ISSN impreso: 0258-5936
ISSN digital: 1819-4087

Ministerio de Educación Superior. Cuba
Instituto Nacional de Ciencias Agrícolas
http://ediciones.inca.edu.cu

RESIDENTS RHIZOBIA IN THE RHIZOSPHERE OF RICE PLANTS (*Oryza sativa* L.) CULTIVAR INCA LP-5

Rizobios residentes en la rizosfera de plantas de arroz (*Oryza sativa* L.) cultivar INCA LP-5

Ionel Hernández Forte✉ and María Caridad Nápoles García

ABSTRACT. The objective of this work was to isolate rhizobia from the rhizosphere of rice plants cultivar INCA LP-5. Rhizobia isolation from rhizospheric soil and rhizoplane of rice plants growing in Petroferric Ferruginous Nodular Gley soil was conducted. The possible taxonomic distribution of the isolates was determinated throught the study of their morpho-cultural and physiological characteristics. *In vitro* nodulation assays were also performed in *Macroptilium atropurpureum* (DC.) Urb. (siratro). Fourteen isolates showed similar cultural and morphological characteristics to rhizobia, of which 28,57 % were obtained from the rhizospheric soil and 71,43 % from rhizoplane of rice plants. All isolates were negative to cetolactase test and produced acid. An isolated showed moderate growth on the culture medium while the rest grew faster. Eleven rhizospherics isolated from rice plants produced effective nodules in siratro plants. A possible member of the *Mesorhizobium* genera and ten possible members of *Rhizobiaceae* family were identified. Four isolates showed a high effectiveness of the nodulation process in siratro plants and one of them significantly increased the root dry mass. The results obtained in this study provide the first evidence in Cuba where interaction of rhizobia-rice is developed.

Key words: isolation, *Rhizobiaceae*, rizobacteria

RESUMEN. El objetivo del presente trabajo fue aislar rizobios de la rizosfera de plantas de arroz cultivar INCA LP-5. El aislamiento se realizó a partir del suelo rizosférico y del rizoplano de plantas de arroz cultivadas en suelo Gley Nodular Ferruginoso Petroférrico. Se determinó la posible distribución taxonómica de los aislados mediante el estudio de sus características morfo-culturales y fisiológicas y se realizaron bioensayos de nodulación *in vitro* en *Macroptilium atropurpureum* (DC.) Urb. (siratro). Se obtuvieron 14 aislados con características culturales y morfológicas similares a las descritas en los rizobios, de los cuales el 28,57 % se obtuvieron del suelo rizosférico y el 71,43 % del rizoplano de las plantas de arroz. Todos los aislados resultaron negativo a la prueba de la cetolactasa y produjeron ácido. Un aislado tuvo un crecimiento moderado sobre el medio de cultivo mientras que el resto crecieron más rápidamente. Once aislados provenientes de la rizosfera de las plantas de arroz produjeron nódulos efectivos en las plantas de siratro. Se identificó un aislado como posible miembro del género *Mesorhizobium* y diez en la familia *Rhizobiaceae*. Cuatro aislados presentaron una elevada efectividad del proceso de nodulación y uno de ellos incrementó significativamente la masa seca radical. Los resultados obtenidos en esta investigación constituyen la primera evidencia en Cuba donde se aborda la interacción de los rizobios con el cultivo del arroz.

Palabras clave: aislamiento, *Rhizobiaceae*, rizobacterias

INTRODUCTION

Rice is one of the most important crops for human consumption, with a world production of 478 million tons[A]. In Latin America, Cuba is one of the countries with the highest per capita consumption, with around 60 kg per year[B].

The INCA LP-5 rice cultivar, obtained at the National Institute of Agricultural Sciences (INCA) is one of the most widely distributed in the country due to its high yields and its resistance to *Pyricularia grisea*, the main pathogen of the crop (1).

Instituto Nacional de Ciencias Agrícolas (INCA), Gaveta postal No.1, San José de las Lajas. Mayabeque, Cuba. CP 32700
✉ ionel@inca.edu.cu

[A] Consejo Internacional de cereales. *Informe de Mercado de Cereales.* Resumen de las perspectivas para cereales y oleaginosas clave, Inst. Consejo Internacional de cereales, 2015, 8 p.

[B] FAO y IAEA. *Programme. Rice production: what Cuba can teach the world? Nuclear Techniques in Food and Agriculture. Inst.* FAO, 2014, Austria, 1 p.

The application of nitrogen fertilizers and phosphates to the soil makes it possible to supply a large part of the nutritional needs of rice cultivation, increasing yields considerably (1). However, the irrational use of these products negatively impacts the different ecosystems and makes the production process more expensive (2). The isolation and characterization of bacteria that are naturally associated with rice cultivation and that promote its growth has allowed the production of environmentally friendly biopreparates that provoke increases in agricultural yields, with the consequent reduction of the costs of the productive process (3).

Azospirillum and Herbaspirillum are among the most representative bacterial genera in the rhizosphere of rice plants, although *Bacillus, Pseudomonas, Azotobacter* and *Burkholderia* are also prominent (4). However, rhizobia, bacteria that have traditionally been studied for their ability to establish symbiosis with leguminous plants from the formation of radical nodules where they perform the Biological Fixation of Nitrogen (BFN) (5), have also been observed colonizing the roots of rice plants and as endophytes of this crop (6). Inoculation of rhizobia strains in various rice cultivars has shown positive effects on development, growth and crop yields (7, 8).

In spite of the above-mentioned antecedents, there have been few studies in the world on the rhizobio-rice interaction and in Cuba so far, there is no documented evidence showing the presence of rhizobia associated with rice cultivation. For this reason, the objective of the present work was to isolate rhizobia from the rhizosphere of rice plants (*Oryza sativa* L.) cultivar INCA LP-5, grown in Gley Nodular Ferruginous Petroferric soil. Having a set of rhizobia obtained from the rhizosphere of rice plants would allow a careful study of the particularities that govern this association. With the most promising rhizobia isolates, biopreparations could be developed to increase rice yield and growth, under the soil and climate conditions of Cuba.

MATERIALS AND METHODS

Sampling and chemical characterization of soil

Rice plants of the cultivar INCA LP-5 were harvested on the terraces of zone three, squares C and H in the Basic Scientific Science Unit “Los Palacios” (UCTB-LP according its acronyms in Spanish), located in Pinar del Río province. Five sites, distant from one another in diagonal, were sampled to obtain a representativeness of the microbiota present in the soil of the studied locality.

Five soil samples were taken at each of the sampled sites, at a depth of less than 30 cm and more than 20 cm from the roots of the plants. For the determination of the sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}). Firstly, they were extracted using the Maslova technique; the content of Na^+ and K^+ was determined by flame photometry and Ca^{2+} and Mg^{2+} using EDTA. The phosphorus content (P) was evaluated by the technique of Oniani (9). The pH (in KCl) was further determined by the potentiometry technique; as well as the percentage content of organic matter by the Walkley-Black method (10).

