



New bacteria genera associated with rice (*Oryza sativa* L.) in Cuba promote the crop growth¹

Nuevos géneros bacterianos asociados al arroz (*Oryza sativa* L.) en Cuba promueven el crecimiento del cultivo

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Abstract

Introduction. The rice cultivar INCA LP-7 is one of the eleven principal cultivars in Cuba due to their high yield potential, salinity tolerance, and pathogens resistance. However, there are not reports about the isolation, identification, and characterization of bacteria associated to this cultivar that promote its growth. **Objective.** To select promising bacteria from the rhizosphere of the INCA LP-7 rice cultivar to promote the crop growth. **Materials and methods.** The investigation was carried out in 2015 and 2016 at the Instituto Nacional de Ciencias Agropecuarias, Cuba and Instituto de Investigaciones Biológicas Clemente Estable, Uruguay. The 16S rRNA gene of bacteria associated with the INCA LP-7 rice cultivar was isolated and identified by sequencing. Characterization was performed as plant growth promoting bacteria and the effect of bacterial inoculation on rice growth was studied. **Results.** Eleven rhizospheric bacteria were isolated from rice plants of the INCA LP-7 cultivar. A phylogenetic analysis showed that for the first time in Cuba *Pantoea*, *Acinetobacter*, and *Mitsuaria* genera were reported associated with rice. *Rhizobium* and *Enterobacter* genera were also informed as rhizospheric bacteria of INCA LP-7 rice cultivar. Some isolates solubilize inorganic phosphates and potassium, produce siderophores and indolic compound, had exo-celulase and protease activity and formed biofilm. The inoculation of isolates belonging to *Pantoea* genus produced an increase in height, root length, and biomass of rice plants under greenhouse conditions. **Conclusions.** *Pantoea* sp. GG1 and *Pantoea* sp. GG2 were the most promising strains for the inoculation of the INCA LP-7 rice cultivar. This is the first report in Cuba that focuses on the characterization of the bacterial microbiota part associated to Cuban rice cultivar INCA LP-7 and reports bacteria of the *Pantoea* genus as crop growth promoters.

Keywords: bacterial isolation, molecular identification, rhizosphere, grass, plant growth promotion.



Resumen

Introducción. El cultivar de arroz INCA LP-7 es uno de los once cultivares principales de Cuba por su alto potencial de rendimiento, tolerancia a la salinidad y resistencia a patógenos. Sin embargo, no existen reportes sobre el aislamiento, identificación y caracterización de bacterias asociadas a este cultivar que promuevan su crecimiento. **Objetivo.** Seleccionar bacterias promisorias provenientes de la rizosfera del cultivar de arroz INCA LP-7 para promover el crecimiento del cultivo. **Materiales y métodos.** La investigación se realizó en el 2015 y el 2016 en el Instituto Nacional de Ciencias Agropecuarias, Cuba y en el Instituto de Investigaciones Biológicas Clemente Estable, Uruguay. Se aisló e identificó por secuenciación el gen *16S rDNA* bacterias asociadas al cultivar de arroz INCA LP-7. Se realizó una caracterización como bacterias promotoras del crecimiento vegetal y se estudió el efecto de la inoculación bacteriana en el crecimiento del arroz. **Resultados.** Se aislaron once bacterias rizosféricas de plantas de arroz del cultivar INCA LP-7. Un análisis filogenético mostró que por primera vez en Cuba se reportaron los géneros *Pantoea*, *Acinetobacter* y *Mitsuaria* asociados con el arroz. Los géneros *Rhizobium* y *Enterobacter* también fueron informados como bacterias rizosféricas del cultivar de arroz INCA LP-7. Algunos aislados solubilizan fosfatos inorgánicos y potasio, producen sideróforos y compuestos indólicos, tuvieron actividad exocelulasa y proteasa y forman biopelículas. La inoculación de aislados pertenecientes al género *Pantoea* provocaron un incremento de la altura, longitud de las raíces y biomasa de plantas de arroz en condiciones de invernadero. **Conclusiones.** *Pantoea* sp. GG1 y *Pantoea* sp. GG2, fueron las cepas más promisorias para la inoculación del cultivar de arroz INCA LP-7. Este es el primer informe en Cuba que aborda la caracterización de parte de la microbiota bacteriana asociada al cultivar de arroz cubano INCA LP-7 e informa a bacterias del género *Pantoea* como promotoras del crecimiento del cultivo.

Palabras clave: aislamiento de bacterias, identificación molecular, rizosfera, gramíneas, promoción del crecimiento vegetal.

Introduction

The consumption of rice (*Oryza sativa* L.) provides 20 % of daily calories to more than 3.5 billion people. In the last years, world cereal production increased by up to 130 %, but this is not enough to satisfy the increasing demand (Fahad et al., 2019). In Cuba, rice is a prioritized crop, since annual consumption is around 72 kg per capita, it is one of highest in Latin America. The imports are the main source of cereal supply in the country (Food and Agriculture Organization [FAO], 2019).

The development of new rice cultivars is a strategy to reduce the rice imports in Cuba. The Cuban cultivar INCA LP-7 has an average yield of 6.7-7.8 t ha⁻¹. It is salinity tolerant and resistant to white mite (*Polyphagotarsonemus latus*) and the fungus *Pyricularia oryzae*, which are two pathogens causing significant damage to crop yields. These qualities have stimulated the distribution of this cultivar in more than 700 ha in the country (8 % of the total area dedicated to rice cultivation) and its commercialization in several countries of America (Pérez et al., 2015).

