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CHARACTERIZATION OF RHIZOBIA ISOLATED FROM COWPEA NODULES, IN CUBAN SALINE SOILS

Caracterización de rizobios aislados de nódulos de frijol caupí, en suelos salinos de Cuba

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ABSTRACT. In soils of Cauto River Valley (Granma, Cuba) affected by salts, six new bacteria isolated from nodules of *Vigna unguiculata* (L.) Walp were obtained. The six isolates were subjected to differents salt levels (0,17- 6,6 dS m^{-1} of NaCl), pH levels (4,5 - 9) and temperatures (28 - 45 °C) with the objective to determine their tolerance to these abiotic stresses. Variation within the 16S rRNA gene, was examined by amplified 16S rDNA restriction analysis (ARDRA) and direct sequencing to show genetic diversity. Three isolates (VIBA-1, VIBA-2 and VIBA-6) achieved similar results as the control with 2,6 and 3,4 dS m^{-1} of NaCl. All of the isolates could grow at pH 7 and 9 and got growth until 40 °C, only two of them (VIBA-4 and VIBA-5) grew at 45 °C. Phylogeny analysis confirmed the close relationship of one isolate with *Bradyrhizobium liaoningense* (VIBA-1) and four isolates with *Bradyrhizobium yuanmingense* (VIBA-2, VIBA-3, VIBA-5 and VIBA-6) and one to *Rhizobium radiobacter* (formely *Agrobacterium tumefaciens*, VIBA-4). All of them, with the exception of VIBA-4, were able to nodulate in the plants when they were inoculated. The survival of these strains in the different abiotic stress (salinity, alkalinity and temperature), evidenced their ability to grow under this specific environment. The findings indicated that under Cuban saline soils there are *Bradyrhizobium* strains able to establish symbiosis with cowpea, but the diversity is still low, being characterized only two different species able to nodulate.

Key words: *Bradyrhizobium* sp, molecular identification, nodulation, salinity, tolerance, *Vigna unguiculata*

RESUMEN. A partir de suelos del Valle del Río Cauto (Granma, Cuba) afectados por sales, se aislaron seis bacterias procedentes de nódulos de *Vigna unguiculata* (L.) Walp. Los aislados se sometieron a diferentes niveles de sales (0,17 - 6,6 dS m^{-1} de NaCl), pH (4,5 - 9) y temperatura (28 - 45 °C), con el objetivo de determinar su tolerancia a estos tipos de estrés. La variación en el gen 16S rRNA, se examinó a través del análisis de restricción de amplificados 16S rDNA (ARDRA) y secuenciación directa. Tres aislados, VIBA-1, VIBA-2 y VIBA-6, mostraron resultados similares al control (0,17 dS m^{-1}) con 2,6 y 3,4 dS m^{-1} de NaCl. Todas las cepas crecieron a pH 7 y 9 hasta 40 °C, solo dos de ellas (VIBA-4 y VIBA-5) crecieron a 45 °C. El análisis de filogenia, confirmó la estrecha relación de la cepa *Bradyrhizobium liaoningense* con el aislamiento VIBA-1, y los aislamientos VIBA-2, VIBA-3, VIBA-5 y VIBA-6, con la cepa *Bradyrhizobium yuanmingense*, y uno de ellos a *Rhizobium radiobacter* (formalmente *Agrobacterium tumefaciens*, VIBA-4). Todos los aislados con excepción de VIBA-4, formaron nódulos en las plantas cuando fueron inoculadas. La sobrevivencia de estas cepas en condiciones de estrés (salinidad, alcalinidad y temperatura), evidencia su habilidad para crecer bajo estas condiciones ambientales específicas. Los resultados indicaron que, en un entorno de suelos salinos de Cuba, existen cepas de *Bradyrhizobium* que pueden establecer simbiosis con Caupí, pero la diversidad de estos microorganismos es aún escasa, debido a que sólo dos especies lograron nodular la leguminosa.

Palabras clave: *Bradyrhizobium* sp, identificación molecular, nodulación, salinidad, tolerancia, *Vigna unguiculata*

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INTRODUCTION

Salinity is one of the main problems in agricultural ecosystems in the world; near 50 % of the planet is affected by this scourge (1, 2). Cuba is not the exception, since around one million hectares of saline soils have been informed. Cauto River Valley, located in the eastern region of the country, has 228 thousand hectares affected by salinity; among them, 28 % are classified as strongly saline and 11 % very strongly saline (3). Numerous damages have been produced on the crops yield, even some cultivation areas have been abandoned, caused by the capacity loss of plants species to grow in these saline environments (3).

On the other hand, it is known that salinity not only impair physiologically and biochemically to the plants. Indeed, the microbial communities, among them rhizobia bacteria, decrease in number, diversity and activity, when the soils give up cultivating and when the environmental stress conditions, like soil salinity, temperature, pH, heavy metals have been found (4,5).

