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Full Length Research Paper

Antagonistic bacteria affecting the Golden cyst potato nematode (*Globoderarostochiensis* Woll.) in the region of Perote, Veracruz, México

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The golden cyst nematode (*Globoderarostochiensis)* presents one of the major problems in potato farming in highlandregionsin variousparts of the world and México is no exception. The high application of chemicals to control the nematode and the related environmental problems have led to the search for alternative control options, such as the use of rhizobacteria, which have indicatednew ways of achieving an increase in agricultural productivity as well asbeing an ecological and economically feasible means to minimize harmful practices in agriculture. The goal of this study was to isolate and evaluate "*in vitro* " the antagonistic capacity of rhizobacteria affecting cysts and J2 stages of *Globoderarostochiensis*. Nematodes were extracted from soil samples from the localities of Perote and Los Altos de Ayahualulco and identified morphometric and molecularly. Potato roots from undisturbed areas in the highland region of Perote were collected for bacterial isolation.The bacteria were isolated on media King B, MB and nutrient agar, and were identified byananalysis ofthe 16S rRNA subunit gene. 102 bacterial isolates were evaluated on J2 and *G. rostochiensis* cysts. Ten of the bacterial isolates were seen to cause distortion and disintegration of J2 nematode individuals. None of the bacterial isolates caused damage in cysts. The cysts provide the female with a defense strategy, which makes them difficult to control. We found that promising bacterial species corresponded to the generas *Providencia, Pseudomonas, Paracoccus* and *Serratia.*

Keywords: Microbial competition, biocontrol, plant parasitic nematodes, rhizobacteria.

INTRODUCTION

The cyst-forming nematodes *Globoderarostochiensis* (Wool.) Skarbilovich and *Globodera pallida* Stone, are important phytophagous organisms in potato farming (Turner and Evans, 1998). They mainly affect the activity of the roots by reducing the volume of soil that the plant can explore in order to absorb water and nutrients and finally, the size and number of tubers also decreases (Trudgill *et al.,* 1998; Aires *et al.,* 2009).

G. rostochiensis and *G. pallida* are widely distributed in countries where potatoes are grown, since they are easily transported to un-infested areas by propagating material (Turner and Evans, 1998), *G. rostochiensis* presents one of the main problems in potato farming (Sullivan *et al.,* 2007). The damage threshold in Mexico and Europe for *G. rostochiensis* is up to 40 cysts per kg^{-1} of soil (EPPO, 1997; NOM-040-FITO-2002).

Among the factors that determine the importance of the nematodes *G. rostochiensis* and *G. pallida* in potato production is the unavailability of effective control measures and the lack of acceptance of these measures by producers (Franco - Ponce and Gonzalez-Verastegui, 2011). This has caused an excessive use of large amounts of nematicidesby the farmers, with unsatisfactory results,since they have not lowered thenematicide application threshold affecting crop development.

Some agricultural activities such as crop rotation and the addition of organic matter to the soil have been employed to promote the activity of native natural enemies present in the rhizosphere and thus lower population densities of the nematode (Wachira *et al.,* 2011). The biological control of plant parasitic nematodes is mainly based on the use of microbial agents such as bacteria and fungi antagonistic to nematodes (Lopez-Llorca *et al.,*2008).

The use of soil microorganisms has provided new options for increased agricultural productivity as an ecological and economically feasible alternative to minimize harmful agricultural practices (Munees and Khan, 2011). While it has been found that plant growth promoting rhizobacteria (PGPR) are capable of potentializing the development of the plant (Kloepper *et al.,* 1980), it has alsobeenshown that when applied to seeds or soils (Verma *et al*., 2010) they can also reduce or eliminate harmful effects caused by pathogens in crops (Hernández-Rodríguez *et al* 2006; Munees and Khan , 2011).