Isolation e identification of the rhizobium presence in the rhizosphere and rhizoplane of rice plants

At the time of sampling, the crop was in the vegetative stage of growth and subjected to flooding, fertilization and irrigation conditions as established in the Technical Instructions for Rice (1) and there was no precedent for the application of inoculants based on Rhizobia. Four rice plants were collected at each of the five soil sampling sites for a total of 20 plants. The whole plants were extracted, taking care not to damage the root system and were stored at 4 °C in polyethylene bags for further processing in the laboratory.

For the isolation of rhizobia from rhizospheric soil, 10 g of roots were taken with rhizospheric soil and placed in a 250 mL Erlenmeyer flask containing 100 mL of sterile distilled water. The Erlenmeyers were kept under stirring for 10 min at 60 r min^{-1}. The roots were then extracted and serial dilutions of sterile distilled water (10^{-1}-10^{-6}) were made from the obtained rhizospheric soil suspension. Isolation of the rhizobia was done by sowing 0,1 mL of the dilutions on plates with yeast-mannitol-agar medium (YMA) (11) with red congo, at pH 6,8. Plates were incubated at 29+1 °C for ten days (12).

For the isolation from the rhizoplane, the roots extracted in the previous procedure were taken and placed again in 250 mL Erlenmeyer flasks containing 100 mL of sterile distilled water, which were maintained under agitation conditions for another 30 min at 150 r min^{-1}. The roots were removed again and the same insulation procedure described for the rhizospheric soil was applied to the suspension obtained.

In parallel, roots were cut in 1-2 cm portions with sterile scalpel blades, and later placed on plates containing Luria-Bertani medium (13), in order to allow the growth of microorganisms more closely adhered to the surface radical. The plates, in both cases, were incubated at 29+1 °C for ten days (14).

For the purification of the possible rhizobia isolates, those colonies that presented cultural characteristics similar to those described for this microbial group were taken (11). Successive passes were performed in YMA medium until pure cultures were obtained. The isolates were stored at 4 °C in test tubes containing said culture medium.

For the rhizobia selection of the set of bacterial isolates obtained from the rhizosphere of rice plants cultivating INCA LP-5, a cultural and morphological characterization was performed. The results of the ketolactose test were also taken into account. The ability of isolates to produce acid/base was determined and *in vitro* inoculation assays were performed on *Macroptilium atropurpureum* (DC.) Urb. (Siratro) plants.

Cultural and morphological characterization of bacterial isolates

The purity of bacterial cultures, as well as their dyeing characteristics and cell morphology was observed by Gram Stain (15). The cell morphological characteristics were studied, bacterial cell form, response to Gram staining and the presence of spores.

For the determination of cultural characteristics, the isolates were cultured by depletion in YMA medium with red congo, at pH 6,8. They were incubated for 10 days at 29±1 °C and the color, mucus production and diameter (mm) of the colonies were determined by stereoscopic microscopy (16). In addition, the growth rate of each isolate was determined by monitoring the occurrence or otherwise of the colonies on the plates every 24 h for ten days of incubation. The isolates whose colonies became visible in the medium at 2-3 days of incubation were considered fast growing; those that appeared from the 4-5 days were considered of moderate growth and those that were observed from the seven days were classified as slow-growing microorganisms (11).

Ketolactase assay

The isolates were cultured in Yeast-Lactose-Agar (YLA) medium (17) and incubated at 29±1 °C for three, five and seven days, according to their growth rate. Subsequently, 10 mL of the Benedict reagent on bacterial growth was added and held for 10 min to observe possible color change of the medium.

Acid and base production

The bacterial isolates were cultured in YMA medium at pH 6,8 with blue indicator of bromothymol. At seven days of incubation at 29±1 °C a change in coloration was observed in the culture medium during the establishment of bacterial colonies on the same.

In vitro inoculation test in siratro plants

Inoculants were prepared from each of the bacterial isolates, for which 20 mL Erlenmeyers were used with 5 mL of liquid MY (mannitol yeast) medium at pH 6,8. They were inoculated with a broth of microorganisms preserved at 4 °C in solid medium. The flasks were kept under stirring conditions in orbital shaker at 150 r min^{-1} for 18-20 h at 29±1 °C.

The identity of the rhizobia was confirmed by their ability to nodulate the siratro legume, traditionally used as a model plant for this type of studies (18). The seeds of this legume were donated by the Experimental Station of Pastures and Forages Cascajal, Villa Clara province.

For the seed scarification they were maintained in 70 % ethanol for five minutes, then washed with distilled water and treated with concentrated sulfuric acid for 10 min. They were then immersed in 25 % (v/v) sodium hypochlorite for 15 minutes and washed six times with sterile distilled water. The seeds were then plated with Agar-Water (0,75 g of agar in 100 mL of distilled water) and incubated at 29 ± 1 °C in the dark for 24 h. Germinated seeds with roots approximately 1-2 cm long were placed in 200 mL flasks containing 50 mL of Norris and Date semisolid (15) media, one seed per flask. Subsequently, the seeds were inoculated with 0,5 mL inoculum of the different isolates at a concentration of 108 CFU mL^{-1}.

The plants grew under controlled conditions, with a photoperiod of 12 h light/12 h dark, at a day/night temperature of 26/22 °C and relative humidity of 70 %. Five weeks later, the number of nodules in the main root was determined; total number of nodules; the effectiveness of nodules and the effectiveness of nodulation (EN) according to the expression:

$$EN = \frac{NNte}{NN\,t} \times 100$$

where: NNt and NNte are the absolute values of the number of total nodules and the number of effective real nodules, respectively. The root dry mass of the plants (g) was also determined.

The effectiveness of the nodules was determined by the visual method, which was done by the dissection of the nodules with scalpel blades of stainless steel, detecting or not the presence of a reddish coloration inside the nodules, characteristic of the protein Leghemoglobin (19).

A completely randomized design was used, using seven replicates per treatment. A control treatment was used, without inoculation. The results from the variables: number of nodules in the main root and total, as well as the number of effective nodules in each case, were submitted to non-parametric analyzes of Kruskal Wallis. In case of significant differences among treatments, the Mann Witney test was used to establish differences among the average ranges of the variables in each of the established treatments (20). The EN variable was not subjected to statistical analysis because it results from the absolute values of total effective nodulation and total nodulation in each of the treatments.

Data from the root dry mass of siratro plants were tested for normality (Bartlett test) and homogeneity of variance (Kormogorov-Smirnov test) (21). Subsequently, simple classification variance analysis was used, using Tukey's mean comparison test for $p < 0,05$ (22); with the aim of determining differences between means (20). These data were plotted using the SigmaPlot 2001 program.

