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REPORT

Bacteria from *Bacillus* genus with potentialities for agricultural sustainability in Cuba

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ABSTRACT

The use of Plant Growth-Promoting Bacteria (PGPB) allows for diminishing the use of chemical products in agriculture. These products are dangerous for environments and human health. The objective of the present work was to characterize isolates from Bacilli class to select potentially efficient strains for their use in sustainable agriculture. To isolate bacteria; the samples from rhizosphere and rice, maize and coffee plants were heated to 80 °C. The isolates were characterized according to PGPB and antagonistic traits. Several strains were selected to study the colonization, diversity and their effect on plant growth. One hundred forty-five aerobic Gram-positive endospore forming bacteria were isolated from rice, maize and coffee. The studied strains showed several traits for plant growth. In thirty-seven selected strains from rice, maize and coffee, antagonistic activity was demonstrated against phytopathogenic fungi. After that, two strains were selected and they show their ability to colonize rice plants, move and form biofilms. The promised strains were identified as belonging to *Bacillus* genus by sequencing the 16S rRNA gene. The molecular study of *Bacillus* from rice demonstrated the high genetic diversity and the occurrence of virulence determinants in few strains. The inoculation of maize and tomato in semicontrolled conditions showed positive effects in plant growth. An integrally characterized bacterial native collection, efficient for plant growth promotion and antagonistic activity is available, which constitutes the base for new bioproducts obtainment. This work proposes an efficient methodology for prospecting and characterizing *Bacillus* for their use in agricultural sustainability. This work received the Annual Award of the Cuban Academy of Sciences for the year 2020.

Keywords: Bacillus, plant growth-promoting bacteria, antagonism, crops of economic interest, agricultural sustainability

RESUMEN

Bacterias del género *Bacillus* con potencialidades para la sostenibilidad agrícola en Cuba. El uso de las bacterias promotoras del crecimiento vegetal (PGPB) permiten disminuir el empleo de químicos en la agricultura, perjudiciales para el ambiente y los seres humanos. El objetivo del presente trabajo fue caracterizar integralmente aislados de la clase Bacilli para la selección de cepas potencialmente eficientes para su uso en la agricultura sostenible. Se aislaron las bacterias calentando a 80 °C las muestras de rizosfera y plantas desinfectadas de arroz, maíz y cafeto. Se caracterizaron según sus atributos como PGPB y antagonistas de patógenos. Se seleccionaron cepas para estudiar la colonización, la diversidad poblacional y el efecto en la promoción del crecimiento. Se aislaron y caracterizaron 145 bacterias aerobias Gram positivas formadoras de endosporas endófitas y rizosféricas. Las cepas estudiadas muestran varios atributos para la promoción del crecimiento. Se demostró el antagonismo de 37 cepas de arroz, maíz cafeto frente a 11 hongos fitopatógenos. Se seleccionaron dos cepas que demostraron su capacidad para colonizar plantas de arroz y de formar biopelículas y de movimiento. Se identificaron las cepas promisorias por secuenciación del gen codificante para el ARNr 16S como pertenecientes al género *Bacillus*. El estudio molecular de la población de *Bacillus* aislados del arroz demostró su elevada diversidad genética y la presencia de determinantes de virulencia en algunas cepas. La inoculación de maíz y tomate en condiciones semicontroladas mostró efectos positivos en el crecimiento de la planta. Conclusiones: Se dispone de una colección microbiana nativa, caracterizada integralmente, eficiente en la promoción del crecimiento vegetal y con actividad antagonista de patógenos, que constituyen la base para la obtención de nuevos bioproductos. Se propone una metodología eficiente para la prospección y caracterización de *Bacillus* para la sostenibilidad agrícola. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2020.

Palabras clave: Bacillus, bacterias promotoras del crecimiento vegetal, antagonismo, cultivos de interés económico, sostenibilidad agrícola

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Introduction

The *Bacillus* genus plays an important role in the biological control of phytopathogens, despite the extended use of promising strains in promoting plant growth. Advantageously, these bacteria form endospores of increased survival in soil, fast growth rate and a wide versatility for metabolite production. The microbial inoculants based on plant growth-promoting bacteria (PGPB) reduce the dependence on high-risk chemical products in agriculture, and are economic and environmentally viable alternatives.

