# Bacteria from the Sonoran Desert with nematicidal potential against phytopathogenic nematodes

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**Research Article**

# Abstract

The use of microorganisms and/or their metabolites (e.g., *Bacillus, Pseudomonas*, etc.) has been proposed as an eco-friendly alternative for controlling phytopathogenic nematodes (PPNs). However, one of the challenges is that microorganisms can thrive in crops affected by climate change, salt stress and drought. Consequently, the search for microorganisms adapted to these conditions has attracted attention. Deserts are characterized by high temperatures, water scarcity, and high salinity, among other harsh conditions; hence, they are a perfect niche for isolating microorganisms adapted to extreme environments. The purpose of this work was to isolate and characterize bacteria from rhizospheric soil samples collected in the Sonoran Desert with the potential for PPN control. Approximately 100 thermotolerant bacteria were isolated, and among them the secretome of the strain identified as *Bacillus paralicheniformis* TB197 showed the highest nematicidal activity (96%) against *Meloidogyne incognita in vitro*. Additionally, in field assays, the TB197 secretome effectively suppressed infections caused by *M. enterolobii*. In greenhouse experiments, TB197 endospores reduced the severity of infection by 81% compared with the control ($p \leq 0.05$). Finally, the efficacy of TB197 endospores at the open field level was demonstrated against *M. incognita* in the tomato crop, where the galling index (GI) was reduced by 84%, and against *Radopholus similis* in the banana crop, where root necrosis was reduced by 81%, both compared with the controls ($p \leq 0.05$). Owing to their efficacy in controlling PNN infections, the endospores and secondary metabolites secreted by the *B. paralicheniformis* TB197 strain can be used to develop effective bionematicides.

# Introduction

Phytopathogenic nematodes (PPNs) are one of the greatest threats to agricultural, ornamental and forestry activities (Archidona-Yuste et al. 2019). According to the American Society of Phytopathology (APS), losses in agricultural production caused by PPNs are approximately 14%, which translates into almost 125 billion dollars annually (Mesa-Valle et al. 2020). A common feature of PPN is the stylet located in the stoma or in the mouth that allows them to cross the host cell wall and inject enzymes that partially digest the cellular content before the nematode sucks it into its digestive system.

According to their life cycle and feeding behavior, PPNs are classified into three groups: endoparasites, ectoparasites and semiendoparasites (Gullino et al. 2020). Endoparasites penetrate inside the plant, where all or part of their life cycle occurs; they are mainly root-knot nematodes (e.g., *Meloidogyne* spp.), encysted nematodes (e.g., *Heterodera* spp., *Globodera* spp.), and root-injuring nematodes (e.g., *Pratylenchus* spp., *Hirschmanniella* spp., *Radopholus* spp.), among others. In contrast, the ectoparasite life cycle occurs entirely outside the plant, piercing the epidermis or the superficial layers of the root cortex of the host plant with the stylet (e.g., *Xiphinema* spp., *Trichodorus* spp., *Paratrichodorus* spp.); therefore, they can also be found in seeds, stems and the aerial part of the plant (leaves and flowers). Finally, semiendoparasites can partially penetrate the plant, leaving part of the nematode inside the plant and another part outside, laying eggs toward the outside (e.g., *Sphaeronema* spp., *Hoplolaimus* spp., and *Helycotylenchus* spp.) (Mesa-Valle et al. 2020).

Any agricultural crop can be affected by the action of some PPNs, and their effect is often underestimated as they produce nonspecific symptoms that are often confused with situations of water stress, nutritional disorders, soil fertility problems or other secondary fungal or bacterial infections (Pires et al. 2022). Additionally, PPNs have fast generation times, high reproductive rates, and cryptic behaviors, contributing to the difficulties experienced in effectively controlling PPN infections (Naz et al., 2021; Horak et al., 2019). Several strategies have been reported for the control of PPN infections, such as nonhost crop rotation, soil solarization and the use of chemical nematicides, which include halogenated aliphatic hydrocarbons (e.g., 1,3-dichloropropene), methyl isothiocyanate, oxamyl, thionazine, and carbofuran. Although the use of chemical nematicides is the most successful strategy for the control of PPN, their long-term repeated use leads to increased nematode resistance, which further exacerbates the difficulty of nematode management (Chen et al. 2020). In addition, there is evidence of their negative impact on the environment, for example, the contamination of groundwater, disturbance of soil fertility, and the accumulation of chemical residues within the trophic levels of soil ecosystems (Engelbrecht et al., 2018). As ecofriendly alternatives, the use of microorganisms and/or their metabolites as biological control agents has attracted scientific interest due to their minimal environmental side effects (He et al. 2020; Engelbrecht et al., 2018).