The mineral fertilization causes a significant increase in rice yield but carrying out this practice irrationally produces serious environmental problems (Vejan et al., 2016). The use of plant growth promoting bacteria (PGPB) is an alternative to mineral fertilization, especially in economically important crops such as rice. These bacteria promote plant growth by direct and indirect mechanisms. The phytostimulation and the increase of availability and absorption of nutrients are known as direct mechanisms. Within the indirect mechanisms, the PGPB can establish an antagonistic activity against phytopathogen by allelochemical compounds or they can induce a systemic resistance in the plant (Singh et al., 2017).

In Cuba, there are few studies about isolation and identification of bacterial community associated with rice. The genera *Bacillus*, *Pseudomonas*, *Burkholderia*, *Azospirillum*, *Azotobacter* and *Herbaspirillum* are highly

present in the rhizosphere of the Cuban rice cultivars INCA LP-5 and J-104 (Rives et al., 2006; 2010; Tejera-Hernández et al., 2013). The genera *Acinetobacter*, *Enterobacter* and *Mitsuaria* were associated at rice in different edaphoclimatic conditions (Gandhi & Muralidharan, 2016; Suman et al., 2016; Zhang et al., 2018). There are few studies about the interaction of these genera with rice in Cuba. Some strains belonging to these genera promote the growth of grasses like wheat and rice, through nitrogen biological fixating and phosphate solubilizing (Mahato & Kafle, 2018). However, there are no studies about the identification of bacterial strains associated with rice cultivar INCA LP-7 that promote its growth. The objective of this research was to select promising bacteria from the rhizosphere rice cultivar INCA LP-7 to promote the crop growth.

Materials and methods

Isolation and identification of bacteria associated to *O. sativa* cv. INCA LP-7

Bacterial isolation was performed at the Department of Plant Physiology and Biochemistry of the Instituto Nacional de Ciencias Agrícolas, Mayabeque, Cuba, between April to May 2015. The molecular identification of isolates was done at the Department of Microbial Biochemistry and Genomics of the Instituto de Investigaciones Biológicas Clemente Estable, Uruguay in October-November 2016.

Strains isolation from rice rhizosphere

This research was part of a previous study that aimed to identify rhizobia associated with the *O. sativa* cultivar INCA LP-7 (unpublished). This cultivar was obtained from *in vitro* culture of mature rice seeds of the Amistad-82 in saline solution (NaCl 4000 ppm). Furthermore, it is one of the eleven planted in Cuba since it has high yield potential and moderate resistance to *Pyricularia oryzae*, the main rice pathogen in the country. In addition, this cultivar is salinity tolerant and the only one in Cuba resistant to white mite (*Polyphagotarsonemus latus*) (Pérez et al., 2015).

At the moment of isolation, the rice plants were 30 days old and were growing under flooded conditions, in a Petroferric Nodule Ferruginous Gleysol soil at Scientific and Technological Unit, Pinar del Rio, Cuba (22° 44' N, 83° 45' W, 60 m.a.s.l.). This soil, with no inoculation history, herbicide application or mineral fertilization, presented the following characteristics: high clay content, poor drainage, acid pH, high organic matter content, and low phosphorus, calcium, magnesium, sodium, and potassium contents (Hernández-Jiménez et al., 2015) (Table 1). The data were donated by Plant Nutrition and Biofertilization Laboratory of the Instituto Nacional de Ciencias Agrícolas, Mayabeque, Cuba.

Five collecting site were established in 3,200 m² of rice field following a W o zig-zag patron and four plants were collected in each collecting site (20 plants in total). The plants were extracted from the soil and stored in polyethylene bags at 4 °C. There were placed 10 g of rhizospheric soil were placed in Erlenmeyer flasks containing 100 mL of sterile distilled water and the suspension was kept under shaking conditions for 10 min at 60 rpm.

The roots were extracted and, from suspension, serial dilutions were made in sterile distilled water (10⁻¹-10⁻⁶). The isolation was performed by spreading culture from 0.1 mL of dilutions on plates with Yeast-Mannitol (LM) solid medium (g L⁻¹: mannitol 10.0, yeast extract 0.5, K₂HPO₄ 0.5, MgSO₄ 7H₂O 0.20, NaCl 0.1, CaCl₃ 0.15, agar 15.0) (Vincent, 1970) since it is the traditional medium for rhizobia isolation. The cultures were incubated at 29 ± 1 °C for ten days. The bacterial colonies were selected following cultural criteria reported for genus *Rhizobium*. It is large whitish colony (2-4 mm) and mucous presence (Vincent, 1970).

Table 1. Chemical description of the Petroferric Nodule Ferruginous Gleysol soil where rice plants were cultured. Instituto Nacional de Ciencias Agrícolas, Plant Nutrition and Biofertilization Laboratory, Mayabeque, Cuba, 2015.

Cuadro 1. Descripción química del suelo Gleysol Nodular Ferruginoso Petroférrico donde se cultivaron plantas de arroz (*Oryza sativa* L.). Instituto Nacional de Ciencias Agrícolas, Laboratorio de Nutrición Vegetal y Biofertilización, Mayabeque, Cuba, 2015.

pH (KCl)	P ₂ O ₅ (mg 100 g ⁻¹ soil)	Organic matter (%)	Ca ²⁺	Mg ²⁺	Na ⁺	K ⁺
			cmol _c kg ⁻¹ Soil			
5.31±0.5	6.60±0.8	4.08±0.9	5.80±0.3	2.88±0.6	0.19±0.07	0.15±0.08

16S rRNA amplification, sequencing and phylogenetic analysis

The *16S rRNA* gene of the isolates was PCR-amplified and sequenced as described by Mareque et al. (2015). For the amplification, the universal primers Eub27f (5'-AGAGTTTGATCMTGGCTCAG-3') and Eub1492r (5-TACGGYTACCTTGTTACGACTT-3) were used. The quality of sequences obtained were checked in the FinchTV program (v.1.4.0) (Geospiza, Inc.), using as an acceptance criterion a quality value equal to or higher than 20 per base. The sequences were assembled using the DNA Baser Sequence Assembler software (v. 4.10.1.13) (Heracle BioSoft SRL, 2020) and the consensus sequences obtained for the isolates were deposited in GenBank under the corresponding accession numbers.

