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Original Article

Isolation and characterization of *Stenotrophomonas asociated* to maize (*Zea Mays* L.) rhizosphere

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ABSTRACT

The inoculation of plant growth-promoting rhizobacteria in agricultural interest crops such as maize constitutes an economically and environmentally friendly alternative to chemical fertilization. *Stenotrophomonas* is a common bacterial genus in many soil types and rhizospheres of different crops, and presents mechanisms for growth promotion such as biological nitrogen fixation, mineral solubilization and phytohormones production. Consequently, the present work aimed to characterize and molecularly identify *Stenotrophomonas* strains from the maize rhizosphere. For this, an isolation was performed from rhizospheric and rhizoplane soil and colonies with cultural characteristics similar to those described for this genus were selected. The microscopic characteristics and the ability

to perform the biological nitrogen fixation solubilize different phosphate and potassium sources and the antagonistic activity against *Fusarium oxysporum* were also taken into account. Molecular identification was performed by 16S rDNA sequencing. Twenty isolates with cultural, morphological and physiological characteristics coinciding with the genus in question were obtained. From these 15 solubilized at least one of the phosphate sources used, two solubilized the potassium sources and six showed antagonism against the pathogen. Seven isolates were identified as *Stenotrophomonas* and the rest were included in three genera, also belonging to the Proteobacteria phylum. The integral isolate in terms of positive attributes evaluated was INCA-FRr1 identified as *Stenotrophomonas*. This could be a promising inoculant for maize and other crops.

Key words: PGPR, FBN, solubilization, antagonism, biofertilization

INTRODUCTION

Corn is an important component in human and animal nutrition. It is cultivated in the most diverse edaphic and ecological conditions given its high plasticity; and its production and consumption worldwide, reach the highest figures compared to other crops [(1)]. However, the availability of certain nutrients, generally caused by mineral fertilization, mainly nitrogen, potassium and phosphorous, is essential to obtain acceptable yields. Although the application of these inputs makes it possible to obtain representative yields, they also constitute a high cost for the production process and negatively influence the agroecosystem [(2)].

The use of biofertilizers based on Plant Growth Promoting Rhizobacteria (PGPR) is a viable alternative to decrease the load of mineral fertilizer applied every year in agriculture. These biopreparations are harmless to the environment, increase crop yields and decrease production costs [(3)].

The *Stenotrophomonas* genus has been as a rhizospheric microbiota of different crops described, including corn [(4-7)], and it is considered a PGPR due to the different mechanisms of promotion of plant growth it presents. These include Biological Nitrogen Fixation (FBN), solubilization of phosphate salts, production of indoles [(6)] and synthesis of ACC deaminase [(8)].

In Cuba, there are no studies related to the *Stenotrophomonas*-corn interaction, nor with the use of the genus for agricultural exploitation, therefore, the present work intends to isolate,

identify and characterize *Stenotrophomonas* from the corn rhizosphere, as well as to determine the effect of their inoculation under controlled conditions.

MATERIALS AND METHODS

Sampling

The sampling was carried out at the El Mulato farm in San José de las Lajas municipality, Mayabeque. The corn was cultivated in Ferralitic Red Leachate Typical eutric [9] with *Phaseolus vulgaris* L. as the preceding crop. Five random sampling points were established and two plants were from each of them taken, for 10. The extraction was carried out taking a volume of soil of approximately 20 cm^3. The samples were placed in separate polyethylene bags, and before two hours were stored in the laboratory at 4 ºC until use.

Isolation of *Stenotrophomonas* from the Corn Rhizosphere

Isolation was made from rhizospheric soil and rhizoplane from corn plants following the methodology proposed by Granada [5]. For rhizospheric soil isolation, 1 g samples were weighed and serial dilutions of 10^{-1} to 10^{-6} were made in sterile distilled water. The 100 µl of the suspensions (10^{-4}-10^{-6}) were by dissemination in LB medium, taken and sown with three replicates each. The plates were at 28 °C for 48 hours incubated.

For rhizoplane isolation, roots were into 1 cm long portions cut and placed in Erlenmeyers bottles with 10 mL of sterile distilled water. These were kept in agitation for 1h at 150 rpm and the same rhizospheric soil methodology was applied to them.

The cut roots were with LB medium plated and incubated at 28 °C for 48 h. Subsequently, roasts were taken from the bacterial growth that was visualized around and on the roots and isolated in the same medium with the same conditions.

