



## Underground biomass of perennial ryegrass with different nitrogenous sources and associated with white clover and red clover



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### Abstract:

This research aimed to evaluate the root biomass of perennial ryegrass (R) (*Lolium perenne*) with different nitrogenous sources and associations with white clover (Wc) (*Trifolium repens*) and red clover (Rc) (*Trifolium pratense*), as well as the nodulation capacity of white or red clover. Two experiments were conducted: (Exp1) monoculture of perennial ryegrass, fertilized with nitrogen (urea, slow-release urea, ammonium nitrate, foliar nitrogen, ammonium nitrate + foliar nitrogen, and a control or without application), and (Exp 2) mixtures of ryegrass with white or red clover, in low and high density, and a control. They were replicated in 2 localities: Tumbaco (Tm) and Machachi (Mc), in Pichincha, Ecuador. The statistical design was a randomized block design (n=3), and a soil analysis was performed at the beginning. Root biomass (Rb) was evaluated on a dry basis in the 2 experiments; for its part, in Exp 2, the number (Nn) and weight of nodules (Nw) were also evaluated. The results indicate that species modify their root growth as an adaptation to environmental conditions. In Exp 1 in Tm, where there was a higher temperature, there was a higher Rb. In Exp 2, the mixtures of R and Rc accumulated greater root biomass. For Nn

and Nw, Wc had the highest values. Based on these results, it can be concluded that the environment plays a preponderant role in root biomass and that species adapt by modifying this trait and the number and dry weight of nodules.

**Keywords:** Nodulation, Roots, Adaptation, Environment.

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The shoots and roots have complementary functions, as the aerial stem harvests radiant energy from the sun, while the roots absorb mineral nutrients and water<sup>(1)</sup>. According to Ramos-Hernández and Martínez-Sánchez<sup>(2)</sup>, underground biomass is usually equal to or greater than aboveground biomass. Poorter and Nagel<sup>(3)</sup> state that, in the face of low nutrient availability, the root has priority in the allocation of carbohydrates over the aerial part. Likewise, the physical, chemical, and biological properties of soils play an essential role in root growth<sup>(4)</sup>. Root systems allow plants to grow and nourish themselves, so they are decisive in the yield of agricultural crops<sup>(5)</sup>. Nevertheless, it is argued<sup>(6)</sup> that, in forage plants, the growth and production of roots is much less studied and understood than that of leaves.

Roots have intense biological activity; among the most important is the symbiotic association between members of the family Leguminosae and soil diazotrophic bacteria (rhizobia), which are well known for their ability to fix atmospheric nitrogen<sup>(7)</sup>. Legumes, therefore, play a key role in the sustainable intensification of agricultural systems, improving biodiversity and ecosystem services, and reducing the dependence of food production on nitrogen fertilizers<sup>(8)</sup>. For this reason, forage legumes have been selected for their nodulation capacity, with white clover developing the greatest capacity<sup>(9)</sup>. Nonetheless, N fixation varies greatly depending on rhizobia; there are several factors that determine the success of the legume-rhizobia symbiotic relationship<sup>(10)</sup>, one of the most important being to match suitable symbionts with the host plants<sup>(11)</sup>.

Therefore, the objective of this research was to assess the root biomass of perennial ryegrass (*Lolium perenne* L.) as a monoculture, with different nitrogenous sources and in mixtures with white clover (*Trifolium repens*) and red clover (*Trifolium pratense*), as well as to evaluate the nodulation capacity of white or red clovers in two contrasting localities.

This research was conducted in Ecuador in the province of Pichincha in two experimental sites, in the parishes of Machachi, Canton Mejía, and Tumbaco, Canton Quito.

According to INEC's<sup>(12)</sup> agricultural census, pastures occupy 58.3 % of the area subjected to agricultural work; therefore, livestock production is carried out in a pastoral system. It is also mentioned that 79.5 % of milk production is from the Sierra region, and in it, the province of Pichincha covers 18 % of the total, being the province with the highest national production.

The climate in the province of Pichincha varies according to altitude; the climate of the locality of Tumbaco is classified as dry mesothermal, while that of Machachi as humid mesothermal<sup>(13)</sup>. Machachi is located at 0°30'36.4" S; 78°34'1.6" W, and has a precipitation of 1,043 mm and an average temperature of 12 °C; for its part, Quito is situated at 0°13'52.5" S; 78°11'0.3" W, and has a precipitation of 954 mm and an average temperature of 16 °C.