RESULTS AND DISCUSSION

The Gley Nodular Ferruginous Petroferric soil at UCTB Los Palacios, Pinar del Rio, was characterized by a pH close to neutrality. The organic matter content and available phosphorus levels were found to be high. However, according to the Manual of the General Directorate of Soils and Fertilizers (23), this soil has a low content of calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^{+}) and potassium (K^{+}) (Table I).

The pH value in the soil (6,87), favors the establishment of rhizobia, which require very similar values for multiplication (24). Although not determinant, the establishment of rice cultivation is optimal at pH close to neutrality (1). The pH of the soil of this locality would positively affect the establishment of the crop and of the rhizobia, as well as the interaction between both organisms.

The contents of calcium, magnesium, sodium and potassium in this soil are low, which could negatively affect the multiplication of rhizobia. However, the high content of phosphorus and organic matter that it possesses, promote the proliferation of these bacteria and the establishment and development of rice plants. Organic matter greatly influences the distribution of the major microbial groups in the soil and enhances their growth in areas where there is greater availability of nutrients (25).

At the time of sampling, INCA LP-5 cultivated rice plants were in flood conditions, propitiated by the irrigation to which the crop was subjected and by the characteristics previously described for this type of soil. It is considered that the soil of the UCTB Los Palacios classifies as Hydromorphic Gley Nodular Ferruginous Petroferric, of fine texture, with a great content of clays and of little drainage (26).

A directly proportional relationship between the soil moisture content and the distance traveled by *Azospirillum* was previously described (27). This suggests that the high humidity present in the sampled soil to which INCA LP-5 cultivated rice plants were subjected could have favored the movement of flagellated bacteria such as rhizobia towards the roots.

The isolation rizhobia process of the rhizosphere of rice cultivars INCA LP-5 was carried out using the cultural characteristics of the described colonies for the rhizobia group as the primary criterion (15). In the YMA medium, fast-growing strains give rise to mucous colonies of 1 to 5 mm after 3 to 5 days of incubation, whereas in slow-growing strains with a drier surface, colonies do not exceed a diameter of 1 mm in a period of ten days (15).

For the selection of the typical rhizobia colonies, another essential element was the lack of absorption of red congo, since the young cultures of these microorganisms generally form translucent, whitish or slightly pink colonies in the center (28). Taking into account the above, 78 colonies were isolated that had the typical size and mucosity characteristics of the rhizobia, as they were observed as large colonies (2-4 mm) and mucosa, at 2-3 days; except an isolate in which dry colonies 1-2 mm in diameter were visualized at 4-5 days of incubation.

Table I. Chemical characteristics of soil sampled at UCTB Los Palacios

pH (KCl)	MO (%)	P_2O_5 (mg 100 g $suelo^{-1}$)	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}
			(cmol kg^{-1})			
6,87±0,37	4,95±0,52	9,19±1,5	6,74±0,34	3,11±0,09	0,19±0,03	0,13±0,04

44,87 % of the 78 bacterial colonies were obtained from the rhizospheric soil samples, while the remaining 55,13 % were found in the rhizoplane of the rice plants. Of the latter group, 90 % of the isolates were detected slightly attached to the rhizoplane, since they were obtained in the serial dilutions that were made with the roots of the plants. The remaining 10% was identified on the epithelial tissue, being observed as a thin, mucous and whitish film covering the roots five days after placing them on the Luria-Bertani medium.

Gram staining was used as the second classification criterion. The results of this determination allowed grouping the 78 isolates in seven groups, considering the morphological characteristics of the bacterial cells, as well as the response to the Gram stain (Table II).

Table II. Bacterial groups identified in the rhizosphere soil and the rhizoplane of rice plants cultivate INCA LP-5, according to their morphological and dyeing characteristics of the bacterial cell

Groups	Micromorphological characteristic	Dyeing characteristics	Proportion (%)
1	Cocobacillus sporulated	negative Gram	42,3
2	Cocobacillus and thick bacilli no sporulated	negative Gram	16,67
3	Coccobacilli, thin bacilli or rounded no sporulated	negative Gram	17,95
4	Bacilli and sporulated cocobacilli	positive Gram	12,82
5	Coccos no sporulated	negative Gram	5,13
6	Curved bacilli not sporulated	positive Gram	3,85
7	Sporulated bacilli	negative Gram	1,28

The morphological and dying characterization allowed identifying three morphological types of cells: one cocobacillar (predominant) and another two where the cells presented the form of cocci or bacilli. Most of the rhizosphere isolates (42,3 %) corresponded to sporulated, Gram negative coccobacilli; while the less representative group (1,28 %) consisted of sporulated Gram negative bacilli.

Gram staining allowed observing a great diversity in the bacterial cells in the rhizosphere of INCA LP-5 cultivating rice plants. The high content of organic matter and phosphorus present in the soil of the rice fields of the UCTB Los Palacios could be favoring the establishment of the bacterial diversity observed in this research.

Rhizobia have been described as fine, Gram negative, non-sporulated bacilli (17). These characteristics are similar to those observed in 17,95 % of bacterial isolates (Table II, group 3); Since they were observed as coccobacilli or fine bacilli, with rounded and sometimes tuned ends, not sporulated and with negative response to Gram staining. This percentage corresponds to 14 isolates, of which 21,43 % (four isolates) were obtained from the rhizospheric soil and 78,57 % (eleven isolates) were obtained from the rhizoplane (Table III).

All the isolates that were studied produced semi-translucent colonies in the YMA medium (pH 6,8); however, a variety of tenuous colorations were observed towards the center of the colonies, especially in those isolated from the rhizospheric soil of rice plants. Results obtained by other authors differ from those obtained in this research, as they obtained whitish colonies in 100 % of the rhizobia isolates studied (29).

The ketolactase test was carried out in order to differentiate the isolates from the genus *Agrobacterium*, since both groups present similar cultural and morphological characteristics. On the other hand, the genus *Agrobacterium* is usually studied for its ability to produce galls or tumors in the roots and stem of the plants, a structure very similar to the nodules that form the rhizobia in leguminous plants (30).

The ketolactase test shows the ability of *Agrobacterium* (not in genera of the *Rhizobiaceae* family) to reduce copper oxide I (Cu_2O) present in the Benedict reagent used in this test (31). In this work all isolates selected as possible rhizobia, due to their cultural and morphological characteristics, resulted in negative ketolactase. This response corroborates the possible taxonomic location of the study isolates the rhizobia (32). In determining the ability to produce acid or base in YMA medium with bromothymol blue, a change in green to yellow coloring around bacterial growth was observed in all cases, indicating the ability of these microorganisms to produce acid.

