

*Full Length Research Paper*

# **Characterization of fluorescent *Pseudomonas* from *Oryza sativa* L. rhizosphere with antagonistic activity against *Pyricularia oryzae* (SACC.)**

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Received 11 April, 2018; Accepted 10 September, 2018

The aim of this work was to select and characterize native Cuban fluorescent *Pseudomonas* from the rhizosphere of *Oryza sativa* with *in vitro* and *in vivo* antagonistic activity against *Pyricularia oryzae*. A total of 200 isolates were recovered with the typical growth and cultural characteristics of fluorescent *Pseudomonas*. The results showed that 12.5% of total isolates were capable of inhibiting mycelial growth, with different levels of inhibition between strains. Six of them (AI03, AI05, AI08, AJ01, AJ13 and AJ29) were able to reduce disease severity and incidence *in vivo* whereas only four (AI05, AJ13, AJ01 and AI08) showed efficient control of *P. oryzae* in greenhouse conditions. *Pseudomonas fluorescens* AI05 and *Pseudomonas putida* AJ13 were able to inhibit *in vitro* the mycelial growth of *P. oryzae* and to reduce symptom severity of *Pyricularia* infection. The production of lytic enzymes, siderophores, hydrogen cyanide (HCN), as well as the detection of genes encoding antibiotics and bacterial motility were also assessed for both strains. They were able to fix nitrogen, produce indolic compounds and to solubilize Pi. These results demonstrate the potential use of *P. fluorescens* AI05 and *P. putida* AJ13 as a biocontrol agent for the protection of rice plants from *P. oryzae* infection.

**Key words:** Biocontrol, rice blast, *Pyricularia*, *Pseudomonas*, plant growth-promoting bacteria.

## **INTRODUCTION**

Rice (*Oryza sativa* L.) is an important crop for human consumption. This cereal is the staple food for over half the world population and is considered the world's largest by the size of the area where it is grown and the number of people who depend on their crop (IRRI, 2002). In Cuba, rice is an important part in the daily diet of the

population and current per capita consumption is among the highest in Latin America, contributing 20% of daily calories consumed (Cárdenas et al., 2007). The area planted with rice in Cuba is approximately 138 455 ha and the production was estimated to be around 250 000 tons per year (MINAG, 2006).

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Controlling diseases that may attack the crop is of great importance to increase productivity in rice growing areas. In particular, fungi have been described as causal agents of various rice diseases (Herrera, 2003). Rice blast, caused by *Pyricularia oryzae* (teleomorph = *Magnaporthe oryzae*) is one of the most important diseases, due to its worldwide distribution, destructiveness and high degree of pathogenicity (Thuan et al., 2006; Rodríguez et al., 2007). Rice blast can cause up to 100% in yield reduction, depending on the cultivar susceptibility, cropping technology and climatic conditions (Prabhu et al., 2009, Magar et al., 2015). Although infections are mostly reported to occur in leaves and stems, seeds and roots may also be infected by *P. oryzae*, using the typical developmental processes of root-infecting fungi (Sesma and Osbourn, 2004).

In Cuba, rice blast is the most relevant disease in rice crop and its incidence is closely related to environmental conditions, such as high relative humidity and temperature (Rodríguez et al., 2007; Almaguer-Chavez et al., 2012). Application of chemical inputs for rice blast control and prevention is not only ineffective, but also hazardous to the environment (Padovani et al., 2006). On the other hand, recent studies have linked nitrogen fertilization with an increase in rice blast severity (Sester et al., 2014).

Plant growth-promoting bacteria (PGPB) could be a good alternative for plant disease management and for reducing the amount of chemical fertilizers applied to rice crop (Adesemoye et al., 2009), as well as a means to cut down production cost (Gupta et al., 2015; Liu et al., 2018). Within PGPB, fluorescent *Pseudomonas* are often described as excellent plant growth promotion and biocontrol agents (Höfte and Altier, 2010; Pathma et al., 2011; Ghirardi et al., 2012). The main microbial mechanisms involved in control of pathogens include direct antagonism, through the production of antimicrobial metabolites (antibiotics, cyclic lipopeptides, volatile compounds, siderophores and a wide variety of lytic enzymes) and competitive exclusion of other microbial groups (Pathma et al., 2011). Competition for nutrients is another mechanism that contributes to plant protection by PGPB (Acebo-Guerrero et al., 2015).

The efficiency of plant growth-promoting rhizobacteria (PGPR) as a biocontrol strategy to fight against fungal diseases in rice has been documented (Filippi et al., 2011; Sharifi-Noori et al., 2015; Prasanna Kumar et al., 2017). These potential biocontrol agents have been mainly focused on bacteria of the genera *Bacillus* (Chaiham et al., 2009; Rojas-Badía et al., 2011) and *Pseudomonas* (De Vleeschauwer et al., 2006; Hernández-Rodríguez et al., 2010). Fluorescent *Pseudomonas* are one of the microbial groups widely used as bacterial antagonists of *P. oryzae* (Gohel and Chauhan, 2015; Sharifi-Noori et al., 2015). However, these reports are often limited to the assessment of *in*

*vitro* antagonistic activity and lack a thorough identification and physiological characterization of the antagonists. In addition, to achieve an efficient bacterial product, native strains of each edaphoclimatic regions must be selected.

The aims of this work were to select and characterize fluorescent *Pseudomonas* from the rhizosphere of *O. sativa* L. with antagonistic activity against *P. oryzae* (SACC.), and to evaluate their *in vivo* biocontrol efficiency.

