Cultivos Tropicales, 2021, vol. 42, no. 1, e08
ISSN impreso: 0258-5936
ISSN digital: 1819-4087

http://ediciones.inca.edu.cu

enero-marzo
Ministerio de Educación Superior. Cuba
Instituto Nacional de Ciencias Agrícolas

Short communication

Approach in the identification of rhizospheric rhizobia of rice (*Oryza sativa* L.) cultivar "INCA LP-7"

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ABSTRACT

There are few studies about rhizobia rice interaction, in Cuba. The aim of this work was to characterize possible rhizobia from rice plant rhizosphere of cultivar "INCA LP-7". Sixteen bacterial isolates were characterized from a cultural, morphological and biochemical point of view. All bacterial isolates produced mucous in mannitol yeast medium; however, they had different size and color. Gram stain revealed that most of these isolates were Gram-negative and non-sporulated coccobacilli. Some isolates did not produce for cetolactase enzime, produced acid and grew in nitrogen-free media. It is concluded that in rice plant rhizosphere of cultivar "INCA LP-7", reside rhizobia populations from Rhizobiaceae family presumably, with potentials in promoting plant growth.

Key words: phenotype, nitrogen fixation, grasses, *Rhizobium*

INTRODUCTION

In Cuba, an annual average of 72 kg of rice (*Oryza sativa* L.) is per person consumed, one of the highest in Latin America [(1)]. Cereal imports cover 66 % of the national demand [(2)]. This problem motivated the development of new varieties of rice, more resistant to phytopathogens and with greater yield potential. The rice cultivar "INCA LP-7", developed at National Institute of Agricultural Sciences (INCA), and it is cultivated in 8 % of total area dedicated to rice cultivation in the country (682 ha) [(3)].

Rice has the physiological capacity to absorb only 50 kg N ha^{-1} [(4)]. The rest of the nitrogen fertilizer applied to the crop produces serious contamination problems [(5)]. The use of Plant Growth

Promoting Bacteria (PGPB) has been used as an alternative to mineral fertilization [6]. Rhizobia are PGPB that have been studied for their symbiotic association with legume plants [7]. However, in recent years it has been found that these microorganisms also promote the growth of grasses such as rice [8].

Currently, there is no scientific evidence in Cuba about on the presence of rhizobia in the rhizosphere of rice cultivar "INCA LP-7", which makes it difficult to develop bioproducts based on these microorganisms that allow reducing mineral fertilization and increase yields of this crop. The objective of this research was to characterize possible rhizobia from rhizosphere of rice plant cultivar "INCA LP-7".

MATERIALS AND METHODS

Sixteen bacterial isolates were used, isolated from the rhizosphere of rice plants cultivar "INCA LP-7" and which presumably belong to rhizobia group [9].

Cultural and morphological characterization of rhizospheric bacteria from "INCA LP-7" cultivar rice plants

For the cultural characterization, isolates were cultured by exhaustion on Yeast Mannitol (YM) medium [10] with Congo red. The cultures were incubated for 48 h at 30 °C. The parameters to take into account were color, mucus and colony size, large 2-4 mm, medium 1-2 mm small 1 mm. The morphological characterization of bacterial isolates was carried out by Gram stain and the cell morphology, response to staining and presence of endospores were determined.

Biochemical characterization of rhizospheric bacteria from cultivar "INCA LP-7" rice plants

Cetolactase test

Bacterial isolates were cultured by exhaustion and in Yeast-Lactose (YL) solid medium [11] and incubated at 30 °C for seven days. Subsequently, 10 mL of Benedict's reagent were added on the bacterial growth and changes of the medium color were observed after 10 min at room temperature. The positive result was considered when the medium changed from blue to yellow, and negative if the medium maintained the blue coloration.

Acid and base production

Bacterial isolates were by cultured exhaustion and in YM solid medium with bromothymol blue indicator (0.5 % in 0.016N NaOH) and the plates were for three days at 30 °C. The change in the

color of the medium from green to yellow was interpreted as the acid production, and from green to blue as base production [12].

Growth in nitrogen-free culture media

Bacterial suspensions were prepared. To this, several colonies were re-suspended in 1 mL of sterile NaCl solution (0.9 % (m/v)). One hundred microliters of the suspensions were inoculated into flasks containing 10 mL of the semi-solid nitrogen-free media JMV and Rennie [13,14]. The inoculation was carried out by introducing the tip of a micro pipette inside the culture media. Flasks were inoculated with a suspension of *Bradyrhizobium elkanii* ICA 8001 strain which is a reference strain in the Biological Nitrogen Fixation (BNF) were used as positive control [15].

RESULTS AND DISCUSSION

Rhizospheric bacterial populations from plant rice cultivar "INCA LP-7", similar from the cultural point of view, differ in their morphological characteristics

All isolates produced mucous colonies in YM solid medium. However, they did differ in size and coloration (Table 1). Similar results were found in previous studies with bacterial isolates from Chilean beans (*Lablab purpureus* (L.) Sweet) and pea (*Pisum sativum*) [16,17].