The organic acids produced by rhizosphere microorganisms increase the availability of micronutrients such as phosphorus, iron, zinc and manganese in the soil, as pH decreases in the rhizosphere, or by the chelation of these micronutrients. The detoxification of metals in the soil has been reported as another function performed by the organic acids written by these microorganisms (33).

Table III. Isolates obtained from rice plants cultivating LP-5, morphological characteristics, dyeing and response to nodulation in siratro plants

Isolated	Site of isolation in the rhizosphere	Cultural characteristics of the colonies	Characteristics micromorphological and dyeing	Growth rate (days)	Effective nodulation in siratro
Rf1	S. rhizospheric	Semitraslucid, pale pink towards the center, mucous membranes, 2-3 mm	Cocobacilli, G-, not sporulated	2	+
Rf7	S. rhizospheric	Semitraslucid, pale red towards the center, mucous membranes, 1-2 mm	Fine bacilli, G-, not sporulated	4-5	+
Rf13	S. rhizospheric	Semitraslucid, pale orange toward the center, mucous membranes, 2-3 mm	Fine bacilli, G-, not sporulated	2	-
Rf33	S. rhizospheric	Semitraslucid, mucous, 2-3 mm	Cocobacilli, G-, not sporulated	2	-
Rpd3	Rhizoplane (DASR)	Semitraslucid, pale red toward the center, mucous membranes, 2-3 mm	Cocobacilli, G-, not sporulated	2	+
Rpd7	Rhizoplane (DASR)	Semitraslucid, pale red toward the center, mucous membranes, 2-3 mm	Cocobacilli, G-, not sporulated	2	+
Rpd8	Rhizoplane (DASR)	Semitraslucid, mucous, 2-3 mm	Cocobacilli, G-, not sporulated	2	+
Rpd10	Rhizoplane (DASR)	Semitraslucid, mucous, 2-3 mm	Cocobacilli, G-, not sporulated	2	-
Rpd16	Rhizoplane (DASR)	Semitraslucid, pale pink to the center, mucous membranes, 2-3 mm	Cocobacilli, G-, not sporulated	2	+
Rpd38	Rhizoplane (DASR)	Semitraslucid, mucous, 2-3 mm	Cocobacilli, G-, not sporulated	2	+
Rpr1	Rhizoplane (FASR)	Semitraslucid, mucous, 2-3 mm	Cocobacilli, G-, not sporulated	2	+
Rpr2	Rhizoplane (FASR)	Semitraslucid, mucous, 2-3 mm	Fine bacilli , G -, not sporulated	2	+
Rpr3	Rhizoplane (FASR)	Semitraslucid, mucous, 2-3 mm	Fine bacilli, G -, not sporulated	2	+
Rpr11	Rhizoplane (FASR)	Semitraslucid, mucous, 2-3 mm	Cocobacilli, G-, not sporulated	2	+

S. rhizospheric, Rhizospheric soil; FASR, strongly adhered to the radical surface (according to its acronyms in Spanish); DASR (according to its acronyms in Spanish), weakly attached to the root surface; G-, Gram negative; G +, Gram positive; (+), Nodule formation; (-), No nodule formation

Studies of the production of organic acids by rhizobia mainly focus on the plant growth promotion, such as 3-indoleacetic acid (AIA) and those that are related to the solubilization of phosphates in soils (34, 35). Rate of growth of rhizobia is one of the aspects to be taken into account during taxonomic studies, since its ability to multiply rapidly, moderately or slowly in the medium of cultivation, is a phenotypic characteristic closely related to the genus to which the species belong of this bacterial group (28).

The study of the growth rate of the different isolates on the YMA medium at pH 6,8 made it possible to detect rapid growth in most isolates, since at least one colony of these microorganisms could be detected on the culture medium at 48 hours of incubation.

The Rf7 isolate, unlike the rest, presented a moderate growth as its colonies were started to visualize from the fourth-fifth day of incubation (Table III). Only 8 to 20 % of the root surface is colonized by bacteria. A higher microbial establishment is observed in areas with greater exudation (36). Greater effectiveness in the colonization of the rhizosphere by certain microorganisms is due, among other factors, to its rapid multiplication. This ability allows them to use a large amount and diversity of nutrients present in radical exudates such as amino acids, organic acids, sugars, vitamins, enzymes, inorganic ions and gaseous molecules as sources of carbon, oxygen, nitrogen, phosphorus and sulfur (37).

The ability to produce nodules as part of the rhizobium-legume symbiosis is one of the premises to be taken into account for the taxonomic location of a rhizobacteria in the rhizobia group (8). Siratro is a model plant to evaluate the infection capacity and the effectiveness of nodulation of bacteria isolates under controlled conditions. This plant is used in research of this type, as it responds as a host to a broad spectrum of rhizobia strains from a great variety of legumes (38).

The *in vitro* inoculation test in siratro plants allowed observing that all but Rf13, Rf33 and Rpd10 produced effective nodules in the roots of these plants (Table III). The assay allowed to conclude that 11 bacterial isolates from the rhizosphere soil and the rhizoplane of rice plants INCA LP-5 belong to the group of rhizobia. These results constitute the first evidence in Cuba of the interaction of bacteria from the rhizobia group with rice cultivation.

The results obtained in this research were similar to those found in the first studies on the interaction of rhizobia with rice cultivation (39). In these investigations, it was demonstrated, through bioassays with legumes white clover (*Trifolium repens* var. Dutch) and alfalfa (*Medicago sativa* var. Gemini), that rhizobia were the endophytes that were found in rice plants. These microorganisms produced nodules in these legumes.

Advances in molecular techniques have allowed a generic relocation of bacterial species within the group of rhizobia, taking into account their ability to perform BNF and to form root nodules in leguminous plants. Currently 14 bacterial genera comprise the group of rhizobia, of which 11 are *Rhizobium, Mesorhizobium, Ensifer, Bradyrhizobium, Phyllobacterium, Microvirga, Azorhizobium, Ochrobactrum, Methylobacterium, Devosia* and *Shinella*, in the alpha-proteobacteria class; two: *Burkholderia* and *Cupriavidus*, within the beta-proteobacteria class and the genus *Pseudomonas* in the gamma-proteobacteria class (5).

In spite of the fact that the characterization that was carried out in this work is not sufficient to locate taxonomically the rhizobia isolates, in all of them; With the exception of Rf7, phenotypic aspects of the *Rhizobiaceae* family were identified (40). The formation of effective nodules in the BNF, the production of acid and rapid growth in YMA medium, would allow classifying them into the genera *Rhizobium*, *Ensifer* or *Shinella*, of the family *Rhizobiaceae*.

The Rf7 isolate, which also induces the formation of nodules, produces acid and has a moderate growth rate, could be located within the genus *Mesorhizobium*, in the family *Phyllobacteriaceae* (41, 42).

