DOI: 10.13140/RG.2.1.1014.7446
http://dx.doi.org/10.13140/RG.2.1.1014.7446
Cultivos Tropicales, 2016, vol. 37, no. 1, pp. 40-47 January-March

ISSN print: 0258-5936
ISSN online: 1819-4087
Ministry of Higher Education. Cuba
National Institute of Agricultural Sciences
http://ediciones.inca.edu.cu

ISOLATION AND CHARACTERIZATION OF RHIZOBIA FROM *Crotalaria* sp. IN SOUTHERN ECUADOR

Aislamiento y caracterización de rizobios de *Crotalaria* sp. en el sur de Ecuador

Francisco Guamán Díaz[1]✉, Roldán Torres Gutiérrez[2], Kléver Granda Mora[3] and María C. Nápoles García[4]

ABSTRACT. The present investigation was aimed to isolate and characterize native diazotrophic bacteria strains and assess their effect on nodulation and growth of *Crotalaria* sp. For the isolation, samples were taken from *Crotalaria* plants with root nodules, under different agro-climatic conditions of Loja province, Ecuador. A morpho-cultural, biochemical and physiological characterization of isolates was performed, also evaluating the effect of each isolate on the nodulation, growth and biomass of *Crotalaria* under greenhouse conditions. From these isolates, 10 strains with Rhizobial characteristics were obtained. All of them showed the ability to nodulate host plant. Results were remarkable in three isolates, due to a higher nodulation ability and efficiency on plant inoculation. These results are a starting point to obtain inoculants and its possible effect on *Crotalaria* plants used as N_2 fixers under field conditions.

Key words: inoculation, legumes, nodulation, selection

RESUMEN. La presente investigación tuvo como objetivos aislar y caracterizar cepas nativas de bacterias diazotróficas y evaluar su efecto en la nodulación y el crecimiento de *Crotalaria* sp. Para el aislamiento se tomaron muestras de plantas de *Crotalaria* con presencia de nódulos en sus raíces, en diferentes condiciones agroclimáticas de la provincia de Loja, Ecuador. Se realizó una caracterización morfo-cultural, bioquímica y fisiológica de los aislados y se evaluó el efecto de cada uno de los aislados sobre las variables de nodulación, crecimiento y biomasa de *Crotalaria* en condiciones de invernadero. De los aislamientos realizados se obtuvieron 10 cepas con características correspondientes al orden Rhizobiales. Todos los aislados evidenciaron la capacidad de nodular la planta hospedante. Se destacaron los resultados en tres de los aislados por su mayor capacidad de nodulación y eficiencia en la inoculación de las plantas. Estos resultados constituyen un punto de partida en la obtención de inoculantes y su posible efecto sobre plantas de *Crotalaria,* utilizadas como fijadoras de N_2 en condiciones de campo.

Palabras clave: inoculación, leguminosas, nodulación, selección

INTRODUCTION

The establishment of efficient legumes in the use of nitrogen (N) in agricultural production systems, is one of the main objectives, which many researchers have focused to achieve sustainability and stability of agricultural production (1). These plants play a key role in natural ecosystems, as they are responsible for a substantial part of the overall flow of atmospheric nitrogen (N_2) to assimilable forms (2).

Because of its ability to fix N^2 in symbiosis with rhizobia, legumes are excellent colonizers in poor environments of this element[A]. Within legumes, Crotalaria genus is of vital importance, for use as green manure in sustainable agricultural systems in tropical and subtropical regions (3, 4). Their contribution not only evidenced by the high amounts of N fixed (300-350 kg N ha^{-1} yr^{-1}) and the consequent effect on the growth of plants which benefit by incorporating this green manure, but the time by eliminating nematodes,

[1] Universidad Nacional de Loja, "La Argelia", PBX: 072546671, Loja-Ecuador.

[2] Proyecto Becas Prometeo, SENESCYT, Ecuador.

[3] Centro de Biotecnología, Universidad Nacional de Loja, Ciudadela Guillermo Falconi, "La Argelia", PBX: 072547252, Casilla Letra "S", Loja-Ecuador.

[4] Instituto Nacional de Ciencias Agrícolas (INCA), gaveta postal 1, San José de las Lajas, Mayabeque, Cuba, CP 32700.