The closest type strains sequences were selected and obtained by comparing the consensus sequences with the database of National Center for Biotechnology Information (NCBI) database. Sequence alignments were performed using ClustalW multiple sequence alignment tool (Gómez-Padilla et al., 2017), in the MEGA-X software (v. 10.0.4) (Kumar et al., 2018). The phylogenetic tree was generated using the same program by the Maximum-Likelihood statistical method, with 1000 bootstrap replicates and the Tamura-Nei substitution model with a discrete Gamma distribution. The bootstrap value in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Bacillus campisalis* SA2-6 was used as the outgroup.

Screening for Plant Growth Promoters traits

The characterization of strains from rice rhizosphere was performed. Phosphate and potassium solubilizing, indolic compounds production, hydrolytic activity, and biofilm formation was evaluated. All this experiment was carried out in October-November 2016

Phosphate and potassium solubilizing capabilities and siderophore production

The bacteria were grown in Tryptone-Yeast (TY) medium (g L⁻¹: Tryptone 5.0, yeast extract 3.0, CaCl₂ 0.68) with an optical density of 0.2 at 620 nm. In triplicate, 10 µL of each culture were spotted in GL glucose-rich medium supplemented with calcium and phosphates salts (g L⁻¹: glucose 10.0, yeast extract 2.0, agar 15.0 and two salt solutions: K₂HPO₄ 10 % 50 mL L⁻¹, CaCl 10 % 100 mL L⁻¹; pH medium 5.8) and National Botanical Research Institute's phosphate (NBRIP) medium (g L⁻¹: glucose 10.0, Ca₃(PO₄)₂ 5.0, MgCl₂ 6H₂O 5.0, MgSO₄ 7H₂O 0.25, KCl 0.2, (NH₄)₂SO₄ 0.1, agar 18.0) to evaluate the phosphate solubilization capacity; whereas Aleksandrov medium (g L⁻¹: sucrose 10.0, K₂HPO₄ 1.5, MgSO₄ 7H₂O 0.5, CaCO₃ 1.0 g, agar 15.0 pH medium 7.5) was used to evaluate the potassium solubilization. In both assays, positive strains were detected by the presence of a translucent halo around the colony after 72 h of growth at 30 °C. In that moment, translucent halos on the NBRIP medium were

measured (cm) and the solubilizing index was calculated. *Pantoea* sp. UYSB45 was used as a positive control for phosphate and potassium solubilization (Mareque et al., 2015).

Siderophore-producing bacteria were detected using the chromeazuroil agar (CAS) assay (Schwyn & Neilands, 1987). The bacterial strains were grown in TY medium for 16 h and aliquots of 10 μL of bacteria inoculum were put on the CAS medium. Positive strains were detected by the presence of a yellow halo around the colony after 72 h of growth at 30 °C. *Herbaspirillum seropedicae* Z67 was used as positive control.

Indolic compounds production

The colorimetric method was used to test the entire collection of isolates with the aim to detect the production of indolic compounds as described previously (Taulé et al., 2011). The strains were grown in TY medium with and without tryptophan (200 $\mu\text{g mL}^{-1}$) as inductor of synthesis to indolic compounds. Positive strains were detected by pink coloration of medium after 72 h of growth at 30 °C. *Gluconoacetobacter diazotrophicus* Pal5 was used as positive control. This determination was carry out twice and three replicates per strain were used.

Hydrolytic activity

For determination of exo-celulase, hemicellulase and protease activities, the isolates were grown in plates containing TY solid medium supplemented with 0.2 % carboximetyl cellulase or 0.5 % Avicel (Sigma-Aldrich, Germany) (Kim et al., 2008), respectively. In the case of protease activity detection, plates containing Triptone-Soy Agar diluted medium (1:100) were supplemented with 10 % (w/v) of skimmed milk (Martínez-Rosales & Castro-Sowinski, 2011). Positive strains, producing exo-celulase, hemicellulose, and protease enzymes, were detected by the presence of a yellow halo around the colony after 48 h growing at 30 °C. Strain *Acinetobacter* sp. UYSB41 (Mareque et al., 2015) was used as positive control in hemicellulase and cellulase hydrolysis assays and the strain *Pseudomonas* sp. UYFA214 (de los Santos et al., 2015) for protease activity.

Biofilm formation

Biofilm formation was detected using the crystal violet (CV) staining method. Isolates were first grown at 30 °C with agitation, in 96-microwell plates containing 200 μL of TY medium, until an optical density of 0.2 at 620 nm. Plates were then incubated 48 h at 30 °C without agitation, the supernatant was removed, and the plates were washed with phosphate buffered saline. For staining, a 0.1 % (w/v) CV solution was added (200 μL per well) and the plates were incubated for 20 min. The CV excess was removed by washing the plates under running tap water while the bound CV was released from the cells by adding 200 μL of 95 % (v/v) ethanol. The suspension absorbance was measured at 570 nm. All steps were carried out at room temperature (Peeters et al., 2008).