The determination of the presence of 11 rhizobia isolates in the rhizosphere of INCA LP-5 cultivating rice plants, results shown in this research, constitutes the first documented evidence in Cuba on the interaction of rhizobium-rice. The implementation of these results in basic research and in the production of bioproducts for rice cultivation opens new doors to the knowledge of this interaction in the country.

The isolation of rhizobia from the rhizosphere of INCA LP-5 cultivated rice plants in this study allowed the identification of a greater quantity and diversity of these microorganisms in the rhizoplane than in the rhizospheric soil of these plants (Table III). The rhizoplane is the rhizosphere zone most influenced by radical exudates and where the concentration of these compounds is maximal, because once in the soil, the exudates are dispersed (43). The presence of high concentrations of nutrients in the rhizoplane makes it an attractive area for microbial colonization. This could explain the greater number and diversity of rhizobia in the rhizoplane with respect to the rhizospheric soil.

Radical exudates establish a selective environment in the rhizosphere, in a way that favors the colonization of certain microorganisms and disfavor the development of others (38). Differences in the nutritional requirements of microorganisms in the rhizosphere, as well as their chemotactic responses, are of vital importance in the specificity between plants and microorganisms (44).

Differences in the mobility of each isolated rhizobium, its chemotactic response to radical exudates, the selective effect exerted by the specificity of these compounds, as well as the intrinsic genotypic and physiological characteristics of the INCA LP-5 rice cultivar may have influenced in the location of rhizobia in the rhizosphere of the plants studied.

In this research, it was possible to verify differences between the rhizobia isolates obtained from the rhizobium of the plants, taking into account their ability to adhere to the root surface. One group of these isolates was characterized by adhering more closely to the epidermal cells of the root and another maintained a less close relationship with the root surface (Table III). Four of the rhizobia isolates (Rpr1, Rpr2, Rpr3 and Rpr11) were strongly bound to the root surface. The capacity of rhizobia for the formation of biofilms on the rhizoplane has been documented, a mechanism by which it maintains a close relation with the exudates of the roots of the plants (45).

Genomic studies have provided important elements that explain the mechanisms involved in the expression of RapA1 genes. This protein has a crucial importance in the union of the rhizobia to the rhizoplane and in the formation of biofilms in the plant roots (39). The presence of flagella in the rhizobia, as well as the production of exopolysaccharides, also play an important role in the formation of these associations, since mutations in genes related to the synthesis of these components affect the stability of biofilms (40).

The inoculation effect of the 11 isolates that produced effective nodules in siratro plants and that are considered rhizobia is shown in Table IV.

In the plants inoculated with the Rpr11 isolate, the best results were observed in the number of nodules. Significant differences were observed between Rpr11 and the rest of the isolates except for Rpr3 in the number of total nodules. Both isolates also showed the highest percentages of EN.

In addition to the aforementioned isolates, Rpr1 and Rpr2 were outstanding, which surpassed the rest in the variables number of total nodules, number of effective total nodules and in the effectiveness of the process.

The abundant nodulation in the siratro plants that were inoculated with Rpr1, Rpr2, Rpr3 and Rpr11, microorganisms that were strongly adhered to the radical surface, allows suggesting the presence of mechanisms in these microorganisms that allow them to colonize more effectively different points of the root and invade the radical hairs of siratro plants. Both phases constitute the first stages of nodule formation (46, 47).

The nodules that are located on the main root have a greater effectiveness in the fixed nitrogen supply, which translates into an increase in the leaf development and yields of the crop. The proximity of these structures to the conducting vessels makes the microsymbiont have a greater access to the photosynthates, and the plant to the nitrogen from the BNF made by the rhizobia (48).

Table IV. Effect of the inoculation of rhizobial isolates obtained from the rhizosphere of rice cultivars INCA LP-5 on the nodulation of siratro plants and the nodulation process effectiveness, obtained from absolute values of the number of total nodules and the number of effective total nodules. The average range of each treatment in the variables related to the number of nodules and their effectiveness, resulting from the Mann-Witney test

Treatments	NNrp	NNerp	NNt	NNte	EN (%)
Control	29,00 d	31,50 d	15,50 e	24,50 e	0,00
Rf1	40,00 c	42,36 c	40,14 c	39,50 cd	33,71
Rf7	45,14 c	41,64 c	33,00 c	32,36 d	52,29
Rpd3	33,86 c	36,57c	27,50 cd	28,43 d	51,10
Rpd7	29,00 d	31,58 d	27,50 cd	28,43 d	51,11
Rpd8	33,86 c	31,53 d	29,50 cd	29,14 d	52,01
Rpd16	29,00 d	31,61d	27,50 cd	28,43 d	50,00
Rpd38	33,86 c	31,70 d	30,21 cd	28,43 d	26,14
Rpr1	45,07 c	41,93 c	62,86 b	58,14 c	73,09
Rpr2	42,86 c	41,07 c	56,50 b	57,64 c	75,54
Rpr3	67,36 b	67,93 b	73,79 a	74,00 b	94,42
Rpr11	128,52 a	135,86 a	75,45 a	95,32 a	87,37

NNrp, number of nodules in the main root; NNerp, number of effective nodules in the main root; NNt, number of total nodules; NNte-Number of total effective nodules; EN, effectiveness of nodulation. Equal letters do not differ significantly (Tukey $\alpha = 0.05$ and $n = 7$)

The ammonium that is produced during the fixation of atmospheric nitrogen by the rhizobia cells is used by the legume for the synthesis of amino acids, enzymes and structural proteins (49). The dissection of the nodules presents in the roots of the siratro plants allowed to observe that all the bacterial isolates produced effective nodules, since a reddish coloration was observed inside these structures, leghemoglobin presence characteristic. This protein is an indicator of the effectiveness of nodules to perform the nitrogen fixation process and the successful establishment of rhizobioleguminous symbiosis (20). The presence of a large number of nodules in the main root of the syrinx plants inoculated with Rpr11, many of which were effective in the BNF, could explain the positive effect of this isolate on the dry mass of plant roots (Figure).