✉ fguaman100@gmail.com

[A] Camarillo, F. del R. *Evaluation of the nitrogen needs and efficiency of Rhizobia strains to provide nitrogen to Chiplin* (*Crotalaria longirosrata* Hook and ARN). [Master thesis], University of Massachusetts, U.S., 2014.

its high tolerance to drought, being host of various kinds of beneficial insects and their antioxidant properties demonstrated recently (5, 6).

The degree to which a culture can benefit from the contribution of N by legumes depends on the amount of biologically fixed N, which is incorporated into the system by establishing symbiotic association with rhizobia. It is estimated that this symbiosis reaches 20 % of the total amount of N, determined annually on the planet (7). This association determines the proportion of residual N left for the next crop and utilization efficiency (8). The evidences show the importance of achieving efficient symbiotic relationships between plant species and related microsymbionts.

For effective isolation of rhizobia native strains, it is recommended that the direct extraction of nodules of plants under study, since you can get an isolated high proportions and clearly compatible with the plant (9). In Ecuador studies devoted to the diazotrophic microbial populations associated with *Crotalaria* legume are very scarce. The seasonally dry areas of the province of Loja, in the south of the country, are characterized by soils with low fertility[B], which affects the decrease of agricultural yields of various crops of economic importance.

It is interesting to study and use the benefit of the rhizobia-*Crotalaria* interaction, so the study of diazotrophic symbiotic microbiota associated to this culture and its effect on the host plant is essential. Thus, the objectives of this research were designed to isolate and characterize native rhizobia strains from plants of *Crotalaria* sp., and evaluate its effect on nodulation and growth of this legume.

MATERIALS AND METHODS

Sampling and isolation of symbiotic diazotrophic bacteria

Sampling was conducted at the Paltas Canton of the province of Loja, Ecuador. Six communities were sampled according to their altitude floors, which were geopositioned use of the Global Positioning System (GPS) (for its acronym in English). Table I shows the geopositioning of collection sites where it is observed that the areas sampled 66,7 % belongs to the altitudinal floor of 1200 to 1700 m s. n. m. (Floor 1) and 33,3 % owned by altitudinal floor of 2000 to 2200 m s. n. m. (Floor 2).

[B] Valdivieso, E. *Estrategias de Desarrollo en base a la agrobiodiversidad y los sistemas productivos para la soberanía alimentaria en la Comuna* Collana Catacocha. [Tesis de Maestría], UNLoja, Ecuador, 2013, 87 p.

Table I. Geopositioning of nodulated *Crotalaria* sampling areas in southern Ecuador

Origin	Geopositioning		
	S	O	height (m s. n. m.)
Chapango	4°02'24,4"	79°38'10,3"	1600
Palomontón	4°04'49,45"	79°40'16,62"	1502
San Fernando	4°03'18.69"	79°41'12,43"	1200
Copasombrero	4°02'59,62"	79°37'54,03"	2010
Guinuma	4°05'23,29"	79°39'50,60"	1673
Puritaca	3°58'39,77''	79°34'01,63"	2200

S = South Latitude, Longitude West O

Crotalaria sp plants were collected that were growing wild with nodules on their roots. Using a peak tool, plants whithout roots were extracted and deposited in Ziplog bags with adequate moisture. Samples were transported to the Microbiology Laboratory of the Biotechnology Center at the National University of Loja, for the realization of the isolations.

To isolate microsymbionts were taken 15 to 20 nodules located in the taproot, for each sampling area, which is carefully separated from the roots and washed with plain water to remove residual soil. Subsequently, the nodules are superficially disinfected by immersing in ethanol (70 %, v/v) for one minute and rinsed three times with sterile distilled water, to remove residual alcohol. Sodium hypochlorite (NaOCl, 3 %, v/v) was then added for three minutes and washed with plenty of these sterile distilled water. After washing, maceration of nodules was performed in 2 mL microtubes containing 300 µL of sterile distilled water.

The sowing of the nodule content was proceeded by the method of exhaustion striations in Petri dishes with medium Yeast Mannitol Agar (LMA) (10). The plates were incubated at 30 °C for seven days. The isolates grown were purified in culture medium by Congo Roj + LMA through striatum. Conservation was conducted in culture tubes containing YMA in inclined plane, at the temperature of 4 °C and glycerol (50 % v/v) at -80 °C.

Morphological, biochemical and physiological characterization of isolates

The morphology of the colonies obtained by stereomicroscopy was studied, evaluating the main features: form, color, runny nose, edges and lift the colonies. Furthermore, the growth rate in this medium, by monitoring from 24 hours of incubation and the morphology of the cells and the response to the Gram stain with the use of optical microscopy (Olympus ID 81617, Germany) was evaluated.