The origin of soils in the inter-Andean region is volcanic, and climate is the most critical factor in their development, so the specific environmental conditions of each site have given rise to different processes that have influenced soil formation<sup>(14)</sup>. Based on this, soil samples were taken from each locality, in which the following were determined: organic carbon (by wet combustion, Walkley-Black Method), pH (with a potentiometer, in aqueous solution), electrical conductivity (using a conductivity meter with a saturated paste extract), total N (by calculation based on the percentage of organic matter), available P (with a photolorimeter, modified Olsen Method), and exchangeable cations (K, Ca, Mg; atomic absorption spectrophotometry) (Table 1).

**Table 1:** Physicochemical characteristics of the soils of the experimental sites

Locality	pH	EC (dS/m)	SOC (%)	SOM	Total N	P (ppm)	K (cmol/kg)	Ca	Mg
Machachi	7.56	0.56	4.05	7.67	0.38	73.5	1.02	16.46	2.7
Tumbaco	6.7	0.59	1.56	2.95	0.15	31.4	1.5	9.79	4.72

EC= electrical conductivity; SOC= soil organic carbon; SOM= soil organic matter; dS= deciSiemens; ppm= parts per million; cmol= centimoles.

**Experiment 1:** Effect of different nitrogen sources on perennial ryegrass (R) monoculture. The following was used: a randomized block design (n=3, plots of 7 x 4 m), 30 kg ha<sup>-1</sup> as planting density, and fertilization with nitrogen sources with the sources specified in Table 2.

**Table 2:** Type and concentration of fertilizers used in perennial ryegrass monoculture in the two experimental sites

Treatment	Fertilizer	kg N ha <sup>-1</sup> cut <sup>-1</sup>	kg fertilizer ha <sup>-1</sup> cut <sup>-1</sup>	Chemical composition
T0	No fertilizer	0	0	Control
T1	Urea	20	43.5	46 % N, urea
T2	Protected urea	20	50	40 % N, PSCU-coated urea <sup>(1)</sup>
T3	Ammonium nitrate	20	59.7	33.5 % N, 50 % in nitric form and 50 % in ammoniacal form
T4	Ammonium nitrate + foliar N	20	61-2	33.5 % N, 50 % in nitric form and 50 % in ammoniacal form, + 18.7 % w/v of calcium oxide; 25.5 % of w/v of N
T5	Foliar N	0.3	1.5	18.7 % w/v of calcium oxide; 25.5 % of w/v of N

(1)= sulfur-rich polymer that allows a controlled release of urea, limiting leaching.

**Experiment 2:** Effect of forage mixtures, ryegrass with white clover and red clover at different planting densities. The following was used: a randomized block design (n=3, plots of 7 x 4 m) and mixtures of grasses and legumes, as detailed in Table 3. These two clover species were selected for their different growth habits: red clover has more erect growth, whereas white clover is more creeping. The clovers were not inoculated with bacteria of the genus *Rhizobium*, as this is not a common practice in Ecuador, since there are native strains, but they have been little studied<sup>(15)</sup>. Clover planting densities were adjusted according to León *et al*<sup>(16)</sup>, who consider that they should range between 2.5 and 5 kg per hectare.

**Table 3:** Forage species used in experiment 2 and their planting densities

Treatments	Species	Grasses (kg ha <sup>-1</sup> )	Legumes (kg ha <sup>-1</sup> )
T0	Perennial ryegrass (control)	30	0
T1	Perennial ryegrass + white clover	25	2.5
T2	Perennial ryegrass + white clover	25	5
T3	Perennial ryegrass + red clover	25	2.5
T4	Perennial ryegrass + red clover	25	5
T5	Perennial ryegrass + white clover + red clover	25	5 (2.5 + 2.5)

Based on the information provided by a tensiometer, the experimental plots of both experiments were maintained at field capacity through the use of a sprinkler irrigation system. At the beginning of the experiments, fertilization was carried out with 80 kg P ha<sup>-1</sup> (triple superphosphate plus microelements) and 60 kg K ha<sup>-1</sup> (potassium muriate). Both experiments were conducted at each experimental site, and they were planted in August 2021 using the broadcast seeding method. During 2022, the aerial growth of the experiments in the two localities was evaluated monthly, and the underground biomass was assessed in February 2023.