In this situation, a natural and ecological option to diminish the harmful effect of salts, and to keep the production under saline-stress, may be the selection and introduction of plants and/or microorganisms adapted to these conditions (6).

In this sense, the legume cowpea is an option reported as rustic specie against adverse environments such as drought tolerance, high temperature, heavy metals and saline stress, but rather also may contribute with appreciable amount of nitrogen (150 kg N ha^{-1}) from symbiotic fixation to the ecosystem (7,8). Nevertheless, the isolation of native rhizobial strains fitted to specific abiotic stresses and able to establish efficient symbiosis with cowpea is a compulsory practice in saline areas of Granma province (Cauto River Valley) Cuba, since there are not commercial strains to cover the *Vigna* inoculation for these soils. Moreover, the inoculation of cowpea seeds and seedlings with appropriate native bradyrhizobia can guarantee root nodulation and have demonstrated to enhance plant production (9).

This study was carry out to isolate, evaluate the abiotic stress-tolerance (saline, pH and high temperature) and to perform the genetic identificacion of natives rhizobial bacteria from saline soils to gain a collection of cowpea bioinoculants from saline soils of Cauto River Valley in Cuba.

MATERIAL AND METHODS

Bacterial isolation

The isolates used in this study were obtained from root nodules of Cowpea varieties [*Vigna unguiculata* (L.) Walp.] grown in saline soils of Babiney (with 4,76 dS m^{-1}) of electrical conductivity (E.C) and Jiguaní (5,8 dS m^{-1} E.C) municipalities, two localities of Cauto River Valley (Granma Province, Cuba). 12 reference strains from international collection were used as control pattern. The isolates, origin sites, and the reference strains are listed in Table I. The isolation of bacteria was performed from nodules recovered directly from the cowpeas established in experimental areas. All nodules were desinfectedon the surface with ethanol (95 % v/v) for 30 s, then subjected to $HgCl_2$ 0,1 % (p/v) for 45 s, and washed with sterile distilled water. Nodules were then cut, and a loopful of infected cell transferred to solid yeast extract mannitol (YEM) medium (10). Single colonies were obtained and checked for purity by repeated streaking and by microscopic examination. Isolates were routinely cultivated at 28 ºC in liquid YEM medium (10). Reference strains from international collections were used as control pattern (Table II).

Table I. Survival of isolates obtained from root nodules of cowpea and reference bradyrhizobial strain under high temperature conditions

Cepas	Temperatura (ºC)[a]			
	28	37	40	45
VIBA-1	+ +	+ +	+ +	-
VIBA-2	+ +	+ +	+ +	-
VIBA-3	+ +	+ +	+ +	-
VIBA-4	+ +	+ +	+ +	+
VIBA-5	+ +	+ +	+ +	+
VIBA-6	+ +	+ +	+ +	-
Bradyrhizobium yuanmingense CCBAU 10071[T]	+ +	+ +	+ +	-

[a] Growth was represented as -, no growth; +, good growth (40-80% compared to the control); + +, very good growth (equal to the control). (Values represent the average of three experiments with three replicates each time). [T] Type strain

Table II. Isolated strains from Gramma province and rhizobia used as controls studies, provenance, original host, ARDRA groups and genetic characterization based on chromosomal 16S rRNA gene

Aislados/especies	Origen geográfico/Coordenadas [a]	Hospedero original	Caracterización genetica [b]	
			ARDRA	16S rRNA gene
VIBA-1[c]	Jiguaní/20º 22.3´ 12´´N, 76º 27´ 56´´ W	*V. unguiculata*	1	*Bradyrhizobium liaoningense*
VIBA-2[c]	Babiney/20º 26´ 58´´N, 76º 32´ 15´´W	*V. unguiculata*	2	*B. yuanmingense*
VIBA-3	Babiney/20º 26´ 45´´N, 76º 32´ 9´´W	*V. unguiculata*	2	*B. yuanmingense*
VIBA-4	Jiguaní/20º 22.3´ 53´´N 76º 27´ 45´´ W	*V. unguiculata*	3	*Agrobacterium tumefaciens*
VIBA-5	Babiney/20º 26´ 38´´N, 76º 32´ 35´´W	*V. unguiculata*	2	*B. yuanmingense*
VIBA-6	Babiney/20º 26´ 40´´N, 76º 32´ 43´´W	*V. unguiculata*	2	*B. yuanmingense*
Cepas de referencia[d]				
CECT 4651 [T]	México	*Phaseolus vulgaris*	4	*Rhizobium etli*
CECT 4846 [T]	España	*Cicer arientinum*	5	*Mesorhizobium ciceri*
CECT 530 [T]	Japón	*Glycine hispida*	6	*B. japonicum*
CIFA ISLU-16	España	*Lupinus albus*	7	*Bradyrhizobium. sp*. (*Lupinus*)
CIFA GR-4	España	*Medicago sativa*	8	*Sinorhizobium meliloti*
NZP 2037	Nueva Zelanda	*Lotus divaricatus*	9	*Mesorhizobium loti*
CECT 4113 [T]	USA	*Pisum sativum*	4	*R. pisi*
CCMA GH-1	España	*P. sativum*	10	*R. leguminosarum* bv. *Viceae*
CCMA HSV-1	España	*Vicia faba* L.	11	*R. leguminosarum* bv. *Viceae*
CECT 4116[T]	USA	*Trifolium praetense*	11	*R. leguminosarum* bv. *Trifolii*
CCBAU 10071[T]	China	*Lespedeza cuneata*	2	*Bradyrhizobium yuanmingense*
USDA 76	USA	*Glycine soja*	12	*B. elkanii*