Biochemical assays were performed to assess the ability of isolates to ferment glucose and lactose sugars by growth on Kligler and MacConkey medium agar and Peptone Glucose Agar (PGA), incubated for 48 hours at 28 °C (11) and the presence of catalase, hydrogen peroxide using 3 % (12). The production of acid or base is determined by sowing by means YEM exhaustion at pH 6.8, with addition of bromothymol blue indicator (0,5 % in NaOH 0,016N) and pH 5,5 with bromocresole purple in equal concentration. The cultures were incubated at 30 °C for seven days. From this date the color change was observed in the medium (12). Finally, the growth of the strains was determined at different concentrations of NaCl (0,1, 1 and 2 %), pH levels (4,0; 5,0; 6,8 and 9,0) and temperatures (10, 35 and 40 °C).

Effect of inoculation on plants of Crotalaria

A trial was conducted under greenhouse conditions to check that isolates studied were capable of forming nodules in plants of Crotalaria and analyze the effectiveness of each of them on this variable and other growth and biomass. Crotalaria seeds were immersed in boiling water for 10 seconds for scarification and removal of pollutants (13). Subsequently, they were germinated in Petri dishes containing agar-water (1,5 %) and incubated at 28 °C for seven days.

The bacterial inoculum was obtained by the growth of all isolates in the middle Yeast Mannitol (LM) for 24 hours at 30 °C in orbital shaker (Techine TS1500, USA) at 250 rpm. The optical density of each culture was measured by spectrophotometry UV/VIS at 595 nm (Jenway 6505 UV / VIS, UK), to standardize the cell concentration to 10^8 CFU (colony forming units) mL^{-1}.

The mixture of substrate (soil, sand and peat, 2:1:1) was previously subjected to sterilization by Tyndalization for two hours for three consecutive days. Then 1 kg of this sterile substrate was placed in plastic pots. Each pot was watered with distilled water, to corresponding field capacity moisture. Using forceps, holes were made, in which two pregerminated seeds were carefully deposited, which are subsequently thinned to leave one plant per pot. The seeds were inoculated with 1 mL of each isolated as each treatment.

The experiment consisted of 12 treatments: 10 inoculated isolates, an uninoculated treatment but fertilized with N at 60 kg ha^{-1} and a treatment with no application (control). The plants were watered with distilled water every two days at the start and then three times a week, throughout the test.

Evaluations and statistical analysis

For morphological, biochemical and physiological study, duplicate tests of each isolates were performed. It was used a completely randomized design in testing inoculation with 10 replicates per treatment. At 75 days after sowing, the total number of nodules was evaluated; the plant height (cm); the number of leaves; fresh and dry foliage and root weight (mg). To determine the dry weight, the plants were dried in an oven at 70 °C for 72 h.

The data obtained were analyzed using the statistical package STATGRAPHICS v. 15 (14). The normality of the data for the use of Simple Statistical Analysis of Variance (One-Way ANOVA) and Tukey HSD test with significance level of $P < 0,05$ was analyzed.

RESULTS AND DISCUSSION

Samplings of Crotalaria and obtention of diazotrophic symbiotic bacteria

From the samplings were able to obtain nodulated *Crotalaria* plants in the towns of San Fernando, Palomontón, Chapango, Guinuma, Copasombrero and Puritaca.

Microsymbionts isolation, from the extracted nodes, 19 different bacterial colonies were obtained. After the purification process YMA with the addition of Congo Rojo (LMA-RC), the number of isolates was reduced to 10, which met the eligible criteria for classification within the group of rhizobia, which were named as shown in Table II.

Table II. Isolates obtained from and their nomenclature

Community	Number of isolates	Nomenclature
Palomontón	2	P2.2a P2.2b
San Fernando	2	S.F3.1 S.F3.2
Copasombrero	3	C4.1 C4.2a C4.2b
Guinuma	2	G5.1 G5.2
Puritaca	1	Pu6.2

Morphological, biochemical and physiological characterization of isolates

In Table III the morphological characteristics of the 10 isolates obtained after purification observed. It is appreciated that there is a high variability among these, according to the parameters evaluated; however, with regard to growth, the production of mucus (extracellular polysaccharides) and elevation of the colonies, differences among isolates from the same locality are evident. Isolates obtained in Copasombrero, San Fernando and Palomontón locations are an example of what it is described above, where the growth of C4.2a isolated as abundant stands while C4.2b is moderate and C4.1 is light. Similar results are obtained in the production of mucus and lift the colonies. These results show that not all microbial isolates from the same location and the same host have the same morphological characteristics. In this regard, other authors report variability morphological characteristics of isolated culture of *Rhizobium* common bean (*Phaseolus vulgaris*) in the central provinces of Cuba[C].