Previous studies have shown that certain plant-associated rhizosphere microorganisms belonging to the *Pasteuria, Pseudomonas, Streptomyces* and *Bacillus* genera control and antagonize PPN through their ability to colonize the rhizosphere and secrete enzymes and/or secondary metabolites that are toxic to PPNs and/or elicit an induced systemic response (ISR) in plants (Berini et al. 2018; Zhao et al., 2018, Horak et al., 2019). However, in addition to their nematode control capabilities, this kind of microorganism must thrive under adverse conditions such as droughts, high temperatures and/or soil salinity promoted by climate change, which has dramatically accelerated in recent years (Bokhari et al., 2020). Indeed, recent research has focused on the bioprospecting of plant-associated beneficial microorganisms capable of adapting to adverse conditions (Bokhari et al., 2020; Engelbrecht et al., 2018).

Desert soils are characterized by extreme conditions, such as high temperatures, water scarcity, high soil salinity, low nutrient levels and high UV radiation (Franklin et al., 2021; Alsharif et al., 2020). In addition, the survival of plants in desert areas is intrinsically related to their microbial communities (Sayed et al., 2019; Bokhari et al., 2020). Alsharif et al. (2020); Bokhari et al. (2020); and Franklin et al. (2021) showed that desert microorganisms not only relieve water stress in plants and promote growth but also protect the plant against pathogens, such as PPN. Moreover, Viljoen & Labuschagne (2019) and Abd-elgawad (2020) found that *Streptomyces* and *Bacillus* strains isolated from the Egyptian desert are capable of effectively controlling PPN and surviving in extreme conditions of drought and water stress. In this way, the plant-associated microbiota from deserts is a perfect niche to isolate new microorganisms that are well adapted to extreme conditions and have potential for the biological control of phytopathogens, including PPN.

The Sonoran Desert is one of the warmest regions in North America and the world, where temperatures above 50°C have been recorded and cover a territory of 260,000 km$^2$, including southern Arizona;

southeastern California; northeast, north and south-central Baja California; and north-central and western Sonora, Mexico (Zolotokrylin et al. 2020, Flower et al. 2021). However, unlike other deserts, the Sonoran Desert is the most biodiverse; this is the case for flora, with approximately 2,500 species identified (CONAGUA, 2020; Andrew et al. 2012; Franklin et al., 2021). This is relevant under the premise that the microbial communities are important for the survival of these plants, constituting a diverse and untapped pool of microorganisms with the potential to control phytopathogenic agents, such as PPN, and to support extreme biotic and abiotic conditions or to synthesize thermostable or operatively stable metabolites. For example, Galaviz et al. (2018) and Bashan et al. (2012) found that the Sonoran Desert is home to plant growth-promoting rhizobacteria (PGPRs), such as *Azospirillum brasilense* and *Bacillus pumilus*. However, research on the potential of microorganisms isolated from this ecological niche for biological control of PPNs is scarce. Therefore, the objective of this work was to isolate and characterize bacteria from the rhizosphere of plants growing in the Sonoran Desert with the potential for PPN control.

# Materials And Methods

### Reagents

Malt extract, yeast extract, and bacteriological agar were purchased from BD Difco (Sparks, MD, USA). Methanol and chloroform were provided by J.T. Baker (Xalostoc, Edo. Mex., Mexico). Glucose was provided by Faga Lab (Guamúchil, Sin., México). Verango® (fluopyram, 41.7% (w/v)), Lila Plus® (*Paecilomyces lilacinus* 3% (w/w)) and Nemacem® (Aqueous extract of *Tagetes erecta*, Alpha terthienyl 10% (w/w)) were provided by a local supplier.

### Rhizospheric soil sample collection from the Sonoran Desert

Three zones of the Sonoran Desert were chosen for soil sampling: the Pinacate Biosphere Reserve and the Great Desert Altar (latitude: 31°31'55.01"N, longitude: 113°25'40.05" W), La Primavera (latitude: 28°48'09.70" N, longitude: 111°12'13.20" W) and El Apache (28°18 '59.60" N, 111°14'40.60" W). Two kilograms of rhizospheric soil samples from at least 10 points in every selected zone were randomly taken in sterile plastic bags at a depth of 30 cm and stored and transported refrigerated to the lab to be processed on the same day.

### Isolation of bacteria

Ten grams of soil samples were suspended in 150 mL Erlenmeyer flasks containing 90 mL of sterile distilled water and were incubated at 200 rpm and 27 °C for 1 h (Innova 44, New Brunswick Scientific). Then, the soil sample suspensions were diluted in sterile distilled water (up to $10^{-4}$). After that, 0.5 mL of the diluted suspensions was inoculated into petri dishes containing ISP2 agar (10 g/L malt extract, 4 g/L yeast extract, 4 g/L glucose and 20 g/L bacteriological agar) and incubated at 40 °C for 72 h. Isolated colonies were transferred to new Petri dishes with fresh ISP2 agar and incubated again at 40 °C for 72 h. Axenic cultures were stored at 4 °C for further analysis.

