Rhizospheric rhizobia identification in maize (*Zea mays* L.) plants

Identificación de rizobios rizosféricos en plantas de maíz (*Zea mays* L.)

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ABSTRACT

Rhizobia have been studied for the symbiosis that they establish with the roots of legumes. However, the colonization and promotion of growth in non-leguminous plants has also been demonstrated. The aim of this work was the biochemical and molecular identification of rhizosphere rhizobia present in the rhizosphere of two commercial maize cultivars. Cultivable isolates were obtained in yeast-mannitol-agar (YMA) medium from rhizospheric soil and the rhizoplane. The cultural (size, color, mucus, etc.), morphological, and staining (cell shape, response to staining and sporulation) characteristics were determined as well as isolate responses to eight biochemical tests (acid-base production, citrate, oxidase, catalase, H_2S production, urease, gelatinase and the oxidative-fermentative assay) that are valuable for rhizobia identification. The genus was determined by 16S rDNA gene sequencing. We obtained 81 total isolates of which 30.86% showed the cultural, morphological and staining characteristics expected for rhizobia and only 20% of these corresponded to the genus *Rhizobium*.

Key words: rhizobacteria, rhizosphere, molecular identification, *Rhizobium*.

RESUMEN

Los rizobios han sido estudiados por la simbiosis que establecen con las raíces de las leguminosas. Sin embargo, la colonización y promoción del crecimiento en plantas no leguminosas también ha sido demostrada. Este trabajo tuvo como objetivo la identificación bioquímica y molecular de rizobios rizosféricos presentes en la rizosfera de dos cultivares comerciales de maíz. Se obtuvieron aislamientos cultivables en medio Levadura-Manitol-Agar (YMA) a partir del suelo rizosférico y rizoplano de las plantas. Se determinaron las características culturales del cultivo (tamaño, color, mucosidad, etc.) y morfo-tintoriales (forma celular, respuesta a la tinción y esporulación), así como la respuesta de los aislados a ocho pruebas bioquímicas (producción ácido-base, citrato, oxidasa, catalasa, producción de H_2S, ureasa, gelatinasa y ensayo oxidativo-fermentativo) con valor predictivo para la identificación de rizobios. Se determinó el género mediante la secuenciación del gen ADNr-16S. Se obtuvieron 81 aislados totales de los cuales el 30.86% mostró las características culturales, morfológicas y tintoriales esperadas para los rizobios y solamente el 20% de estos correspondió al género *Rhizobium*.

Palabras clave: rizobacterias, rizosfera, identificación molecular, *Rhizobium*.

Introduction

From a nutritional point of view, maize (*Zea mays* L.) is one of the most important cereals in the world. Maize constitutes the nutritional basis for humans in many developing countries, and it is also the energy concentrate par excellence of intensive poultry and livestock feeding systems (Permuy, 2013). In Cuba, maize grain production has been affected by economic problems and by the use of inadequate soil practices and crop management that sometimes leads to significant environmental degradation.

Obtaining acceptable crop yields requires the application of large quantities of mostly nitrogen fertilizers. The use of chemical compounds as a means of fertilization in agriculture is a common practice among farmers. However, its indiscriminate use significantly alters the balance of the organic and living soil constituents (Vidal *et al.*, 2015). In agricultural production systems based on crop rotation, maize is intercropped with some leguminous crops. This technique allows the maize to take advantage of the benefits provided by microbial communities that are associated with the leguminous rhizosphere. This nutrient

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contribution is one of the main benefits helping to decrease the application of the mineral fertilizer load (Martín and Rivera, 2015).

Biofertilizers based on microorganisms that promote plant growth are considered a component of integrated plant nutritional management (Vessey, 2003) and allow substituting part of the inorganic fertilizers in many crops. The success in the use of these biopreparations lies (besides other aspects) in obtaining strains that are compatible with the crop of interest (Hernández *et al.*, 2004) and that are efficient for obtaining the desired yields.

The rhizobia constitute a group of diazotrophic microorganisms that have been mainly studied for their symbiotic association with leguminous plant roots and on which differentiated organs called nodules are produced. In these structures, these bacteria perform biological nitrogen fixation (BNF), mediated by nitrogenase protein complex activity (Luyten and Vanderleyden, 2000). Currently, rhizobia are considered plant growth promoting rhizobacteria (PGPR) because they have the ability to promote non-leguminous plant growth at the rhizosphere level or as endophytes without nodule formation (Sessitsch *et al.,* 2002). Several studies demonstrate these microorganisms' capacity to colonize non-leguminous plant rhizospheres such as rice, lettuce, tomato, pepper, wheat and maize (Bécquer *et al.*, 2011; García-Fraile *et al.*, 2012; Flores-Félix *et al.*, 2013; Hernández, 2015). However, rhizobia used in these trials were isolated from legume nodules and not from the crop rhizosphere itself.