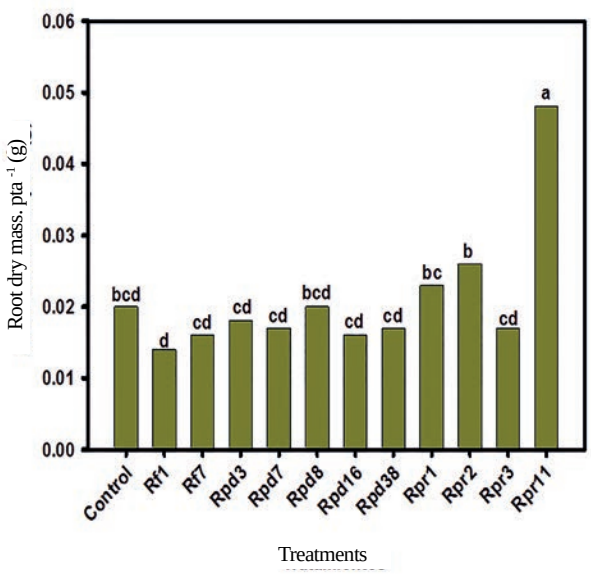

Figure. Effect of inoculation of rhizobial isolates from the rhizosphere of INCA LP-5 rice plants in the root dry mass of siratro plants. Equal letters do not differ significantly ES ** = 0.003, n = 7

The number and effectiveness of plant nodules inoculated with Rpr3 exceeded those where Rpr2 was used. However, this microorganism produced a greater effect on radical growth. The behavior may be due to increased activity of the nitrogenase enzyme in Rpr2. This isolate would then produce higher ammonium levels than Rpr3, translating into an increase in the root growth of siratro plants.

Similar results were found in soybeans, as increases in plant height were observed in response to an increase in the activity of the nitrogenase enzyme (50). The isolates that produced the highest number of nodules in the roots of the plants (Rpr1, Rpr2, Rpr3 and Rpr11) coincided with those with the highest percentages of EN, and in the case of Rpr11 also with a higher effect on mass Dry radical. This assay confirms the membership of these isolates to the group of rhizobia and some are outstanding in their ability to nodulate siratro plants.

CONCLUSIONS

The characteristics of the Gley Nodular Ferruginous Petroferric soil of the UCTB "Los Palacios", Pinar del Río, led to the proliferation of rhizobia populations in the rhizosphere of rice plants cultivar INCA LP-5. This research allowed having new isolates of rhizobia that they defended in the interaction level that they established in the rhizosphere of the rice plants and in their effectiveness in the siratro nodulation. These findings constitute the first evidence in Cuba that addresses the interaction of rhizobia with rice cultivation.

BIBLIOGRAPHY

1. MINAGRI. *Instructivo Técnico del Arroz*. Ed. Centro Nacional de Sanidad Vegetal, Instituto de Investigaciones del Arroz, 2005, Cuba, 22 p., ISBN 959-246-037-X.
2. Biau, A.; Santiveri, F.; Mijangos, I. y Lloveras, J. "The impact of organic and mineral fertilizers on soil quality parameters and the productivity of irrigated maize crops in semiarid regions". *European Journal of Soil Biology*, vol. 53, 2012, pp. 56-61, ISSN 1164-5563, DOI 10.1016/j.ejsobi.2012.08.008.
3. Mehnaz, S. "Azospirillum: A Biofertilizer for Every Crop" [en línea]. En: ed. Kumar N. A., *Plant Microbes Symbiosis*: Applied Facets, Ed. Springer India, New Delhi, 2015, pp. 297-314, ISBN 978-81-322-2067-1, [Consultado: 16 de noviembre de 2016], Disponible en: <http://link.springer.com/10.1007/978-81-322-2068-8_15>.
4. Hernández, R. A.; Rives, R. N.; Acebo, G. Y.; Díaz, de la O. A.; Heydrich, P. M. y Divan, B. V. L. "Potencialidades de las bacterias diazotróficas asociativas en la promoción del crecimiento vegetal y el control de *Pyricularia oryzae* (Sacc.) en el cultivo del arroz (*Oryza sativa* L.)". *Revista de Protección Vegetal*, vol. 29, no. 1, 2014, pp. 1-10, ISSN 1010-2752.
5. Berrada, H. y Fikri-Benbrahim, K. "Taxonomy of the Rhizobia: Current Perspectives". *British Microbiology Research Journal*, vol. 4, no. 6, 2014, pp. 616-639, ISSN 2231-0886, DOI 10.9734/BMRJ/2014/5635.
6. Chen, C. y Zhu, H. "Are common symbiosis genes required for endophytic rice-rhizobial interactions?". *Plant Signaling & Behavior*, vol. 8, no. 9, 2013, ISSN 1559-2324, DOI 10.4161/psb.25453, [Consultado: 16 de noviembre de 2016], Disponible en: <http://www.tandfonline.com/doi/abs/10.4161/psb.25453>.