Particularly, the biodiversity and the prospective studies of *Bacillus* species may be of special interest, due to high potential of this genus for sustainable food production, of vital importance for the development of our country. Most of domestic and international studies of this genus analyze pest control using

1. Rojas MM, Tejera B, Bosch DM, Ríos Y, Rodríguez J, Heydrich M. Potencialidades de cepas de *Bacillus* para la promoción del crecimiento en el cultivo del *Zea mays* L. Cuban J Agric Sci. 2016;50(3):485-96.

Bacillus thuringiensis. In other cases, studies covered isolates and partial characterizations concerning their attributes as plant growth promoter or pathogen antagonists. Therefore, this work was aimed to fully characterize isolates of the Bacilli class, for the selection of potentially efficient strains that can be used in sustainable agriculture.

Materials and methods

Bacilli isolates

Samples of the rizosphere and rice, corn and coffee plants were taken from different locations of not biofertilized areas. Diluted samples were heated at 80 ºC for 30 min, cultured in Tryptone Soy Agar or Nutrient Agar and then subjected to Gram staining. Gram positive endospore-forming bacilli with aerobic growth were selected.

Determination of the growth-promoting attributes

Indoles of the indoleacetic acid (IAA)-type, the capacity of growing in a nitrogen free medium and the solubilization of phosphates, were determined according to Rojas *et al*. [1]. Phosphatases production was determined using p-nitrophenyl sodium phosphate at different pH, depending on whether they were acid or alkaline, and measuring p-nitrophenol production through spectrophotometry. For ammonium production, the Nessler reagent was applied and for the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (ACCd) cultures were established in minimal medium with ACC as the single nitrogen source.

The methods for obtaining, validating the polyclonal antisera and the immunochemistry technique used for IAA detection are described in Rojas *et al.* [2]. Moreover, produced indoles were characterized by thin layer chromatography and the effect of IAA on plant growth *in vitro* [3] and the effect of this hormone in the growth of *Bacillus* strains [4] were established.

Determination of the *in vitro* antagonistic activity and the possible mechanisms involved

The dual-culture methodology was used to determine the antagonistic effect of isolates against the 11 phytopathogenic fungi affecting crops of economic interest [5, 6]. Biosurfactants were characterized by the drop collapse test, and the hydrogen cyanide (HCN) method through its reaction with calcium carbonate in picric acid. Different lytic enzymes were evaluated (glucanases, proteases, lipases, chitinases and amylases) by measuring the hydrolysis halo produced in a medium containing the appropriate substrate, according to the enzyme (cellulose, skim milk, Tween 80, chitinase colloidal substrate and starch, respectively). When required, Congo Red and Lugol solution dyes were used to develop glucanases and amylases reactions, respectively.

Identification of selected isolates by their potential

The gene coding for the ribosomal RNA 16S (16S rRNA) was sequenced and compared to sequences at the GenBank database [6]. The 32 *Bacillus* sequences obtained from GenBank were searched *in silico* for a fragment of subunit A gyrase (*gyrA*) gene and aligned using the ClustalW software. A new pair of primers was designed for the amplification of this fragment, and species were identified by sequencing.

Colonization and its attributes

The crystal violet method was used to quantify biofilm production [1]. The motile capacity was tested, of both superficial by swarming (flagellum movement) and swimming, through superficial inoculation or by puncture in the center of the culture with 0.6 and 0.3 % agar, respectively. Colonization dynamics was studied by the inoculation of rice seeds with strains double-mutant to the antibiotics rifampicin and nalidixic acid (RP27 and EAI5, respectively); they were quantified in selective media with antibiotics one week after inoculation. Furthermore, the presence of the bacteria in the roots and stems of rice plants were visualized through fluorescence microscopy, using these strains labeled with the green fluorescent protein.

Molecular study of *Bacillus* populations from rice

The diversity of the 17 *Bacillus* spp. strains associated to rice was characterized through the combined analysis of four random amplified polymorphic DNA (RAPD) markers. Furthermore, these strains were characterized by PCR for the presence of eight virulence determinants for humans using specific primers.

Effect of the selected strains on plant growth under semi-controlled conditions

Inoculation bioassays were applied in corn, tomato and carrots under semi-controlled conditions, as described by Rojas *et al*. [7].