In Cuba, the presence of rhizobia associated with maize plants has not been reported; and, therefore, there are no biopreparations based on these microorganisms for crop biofertilization. Taking into account this background, the aim of this study was to identify maize rhizospheric rhizobia.

Materials and methods

Studies were carried out in the Department of Plant Physiology and Biochemistry at the Instituto Nacional de Ciencias Agrícolas (INCA) in Cuba and at the Centro de Investigaciones Biotecnologicas del Ecuador (CIBE), Escuela Superior Politecnica del Litoral (ESPOL).

Sampling

Two maize cultivars were sampled: Raúl and Canilla. They were cultivated in a Ferralitic Red Leached soil (Hernández

et al., 2015) at "El Mulato" farm and at the Centro Nacional de Sanidad Agropecuaria (CENSA), respectively. Both centers are located at San Jose de las Lajas, Mayabeque province, Cuba. The bean (*Phaseolus vulgaris* L.) was the rotation crop. At both sites five random sampling points were established and two plants were taken from each of them for a total of 10 plants per cultivar. The extraction was performed taking a volume of soil of 20x20x20 cm. Samples were placed separately in polyethylene bags and stored in the laboratory at 4ºC until they were used.

Rhizobia isolation from Raúl and Canilla maize cultivar rhizospheres

Rhizobia isolation was conducted from rhizospheric soil and the maize rhizoplane using the methodology of Knief *et al.* (2012). For rhizospheric soil isolation, 1 g samples of soil were taken and serial dilutions were performed (10^{-1}-10^{-6}). One hundred µl suspensions were grown in YMA (Yeast Mannitol Agar) medium at pH 6.8 with congo red. Plates were incubated at 28°C for 10 d.

For rhizoplane isolation, roots were cut into 1 cm long portions and placed in Erlenmeyer flasks with 10 ml of sterile distilled water. Flasks were maintained while stirring for 1 h at 150 rpm. The same methodology was used later with dilutions from rhizospheric soils.

Roots from the previous methodology were placed on plates with LB (Luria Bertani agar) medium and incubated at 28ºC for 24 h. Subsequently, visible bacterial growth around the roots was collected and cultivated in YMA medium at pH 6.8 with Congo red. Plates were incubated at 28°C for 10 d. Strains obtained were stored at -20ºC in the INCA bacterial culture collection.

Rhizospheric rhizobia characterization of the maize cultivars Raúl and Canilla

Cultural and morphological characterization

The color, size (mm) and mucus of the colonies were determined as proposed for rhizobia (Wang and Martínez-Romero, 2001). Colonies with whitish coloration, semi-translucent, mucous or dry, and all those that absorbed YMA medium red pigment at its center were selected at different culture stages. Cultures that grew over a period of 1-3 d were considered as fast growth, and those up to 10 d as slow growth. Colonies with diameters between 1-2 mm and 2-4 mm were considered small or large, respectively. These were purified by successive passes and kept at 4°C in tubes with YMA medium. Cell morphology and the presence of spores were determined by gram staining.

Biochemical characterization

Eight biochemical tests were applied to each isolate: acid-base production, citrate utilization, oxidase, catalase, urease, hydrogen sulfide production (H_2S), gelatinase, and an oxidation-fermentation test.

To determine acid-base production isolates were cultured in YMA medium at pH 6.8 with bromothymol blue indicator (0.5% in sodium hydroxide (NaOH) 0.016 N). Subsequently, plates were incubated at 28°C for 10 d. Cultures that changed medium coloration from green to yellow were taken as positive results for acid production. Base production was found in the isolates that changed medium coloration from green to blue.

The use of citrate as carbon and energy sources was determined in tubes with Simmons citrate agar medium (Koser, 1923). Tubes were incubated at 28°C for 10 d. A change in medium coloration from green to yellow was interpreted as a positive result.

Determinations of oxidase and catalase activity were performed by the methods of Kovaks (1956) and Graham-Parker (1964), respectively.

To determine urease enzyme production, Christensen's urea agar medium (Christensen, 1946) was used. Tubes were incubated at 28°C for 10 d. A change in medium coloration from yellow to pink was considered positive urease and negative urease was considered when the medium remained yellow.