[a] Geographic location from different areas of Gramma province (Cuba).
[b] Specific patterns obtained from ARDRA of 16S rDNA digested with endonucleases *Msp*I, *Hinf*I, *Dde*I and *Hha*I, respectively. Different numbers were assigned to represent each ARDRA group. 16S rRNA gene determined by the comparison of the full nucleotide sequence with Genbank database.
[c] Previously identified and strains employed in field experiments (VIBA-1, FJ941843; VIBA-2, FJ941844).
[d] CECT, Colección Española de Cultivos Tipo; CIFA, Centro de Investigación y Formación Agraria-Las Torres-Tomejil, Sevilla; NZP, Division of Scientific and Industrial Research, Palmeston North, New Zealand; CCMA, Centro de Ciencias Medioambientales, CSIC; CCBAU, Culture Collection of Beijing Agricultural University, Beijing, People's Republic of China; USDA, United States Department of Agriculture, Beltsville, Md.
[T] Type strains

Phenotypic characterization

In order to evaluate their tolerance capacity to abiotic stresses (VIBA-1, VIBA-2, VIBA-3, VIBA-4, VIBA-5 and VIBA-6) and the reference strains CCBAU 10071 (*Bradyrhizobium yuanmingense*) were subjected to salt, pH and temperature extremes (11).

A standard culture to carry out the experiments was used (50 µl of culture in exponential phase on 5 ml of final culture, dilution 1:100). Five ml of liquid sterile YEM medium were put in sterilized Vimex glass tubes.

Each strain was sowed under sterile conditions and then, put it in incubator at 28 ºC and 100 rpm until reaching the exponential growth.

Saline-stress tolerance

Each isolate was subjected to 0,17; 2,6; 3,4; 4,2; 5,00; 5,8 and 6,6 dS m^{-1} of NaCl respectively and then placed in three glass tubes. NaCl level recommended to *Rhizobium* cultures (0,1 g L^{-1}=1,7 mM= 0,17 dS m^{-1}) was considered as control (10). The microbial growth of each treatment was determined to 120 h after the cultures were sown (stationary phase) through optic density (absorbance measurement to 680 nm) by using a spectrophotometer Spectronic 2000 (Bausch&Lomb).

pH-stress tolerance

The isolates were subjected to three treatments from culture YEM medium buffered to pH 4,5, 7 and 9. The 2.2-dimethylsuccinic acid (30 mM) and tris-hydrochloride (50 mM) were used to obtain solutions at pH 4,5 and pH 9, respectively. The pHs were checked after autoclaving and did not change by more than ± 0,1 unit. The microbial growth of each treatment was followed as in salinity assays.

Temperature-stress tolerance

The isolates were cultured in Petri plates on solid YEM medium (10, and subjected to 28 (control), 37, 40, 45 ^{o}C, in which their growth were observed every 24 hours for 12 days (11).

Nodulation test

An inoculation test to evaluate the isolates ability to form roots nodules was carried out (10). The plants of Cowpea were inoculated with all native strains, a control without inoculation was used. The seedlings were grown in a growth chamber under 14 h light (23 ºC)/10 h dark (20 ºC) photoperiod during one month. Plants were watered with sterlized distilled water during the first week after sowing and with nutrient solution without nitrogen the rest of the time.

Molecular characterization

Isolation of genomic DNA

A collection of known rhizobial strains was employed as reference (Table II).

A loopful of each isolate was used to inoculate 20 ml of YEM broth. After incubation (10), the cells were harvested, washed twice with phosphate buffered saline (PBS) and DNA was isolated using UltraClean TM Microbial DNA Isolation Kit (MOBIO). The concentration and integrity of DNA was analyzed by electrophoresis with 0,8 % agarose gels and compared to known amounts of phage lambda DNA (12).

PCR amplification of 16S rDNA

The 16S rDNA gene of all strains was amplified by PCR. The primers fD1 and rP2 were employed at final concentration of 1µM and synthesized by Genotek (Spain). The amplification was developed using a thermocycler (Veriti 96 Well, Applied Biosystems) (13).