## MATERIALS AND METHODS

### Isolation and screening of *in vitro* antagonistic activity against *P. oryzae*

The isolation of fluorescent *Pseudomonas* was done using the Microcosm model (Kabir et al., 1995) from rice rhizosphere samples. The rhizosphere samples were taken from 15-day-old plants (*O. sativa* L. cv. J-104) sown in field that had never been biofertilized. A soil sample was analyzed by the Chemical Analysis Laboratory from the National Agricultural Science Institute according to Paneque (2000). Some of the agrochemical characteristics of the soil were the following: pH 7.2, organic matter percentage 3.92%, phosphorus 125 ppm, potassium 0.76 cmol·kg<sup>-1</sup>, calcium 28.5 cmol·kg<sup>-1</sup>, magnesium 3.5 cmol·kg<sup>-1</sup>.

All the bacterial isolates were screened for *in vitro* antagonism against *P. oryzae*. To determine *in vitro* antagonistic activity, five strains of *P. oryzae* (AAM-275, AAM-361, AAM-403, AAM-425 and AAM-903) previously isolated from the aerial ecosystem of rice fields by Almaguer-Chavez et al. (2012) that belonged to the Fungi Culture Collection of the Faculty of Biology of the University of Havana were used in this study. The fungal strains were cultured in Potato Dextrose Agar (PDA; Merck, Germany) for 5 days at 28°C. The selected bacterial isolates were grown in King B (KB) broth (Merck, Germany) for 24 h at 28°C at 200 rpm. The cell concentration was adjusted to 10<sup>8</sup> cfu·mL<sup>-1</sup> and 100 µL of each bacterial culture was spread on 9-cm-diameter Petri dishes containing KB Agar (Merck, Germany) and straightaway, a 5-mm plug from the leading edge of a 5-days-old fungal culture was placed in the centre of each Petri dish. The control was set by inoculating only the fungus in KB Agar. The plates were incubated for 7 days at 28°C. The fungal inhibition was scored by measuring radial growth of the fungus (in mm) in every plate and the inhibition percentage was calculated through a comparison with the control plate, according to Bashan et al. (1996). The experiment was repeated three times with five replicates per treatment. For each isolate, an inhibitory efficiency index (IEI) was calculated against all 5 fungal strains as the sum of the inhibition frequency of each isolate of the fungal strains and the average inhibition for each bacterial strain (Hernández-Rodríguez et al., 2010). The isolates with an IEI higher than 1.0 were selected for further studies.

### *In vivo* evaluation of antagonistic activity of selected isolates

The bioassay was conducted on *O. sativa* cv. J-104. For disinfection, seeds were surface decontaminated with calcium hypochlorite according to Hernández-Rodríguez et al. (2008). Some seeds were placed in a Nutrient Agar plate to confirm the disinfection process. *P. oryzae* AAM-275 was selected for the assays in plants, with the intention to assess if the antagonistic isolates were able to control the most common strain pathogen in

the rice agroecosystem (Almaguer-Chávez et al., 2012).

The fungal inoculum was prepared from Potato Dextrose Broth (PDB; Merck, Germany) liquid cultures incubated at 30°C for 7 days in intervals of light/darkness (12 h/12 h) at 250 rpm. The cultures were then centrifuged and the supernatant containing the spores was kept for further use. Part of the soil was infected by mixing it thoroughly with the fungal inoculum adjusted to a final concentration of  $10^5$  spores/g of soil. The bacterial inocula were prepared by culturing the isolates overnight in KB Broth (30°C, 250 rpm) and adjusting the cell density to  $10^8$  cfu·mL<sup>-1</sup> and they were then inoculated on the seeds through their immersion in the culture broth for 45 min. Then the following treatments were established: T1, non-inoculated seeds in uninfected soil control; T2, non-inoculated seeds in fungus-infected soil control; T3 to T14, seeds inoculated with the selected bacteria in the fungus-infected soil. The plants were kept at 30°C, with a 16 h light photoperiod. The antagonistic effect was evaluated 21 days after seed germination. A completely randomized design was established with nine replicates per treatment and three repetitions of the experiment. Fungal suppression was based on observations of symptoms of fungal disease in the roots, since Sesma and Osbourn (2004) demonstrated that *P. oryzae* is able to infect rice roots using the typical developmental processes of root-infecting fungi. The disease incidence was based on the percentage of roots with disease symptoms in nine randomly selected rice plants. Disease severity was assessed using the following scale (Bigirama et al., 2000): scale 1, no symptoms shown; scale 3, infected roots up to 1%; scale 5, infected roots up to 5%; scale 7, infected roots up to 10%; scale 9, more than 25% of the roots exhibited disease symptoms and the seeds were severely damaged. Disease severity and control efficacy were calculated as follows: Disease severity =  $[\sum(\text{The number of diseased plants in this index} \times \text{Disease index}) / (\text{Total number of plants investigated} \times \text{The highest disease index})] \times 100\%$ . Control efficacy =  $[(\text{Disease severity of control} \times \text{Disease severity of treated group}) / \text{Disease severity of control}] \times 100\%$  (Yang et al., 2009).

#### **In planta disease severity assay**

The strains that were effective in the control of the disease symptoms in the *in vivo* preliminary test were selected to perform the experiment in greenhouse conditions. Disinfected rice seeds from cv. J-104 were used. Bacterial and fungal inocula were prepared and the inoculation of bacteria in the seeds was carried out as described earlier. Then they were planted in 30-cm-diameter pots in a greenhouse at 30°C, with a photoperiod of 16 h of light. The fungus was inoculated by foliar spraying of a dilution of  $10^5$  spores·mL<sup>-1</sup> when plants were 60 days old. Treatments of 20 replicates comprising one plant per pot were established. The treatments were: T1, non-inoculated control; T2, fungus-inoculated control; T3-T8, seeds inoculated with the selected bacterial strains and then inoculated with the fungal strain.