Hydrogen sulfide production (H_2S) was determined in TSI agar medium (Triple Sugar Iron) (MacFaddin, 2000). Tubes were incubated at 28°C for 10 d. The presence of a black precipitate at the bottom of the tube was interpreted as a positive result.

The gelatinase activity test was performed according to Leboffe and Pierce (2010) in nutrient gelatin medium (Difco Laboratories, 2009). Tubes were incubated at 28°C for 48 h. Those isolates that hydrolyzed the medium were taken as positive gelatinase and the others that maintained the medium in the solid state were considered as a negative gelatinase.

The type of energy metabolism (respiratory or fermentative) was performed following Hugh-Leifson's method (Hugh and Leifson, 1953) in O-F basal medium (Winn

et al., 2006). Two tubes were used per isolate, one under anaerobic and the other one under aerobic conditions. Tubes were incubated at 28°C for 48 h. Isolates that totally changed medium coloration from green to yellow in both tubes were considered positive for anaerobic metabolism. Those that remained green in the anaerobic tube and then changed partially or totally to yellow in the other tube were considered positive for oxidative metabolism.

Identification by 16S rDNA gene sequencing

DNA extraction

Extraction of the genetic material was performed by alkaline lysis (von Post *et al.*, 2003). A colony of the axenic culture was taken and placed in an Eppendorf tube with 40 µl of NaOH at 0.20 M. It was then heated in a microwave at 10% power or 700 W for 1 min.

Nucleic acid quantification by spectrophotometry was performed using the NanoDropTM 2000 at 260 nm; the ratios 260/280 and 260/230 were calculated in order to determine DNA concentration and quality.

PCR amplification

Universal primers forward 27F (5'-AGAGTTTGATCCT-GGCTCAG-3') and reverse 1492R (5'-GGTTACCTT-GTTACGACTT-3') (Criollo *et al.*, 2012) were used for 16S rDNA amplification. This procedure was carried out in a mini MS thermal cycler (Major Science, USA) under the following amplification parameters: initial denaturation 95°C for 10 min, additional denaturation for 1 min at 92°C and 35 cycles of 72°C each for 2 min. Amplification results were verified by electrophoresis in 1% agarose gel. PCR product sequencing was carried out by the Sanger method of Macrogen® (Republic of Korea).

Editing sequences and identification

Sequence quality analysis was performed through the FinchTV program (version 1.4.0, Geospiza Inc., Seattle, WA, USA). Consensus sequences were obtained with the MEGA-X software (version 10.0.4, Molecular Evolutionary Genetics Analysis) (Kumar *et al.,* 2018) and compared with the NCBI (National Center for Biotechnological Information) database, using the BLASTn (Basic Local Alignment Search Tool - nucleotide, Maryland, USA) program (Benson *et al.,* 2015). Alignments with an e-value lower than 1×10^{-5} and with a similarity and coverage greater than 80% of amplified region were considered as a hit.

Results and discussion

Rhizobia isolation from the maize cultivars Raúl and Canilla rhizosphere

A total of 81 isolates were obtained consisting of 46 strains of the Raúl cultivar and 35 strains of the Canilla cultivar. Forty-seven came from the rhizoplane equaling 58.3% and the rest came from rhizospheric soil. The rhizoplane is the rhizosphere zone most influenced by radical exudates and where their concentration is at a maximum (Atlas and Bartha, 2005). This is an attractive area for microbial colonization (Kapulnik, 2002). In addition, radical exudates from grasses provoke chemotactic responses in rhizobacteria such as rhizobia (van Rossum *et al.*, 1995). Vanillic, p-coumaric, m-coumaric and cinnamic acids excreted by rice plants (and others) are important carbon sources for rhizobia and allow their saprophytic survival in this crop rhizosphere (Heidarzade *et al.*, 2010).

Rhizospheric rhizobia characterization of the maize cultivars Raúl and Canilla

Cultural and morphological characterization

The 81 isolates complied with cultural descriptions proposed for rhizobia (Wang and Martínez-Romero, 2001). Taking into account the large number of isolates with similar morpho-cultural characteristics to those described for the rhizobia group, gram stain was used as a second discrimination criterion. Cellular morphology and staining responses allowed separating isolates into seven groups (Tab. 1).

Group number V was the most representative with a 30.9% of total bacteria detected. Coincidentally, morphological and staining isolate characteristics included in this group agree with those described by Frioni (1990) for rhizobia. In addition, these isolates showed a rapid growth rate (24 h) under used conditions. Rhizobia usually show fast growth rates with the exception of the genus *Bradyrhizobium*, which can take 7 to 10 d to grow (Berrada and Fikri-Benbrahim, 2014).