Restriction analysis (ARDRA)

The restriction endonucleases *Msp*I, *Hinf*I, *Dde*I and *Hha*I (New England Biolabs) were used in separate digestion reactions with PCR-amplified 16S rDNA from all isolated listed in Table II. A 15 µl portion of amplification reactions was digested according to the manufacturer's recommendations and restriction analyses of amplified 16S rDNA (ARDRA) (13).

16S rDNA sequence

The sequences of PCR products were obtained with a sequencer ABI PRISM 3700 (Applied Biosystems) using the Taq Dyedeoxi Terminator cycle systems (Automatic Sequencing Service SAAD, CIB, CSIC, Madrid). There were used 14 µl of PCR product for sequencing reactions and 1,4 µl of the primers 1050R, 800R, 800F e IRF1 at 5 µM (12). The sequence of each isolate was determined by pairwise alignments using the Clustal W analysis. The similarities sequences were identified and analyzed by BLASTN program on the network service of the GeneBank. The percentage values of similarity with respect to the 16S rRNA gene were calculated by Clustal W multiple alignments (13).

Phylogenetic analysis

Phylogenetic analyses were performed using the MEGA program version 4 (14). The phylogenetic tree was constructed using the neighbor-joining method based on the two parameter distance model of Kimura (15). To assess the relative support for each clade, bootstrap values were calculated from 1,000 replicated analysis.

Experimental design and statistical analysis

The experiments were arranged in completely randomized designs and repeated at least twice, with three replicates each time. Data of tolerance to saline-stress and to pH-stress experiments were analyzed by one-way analysis of variance ($p<0,05$). The treatment means were compared by using the Tukey test (16). All statistical analyses were performed with Statistica for Windows, version 10 (17).

RESULTS

Phenotypical characterization

All isolated were Gram negative and a considerable amount of polysaccharide was produced in VIBA-2. The growth period of the strains was different, VIBA-1 still there was no genetic characterization) began to grow at fifth day after sowing, and therefore took the longest time period. The rest of the strains showed an intermediated growth in the range of three or four days (data not shown).

Saline-stress tolerance

The increase of salinity concentration in culture medium induced significant growth reduction on strains, particularly for levels higher than 4,2 dS m^{-1} where absorbance values decreased with the increase of salt concentration (Figure 1).

The isolated VIBA-1 and VIBA-2, showed the highest growth in the range from 2,6 to 3,4 dS m^{-1} of NaCl, with similar results to the control. The growth of all strains were significantly reduced with 4,2 dS m^{-1} of NaCl.

On the other hand, CCBAU 10071 (*Bradyrhizobium yuanmingense*) revealed a higher growth than the control salinity level (0,17 dS m^{-1}) at 2,6 dS m^{-1} of NaCl. Even this strain reached similar growth to the control with the highest salt levels (6,6 dS m^{-1}) (Figure 1). This strain could be considered as the most saline-stress tolerant.

pH-stress tolerance

None of the strain were able to grow at pH 4,5. The growth of the isolated VIBA-1, VIBA-2, VIBA-3 and VIBA-6 were significantly increased at pH 7 in comparison with pH 9. However, the isolated VIBA-4, VIBA-5 and the reference strain CCBAU 10071 were similar for both pH levels, even this last one, had the tendency to grow better in pH 9.

Temperature-stress tolerance

All isolates, including the reference strain could grow until 40 ºC at the same rate as 28 ºC (Table I). The exceptions were VIBA-4 and VIBA-5 which grew at 45 ºC although at less rate. These results suggest a very good adaptation of these isolates to the high temperatures which are characteristics of the areas where the bacteria were isolated. These results suggest a good adaptation of the isolates to the high temperatures which are characteristics of the areas where the bacteria were isolated.