Effect of bacterial inoculation in rice growth

Inoculation assays were conducted out only with the more promising strains according the screening for plant growth promoters' traits and the assays were done twice. Thus the treatment's number was ajusted to the selected strains. The experiment was carried out in February-June 2016. Seeds of *O. sativa* cv. INCA LP-7, donated for Scientific and Technological Unit, Pinar del Rio, Cuba; were surfaced disinfected according to García et al. (2008), using sodium hypochlorite. The disinfected seeds were placed in Petri dishes containing 0.8 % (w/v) agar in water and incubated in the dark at 30 °C, for three days. Three seedlings were sown into a pot containing 1.2 kg of

Petroferric Nodule Ferruginous Gleysol soil (non-sterile) as substrate. Non-sterile soil was used since the interest that to know the inoculation effect in presence of the resident microbiota in the same soil that the strains come from.

The soil substrate presents the following characteristics: high clay content, poor drainage, acid pH, high organic matter content, and low phosphorus, calcium, magnesium, sodium and potassium contents (Hernández-Jiménez et al., 2015) (Table 2). The dates were obtained in Plant Nutrition and Biofertilization Laboratory of the Instituto Nacional de Ciencias Agrícolas, Mayabeque, Cuba.

Table 2. Chemical description of the substrate of the Petroferric Nodule Ferruginous Gleysol soil used in rice plant inoculation (*Oryza sativa* L.) assays. Instituto Nacional de Ciencias Agrícolas, Plant Nutrition and Biofertilization Laboratory, Mayabeque, Cuba, 2015.

Cuadro 2. Descripción química del sustrato de suelo Gleysol Nodular Ferruginoso Petroférrico utilizado en ensayos de inoculación de plantas de arroz (*Oryza sativa* L.). Instituto Nacional de Ciencias Agrícolas, Laboratorio de Nutrición Vegetal y Biofertilización, Mayabeque, Cuba, 2015.

pH (KCl)	Organic matter (%)	P ₂ O ₅ (mg 100 g ⁻¹ soil)	Ca ²⁺	Mg ²⁺	Na ²⁺	K ⁺
			(cmol kg ⁻¹)			
6.57±0.27	3.05±0.70	75.1±6.5	11.62±0.97	4.75±0.1	Traces	0.80±0.02

The pots were placed in trays with diluted Hoagland nutrient solution (1:2) and were maintained under this condition during the experiment. Different trays were used for each treatment avoided contamination between treatments. Each seedling was inoculated with 300 µL (5x10⁹ CFU mL⁻¹) of each bacterial strain grown in liquid TY medium. A negative control without bacterial inoculation and fertilization was included. At 7 days' post inoculation (dpi), two plants were discarded and only one per pot was grown, to allow adequate plant development, principally in posterior moments of experiment.

The plants grew under greenhouse following a randomized design and using 10 replicates per treatment. Plants were grown with a photoperiod of 12 h light / 12 h darkness conditions during 50 days. At that time, plants were harvested and plant height (cm) and root length (cm) were determined with a graduated ruler. Furthermore, the biomass (g) of roots and aerial parts dried at 70 °C until constant weight was recorded, using an analytical balance.

Statistical analysis

Data from the screening for phosphate solubilizing ability, indolic compound, and rice growth in greenhouse conditions were subjected to normality test (Bartlett test) and variance homogeneity (Kolmogorov-Smirnov test). Simple classification analysis of variance was applied, with the Tukey HSD mean comparison test for p<0.05. The Statgraphic Plus version 5.0 software was used for the statistical processing of the data and the Microsoft Excel 2016 program for its representation.

Results

Proteobacteria strains reside in the rhizosphere of rice cultivar INCA LP-7

The partial *16S rRNA* sequencing and phylogenetic analysis showed that isolates belong to Proteobacteria class and were distributed in five genera: *Rhizobium*, *Mitsuaria*, *Acinetobacter*, *Enterobacter* and *Pantoea* (Table 3).

Table 3. *16S rRNA* nucleotide sequence similarities of bacterial isolates from rice (*Oryza sativa* L.) cv. INCA LP-7. Microbial Biochemistry and Genomics Laboratory, Montevideo, Instituto Nacional de Ciencias Agrícolas Clemente Estable, Uruguay. 2016.

Cuadro 3. Similitud de la secuencia nucleotídica del gen *ARNr 16S* de cepas bacterianas provenientes de arroz (*Oryza sativa* L.) cv. INCA LP-7. Laboratorio de Bioquímica y Genética Microbiana, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay. 2016.

Name	Isolate		Best hist ^c				
	Identification ^a	Accession number ^b	Name	Accession number	Coverage	E value	Maximum Identity (%) ^d
1AA	<i>Rhizobium</i> sp.	MT759825	<i>Rhizobium alamii</i> LMG 24466	MF977621.1	99 %	0.0	98.6 (1323/1341)
1DD1	<i>Pantoea</i> sp.	MT759826			100 %	0.0	99.7 (1311/1315)
1DD2	<i>Pantoea</i> sp.	MT759827			100 %	0.0	99.8 (1238/1240)
II2	<i>Pantoea</i> sp.	MT759834			100 %	0.0	99.6 (1231/1235)
5O	<i>Pantoea</i> sp.	MT759830	<i>Pantoea dispersa</i> DSM 30073	AB907780.1	100 %	0.0	99.7 (1310/1314)
GG1	<i>Pantoea</i> sp.	MT759832			100 %	0.0	97.1 (1238/1275)
GG2	<i>Pantoea</i> sp.	MT759833			100 %	0.0	99.7 (1238/1241)
4S	<i>Pantoea</i> sp.	MT759828			100 %	0.0	99.7 (1238/1241)
3W	<i>Acinetobacter</i> sp.	MT764778	<i>Acinetobacter lwoffii</i> JCM 6840	NR_113346.1	100 %	0.0	99.0 (1346/1359)
II3	<i>Mitsuaria</i> sp.	MT759835	<i>Mitsuaria noduli</i> HZ7	NR_159302.1	100 %	0.0	98.7 (1175/1190)
5FF1	<i>Enterobacter</i> sp.	MT759829	<i>Enterobacter cloacae</i> LMG 2683	NR_044978.1	99 %	0.0	99.8 (1206/1208)

^aTaxonomic identification based on the *16S rRNA* gene similarity. / ^aIdentificación taxonómica basada en la similitud del gen *ARNr 16S*.