7. Mia, M. A. B.; Shamsuddin, Z. H. y Mahmood, M. "Effects of rhizobia and plant growth promoting bacteria inoculation on germination and seedling vigor of lowland rice". *African Journal of Biotechnology*, vol. 11, no. 16, 2012, pp. 3758–3765, ISSN 1684-5315, DOI 10.5897/AJB09.1337.
8. Hasan, M.; Bano, A.; Hassan, S. G.; Iqbal, J.; Awan, U.; Rong-ji, D. y Khan, K. A. "Enhancement of rice growth and production of growth-promoting phytohormones by inoculation with *Rhizobium* and other rhizobacteria". *World Applied Sciences Journal*, vol. 31, no. 10, 2014, pp. 1734-1743, ISSN 1818-4952, DOI 10.5829/idosi.wasj.2014.31.10.364.
9. Oniani, O. G. "Determinación del fósforo y potasio del suelo en una misma solución de los suelos Krasnozen y Podsólicos en Georgia". *Agrojima*, vol. 6, 1964, p. 25.
10. Walkley, A. y Black, I. A. "An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method."*Soil Science*, vol. 37, no. 1, 1934, pp. 29–38, ISSN 0038-075X, 1538-9243.
11. Wang, T.; Romero-Martínez, J. y López-Lara, I. "*Rhizobium* y su destacada simbiosis con plantas". En: *Microbiología en línea,* Ed. Universidad Nacional Autónoma de México, México, 2001, pp. 344-386, ISBN 978-968-36-8879-8.
12. Vincent, J. M. *A manual for the practical study of root-nodule bacteria*. (ser. IBP Handbook, no. ser. 15), Ed. International Biological Programme, 1970, Oxford, 164 p., ISBN 978-0-632-06410-6.
13. Bertani, G. "Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli". *Journal of Bacteriology,* vol. 62, no. 3, 1951, pp. 293-300, ISSN 0021-9193.
14. Knief, C.; Delmotte, N.; Chaffron, S.; Stark, M.; Innerebner, G.; Wassmann, R.; von Mering, C. y Vorholt, J. A. "Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice". *The ISME Journal*, vol. 6, no. 7, 2012, pp. 1378-1390, ISSN 1751-7362, DOI 10.1038/ismej.2011.192.
15. Norris, D. O. y Date, R. A. "Legume bacteriology". En: Shaw N. H. y Bryan W. W., Tropical pasture research: principles and methods, (ser. Commonwealth Bureau of Pastures and Field Crops, no. ser. 51), Ed. *Commonwealth Agricultural Bureaux,* Farnham Royal, England, 1976, pp. 134-174, ISBN 978-0-85198-358-5.
16. Frioni, L. "Ecología microbiana del suelo". En: *Procesos Microbianos*, Ed. Editorial de la fundación universidad nacional de rio cuarto, Montevideo, 1990, pp. 205–253, ISBN 950-665-110.
17. Thies, J. E.; Singleton, P. W. y Bohlool, B. B. "Modeling Symbiotic Performance of Introduced Rhizobia in the Field by Use of Indices of Indigenous Population Size and Nitrogen Status of the Soil". *Applied and Environmental Microbiology,* vol. 57, no. 1, 1991, pp. 29-37, ISSN 0099-2240, 1098-5336.
18. López-López, A.; Rogel-Hernández, M. A.; Barois, I.; Ortiz Ceballos, A. I.; Martínez, J.; Ormeno-Orrillo, E. y Martínez-Romero, E. "*Rhizobium grahamii* sp. nov., from nodules of *Dalea leporina*, *Leucaena leucocephala* and *Clitoria ternatea*, and *Rhizobium mesoamericanum* sp. nov., from nodules of *Phaseolus vulgaris*, siratro, cowpea and *Mimosa pudica*". *International Journal of Systematic and Evolutionary Microbiology*, vol. 62, no. 9, 2012, pp. 2264-2271, ISSN 1466-5026, 1466-5034, DOI 10.1099/ijs.0.033555-0.
19. Vázquez-Limón, C.; Hoogewijs, D.; Vinogradov, S. N. y Arredondo-Peter, R. "The evolution of land plant hemoglobins". *Plant Science*, vol. 191-192, 2012, pp. 71-81, ISSN 0168-9452, DOI 10.1016/j.plantsci.2012.04.013.
20. Sigarroa, A. *Biometría y Diseño Experimental*. 1.ª ed., Ed. Pueblo y Educación, 1985, La Habana, Cuba, 393 p.
21. Allen, M. E. *Kolmogorov-Smirnov test for discrete distributions* [en línea]. Master Thesis, Naval Postgraduate School, marzo de 1976, Monterey, California, 93 p., [Consultado: 26 de mayo de 2016], Disponible en: <http://calhoun.nps.edu/handle/10945/17830>.
22. Tukey, J. W. "Bias and confidence in not quite large samples". *The Annals of Mathematical Statistics*, vol. 29, no. 2, junio de 1958, pp. 614-623, ISSN 0003-4851, DOI 10.1214/aoms/1177706647.
23. Mesa, A.; Naranjo, M.; Cancio, R.; Martín, A.; Clemente, B.; Suárez, O. y Pacheco, E. Manual de interpretación de los índices físico-químicos y morfológicos de los suelos cubanos. 2.a ed., Ed. Pueblo y Educación, 1984, La Habana, Cuba, 10 p.
24. Marquina, M. E.; González, N. E. y Castro, Y. "Caracterización fenotípica y genotípica de doce rizobios aislados de diversas regiones geográficas de Venezuela". *Revista de Biología Tropical*, vol. 59, no. 3, 2011, pp. 1017-1036, ISSN 0034-7744.
25. Ruiz, S. "Influencia de microorganismos sobre caracteristicas fisicoquimicos de los suelos de cultivo de cacao (*Theobroma cacao* L.), en tingo maria". *Revista Investigación y Amazonía,* vol. 2, no. 1,2, 2013, pp. 67-74, ISSN 2224-445X.
26. Hernández, A.; Ascanio, M. O.; Morales, M. y León, A. *La historia de la clasificación de los suelos de Cuba*. Ed. Félix Varela, 2006, La Habana, Cuba, 98 p., ISBN 959-07-0145-0.
27. Bashan, Y.; Holguín, G. y Ferrera-Cerrato, R. "Interacciones entre plantas y microorganismos benéficos. III. Procedimientos para el aislamiento y caracterización de hongos micorrízicos y rizobacterias promotoras del crecimiento de plantas". *Terra*, vol. 14, no. 2, 1996, pp. 159-194, ISSN 0187-5779.
28. Durán, D.; Rey, L.; Sánchez-Cañizares, C.; Jorrín, B.; Imperial, J. y Ruiz-Argüeso, T. "Biodiversity of Slow-Growing Rhizobia: The Genus Bradyrhizobium". En: eds. Rodelas G. M. B. y Gonzalez-López J., *Beneficial plant-microbial interactions: ecology and applications*, Ed. CRC Press, Boca Raton, FL, 2013, pp. 20-37, ISBN 978-1-4665-8717-5.
29. Mohamed, A. T. H. y Saifelden, A. M. "Diversity of *Rhizobium leguminosarum* bv. viceae Strains Isolated from Different Schemes in Shendi Area". *Extensive Journal of Applied Sciences,* vol. 3, no. 1, 2015, pp. 1-10, ISSN 2409-9511.
30. Peix, A.; Ramírez-Bahena, M. H.; Velázquez, E. y Bedmar, E. J. "Bacterial Associations with Legumes". *Critical Reviews in Plant Sciences*, vol. 34, no. 1-3, 2015, pp. 17-42, ISSN 0735-2689, 1549-7836, DOI 10.1080/07352689.2014.897899.
31. Clark, A. G. "A Selective Medium for the Isolation of Agrobacterium Species". J*ournal of Applied Bacteriology,* vol. 32, no. 3, septiembre de 1969, pp. 348-351, ISSN 0021-8847, DOI 10.1111/j.1365-2672.1969.tb00982.x.
32. Lloret, L. y Martínez-Romero, E. "Evolución y filogenia de *Rhizobium*". *Revista Latinoamericana de Microbiología,* vol. 47, no. 1-2, 2005, pp. 43–60, ISSN 0034-9771.
33. Singh, Y.; Ramteke, P. W. y Shukla, P. K. "Characterization of *Rhizobium isolates* of pigeon pea rhizosphere from Allahabad soils and their potential pgpr characteristics". *International Journal of Research in Pure and Applied Microbiology*, vol. 3, no. 1, 2013, pp. 4–7, ISSN 2277-3843.