Nodulation test

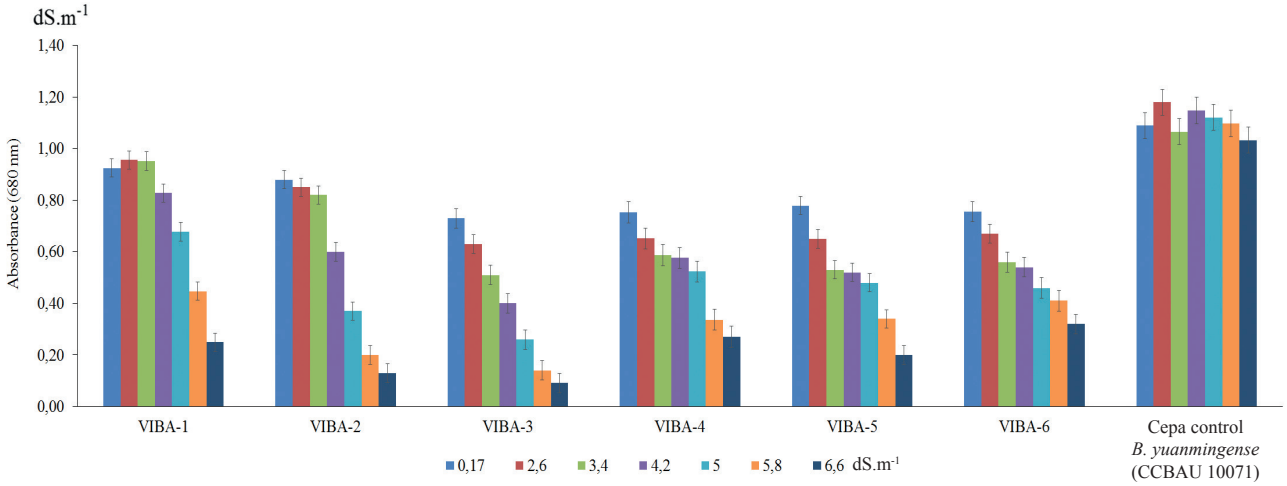
The plants inoculation with VIBA-1, VIBA-2, VIBA-3, VIBA-5 and VIBA-6, induced nodules formation Which indicated Which indicated that these strains belong to *Rhizobiaceae* family. Furthermore, the pink color inside the nodules revealed their activity fixing nitrogen, due to the presence of leghemoglobin in infected zone (18). Nevertheless, VIBA-4 was not able to induce nodulation in the three times that the experiment was carried out.

Molecular characterization

Restriction analyses of amplified 16S rDNA (ARDRA)

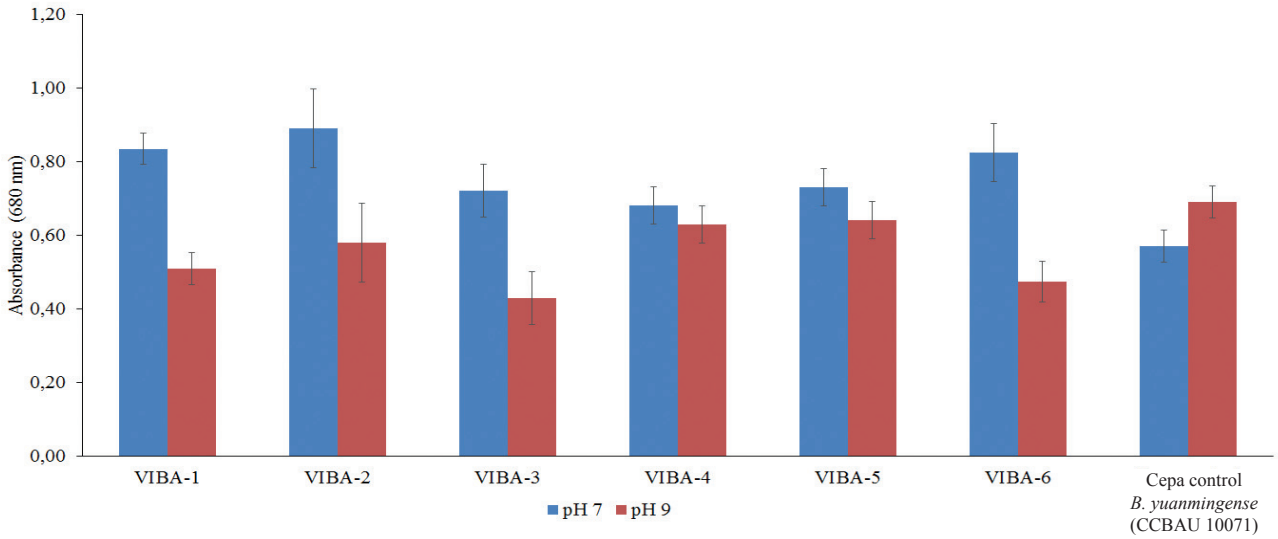
The 16S rDNA from each isolate and the reference strains were amplified by producing one characteristic band of 1,500 bp. Restriction analysis showed four to eight patterns for each enzyme used (restriction patterns not shown) Twelve ARDRA genotypes (1–12), representing the different combinations of restriction patterns, were retrieved from all the strains investigated (Table II).

The six cowpea isolates were classified into three different rDNA genotypes. VIBA-2, VIBA-3, VIBA-5 and VIBA-6 exhibited the same genotype pattern as *Bradyrhizobium yuanmingense* CCBAU 10071 (Table II), while the genotype of VIBA-1 and VIBA-4 did not match with any reference strain. Using the ARDRA reference results, the determination of 16S rDNA gene sequences was performed to achieve the species affiliation and their genetic identity.