^bAccession number of consensus sequences deposited in GenBank. / ^bNúmero de accesión de la secuencia consenso depositada en el GenBank.

^cFirst reference strain that appears in the BLASTn with the highest percentage of identity. / ^cPrimera cepa de referencia que aparece en el BLASTn con mayor porcentaje de identidad.

^dFractions in parentheses represents the number of identical nucleotides over the total of compared nucleotides / ^dDentro del paréntesis aparece el número de nucleótidos idénticos en relación al total de nucleótidos comparados.

Results indicated that only isolate 1AA belonged to genus *Rhizobium* and formed a well-supported cluster closely related to some type strains of *Rhizobium* (Figure 1) and with a 98.66 % of identity with *Rhizobium alamii* LMG 24466T (Table 3).

The strains II3, 3W and 5FF1 were closely related to the type strains *Mitsuaria noduli* HZ7^T, *Acinetobacter lwoffii* JCM 6840^T and *Enterobacter cloacae* ATCC 23373^T, respectively (Figure 1, Table 3). The other seven strains were related with *Pantoea* genus and were closely related to the reference strain *Pantoea dispersa* DSM 30073^T (Figure 1) with a high percentage of identity (Table 3).

Bacteria strains from rice plants cultivar INCA LP-7 have biofertilization, phytostimulation and plant infection potentialities

The characterization of the eleven rizospheric strains allowed distinguishing some potentialities as PGPB. The results showed that 54.5 % and 27.3 % of them solubilized calcium phosphate in NBRIP and GL media, respectively; since translucent halos around the growth were showed (Table 4).

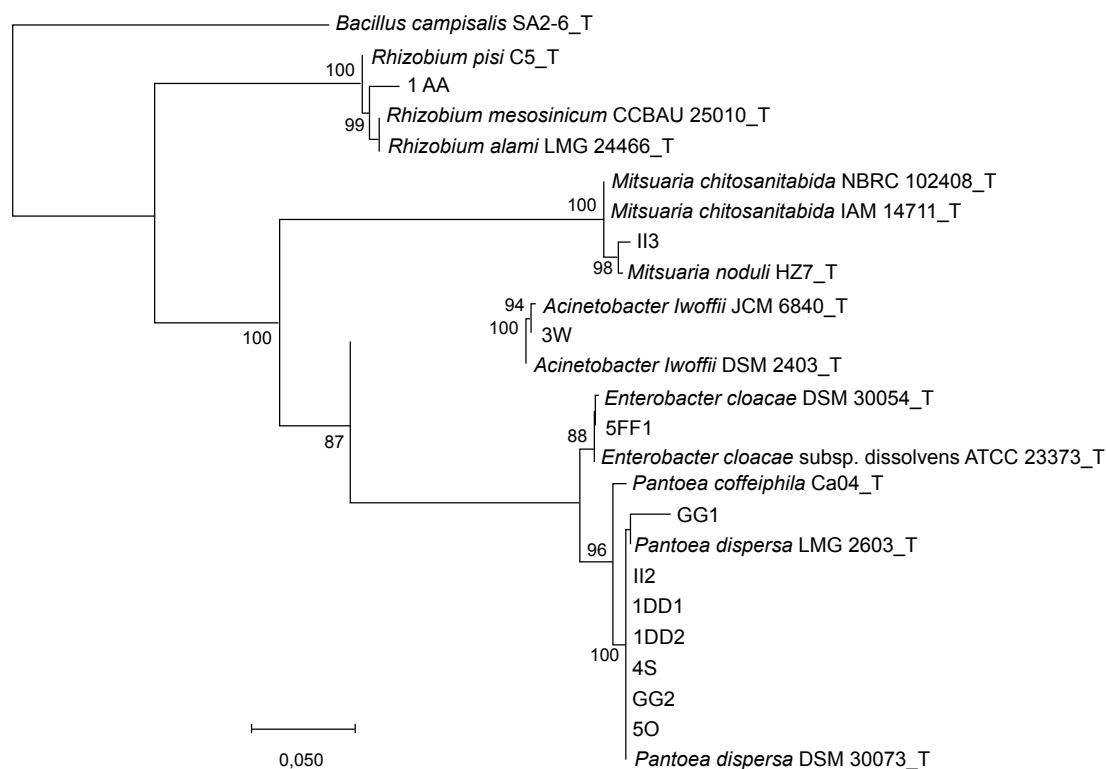


Figure 1. Phylogenetic tree with eleven 16SrRNA sequences of the ARNr16S gene of bacteria from the rhizosphere rice plants (*Oryza sativa* L.), cultivar INCA LP-7. Instituto de Investigaciones Biológicas Clemente Estable, Microbial Biochemistry and Genomics Laboratory, Montevideo, Uruguay. 2016.

Figura 1. Árbol filogenético con once secuencias del gen *ARNr16S* de bacterias provenientes de la rizosfera de plantas de arroz (*Oryza sativa* L.) cultivar INCA LP-7. Instituto de Investigaciones Biológicas Clemente Estable, Laboratorio de Bioquímica y Genómica Microbiana, Montevideo, Uruguay. 2016.

Strains 1DD2, GG1, and GG2 solubilized the salt in both media used. The size of halos observed around the strains 1DD1, 1DD2, 5O, GG1, GG2, and 5FF1 were similar to that obtained for *Pantoea* sp. UYSB45 used as positive control (Table 4).