Biochemical characterization

Acid production in YMA medium with bromothymol blue was determined in 24 isolates, and only one showed base production (results not shown). Acid excretion is a characteristic shared by some rhizobia genera with fast growth rates, such as *Rhizobium*, *Allorhizobium* and *Sinorhizobium* (Berrada and Fikri-Benbrahim, 2014). One of the organic acids produced by rhizobia is indoleacetic acid (IAA), which promotes plant growth, since it allows cellular elongation, root initiation, and the formation of root hairs (van Loon, 2007).

Phosphate solubilization in soils constitutes another important function of these acids, which increase nutrient availability, an aspect that is advantageous for the plant (Zúñiga *et al.*, 2013). Regarding base production, although less frequent, it has taxonomic value in slow-growing rhizobia identification, such as the genus *Bradyrhizobium*. In addition, this phenomenon intervenes in other mineral solubilization (Sugumaran and Janarthanam, 2007).

Eight isolates grew and produced a change of color in Simmons medium (Tab. 2), which indicates the capacity to metabolize sodium citrate (MacFaddin, 2000). Generally, rhizobia respond negatively to this assay *(Sadowsky et al.,* 1983); this could be due to citrate molecule complexity in terms of its functional groups volume (three carboxyl groups), compared to other carbon sources used by these microorganisms that basically include sugars, alcohols and organic acids (Gaurav *et al.*, 2016). The YMA medium includes mannitol, sucrose or glycerol as the sole carbon sources. These guarantee rhizobia multiplication with a suitable nitrogen source and under certain parameters of pH and temperature (Hernández and Nápoles, 2018).

TABLE 1. Bacterial groups established according to their morphological and staining characteristics.

Groups	Morphological and staining characteristics	Quantity	%
I	Gram-negative cocci, not sporulated	5	6.2
II	Gram-positive cocci, not sporulated	17	21.0
III	Gram-positive cocobacilli, not sporulated	4	4.9
IV	Coccobacilli and gram-negative long/short bacilli, sporulated	8	9.9
V	Coccobacilli and short Gram-negative bacilli, not sporulated	25	30.9
VI	Long bacilli, Gram-negative, not sporulated	20	24.7
VII*	White lumps with pink edges	2	2.5

*There was no response to gram staining. Probably not bacteria.

TABLE 2. Isolate rhizosphere biochemical characterization of the maize cultivars Raúl and Canilla.

Isolate	Provenance	Citrate	Oxidase	Catalase	H₂S production	Gelatinase	Oxidative/ Fermentative Metabolism
Zea mays L. cv. Raúl							
R1	RS	-	+	+	-	-	Oxidative
R2	RS	-	+	+	-	-	Fermentative
R3	RS	+	+	+	-	-	Oxidative
R4	RS	+	+	+	-	-	Oxidative
R5	RS	-	+	+	-	-	Oxidative
R6	RS	-	-	-	-	-	Oxidative
R7	RS	+	-	+	+	-	Oxidative
R8	RP	-	+	+	-	-	Oxidative
R9	RP	-	-	-	+	-	Oxidative
R10	RP	-	+	-	-	+	Oxidative
R11	RP	+	-	+	-	-	Oxidative
R12	RP	+	+	+	-	-	Fermentative
R13	RP	-	+	+	-	-	Oxidative
R14	RP	-	+	+	+	-	Oxidative
R15	RP	+	+	-	-	-	Oxidative
R16	RP	+	+	+	-	-	Fermentative
Zea mays L. cv. Canilla							
C1	RP	-	+	+	-	+	Oxidative
C4	RP	-	+	+	-	+	Oxidative
C6	RP	-	+	+	-	+	Oxidative
C8	RP	-	+	-	-	+	Fermentative
C16	RS	-	+	+	+	+	Oxidative
C19₂	RS	-	-	+	-	-	Oxidative
C21	RS	-	+	+	-	-	Oxidative
C22	RP	+	+	+	-	-	Oxidative
C24	RP	-	+	+	-	-	Oxidative

RP: rhizoplane, RS: rhizospheric soil.
Rhizobia are: citrate negative (-), oxidase positive (+), catalase positive (+), no H_2S production (-), gelatinase positive (+), urease positive (+), and have oxidative metabolism (usually).