Vertical bars (I) indicate Confidence Intervals for 1-α=0,05 1-α=0,05 and n=3

Figure 1. Effects of different salt concentrations (NaCl) liquid yeast-mannitol medium, on growth of strains



Vertical bars (I) indicate Confidence Intervals for 1-α=0,05 and n=3

Figure 2. Effect on strains growth of different pH in liquid yeast-mannitol medium

16S rDNA sequence analysis

The sequences were introduced in GeneBank database under the following access numbers: FJ941843, VIBA-1; FJ941844, VIBA-2; FJ941845, VIBA-3; GU784791, VIBA-4; GU784792, VIBA-5 and VIBA-6. The alignments carried out with Clustal W Multiple Alignments, comprised all of these new strains and employed 1434 nucleotides from VIBA-1, 1279 from VIBA-2, 1252 from VIBA-3, 1432 from VIBA-4, and 1261 from VIBA-5 and 6. The Blast N analysis revealed that all isolates belonged to rhizobial lineages of α-Proteobacteria. The nearby strain to VIBA-1 with a homology of 99,80 % was *Bradyrhizobium liaoningense* LYG2, (accession number DQ497619), while the closest type specie/strain was *B. liaoningense* USDA 3622 (accession AF208513) with 99,70 % of similarity. The VIBA-2 and VIBA-3 isolates presented a similarity of 99,90 % and 99,80 %, respectively, with *Bradyrhizobium yuanmingense* TSC10 (FJ540961), while the homology with type specie/strain *B. yuanmingense* CCBAU 10071 (AF193818) was 99,53 % and 99,52, respectively. In the same way, VIBA-5 and 6 presented 99,30 % of similarity with *Bradyrhizobium yuanmingense* M11 (accession number AB601666) and a homology of 98,95 % with the type specie/strain *Bradyrhizobium yuanmingense* CCBAU 10071, (accession number AF193818). On the other hand, VIBA-4 had a closeness of 100 % with *Agrobacterium* sp. JS71 (accession number AY174112) and 99,10 % with the type specie/strain *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*) NCPPB2437 (accession number D14500).