Biometric analysis

Experimental designs were custom-made for all the assays. The *in vitro* experiments were performed in triplicate and the *in vivo* experiments had 10 replicates each. The normal distribution and homogeneity of the variance were tested to guarantee compliance with the premises established for parametric analyses. Depending on their results, parametric or non-parametric tests were selected and applied for results comparison. The STATISTICA software version 8.0 for Windows was used for data processing.

Results and discussion

Using isolates of rice, corn and coffee, 145 native *Bacillus* isolates were collected from the rhizosphere and endophytes, from the roots and aerial parts of the plants, the most abundant in the rhizosphere (Table 1). These isolates were Gram-positive endospore forming bacilli with aerobic growth, being classified as belonging to the Bacilli class.

Characterization of growth-promotion attributes

The isolated strains were capable of solubilizing calcium, iron and aluminum phosphate; they mineralize organic phosphorus through acid and alkaline phosphatases. They also produce indoles such as the 3-IAA, ammonium, the ACCd enzyme and fix atmospheric nitrogen [1, 5, 8]. All these studies supported

2. Rojas MM, Hernández A, Rives N, Tejera B, Acebo Y, Heydrich M. Producción de un antisuero para la detección de ácido indoalcético en cultivos de bacterias promotoras del crecimiento vegetal. Acta Biol Colombiana. 2012;17(2):271-80.

3. Sánchez D, González L, del Monte-Martínez A, Rojas MM. Factibilidad biológica del uso de cepas de Bacilli productoras de ácido 3 indolacético en el crecimiento *in vitro* del cultivo del arroz. Rev Cub Cienc Biol. 2019;7(1):1-10.

4. Rojas MM, Rodríguez AJ, González L, Heydrich M. Influencia de diferentes factores en el crecimiento de bacterias endófitas de caña de azúcar. Rev Colombiana Biotecnol. 2015;XVII(2):149-55.

5. Rojas MM, Tejera B, Larrea JA, Mahillon J, Heydrich M. Aislamiento y caracterización de cepas de *Bacillus* asociadas al cultivo del arroz (*Oryza sativa* L.). Rev Bras Agroecol. 2011;6(1):90-9.

6. Rojas MM, Sánchez D, Rosales K, Lugo D. Antagonismo de *Bacillus* frente a hongos fitopatógenos de cultivos hortícolas. Rev Protección Veg. 2017;32(2):1-9.

7. Rojas MM, Bello MA, Ríos Y, Lugo D, Rodríguez J. Estimulación del crecimiento de cultivos de interés económico por cepas de *Bacillus*. Acta Agron. 2020;69(1):54-60.

8. Tejera B, Heydrich M, Rojas MM. Aislamiento de *Bacillus* solubilizadores de fosfatos asociados al cultivo del arroz. Agron Mesoam. 2013;24(2):357-64.

the selection of strains showing most of these attributes (Table 2).

On the other hand, antisera were obtained in rabbits using the IAA adsorpted to nitrocellulose membranes, showing a high titer and specificity. The production of this auxin was detected through immunoadsorption, equivalent to its quantification by spectrophotometry. This enabled the validation of this assay for the rapid and efficient detection of this metabolite [2]. Furthermore, it was demonstrated through thin layer chromatography that IAA is the main indole produced by the analyzed strains [3] and how it can affect the growth of the selected strains of this genus [4].

Characterization of attributes for pathogen antagonism

The antagonism of 15 strains from rice [5, 9], two from corn and 20 from coffee [6] was tested against different phytopathogenic fungi, accordingly: *Pyricularia oryzae*, *Alternaria solani*, *Curvularia* sp., *Fusarium* sp., *F. chlamydosporum*, *F. oxysporum*, *F. moniliforme*, *F. incarnatum*, *Corynespora cassicola*, *A. brassicicola*, and *Cladosporium fulvum*. This would contribute to establish comprehensive measures for improving plant health. It was demonstrated the production of lytic enzymes (glucanases, proteases, chitinases, lipases and amylases), HCN and biosurfactants, thus sustaining the antagonistic activity of these strains. The most promising strains were selected for the biological control of the fungi studied (Table 2).