Cytochrome oxidase enzyme activity was observed in 20 isolates and the same number for catalase activity (Tab. 2). Similar results were obtained by Sadowsky *et al.* (1983), which show that both slow-growing and fast-growing rhizobia share the positive results of these trials. However, urease enzyme production that is positive for rhizobia (Sadowsky *et al.*, 1983), is negative in all isolates (results not shown).

Hydrogen sulfide precipitation was seen in four isolates. Rhizobia lack the capacity to reduce the sodium thiosulfate present in TSI medium, so there is no production of H_2S. Gelatinase activity was seen in six cases (Tab. 2), which could correspond to some types of rhizobia, since this is a biochemical characteristic of the group (Sadowsky *et al.*, 1983).

The ability to metabolize glucose by fermentation was seen in four isolates that produced a change in medium coloration produced by organic acid excretion (Tab. 2). Oxidative metabolism was much superior to the previous variant with 21 isolates grown in aerobic conditions. Some gram-negative bacteria metabolize glucose through aerobic respiration; and, as a result, small amounts of weak acids are formed during Krebs cycle and the Entner Doudoroff pathway (Winn *et al.*, 2006) that promote medium color changes, from green to yellow. In addition, this test allowed the determination of motility, since acid production is low, color changes are only observed in the tube's upper levels. In this case, total medium discoloration to yellow was observed in 100% isolates, which shows bacterial displacement throughout the tube.

Identification by 16S rDNA gene sequencing

In biochemical tests performed on rhizospheric isolates, null urease enzyme activity was determined that contradicts the biochemical pattern usually present in rhizobia. In this way, it could be concluded that none of the isolates belong to this group of microorganisms. However, molecular biology confirmed these results (Tab. 3).

Eight bacterial genera were identified from twenty-five isolates; five corresponded to the genus *Rhizobium* for 20% (Tab. 3.).

The most representative genera were *Pseudomonas* and *Stenotrophomonas* with 32 and 28%, respectively. The genera *Enterobacter, Starkeya, Achromobacter, Delftia* and *Flavobacterium* were identified, each one with 4% representation.

Most of these genera have been described as part of various crops' rhizospheric microbiota, including maize. These include mainly *Pseudomonas*, *Stenotrophomonas* and *Enterobacter* (Hernández *et al.*, 2004; Morales-García *et al.,* 2011; Granada *et al.*, 2015; Yang *et al.,* 2017; Gaviria-Giraldo *et al.*, 2018).

The capacity of *Rhizobium* for rhizospheric and endophytic colonization of horticultural crops (García-Fraile *et al.*, 2012; Flores-Félix *et al.*, 2013) and grasses (Chabot *et al.*, 1996; Gutiérrez-Zamora and Martínez-Romero, 2001; Sessitsch *et al.*, 2002) has been studied. However, microorganisms that were used in these studies were isolated from legume nodules and not from the crop's rhizosphere.

Isolates obtained in this study come from Red Ferralitic soils, which have a low percentage of organic matter, and this translates into poor nitrogen content (Hernández *et al.*, 2015). Maize requires high doses of nitrogen for growth and development, so it is common to rotate it with leguminous crops that enrich soil nitrogen concentration (Martín and Rivera, 2015). The presence of beans (*Phaseolus vulgaris* L.)

TABLE 3. Molecular identification of the isolates based on the 16S subunit rDNA sequencing.

Isolate	Genus	Possible identification	Identity%	Access Number
R1	*Stenotrophomonas*	*Stenotrophomonas rhizophila*	99%	NR _ 121739.1
R4		*Stenotrophomonas rhizophila*	100%	MH828345.1
R9		*Stenotrophomonas* sp.	99%	MH396743.1
R10		*Stenotrophomonas* sp.	95%	AM745261.1
R13		Stenotrophomonas rhizophila	99%	NR _ 121739.1
R16		Stenotrophomonas rhizophila	100%	MH828345.1
C16		Stenotrophomonas bentonitica	99%	NR _ 157765.1
C24		Stenotrophomonas pavanii	93%	NR _ 116793.1
R2	*Pseudomonas*	Pseudomonas graminis	99%	NR _ 026395.1
R3		Pseudomonas hibiscicola	97%	KX527638.1
R5		Pseudomonas putida	100%	MF952434.1
R6		Pseudomonas sp.	100%	MG833395.1
R8		Pseudomonas sp.	99%	KF542910.1
R14		Pseudomonas graminis	99%	NR _ 026395.1
R15		Pseudomonas hibiscicola	97%	KX527638.1
R7	*Rhizobium*	Rhizobium sp.	88%	MH899428.1
C1		Rhizobium mesosinicum	99%	NR _ 043548.1
C4		Rhizobium aegyptiacum	100%	NR _ 137399.1
C8		Rhizobium mesosinicum	99%	NR _ 043548.1
C19$_2$		Rhizobium sp.	99%	MK092996.1
R11	*Achromobacter*	Achromobacter xylosoxidans	99%	MK089550.1
R12	*Enterobacter*	Enterobacter bugandensis	99%	NR _ 148649.1
C6	*Starkeya*	Starkeya novella	99%	NR _ 074219.1
C21	*Flavobacterium*	Flavobacterium anhuiense	99%	NR _ 044388.1
C22	*Delftia*	Delftia lacustris	91%	NR _ 116495.1

