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RESEARCH ARTICLE

First isolation and characterisation of the *Steinernema carpocapsae/Xenorhabdus nematophila* complex in Hidalgo, Mexico: Morphometric, molecular, and virulence insights

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Abstract

This is the first report on the isolation and full characterisation (i.e., morphological, molecular, virulence) of the entomopathogenic nematode/symbiotic bacterium complex, *Steinernema carpocapsae* / *Xenorhabdus nematophila*, in soils of the State of Hidalgo, Mexico. The nematode was characterised based on morphometrics with the aid of both light and scanning electron microscopy, and analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA. The symbiotic bacterium was characterised based on its phenotypic characteristics (i.e., growth on nutrient agar plus bromothymol blue and triphenyltetrazolium chloride, and 17 biochemical tests) and analysis of the 16S rDNA gene sequencing. The constructed phylogenetic trees of both nematodes and bacteria were associated with bootstrap values of 99-100. The virulence of the infective juvenile (IJ) stages of the nematode was tested against larvae of *Galleria mellonella* at doses (IJ:larva): 1:1, 5:1, 10:1, 30:1, 60:1, and 100:1, resulting in mortality rates ranging from 22% (standard deviation, SD = 2) to 100% (SD = 1) in three days.

Keywords: *Biological control*; *Morphometrics*; *Nematoda: Rhabditida*; Symbiotic Bacterium; Bioassays.

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1. Introduction

Nowadays, great productivity in agriculture is made possible, in part, by the extensive use of chemical insecticides, herbicides, fertilisers, and other chemical products. It was estimated that 3.92×10^6 Ton of active ingredients (mainly herbicides, insecticides, fungicides and bactericides) were used worldwide in 2024 (FAO, 2026). However, it has been reported that the use of chemical insecticides has some widespread ecological consequences, including soil and water contamination, impacts on the food chain, and healthcare concerns for humans (Chang et al., 2025; Kreick et al., 2025; Veres et al., 2020). As a result, it is necessary to search for more environmentally benign alternatives where biological control within integrated pest management (IPM) strategies is viable for controlling a wide range of pests, including insects (Gontijo et al., 2025). Specifically, the use of infective juvenile stages (IJ) of entomopathogenic nematodes (EPN) (mainly individuals belonging to both genera *Steinernema* and *Heterorhabditis*) has been effective in controlling various pest species (Shapiro-Ilan & Lewis, 2024). Both steinernematids and heterorhabditids have established symbiotic relationships with Gram-negative bacteria (SB) from the genera *Xenorhabdus* and *Photorhabdus*, respectively. SB can occur in two forms, with one of them -called Phase I- essential for the effective killing of the insect host. The bacteria are carried in the gut of the IJs, a specialised resistant developmental stage that can survive outside the host. The IJ stage can seek out an insect host within the soil, invade it and release its SB within the haemolymph, where the bacteria can proliferate and produce a wide range of toxins and hydrolytic exoenzymes, which are responsible for the death and bioconversion of the insect larva into a nutrient broth that is ideal for

nematode growth and reproduction. The nematodes reproduce until the nutrient supply becomes limiting, at which time a new generation of IJs is induced, ready to leave the insect carcass to find new hosts (Shapiro-Ilan & Lewis, 2024). Therefore, IJs are very attractive for the biological control of some pests.

On the other hand, in recent times, there has been intensive research to isolate, identify and test the entomopathogenic capacity of indigenous EPNs with two primary purposes: a) to increase the knowledge of the biodiversity of species within different ecosystems, and b) to explore the potential uses of the newfound species and strains. Furthermore, the use of indigenous EPNs is frequently more effective in controlling local pest species compared to the results obtained when using exotic specimens. In this sense, concerning the isolation of indigenous EPNs, there are abundant reports of new isolates in different places all over the world: *Steinernema feltiae*, *Steinernema akhursti*, *Oscheius myriophilus*, *Oscheius chongmingensis*, China (Xi et al., 2027); *Steinernema yirgalemense*, South Africa (Ansen et al., 2024); *Heterorhabditis bacteriophora*, South Africa (Claasen et al., 2024); *Heterorhabditis* sp., India (Kaur et al., 2024); *Oscheius colombianus*, Philippines (Suan et al., 2025); *Heterorhabditis indica*, India (Singh et al., 2025), among others.

Once an appropriate EPN species is isolated and proposed as a candidate for the biocontrol of a specific pest species, it is possible to move to the EPN mass production, mainly through the submerged monoxenic culture technology, to provide enough quantity of IJs to be used for field applications (Cortés-Martínez & Chavarría-Hernández, 2020).

Based on the aforementioned information, the present study focuses on the isolation and characterisation of an EPN/SB complex within the State of Hidalgo, Mexico, making this report the first of its kind in Hidalgo. Specifically concerning Mexico, there are few reports about EPN isolation to date: *Heterorhabditis indica* (Avila-Lopez et al., 2021); *Steinernema ralatorei* (Grifaldo-Alcantara et al., 2017); *Heterorhabditis atacamensis*, *Heterorhabditis bacteriophora* (Chandrakasan et al., 2025); *Steinernema texanum*, *Heterorhabditis atacamensis* (Villanueva-Sánchez et al., 2022); *Steinernema* spp., *Heterorhabditis* spp. (Molina-Ochoa et al., 2003); *Heterorhabditis bacteriophora*, *Steinernema carpocapsae* (Treviño-Cueto et al., 2023), mainly. We believe Mexican researchers must intensify efforts to isolate and characterise native EPN with potential applications in biological control.

2. Methodology

2.1 Culture media

NBTA medium (Chavarría-Hernández & de la Torre, 2001). Each litter of medium contained 23 g nutrient agar, 0.25 g Bromothymol Blue, and 0.04 g triphenyltetrazolium chloride, and was pH adjusted to 8.3. STB medium (Chavarría-Hernández & de la Torre, 2001). The medium contained 30 g soy trypticase broth and 5 g yeast extract per litter and was adjusted to pH 7. LNA medium. Each litter of medium contained 120 g beef liver, 23 g nutrient agar, and 20 mL corn oil, and was pH adjusted to 7. All culture media were autoclaved after the pH adjustment.

2.2 Soil samples

Three soil samples of a total mass of 2 kg were collected from an area of 10 m^2 of non-arable land (random fashion, taken at a depth of 10 - 20 cm) near a reservoir within the town of Alcholoya in Acatlán, State of Hidalgo, Mexico (Latitude/Longitude/Altitude: 20° 13' 30.00" N, 98° 36' 54.00" W, 2080 m; Google Maps, 2025) (Figure 1) during August and September (i.e., rainy season). This territory has an average annual temperature of 14 °C and receives approximately 600 mm of rainfall (Gobierno del Estado de Hidalgo, 2002). The obtained samples were placed in plastic bags and transported to the laboratory for analysis. Once in the laboratory, the samples were homogenised and divided into two portions, each placed into a 1 L transparent plastic cylindrical container (lower diameter, 9 cm; upper diameter, 11.4 cm; height, 14 cm). The containers were stored at the laboratory temperature on a shelf.

pH measurement. 20 mL of distilled water was added to 10 g of soil and agitated every 10 min for 30 min. Then, pH suspension was checked using a potentiometer (pH 510 Series, Oakton) in triplicate.