Plants were arranged in a completely randomized block and watered three times a week with sterile, distilled water. The percentage of plants showing the characteristic blast ellipsoidal lesions on leaves was determined 7 days post-inoculation with the fungal strain (Ribot et al., 2008). Then the reduction of disease symptoms was determined as follows: the percentage of plants showing disease symptoms was evaluated, considering the fungus-inoculated control as 100%, and the resulting value of the subtraction from this 100% and the percentage obtained with the rest of the treatments was considered to be the reduction of disease symptoms for each treatment (Hernández-Rodríguez et al., 2008). The experiment was repeated three times. The best performing strains were selected to continue their further characterization.

#### **Molecular characterization of the selected isolates**

For the most promising strains, partial regions of the *16S rRNA* were sequenced, using the primers 27f and 1492r (Table 1). Amplification was performed, with the following temperature program: initial denaturing at 95°C for 5 min, 30 cycles consisting of 95°C for 1 min, 55°C for 1 min and 72°C for 1.5 min, followed by a final extension step of 10 min at 72°C. Polymerase chain reaction (PCR) products were visualized by electrophoresis in agarose gel (1.5%). PCR products were cloned using the TOPO TA Cloning Kit®, according to the manufacturer's instructions (Invitrogen Life Technologies SA). The selected clones (three per strain) were sequenced by GATC Biotech® (Germany).

Phylogenetic analysis of *16S rRNA* gene sequences was carried out using these isolates and the *Pseudomonas* type strains with the most similar sequences retrieved from GenBank. Sequence alignment was carried out using the program MEGA 6 (Tamura et al., 2013) and corrected manually. A maximum likelihood tree was used to build an individual tree for 16srDNA with the models that best fitted the data, and confidence analysis was undertaken using 1000 bootstrap replicates.

#### **Physiological characterization of selected isolates**

Lipase activity was detected according to Poritsanos (2005) in Luria Bertani (LB; Merck, Germany) agar medium supplemented with Tween 80 (2% v/v) and CaCl<sub>2</sub> (0.22 g·L<sup>-1</sup>). Protease activity was detected in Nutrient Agar supplemented with 2.5% (w/v) skimmed milk (Nestlé) according to Perneel et al. (2007).

Pyoverdine production was quantified according to Meyer and Abdallah (1978), using overnight cultures of the bacteria in Casamino Acid (CA) Broth (Bacto Casamino Acid, BD, 5 g·L<sup>-1</sup>; K<sub>2</sub>HPO<sub>4</sub> 1.18 g·L<sup>-1</sup>; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.25 g·L<sup>-1</sup>) (28°C at 170 rev min<sup>-1</sup>).

The ability to produce hydrogen cyanide was detected in Tryptic Soy Agar (Merck, Germany) amended with glycine (4.4% w/v), using the picrate/Na<sub>2</sub>CO<sub>3</sub> saturated filter paper, fixed to the underside of Petri dish lids, which were sealed with parafilm before incubation at 28°C for 96 h (Kremer and Souissi, 2001).

The screening for *phlD*, *prnC*, *phzCD* and *pltB* (involved in the production of the antibiotics 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (PRN), phenazines (PHZ), pyoluteorin (PLT), respectively) was carried out by PCR, according to Mavrodi et al. (2001) and Calderón et al. (2013). Primers are listed in Table 1. Antibiotic production was confirmed by TLC, as described elsewhere (Whistler et al., 2000; Cazorla et al., 2006). Briefly, bacterial cultures (15 ml) of 4 days in King B broth at 28°C without shaking were centrifuged at 3800 g for 15 min. The supernatants were extracted twice with 5 ml ethyl acetate, left to dry out and resuspended in 100 ml of methanol. The metabolites were detected by thin layer chromatography using silica RP-18F254S TLC plates (Merck AG, Germany). *Pseudomonas protegens* Pf-5 was used as control for PLT, PRN, and DAPG; *Pseudomonas chlororaphis* 30 to 84 for PHZ.

Additional analyses were carried out using high-pressure liquid chromatography (HPLC) and mass spectra (MS) to identify bioactive compounds by comparison with the UV spectra and molecular weight of reference compounds. Selected strains were grown in Casamino Acids Agar (CAA) for 96 h at 28°C (seven plates per strain). Agar (with bacteria) was removed from the plates, placed in glass bottles and 300 mL of ethyl acetate were added. Extraction was carried out for 3 h while shaking at room temperature and the organic phase was transferred to a round-bottom glass flask and flash evaporated, and the residue was dissolved in 1 ml of HPLC-grade methanol for the analyses. Analytical HPLC was performed by using a Waters apparatus equipped with a 626 pump, a 626