as a predecessor crop to maize from which the isolation was performed and the type of soil characteristics constitute two important factors that explain the rhizobia population presence associated with this plant rhizosphere. Bean establishes a greater symbiotic interaction with Rhizobiaceae family representatives, fundamentally with the genus *Rhizobium* (Ramírez-Bahena *et al.*, 2008; Dall'Agnol *et al.*, 2013), an aspect that could explain the presence of this genus in the crop's rhizosphere. In this way, *Rhizobium* can be demonstrated as a common component of maize rhizospheric microbiota.

Conclusions

In the maize rhizosphere, planted in Red Ferralitic soil in Mayabeque province, Cuba, there are rhizobia populations of the genus *Rhizobium* spp. Biochemical characterizations are important elements for microorganism identification. However, with the development of molecular techniques, a more accurate and reliable taxonomic diagnosis can be made.

Acknowledgments

Literature cited

Atlas, R. and R. Bartha. 2005. Ecología microbiana y microbiología ambiental. Addison Wesley, Madrid.

Bécquer, C.J., B. Salas, D.J. Archambault, J.J. Slaski, and A. Anyia. 2008. Selección de rizobios adaptados a ecosistemas ganaderos de Sancti Spíritus, Cuba, inoculados en maíz (*Zea mays* L.). Fase I: Invernadero. Pastos y Forrajes 31(3), 229-246.

Bécquer, C.J., U. Ávila, L. Palmero, A. Nápoles, L. Ulloa, Y. Suárez, and O. Colina. 2011. Selección de cepas aisladas de rizobios, inoculadas en maíz (*Zea mays* L.), en condiciones de campo en ecosistemas ganaderos de Sancti Spíritus, Cuba. Rev. Cub. Cienc. Agric. 45(4), 445-449.

Benson, D.A., K. Clark, I. Karsch-Mizrachi, D.J. Lipman, J. Ostell, and E.W. Sayers. 2015. GenBank. Nuc. Acids Res. 43(Database issue), D30-5. Doi: 10.1093/nar/gku1216

Berrada, H. and K. Fikri-Benbrahim. 2014. Taxonomy of the rhizobia: current perspectives. Br. Microbiol. Res. J. 4(6), 616-639. Doi: 10.9734/BMRJ/2014/5635

Chabot, R., H. Antoun, and M.P. Cescas. 1996. Growth promotion of maize and lettuce by phosphate-solubilizing *Rhizobium leguminosarum* bv. *phaseoli*. Plant Soil 184(2), 311-321. Doi: 10.1007/BF00010460

Christensen, W.B. 1946. Urea decomposition as a means of differentiating *Proteus* and Paracolon cultures from each other and from *Salmonella* and *Shigella* types. J. Bacteriol. 52(4), 461-466.

Criollo, P.J., M. Obando, L. Sánchez, and R. Bonilla. 2012. Efecto de bacterias promotoras de crecimiento vegetal (PGPR) asociadas a *Pennisetum clandestinum* en el altiplano cundiboyacense. Cienc. Tecnol. Agropec. 13(2), 189-195. Doi: 10.21930/rcta.vol13_num2_art:254

Dall'Agnol, R.F., R.A. Ribeiro, E. Ormeno-Orrillo, M.A. Rogel, J.R.M. Delamuta, D.S. Andrade, and M. Hungria. 2013. *Rhizobium freirei* sp. nov., a symbiont of *Phaseolus vulgaris* that is very effective at fixing nitrogen. Int. J. Syst. Evol. Microbiol. 63(11), 4167-4173. Doi: 10.1099/ijs.0.052928-0

Difco Laboratories. 2009. Difco & BBL manual: manual of microbiological culture media. Becton Dickinson and Company, Sparks, USA.