Moisture determination. Samples of three grams of soil were dried in an oven (Shel-Lab 1380FX) for 24 h at 100 °C. Then, the dry base moisture content was determined (%H = (fresh weight - dry weight)/dry weight × 100) in triplicate.

2.3 EPN isolation

Last instar larvae of *Galleria mellonella* were used as baits to be infested by EPNs present within the soil samples (Woodring & Kaya, 1988). Six *G. mellonella* larvae were placed at the bottom of each soil sample container, which was stored at lab temperature on a shelf. Larvae were checked every three days, and cadavers that looked like they were infested by EPNs (i.e., exhibiting uniform cream or red colour

for steinernematids and heterorhabditids EPNs, respectively, as well as not offensive odours) were placed into White traps, which were checked daily until IJ emergence. IJs were collected for one week and washed with sterile 0.9% w/v NaCl to be stored in sterile bottles (50 mL suspension, 250 mL total volume) at room temperature until further experimentation.

2.3.1 EPN molecular characterisation. The isolated EPN was molecularly characterised by analysis of the internal transcribed spacer (ITS) region of the large subunit (LSU) ribosomal DNA (rDNA), using the primer sequences reported by Vrain et al. (1992) (Table 1).

Table 1
DNA sequences for primers used for amplification of nematode genes in this study (Vrain et al., 1992)

Primer set (forward-reverse)	Sequence (5′ – 3′)
18S	TTGATTACGTCCCTGCCCTTT
26S	TTTCACTCGCCGTTACTAAGG

The procedure was as follows: one loop full of a colony of the phase I-SB growing on an NBTA plate was transferred to 30 mL of STB in a 250 mL Erlenmeyer flask and incubated for 24 h at 30 °C and 200 rpm (MaxQ5000, Barnstead). Then, 1 mL of this fermentation broth was streaked onto an LNA plate and incubated for 48 h at 30 °C. Later, the LNA bacterium culture was inoculated with approximately 1,000 surface-sterilised IJs and incubated at room temperature (approximately 22 °C). After five days, first-generation adult EPNs were recovered using sterile deionised water (SDW) until an EPN pellet of 300 µL was obtained in a 1.5 mL vial. Thereafter, EPNs were resuspended in 2 volumes of worm lysis buffer (0.2 M NaCl, 0.1 M Tris-HCl pH 8, 0.05 M EDTA, 0.5% v/v SDS) and 10 µL fungal proteinase K (20 mg/mL; Invitrogen). The former mixture was incubated overnight at 55 °C. Then, 3-stage DNA extraction was carried out, with centrifugation steps between extraction stages (12,000 rpm at 8 °C, 8 min) to recover the supernatant, starting with the phenol addition, then a phenol-chloroform-isoamyl alcohol mixture (25:24:1) and finally a chloroform-isoamyl alcohol mixture (24:1), followed by DNA precipitation using 100% ethanol and 5 M NaCl. Later, the DNA was rinsed with 75% ethanol, precipitated and dried for 10 min at 50 °C in an oven (Shel-Lab 1380 FX). Finally, the DNA was resuspended in a specific volume of SDW, depending on the DNA pellet size, and then frozen at -18 °C until amplification.

EPN-DNA amplification. While keeping all tubes on a frozen conventional thermoblock, the following PCR mixture was added to each tube: 19.35 µL of SDW, 2.5 µL of 10×PCR Buffer, 1 µL of 50 mM $MgCl_2$, 0.5 µL of dNTP mixture (2.5 mM each one), 0.25 µL of 25 µM forward and reverse primers, 0.15 µL of Taq DNA polymerase (5 U/µL) and 1 µL of template DNA. Thermal cycling (Master Gradient, Eppendorf, Germany) was performed as follows: an initial step at 94 °C, 5 min; 35 cycles at 94 °C, 40 s; then, 55 °C, 30 s, followed by 1 min at 72 °C; and a final step at 72 °C for 7 min. The quality of the PCR products was assessed by agarose gel electrophoresis. The PCR products were purified using a Pure Link Purification Kit (Invitrogen). Once it was confirmed that the amplified DNA was feasible, it was sent to STAB VIDA, Lda (2015) for sequencing. Then, the obtained sequences were aligned using the Clustal W option in the MEGA12 software package (MEGA, 2025; Kumar et al., 2024).

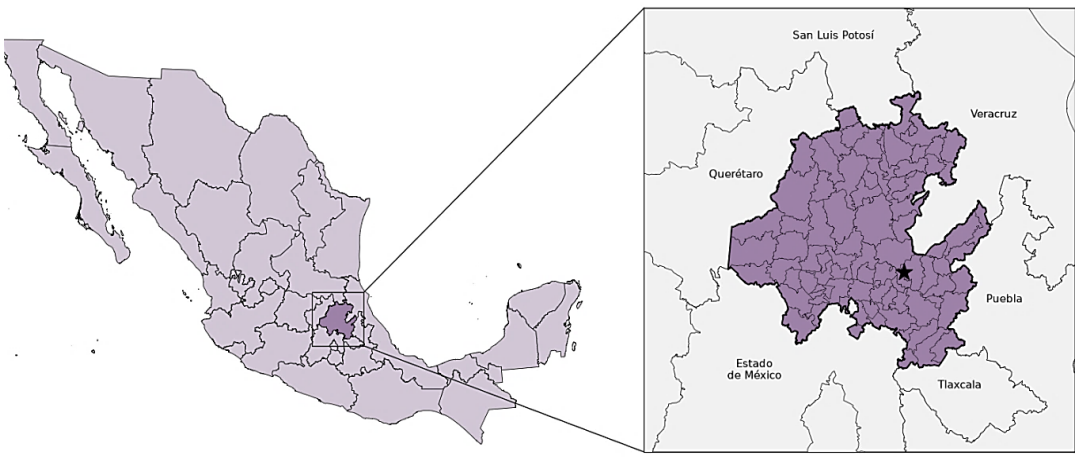


Figure 1. The town of Alcholoya (★), where soil samples were taken to isolate EPNs, is part of the State of Hidalgo (black-filled area), Mexico. Figure created using geostatistical data from INEGI (https://www.inegi.org.mx/default.html)

Thereafter, the obtained DNA sequences were compared to a library of 31 EPN species available at NCBI (2025) through phylogenetic tree construction, considering the evolutionary history based on the Neighbor-Joining method (Saitou & Nei, 1987) with a bootstrap of 500 replicates and the nucleotide: p-distance model within MEGA12. The consensus sequence was deposited in the GenBank database with the accession number PV917524.