**Table 1.** Primers used in this study.

Primer	Sequence (5'-3')	Amplicon size (bp)	Amplified gene	Annealing PCR temperature (°C)	Reference
27f 1492	ACTCCTACGGGAGGCAG GGCGTCTGTACAAGGCCCGG	~1500	16s rDNA	55	Lane (1991)
PhI2a PhI2b	GAGGACGTCTGAAGACCACCA ACCGCAGCATCGTGTATGAG	745	<i>phlD</i>	58	Mavrodi et al. (2001)
PrnCf PrnCr	CCACAAGCCCGGCCAGGAGC GAGAAGAGCGGGTCGATGAAGCC	719	<i>prnC</i>	58	Mavrodi et al. (2001)
PHZ1 PHZ2	GGCGACATGGTCAACGG CGGCTGGCGGCGTATTC	1400	<i>phzCD</i>	56	Delaney et al. (2001)
PltBf PltBr	CGGAGCATGGACCCCGCAGC GTGCCCGATATTGGTCTTGACCGAG	790	<i>pltB</i>	58	Mavrodi et al. (2001)
30-84/1(F) 30-84/2(R)	CAGTTCATCCGGCGGGCTGCA CCCGTTTCAGTAAGTCTTCCATGATGCG	1200	<i>phzX</i>	58	Mavrodi et al. (1998)

controller and a 996-photodiode array detector. Samples were analysed on an HPLC apparatus (Waters 600 system) coupled to both UV (Waters 2487 detector) and mass (Micromass Waters VG Quattro II mass spectrometer) detectors. A BIO Wide Pore C-18 column (250 × 4.6 mm; 5 µm) was used and the solvent elution consisted of a linear gradient of water and acetonitrile (from 5 to 100% acetonitrile in 30 min) at a flow rate of 1 ml/min. After UV detection at 215 and 254 nm, the column elute was split (LC Packings splitter), and 0.1 ml.min<sup>-1</sup> was directed to the mass spectrometer fitted with an ESI interface. For mass detection, analyte ionization was achieved by using the positive electrospray mode. The ESI parameters used were: nebulizing gas (N<sub>2</sub>, 20 l.h<sup>-1</sup>), drying gas (N<sub>2</sub>, 250 l.h<sup>-1</sup>), source temperature (80°C), cone voltage (35 V) and capillary voltage (35 kV). The total ion current scanning was from m/z 115 to 1000 with 1 s/scan. Nitrogenase activity was assessed by the acetylene reduction assay (ARA), described by Boddey et al. (1990). The bacteria were grown in 15-ml bottles containing 5 ml of semi-solid Nfb medium without pH indicator and capped with cotton stoppers. These flasks were incubated at 30°C for 48 h. After the formation of the film, cotton stoppers were removed aseptically and replaced with sterile pierceable rubber stoppers (Suba-Seal, Sigma-Aldrich, USA). Immediately after, 1 ml of air was taken from each bottle with a syringe and 1 ml of acetylene was injected in the bottles and they were incubated at 30°C for an hour. To assess the concentration of ethylene in the flasks, 0.5 ml of the gas phase was injected into a gas chromatograph with flame ionization (Perkin Elmer model F11).

Indole production was analyzed by the microplate method (Sarwar and Kremer, 1995). Bacteria were grown for 42 h in 20 ml of DYGS broth supplemented with L-tryptophan (200 µg.ml<sup>-1</sup>) in the dark, at 33°C and with 150 rpm. Aliquots of 1 ml were centrifuged at 10,000 rpm for 15 min and 150 µl of the supernatant were mixed with 100 µl of Salkowski reagent (1 ml of 0.5 M FeCl<sub>3</sub> in 49 ml of 35% perchloric acid) in U-type 96-well plates. The samples remained in the dark for 30 min at room temperature. The absorbance (A<sub>540</sub>) was measured with a microplate reader (Labsystem reader MF, LabSystem). The quantification of

indole compounds was achieved using a calibration curve prepared with serial dilutions of IAA standards. The results were expressed in µg.ml<sup>-1</sup> of IAA per unit of protein, using 3 replicates for each strain.

The ability of the strains to solubilize Pi was tested on NBRIP plates containing 5% tricalcium phosphate (Nautiyal, 1999). The diazotrophic *Gluconacetobacter diazotrophicus* strain PAL5<sup>T</sup> (BR 11281) (Maheshkumar et al., 1999) was used as a positive control. The assay was carried out using a randomized design with three replicates. Four 20-µl drops of cell suspension (log-phase) were inoculated on the surface of NBRIP Agar plates and incubated for 15 days at 30°C. The diameter of the solubilization halo (translucent area around the colony) and the colony diameter were used to calculate the solubilization index (SI), according to Kumar and Narula (1999).

The quantification of soluble P was carried out with the phosphomolybdenum blue method according to Chen et al. (2006). The *G. diazotrophicus* strain PAL5<sup>T</sup> was utilized as a positive control. The strains were grown in liquid DYGS medium (Rodrigues et al., 2006) for 24 h at 30°C and 150 rpm. The cultures were adjusted to 0.9 to 1 (OD<sub>600</sub>) with saline solution and 5 mL was used to inoculate 250 mL Erlenmeyer flasks containing 45 mL NBRIP medium supplemented with 0.05% Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> (tricalcium phosphate) as a phosphorus source. The flasks were incubated in a shaker at 30°C and 150 rpm. The experiment was carried out using a randomized design with three replicates. Samples were taken 5 days after inoculation. Changes in the pH of the culture medium as well as the bacterial population, measured by the microdrop technique (Spencer and Ragout, 2001) were monitored during the growth period.

Swarming and twitching motilities were tested in LB medium (Merck, Germany) with Agar concentrations of 0.6 and 1%, respectively, according to Rasamiravaka et al. (2013), with slight modifications. The inoculum was 5 µl of an overnight culture of the strains in KB broth (28°C at 170 rev min<sup>-1</sup>) diluted to an OD<sub>600</sub> of 1. To assess swarming, the inoculum was placed on the Agar surface and swarming motility was recorded as the diameter of bacterial growth in mm after 48 h of incubation at 28°C and any diameter

higher than twofold the inoculation diameter (5 mm) was considered positive. For twitching, the inoculum was deposited in between the Agar surface and the bottom of the plate. Twitching was recorded as the diameter of the stained area and any bacterial strain that had a detectable twitching zone upon visible inspection was scored as positive. Three replicates were established by strain and the experiment was repeated three times.