34. Panhwar Q. A. "Root colonization and association of phosphate- solubilizing bacteria at various levels of triple supper phosphate in aerobic rice seedlings". African *Journal of Microbiology Research*, vol. 6, no. 10, 2012, pp. 2277-2286, ISSN 1996-0808, DOI 10.5897/AJMR11.916.
35. Zúñiga, A.; Poupin, M. J.; Donoso, R.; Ledger, T.; Guiliani, N.; Gutiérrez, R. A. y González, B. "Quorum Sensing and Indole-3-Acetic Acid Degradation Play a Role in Colonization and Plant Growth Promotion of *Arabidopsis thaliana* by *Burkholderia phytofirmans* PsJN". *Molecular Plant-Microbe Interactions*, vol. 26, no. 5, 2013, pp. 546-553, ISSN 0894-0282, DOI 10.1094/MPMI-10-12-0241-R.
36. de la Fe Pérez, Y.; Díaz de la Osa, A.; Restrepo-Franco, G. M.; Diván-Baldani, V. L. y Hernández-Rodríguez, A. "Diversidad de bacterias diazotróficas asociativas potencialmente eficientes en cultivos de importancia económica". *Revista Cubana de Ciencias Biológicas*, vol. 4, no. 1, 2015, pp. 17-26, ISSN 2307-695X.
37. Badri, D. V.; Chaparro, J. M.; Zhang, R.; Shen, Q. y Vivanco, J. M. "Application of Natural Blends of Phytochemicals Derived from the Root Exudates of Arabidopsis to the Soil Reveal That Phenolic-related Compounds Predominantly Modulate the Soil Microbiome". *Journal of Biological Chemistry,* vol. 288, no. 7, 2013, pp. 4502-4512, ISSN 0021-9258, 1083-351X, DOI 10.1074/jbc.M112.433300.
38. Lima, A. S.; Nóbrega, R. S. A.; Barberi, A.; da Silva, K.; Ferreira, D. F. y Moreira, F. M. de S. "Nitrogen-fixing bacteria communities occurring in soils under different uses in the Western Amazon Region as indicated by nodulation of siratro (*Macroptilium atropurpureum*)". *Plant and Soil*, vol. 319, no. 1-2, 2009, pp. 127-145, ISSN 0032-079X, 1573-5036, DOI 10.1007/s11104-008-9855-2.
39. Yanni, Y. G.; Rizk, R. Y.; Corich, V.; Squartini, A.; Ninke, K.; Philip-Hollingsworth, S.; Orgambide, G.; de Bruijn, F.; Stoltzfus, J.; Buckley, D.; Schmidt, T. M.; Mateos, P. F.; Ladha, J. K. y Dazzo, F. B. "Natural endophytic association between *Rhizobium leguminosarum* bv. trifolii and rice roots and assessment of its potential to promote rice growth". *Plant and Soil*, vol. 194, no. 1/2, 1997, pp. 99-114, ISSN 0032-079X, DOI 10.1023/A:1004269902246.
40. Santi, C.; Bogusz, D. y Franche, C. "Biological nitrogen fixation in non-legume plants". *Annals of Botany*, vol. 111, no. 5, 2013, pp. 743-767, ISSN 0305-7364, 1095-8290, DOI 10.1093/aob/mct048.
41. Degefu, T.; Wolde-meskel, E.; Liu, B.; Cleenwerck, I.; Willems, A. y Frostegard, A. "*Mesorhizobium shonense* sp. nov., *Mesorhizobium hawassense* sp. nov. and *Mesorhizobium abyssinicae* sp. nov., isolated from root nodules of different agroforestry legume trees". *International Journal of Systematic and Evolutionary Microbiology*, vol. 63, no. 5, 2013, pp. 1746-1753, ISSN 1466-5026, 1466-5034, DOI 10.1099/ijs.0.044032-0.
42. Zheng, W. T.; Li, Y.; Wang, R.; Sui, X. H.; Zhang, X. X.; Zhang, J. J.; Wang, E. T. y Chen, W. X. "*Mesorhizobium qingshengii* sp. nov., isolated from effective nodules of Astragalus sinicus". *International Journal of Systematic and Evolutionary Microbiology*, vol. 63, no. 6, 2013, pp. 2002-2007, ISSN 1466-5026, 1466-5034, DOI 10.1099/ijs.0.044362-0.
43. de Lima, T. E. F.; Bezerra, J. L. y de Queiroz, C. M. A. "Fungi from the rhizosphere and rhizoplane from the grapevine *Vitis labrusca* in Pernambuco, Brazil". *Nova Hedwigia*, vol. 99, no. 3, 2014, pp. 531-540, ISSN 0029-5035, DOI 10.1127/0029-5035/2014/0204.
44. Philippot, L.; Raaijmakers, J. M.; Lemanceau, P. y van der Putten, W. H. "Going back to the roots: the microbial ecology of the rhizosphere". *Nature Reviews Microbiology,* vol. 11, no. 11, 2013, pp. 789-799, ISSN 1740-1526, 1740-1534, DOI 10.1038/nrmicro3109.
45. Nogales, J.; Pérez-Mendoza, D.; Gallegos, M. T. y Soto, M. J. "Importance of Motile and Biofilm Lifestyles of Rhizobia for the Establishment of Symbiosis with Legumes". En: Rodelas G. M. B. y Gonzalez-López J., Beneficial plant-microbial interactions: ecology and applications, Ed. CRC Press, Boca Raton, FL, 2013, pp. 47-55, ISBN 978-1-4665-8717-5.
46. Frederix, M.; Edwards, A.; Swiderska, A.; Stanger, A.; Karunakaran, R.; Williams, A.; Abbruscato, P.; Sanchez-Contreras, M.; Poole, P. S. y Downie, J. A. "Mutation of praR in Rhizobium leguminosarum enhances root biofilms, improving nodulation competitiveness by increased expression of attachment proteins". *Molecular Microbiology*, vol. 93, no. 3, 2014, pp. 464-478, ISSN 0950-382X, DOI 10.1111/mmi.12670.
47. Murray, J. D. "Invasion by Invitation: Rhizobial Infection in Legumes". *Molecular Plant-Microbe Interactions,* vol. 24, no. 6, 2011, pp. 631-639, ISSN 0894-0282, DOI 10.1094/MPMI-08-10-0181.
48. McDermott, T. R. y Graham, P. H. "Bradyrhizobium japonicum Inoculant Mobility, Nodule Occupancy, and Acetylene Reduction in the Soybean Root System". *Applied and Environmental Microbiology*, vol. 55, no. 10, 1989, pp. 2493-2498, ISSN 0099-2240, 1098-5336.
49. Gil-Quintana, E.; Larrainzar, E.; Seminario, A.; Díaz-Leal, J. L.; Alamillo, J. M.; Pineda, M.; Arrese-Igor, C.; Wienkoop, S. y Gonzalez, E. M. "Local inhibition of nitrogen fixation and nodule metabolism in drought-stressed soybean". *Journal of Experimental Botany*, vol. 64, no. 8, 2013, pp. 2171-2182, ISSN 0022-0957, 1460-2431, DOI 10.1093/jxb/ert074.
50. Mubarik, N. R. y Sunatmo, T. I. "Symbiotic of nitrogen fixation between acid aluminium tolerant *Bradyrhizobium japonicum* and soybean". En: Ohyama T., *Advances in biology and ecology of nitrogen fixation*, Ed. Intech, Rijeka, Croatia, 2014, pp. 259-272, ISBN 978-953-51-1216-7.

Received: October 9th, 2015
Accepted: April 22th, 2016