2.3.2 EPN light microscopy. First-generation adults and IJs of isolated EPNs were prepared according to Kaya & Stock (1997) for examination under a light microscope (Nikon Eclipse 80i) and photographed with a digital camera (Nikon DS-Fi1) for further analyses.

2.3.3 EPN scanning electron microscopy (SEM). EPNs (first-generation adults and IJs) were prepared according to Kaya & Stock (1997), subjected to critical point drying (Samdri-780), and gold-coated for one minute (Desk II, Denton Vacuum) for examination under a scanning electron microscope (SEM) (Jeol 35C), with printed photographs taken, to check some morphometric characteristics.

2.4 Symbiotic bacterium (SB) isolation

Ten IJs were taken from an IJ water suspension using a needle and immersed in a drop of 3%v/v NaOCl until the larvae sheath was digested entirely (approximately 5 min). The unsheathed IJs were rinsed twice in sterile water and then transferred into a drop of STB in a Petri dish, where they were cut using a sterilised needle. Then, 8 mL of sterile STB was added, and the Petri dish was sealed with parafilm and incubated for 24 h to 48 h at 30 °C. Thereafter, samples of this culture were streaked onto NBTA plates and incubated at 30 °C for 48 h for bacterial growth. Then, we selected phase I colonies (for example, phase I of *Xenorhabdus* spp. colonies frequently exhibits a dark blue colour on NBTA plates due to their absorption of Bromothymol Blue). The selected bacterium colonies were used to obtain 24 h-old STB fermentation broths, which in turn were used to prepare 15% v/v glycerol conservation vials, which were maintained at -70 °C.

2.4.1 SB molecular characterisation. The isolated SB was molecularly characterised by analysis of the 16S rDNA gene sequence. A volume of 30 mL of STB medium was inoculated with an SB-conservation vial and incubated at 30 °C and 200 rpm for 24 h; then, 1.5 mL of this fermentation broth was centrifuged at 15 °C and 5,000 rpm for 5 min (Hermle Z 323K). The obtained cellular sediment was resuspended and rinsed twice with 1×TE buffer (Sambrook & Russell, 2001); then, DNA extraction was initiated by the addition of 840 µL of phenol-SDS-STE buffer mixture. Next, 420 µL chloroform-isoamyl alcohol (24:1) was added before precipitation using 100% ethanol and 5 M NaCl. The obtained DNA was then rinsed with 75% ethanol, precipitated, and dried (50 °C, 10 min) to be suspended in 1×TE buffer. Later, 2 µL of RNAse A (1 mg/µL; Applied Biosystems) was added to the former suspension and incubated at 37 °C for 30 min. Furthermore, a second DNA purification cycle was performed (i.e., phenol and phenol-chloroform-isoamyl alcohol (25:24:1) mixture was added, followed by 100% ethanol and 5 M NaCl precipitation; then, DNA was rinsed with 75% ethanol, precipitated and dried at 50 °C, 10 min). Finally, the DNA was resuspended in a specific volume of SDW, depending on the pellet size and frozen at -18 °C until use.

SB-DNA amplification. To amplify the 16S rDNA region, the primers, (forward) 5'-GAAGAGTTTGATCATGGCTC-3' and (reverse) 5'-AAGGAGGTGATCCAGCCGCA-3' were used (Tailliez et al., 2006). The PCR mixture (i.e., 19.5 µL of SDW, 2.5 µL of 10×PCR buffer, 1 µL of 50 mM $MgCl_2$, 0.5 µL of dNTP mixture (2.5 mM each one), 0.2 µL of 25 µM forward and reverse primers, 0.1 µL of Taq DNA polymerase (5 U/µL) and 1 µL of template DNA) was added to the extracted SB-DNA. Then, PCR was carried out, involving an initial step at 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 s, then 60 °C for 30 s, and 72 °C for 50 s, with a final step at 72 °C for 8 min. The synthesised PCR products were sequenced at STAB VIDA, Lda, and the obtained data were aligned and analysed in the same manner as the molecular analysis of EPN-DNA samples, involving comparison with 20 *Xenorhabdus* species.

2.4.2 SB phenotypic characterisation. The colony aspect of SB growing on NBTA plates was recorded after 48 h of cultivation at 30 °C. Additionally, some SB biochemical activities were assessed on API 20E, API 20NE, and API 50CH strips (bioMérieux) (Tailliez et al., 2006).

2.5 Bioassay of IJs virulence. Nematode virulence was evaluated according to the mortality caused to last instar larvae of *Galleria mellonella*, based on the methodology reported by Treviño-Cueto et al. (2023) and Cortes-Martínez et al. (2023). Firstly, the IJ stages of the nematode were produced by *in vivo* cultures in *G. mellonella*; then, the produced IJs were washed with sterile distilled water for five times, and the nematode suspension stored at 15-

20 °C till the bioassay, for which various doses, IJ:larva, were considered: 1:1, 5:1, 10:1, 30:1, 60:1, and 100:1. The 1:1 assay was conducted in 2.54 cm-diameter plates (1 larva per well; 36 larvae/assay); and the other assays were in 6 cm-diameter plates (6 larvae per well; 30 larvae/assay). Filter paper disks were placed in each well, and then the IJs were transferred into each well, involving a total volume of 100 µL of water. Later, *G. mellonella* larvae were added according to the doses, and plates were stored at 20 – 25 °C and 10% – 25% relative humidity. The controls were without IJs but with water, and the insect mortality was recorded every 24 h for three days. Samples of insect cadavers were dissected to check the possible presence of nematodes growing within the cadavers, and the insect digested tissues were streaked onto NBTA plates to check the growth of the symbiotic bacterium at 30 °C.

Mortality data were analysed using a two-way analysis of variance, followed by all pairwise comparisons using the Holm-Sidak method ($p \leq 0.05$) in SigmaPlot™ 15.

3. Results and discussion

3.1 Isolation and characterisation of the isolated entomopathogenic nematode, EPN

An indigenous species of steinernematid-EPN was isolated during this study, in soil samples with pH = 7.07 and %H = 34.28. Figure 2 shows some aspects of the first-generation adults and IJs of the isolated steinernematid. Other groups have isolated steinernematids in soils with 5.6 < pH < 7.9 in Turkey (Hazir et al., 2003), 7.4 < pH < 7.5 in Arizona, USA (Stock & Gress, 2006), and 4.9 < pH < 8.3 in La Rioja, Spain (Campos-Herrera et al., 2007), showing important variations in the physical characteristics of the studied soils. Furthermore, EPN likely exhibit a patchy distribution due to the abundance of susceptible hosts (Stuart et al., 2006).