### Statistical analysis

Normality and homogeneity of variance tests were carried out for all the data. Data were processed with the analysis of variance (ANOVA) and means were separated with Tukey's honest significance test (plant growth data) and Fisher's Protected Least Significant Difference (LSD) (disease severity, control efficacy, percentage of infected leaves per plant and disease reduction). Kruskal-Wallis' ANOVA and Mann-Whitney U test were performed for the data that did not fulfill the normality criteria to assess significant differences ( $P < 0.05$ ). A representative repetition of each experiment was used for the figures. Standard deviation was calculated and indicated as vertical bars in the figures. All tests were performed using the 7 software STATISTICA 8.0 (Stat Soft, Inc., Tulsa, OK, USA).

## RESULTS

### Selection of the isolates with *in vitro* antagonistic activity against *P. oryzae*

In this study, from the 200 isolates that showed the typical characteristics of fluorescent *Pseudomonas*, 25 (representing 12.5% of the isolates) were capable of inhibiting mycelial growth of at least one of the five fungal strains (Figure 1). However, the inhibition varied depending on the fungal strain the bacteria were faced with. When *P. oryzae* AAM-275 was tested (Figure 1A), it was shown that 19 of the isolates were capable of inhibiting the mycelial growth, from 54 to 84%. *P. oryzae* AAM-903 and AAM-403 were inhibited by 15 of the isolates, from 27 to 100% and 28 to 100% of mycelial growth inhibition, respectively (Figure 1B and C). *P. oryzae* AAM-425 was inhibited by 11 isolates, with 44 to 84% of mycelial growth inhibition (Figure 1D). The isolates showed less antagonistic effect against *P. oryzae* AAM-361, since only 10 of them were capable of inhibiting fungal growth from 36 to 89% (Figure 1E). The inhibitory efficiency index for each isolate was calculated and the isolates AJ01, AI05, AJ13, AJ30, AI08, AJ28, AJ29, AI03, AI07, AJ26, AJ25 and AI02 were selected, since their index values were higher than 1.0.

### *In vivo* antagonistic activity of selected isolates against *P. oryzae*

The incidence and severity of *P. oryzae* AAM-275 in 21-days-old control plants were of 100 and 78% respectively.

When the rhizobacteria were applied, only six of them were able to reduce disease severity and incidence to some extent (Table 2). The disease incidence ranged from 11 to 33% and disease severity from 11 to 27%, achieving the best results with the strains AI05, AJ01 and AJ13, according to the results of the control efficacy (Table 2).

### Disease severity is reduced in leaves when the selected isolates are applied

Four of the isolates showed efficient control of *P. oryzae* in greenhouse conditions, while the others did not (data not shown). The percentage of infected leaves per plant was considerably lower in all the treatments inoculated with rhizobacteria, as opposed to the control infected with the fungus (43.27 to 30.17%) (Table 3). All the strains exerted biocontrol over *P. oryzae*, as shown by the disease reduction percentage in the rhizobacteria-treated plants (28.23 to 41.33%). According to this research, the strains AI05 and AJ13, AJ01 and AI08 can be used for biocontrol of *P. oryzae* in rice. The strains AI05 and AJ13 were selected for further studies, since these strains were also able to stimulate plant growth in field conditions (data not shown).