Flores-Félix, J.D., E. Menéndez, L.P. Rivera, M. Marcos-García, P. Martínez-Hidalgo, P.F. Mateos, and E. Martínez-Molina. 2013. Use of *Rhizobium leguminosarum* as a potential biofertilizer for *Lactuca sativa* and *Daucus carota* crops. J. Plant Nutr. Soil. Sci. 176(6), 876-882. Doi: 10.1002/jpln.201300116

Frioni, L. 1990. Comunidad del Sur-Edinor. Ecología microbiana del suelo. Colección Reencuentro, Universidad de la República, Montevideo.

García-Fraile, P., L. Carro, M. Robledo, M.H. Ramírez-Bahena, J.D. Flores-Félix, M.T. Fernández, and P.F. Mateos. 2012. *Rhizobium* promotes non-legumes growth and quality in several production steps: towards a biofertilization of edible raw vegetables healthy for humans. PLOS ONE 7(5), e38122. Doi: 10.1371/journal.pone.0038122

Gaurav, K., A.K. Singh, and G. Singh. 2016. Spent wash as an alternative medium for growth of Rhizobium. J. Acad. Indust. Res. 5(6), 81-84.

Gaviria-Giraldo, J., G. Restrepo-Franco, N. Galeano-Vanegas, and A. Hernández-Rodríguez. 2018. Bacterias diazotróficas con actividad promotora del crecimiento vegetal en *Daucus carota* L. Cienc. Agricult. 15(1), 19-27. Doi: 10.19053/01228420.v15.n1.2018.7753

Graham, P.H. and C.A. Parker. 1964. Diagnostic features in the characterization of the root-nodule bacteria of legumes. Plant Soil 20, 383-396. Doi: 10.1007/BF01373828

Granada-Mora, K.I., R. González, Y. Alvarado, A.R. Robles, and R. Torres. 2015. Caracterización de rizobacterias y estimulación de parámetros morfológicos y biomasa en maíz (*Zea mays* L.). Centro de Biotecnología 4(1), 14-22.

Heidarzade, A., H. Pirdashti, and M. Esmaeili. 2010. Quantification of allelopathic substances and inhibitory potential in root exudates of rice (*Oryza sativa*) varieties on Barnyardgrass (*Echinochloa crus-galli* L.). Plant Omics 3(6), 204-209.

Hernández, A., J.M. Pérez, and D. Bosch. 2015. Clasificación de los suelos de Cuba. Ediciones INCA, Instituto Nacional de Ciencias Agrícolas, Cuba.

Hernández, A., N. Rives, A. Caballero, A.N. Hernández, and M. Heydrich. 2004. Caracterización de rizobacterias asociadas al cultivo del maíz en la producción de compuestos indólicos, sideróforos y ácido salicílico. Rev. Colomb. Biotecnol. 6(1), 6-13.

Hernández, I. 2015. Rizobios asociados a plantas de arroz (*Oryza sativa* L.) cultivar INCA LP-5. Efecto en el crecimiento del cultivo. Msc thesis, Universidad de La Habana, La Habana.

Hernández, I. and M.C. Nápoles. 2018. Efecto de diferentes fuentes de carbono sobre el crecimiento de un aislado de rizobio. Cult. Trop. 39(3), 87-90.

Hugh, R. and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative rods. J. Bacteriol. 66(1), 24-26.

Kapulnik, Y. 2002. Plant roots the hidden half. Plant growth promotion by rhizosphere bacteria. Marcel Dekker, New York, USA.

Knief, C., N. Delmotte, S. Chaffron, M. Stark, G. Innerebner, R. Wassmann, and C. von Mering. 2012. Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. Isme J. 6(7), 1378. Doi: 10.1038/ismej.2011.192

Koser, S.A. 1923. Utilization of the salts of organic acids by the colon-aerogenes group. J. Bacteriol. 8(5), 493-520.

Kovaks, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature 178, 703. Doi: 10.1038/178703a0

Kumar, S., G. Stecher, M. Li, C. Knyaz, and K. Tamura. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35(6), 1547-1549. Doi: 10.1093/molbev/msy096

Leboffe, M.J. and B.E. Pierce. 2010. Microbiology laboratory theory and application. Morton Publishing Company, Englewood, USA.

Luyten, E. and J. Vanderleyden. 2000. Survey of genes identified in *Sinorhizobium meliloti* spp., necessary for the development of an efficient symbiosis. Eur. J. Soil Biol. 36(1), 1-26. Doi: 10.1016/S1164-5563(00)00134-5

MacFaddin, J.F. 2000. Biochemical tests for identification of medical bacteria. Lippincott, Williams, and Wilkins. Philadelphia, USA.