Characterization of *Stenotrophomonas* isolated from the corn rhizosphere

Cultural and morphological characterization

For the cultural characterization, the coloration, mucus and morphology of the colony were taken into account. Successive striating in LB medium purified those with translucent coloration, light mucus, smooth edges and flat elevation [5]. Then Gram staining was

performed and gram-negative isolates with bacillary or cocobacillary morphology and non-sporulated were selected, which were subsequently punctured in the nitrogen-free media, Rennie and JMV. The isolates that grew in these media were as nitrofixers classified.

Characterization as PGPR

In the test, the capacity of the isolates to solubilize phosphorus and potassium was evaluated, in addition the antagonistic activity against *Fusarium oxysporum* was determined.

Phosphate solubilization was determined in NBRIP medium using the bromocresol purple indicator, In addition, three different sources of inorganic phosphorus were tested: tricalcium phosphate ($Ca_3(PO_4)_2$), aluminum phosphate ($AlPO_4$) and iron phosphate ($FePO_4$). The bacteria were by spots around 0.5 cm in diameter seeded, at the rate of five isolates per plate, and three replicates were for each one established. The plates were at 28 °C for 48 hours incubated. Those isolates that showed a yellow halo around the growth were taken as positive results, indicating the solubilization of the phosphate salts.

Following the same procedure, the solubilization capacity of potassium in the Alexandrov medium was determined using dipotassium hydrogen phosphate (K_2HPO_4) and potassium oxide (K_2O) as potassium sources. The plates were at 28 °C incubated for 48 h and the isolates that had a solubilization halo around the colonies were as positive results taken.

To determine the antagonistic activity against the *Fusarium oxysporum* EC20-E-GM strain (belonging to the strain of the plant pathology laboratory of the Center for Biotechnological Research of Ecuador), the mycelium was cut with a sterile scalpel. This was in a Falcon tube placed with 20 ml of sterile distilled water and subsequently sterilized steel pellets, 4 mm in diameter were added. The bottle was shaken vigorous and manually for 5 min and the contents were filtered with a 40-µm sieve mounted on a sterile Falcon tube. The 200 µl of the sieved solution were seeded in Potato Dextrose Agar, and the bacterial isolates were spotted on this point at the rate of four isolates per plate. Incubation was at 28 °C for 7 days. The isolates that showed a fungal growth inhibition halo with a radius greater than 0.5 mm were as positive results for the antagonistic activity taken.

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Identification by sequencing of the 16S rDNA gene

Genomic DNA extraction

The extraction of the genetic material was carried out by alkaline lysis. A colony was from the axenic culture taken and placed in Eppendorf with 40 µl of NaOH at 0.20 M. It was then at 10 %, power of 700 W for one minute microwaved and immediately cooled on ice for 5 min. The lysed cells were for 10 min at 10,000 gravities spun. The supernatant was taken and the nucleic acid quantification was performed by spectrophotometry using the NanoDropTM 2000 at 260 nm, and the ratio 260/280 and 260/230 were calculated in search of determining the concentration and quality of the DNA, respectively.

Amplification and sequencing of 16S rDNA

For the amplification, 25 µl of the PCR mixture containing 1 µl of the crude extract containing DNA, 1 µl (10 µM) of the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-) were used. 3') (10), which allow obtaining amplicons of around 1500 bp, and GoTaq® Green Master Mix (Promega Corporation, USA). Amplification was performed in an MS mini thermocycler (Major Science, USA) under the following parameters: initial denaturation 95 °C for 10 minutes and additional denaturation for 1 minute at 92 °C, 35 cycles of 72 °C each, for 2 minutes. Amplification products were verified by 1 % agarose gel electrophoresis (1 g agarose in 150 mL of 1X TAE buffer) at 80 V for 45 min. The reaction products were purified using the prior to sequencing, was performed by the Sanger method at the Macrogen® Company (Republic of Korea)

Editing sequences and identification

A quality analysis was performed of the sequences obtained using the FinchTV program (ver. 1.4.0) (Geospiza Inc.) using as a criterion of acceptance a quality value (Q) equal to or greater than 20 per base. They were subsequently analyzed using the BLASTn program [(11)]. The cutoff value used was 1 x 10^{-5}, with a minimum coverage of approximately 80 %. The debugging and alignment of the obtained sequences were performed using the ClustalW multiple sequence alignment tool [(12)] in the MEGA-X program (ver. 10.0.4) [(13)].