Root biomass (g DM L<sup>-1</sup> soil): Six random samples were taken from each treatment and washed in plastic bags with 3 mm diamond-type perforations, roots were manually separated from the soil and dried with filter paper, the total weight of the roots was determined without separation by crop (fresh weight), and finally they were dehydrated in a forced-air oven at 70 °C for 24 h; they were weighed, and the percentage of dry matter was calculated<sup>(17)</sup>.

Number of nodules (No. of nodules L<sup>-1</sup> soil): Six samples were taken from each treatment, with red or white clover, and the number of nodules was determined by manually separating the roots and nodules<sup>(18)</sup>.

Nodule weight (mg DM L<sup>-1</sup> soil): Six samples were taken from each treatment, with red or white clover; the washed nodules were dehydrated in a forced-air oven at 70 °C for 24 h to determine the dry weight<sup>(19)</sup>.

All measurements were made in February 2023 on samples of 20 x 20 x 20 cm<sup>(20)</sup>; this sampling depth was used because, in perennial ryegrass and clovers, more than 80 % of the roots grow to a depth of less than 20 cm<sup>(21)</sup>.

Statistical analysis. For each variable analyzed, the normal distribution was determined with the Shapiro-Wilks test and homogeneity of variance with the Levene test. Data analysis was performed using analysis of variance, and variables with statistical differences ( $P < 0.05$ ) were compared with a Tukey test. The statistical program INFOSTAT<sup>(22)</sup> was employed.

The mathematical model used for experiment 1 (perennial ryegrass monoculture) was as follows:

$$Y_{ijk} = \mu + L_i + B_j(L_i) + F_k + L_i \times F_k + E_{ijk}$$

Where:

$\mu$  = total mean effect,

$L_i$  = effect of locality  $i$ ,

$B_j(L_i)$  = effect of block  $j$  nested in Locality  $i$ ,

$F_k$  = effect of the fertilization source caused by level  $k$ ,

**Eijk**= experimental error at level i of the cut, level j of the block, and level k of the source.

The mathematical model used for experiment 2 (forage mixture) was as follows:

$$Y_{ijk} = \mu + L_i + B_j(L_i) + M_k + L_i \times M_k + E_{ijk}$$

Where:

**$\mu$** = total mean effect,

**$L_i$** = effect of locality i,

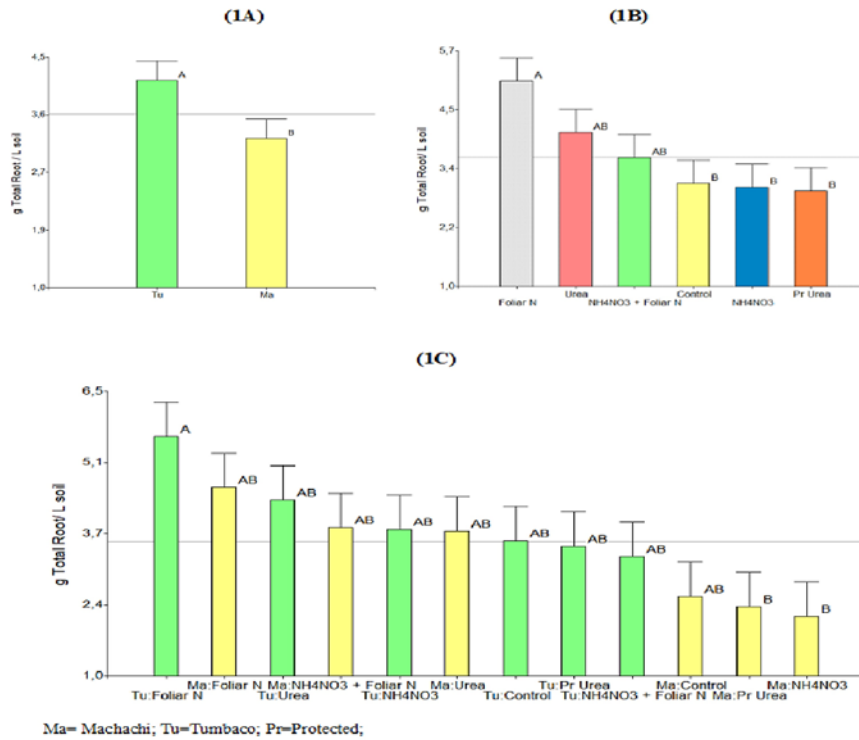
**$B_j(L_i)$** = effect of block j nested in Locality i,

**$M_k$** = effect of ryegrass and clover mixture caused by level k,

**$E_{ijk}$** = experimental error at level i of the cut, level j of the block, and level k of the mixture of ryegrass and clover.