In analyzing the growth of colonies it is observed that three isolates (27 %) had an abundant growth six moderate growth (55 %) and two slight growth (18 %), obtaining 82 % of those among the categories of abundant and moderate; however, all had rapid growth, between 24 and 48 hours, coinciding with the results of other studies, which fast-growing rhizobia isolated from *Crotalaria juncea*, *Crotalaria labumifolia*, *Crotalaria retusa* and *Crotalaria verrucosa* (15).

The presence of colonies with regular edges and an opaque color, and the observation to the optical microscope showed in all cases short Gram-negative bacilli show similar features to those obtained by other authors for bacteria belonging to the group of rhizobia (16).

In Table IV the results of biochemical tests, which shows that the isolates changed the green coloring YMA + bromothymol blue and blue color YMA + bromocresol purple to yellow are shown, indicating that they are acid producers and therefore possible members of the family *Rhizobiaceae*. These results are consistent with other research done in similar crops, where production capacity of acids or bases by strains belonging to the genera *Rhizobium* and *Bradyrhizobium,* respectively (17, 18) was determined.

None of the isolated fermented lactose; however, C4.1, P2.2a and P2.2b fermented it. Results of other studies show that several isolates obtained from *Pueraria phaseoloides* (tropical kudzu) were able to ferment these two sugars (19). However, it is known that neither glucose nor the lactose are preferred carbon sources by rhizobia (9). All isolates were positive to catalase, releasing oxygen as bubbles upon reaction with hydrogen peroxide. This behavior is typical of this bacteria group (17, 20).

Elucidating the physiological parameters evaluated in isolates obtained is vitally important, because these are closely linked to indicators that may have soil, where they could be applied as biofertilizers. Table V shows the physiological characteristics related to the tolerance of isolated three concentrations of NaCl, three pH levels and three temperatures.

The 10 isolates grew adequately in the three concentrations of NaCl and all were framed in the categories of moderate and abundant growth. The salinity tolerance is one of the discussion areas regarding the growth of rhizobia.

Results of other authors in red bean show no growth of any single 2 % of this salt[D], while some found that the total isolates made in cowpea, 21 % failed to grow at any concentration of NaCl, 15 % only they tolerated 1 % and 38 % had the ability to grow by 3 % (17).

At acidic pH values (4 and 5) C4.2a and G5.1 did not grow. These isolates only tolerated alkaline pH. The strain G5.1 is noted that was isolated from a soil pH 5, 97 (data not shown); although it is acid, it is slightly higher than the tested plates 5. Meanwhile, P2.2a, Pu6.2 and SF3.2 not grew in the value 4, but at pH 5. The 10 isolates grew abundantly value pH 9, which means that tolerate well alkalinity and surprisingly, considering that are producers of acid (20). In general, the isolated C4.1, S.F3.1, P2.2b and C4.2b tolerated three pH levels studied. It is well documented that the optimal pH for growth of rhizobia is around neutrality (6), although some strains can grow between 6 and 8 (19). Other papers refer tolerance acid pH values[D]. Sometimes, correlation between soil pH of strains and pH tolerance in culture medium are isolated has been found, such as isolates *P. vulgaris*[D] or *P phaseoloides*[E].

[C] Colás, S. A.; Torres, G. R.; Eichler, L. B.; Granda, M. K. I.; Gutiérrez, S. Y.; Willems, A.; Michiels, J. y Vanderleyden, J. *Genetic identification of Rhizobia isolates. Behavior on bean* (*Phaseolus vulgaris L.*) *genotypes*. Inst. International Research on Food Security, Natural Resources Management and Rural Development, 2011.

[D] Díaz-Alcántara, C. A. *Aislamiento, caracterización y selección de Rizobia autóctonos que nodulan habichuela roja* (*Phaseolus vulgaris L.*), *en la República Dominicana*. [Tesis de Doctorado], Universidad de León, Departamento de Ingeniería y Ciencias Agrarias, República Dominicana, 2010.