Colonization studies

The ability of strains to form biofilms, as well as their motility, offer advantages for the colonization of the rhizosphere and the inner part of plants, thus contributing to their positive effect on plants. Biofilm formation was tested in 19 *Bacillus* strains from rice, 10 from corn and two from coffee. It was determined that the maximum production was reached at between 48 and 72 h for all strains. Both the superficial flagella movement (swarming) and swimming in eight strains of rice, two of corn and two of coffee, reached the maximum values after 48 h. In the inoculation of rice seedlings using double-mutant strains to the antibiotics rifampicin and nalidixic acid (RP27 and EAI5), it was found that they appear at concentrations higher than 10^3 cfu per plant after one week. However, strain RP27 colonizes rice plants better regardless of the variety. Furthermore, it was demonstrated that one week after the inoculation, the bacterium has disseminated in the roots and stems of the rice plants, through fluorescence microscopy using these strains labeled with the fluorescent green protein.

Diversity studies in the *Bacillus* population isolated from rice

The diversity of the 17 strains of *Bacillus* spp. isolated from rice was characterized using RAPD markers. It was demonstrated that groups of two and three strains are formed with more than 70 % homology, and the others remain isolated. The strains of *B. thuringiensis* form a separate group from the rest of the strains, which in general show a high diversity degree within the *Bacillus* population associated to rice production. Furthermore, those of the *B. thuringiensis* strains showed the presence of four virulence determinants, *cyt K2*, *clo*, *nheA* and *hbl*, as well as hemolysis type β, thus excluding these strains from its possible use in agriculture, due to their potential risk for threatening human health.

Table 1. Distribution of the isolates obtained from different cultures

Crop	Cultivar	Bacilli isolates			Location
		Rhizospheric	Endophytic	Total	
Rice (*Oryza sativa* L.) 71 isolates	Iacuba 30	11	13	24	Grain Research Institute (IIG), Bauta, Artemisa
	Perla de Cuba	27	7	34	
	J-104	7	–	7	
	INCA-LPS	6	–	6	
Corn (*Zea mays* L.) 48 isolates	Hybrid P-7928	9	10	19	Institute for Basic Research in Tropical Agriculture "Alexander Humboldt" (INIFAT), Havana
	Jibara	14	4	18	
	Francisco Mejorado	6	5	11	
Coffee tree (*Coffeea arabica* L.) 26 isolates	Caturra yellow	10	–	10	"El Dorado" Farm, Granma
	Caturra	8	–	8	Farm of a small landowner, Quivicán, Artemisa
	Caturra red	8	–	8	Germoplasm collection from the Coffee and Cacao Station, Baracoa, Guantánamo

Identification of promising isolates

The sequencing of the 16S rRNA gene and its comparison with the sequence databases made it possible to cluster 21 *Bacillus* strains isolated from rice and six from coffee within the *Bacillus* genus. Due to the taxonomic complexity of this genus, more than one gene is required for the identification of the species. A pair of new primers for the amplification of a *gyrA* gene fragment for this genus. Four isolated coffee strains were sequenced and identified.

Effect of selected strains when applied under semi-controlled conditions

The biological effect of IAA concentration produced by two strains of *Bacillus* was determined in rice seeds, aimed for verifying the possible action of auxins produced by bacteria. There was a greater growth in the secondary roots with the treatment using strains RC9 and RCQ7 and a lower concentration of IAA (2 mg/mL). This was highly beneficial for plants by increasing root surface for nutrient absorption and root coverage of a greater soil volume [3].

Bioassays were conducted under semi-controlled conditions in corn, tomato and carrot plants used as model plant, demonstrating in greenhouse conditions that the *Bacillus* strains displayed growth-stimulating capacity in corn and tomato plants. These two crops showed the greatest rooting effect, with modifications in plant architecture, which is encouraging because of the role of the root in water and nutrient uptake [7]. These results validate the potential of the *Bacillus* strains in different crops, thus extending their possible future use as bioproducts.

Proposal of a methodology for the prospection, selection and characterization of *Bacillus* genus strains of interest for sustainable agriculture

This study has led us to propose a comprehensive methodology, starting with the isolation of strains, including their comprehensive characterization for the selection of the most promising ones, and the studies of the plant-bacteria interaction under

9. Tejera B, Heydrich M, Rojas MM. Antagonismo de *Bacillus* sp. contra hongos fitopatógenos del cultivo del arroz (*Oryza sativa* L.). Rev Protección Veg. 2012;27(2): 117-22.