The use of bacteria for the control of J2 *Meloidogyne incognita* individuals has been documented by Hashem and Abo- Elyousr (2011). However, their application for controlling pathogenic nematodes has not been well studied, possibly due to the fact that parasitism levels of bacteria on cyst nematodes are apparently lower in places where they have been introduced, compared to the centers of origin of the pathogen itself.This suggests that these pathogens have no local natural enemies and their biological control would bepotentially unfeasible (Sayre and Walter, 1991).

However, nematodes, like all living things, are not exempt from having native natural enemies, including some bacteria (Rodríguez- Kábana ,1991); therefore, we decided to evaluate the antagonistic activity of rhizobacteriaon the native potato grown in the region of Perote, Veracruz Mexico, affecting the golden nematode *G. rostochiensis*.

MATERIAL AND METHODS

Soil sampling and extraction of *Globoderarostochiensis* cysts

The study sites were the localities of Los Altos de Ayahualulco, Ejido El Paisano and Ejido de losPescados, situated in the County of Perote. A sample from each locality was taken on three occasions. Each sample consisted of 100 subsamples of soil per hectare, each sub sample was approximately 30 g of soil, collected at a depth of 20 cm at 10 m distance fromeachanother,a procedure inaccordancewith the NOM- 040 -FITO- 2002. Sub-samples were homogenized, and approximately 2 kg soil from each was placed in a plastic bag and labeled with the batch data before its transfer to the laboratory.

Extractions of female cyst nematodes were performed by the floating or Fenwick funnel technique (Fenwick, 1940). Each soil sample was dried on paper towels and homogenized manually. From each sample, 500 g of soil was placed in an open mesh sieve, which was washed lightly with water over the funnel. Cysts were retained in sieves of 20 and 60 microns, and recovered in a beaker of 100 ml lined with moistened absorbent paper towels. Two drops of Tween 20 were added to the suspension. The paper was removed from the beaker and put to dry at room temperature. Subsequently, cysts whichadhered tothe paper were collectedwith a paint brush No. 1 under a stereoscopic microscope (Carl Zeiss Stemi 100) and placed in porcelain capsules with distilled water.

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The identification of*Globoderarostochiensis*

The morphological identification was made through cuts and mounting fenestra, according to the procedure proposed by Sosa-Moss (1997), which consists of hydrating the cysts for 12 h before making the cuts. After hydration, thecysts were transferred to a slide to make a cut at the height of thelowerthird of the body, making sure the fenestral area remained in the center of the cut section. Ten cuts for each soil sample were analyzed. The fenestral cut was placed on a clean microscope slide and one drop of 45% lactic acid was added to cleanse the tissues attached to the cuticle. Finally, the cut was set up in dehydrated pure glycerin and sealed with wax rings. Each slide was registered with a code assigned to the sample. The preparation was observed with a 100x objective compound microscope (Carl Zeiss).Thefenestra diameter and the distance between the anus and the fenestra were measured for the Graneck radius value (Graneck, 1955). In addition, the number of crosslinks or grooves between the vulva and anal fenestra were counted,and in the case of juveniles, body length, stylet length, the size of thetail and tail hyaline were observed.