[E] Pérez, G. *Selección de aislados de rizobios provenientes de la leguminosa forrajera Pueraria phaseoloides* (*Kudzú Tropical*)*, cultivada en condiciones de acidez*. [Tesis de Maestría], Universidad de La Habana, Ciudad de La Habana, Cuba, 2010.

Table III. Morphological characteristics of the colonies isolated of Crotalaria

Isolates	Growth	Color	Mucus Production	Edges	Elevation	Staining Gram
P2.2.a	+++	Opaco	+++	Regular	+++	-
P2.2.b	++	Opaco	++	Regular	++	-
SF 3.1	++	Opaco	+++	Regular	+++	-
SF.3.2.	+	Opaco	++	Regular	+	-
C 4.1	+	Opaco	+	Regular	+	-
C4.2.a	+++	Opaco	+++	Regular	+++	-
C4.2.b	++	Opaco	+++	Regular	+++	-
G.5.1	++	Opaco	+	Regular	++	-
G.5.2	++	Opaco	++	Regular	+	-
Pu 6.2	++	Opaco	+	Regular	+	-

\+ ligero ++ moderado +++ abundante

Table IV. Results of biochemical evidence of isolates

Isolates	Kligler Agar			LMA+RC	MacConkey	PGA	Acid base		Catalase
	Glucose	Lactose	Hydrogen sufide				LMA+ Bmol Blue	LMA+ Bcresole	
	+	-	-	+	-	+	+	+++	+
	+	-	-	+	-	-	+	-	+
Pu.6.2	-	-	-	+	-	-	+	+++	+
S.F. 3.1	-	-	-	+	-	-	+	+++	+
G.5.1	-	-	-	+	-	-	+	++	+
P. 2.2.b	+	-	-	+	+	+	-	+++	+
C.4.2.a	-	-	-	+	-	-	+	++	+
C.4.2.b	-	-	-	+	-	-	-	++	+
G.5.2.	-	-	-	+	-	-	+	++	+
S.F. 3.2	-	-	-	+	+	-	+	+++	+

\+ light ++ moderate +++ abundant

Table V. Growth in YMA isolates with different concentrations of NaCl, pH values and incubation temperatures

Isolates	Concentrations NaCl (%)			pH			Temperature (°C)		
	0,1	1	2	4	5	9	10	35	40
P2.2a	++	+++	+++	-	+++	+++	-	++	-
P2.2b	+++	+++	+++	++	++	+++	-	+++	++
S.F3.1	++	+++	+++	+++	+++	+++	-	+++	++
S.F3.2	++	+++	+++	-	++	+++	-	+++	-
C4.1	++	++	+++	+++	+++	+++	-	+++	++
C4.2a	++	+++	+++	-	-	+++	-	++	-
C4.2b	+++	+++	+++	++	+++	+++	-	+++	+++
G5.1	++	+++	+++	-	-	+++	-	++	++
G5.2	+++	+++	+++	-	+++	+++	-	+++	+++
Pu6.2	++	+++	+++	-	++	+++	-	++	++

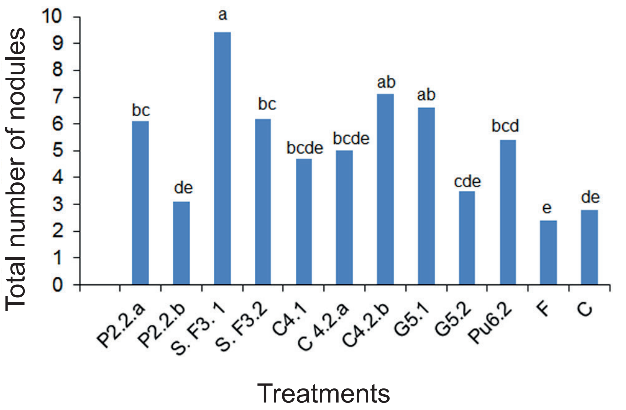
\+ growth: light, ++ moderate, +++ abundant, - negative

When analyzing growth at different temperatures, it was observed that at 10 °C did not grow any of the isolates. Neither grew 40 °C P2.2a, C4.2a, or SF 3.2, which means that these isolates did not tolerate extreme temperatures. It is stated that in temperate climates, temperatures above 37 °C allow to differentiate among some species of *Rhizobium* (21).

The rhizobia optimum growth temperature is at 28-30 °C (22), hence almost all isolates of *Crotalaria* showed abundant growth at 35 °C temperature. According to other reports, there is tolerance of some strains of *Rhizobium*, as *R. tropici* to grow at different temperature ranges, but none of them support thermophilicity or psychrophilia conditions (23).