Table 1. Cultural, morphological and biochemical characterization of bacteria isolates from rhizorphere rice plants cultivar "INCA LP-7"

Isolates	Cultural characteristics	Morphological characteristics	Acid/base production	Growth in semi-solid nitrogen-free media	
				JMV	Rennie
4S	Medium, pale pink with dark pink center, mucous	Coccobacilli, G-, not sporulated	acid	+	+
4U	Large, whitish, mucous	Coccobacilli, G-, not sporulated	acid	+	+
1DD1	Medium, whitish, mucous	Coccobacilli, G-, not sporulated	acid	+	+
1DD2	Medium, whitish, mucous	Coccobacilli, G-, not sporulated	acid	+	+
1AA	Large, whitish, mucous	Coccobacilli, G-, not sporulated	acid	+	+
1LL	Large, whitish, mucous	Coccobacilli, G-, not sporulated	ND	ND	ND
3W	Small, pale pink, mucous	Coccobacilli, G-, not sporulated	acid	+	+
5FF1	Medium, whitish, mucous	Coccobacilli, G-, not sporulated	base	+	+
5FF2	Large, whitish, mucous	Coccobacilli, G-, not sporulated	ND	ND	ND
5O	Medium, translucent, mucous	Coccobacilli, G-, not sporulated	acid	+	+
5P1	Small, whitish, mucous	Coccobacilli, G-, not sporulated	acid	+	+
GG1	Large, translucent, mucous	Coccobacilli, G-, not sporulated	acid	+	+
GG2	Medium, pink, mucous	Coccobacilli, G-, not sporulated	acid	+	+
II1	Small, translucent, mucous	Coccobacilli, G-, not sporulated	acid	+	+
II3	Large, translucent, mucous	Coccobacilli, G-, not sporulated	acid	+	+
II2	Large, translucent, mucous	Coccobacilli, G-, not sporulated	acid	+	+

G-, Gram negative; G +, Gram positive; ND, Not determined

The 87.5 % of isolates were coccobacilli Gram negative and not sporulated. Isolates 1LL and 5FF2, which make up the remaining 12.5 %, presented cultural and even some morphological characteristics similar to the rest. However, both isolates were Gram positive.

The rhizobia are generally non-sporulated and Gram-negative coccobacilli [(18)]. Thus isolates 1LL and 5FF2 isolates were eliminated from the subsequent determinations.

Possible rhizobia with attributes in promoting plant growth reside in rhizosphere in rice plant cultivar "INCA LP-7"

The response to cetolactase test was the third indicator used to determine if the bacterial isolates belong to rhizobia group. This determination was for a long time a reliable phenotypic character, which allowed to differentiate the Rhizobiaceae family from *Agrobacterium* genus. Rhizobia and the genus *Agrobacterium* have similar habitats and cultural and morphological characteristics [19]. *Agrobacterium* forms mucous and semitranslucent colonies, after 2-3 days in YM solid medium and the Gram stain reveals in the form of Gram negative and non-sporulated cells. This genus produces galls or tumors on the roots and stem of plants, very similar to the nodules that form rhizobia in legumes [20].

All isolates selected in this work as possible rhizobia, taking into account the cultural and morphological characteristics, were cetolactase negative test. Scientific evidence has allowed atoxonomic redistribution of some bacteria species, since they constitute exceptions to rule. For example, according to the Bergey Manual, species that were previously identified as *Agrobacterium rhizogenes*, *Agrobacterium rubi*, and *Agrobacterium vitis* are cetolactase negative and *Agrobacterium tumefaciens* is cetolactase positive [21].

Molecular biology methods have allowed a greater clarification of these issues, so that these three species of *Agrobacterium* are currently within the genus *Rhizobium* [22,23]. Taking into account these and other scientific evidences it is necessary to use, in addition to conventional phenotypic tests, *16S rRNA* sequencing to identify bacterial isolates associated with the rice plants cultivar "INCA LP-7".

Acid/base production was another phenotypic criteria studied in this research. Except isolate 5FF1, the rest produced acid in the culture medium. Some microbial acids act as siderophores, which capture iron from the soil [24]. Others allow the phosphorus solubilization and increases its availability of plants [25]. Indole-3-acetic acid and salicylic acid are also acids that produce PGPB, which increase plant growth [26] and induce defensive responses against pathogens [27], respectively.

On the other hand, the growth of bacterial isolates in nitrogen-free culture media was used as an approach to their ability to perform the biological nitrogen fixation (BNF). The 100 % of isolates grew on JMV and Rennie media (Table 1). These results are similar to recent studies with those from other authors [26], with bacteria associated with rice plants.

Nitrogen-free semi-solid media are used isolate diazotrophic microorganisms from soil, rhizosphere, or interior of plant [27]. Thus, it is necessary to apply techniques such as the amplification of the *nifH* gene [28] and the Acetylene Reduction Assay (ARA) [29], which allow confirming the bacteria ability to fix nitrogen.

Biological Nitogen Fixation is one of the direct mechanisms used by PGPB to promote the plant growth [30]. Rhizobia have been studied primarily for their ability to fix nitrogen, symbiosis with legumes [31]. The presence of this attribute in rhizobia isolates studied here would allow their use as biofertilizer allowed the reduction of nitrogen fertilizer to the crop.

CONCLUSIONS

In this work phenotypic aspects of Rhizobiaceae family were identified. In bacterial isolated from rhizosphere of rice plant cultivar "INCA LP-7" characterization allow that this isolated belong to *Rhizobium*, *Ensifer* or *Shinella* genera. However, it is necessary to use molecular techniques to confirm it. Molecular methods and phenotypic studies and constitute what is now known as polyphasic taxonomy, a tool that allows a greater comprehensiveness of microorganism taxonomic studies.

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