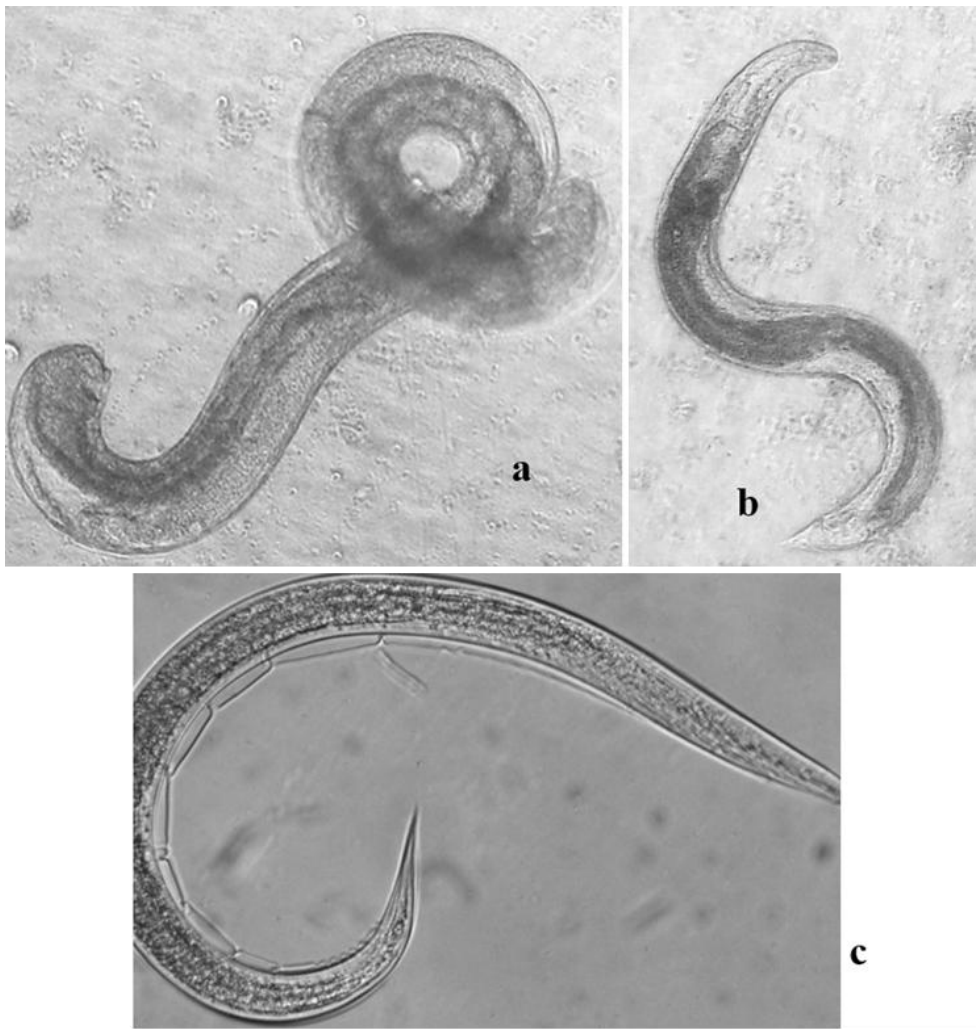


Figure 2. The aspect of some developmental stages of the isolated EPN species within the State of Hidalgo, Mexico. First-generation adults, a) female, and b) male (magnification, 40 ×). c) IJ stage (magnification, 400 ×).

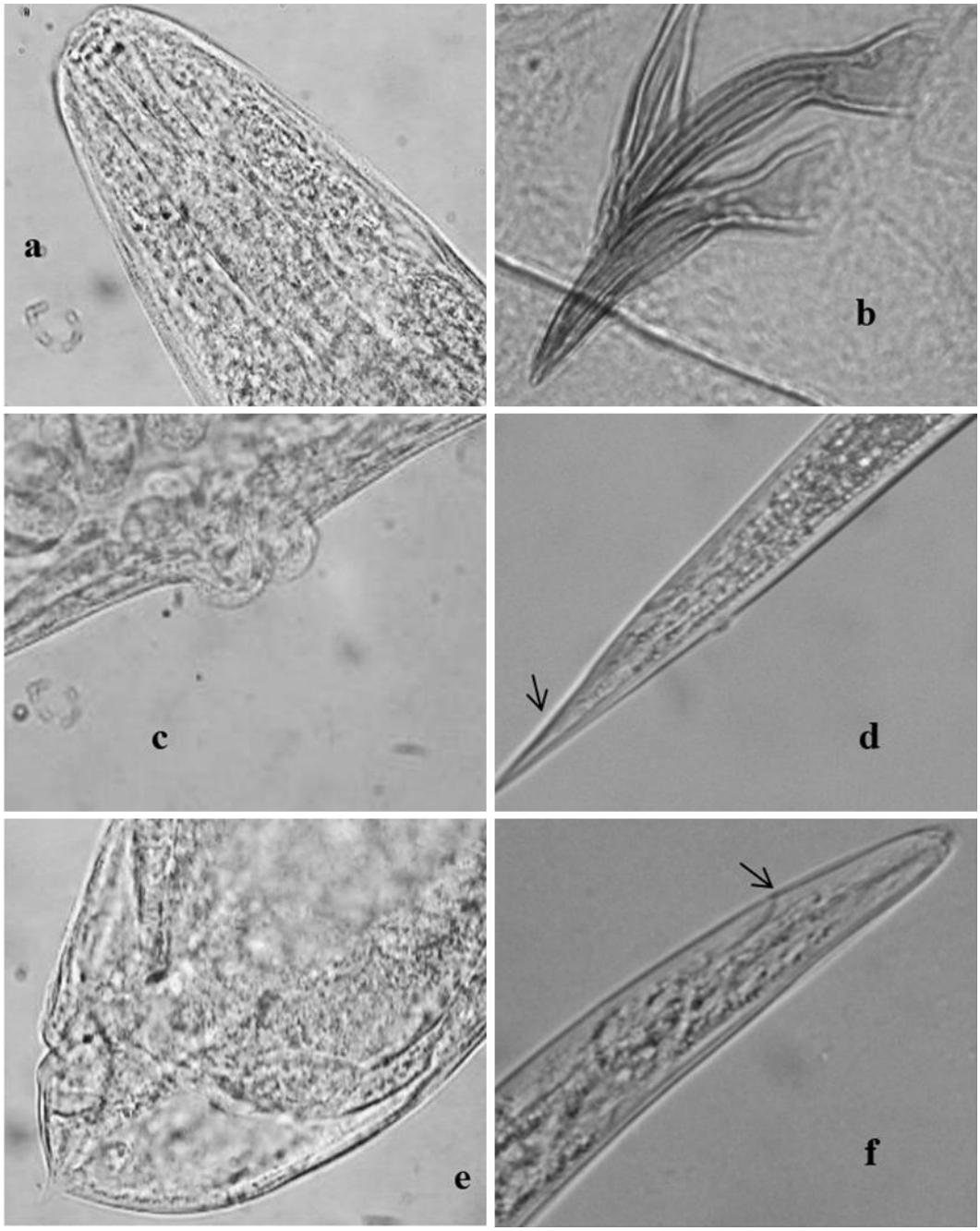


Figure 3. Some morphological details of three developmental stages of the isolated EPN species within the state of Hidalgo, Mexico. First-generation adult female: a) head, c) vulva, and e) tail (magnification, 400 ×). b) Spicules of first-generation adult male (magnification, 1000 ×). IJ stage: d) tail (arrow indicates the hyaline portion), and f) head (arrow indicates the excretory pore) (magnification, 400 ×).