Effect of inoculation on Crotalaria plants

The ultimate tests to prove that isolates made correspond to Crotalaria symbionts, is contacting both agencies and observe the appearance of nodules, provided that there are minimum conditions for interaction. The nodulation of isolated on Crotalaria plants under greenhouse conditions is presented in Figure 10. All isolates were able to form effective nodules on the roots, indicating its authenticity as microsymbiont of this plant species.



Same letter in the columns do not differ statistically for p≤0,05, according to Tukey's test

Total number of nodules formed in *Crotalaria* with inoculation of different isolates, application of mineral fertilizers (F) and a control treatment (C)

The presence of nodules in fertilized treatments was detected and uninoculated, although less than five treatments inoculated showed no differences with P2.2b, C4.1, C4.2a or G5.2 strains.

Although the support employed in this assay was sterilized, the method Tyndalization not guarantee such an effective sterility as autoclaving, as this method does not combine changes in temperature and pressure, but only changes in temperature, so it may not be and yet it has been sufficient to remain resident in the support rhizobia capable to nodulate such plants. However, the marked effect of isolates is seen for example, S.F3.1 with 9,4 nodules per plant; C4.2b with 7,1 nodules and G5.1, with 6,6 nodules per plant.

The *Crotalaria* is classified in the group of legumes that form effective nodules with a wide range of strains, according to studies that characterize their nodulation middle, with large nodules, red internal color and a majority presence of these structures in the principal root[F].

The effect of different treatments on growth variables: fresh and dry foliage weight, fresh and dry root weight, height and number of leaves, is presented in Table VI. There were statistical differences among isolates, favor S.F3.1 and between this and C4.2b isolate, with fertilized treatment for bacteria. Although there were no differences with the control variables radical treatment for dry and fresh weight, height and numbers of leaves, the best results were for the treatment inoculated with isolate S.F3.1, followed by C4.2b isolate, which coincides with the results of nodulation.

It stands to reason that more nodulation on these plants has resulted in outperformance of its biomass, which can indirectly linked to an increased supply of N through symbiotic fixation with these isolates. Interestingly, these isolates surpassed even the fertilized witness in the trial conditions.

Similar results were obtained when evaluating the efficiency of nitrogen requirements against inoculation with *Rhizobium* in chiplin (*Crotalaria longirostrata*), determining that the inoculation with rhizobacteria significantly promoted plant biomass compared to mineral fertilization and treatment controls.

[F] Bisson, A. y Mason, T. *Identification of rhizobia species that can establish nitrogen-fixing nodules in Crotalaria longirostrata*. Bachelor of Science, University of Massachusetts, U.S., 2010, 106 p.

Table VI. Effect of different treatments on growth variables of *Crotalaria*

Treatments	PFF (mg)	PSF (mg)	PFR (mg)	PSR (mg)	height(cm)	Number of leaves
P2.2a	176,56 b	34,68 c	42,46 abc	13,77 ab	7,4 ab	2,3 ab
P2.2b	162,13 b	32,77 c	32,62 bc	11,25 ab	7,3 ab	2,1 ab
S.F3.1	478,22 a	106,96 a	87,82 a	21,34 a	9,2 a	3,8 a
S.F3.2	197,59 ab	44,72 c	45,35 abc	11,72 ab	7,9 ab	2,3 ab
C4.1	300,68 ab	43,74 c	47,12 abc	14,27 ab	8,7 ab	3,2 ab
C4.2a	374,18 ab	44,65 c	47,94 abc	15,16 ab	7,4 ab	2,7 ab
C4.2b	367,74 ab	98,60 ab	65,92 ab	23,12 a	9,0 a	3,7 a
G5.1	229,63 ab	61,20 abc	61,47 abc	14,66 ab	8,6 ab	2,6 ab
G5.2	262,46 ab	46,37 c	53,74 abc	15,26 ab	8,5 ab	2,3 ab
Pu6.2	244,53 ab	50,01 bc	43,42 abc	15,77 ab	8,9 a	2,4 ab
Fertilization	190,77 b	16,93 c	15,32 c	5,74 b	5,6 b	1,6 b
Control	137,62 b	28,47 c	42,0 abc	13,83 ab	6,2 ab	2,1 ab
E.s x̄	59,91***	10,17***	10,45**	2,83*	0,6***	0,3**

PFF: foliage fresh weight; PSF: foliage dry weight; PFR: root fresh weight; PSR: root dry weight z
Equal letters do not differ statistically for p≤0,05, according to Tukey's test

CONCLUSIONS

Crotalaria plants that grow wild in the communities of Loja, are naturally associated with efficient strains of rhizobia, which can contribute to their growth and development.

