Animal Health

Original

Zoo-Nematode Egg Associated Bacterium as a Potential Agent for *Meloidogyne* spp. Biocontrol in Tomato

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ABSTRACT

Background: The aim of this study was to characterize bacterium CIGBTb, isolated from eggs of *Tricostrongylus* for its nematode bio-controlling activity, and its possible action mechanisms, through *in vitro* and pot trials, as well as the detection of its pathogenic attributes.
Methods: The strain was characterized by molecular and conventional methods. Its effectiveness against nematodes and the effect on plant growth, were evaluated in pots containing *Solanum lycopersicum* plants *var* UC-8213. At 40 days, the infestation index, number of knots, length and mass of stems and roots, and the number of eggs per mass, were determined. The *in vitro* effect of *Haemonchus* spp. and *Meloidogyne* spp. on hatching was determined. ANOVA and the Duncan test were used for data comparison.
Results: *Sphingobacterium* sp. CIGBTb inhibited egg hatching of *Haemonchus* spp. (100%) and *Meloidogyne* spp (87%). The number of root knots and eggs per mass were significantly reduced in *Solanum lycopersicum* (58% and 53%, respectively). The growth of *Solanum lycopersicum* increased 0.59-fold in relation to the control. No other reports were found on more strains of this gender with nematicidal activity and a capacity for stimulating plant growth, simultaneously.
Conclusions: CIGBTb might allow for biological control of nematodes by not only reducing egg hatching, but also the number of eggs per mass.
Key words: biological control, *Solanum lycopersicum*, Nematoda, *Sphingobacterium, Haemonchus* (Source: *MeSH)*

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INTRODUCTION

Meloidogyne spp. is the most harmful of all plant nematode genera, which attacks tomato in tropical and subtropical regions, causing production loses of up to 30%. (Netscher and Sikora, 1990). In Cuba, this root knot nematode (RKN) is also responsible for one of the main plant health problems observed in protected crops (Gómez, Rodríguez, Enrique, Miranda, and González, 2009). Chemicals can control this pest quickly and with higher effectiveness (Whitehead, 1997). However, the damage caused to the ecosystem, and the residual effects have increasingly encouraged researchers to work on safer alternatives.

In that sense, novel and more selective methods are being promoted to control *Meloidogyne.* sp. The search of natural antimicrobial antagonists may offer new alternatives to biological control of nematodes. In Cuba, several microbial species, like *Pochonia chlamydosporia* (Manzanilla-López and Lopez-Llorca, 2017) and *Tsukamurella paurometabola* (Mena *et al*., 2003), which are used on several crops, have demonstrated high efficacy to control this kind of plant pathogen under certain management conditions and soil types. Other isolates (*Stenotrophomonas* sp. CIGB G1, and *Sphingobacterium* sp. (CIGBTb) have demonstrated some *in vitro* activity against nematodes in assays (Sánchez *et al*., 2003), and they have shown a potential as biocontrols (Sánchez *et al.*, 2018).

Genus *Sphingobacterium* is particularly appealing due to its versatility under different environmental conditions. It has been isolated from Antarctic soil, maize roots, cattle feces, forest soils, cropland, activated mud, nematodes, and tissue from leaves of *Nicotiana tabacum* (Yabuuchi, Kaneko, Yano, Moss, and Miyoshi, 1983; Shivaji *et al*., 1992; Holmes, Owen, and Hollis, 1982; Kim, Ten, Liu, Im, and Lee, 2006; Mehnaz, Weselowski, and Lazarovits, 2007; Zhang *et al*., 2012; Liu *et al*., 2012). Although it has been isolated from the soil with repressive effects on nematodes, few reports have been published on its application to control the pathogen in tomato.

Accordingly, the aim of this study was to characterize bacterium CIGBTb, isolated from eggs of *Tricostrongylus* for its nematode bio-controlling activity, and its possible action mechanisms, by means of pot and *in vitro* trials and the detection of its pathogenic attributes.