Martín, G.M. and R. Rivera. 2015. Influencia de la inoculación micorrízica en los abonos verdes. Efecto sobre el cultivo principal. Estudio de caso: el maíz. Cult. Trop. 36, 34-50.

Permuy, A.N. 2013. El cultivo de maíz. Conferencia sobre el cultivo de maíz. Estación territorial de investigaciones agropecuaria de Holguín (ETIAH), Holguín, Cuba.

Ramírez-Bahena, M.H., P. García-Fraile, A. Peix, A. Valverde, R. Rivas, J.M. Igual, P.F. Mateos, E. Martínez-Molina, and E. Velázquez. 2008. Revision of the taxonomic status of the species *Rhizobium leguminosarum* (Frank 1879) Frank 1889AL, *Rhizobium phaseoli* Dangeard 1926AL and *Rhizobium trifolii* Dangeard 1926AL. *R. trifolii* is a later synonym of *R. leguminosarum*. Reclassification of the strain *R. leguminosarum* DSM 30132 (=NCIMB 11478) as *Rhizobium pisi* sp. nov. Int. J. Syst. Evol. Microbiol. 58(11), 2484-2490. Doi: 10.1099/ijs.0.65621-0

Sadowsky, M.J., H.H. Keyser, and B.B. Bohlool. 1983. Biochemical characterization of fast- and slow-growing rhizobia that nodulate soybeans. Int. J. Syst. Bacteriol. 33(4), 716-722. Doi: 10.1099/00207713-33-4-716

Sessitsch, A., J.G. Howieson, X. Perret, H. Antoun, and E. Martinez-Romero. 2002. Advances in Rhizobium research. Critical Rev. Plant Sci. 21, 323-378. Doi: 10.1080/0735-260291044278

Sugumaran, P. and B. Janarthanam. 2007. Solubilization of potassium containing minerals by bacteria and their effect on plant growth. World J. Agricul. Sci. 3(3), 350-355.

van Loon, L.C. 2007. Plant responses to plant growth-promoting bacteria. Eur. J. Plant. Pathol. 119, 243-254. Doi: 10.1007/s10658-007-9165-1

van Rossum, D., F.B. Schuuramns, M. Gilli, A. Muyotcha, H.W. van Versveld, A.H. Stouthamer, and F.C. Boogerd. 1995. Genetic and phenetic analyses of Bradyrhizobium strains nodulating peanut (*Arachis hypogaea* L.) roots. Appl. Environ. Microbiol. 61(4), 1599-1609.

Vessey, J.K. 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255(2), 571-586. Doi: 10.1023/A:1026037216893

Vidal, M., R. Ruiz, A. Antúnez, and C. Araya. 2015. Antecedentes nutricionales del cultivo de maíz en Chile. pp. 83-93. In: Antúnez, A., M. Vidal, S. Felmer, and M. González (eds.). Riego por pulsos de maíz grano. Boletín INIA no. 312. Instituto de Investigaciones Agropecuarias INIA, Rengo, Chile.

von Post, R., C. Dayteg, M. Nilsson, B. Forster, S. Tuvesson, and L. von Post. 2003. A high-throughput DNA extraction method for barley seed. Euphytica 130, 255-260. Doi: 10.1023/A:1022863006134

Wang, E.T. and J.C. Martínez-Romero. 2001. Taxonomía del Rhizobium. Centro de investigaciones sobre fijación de Nitrógeno. Microbios, Universidad Nacional Autónoma de México, Mexico.

Winn, W., S. Allen, W. Janda, E. Koneman, G. Procop, P. Schreckenberger, and G. Woods. 2006. Color atlas and textbook of diagnostic microbiology. Lippincott Williams and Wilkins, Philadelphia, USA.

Yang, Y., N. Wang, X. Guo, Y. Zhang, and Y. Boping. 2017. Comparative analysis of bacterial community structure in the rhizosphere of maize by high-throughput pyrosequencing. PLOS ONE 12(5), e0178425. Doi: 10.1371/journal.pone.0178425

Zúñiga, A., M.J. Poupin, R. Donoso, T. Ledger, N. Guiliani, R.A. Gutiérrez, and B. González. 2013. Quorum sensing and indole-3-acetic acid degradation play a role in colonization and plant growth promotion of *Arabidopsis thaliana* by *Burkholderia phytofirmans* PsJN. Mol. Plant Microbe. Interact. 26(5), 546-553. Doi: 10.1094/MPMI-10-12-0241-R