The 54.5 % of the strains produced siderophores and 63.6 % indole compounds. The strain *G. diazotrophicus* PAL5 produced higher indolic compound than the rest of strains in culture medium with tryptophan (Table 4). The results showed other PGP potentialities such as potassium solubilizing (strain 3W) and exo-cellulase (strain 4S) and protease activity (strain 1DD1). Hemicellulase activity was not detected in any isolates.

The 72.7 % of strains formed biofilms in 96-microwell plates. The strain 1AA did not show any of attributes, and the strains 4S and II2 only one; so, they were not used in subsequent experiments.

***Pantoea* promote the rice plants cultivar INCA LP-7 growth in greenhouse conditions**

Three strains (1AA, 4S and II2) were fewer promising bacteria according the screening for plant growth promoters' traits. Only eight strains were used in inoculation assay and control plants without bacterial inoculation

Table 4. Plant growth promotion attributes of bacterial isolates from rice (*Oryza sativa* L.) cv. INCA LP-7. Instituto de Investigaciones Biológicas Clemente Estable, Microbial Biochemistry and Genomics Laboratory, Montevideo, Uruguay. 2016.

Cuadro 4. Atributos de promoción de crecimiento vegetal de los aislados bacterianos provenientes de arroz (*Oryza sativa* L.) cv. INCA LP-7. Instituto de Investigaciones Biológicas Clemente Estable, Laboratorio de Bioquímica y Genómica Microbiana, Montevideo, Uruguay. 2016.

Isolate	Identification ^a	Biofertilization and Phytostimulation					Infection			
		Phosphate Solubilization		Solubili- zation K	Sidero- phores	Indolic Compound ($\mu\text{g mL}^{-1}$)	Exo-ce- llulase	Hemice- llulase	Protease	Biofilm
		National Botanical Research Institute's Phosphate medium*	Glucose yeast ex- tract rich medium							
1AA	<i>Rhizobium</i>	-	-	-	-	0.0 c	-	-	-	-
1DD1	<i>Pantoea</i>	1.6	-	-	+	24.4 b	-	-	+	+
1DD2	<i>Pantoea</i>	1.4	+	-	+	25.2 b	-	-	-	+
4S	<i>Pantoea</i>	-	-	-	-	0.0 c	+	-	-	-
5O	<i>Pantoea</i>	1.6	-	-	+	26.9 b	-	-	-	+
GG1	<i>Pantoea</i>	1.5	+	-	+	23.8 b	-	-	-	+
GG2	<i>Pantoea</i>	1.6	+	-	+	25.3 b	-	-	-	+
II2	<i>Pantoea</i>	-	-	-	-	0.0 c	-	-	-	+
5FF1	<i>Acinetobacter</i>	1.7	-	-	+	0.0 c	-	-	-	+
3W	<i>Mitsuaria</i>	-	-	+	-	24.6 b	-	-	-	-
II3	<i>Enterobacter</i>	-	-	-	-	21.4 b	-	-	-	+
Reference strains										
	<i>Pantoea</i> sp. UYSB45	1.2	+	+	nd	nd	nd	nd	nd	nd
	<i>H. seropedicae</i> Z67	nd	nd	nd	+	nd	nd	nd	nd	nd
	<i>G. diazotrophicus</i> Pal5	nd	nd	nd	nd	37.5 a	nd	nd	nd	nd
	<i>Acinetobacter</i> UYSB41	nd	nd	nd	nd	nd	+	+	nd	nd
	<i>Pseudomonas</i> sp. UYFA214	nd	nd	nd	nd	nd	nd	nd	+	nd
	SEx	0.09 ns				1.14				

^aTaxonomic identification was based on the *16S rRNA* similarity. / ^aIdentificación taxonómica basada en la similitud del gen *ARNr16S*. nd: not determined; (+): positive; (-): negative; (*): solubilization index calculated according to Kumar and Narula (1999). / nd: no determinado; (+): positivo; (-): negativo; (*): índice de solubilización calculado según Kumar y Narula (1999).

Mean of three replicates (n=3). Same latter in the same column were not statistically different between means (Tukey HSD p<0.05). / Medias de tres replicas (n=3). Letras iguales en la misma columna no difirieron estadísticamente entre las medias (Tukey HSD p<0.05).

and without fertilization; thus, nine treatments in total were done. Results exhibited that the treatments inoculated with 1DD2, II2, GG1 or GG2 increased significantly the plant height. The inoculation of strain GG1 produced the higher value at 50 dpi in greenhouse conditions (Figure 2A).

Furthermore, the plants inoculated with strains 1DD1, GG1, and GG2 increased the root length. The treatment inoculated with strain GG2 produced the higher increased of this variable (Figure 2A).

The evaluation of the inoculation effect on plants biomass, showed that the treatment inoculated with strain GG1 favored the growth of aerial dry weight. Additionally, the plants inoculated with strains GG1 and GG2 increased significantly the root dry weight (Figure 2B).