The morphological identification was confirmed by analyzing the ITS1-5.8S-ITS2 rDNA gene region following the "Protocol for diagnosis of nematodes of quarantine importance in potatoes proposed by the National Phytosanitary Reference Centre of the General of Plant Health (SAGARPA, 2014). For thispurpose, three cysts were cleaned of adhering soil residues and separated. Each cyst was placed in a 1.5 sterilized micro-centrifuge tube, where150 mL of extraction buffer was added (200 mMTris-HCl, pH 8.0, 250 mMNaCl, 25 mM EDTA, 0.5% SDS). Each sample was macerated with a sterile pestle attached to a hand drill and incubated at -40 °C for 10 min. Later, the suspension was defrosted and was macerated once more with a new sterile pestle. Afterwards, 0.5 vol. of 3 M sodium acetate pH 5.8 was added, mixed by inversion and incubated at -20 °C for 10 min. After the incubation, each sample was defrostedand mixed by vortex. It was centrifuged at 13,200 rpm for 5 min and the supernatant was transferred to a fresh micro-centrifugesterile tube 1.5 Mland immediately, 1 vol.of cold isopropanol (4-6 °C) was added, mixed by inversion and incubated at -20 °C for 20 min. It was centrifuged at 13,200 rpm for 15 min. The supernatant was removed and the pellet was washed with200 ml of 70% ethanol at -20 °C. It was again centrifuged at 12,000 rpm for 5 min and the supernatant was removed leaving the DNA. This process was performed twice.200 mL of 100% ethanol at -20 °Cwas added to the precipitated pelletandcentrifuged at 12,000 rpm for 5 min. The supernatant was removed and the precipitate was dried at room temperature in a laminar fume hood for one hour. The pellet was re-suspended in 20 uL of nuclease-free water. DNA integrity was checked by electrophoresis on 1.2% agarose gels stained with ethidium bromide (10 mg mL-1); the amount of DNA was measured by the spectrophotometric method with Bio-Photometer (Eppendorf) equipment.

For amplification of the region ITS1-5.8S-ITS2 rDNA gene primers AB28 (ATATGCTTAAGTTCAGCGGGT) and TW81 (GTTTCCGTAGGTGAACCTGC) were used. The amplification was carried out in 50 mL of final reaction volume and consisted of PCR buffer 1x, MgCl23 mM, dNTPs 0.2 mM, Taq DNA polymerase 1.25 U, primers AB28 0.4 mM, TW81 0.4 mM and 5.0 µl of DNA total. Thermal cycling consisted of an initial denaturation at 95 °C for 3 min, followed by 35 thermal cycles at 95 °C for 45 sec, 53 °C for 30 sec, 72 °C 1:30 min and a final extension at 72°C for 5 min. Amplification products were verified by electrophoresis on 1.4% agarose gel in 1X TBE buffer. Visualization of the amplified product was carried out in the photo documentor (MicrOBIS). Positive controls were provided by the National Phytosanitary Reference Centre, part of the National Health Service, Food Safety and Quality.

Isolation of bacteria associated with the creole potato rhizosphere

Roots of 20 vigorous potato plants without apparent damage where collected fromthe ElConejo locality, in Perote Veracruz. In order toremove excess soil from the roots, two rinses were performed with running water for 5 min.TheRoots were disinfected with sodium hypochlorite at 4% for 3 min and rinsed twice with sterile water.

For each sample, 5 g of rootswere macerated in 500 µl of sterile distilled water in a sterile mortar. From this suspension, four decimal dilutions were used for obtaining microbial cultures. For thisprocess,we used the technique of emptying on a plate, using 400 µl of the suspension. Culture media King B (KB), Nutrient agar (NA) and Bacillus medium (MB) were used andthe cultures were incubated for 24 hours at 28ºC. Subsequently, subcultures were made in order to obtain pure bacterial cultures based on their colonial morphology.

Evaluation "*in vitro*" of the bacterial activity of*Globoderarostochiensis* in two phases: juveniles and females

The bacterial activity tests oncysts and J2 of *G. rostochiensis* were performed in 96-well immune plates. First, from each pure bacterial culture, aqueous cell suspensions were prepared at a concentration of 1.2 x 108 UFC.

Table 1. Average morphometric data of 60 females of *G. rostochiensis* drawn from the three locations studied.

Data	Granekradius	Crosslink between the fenestra and anus (µm)	Distance between the anus and fenestra(µm)	Fenestradiameter(µm)
Averages	4.07	18.22	74.46	18.26
Minimum	3.50	16	58	17
Maximum	4.50	32	70	19

Table 2. Average morphometric data of 60 juveniles (J2) of *G. rostochiensis* drawn from the three locations studied.

Data	Bodylength (µm)	Styletlength (µm)	Sizetail (µm)	Hyalinetail (µm)
Averages	446.02	23.67	44.29	26.8
Minimum	445	22	41	25
Maximum	494	23	51	28

The measurements registered are result of average measurements of juveniles drawn from the three localities.