BIBLIOGRAPHY

1. Daimon, H. ''Traits of the Genus *Crotalaria* Used as a Green Manure Legume on Sustainable Cropping Systems''. *Japan Agricultural Research Quarterly: JARQ*, vol. 40, no. 4, 2006, pp. 299-305, ISSN 0021-3551, DOI 10.6090/jarq.40.299.
2. Marschner, H. *Mineral Nutrition of Higher Plants*. edit. Academic Press, 8 de agosto de 2011, 668 p., ISBN 978-0-12-384906-9.
3. Miyazawa, K.; Murakami, T.; Takeda, M. y Murayama, T. ''Intercropping green manure crops-effects on rooting patterns''. *Plant and Soil*, vol. 331, no. 1-2, 18 de diciembre de 2009, pp. 231-239, ISSN 0032-079X, 1573-5036, DOI 10.1007/s11104-009-0248-y.
4. Prager, M. M.; Sanclemente, R. O. E.; Sánchez, de P. M.; Miller, G. J.; Sánchez, Á. y Iván, D. ''Abonos verdes: Tecnología para el manejo agroecológico de los cultivos''. *Agroecología*, vol. 7, no. 1, 2012, pp. 53-62, ISSN 1989-4686.
5. Kimenju, J. W.; Kagundu, A. M.; Nderitu, J. H.; Mambala, F.; Mutua, G. K. y Kariuki, G. M. ''Incorporation of green manure plants into bean cropping systems contribute to root-knot nematode suppression''. *Asian Journal of Plant Sciences*, vol. 7, no. 4, 2008, pp. 404–408, ISSN 1682-3974.
6. Deepha, V.; Praveena, R.; Sivakumar, R. y Sadasivam, K. ''Experimental and theoretical investigations on the antioxidant activity of isoorientin from *Crotalaria globosa*''. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 121, 5 de marzo de 2014, pp. 737-745, ISSN 1386-1425, DOI 10.1016/j.saa.2013.12.025.
7. Martínez, V. R. y Dibut, A. B. *Biofertilizantes bacterianos*. edit. Editorial Científico Técnica, La Habana, Cuba, 2012, 279 p., ISBN 978-959-05-0659-8.
8. Giller, K. E. *Nitrogen Fixation in Tropical Cropping Systems*. Second edition ed., edit. CABI, Wallingford, Oxon, UK ; New York, NY, USA, 6 de julio de 2001, 352 p., ISBN 978-0-85199-417-8.
9. Hernández, J. L.; Cubillos-Hinojosa, J. G. y Milian, P. E. ''Aislamiento de cepas de *Rhizobium* sp., asociados a dos leguminosas forrajeras en el Centro Biotecnológico del Caribe''. *Revista Colombiana de Microbiología Tropical*, vol. 2, 2012, pp. 51–62, ISSN 2215-8375.
10. Vincent, J. M. *A Manual for the Practical Study of Root-nodule Bacteria*. vol. 15, edit. International Biological Programme, Blackwell Scientific, London, 1 de enero de 1970, 202 p., ISBN 978-0-632-06410-6.
11. Bécquer, C. J.; Prévost, D. y Prieto, A. ''Caracterización fisiológica-bioquímica de cepas de rizobios, aislados en leguminosas forrajeras''. *Revista Biología*, vol. 14, no. 1, 2000, pp. 57-65, ISSN 0864-3490.
12. Sosa, A.; Elías, A.; García, O. A. y Sarmiento, M. ''Aislamiento y caracterización fenotípica parcial de cepas de rizobios que nodulan leguminosas rastreras''. *Revista Cubana de Ciencia Agrícola*, vol. 38, no. 2, 2004, pp. 197-201, ISSN 2079-3472.
13. Sanabria, V. D.; Silva, A. R.; Alfaro, C. y Oliveros, M. ''Escarificación térmica de semillas de tres accesiones de *Leucaena leucocephala*''. *Zootecnia Tropical*, vol. 15, no. 2, 1997, pp. 67–80, ISSN 0798-7269.
14. StatPoint Technologies. *Statgraphics Centurion* [en línea]. (ser. Centurion), versión 16.1 (XV), [Windows], 21 de mayo de 2010, Disponible en: <http://statgraphics-centurion.software.informer.com/download/>.