Figure 1 presents the root biomass (Rb) data from experiment 1, expressed as grams of DM of roots per unit volume (L) of soil. Section 1A shows the differences between localities on the amount of Rb, with Tumbaco having a higher value, 4.04 g DM of roots L<sup>-1</sup> soil, whereas Machachi registered 3.22 g. Section 1B illustrates the data by treatment; the foliar N treatment had the highest Rb, 5.13 g, followed by urea, ammonium nitrate plus foliar N, and the control (T0) (4.09; 3.58; 3.07 g), and in lesser amounts, ammonium nitrate and protected urea (2.99 and 2.92 g). Section 1C presents the interaction (locality x source); the leaf N had the highest Rb in the two localities, 4.64 g DM of roots L<sup>-1</sup> soil in Machachi and 5.62 g in Tumbaco, whereas the control and ammonium nitrate obtained the lowest Rb values in Machachi (2.34 and 2.15 g).

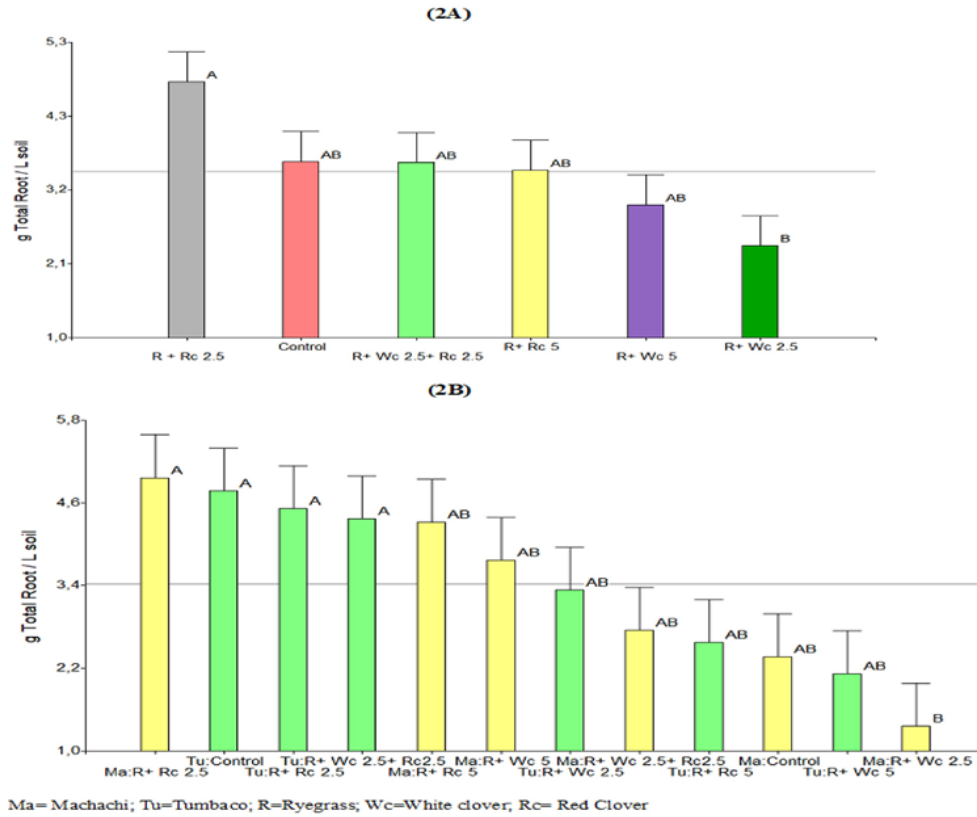
**Figure 1:** Effect of nitrogen fertilizer sources on root biomass (g DM of roots L<sup>-1</sup>): (1A) effect of the localities of Machachi and Tumbaco; (1B) effect of the treatments of the sources evaluated; (1C) locality x treatment interaction



The values are means + 1 standard error. Different letters indicate significant differences between treatments ( $P < 0.05$ ). In each figure, the general means are represented by solid lines.