MATERIALS AND METHODS

Bacteria

CIGBTb was isolated from eggs of *Tricostrongylus* spp. with an altered morphology, which were disinfected with 0.5% hibitane, and were placed in Triptona Soy Agar (TSA). The isolate was cryopreserved at -70 ºC in the collection of the Center of Genetic Engineering and Biotechnology of Camaguey, Cuba, and it was identified using the API 20NE system, along with analysis of the

16S rRNA gene sequence (access number GenBank: MG461604), achieved by PCR through universal primers 27 F and 1492R (Sánchez *et al.*, 2018). The phylogenetic analysis was done using the MEGA suite, version 6.06, following multiple data alignment, with CLUSTAL - X (Thompson *et al.*, 1997). The evolutionary distances of strain CIGBTb were calculated according to the model of two parameters, of Kimura (Kimura, 1980), grouping was based on the maximum probability method (Felsenstein, 1981). The bootstrap technique (1 000 repetitions) was used to evaluate the typology of the tree (Felsenstein, 1985). The experimental strain was grown in Triptona Soy Broth (TSB) (Oxoid; 30 g l−1), at 30 ºC, for 24 h, at 250 rpm in an orbital shaker.

The positive control used was nematicide Hebernem®, from bacterium C924 (deposited on August 8, 1995, according to the Budapest Initiative, at the Centraalbureau voor Schimmelcultures, Baarn,, The Netherlands, deposit number, CBS 613.95.

Eggs from *Haemonchus* sp. were collected from adult females in the abomasum of a sheep.

The specimens were washed with sodium chloride solution (0.9%), and were disinfected with 0.5% Hibitane, for 1 minute. Then they were incubated in nutrient broth (Oxoid), at 37 °C, for 24 hours. The samples were sieved (300, 60, and 30 µm diameter, successively), and the eggs were retained on the 30 µm sieve. Then the eggs were disinfected with 0.5% Hibitane, for 5 minutes, and washed twice in LB medium (8) immediately after, in a 1/10 dilution with distilled sterile water (DLB). Handling was made under aseptic conditions. An optical microscope (Olympus) was used for egg and larval counts.

Meloidogyne spp. was collected from tomato plants in protected crops in the province of Ciego de Avila, Cuba. The population of *Meloidogyne* spp. was propagated in plants of *Solanum lycopersicum,* variety UC-8 213, at the CIGB of Camagüey, Cuba. The egg masses were collected from the roots of *S. lycopersicum.* The eggs were separated from their masses using sodium hypochlorite (0.5%), and were dipped in distilled water at 80 ºC, until the trial was conducted. Then the eggs were counted through an inverted Olympus CK 2 binocular microscope (x40). Counts of egg per mass were done in the same way, but in every treatment, a mass of eggs was placed in three wells of a polystyrene dish. Later, the eggs were separated, and finally, they were counted.

Pot trials

Nylon bags (8 cm diameter x 15 cm deep) were filled with 1 000 cm^3 of substrate (1:1 sterile sand: sterile enriched substrate (Terraplant). The substrate was infested with 1 500 eggs of *Meloidogyne* spp. placed in the bags, at 3 cm deep.

After 5 days, 50 mL of the *Sphingobacterium* CIGBTb culture were added (10^6 cfu/mL). The positive control used was nematicidal Hebernem®, whereas the TSB medium was utilized as negative control. A completely randomized experimental design with ten replications for each

treatment, was applied. The plants of *Solanum lycopersicum* UC-8213 were transplanted into the pots seven days after. At day 40, the galling index, number of knots per root gram, plant height, root and plant weight, and the number of eggs per mass, were determined (Bridge and Page, 1980).