RESULTS AND DISCUSSION

Isolation of *Stenotrophomonas* from the corn rhizosphere

The 59 total isolates were obtained, of which 72.9 % came from the rhizoplane and 27.1 % from the rhizospheric soil of corn plants. The nutritional wealth that the rhizoplane represents makes it an attractive area for the establishment of microbial populations. Radical exudates, whose concentrations are highest at the rhizoplane level, constitute important sources of carbon for many edaphic microorganisms [(14)].

Characterization of isolated *Stenotrophomonas* from the corn rhizosphere

Cultural and morphological characterization

The 59 isolates obtained presented flat, translucent, mucous colonies with smooth edges, characteristics similar to those described by other author [(5)]. From these, 86.4 % (51 isolates) were gram-negative and, in turn, 94.1 % (48 isolates) coincided with the proposed morphological specifications for the genus *Stenotrophomonas*: gram-negative bacilli or cocobacilli not sporulated. Many bacterial genera develop in the soil, however, in absolute numbers; there is a predominance of gram-negative bacteria over gram-positive bacteria [(15)]. In the particular case of the rhizosphere, the roots have a direct influence on the composition and density of the soil microbiota. The release of organic plant substances favors the establishment of heterotrophic microbial populations that, for the most part, comprise gram-negative bacilli and actinomycetes [(15)].

The genus *Stenotrophomonas* presents the ability to perform the FBN, so the Rennie and JMV media were used to rule out those isolates that do not perform this function; and 41.7 % (20 isolates) grew in the media. Red Ferralitic soils are the most common in the Habana-Matanzas plain, an area that includes the sampled area. In general, the productivity of these soils is high due to the depth and characteristics of their horizons; however, they are poor in organic matter and have low fertility [(9)], which can translate into poor nitrogen concentrations. These characteristics could favor the proliferation and activity of diazotrophic microorganisms such as *Stenotrophomonas*. FBN occurs as a function of the concentration of the element in the medium, responding to the principle of cellular economy. The presence of high concentrations of nitrogenous compounds inhibits the synthesis of nitrogenase, the enzyme responsible for the process, by repressing the expression of the *nif*

genes that encode it. In this way, the microorganism preferably uses these elements as a nitrogen source, instead of carrying out a process that involves high-energy consumption, such as FBN. On the other hand, it is argued that any deficiency in organic and inorganic nitrogenous compounds stimulates N_2 microbial fixation [16, 17].

The means used in the isolation of nitrofixing bacteria were completely devoid of nitrogen sources and included in its composition, sodium molybdate salt dihydrate ($Na_2MoO_4.2H_2O$) as a source of molybdenum, a structural component of the enzyme nitrogenase [17]. Both aspects favored the synthesis and activity of the enzyme, and therefore allowed microbial growth under these conditions.

Characterization as PGPR

The 20 isolates that showed the ability to perform the FBN, grew in the NBRIP medium. The carbon source present in said medium is glucose, which constitutes the sugar most used as a carbon source in culture media; and there are few microorganisms with the inability to metabolize it [17].

The 75 % (15 isolates) of these isolates developed a solubilization halo against the different phosphorus sources used (Table 1).

Table 1. Qualitative characterization as PGPR. Solubilization of phosphorus, potassium and antagonistic activity against *Fusarium oxysporum*

Isolated	PO_4^{-2} source			K^+ source		Antagonism
	$Ca_3(PO_4)_2$	$AlPO_4$	$FePO_4$	K_2HPO_4	K_2O	
INCA-FRr1	+	+	+	+	+	+
INCA-FRr2	+	-	+	-	-	+
INCA-FRr3	+	+	+	nc	nc	+
INCA-FRr4	-	-	+	nc	nc	-
INCA-FRr5	+	-	+	+	+	-
INCA-FRr6	+	+	+	-	-	-
INCA-FRr7	+	+	+	nc	nc	+
INCA-FRr8	+	-	+	nc	nc	+
INCA-FRr9	+	+	+	nc	nc	-
INCA-FRr10	-	-	-	-	-	-
INCA-FRr11	+	+	+	nc	nc	-
INCA-FRr12	+	+	+	-	-	-
INCA-FRr13	+	-	+	nc	nc	-
INCA-FRr16	+	+	+	nc	nc	-
INCA-FRc1	-	-	-	-	-	-
INCA-FRc4	-	-	-	-	-	-
INCA-FRc8	-	-	-	-	-	-
INCA-FRc16	+	+	+	-	-	-
INCA-FRc19	-	-	-	-	-	-
INCA-FRc24	-	-	+	-	-	+