15. Wang, E. T. y Martínez-Romero, J. ''Taxonomía del *Rhizobium*''. En: eds. Martínez-Romero E. y Martínez-Romero J. C., *Microbios*, edit. Centro de Investigaciones sobre Fijación de Nitrógeno. Universidad Nacional Autónoma de México, México, 2001, p. 264, ISBN 968-36-8879-9.
16. Granda-Mora, K. I.; Ochoa, M. A.; Ruviela, V.; Guamán-Díaz, J. F. y Torres-Gutiérrez, R. ''Evaluación de cepas nativas de Rhizobium sobre parámetros fenotípicos en fréjol común (*Phaseolus vulgaris* L.)''. *Centro de Biotecnología*, vol. 3, no. 1, 2013, pp. 25-37, ISSN 1390-7573.
17. Cuadrado, B.; Rubio, G. y Santos, W. ''Caracterización de cepas de *Rhizobium* y *Bradyrhizobium* (con habilidad de nodulación) seleccionados de los cultivos de fríjol caupi (*Vigna unguiculata*) como potenciales bioinóculos'' *Revista Colombiana de Ciencias Químico-Farmacéuticas*, vol. 38, no. 1, enero de 2009, pp. 78-104, ISSN 0034-7418.
18. Lei, Z.; Jian, P. G.; Shi, Q. W.; Ze, Y. Z.; Chao, Z. y Yongxiong, Y. ''Mechanism of acid tolerance in a rhizobium strain isolated from *Pueraria lobata* (Willd.) Ohwi''. *Canadian Journal of Microbiology*, vol. 57, no. 6, 1 de junio de 2011, pp. 514-524, ISSN 0008-4166, DOI 10.1139/w11-036.
19. Boboye, B. E. y Ajayi, G. O. ''Biodegradation of Selected Nigerian Fruit Peels by the use of a Non-pathogenic *Rhizobium* species CWP G34B''. *The Open Microbiology Journal*, vol. 6, 2 de noviembre de 2012, pp. 88-93, ISSN 1874-2858, DOI 10.2174/1874285801206010088.
20. Moscatiello, R.; Baldan, B.; Squartini, A.; Mariani, P. y Navazio, L. ''Oligogalacturonides: Novel Signaling Molecules in *Rhizobium*-Legume Communications''. *Molecular Plant-Microbe Interactions*, vol. 25, no. 11, 26 de julio de 2012, pp. 1387-1395, ISSN 0894-0282, DOI 10.1094/MPMI-03-12-0066-R.
21. López, L. G.; Tabche, M. L.; Castillo, R. S.; Mendoza, V. A.; Ramírez, R. M. A. y Dávila, G. ''RNA-Seq analysis of the multipartite genome of *Rhizobium etli* CE3 shows different replicon contributions under heat and saline shock''. *BMC Genomics*, vol. 15, no. 1, 8 de septiembre de 2014, p. 770, ISSN 1471-2164, DOI 10.1186/1471-2164-15-770.

22. Reina, B. M.; Argandoña, M.; Nieto, J. J.; Hidalgo, G. A.; Iglesias, G. F.; Delgado, M. J. y Vargas, C. "Role of trehalose in heat and desiccation tolerance in the soil bacterium *Rhizobium etli*". *BMC Microbiology*, vol. 12, no. 1, 17 de septiembre de 2012, p. 207, ISSN 1471-2180, DOI 10.1186/1471-2180-12-207.

23. Ormeño, O. E.; Menna, P.; Almeida, L. G. P.; Ollero, F. J.; Nicolás, M. F.; Rodrigues, E. P.; Nakatani, A. S.; Batista, J. S. S.; Chueire, L. M. O.; Souza, R. C.; Vasconcelos, A. T. R.; Megías, M.; Hungria, M. y Martínez, R. E. "Genomic basis of broad host range and environmental adaptability of *Rhizobium tropici* CIAT 899 and *Rhizobium* sp. PRF 81 which are used in inoculants for common bean (*Phaseolus vulgaris* L.)". *BMC Genomics*, vol. 13, no. 1, 27 de diciembre de 2012, p. 735, ISSN 1471-2164, DOI 10.1186/1471-2164-13-735.

Received: November 19th, 2014
Accepted: January 27th, 2015