This report is the first concerning the isolation and characterisation of EPN in the State of Hidalgo, Mexico. Similar reports are scarce in this country; for example, the EPN isolation reported in the Mexican States of Colima, Chihuahua, Jalisco, Michoacán, Oaxaca, Sonora, Tamaulipas, Veracruz, Yucatán and Zacatecas (Avila-Lopez et al., 2021; Georgis & Hom, 1992; Grifaldo-Alcantara et al., 2017; Molina-Ochoa et al., 2003; Nguyen et al., 2004; Ruiz Vega et al., 2003; Stock et al., 2009).

The first-generation adult female exhibits a round and short anterior region (Figure 3a) as well as a vulva with protuberant lips and no epiptygma, positioned at the middle of the female's body (Figure 3c). The first-generation adult male exhibits a pair of separated symmetric spicules and gubernaculum without bursa (Figure 3b). The IJ stage presents an excretory pore in the anterior region (Figure 3f), and a filiform tail with a hyaline portion (Figure 3d). Former characteristics are in

agreement with that corresponding to individuals of the *Steinernema* genus according to different authors (Nguyen et al., 2008; Qiu et al., 2005; Stock & Hunt, 2005).

Additionally, SEM photographs of the isolated EPN (Figure 4) suggest that the nematode belongs to the Steinernematidae family, based on its distinctive characteristics. Figure 4f shows the lateral view of the posterior region of a first-generation adult male exhibiting some genital papillae. Steinernematid adult males exhibit one single midventral and 10 - 14 pairs of genital papillae, of which 7 - 10 pairs are precloacal (Stock & Hunt, 2005).

Table 2 presents the morpho-metrics corresponding to the IJ stage based on the key for steinernematid IJs proposed by Poinar (1990). The isolated nematode resembles *Steinernema carpocapsae* (Weiser, 1955) (Nematoda: Rhabditida) based on morphometrics ratios: $L_{\text{isolated EPN (iEPN)}}/L_{\text{S. carpocapsae Wiser (ScW)}}$ = 593.19/558 = 1.06; W_{iEPN}/W_{ScW} = 28/25 =1.12; HP_{iEPN}/HP_{ScW} = 38.78/38 = 1.02; HF_{iEPN}/HF_{ScW} = 130.27/120 = 1.08; T_{iEPN}/T_{ScW} = 59.25/53 = 1.11; D_{iEPN}/D_{ScW} = 0.29/0.26 = 1.11, and E_{iEPN}/E_{ScW} = 0.65/0.60 = 1.08 times. The ratio variations observed would be related, for example, to the day of IJ emergence from the larval cadavers (i.e., average IJ length 450 - 600 µm; Therese & Bashey, 2012) and the species of the host tested for *in vivo* nematode production (Costa et al., 2007), among other factors. *S. carpocapsae* was initially described as *Neoaplectana carpocapsae* by Weiser (1955) in Czechoslovakia and later redescribed by Poinar in 1967. Furthermore, *S. carpocapsae* has been reported to be distributed in Europe, North and South America, Australia and New Zealand, among other regions (Poinar, 1990; Shishiniova et al., 1998).

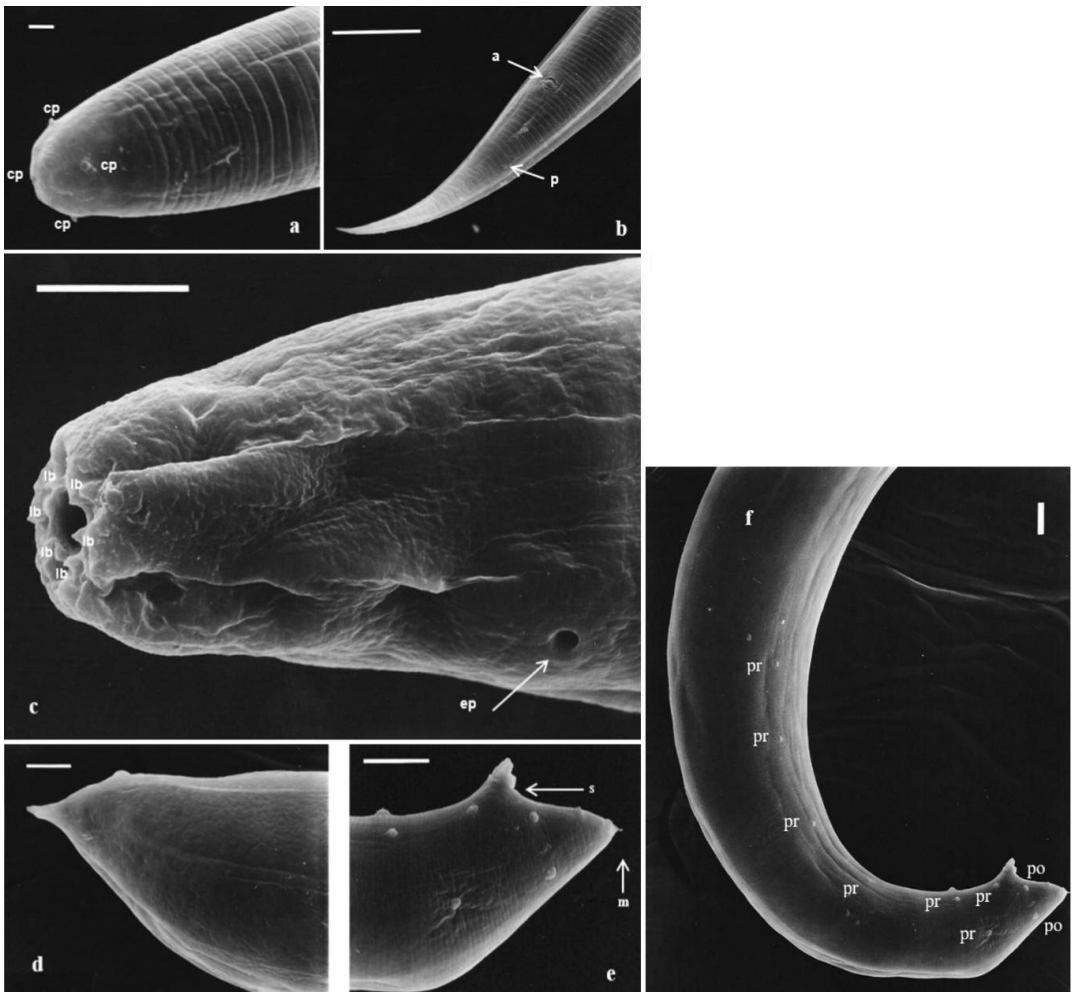


Figure 4. Scanning electron microphotographs showing some morphological details of three developmental stages of the isolated EPN species within the State of Hidalgo, Mexico. IJ stage: a) anterior region showing cephalic papillae (cp), and b) posterior region showing both the anus (a) and phasmid (p). First-generation adult female: c) anterior region showing six labial papillae (lb) and the excretory pore (ep), and d) posterior region (lateral view). First-generation adult male, posterior region: e) spicules (s) and mucron (m), and f) lateral view of tail showing various precloacal and postcloacal papillae [bar length in all cases, 10 µm, except a), which is 1 µm].