Figure 2 shows the root biomass (Rb) of the different forage mixtures of perennial ryegrass (R) with/without white clover (Wc) and with/without red clover (Rc). There were no significant differences between localities. In contrast, differences ( $P < 0.05$ ) between treatments are observed in 2A; R + 2.5 Rc had the highest root biomass (4.76 g DM of roots L<sup>-1</sup> soil), and the lowest value was recorded in R + 2.5 Wc (2.35 g). 2B exhibits the locality x mixture interaction, showing that R + Rc 2.5 kg had the highest Rb in both localities, 4.98 g in Machachi and 4.53 g in Tumbaco. The control was one of the highest in Rb in Tumbaco (4.79 g), and the lowest value of Rb was observed in Machachi, in the plots with R+ Wc 2.5 kg (1.37 g). Likewise, the root biomass in mixtures of ryegrass with clover varied depending on the species, observing that mixtures with red clover had a higher root biomass, 4.76 g, compared to that of ryegrass with white clover, 2.35 g DM of roots L<sup>-1</sup>.

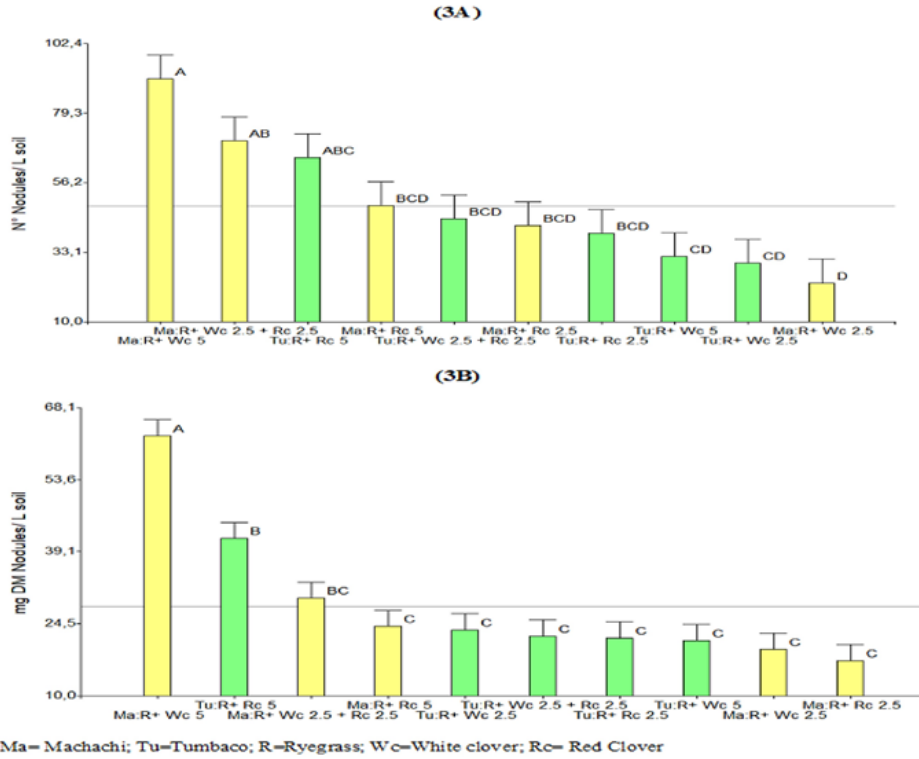
**Figure 2:** Effect of mixtures of ryegrass and white or red clover on root biomass (g DM of roots L<sup>-1</sup>) (2A) differences between mixtures; (2B) locality x mixture interaction



The values are means + 1 standard error. Different letters indicate significant differences between treatments ( $P < 0.05$ ). A solid line on the Y-axis represents the general mean. R= ryegrass, Tb= white clover (Wc), and Tr= red clover (Rc).

Figure 3A shows the statistical differences ( $P < 0.05$ ) for the number of nodules (Nn) in the locality x mixture interaction. In Machachi, the mixture of R + Wc 5 kg had 90 Nn L<sup>-1</sup> soil, and the mixture of R + Wc 2.5 kg + Rc 2.5 kg had 70 Nn, the highest Nd, whereas in Tumbaco, it was R + Rc 5 kg, which had 64 Nn. Nodule weight (Nw) showed differences in the locality x mixture interaction ( $P < 0.05$ ). In 3B, it is observed that, in Machachi, the mixture of R + Wc 5 kg had 62 mg DM of nodules L<sup>-1</sup> soil, being the highest Nw; in contrast, in Tumbaco, it was the mixture of R + Rc 5 kg, which had 41 mg nodules.