Hatching inhibition bioassay

Approximately 90-100 eggs in 900 µL of water and 0.1% peptone (*Meloidogyne* spp.) in diluted LB medium in 1/10 water (*Haemonchus*) were placed in the wells of a 24-well Petri dish. Then, 100 µL of bacteria in water/TSB medium were added, and three concentrations of *Sphingobacterium* sp were tested: CIGBTb (10^8, 10^7, 10^6 cfu/mL). The positive control used was Hebernem® in the same concentration. The negative control received 100 µL of a water/TBS solution. Then the dish was coated and incubated at 28 ºC. The nematicidal effect was determined through a binocular microscope, by count of the J2s that failed to hatch at 72 h of treatment. Every treatment had three replications. The inhibition per cent was calculated according to: PIH = $[(C - T)/C] \times 100$, where C was the control hatching per cent, and T was the treatment hatching per cent.

Determination of extracellular enzymes

Growth in M9 medium supplemented with colloidal chitin (Shimahara and Takiguchi, 1988) indicated the production of chitinase. Chitosanases were determined by the growth of the organism in the dishes, with Chitosanase Detection Agar (CDA) (Cheng and Li, 2000). The proteases were determined through cultures in Nutrient Agar dishes and 0.5% gelatin, followed by detection with Frazier's reagent, and casein hydrolysis in Nutrient Agar Medium with 1% skim milk (VanDemark & Lee, 1991). The API ZYM kit was used for the detection of phosphates, esterases, lipases, glucoronosidases, galactosidases, mannosidase, arylases, β-N-acetyl-glucosaminidase, and naphthol-AS-BI- phosphorylase.

The microorganisms were inoculated in tubes containing 8 mL of nutrient broth, and were grown at 30 ºC, for 12 h. A embedded lead-acetate strip was placed around the mouth of the tube. The reaction was positive when the strip turned darker.

Data analysis

Data normality was corroborated through the Kolmogorov-Smirnov test. Analysis of variance was performed (Rodríguez *et al.*, 2005) to all the data, in order to determine the significant differences between the treatments. The means were compared according to Duncan ($P<0.05$). Statgraphics Plus 5.0, for Windows, was used.

RESULTS

CIGBTb showed yellow, convex, and circular colonies after a few days of incubation. The cells were Gram-stain-negative, short rods, non-motile and non-spore-forming. The API 20 NE kit identified strain CIGBTb as *Sphingobacterium spiritivorum*. The phylogenetic analysis of the 16S rRNA sequences confirmed that it belongs to genus *Sphingobacterium*. The most probable evolutionary tree showed that CIBGTb formed a coherent group with members of genus *Sphingobacterium,* and an intra genus branch with *Sphingobacterium spriritivorum* NCTC 11386 (Fig. 1). However, the strain showed a genic sequence value below 97% (95.58%); therefore, it does not belong to that species.