### The selected isolates were identified as *Pseudomonas putida* and *Pseudomonas fluorescens*

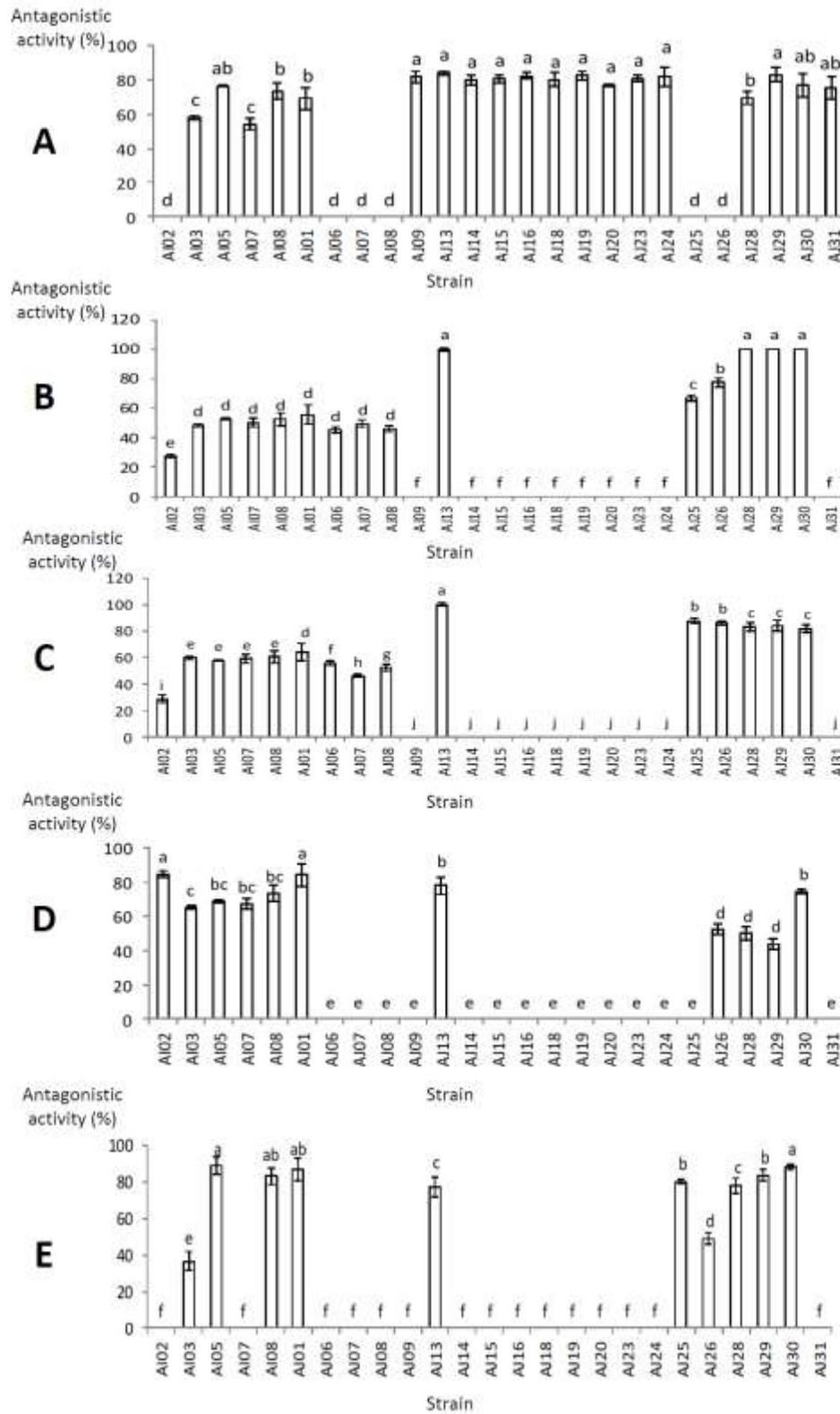
The obtained sequences (GenBank access no. HQ446870 and HQ446871) were compared to the retrieved sequences from GenBank. According to the sequences of the 16s *rDNA* gene, the strains AJ13 belonged in the *Ps. putida* cluster and AI05 is in *P. fluorescens* cluster (Figure 2).

### Physiological characterization of the selected isolates

Some traits that could be involved in biocontrol and/or plant growth promoting activity were tested and the results are shown in Table 4. The strains AI05 and AJ13 were positive for exoprotease and lipase activities, piochelin, hydrogen cyanide (HCN) and pyoluteorin production. When pyoverdine production was quantified, AJ13 produced almost twice as much pyoverdines as AI05.

Antibiotic genes were identified by PCR and their corresponding products were detected by TLC. In both strains, a 790-bp *pltB* fragment was amplified, suggesting that they produce pyoluteorin. The detection of a 719-bp (*prnC*) and a 1200-bp (*phzX*) bands were amplified for AJ13 but not for AI05, indicating that AJ13 could produce pyrrolnitrin and phenazine-1-carboxylate.

When the extracts of AI05 and AJ13 strains in CAA



**Figure 1.** Antagonistic effect of bacterial isolates against *P. oryzae*. Percentage of inhibition of mycelial growth of fungal strains. **(A)** AAM275; **(B)** AAM903; **(C)** AAM403; **(D)** AAM425; **(E)** AAM361. Non common letters suggest significant differences according to Bonferroni correction ( $l =$  Standard deviation of the mean).

**Table 2.** Control of rice blast by rhizobacteria in 21-days-old rice plants.

Treatment	Disease incidence (%)	Disease severity (%)	Control efficacy (%)
NIC	0 <sup>e</sup>	0 <sup>d</sup>	-
FIC	100 <sup>a</sup>	78 <sup>a</sup>	-
FS + AI03	33 <sup>b</sup>	27 <sup>b</sup>	65 <sup>c</sup>
FS + AI05	11 <sup>d</sup>	11 <sup>c</sup>	86 <sup>a</sup>
FS + AI08	22 <sup>c</sup>	22 <sup>b</sup>	72 <sup>b</sup>
FS + AJ01	33 <sup>b</sup>	11 <sup>c</sup>	86 <sup>a</sup>
FS + AJ13	33 <sup>b</sup>	13 <sup>c</sup>	83 <sup>a</sup>
FS + AJ29	33 <sup>b</sup>	22 <sup>b</sup>	72 <sup>b</sup>

NIC, Non-inoculated control; FIC, fungus-inoculated control; FS, fungus-inoculated soil. Values in columns followed by similar letters were not significantly different according to Fisher's protected LSD test (P = 0.05).

**Table 3.** Ability of potential antagonistic rhizobacteria to suppress *P. oryzae* in 60-days-old rice plants in greenhouse study.

Treatment	Infected leaves per plant (%)	Disease reduction (%)
NIC	25.00 <sup>e</sup>	-
FIC	71.50 <sup>a</sup>	0 <sup>d</sup>
FI + AJ01	38.50 <sup>c</sup>	33.00 <sup>b</sup>
FI + AI05	30.52 <sup>d</sup>	40.98 <sup>a</sup>
FI + AJ13	30.17 <sup>d</sup>	41.33 <sup>a</sup>
FI + AI08	43.27 <sup>b</sup>	28.23 <sup>c</sup>

NIC, Non-inoculated control; FIC: fungus-inoculated control; FI, fungus-inoculated. Values in columns followed by similar letters were not significantly different according to Fisher's protected LSD test (P = 0.05).

Agar were analysed through HPLC-MS, the results showed that both strains were able to produce pyoverdines (1160 Da) and different isoforms of pyochelin (324 Da). Additionally, the production of ornibactin (734 Da) was detected for the strain *P. fluorescens* AI05.