The comparison of the obtained nucleotide sequences with those available in GeneBank (NCBI, 2025) rendered up to 99% - 100% similarity with *S. carpocapsae* through the Basic Local Alignment Search Tool (BLAST). Furthermore, the isolated EPN strain clusters with high confidence within the monophyletic *S. carpocapsae* clade (Figure 5) due to node statistics values ≥ 99 of the associated taxa clustering two *S. carpocapsae* isolates with GenBank accession numbers KJ950291 and AF331913, and *Steinernema* sp. Hidalguense, suggests that the isolated nematode is a local variant of *S. carpocapsae*. These nodes have a bootstrap support of 99.

3.2 Isolation and characterisation of the isolated symbiotic bacterium, SB

Figure 6 shows the microscopic aspect of the phase I-*Xenorhabdus* sp.-SB (Figure 6a) growing in STB medium (where cells look like rods of 1.2 µm to 9 µm in length with a width of approximately 1.1 µm), as well as its colonies growing on NBTA medium, which exhibits a reddish core surrounded by a dark blue stripe, aspect which is frequently associated to the phase I of various *Xenorhabdus* species (Figure 6b).

Table 3 presents some phenotypic characteristics of the isolated *Xenorhabdus* sp.-SB, based on biochemical reactions using API 20E, 20NE, and 50CH strips. The mentioned table also includes the corresponding results for 16 strains of *X. nematophila* reported by other authors. Although phenotypic variation is important among the compared SBs, it can be seen that all 17 strains are common in a) negative aesculin hydrolysis, and b) negative acid production from gluconate; also, most of them can use inositol as a carbon source.

The documented results and variations reflect genetic and phenotypic factors involved in complex interactions within the whole ecosystem; at this stage, our results contribute data to enrich common knowledge and support future work.

Table 2

Morphometrics of the infective juvenile stage of the isolated EPN species within the State of Hidalgo, Mexico

Key	Length (µm) (n=25)*			
	Minimal	Maximum	Mean	SD**
Total length (L)	495.71	691.86	593.19	46.30
Maximum width (W)	25.62	30.50	28.00	1.44
Distance Head–excretory pore (HP)	33.44	46.58	38.78	3.52
Distance Head–nerve ring (HR)	85.28	116.58	101.16	6.97
Distance Head–pharynx base (HF)	114.79	141.41	130.27	7.73
Tail length (T)	51.39	72.27	59.25	5.14
Ratio D (=HP/HF)	0.24	0.35	0.29	0.02
Ratio E (=HP/T)	0.52	0.86	0.65	0.08

* n, the sample of 25 IJ individuals; ** SD, Standar deviation, $\sqrt{\Sigma(x-x_{average})^2/(n-1)}$

Table 3

Some phenotypic characteristics of *Xenorhabdus* sp. isolated from *Steinernema* sp. Hidalguense, in comparison with that of some *X. nematophila* strains reported by other groups

Characteristic	Isolated SB (Present work)	13 strains (Tailliez et al., 2006)	3 strains (Fischer-Le Saux et al., 1999)
Aesculin hydrolysis	-	-	-
L- Tryptophan	-	nr*	nr
Simmons' citrate	-	v**	+
Cytochrome oxidase	-	nr	nr
Acid production from:			
Inositol	-	v	+ (w)***
Ribose	+	v	+ (w)
Salicin	-	nr	-
Gluconate	-	-	-
Acetoine	+	nr	nr
Assimilation of:			
Inositol	+	v	+ (w)
Ribose	-	-	v
L-arabinose	+	nr	nr
N-acetil-glucosamine	+	nr	nr
Glycerol	+	nr	nr
D-galactose	+	nr	nr
D-fructose	+	+	nr

*nr, not reported; **v, variable; ***w, weak.

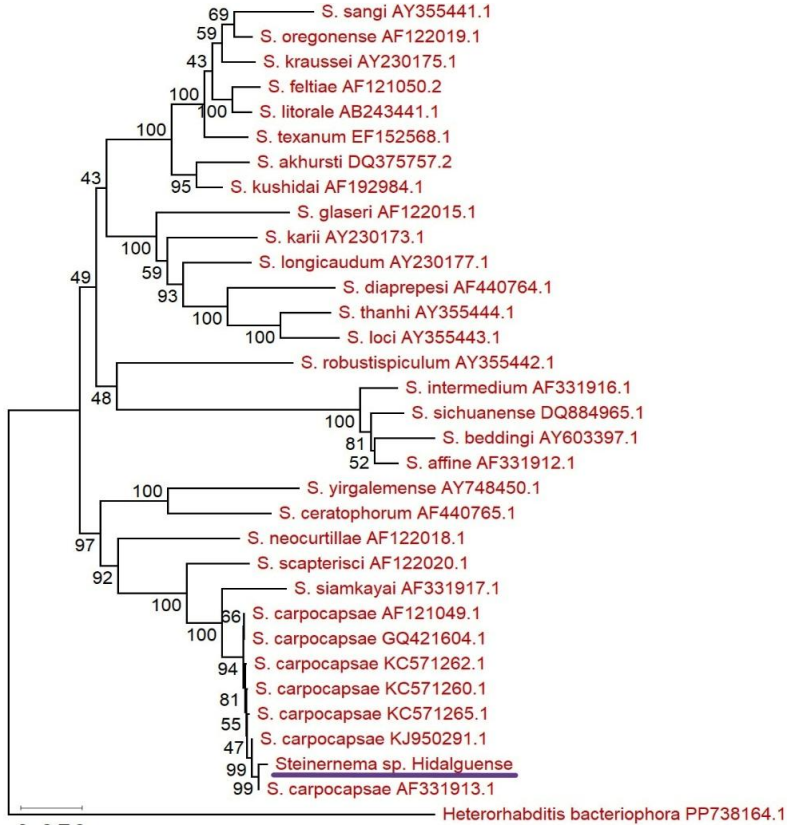


Figure 5. Phylogenetic tree involving the relationships of *Steinernema* sp. Hidalguense, isolated in Mexico, with 31 *Steinernema* species based on the ITS region of ribosomal DNA. Analysis was conducted using MEGA12 (Kumar et al., 2024), employing the Neighbor-Joining method with a bootstrap of 500 replicates. NCBI accession numbers are listed to the right of each species used during the comparison analysis.