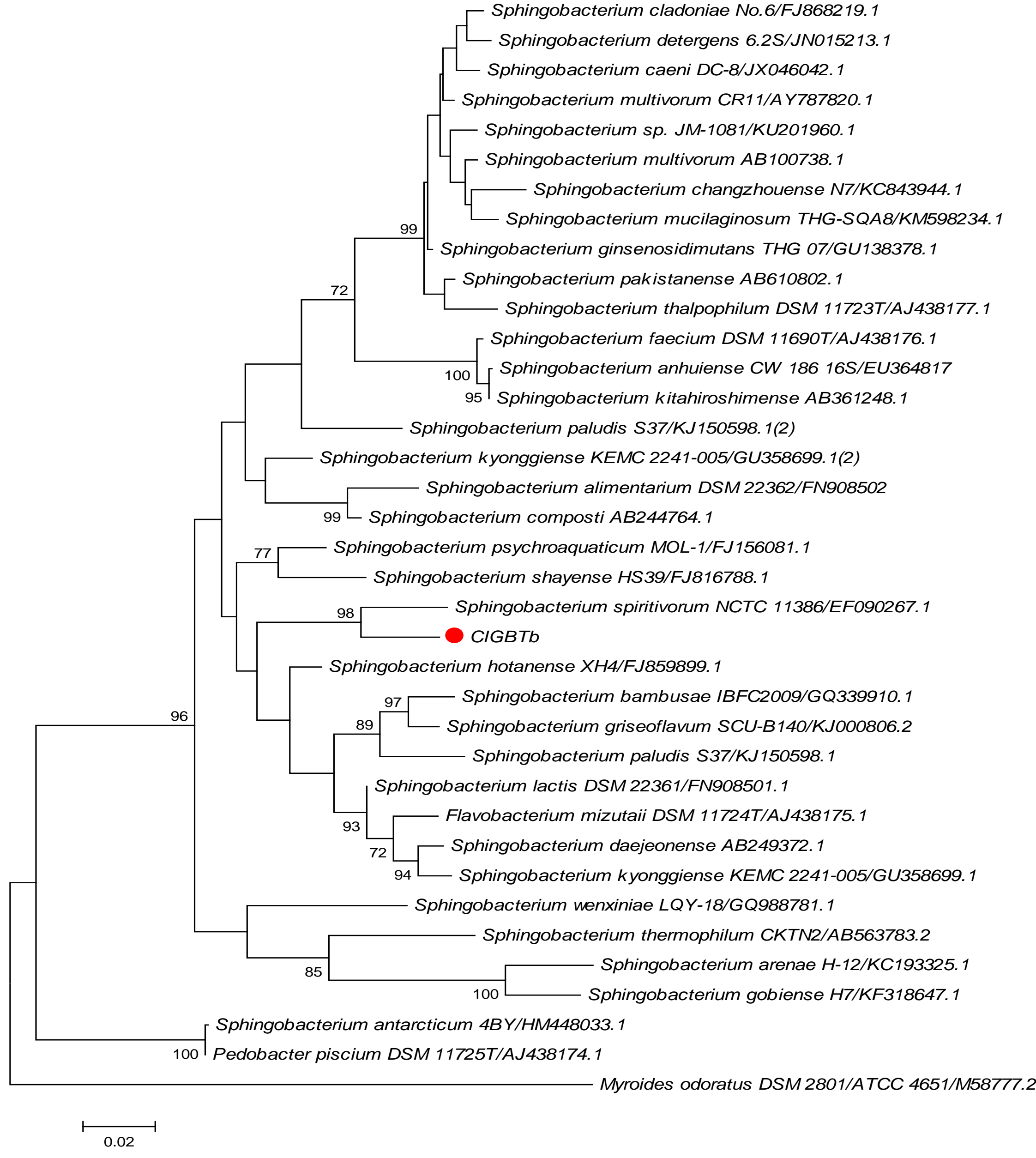
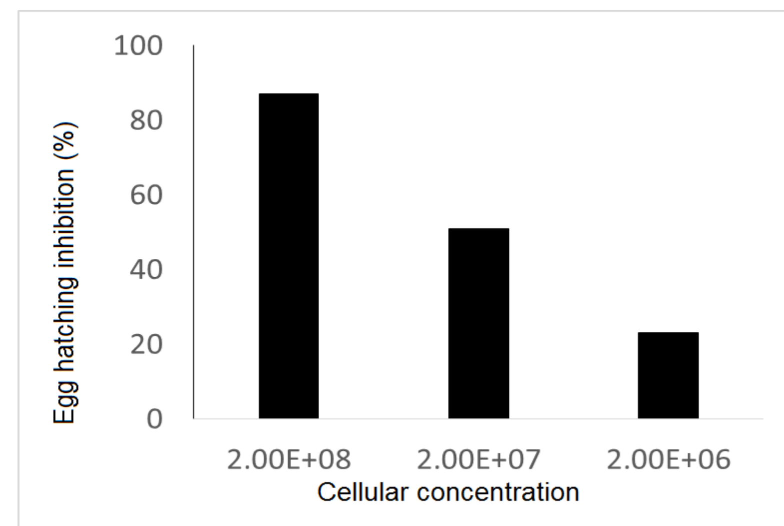


Fig. 1. Phylogenetic tree according to the maximum probability method based on the 16S rRNA sequence The tree shows the relation between strain CIGBTb and some representative members of family *Sphingobacteriaceae*. The bootstrap values (expressed in per cents of 1 000 repetitions), over 70% appeared on the plant´s knots. The bar represents two substitutions per day in every 100 nucleotides.