**Figure 3:** (3A) Effect of the mixture of ryegrass and white or red clover on the number of nodules (No. of nodules/L soil) in the locality x mixture interaction. (3B) Effect of the mixture of ryegrass and white or red clover on nodule weight (mg nodules on a DM basis/L soil) and of the locality x mixture interaction



The values are means + 1 standard error. Different letters indicate significant differences between treatments ( $P < 0.05$ ). A solid line on the Y-axis represents the general mean. R= ryegrass, Tb= white clover (Wc), and Tr= red clover (Rc).

The differences in temperatures, 16 °C in Tumbaco and 12 °C in Machachi, could be responsible for the higher root biomass in the former compared to the latter locality (4.04 vs. 3.02 g DM of roots L<sup>-1</sup> soil, respectively). These results are related to Walne and Reddy<sup>(23)</sup>, who determined that temperature is a critical environmental factor that regulates crop growth and yield. For example, in corn (*Zea mays*) crops, when temperatures increased, they detected longer, thicker, and denser roots. Similarly, in crops of perennial forage plants, plant growth was activated by temperature<sup>(24)</sup>. In megathermal forage plants, it has been established that species adapt to the environment by modifying the depth and length of their root development<sup>(25)</sup>. This is corroborated by Cougnon *et al*<sup>(26)</sup>, who, when studying temperate climate pastures, such as perennial ryegrass and fescue (*Festuca arundinacea* Schreb), determined that they have a higher root biomass when soil temperatures increase, such as in spring.

The root biomass in mixtures of ryegrass and clover varied according to the species, observing that mixtures with red clover had a higher root biomass, 4.76 g DM of roots L<sup>-1</sup>, compared to that of ryegrass with white clover, 2.35 g. An important factor in the development and growth of white clover roots is, according to Nichols *et al*<sup>(27)</sup>, the death of the taproot, which occurs between 12 and 18 mo after the formation of the plant, after which the plant depends only on the nodal roots. The nodal roots are smaller and shallower, which limits the search for water and nutrients, making white clover more vulnerable<sup>(28)</sup>.

The nodulation of legumes is a critical factor in the ability to fix N, as stated by Echeverría and Sainz Rosas<sup>(29)</sup>, who mentioned that the bacteria in the nodules could fix between 25 and 80 % of the N required by the plant, and that this variability was due to environmental and soil factors and rhizobia strains. It should also be borne in mind that nitrogen transfer between legumes and grasses in mixed grasslands played a vital role in the nitrogen cycle of sustainable agricultural systems<sup>(30)</sup>. Reilly *et al*<sup>(31)</sup> stated that the biological fixation of N by legumes increased over time, and that the combination of grasses and legumes increased the total biomass yield compared to monocultures.

The average number of nodules was higher in Machachi, 53.82 nodules L<sup>-1</sup> soil, whereas in Tumbaco, it was 41.86. While the number of nodules could indicate the legume's ability to fix N, the fixation of N<sub>2</sub> by rhizobia has a high energy cost for the plant. According to Dicenso *et al*<sup>(32)</sup>, fixing an atmospheric N<sub>2</sub> molecule requires the investment of 16 ATP molecules; however, other researchers<sup>(33)</sup> stated that if all the processes associated with biological fixation are considered, the energy cost amounts to 30 ATP molecules. In any case, the high energy cost would force the legume to control the number of nodules, avoiding excessive energy expenditure that would compromise its development and survival<sup>(34)</sup>. On the other hand, if there are areas rich in assimilable nitrogen in the soil, legumes obtain this nutrient through a non-symbiotic pathway, avoiding extra energy expenditure<sup>(35)</sup>.

Likewise, the plant provides the nodule with constant amounts of phosphate for the development of the nodule, so phosphate deficiencies activate a process of self-regulation of nodules in legumes<sup>(35,36)</sup>. The availability of P in its soils has a regulatory effect on nodulation<sup>(37)</sup>, so the greater availability of P in the Machachi soil could explain the greater number of nodules in this locality, since P deficiencies reduce the rhizobial infection process.