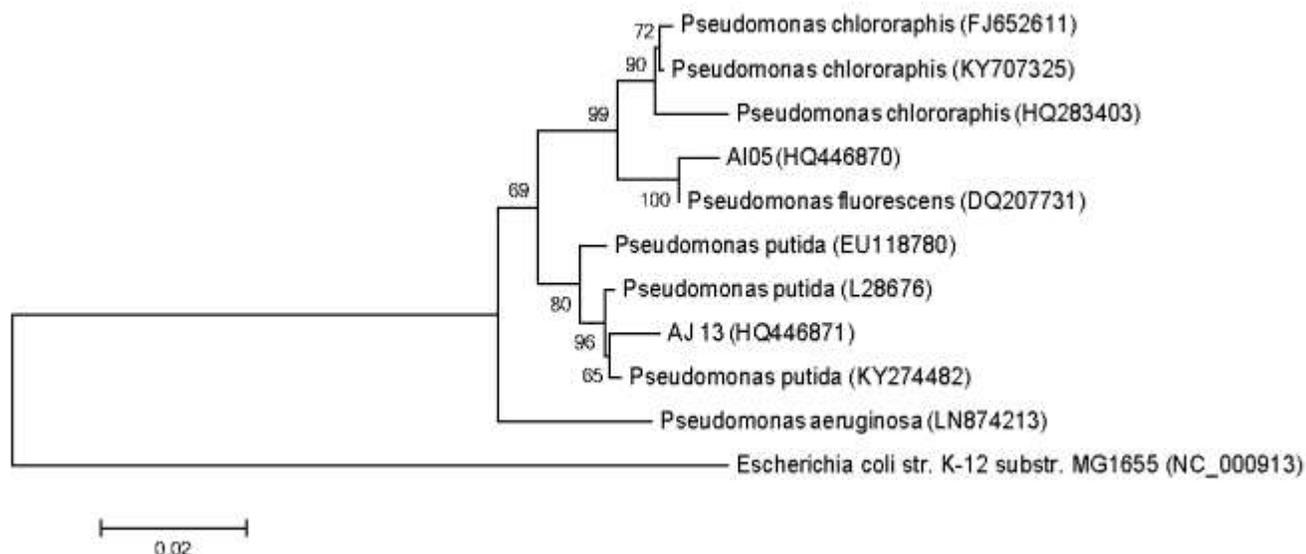
The analysis showed that both strains were able to fix nitrogen, produce indolic compounds and to solubilize Pi when grown on NBRIP (pH 7.0) Agar medium, as indicated by the presence of a visible halo that was used to calculate a solubilization index (SI). The strains AI05 and AJ13 showed maximum Pi solubilization at 325 and 348 mg.L<sup>-1</sup>, respectively. AI05 and AJ13 exhibit swarming motility, since their diameters were significantly higher (81.2 ± 0.17 and 45.1 ± 0.14, respectively) than the inoculation diameter (5 mm). However, AJ13 showed the highest diameter (5.65 ± 0.03 mm) in twitching assay.

## DISCUSSION

In the present study, of the 200 isolates of fluorescent *Pseudomonas* evaluated, 25 of them showed *in vitro*

antagonistic activity against *P. oryzae*. Although in comparison with other studies, the number of recovered antagonists may be considered as high (Acebo-Guerrero et al., 2015); there are several factors that may contribute to the frequency of antagonistic bacteria, such as the soil type, plant species and environmental conditions (Lugtenberg and Kamilova, 2009; Filippi et al., 2011).

This research revealed that these Cuban native strains of fluorescent *Pseudomonas* from rice rhizosphere have *in vitro* antagonistic activity, although only four of them (AI05, AJ13, AJ01 and AI08) are able to control *P. oryzae* in greenhouse conditions. In the preliminary *in vitro* dual culture experiments, the antagonistic activity of the isolates was assessed against five strains of *P. oryzae* (AAM-275, AAM-361, AAM-403, AAM-425 and AAM-903), showing a differential antagonistic effect of the rhizobacteria against the phytopathogenic strains. These results may be explained regarding the differences between the strains within species and also the specificity of the different physiological biovars of a pathogen (Almaguer-Chavez et al., 2012), that correspond with different levels of resistance to an antagonist (Karthikeyan and Gnanamanickam, 2008). Jaiganesh et



**Figure 2.** Phylogenetic tree of the isolates and strains of *Pseudomonas* species inferred from *16S rRNA* gene sequences. Numbers at the nodes are percentage bootstrap values based on 1000 resampled datasets, with only bootstrap values  $\geq 50\%$  shown. The *16S rRNA* gene sequence of *E. coli* K12 (NC000913) was used as outgroup. The evolutionary distances were computed using the Kimura 2-parameter method and the rate variation among sites was modelled with a gamma distribution. The scale bar indicates a genetic distance of 0.005 nt substitutions per site.

**Table 4.** Overview of the distribution of bacterial metabolite production and motility among the antagonistic *Pseudomonas* strains.