The *in vitro* treatment using *Sphingobacterium* sp. CIGBTb at concentrations of 10^8 cfu/mL led to 87% inhibition of *Meloidogyne* sp. egg hatching, and 100% inhibition of *Haemonchus* sp. egg hatching (Fig. 2). A large vacuolization area and little motility was observed in the larvae that managed to emerge.

A



B

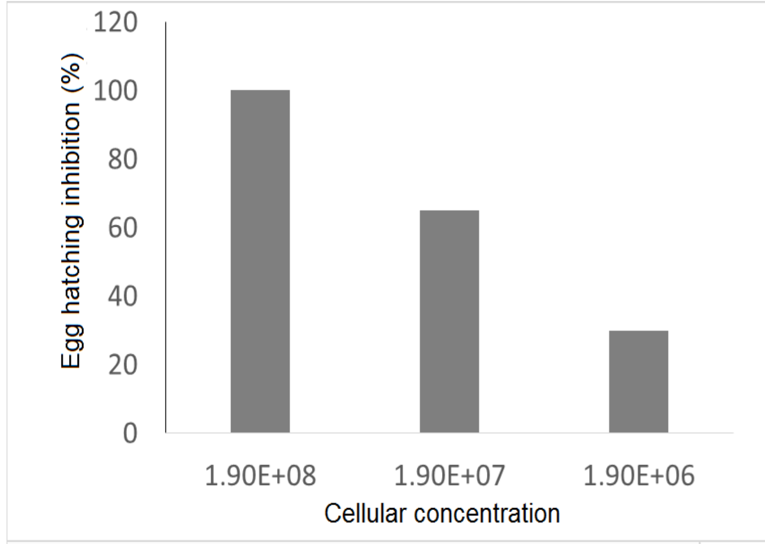


Fig 2. Inhibition of *Meloidogyne* spp. egg hatching (A), and *Haemonchus* spp. (B), at different concentrations of *Sphingobacterium* CIGBTb

In the pots, *Sphingobacterium* sp. CIGBTb significantly reduced (P<0.05) the galling index, from 5.3 to 2.9. No significant differences were observed in the positive control. Moreover, *Sphingobacterium* sp. CIGBTb significantly reduced galling formation (58%) in the roots of *Solanum lycopersicum* (Table 1).

Table 1. Effect of *Sphingobacterium* sp. CIGBTb on the roots of *S. lycopersicum* UC-8213 infested with *Meloidogyne* spp.

Treatment	Knot severity	
	Galling index	**Knots/root gram**
CIGBTb	2.9 ± 1.5 b	27.8 ± 4.8 b
Control	5.3± 1.2 a	47.8 ± 2.8 b

Unequal letters represent significant differences in the Duncan test (P < 0. 05).

An evaluation of the number of eggs per mass isolated from the tiny knots formed, showed that *Sphingobacterium* sp. CIGBTb reduced the number of eggs in all the masses (53%), whereas no changes were observed in the controls (Fig. 3).