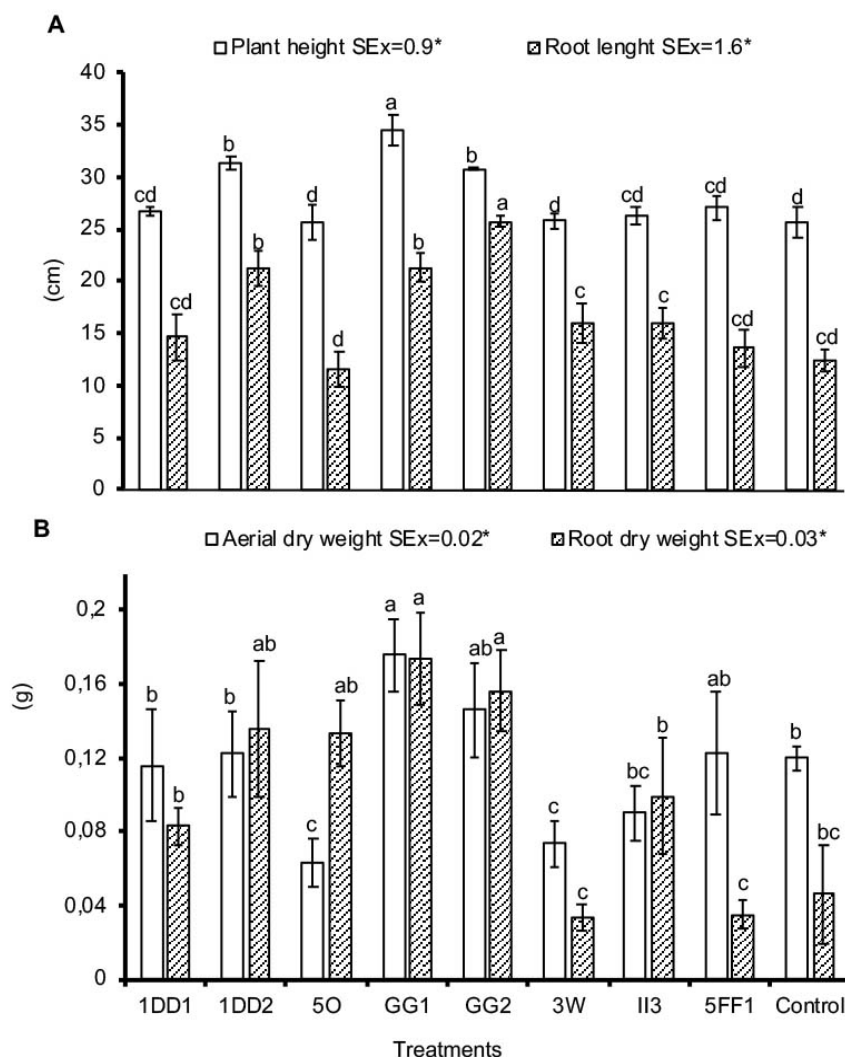


Figure 2. Effect of bacterial strains inoculation on rice plants (*Oryza sativa* L.) cv. INCA LP-7 growth under greenhouse conditions. Plant height and root length (A); aerial dry weight and root dry weight (B) 50 days post inoculation (dpi). Instituto Nacional de Ciencias Agrícolas, Physiology and Biochemistry Laboratory, Mayabeque, Cuba, 2016.

The control treatment consisted of non-inoculated plants. The reported data are the means \pm the standard errors (SE) of the mean from ten sample replicates (Tukey HSD $p < 0.05$, $n = 10$).

Figura 2. Efecto de la inoculación de cepas bacterianas sobre el crecimiento de plantas de arroz (*Oryza sativa* L.) cv. INCA LP-7, en condiciones de invernadero. Altura de la planta y longitud de la raíz (A); peso seco parte aérea y peso seco de la raíz (B) 50 días después de la inoculación (ddi). Instituto Nacional de Ciencias Agrícolas, Laboratorio de Fisiología y Bioquímica, Mayabeque, Cuba, 2016.

El tratamiento control consistió en plantas no inoculadas. Los datos reportados son las medias \pm los errores estándar (ES) de la media provenientes de diez réplicas (Tukey HSD $p < 0,05$; $n = 10$).

Discussion

A group of bacteria with similar cultural characteristics to *Rhizobium* genus was isolated from rice plant cultivar INCA LP-7 rhizosphere. However, before the *16S rRNA* sequencing was determinate that they belonged

to three classes: Alphaproteobacteria (*Rhizobium*), Betaproteobacteria (*Mitsuaria*), and Gammaproteobacteria (*Acinetobacter*, *Enterobacter* and *Pantoea*). The phenotypic studies can be complemented with molecular tools such as *16S rRNA* sequencing (polyphasic taxonomy) for bacterial identification. This is the first report that studies part of the bacterial community associated to Cuban rice cultivar INCA LP-7.

The flood conditions where the rice plants cultivar INCA LP-7 grew at the isolation moment and the soil acidity could explain the number and diversity of genera identified in this study. Soil moisture is a factor that determines the rhizosphere colonization by soil bacteria (Classen et al., 2015). The fine texture and high clay content of Petroferric Nodule Ferruginous Gleysol soil maintain flooding conditions in rice fields (Hernández-Jiménez et al., 2015). Additionally, is known the negative effect of acidity on the multiplication and viability of soil bacteria, which negatively influence rhizosphere colonization (Bellabarba et al., 2019). Previously, a similar number of bacteria isolated from rice Cuban cultivar (INCA LP-5) was reported in the same soil and similar culture conditions (Hernández Forte & Nápoles García, 2017).

A *Rhizobium* strain was identified for first time associated with rice plants cultivar INCA LP-7. Recent studies showed that rhizobia colonize the rhizosphere of rice plants in countries like Egypt (Yanni & Dazzo, 2015). The *R. alamii* strains that are phylogenetically close to strain IAA were identified from the rhizosphere of sunflowers (Berge et al., 2009).

Previously, *Enterobacter* genus was identified from Cuban rice cultivar and promotes the crop growth (Hernández-Rodríguez et al., 2016). This is a first report in Cuba that informs the association *Enterobacter*-rice cv. INCA LP-7 and between the genera *Mitsuaria* and *Acinetobacter* with rice plants.