(+) Production of solubilization halo or inhibition halo in case of antagonism; (-) bacterial growth without halo formation of solubilization or inhibition in case of antagonism; (nc) there was no bacterial growth

Aluminum phosphate was the least consumed source, with nine isolates; while tricalcium phosphate and iron phosphate were more successful with 13 and 15 isolates respectively. The iron content in Red Ferralitic soils is high [(9)], making it more likely that phosphate will establish bonds with ferric ions compared to other ions, and in turn, the microorganisms present in these soils are more familiar with these compounds.

The formation of the solubilization halos occurs because of the cation exchange carried out by organic acids secreted by the bacteria. These compounds convert insoluble phosphate to soluble forms of the salt [(18)].

On the other hand, the presence or not of the halo around the colony is not a sufficient criterion to classify the microorganisms as phosphorus solubilizers [(19)]. The solubilization of phosphorous salts could occur by the binding of a chelating agent to the cations, displacing

the phosphate groups. These reactions, although they allow the phosphate to be solubilized, do not produce halo in the culture medium. Therefore, although the possibility that some of the isolates studied here may not have the ability to solubilize any of the phosphate salts used, it cannot be ruled out that there may be isolates with solubilizing potentialities and, due to the methodology used, it has not been possible to determine [20].

Regarding the solubilization of potassium, 60 % (12 isolates) of the isolates grew in Aleksandrov's medium and of these, 16.6 % (2 isolates) developed a halo of solubilization. This medium has as its carbon source and the main component sucrose. Disaccharide that not constitute the most attractive carbon source for most microorganisms.

Some authors associate the solubilization of potassium with the production of acids of microbial origin [21]. This could have been the mechanism used to form the solubilization halo. However, as in the phosphate solubilization, there are studies that indicate that this occurs without the release of acids into the medium, and therefore without the decrease in pH [22].

The antagonistic activity against *Fusarium oxysporum* was positive in 30 % (6 isolates) of the isolates. A large number of pathogens that cause significant economic damage to its production attacks corn in tropical environments. These include species of the genus *Fusarium* [23] that cause ear rot, which, in addition to reducing yield, cause deterioration and poor quality of grains, and due to the ability to produce mycotoxins, they are also to diseases in their diners related [24].

Identification by sequencing of the 16S rDNA gene

Four bacterial genera belonging to the Phylum Proteobacteria were identified, a group especially abundant in the rhizosphere during the different phenological stages of maize [25,26]. These included *Enterobacter*, *Pseudomonas*, *Rhizobium* and *Stenotrophomonas* with a percentage of identity greater than or equal to 97 in 95 % of cases (Table 2).

Table 2. Comparison of the sequences obtained, with the NCBI database using the BLASTn tool

Isolated	Putative species	Identity
INCA-FRr16	*Enterobacter bugandensis*	99 %
INCA-FRr5	*Pseudomonas brenneri*	98 %
INCA-FRr3	*Pseudomonas gessardii*	99 %
INCA-FRr2	*Pseudomonas graminis*	99 %
INCA-FRr4	*Pseudomonas hibiscicola*	97 %
INCA-FRr8	*Pseudomonas putida*	100 %
INCA-FRr9	*Pseudomonas* sp.	100 %
INCA-FRr11	*Pseudomonas sp.*	99 %
INCA-FRc4	*Rhizobium aegyptiacum*	100 %
INCA-FRc1	*Rhizobium mesosinicum*	99 %
INCA-FRc8	*Rhizobium mesosinicum*	99 %
INCA-FRr10	*Rhizobium sp.*	88 %
INCA-FRc19	*Rhizobium sp.*	99 %
INCA-FRr12	*Stenotrophomonas maltophilia*	100 %
INCA-FRr13	*Stenotrophomonas maltophilia*	100 %
INCA-FRc16	*Stenotrophomonas maltophilia*	98 %
INCA-FRr7	*Stenotrophomonas pavanii*	98 %
INCA-FRc24	*Stenotrophomonas pavanii*	99 %
INCA-FRr1	*Stenotrophomonas rhizophila*	99 %
INCA-FRr6	*Stenotrophomonas rhizophila*	100 %

The most representative genera were *Stenotrophomonas* and *Pseudomonas* for 35 % of the total in each case (7 isolates). Similar results were obtained in the *Daucus carota* L. rhizosphere [(6)]. To a lesser extent, the genera *Rhizobium* and *Enterobacter* were identified with 25 % (5 isolates) and 5 % (1 isolate) of appearance respectively.