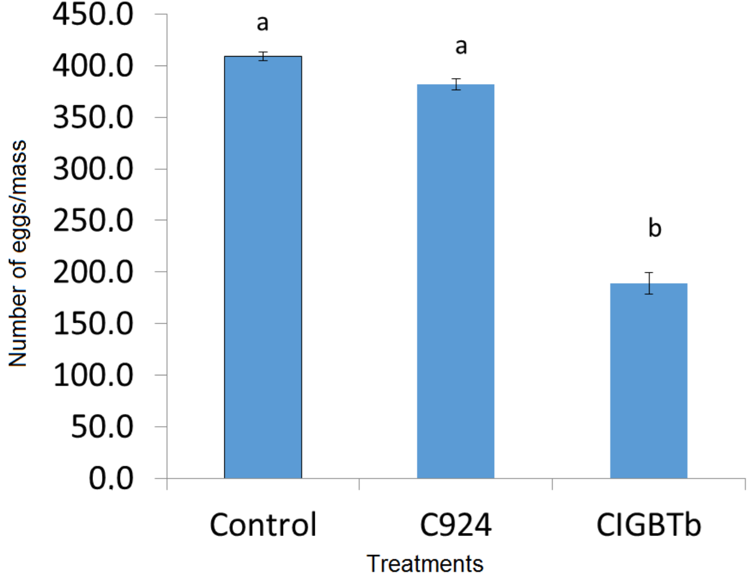


Fig 3. Number of eggs per mass in the plants treated with Triptona Soy Broth (control) *Sphingobacterium* sp. CIGBTb (CIGBTb) and Hebernem® (C924)
Unequal letters represent significant differences in the Duncan test (P < 0. 05).

A concentration of approximately 10^5 cfu/mL of *Sphingobacterium* sp. CIGBTb also stimulated growth of *Solanum lycopersicum.* Besides, the weight of the plant increased 0.59-fold above the weight of the control plants without the bacterium (Fig. 4).

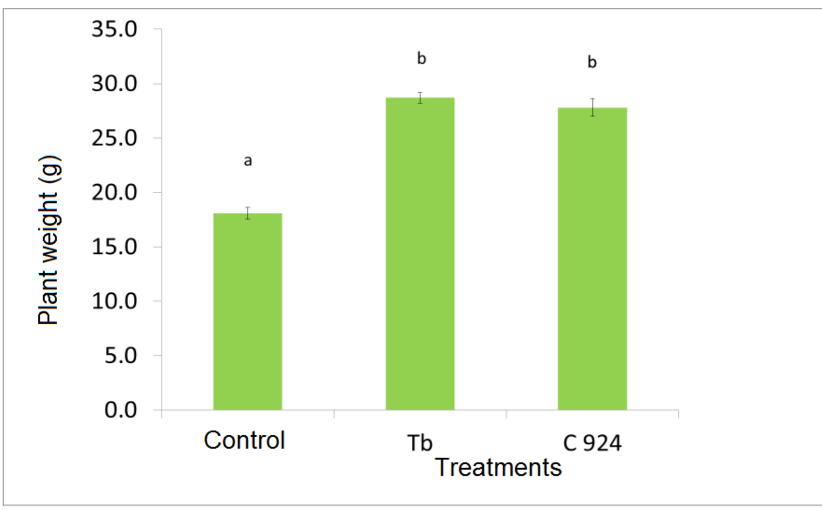


Fig 4. Weight of *S. lycopersicum* UC-8213 plants infested with *Meloidogyne* spp., 40 days after the treatment with Triptona Soy Broth (control), *Sphingobacterium* sp. CIGBTb (CIGBTb) and Hebernem® (C924) Unequal letters represent significant differences in the Duncan test (P < 0. 05).

CIGBTb showed some pathogenic attributes, such as trypsin, esterases, and lipase-esterase enzymes. Hydrogen sulfur and extracellular proteases (gelatin and casein as substrates) were not detected through the conventional test, and the strain was able to grow slowly in minimum culture medium with chitin and chitosan, though no halo of hydrolysis was observed. Moreover, the results provided by the API ZYM kit were positive for acid phosphatase and alkaline phosphatase, two important enzymes for bacterium-mediated solubilization of phosphorous in the soil.