Characteristics	Strains	
	AI05	AJ13
<b>Lipase</b>	+	+
Protease	+	+
Pyoverdines	$0.15 \pm 0.09$	$0.33 \pm 0.09$
Pyochelin	+	+
Ornibactin	+	-
HCN	+	+
<b>Antibiotic production*</b>		
PHZ	-	+
PLT	+	+
PRN	-	+
ARA (nmol C <sub>2</sub> H <sub>2</sub> /mg protein/h <sup>3</sup> )	$68.9 \pm 0.09$	$63.5 \pm 0.11$
Indole production ( $\mu\text{g/mL}$ )	$9.11 \pm 1.09$	$12.61 \pm 1.06$
Solubilisation Index (S.I.) Medium NBRIP (pH 7.0)	$2.88 \pm 0.03$	$2.42 \pm 0.13$
Soluble-P ( $\text{mg.L}^{-1}$ )	$325 \pm 4.68$	$348 \pm 2.47$
<b>Motility</b>		
Swarming**	$81.2 \pm 0.17$	$45.1 \pm 0.14$
Twitching***	$0.25 \pm 0.01$	$5.65 \pm 0.03$

+, positive reaction; -, negative reaction. Non-common letters indicate significant differences according to Tukey test ( $P < 0.05$ ). PHZ, Phenazines; PLT: pyoluteorin; PRN, pyrrolnitrin. \*The antibiotic genes were detected by PCR and the antibiotic production was detected by TLC. \*\*Value represents the diameter of the swarming area (the bacterial growth diameter minus the inoculation diameter (5 mm)). \*\*\*Value represents diameter of the stained area (mm) \_ standard deviation.

al. (2007) suggested that when an interaction is established between the antagonists and phytopathogens, there is a set of strain-dependent responses, the biocontrol activity being the sum of various mechanisms.

In this work, two strains (*P. fluorescens* AI05 and *P. putida* AJ13) isolated from *O. sativa* showed potential to be used as biocontrol agents, since they were able to inhibit *in vitro* the mycelial growth of *P. oryzae* and to reduce symptom severity of *Pyricularia* infection. These strains also have the ability to promote plant growth under field conditions. *Pseudomonas* has been described as highly adaptable in several ecosystems, prevailing in some of them and largely known as a plant growth promoting agent (Kang et al., 2014; Heng et al., 2017), as a biocontrol agent (Kumar et al., 2015; Sun et al., 2017), as well as a bioremediating agent (Nelson et al., 2002; Zhang et al., 2016).

The most commonly reported mechanisms of biocontrol by *Pseudomonas* include production of antibiotics, hydrogen cyanide, lytic exoenzymes (Mishra and Arora, 2017), cyclic lipopeptides (Flury et al., 2017), competition for nutrients and niches (Saber et al., 2015), competition for iron mediated by siderophores (Ahmad et al., 2008; Buataité et al., 2017) and induced systemic resistance (Kumar et al., 2015). In this particular study, the strains *P. putida* AJ13 and *P. fluorescens* AI05 were able to produce siderophores, such as pyoverdine and pyochelin and in the case of AJ13, the production of ornibactin was also detected. Siderophore production could have played a major role in antagonistic activity, since the King B medium is iron-limited, which favors bacterial siderophore production and iron uptake over fungal siderophores, and has been reported as a desirable trait for the biocontrol of phytopathogens (Cornelis, 2010).

Fluorescent *Pseudomonas* produce an array of extracellular metabolites with antifungal activity (Park et al., 2011). In this study, AJ13 and AI05 have potentialities to produce metabolites that have been reported as involved in biocontrol activity, such as pyoluteorin for both strains and for AJ13, the additional production of pyrrolnitrin and phenazine-1-carboxylate was predicted. The production of these metabolites and the siderophores could partially explain the beneficial effects observed in greenhouse conditions, although other studies are needed to confirm their involvement in this particular plant-microorganism interaction.

In addition to the production of antagonistic metabolites, *Pseudomonas* have been reported to stimulate plant growth by increasing the availability and uptake of mineral nutrients or by enhancing root growth and morphology *via* the production of phytohormones such as auxins (Ghirardi et al., 2012; Mehnaz, 2013). In this study, both AJ13 and AI05 were able to produce auxins and to fix atmospheric nitrogen which suggests that they may be excellent plant growth promoting agents. In this context, with the application of these rhizobacteria as a microbial inoculant, a greater contribution of atmospheric

nitrogen to the plant is achieved, which reduces the use of chemical products. These reductions in the application of nitrogenous fertilizers could affect the physiology of *P. oryzae* that needs this element for its own nutrition (Agrios, 2015), which would cause a decrease in the concentrations of this phytopathogen and a lower incidence of the disease in field conditions (Sester et al., 2014). At the same time, these bacteria release metabolites in the rhizosphere that contribute to the ecological balance of the soil. However, the demonstration of this hypothesis will require future research.

This study reports for the first time the ability of the strains AI05 and AJ13 to produce siderophores and to solubilize inorganic phosphate. In this sense, a recent study by Estrada et al. (2013) related the *in vitro* fixation of nitrogen and the solubilization of inorganic phosphorus with the ability to promote plant growth in rice for *Bacillus* and *Herbaspirillum* strains. On the other hand, the production of auxins by *Pseudomonas* has also been related to plant stress alleviation (Egamberdieva and Lugtenberg, 2014).

These results demonstrate that rizosphere bacteria can be used to reduce the damage caused by *P. oryzae* in *O. sativa* L. Specifically, the isolates *P. putida* AJ13 and *P. fluorescens* AI05 showed promising potentialities for biocontrol and plant growth promotion. However, there was a difference in behavior between the strains, which suggest that the mechanisms of action against *P. oryzae* are probably different.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ACKNOWLEDGEMENT

This research was supported by Project of National Program of Human Food: Potentialities of use of plant growth promoting diazotrophic bacteria in sustainable production of rice crop (*Oryza sativa* L.) financed by Cuban Ministry of Science, Technology and Environment (CITMA). The authors of this work are grateful for the sponsorship received from the Research and Postgraduate Department of the Universidad Católica de Manizales, Colombia.

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