It is common to find reports of pathogenicity associated with the genera *Stenotrophomonas*, *Pseudomonas* and *Enterobacter*, in humans, animals and even plants [(27-30)]. However, on many occasions, the presence of virulence factors and the pathogenicity of microorganisms are restricted to species, and even to specific strains within a genus [(17)]. The pathogenic character that some of these genera may present does not detract from the biostimulant potentialities that they develop in plants; although they are not exempt from the eco-toxicological tests established for the agricultural exploitation of microorganisms.

The genus *Pseudomonas* have been widely reported as PGPR of various plants including grasses [(5,6,15,31)]. Populations of *Pseudomonas* frequently stand out above other diazotrophic genera due to their short latency period, rapid growth rate, and metabolic versatility [(32)]. On

the other hand, *Enterobacter* has been described as a rhizospheric microorganism and, above all, as an endophyte of different plant species; standing out for its growth promoting characteristics [33,34]. In the case of the *Rhizobium* genus, isolations have been made as endophytes of corn [35] and to a lesser extent of the rhizosphere [36]; mainly in soils where the crop rotation system is applied with some legume species. The use of *Phaseolus vulgaris* L. as a rotation culture in this study, may have favored the establishment of rhizobia populations, since this legume presents greater susceptibility to colonization and symbiosis with species of the *Rhizobium* genus with respect to other genera rhizobia [37].

Stenotrophomonas has been isolated from various environments, from different soil types to animal intestinal tracts [38], which highlights the ubiquitous character of this bacterium. The *Stenotrophomonas pavanii* species is in bioremediation processes at industrial [39] and agricultural [40] levels used, while *Stenotrophomonas maltophilia* has a contrasting behavior in the different habitats it occupies. This species is common in hospital settings and some strains are as powerful nosocomial agents described; however, it is described as part of the normal edaphic microbiota and is associated with many crops of agricultural interest without causing damage to them [41]. Likewise, it occurs with *Stenotrophomonas rhizophila*, which shows great potential to promote the growth of plants such as soybeans [42], cotton, tomato and pepper, among others; and it is considered a planktonic microbiota of marine environments [43].

In this study, the most comprehensive isolate in terms of the plant growth promotion mechanisms evaluated was INCA-FRr1, identified as *Stenotrophomonas rhizophila*. The ability to solubilize different sources of phosphorus and potassium, to perform FBN and antagonistic activity against phytopathogens, in addition to other positive characteristics that were not in this study demonstrated but are known [6]; they represent clear advantages over other rhizospheric bacterial genera [43]. On the contrary, they use a strain of *Stenotrophomonas* rhizophila that does not present the ability to perform the FBN and still obtain important results in terms of development of variables such as height, stem and root length, dry area and leaf biomass in basil plants. This could suggest the presence of other mechanisms for promoting plant growth. In co-inoculation tests of *Stenotrophomonas rhizophila* with Arbuscular Mycorrhizal Fungi (AMF), compared to simple inoculations, an increase in plant growth and chlorophyll content is observed [44]; consequently, the bacterium

is also classified as a mycorrhizal enhancer (term known as Mycorrhiza Helper Bacteria, MHB) [45, 46].

The success of bioproduct use lies, among other aspects, in obtaining strains compatible with the crop in question [47] and efficient for achieving the desired yields. In this way, the use of native PGPRs as inoculants promotes ecological-sustainable management of agroecosystems as well as, could improve corn production. The ability of native strains to interact positively with the resident soil microbiota and their adaptability to local climatic and agro-ecological conditions often enhances their performance compared to non-native strains.

CONCLUSIONS

- Diazotrophic bacterial populations coexist in the genus *Stenotrophomonas*, *Pseudomonas*, *Rhizobium* and *Enterobacter*. In an integral way, the INCA-FRr1 isolate corresponding to the genus *Stenotrophomonas*, stands out for its characteristics that promote plant growth and could constitute a promising inoculant for crops of agricultural interest and for the cultivation of corn itself.
- The study of the rhizosphere and the knowledge of its microbial composition is key to the conception of efficient and compatible biological products within a sustainable agriculture.

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