For testing with the cysts, five wells per plate were used to assess bacterial isolation; one cyst per well. For the test with J2 specimens, five wells were used with five J2 individuals per well. 100 µl of bacterial suspension was employed. Cysts and J2 stadiums were tested on separate plates. Control treatments consisted of Wells where the nematode (cysts or J2) were put into 100 µl of sterile water and single wells containing only 100 µl of sterile distilled water. The plate was incubated for 3 days at 28 ± 2 °C. Observations were made on the compound and stereoscopy microscopes to analyze damage evidence to organisms. The bacterial isolates that caused nematode damage in each test were evaluated twice more to confirm the results.

Molecular analysis for the identification ofthe bacteria

Only those bacterial isolates that produced damage in individuals J2 *G.rostochiensis* were identified by analysis ofthe 16S rRNA subunit gene. Total DNA extraction from bacteria was performed using the DNeasy Plant Mini Kit protocol (Qiagen) and amplification was carried out according to Tambong *et al.* (2009) using the forward primers5'GATCCTGGCTCAGGATGAAC 3 'and reverse primers 3 5ˈGGACTACCAGGGTATCTAATC3'. The reaction mixture was prepared to a final volume of 25 uL containing 40 ng of DNA, 1X Taq buffer, 2 mM MgCl2, 0.25 mM dNTPs, 15 pM primers (each) and 1.5 units of Taq DNA Polymerase (Promega). The amplification reaction was carried out at a temperature of initial denaturation at 94 for 1 min and 34 cycles of denaturation at 94 ° C for 1min, 55 ° C annealing for 2 min and elongation at 72 for 3 min, with a final elongation at 72 for 7 min in a thermocycler Master cycler (Eppendorf).

The amplification products were analyzed on agarose gel 1.8 % in 1X TBE buffer and visualized on an UV light trans illuminator. They were then purified using the Wizard SV Gel and PCR Clean -Up System (Promega) system following the manufacturer's instructions. They were sent to the sequencing service of the Institute of Biotechnology of the UNAM. The sequences were compared to the Gen Bank database of NCBI (National Center for Biotechnology Information), lined and analyzed with Bio. Edit 7.0.0 (Isis Pharmaceuticals, Inc.) and MEGA 4.1 programs.

RESULTS

Morphological identification of *G. rostochiensis*

In total, 60 female fenestral cuts and juveniles were analyzed, 20 females and 20 juveniles per sampling location. The average results of these measurements are shown in Tables 1 and 2.

The morphometric analysis of the data indicated that individuals found at the study sites correspond to the species *G. rostochiensis* according to the descriptors indicated by Mulvey (1973), Stone (1975) , Mulvey and Golden (1983) and Sosa- Moss (1997), these being: Graneck radius of 3.6 ± 0.8 µm; fenestra diameter of 19 µm ± 2; 16-31 grooves between the fenestra and anus (average of 22); and finally, the distance between the anus and the fenestra of 66.5 ± 10.3 µm, for juveniles (J2), body