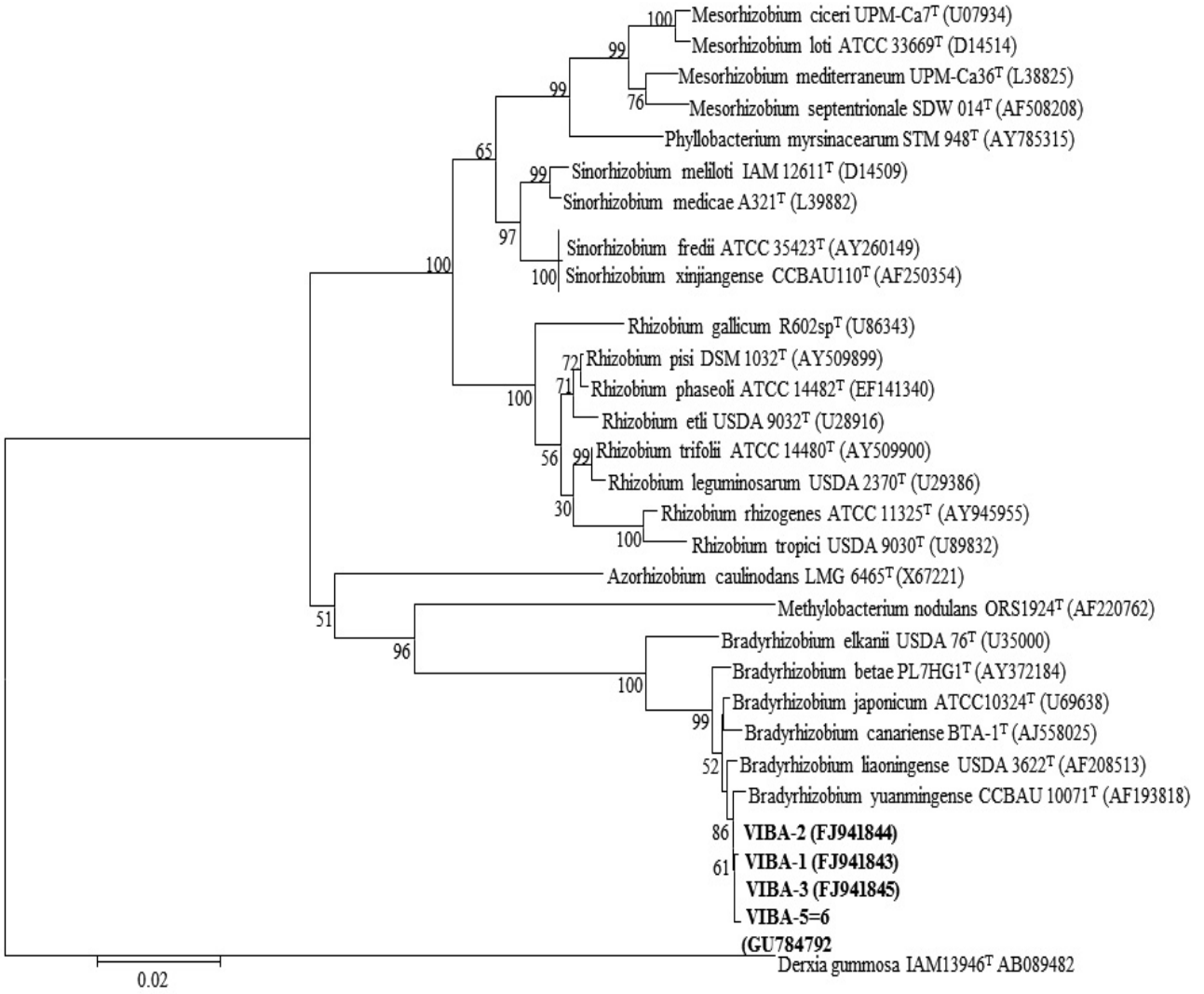
Phylogenetic analysis

The phylogenetic analysis indicated evolutionary relationship among isolates and the most important strains collection (Figure 3).

The VIBA-1 strain was located in the same branch with type specie/strain *Bradyrhizobium liaoningense*, USDA 3622, while VIBA-2, VIBA-3 and VIBA-5 and 6 were located in the same branch of *Bradyrhizobium yuanmingense*, CCBAU 10071, which is similar to ARDRA genotype results.

DISCUSSION

The fact that the native's strains were grown in saline culture medium (6,6 dS m^{-1} of NaCl) implies some adaptation characteristics to salt-stress, although their growth were obviously smaller than the type specie/strain CCBAU 10071. Indeed, the salt-tolerance of these strains was less than other reports, even being isolated from the same Cuban soils reported as highly saline (Babiney with E.C 4,76 dS m^{-1}) and very highly saline (Jiguaní 5,8 dS m^{-1}) (19). Concerning rhizobial species, have been documented the high salt-tolerance level of *Phyllobacterium myrsinacearum,* which can grow at 2,5 to 3 % of salinity (11). While *Mesorhizobium ciceri* (strain GA-2) and *Ensifer* (*Sinorhizobium*) *meliloti* (strains AFL-2 and AFL-3) grew at 1,5 and 2 % of salt levels, contrary to *Bradyrhizobium liaoningense* and *Bradyrhizobium canariense*, by showing a true development only at 0,1 % of salt level (11).



Values of bootstrap based on 1000 replies (showed only > 50 %). Scale, 2 nt substitution/100 nt

Figure 3. Phylogenetic tree derived from 1484 pb alignment of 16S rDNA gene from isolated and the most representative strain types

Nevertheless, *Bradyrhizobium japonicum* (E109) can grow in media with NaCl concentrations of up to 75 mM, showing that salt sensitivity in this specie is affected by the growth phase of the culture and the type of test used to measure the salt-tolerance (20, 21).

Other abiotic stresses wide known is the pH variation (acid or alkaline), which may provoke damages in rhizobial bacteria, mainly on survival, infection capacity and its diversity (11,22). Our results confirm these previous findings in a way; due to strains were not able to develop under acid medium (pH 4,5), but contrary all isolated grew under alkaline conditions (pH 9). Although these results seem to be the tendency, rhizobial bacteria adapted to acid environments, as *Bradyrhizobium canariense*, isolated from acid soils of Spain (pH 5,3 and 4,8), have been informed by other authors (11). Furthermore, there are reports in order to the adaptation of rhizobia to alkaline conditions too; even the isolated have the trend to be more tolerant to alkalinity than the acidity (22). Nevertheless, there are differences among the genera. Several informs revealed that *Bradyrhizobium* sp. is more acid-tolerant than *Rhizobium* sp. However, it has been demonstrated their capacity to grow in alkaline medium too (23, 24). In spite of, have been found and characterized *Bradyrhizobium* strains with tolerance to pH 11 (25), while others, obtained growth in most of the isolates at pH 9 and a few strains reached their development at pH 4 (11).

A possible explanation to acidity or alkalinity tolerance, is based on the ability of microsymbiont to maintain internal pH near to neutrality, which could be related to protons exclusion, increase the cytoplasm buffer capacity, the maintenance of high potassium and glutamate concentrations. (26, 27). However the demontrated growth of these strains at alkaline pH is a good response as the soils of Cauto River Valley are slightly alkalines (9).

On the other hand, the results of temperature variations indicate that the strains not only have the ability to be developed in saline environment and pH variations, but rather have also capacity to support high temperatures. The adaptation to high temperature of these strains may be related with the region where they were isolated, which has long drought periods and high temperatures (28, 29), being this a good characteristic, in order to get an improvement of the symbiotic process in cowpea plants (30-32). Likewise, other authors have found a high variability in the capacity to grow with temperatures above 35 °C, although the tendency was to decrease in the cell number (30,33).