The average weight of the nodules was higher in Machachi than in Tumbaco, 30.24 vs. 25.97 mg of nodules on a dry basis L<sup>-1</sup> soil. According to Dubach and Russelle<sup>(38)</sup>, when comparing the weight of nodules in alfalfa (*Medicago sativa*) and bird's foot clover (*Lotus corniculatus*), the weight of the nodules was related to the higher concentration of N. In another report<sup>(39)</sup>, by evaluating peas (*Pisum sativum* ssp. *sativum*), they stated that the weight of the nodules was positively related to the green matter yield of the crop. On the other hand, in peanut

(*Arachis hypogaea* L.) crops, the presence of mycorrhizae influenced the weight of the nodules<sup>(40)</sup>.

It has been suggested that legume root nodules establish an ecological niche for the survival and growth of other bacteria<sup>(41,42)</sup>. Wigley *et al*<sup>(43)</sup> concluded that nodules contain various bacterial species in alfalfa (*Medicago sativa*) and that the population density of these bacteria is in the range of  $10^4$  viable bacteria per gram of fresh legume nodule tissue<sup>(44)</sup>.

The term consortium of microorganisms (CMs) has been assigned to the mixture of two or more microbial species or strains that live together symbiotically; a CM generally works better than the inoculation of a single species<sup>(45)</sup>. Ye *et al*<sup>(46)</sup> also found a higher abundance of microbe-derived molecules in legume plantations. By evaluating the impact of the co-inoculation of *Rhizobium* sp. and *Azospirillum* sp. on red clover growth, they obtained greater nodule development than by inoculating *Rhizobium* alone<sup>(47)</sup>. The interaction of these strains forms a CM that enhances root growth and crop yields<sup>(48)</sup>. On the other hand, it is claimed<sup>(49)</sup> that the organic carbon content of the soil improves the activities of microorganisms, and that it acts as a reservoir of soil nutrients and biologically increases the energy supply to microbes<sup>(50)</sup>.

Therefore, the greater weight of the nodules in the Machachi locality could be due to the better availability of phosphorus and organic carbon in the soil, allowing the development of microorganisms in general, and they enhanced the legume-*Rhizobium* association.

The inoculation of forage legumes should be a strategy to improve N fixation, since soils may be deficient in the number and quality of native rhizobia to improve plant productivity<sup>(51)</sup>. Studies carried out in Ecuador inoculating native strains of *Bradyrhizobium japonicum* in soybean (*Glycine max* L.) crops showed greater crop growth<sup>(52)</sup>. Granda-Mora *et al*<sup>(15)</sup> selected native strains of *Rhizobium* sp. for beans (*Phaseolus vulgaris* L.) in Ecuador, assuring that the bacterial inoculant obtained stimulated the growth and yield of beans. This opens the door to the potential use of native strains for forage plants, such as in Argentina, where they obtained improvements of 73 % in *Desmanthus virgatus*, a native forage plant inoculated with native rhizobia<sup>(53)</sup>, or in Brazil, where they identified native strains for *Leucaena leucocephala* and obtained greater biomass production and accumulation of N<sup>(54)</sup>.

Enhancing the N fixation capacity in legumes is a tool that allows reducing the use of N fertilizer in pasture production<sup>(55)</sup>. Forage associations of grasses and legumes increase the production and quality of forage for livestock<sup>(56)</sup>. They also have advantages over nitrogen fertilization, which has adverse side effects: water contamination by  $\text{NO}_3^-$ <sup>(57)</sup> and production of  $\text{N}_2\text{O}$ , a greenhouse gas<sup>(58)</sup>.

Temperature plays a vital role in ryegrass monoculture. Differences were detected between species regarding the accumulation of root biomass: higher in red clover. In soils of greater fertility, such as those of Machachi, a greater number and weight of nodules were observed. Nodulation capacity was different among legumes; in general terms, white clover had a greater number and weight of nodules than red clover. The species were able to adapt to the different environments; the red clover had better nodulation in the conditions of Tumbaco, whereas the white clover was better in Machachi. The most advisable amount of clover seed to use in the mixture with perennial ryegrass is 5 kg per hectare, which makes it possible to achieve a greater number and weight of nodules; likewise, it is also recommended to make mixtures with white and red clover in 2.5 kg of each.

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### Conflict of interest

The authors state that there are no conflicts of interest in this study.

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