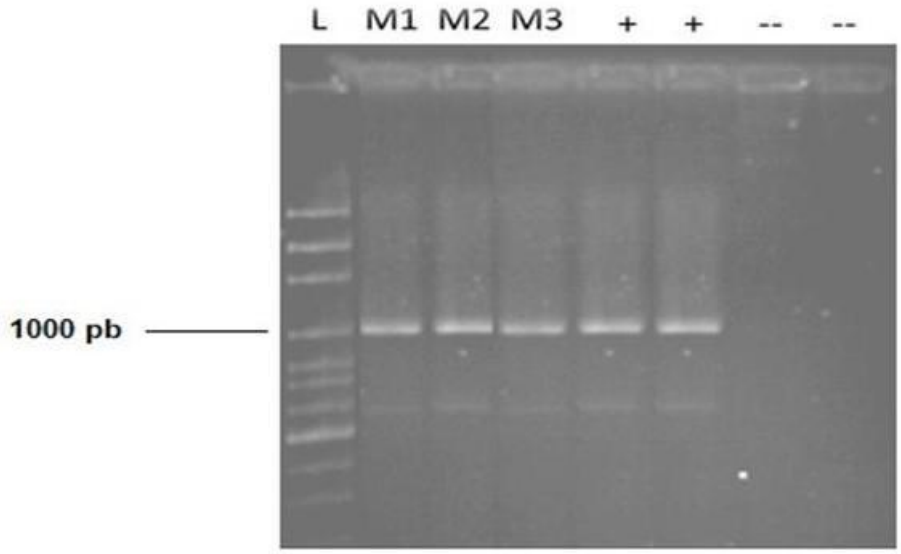

Figure 1. Amplified DNA products of three *G. rostochiensis* cysts . Lane 1 L = Ladder (molecular weight marker) ; lanes 2 through 4, amplification products of *G. rostochiensis* cysts ; lanes 5 and 6 , positive controls ; lanes 7 and 8 , negative controls.

Table 3. Juveniles J2 of *G. rostochiensis* showing damage to the activity of bacterial isolates.

Key	***Bacterialspecies***	***Distortion***	***Desintegration***
B17	*Serratiamarcescens*	*25*	*-*
B8	*Serratiaplymuthica*	*-*	*25*
A3	*Providencia alcalifaciens*	*25*	*-*
A20	*Pseudomonassyringae*	*25*	*-*
E4	*Serratialiquefaciens*	*-*	*25*
E3	*Serratialiquefaciens*	*25*	*-*
D4	*Paracocusmarcusii*	*-*	*25*
Z4	*Serratiaficaria*	*25*	*-*
F3	*Serratialiquefaciens*	*-*	*25*
F12	*Serratiamarcescens*	*-*	*25*

length 468 µm± 100 µm, stylet length 22 µm ± 0.7, tailsize 44 µm± 12 µm and tailhyaline 26.5 µm ± 2 µm.

Molecular identification of *G. rostochiensis*

The amplification reaction ofthe ITS1-5.8S - ITS2 region ofthe rDNA gene was successful for the three cysts of *G. rostochiensis* analyzed. The amplification generated a fragment of approximately 980 bp (Figure 1), consistent with that reported for *G. rostochiensis*, according to the Diagnostic Protocol on Quarantine Important nematodes for potato in Mexico (SAGARPA, 2014). **Figure 1**

Evaluation "*in vitro*" of the bacterial activity in juveniles and females of *G. rostochiensis*.

Of the 102 bacterial isolates analyzed, only 10 individuals generated damage on J2 of *G. rostochiensis*. Damage consisted of distortion or disintegration of the organism (Table 3). No bacterial strain caused damage to female cysts. **Table 3**

The molecular identification of ten bacterial isolates indicated the following: A3 strain with a sequence of 1061 nucleotides presented a 99% similarity with *Providencia alcalifaciens*, coinciding with the test Api 20E; A20 strain with 1050nucleotides presented a 98% similarity with the

species *Pseudomonas syringae* pathovars *syringae* and *phaseolicola*; B8 strain with 1040 nucleotides was 99% similar to the species *Serratiaplymuthica*; strain B17 with 1072 nucleotides was 99% similar to the genus *Serratia* and API 20E tested positive for the species *S. marcescens*; E3, E4 and F3 strains with1007, 1046 and 854 nucleotides, respectively, showed99% similarity to the species *Serratialiquefaciens,* which was confirmed with the API 20E test; D4 and F12 strains with1040 and 980 nucleotides were 97% and 99% similar to *Paracococus Marcusi* and *Serratiamarcescens*, respectively; and finally, the Z4 strain with 1041 nucleotides showed a 99% similarity to the genus *Serratia* sp, and withthe API 20E tests confirmed as *S. ficaria*.