The results of this experience were similar to obtained by others researchers, who isolated and characterized *Bradyrhizobium* sp. strains from *Centrosema* sp., which grew at 40 °C (29).

In this study, we achieved the isolation of four native *Bradyrhizobium* strains from nodules of cowpea plants grown on soil affected by saline stress. Nevertheless, only two different species were represented by the isolated *Bradyrhizobium* strains, which were obtained from the 16S rDNA sequence. Isolate VIBA-1 was located in *Bradyrhizobium liaoningense* species, while VIBA-2, VIBA-3, VIBA-5 and VIBA-6 in *Bradyrhizobium yuanmingense* species. However, VIBA-4 was identified as *R. radiobacter* (formerly *Agrobacterium tumefaciens*).

The fact that only two *Bradyrhizobium* species were found in our research, show a low diversity of *Bradyrhizobium* genus in this region. The reduction of the bacterial populationin saline soils may be attributed to the scarce use of leguminous species (29-33). The search and selections of new isolates represented by a major number of rhizobial species is then needed in order to improve the bacterial populations and the symbiosis quality.

The *Bradyrhizobium liaoningense* species was initially isolated it from soybeans plants (34), while *Bradyrhizobium yuanmingense* was isolated from *Lespedeza* nodules (35). This showed the capacity to form nodules in *Medicago sativa* y *Melilotus albus,* while it was not possible to found nodules in soybean.

However, have been found that *B. yuanmingense* is able to infect roots and establish symbiosis in Cowpea plants, in contrast with *B. liaoningense* which was not able to produce nodules (36). In Cuba, these species have not been reported in cowpea plants grown under saline soil conditions. Nevertheless, *B. liaoningense* and *B. yuanmingense* have been isolated from saline, alkaline and sodic soils in dry (37, 38).

One strain of *Rhizobium radiobacter* (formely *Agrobacterium tumefaciens*, VIBA-4), from the nodules sampled was also isolated; nevertheless, this isolate was not capable to form nodular structures in cowpea plants when these were inoculated again. This species has also been isolated from nodules of *Retama sphaerocarpa* (11), nevertheless *A. tumefaciens* strains miss the ability to form nodules, which indicates that these kind of strains can belong to a procession of endophytic bacteria that slip into nodules for protection (39, 40).

Several findings have reported the presence of others microorganism into the nodules as non-symbiotic proteobacteria, denitrificant, pathogen and phototropic bacteria from numerous genus among it can find the *Agrobacterium* (11, 41, 42). In the same way, isolated of *Agrobaterium* strains have been obtained from nodules of different legumes but most of the *Agrobacterium* strains isolated failed to nodulate on their original hosts, because they did not hybridize to *nif* and *nod* gene probes by verifying that were not symbiotic bacteria (43-45). Nonetheless, other authors have reported that some *Agrobacterium* strains could form effective nodules on some legume plants (46- 48).

The *Bradyrhizobium yuanmingense* specie used in the experiments was firstly isolated from *Lespedeza cuneata* nodules in the north of China (36). This specie has been isolated in a wide range of temperature, dry and hot regions of southeastern Asian, and the south of Africa, as well as, different host plants. For this reason, this strain has been used as a reference to identify new isolates, as well as, to research strain adaptation in stress conditions, due to its tolerant characteristics and easy adaptation to low and high temperature, different pH and hyperosmotic stress (49-52).

Lastly, the results demonstrated that in salt-affected soils of Cauto River Valley, there are native *Bradyrhizobium* strains able to grow under different stress conditions such as, salinity, pH and high temperature. This means the possibility to use new inoculants from strains adapted to these environmental conditions and to improve the symbiotic nitrogen fixation in Cowpea (9).

Furthermore, the fact that only two bradyrhizobial species (*B. yuanmingense* and *B. liaoningense*) were found puts emphasis on the need to find, and to characterize new rhizobial strains from saline soils of Cauto River Valley in order to increase the diversity.-

CONCLUSIONS

- Six rhizobial strains of *Bradyrhizobium* genera were isolated and characterized from cowpea nodules grown in saline soils of Cauto River Valley. *Bradyrhizobium liaoningense* and *Bradyrhizobium yuanmingense* were the representative species, which were reported for the first time in these areas.
- The strains showed good responses to different abiotic stresses (salinity, high temperature and alkaline pH) which point out their adaptation to the environmental conditions.
- The creation of the first collection of *Bradyrhizobium* strains adapted to the soils of Cauto River Valley, is an important hit to achieve new inoculants for improve the fixation of symbiotic nitrogen and consequently the yield of the cowpea plants.
- This research is a step beyond to get new bioinoculants/biofertilizers to invigorate the Cuban saline soils and to contribute to the development of organic farming in this region.

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