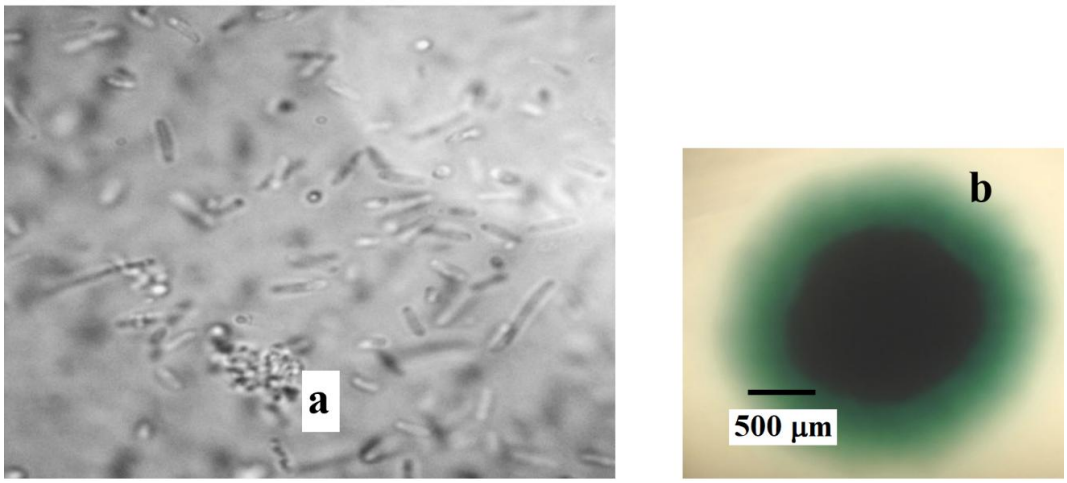


Figure 6. Aspects of the phase I- *Xenorhabdus* sp., which is the symbiotic bacterium of *Steinernema* sp. Hidalguense, isolated in soils of the State of Hidalgo, Mexico. a) Cells in a 12 h old-STB culture broth at 30 °C (magnification, 1000 ×); b) a colony in a 48 h old-NBTA solid culture at 30 °C.

Figure 7 presents the phylogenetic relationship of the isolated *Xenorhabdus* sp.-SB with other *Xenorhabdus* species based on the 16S rDNA gene sequence analysis, clustering them within the monophyletic clade of *X. nematophila*, with a bootstrap support value of 100, indicating a very close phylogenetic relationship. It suggests that the isolated SB is a local strain of *X. nematophila* (i.e., *X. nematophila* Hidalguense). Furthermore, this result supports that the isolated EPN is probably a strain of *S. carpocapsae*.

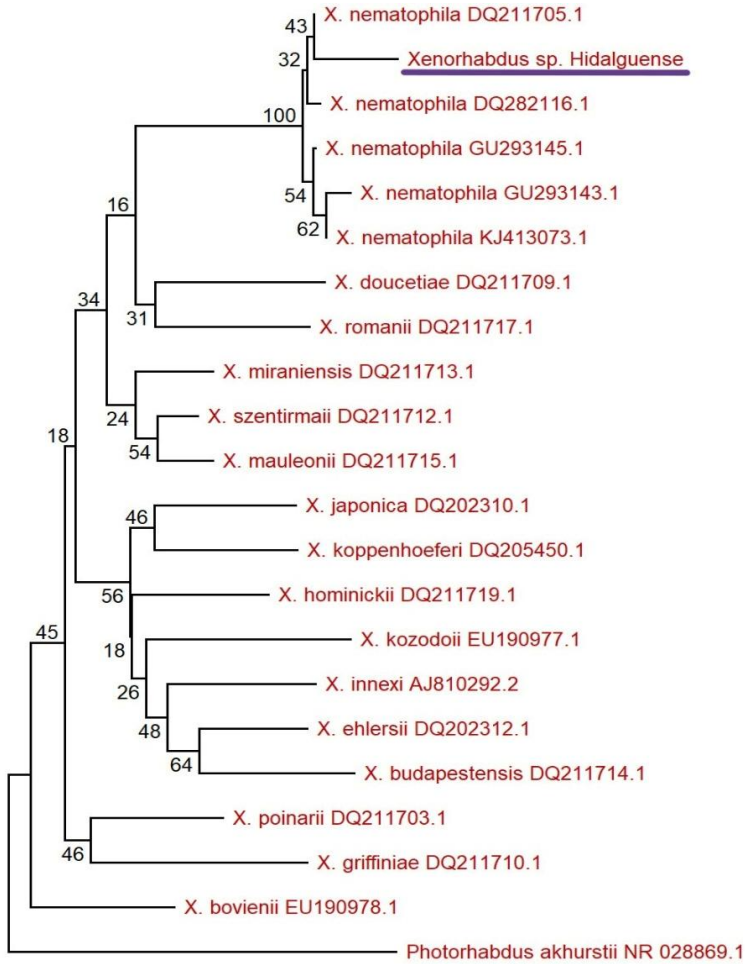


Figure 7. Phylogenetic tree involving the relationships of *Xenorhabdus* sp., the symbiotic bacterium of *Steinernema* sp. Hidalguense, isolated in Mexico, with 20 *Xenorhabdus* species based on 16S rDNA sequence. Analysis was conducted using MEGA12 (Kumar et al., 2024), employing the Neighbor-Joining method with a bootstrap of 500 replicates. NCBI accession numbers are listed to the right of each species considered during the comparison analysis.

3.3 Entomopathogenic nematode virulence. At the lower tested doses of IJ stages per larva of *G. mellonella* (i.e., IJ:larva, 1:1, and 5:1), insect mortality was recorded at 48 h of exposure (Figure 8a, b), and the lowest mortality occurred in 1:1 treatment with ~25% at 72 h. In the one-on-one assay, Kazimierczak et al. (2017) recorded ~50% mortality of *G. mellonella* with *S. carpocapsae*-IJs at 168 h of exposure. In our study, higher doses, like 5:1, 10:1, and 30:1 rendered insect mortality values of ~90%, ~96%, and ~100%, respectively (Figure 8c). Moreover, testing *S. carpocapsae* M5, isolated in Coahuila, Mexico, Treviño-Cueto et al. (2023) recorded similar results to the ones reported in this study, for (IJs/larva *T. molitor*) of 10, 25, 50, 100 and 200, with mortalities of 84 for the lowest dose, and 100 for the others, at 72 h postexposure time, respectively. In contrast, Susurluk & Ulu (2015) studying *S. carpocapsae*-IJ-virulence against *G. mellonella*, reported 0% and 50% mortality for doses (IJ:larva) of 1:1 and 5:1, respectively, at exposure times of 96 h. Furthermore, in this study, the death of larvae appeared to be due to the action of the nematode/bacterium complex, as evidenced by the growth of the symbiotic bacterium in NBTA streaked with dead larval tissues, and the presence of adult nematodes within the dissected insect cadavers. Moreover, two weeks after larval death, IJs emerged, confirming the entomopathogenic behaviour of the isolated nematode.