## Submerged culture to obtain secretomes

Submerged cultures of isolated strains were established in 250 mL Erlenmeyer flasks containing 50 mL of ISP2 broth. The culture media were inoculated with a single colony of the axenic strains and incubated at 37 °C at 180 rpm for 72 hours (Innova 44, New Brunswick Scientific). Then, the culture media were centrifuged at 10,034 x g and 4 °C for 20 minutes (Allegra 64R Centrifuge, Beckman Coulter), and supernatants (cell-free) containing secretomes were stored at -20 °C for further chemical analysis or bioassays.

## Chemical characterization of secretomes by thin-layer chromatography (TLC)

The selected secretomes with the highest nematicidal activities were freeze-dried (Yamato DC401 freeze dryer), and metabolites contained in the lyophilized powder were further extracted with methanol. To this end, 300 μL of each solvent was added to 8 mg of lyophilized secretome and sonicated for 15 minutes in an ultrasonic bath (Branson 2510). Then, the samples were centrifuged (Eppendorf Centrifuge 5417R) at 9,279 x g and 4 °C for 10 minutes, and the supernatants were collected for TLC analysis. Eight microliters of the extracts were spotted on silica gel plates (10 x 10 cm, TLC Silica gel 60 $F_{254}$, Merck) and eluted with a mobile phase containing chloroform:methanol:distilled water (65:25:4 v/v). After elution, compounds were visualized with UV light (A: 254, and C: 365 nm), ninhydrin (sprayed with 0.1% w/v ethanol and heated at 60 °C for 15 min) or iodine vapors.

## Molecular identification of bacterial strains

### Genomic DNA extraction

Genomic DNA was extracted from a liquid culture of isolate TB 197 grown aerobically for 24 h at 30 °C. using the PowerSoil® DNA isolation kit (MO BIO Laboratories Inc.) according to the standard protocol provided by the manufacturer.

### PCR amplification of the 16S rRNA, gyrA, rpoB, purH, and groEL genes

PCR amplification of marker genes was performed using OnePCR™ Ultra Supermix with Fluorescent Dye (Bio-Helix) according to Ben Gharsa (2021) methodology with some modifications. The primer pairs used for the 16S *rRNA*, *gyrA*, *rpoB*, *purH* and *groEL* genes are detailed in Table 1. For all markers, a Studio™ 5 Real-Time PCR System was used (Applied Biosystem by Thermo Fisher Scientific), with a unified PCR program that consisted of 1) an initial denaturation phase at 95 °C for 5 min; 2) 30 cycles of denaturation at 95 °C for 40 s, annealing at 56 °C (for the 16S region) or 55 °C (for the other genes) for 1 min and elongation at 72 °C for 30 s; and 3) final elongation at 72 °C for 2 min.

The amplified 16S *rRNA*, *gyrA*, *rpoB*, *purH* and *groEL* products were confirmed by horizontal agarose gel electrophoresis with 1Kb Plus DNA ladder RTU (Bio-Helix). The gels were visualized on a UVP® High-Performance UV Transilluminator (Thermo Fisher Scientific).

**Table 1.** Primers used for the molecular identification of bacterial strains

| Gene | Primer | Primer Sequence (5′-3′) | Reference |
|------|--------|--------------------------|-----------|
| *16S rRNA* | 27F | AGAGTTTGATCMTGGCTCAG | Ben Gharsa et al. 2021 |
| | 1492R | TACGGYTACCTTGTTACGACTT | |
| *gyrA* | 42F | CAGTCAGGAAATGCGTACGTCCTT | Ben Gharsa et al. 2021 |
| | 1066R | CAAGGTAATGCTCCAGGCATTGCT | |
| *rpoB* | 2292F | GACGTGGGATGGCTACAACT | Ben Gharsa et al. 2021 |
| | 3354R | ATTGTCGCCTTTAACGATGG | |
| *purH* | 70F | ACAGAGCTTGGCGTTGAAGT | Ben Gharsa et al. 2021 |
| | 1013R | GCTTCTTGGCTGAATGAAGG | |
| *groEL* | 550F | GAGCTTGAAGTKGTTGAAGG | Ben Gharsa et al. 2021 |
| | 1497R | TGAGCGTGTWACTTTTGTWG | |

*Sequencing and phylogenetic analysis of the 16S rRNA, rpoB, gyrA and groEL genes*