DISCUSSION

Previously, genus *Sphingobacterium* had been reported as a component of grapevine rhizosphere, with nematode-suppressing properties (Vargas-Ayala, Rodríguez-Kábana, Morgan-Jones, McInroy, & Kloepper, 2000; Aballay, Mårtensson, & Persson, 2011). These authors demonstrated the *in vitro* effectiveness of strain *Sphingobacterium spiritivorum* 64, and another strain of the same genus (*Sphingobacterium nematocida*), which had been isolated in China during a study of the diversity of endophytic nematicidal organisms (Liu *et al*., 2012). However, there is little information on the pot trials, and the possible action mechanisms of these bacterium genera. Mena *et al*. (1996) patented strain C926 of *Sphingobacterium spiritivorum*, and demonstrated its effectiveness to control *Radopholus similis and Meloidogyne incognita* in field and pot trials. Nonetheless, *Sphingobacterium* CIGBTb not only has a nematicidal activity, but also stimulates the growth of tomato plants, possibly due to the solubilization of soil sulfates, through the production of acid and alkaline phosphatases, though other mechanisms have not

been studied. Previously, the plant growth stimulating activity of *Sphingobacterium canadense* (Mehnaz *et al*., 2007), and *Sphingobacterium pakistanense*, were known (Ahmed *et al*., 2014).

There are various mechanisms used by bacteria during antagonistic interactions with nematodes. One of them is the role played by hydrolytic enzymes, which is well documented in a series of organisms with hyperparasitic activity (Chernin & Chet, 2002). In the case of *Sphingobacterium* CIGBTb, the strain had chitinase, chitosanase, trypsin, esterase, and esterase lipase activities. These pathogenic attributes might bring about enzymatic changes in the layers of chitin, proteins, and other lipids that make up the egg covering, and other structures of *Meloidogyne* sp., and therefore, facilitate the occurrence of parasitism.

Several strains of *Sphingobacterium* produce chitosanases which are similar to *Mitsuaria* ChoA (Yun, Amakata, Matsuo, Matsuda, & Kawamukai, 2005), and this particular strain may be part of them. Moreover, CIGBTb releases esterases C4 and C8, like *Sphingobacterium nematocida* (Liu *et al*., 2012), which might explain the *in vitro* inhibition of *Meloidogyne* sp. eggs. Nevertheless, the larvae that managed to emerge and parasite the tomato plants in the pot trials, not only produced a low number of knots, but also smaller knots with fewer eggs in the masses than the control. The silencing of a parasitizing gene, 16D10, expressed in the cells of subventral glands of *M. incognita*, also caused a similar effect in *Arabidopsis*. A substantial reduction in the number (63 and 90%) and size of knots is produced (Huang, Allen, Davis, Baum,and Hussey, 2006).

CONCLUSIONS

Zoonematodes are an appropriate source to search for plant nematode biocontrollers. *Sphingobacterium* sp. CIGBTb, isolated from *Tricostrongylus* sp. eggs, is a novel alternative for biological control. The strain diminishes the number of eggs per mass, contrary to the active ingredient of C924 in nematicidal product Hebernem®, which releases chitinases and hydrogen sulfur. The results of this study corroborate the effectiveness of the native isolate as an antagonist, and its potential as a plant biofertilizer and biological control of *Meloidogyne* sp., a root-knot nematode, in *Solanum lycopersicum*.

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AUTHOR CONTRIBUTION

ISO: Design and supervision of isolation and identification of strains, *in vitro*in vitro and *in vivo* experimental design, and redaction of the manuscript. RMV: Design, formulation, and supervision of the experiment, redaction and review of the manuscript. IAL: Experimental data collection and redaction of the manuscript. IWP: Experimental data collection and redaction of the manuscript. DSS: Collection of *in vitro* experimental data and redaction of the manuscript. EPV: Design and supervision of experiments and redaction of the manuscript. JMC: Statistical analysis of data and redaction of the manuscript, MRB, statistical analysis of data and redaction of the manuscript.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.