapt to each place. Since no soil analysis data are available for the collection sites, no definitive conclusions can be drawn in this regard. To elucidate genotype variations, a corresponding molecular analysis should be performed.

Given the unequal sample sizes between the Cotopaxi ($n = 6$) and Pichincha ($n = 24$) strains, a non-parametric Kruskal Wallis test followed by Dwass Steel Critchlow Fligner multiple comparisons was performed as a complementary analysis to assess the robustness of the regional comparisons. Unlike the one-way ANOVA followed by Tukey's HSD, this approach did not detect statistically significant differences among strains, indicating that the apparent differences observed in the parametric analysis should be interpreted with caution when accounting for the imbalance in sample size and the potential influence of distributional assumptions. Nevertheless, one strain consistently exhibited a markedly distinct physiological profile, with substantially different values across the variables evaluated, suggesting that this response may represent a biologically relevant phenotype rather than solely a statistical artifact. Therefore, although the non-parametric analysis provides a more conservative assessment and does not support statistically significant differences among the strains, the consistency and magnitude of the response observed for this particular strain warrant further investigation using balanced sampling and larger numbers of strains per geographic region.

Principal Component Analysis

Principal component analysis (Figure 6) detected a close relationship between the number of nodules, the amount of nitrogen per plant and the dry weight of the shoot, and between the diameter of the nodules and the percentage of nitrogen in the shoot. The two components, CP1 and CP2, add up to 89.9%, which corresponds to the variation explained by the two axes. This could be interpreted in the sense that the number and diameter of nodules observed in this research correspond to the appropriate values for the species *T. repens* [31] and therefore are efficient nodules for biological nitrogen fixation. The presence of leghemoglobin inside the nodules, which is an indication of active nitrogen fixation [28, 31], would corroborate the efficiency of the nodules in N fixation.

Additionally, the greater development and intense green color of the foliage of the white clover plants inoculated with the strains that stood out in this research, in contrast to the control not fertilized with nitrogen, would indicate a healthy state for the former and therefore an adequate supply of nitrogen fixed by the Legume-*Rhizobium* symbiosis (Fig. 7). Coincidentally, [12], found that the fresh weight and nitrogen percentage of dry shoot are the parameters that best show the effect of *Rhizobium* inoculation on plant growth and productivity. [43] found a positive correlation between the dry matter of *T. repens* and the percentage of atmospheric N fixed by the legume symbiosis *Rhizobium*.



Fig. 7: White clover plants inoculated with *Rhizobium* strains collected in Pichincha and Cotopaxi. From left to right: plant inoculated with the CO60 strain; control fertilized with KNO₃: 0.75 g L⁻¹, and control not fertilized with nitrogen

A limitation of this study is that it was conducted under greenhouse conditions; therefore, validation of the selected promising strains is necessary under field conditions and at different locations. Another limitation is that the chemical characterization of the soils from the collection sites is not available, as it was not planned at the beginning of the study. Such information may provide insights into the competitiveness and persistence of the strains in situ. This limitation will be addressed in the subsequent stages of the research. Finally, molecular analysis, including sequencing of the 16S rRNA gene and/or constitutive genes necessary for specific strain identification is underway, and will be concluded before strain validation field experiments.

Conclusion

Of the 30 strains of *Rhizobium* collected in pastures in the provinces of Pichincha and Cotopaxi and evaluated in greenhouses to determine their nitrogen-fixing capacity, seven strains of Pichincha (PI44, PI42, PI41, PI40, PI36, PI34 and PI20) and the six of Cotopaxi (CO63, CO60, CO55, CO54, CO49 and CO46) stood out in relation to the others, based on the five variables analyzed.

Significant differences were found between the *Rhizobium* strains evaluated and with respect to the nitrogen-fertilized or non-nitrogen controls, in the five variables analyzed, number and diameter of nodules, dry weight and nitrogen content of the shoot.

These results show the need to carry out studies of native strains to determine their nitrogen-fixing capacity and their adaptability to different environments to use them as inoculants in pastures.

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Author's Contributions

Daniela Michell Chuquimarca Mamarandi: Conducted the investigation, collected and organized the data, and performed the literature review.

Venancio Saúl Arahana Bonilla: Conceptualization, interpretation of results, preparation of the original draft, and review and editing of the manuscript.

Paulina Andrea Cevallos Hidalgo: Research supervision and validation, review and editing of the manuscript, and correspondence with the journal.

Aníbal Pozo Bonilla: Project administration, methodological development, supervision, technical evaluation, interpretation of results, and critical review of the manuscript.

Mayra Alejandra Guamán Rodríguez: Literature review, preparation of tables and figures, and drafting of the original manuscript.

Francisco Adolfo Gutiérrez León: Sample collection and preparation, nitrogen content analysis, interpretation and validation of the results.

Carlos Andrés Bolaños Carriel: Statistical analysis, interpretation and validation of the results, critical review of the manuscript, and approval of the final version.

Ethics

The authors acknowledge their responsibility to address any ethical issues that may arise after the publication of this manuscript.

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