The genus *Pantoea* was another of those associated to rice plants cultivar INCA LP-7. This genus colonizes the rhizosphere of grasses such as rice and sugarcane (Cui et al., 2020; Walitang et al., 2018). In Cuba only one report informed the association *Pantoea*-plants and it was not even with rice but with sugarcane (Fernández et al., 2004). Moreover, *P. dispersa* LMG2603, a specie that formed a well-supported cluster with all the strains belonging to the genus *Pantoea* studied here, colonizes the endosphere and rhizosphere of maize and rice, promoting their growth even with mineral fertilization and salinity condition (Marag & Suman, 2018; Shahzad et al., 2017).

The characterization of rhizospheric strains of rice cultivar INCA LP-7 showed some PGP and interaction traits *in vitro* that allow to select those most promising for crop inoculation. Additionally, it is important the fact that these bacteria were isolated from Gleysol Nodular Ferruginous Petroferric soil. The strains adaptation to chemical and physical characteristics of the soils is important to select promising strains for effectiveness inoculants (Kaminsky et al., 2019). These soils have iron nodules and the calcium phosphates insolubility increases since flood conditions which rice is subjected (Hernández-Jiménez et al., 2015). This edaphic condition justifies searching bacterial strains with potentialities in the phosphate solubilization and siderophores production such as some *Pantoea* and *Enterobacter* strains studied here. The essential role of calcium in the host plant is well known as it produces molecular changes in root cells and act as a signaling agent between the plant and bacteria (Groth et al., 2010). Recent studies demonstrate an increase in phosphorous and iron in the plant inoculated with *Pantoea* and *Enterobacter* (Ferreira et al., 2019; Jiang et al., 2019).

Potassium is another essential element for rice growth since the plant uptake K in larger amounts compared to N and P (Islam & Muttaleb, 2016). However, the amount of soluble potassium in soils is usually very low such as Gleysol Nodular Ferruginous Petroferric soil (Hernández-Jiménez et al., 2015). Recent research shows that the inoculation with potassium solubilizing *Acinetobacter* spp. enhances the absorption of this element by rice plants (Bakhshandeh et al., 2017). This could be the case of the *Acinetobacter* sp. 3W strain associated to rice cultivar INCA LP-7.

The indole compounds production by *Pantoea*, *Acinetobacter*, and *Mitsuaria* strains was demonstrated in this investigation in medium with tryptophan. This amino acid induces the indole compounds production such as indol acetic acid, which has auxin activity in plants and promotes their growth (Fang et al., 2019). In legumes, for example, some auxins are related with regulator genes of infection process and the nodule organogenesis (Liu et al., 2018).

Microbial hydrolytic enzymes have been studied as they constitute mechanisms that enable plant colonization and present antagonistic activity against phytopathogenic fungi. The endoglucanases and proteases produced by *Pantoea*, *Mitsuaria*, and *Enterobacter*, studied here, were reported previously (Das et al., 2017; Fan et al., 2016). These enzymes degrade the fungi cell wall, decreasing their populations in the soil and damaging effects on the plant (Jayasekara & Ratnayake, 2019; Rajeswari & Kapoor, 2017).

The biofilm formation is another mechanism used by PGPB for plant colonization, it is a strategy to enhance the plant-bacteria interaction and are resistance structures against microbial antibody, aggressive substance from plants and abiotic factors such as ultraviolet light, osmotic stress and pH changes (Das et al., 2017; Paula et al., 2020). The crystal violet method showed biofilm formation in polystyrene plates with inoculants of *Pantoea*, *Mitsuaria*, and *Enterobacter*, which could represent a potentiality of these bacteria to colonize the rice plant and survive under stress conditions.

The characterization of rhizospheric strains from rice plants cultivar INCA LP-7 allowed selecting some as the most promising for inoculation tests under greenhouse conditions. The *Pantoea* sp. strains promoted the plant height, root length and dry weight. Previously, these beneficial effects were demonstrated when rice plant inoculated with *P. ananatis* and *P. dispersa* promoted the grass growth, enhanced the formation of root hairs and gravitropic response of root (Megías et al., 2016; Shahzad et al., 2017).

In Cuba, there are previous works about PGPB inoculation to promote rice growth and the genera *Azospirillum*, *Herbaspirillum* and *Enterobacter* have been used. The inoculation of *Azospirillum* increases the plant height and aerial dry weight by 21.8 and 102.1 %, respectively, and allows reducing the nitrogen fertilization by 33 % (García et al., 2010; Velazco & Castro, 1999). Additionally, the inoculation of one Cuban rice variety (J-104) with *Herbaspirillum* increases the total nitrogen content in grains (Rives et al., 2007). More recent works show that rice inoculation with *Enterobacter* promotes the crop growth (Hernández-Rodríguez et al, 2016). However, the positive effect of *Pantoea* inoculation on Cuban rice cultivars had never been proven before. This is the first report in the country that shows the beneficial effect of *Pantoea* inoculation to promote rice growth.

Phytostimulation by indole compounds production and increase nutrients availability by phosphates solubilizing and siderophores production are some of the mechanisms used by *Pantoea* to promote the rice and wheat growth (Gholamalizadeh et al., 2017; Tahir et al., 2020). The biofilms formation enhances the rhizosphere colonization and beneficial effects of microbial metabolites on the plant growth (Ansari & Ahmad, 2018). These mechanisms were identified in *Pantoea* strains GG1 and GG2 that promoted the rice cultivar INCA LP-7 growth.

Conclusions

For first time in Cuba the genera *Pantoea*, *Acinetobacter*, and *Mitsuaria* are reported associated with rice, and *Rhizobium* and *Enterobacter* are informed as rhizospheric bacteria of plant rice cultivar INCA LP-7. These genera show some PGP traits and promote the rice growth. *Pantoea* sp. GG1 and *Pantoea* sp. GG2 are the most promising strains for inoculation of rice cultivar INCA LP-7.

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