Table 2. Summary of plant growth-promoting attributes and antagonistic activity against fungal pathogens of selected *Bacillus* strains isolated from different crops

Plant growth-promoting attributes										
Strains	IAA-type indoles (mg/mL)	Phosphorous solubilization (index at 7 days)			Ammonium	ACCd	Phosphatase enzymes (μmol/L•h		Growth in medium without N	Potassium solubilization (index at 10 days)
		Ca-P	Al-P	Fe-P			Acid	Alcaline		
RC9	6.51	1.53	2.50	2.50	+	+	84.18	2.84	+	1.33
RC15	15.96	1.46	2.59	2.50	+	+	63.93	0.899	+	1.50
RP12	10.19	1.50	1.56	1.16	+	–	167.90	0.40	+	ND
RP20	9.22	1.28	1.37	1.16	+	+	47.22	3.54	+	ND
RM5	17.03	1.66	1.00	1.66	+	+	21.46	2.02	+	0

Pathogen-antagonistic attributes											
Strains	Proteases	Glucanases	Chitinases	Amilases	HCN	Biosurfactants	Cc	Fc	Fi	Ab	Cf
RC9	+	+	ND	ND	ND	–	36.17	39.65	21.48	51.35	13.97
RC15	+	+	–	+	–	+	71.99	42.22	25.92	36.94	40.86
RP12	+	+	+	+	–	–	24.73	47.91	17.77	65.76	42.10
RP20	+	+	+	+	+	+	34.54	28.89	19.25	38.73	17.51
RM5	+	+	–	+	+	–	39.46	27.40	17.77	41.44	0

* Cc: *Corynespora cassicola*, Fc: *Fusarium chlamydosporum*, Fi: *F. incarnatum*, Ab: *Alternaria brassicicola*, Cf: *Cladosporium fulvum*, HCN: *cianuro de hidrógeno*, ND: not determined.

semi-controlled conditions. This supports the recommendation of the most efficient strains for further scaling-up studies and field trials (Figure). In this study, it was established a native microbial collection which was comprehensively characterized, and showing plant growth promoting capacity and pathogen antagonist activity in economically relevant crops. Our results support the generation of new bioproducts that could decrease the application of chemicals in agriculture, with a positive impact on food production and environmental protection.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

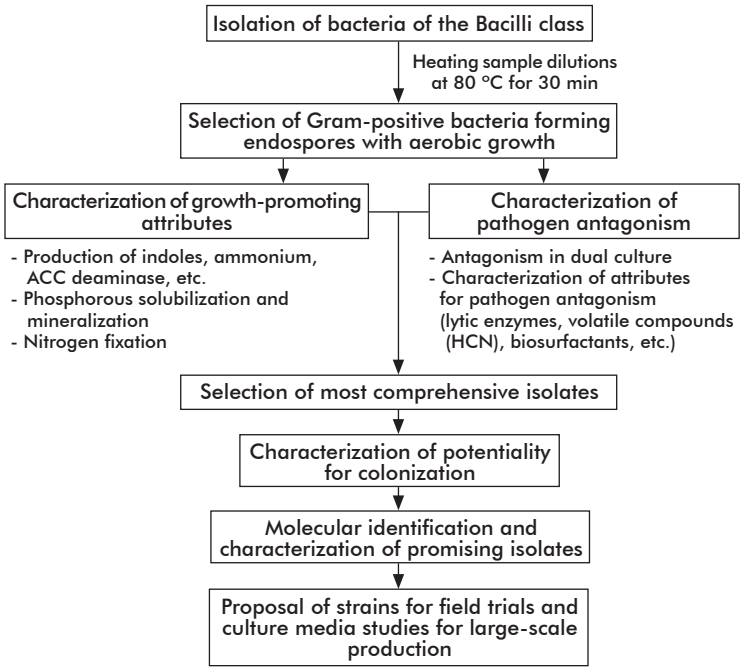

Figure. Proposed methodology for the prospection and characterization of *Bacillus* strains of interest for agricultural sustainability. ACC: 1-aminocyclopropane-1-carboxylic acid (ACC).

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