DISCUSSION

The development of populations and activities of plant parasitic nematodes in the soil are limited by antagonistic organisms. Some bacterial species and other prokaryotes areobliged parasites of plants that can kill or inhibit nematodes through the production of toxins, metabolites or enzymes that destroy the eggs or other life stages of these organisms (Rodríguez- Kábana, 1991).

Many of the species *Serratia* are chitinolytic, providing them with the ability to destroy parts of the outer membranes of the tylenchid eggs (Sayre and Starr, 1998), such as those from *G. rostochiensis* .In the case of the species *S. marcescens* and *S. liquefaciens* isolated in this study, these bacteria have been reported to degrade protein and carbohydrates, compounds present in the nematode wall (Bosa and Cotes, 2004 ; Lee, 2010), effects seen "*in vitro*" of juveniles (J2) of *G. rostochiensis* in this article (Table 3). Added to this,Zeinat *et al*. (2010), found that *S. marcescens* reduced 91% of *Meloidogyne incognita*juvenile population on bean plants in greenhouses, indicating that could prove tobe a good control agent.

Similarly, Almaghrabi *et al.*(2013) found that *S. marcescens* showed greater efficiency as PGPR in greenhouse tomato plants, 45 days after inoculation with nematodes of the genus *Meloidogyne* , analyzing variables, dry weight, plant height, plant yield and fewer J2

/ 10 g soil , compared to treatments *Pseudomonas putida , P fluorescens , Bacillus amyloliquefaciens , B subtilis, B cereus* andthenematode infested control.However, in our study,one strain of the ten showing bioactivity with *G. Rostochiensis* was seen to be *Pseudomonas syringae*.

Additionally, *Serratia* species are commonly found as endophytes in plant roots. The endophytic bacteria play a wide variety of ecological roles, such as promoting plant growth and acting as biological control agents on a variety of pathogens. These microorganisms are an inexhaustible source of more than 20,000 biologically active compounds which have a direct bearing on the performance and survival of host plants (Perez and Chamorro, 2013). Therefore, *Serratiaplymuthica* endophytic bacteria isolated from the rhizosphere of potato in the highland region of Perote, is used as an alternative control for disease management in various crops, such as strawberries and rapeseed, probably due to their ability to degrade chitin, a compound found in nematode eggs. In accordance with our results, a strain of this species proved capable of disintegrating the 25 juvenilesJ2 tested (Grimont*et al.,* 1981;Kurse and Berg, 2001; Muller and Berg, 2008).

In recent years, several species of plant pathogenic bacteria have been isolated and used as pesticides and used successfully inthe biological control of pests in different crops in various parts of the world (Demir*et al.,* 2012). This study found that *Pseudomonas. syringae* caused the disintegration of the J2 individuals of *G. rostochiensis*, suggesting promise as a biological control agent.However, it should be noted that this agricultural area is used for growing peas and beans, and other crops that are susceptible to phytopathogenic bacteria. Among the main reservoirs of the bacteria is *Vicia sativa* (Rodríguez, 2010) used as a cover crop after growing potatoes by producers of the upper region of Perote.

Species such as *Providencia alcalifaciens, Paracocusmarcusii, Serratiaficaria*are not reported as control agents against plant pathogenic nematodes. This paper is the first to report on their nematicide activity.

CONCLUSIONS

Ten bacterial strains showing antagonistic activity affecting the golden nematode of potatoes (*G. rostochiensis*) from potato roots of the region of Perote, Veracruz were obtained. The bacteria were able to cause significant body distortion and degradation in the J2 stage of the nematode. The bacteria identified corresponded to *Providence alcalifaciens*, *Pseudomonas syringae*, *Paracoccusmarcusii*, *Serratiamarcescens, Serratialiquefaciens, Serratiaplymuthica* and *S.ficaria*. Despite the promise which these bacteria speciess how, their efficacy and innocuity must be verified infurtherfield studies.

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