This report is the third concerning the isolation of *S. carpocapsae* in Mexico; the first one was conducted by L. Caltagirone in Allende, Chihuahua, some years ago (Georgis & Hom, 1992), and the second by Treviño-Cueto et al. (2023) in agricultural soils of Coahuila.

Other EPNs isolated in Mexico include *S. feltiae* Filipjev within the Cañada region in Oaxaca (Ruiz Vega et al., 2003); *Heterorhabitidis mexicana* n. sp. in soils of Tamaulipas (Nguyen et al., 2004), *H.*

sonorensis n. sp. in the desert of Sonora (Stock et al., 2009), *S. ralatorei* n. sp. isolated at Veracruz (Grifaldo-Alcantara et al., 2017), and *H. indica* in soils of Yucatán (Avila-Lopez et al., 2021), mainly. Nonetheless, there have been other efforts to identify isolated EPNs that have been classified at the genus level but not at the species level: five *Steinernema* spp. isolated in Colima, Jalisco and Michoacán, and two *Heterorhabditis* spp. isolated in Colima and Michoacán (Molina-Ochoa et al., 2003); in Oaxaca, one *Heterorhabditis* sp. in Sierra Sur and one *Steinernema* sp. in Valles Centrales (Ruiz Vega et al., 2003); and in Yucatán, two *Heterorhabditis* spp. isolated (Avila-Lopez et al., 2021).

On the other hand, *S. carpocapsae*, as well as *S. feltiae*, *H. bacteriophora* and *H. indica* are considered ubiquitous; specifically, *S. carpocapsae* has been reported to be present in soils of Canada, USA, Argentina, Brazil, China, Korea, Taiwan, Australia, the old Czechoslovakia, Austria, Portugal, France, Georgia, Germany, Italy, Poland, Russia, Spain, Sweden, Switzerland, England, and Mexico, among others (Hominick, 2002).

We considered our results to be important, among other reasons, for pest-biocontrol perspectives in Mexico. In fact, *S. carpocapsae* is the second most commercially produced species globally, with 57 bioproducts currently available on the market. The first one is *S. feltiae* with 68 bioproducts (Ramakuwela et al., 2025). We continue research to test the biocontrol potential of *S. carpocapsae* Hidalguense against different pests, and to test the potential of symbiotic bacteria as producers of different metabolites with biotechnological potential, linked to specific genes they have.

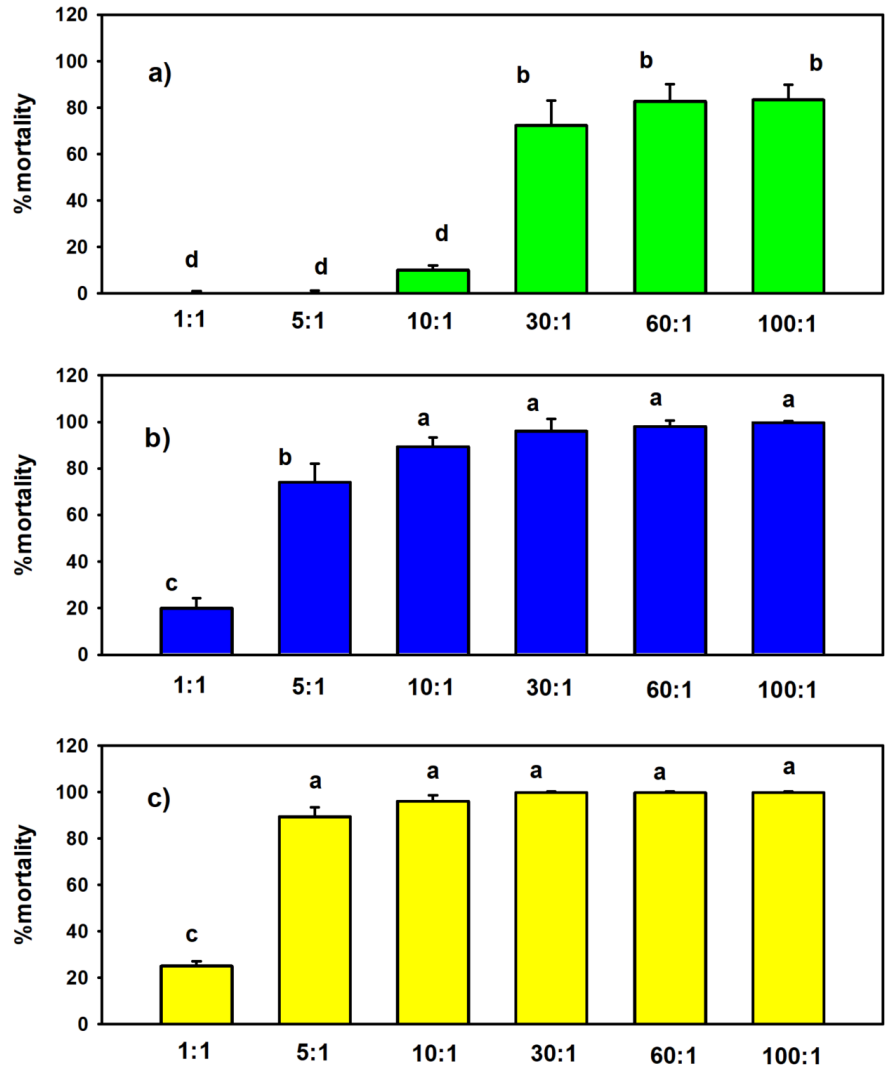


Figure 8. Virulence of the IJ stages of *Steinernema* sp. Hidalguense, against last instar larvae of *Galleria mellonella*, expressed as the percentage of mortality at a) 24 h, b) 48 h, and c) 72 h. Different doses (IJ:larva): 1:1, 5:1, 10:1, 30:1, 60:1, and 100:1. A control without IJs did not exhibit any mortality. The same letters in the columns indicate non-significant differences ($p \leq 0.05$).

4. Conclusions

This report presents the first isolation and characterisation of the complex *S. carpocapsae/X. nematophila* from soil samples from the town of Alcholoya, Hidalgo, Mexico, covering morphological, phenotypic, genetic, and virulence aspects. Moreover, the virulence shown by the IJs against larvae of *G. mellonella* in laboratory bioassays suggests an attractive potential for agroforestry applications for pest control, as well as non-agricultural purposes; however, these should be experimentally validated for specific cases in Mexico, like the Agave Weevil, *Scyphophorus acupunctatus*; white grubs in maize; *Aedes aegypti* mosquito due to dengue spread, among others, where *S. carpocapsae* has been tested for biocontrol.

Conflict of interest

The authors declare no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical concerns

There are no ethical concerns.

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Author's contribution

MAIL: Formal analysis, Data curation, Writing. **AIRH:** Project administration, Formal analysis, Writing. **NCH:** Conceptualisation, Project administration, Formal analysis, Data curation, Writing.

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