The PCR products were purified and analyzed by PSOMAGEN. Raw sequence data were combined into a single consensus sequence for each marker gene using the MEGA program version 10.0.05 (Kumar et al. 2018). The consensus sequences obtained were used as queries in GenBank database searches using the BlastN algorithm (NCBI GenBank database). Phylogenetic analysis was performed using MEGA software version 10.0.05 (Kumar et al. 2018). Evolutionary distances were calculated by Kimura's two-parameter model (Kimura, 1980). Phylogenetic trees based on the 16S *rRNA*, *gyrA*, *rpoB*, *purH* and *groEL* sequences of the isolates and different strains retrieved from NCBI GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were constructed using the neighbor-joining method (Saitou & Nei 1987) with bootstrap values based on 1000 replicates. The obtained 16S *rRNA*, *gyrA*, *rpoB*, *purH* and *groEL* gene sequences of the isolates were submitted to the GenBank database.

### Collection and synchronization of larval and egg stages of phytopathogenic nematodes

Nematode synchronization was performed based on the Zhao et al. (2020) methodology with some modifications. Tomato roots highly infested with *Meloidogyne incognita*, *M. enterolobii* or *Radopholus similis* were collected and vigorously washed with water to remove any adhered soil. The roots were carefully cut into 2–4 cm long pieces and placed into a blender containing a NaOCl solution (2% w/v) to grind for 30 s. The crushed roots were rinsed with tap water and passed through 100-, 325- and 500-mesh sieves. Second-stage (J2) juveniles of PPN retained on the 325-mesh sieves and eggs retained in the 500-mesh sieves were suspended in sterile tap water and stored at 15 °C for further analysis. On average,

approximately 200 J2s or eggs per milliliter were collected by this procedure (five counts visualized at 10X, MOTIC, AE 2000, inverted microscope).

## In vitro screening for nematicidal activity

*In vitro* nematicidal activity bioassays employing *M. incognita* J2 larvae in aqueous suspension were performed according the methodology of Ala et al. (2020) with some modifications. Twenty microliters of secretomes obtained from submerged cultures were added to 96-well flat-bottom sterile polystyrene microplates (Corning® Costar® 3595) containing 40-60 J2 larvae suspended in 200 µL of sterile tap water per well. Subsequently, the plates were sealed with parafilm and incubated at 25 °C in the dark for 48 h, and motile and immotile larvae (considered death) were counted at 10X amplification (MOTIC, AE 2000, inverted microscope).

All experiments were performed considering three biological replicates (with three technical repeats each). Nematicidal activity was estimated according to Eq. 1.

$$\% \, \textbf{\textit{Mortality}} = \frac{dead \, J2}{total \, J2} * 100\% \, (\text{Eq. 1})$$

## Nematicidal activity evaluation of secretomes in field assays

The evaluation was performed on a tomato (*Solanum lycopersicum* L.) crop within the presence of a shade mesh ($\sim$50% reduction of sunlight radiation) at Agroindustrias Tombell (Culiacan, Sinaloa-Mexico). The experimental area was selected based on previous analyses of the nematode populations, and areas with the highest level of *M. enterolobbi* infestation were selected. Tomato seedlings of the commercial Dionysus® hybrid (Ahern Seeds) were transplanted into cultivation plots, and the following three treatments were applied: 1) conventional management (undisclosed by the farmer), 2) secretome of the selected strain from *in vitro* screening and 3) Verango® (fluopyram, 41.7%, as a positive control). The secretome of the selected strain was applied at a concentration of 8 L/ha, while Verango® was applied at 1 L/ha. A total of 12 applications were made using a drench system with intervals of eight days between them for a period of 90 days. Each experimental unit consisted of three plots (1.80 m between them) and was 50 m long (270 m$^2$ per treatment). Only the central plot of each treatment was evaluated to avoid the influence of adjacent treatments.

To evaluate the root damage produced by nematodes, root washings were performed at 30, 60 and 90 days after transplantation, for which 10 plants were selected randomly from the central plot of each treatment. The galling index (GI) in tomato roots was determined based on the visual scale proposed by Baker (1978) (Table 1S and Fig. 1S) in a range of 0-5, where 0 represents 0% galling and 5 represents greater than 80%.

## Biological control in greenhouse assays

The assays were performed at Centro de Investigación en Alimentación y Desarrollo, (Culiacan, Sinaloa, Mexico) in the spring of 2020 (March and April). Taureg hybrid tomato seeds were sown directly in plastic pots under aseptic conditions at a greenhouse temperature of ~25 °C and 16 h of daylight. A cluster of 4 to 6 seeds was sown approximately 1.5 inches deep in a 15-cm-wide pot, which was filled with 1.0 kg of a high-quality well-drained potting mix. The plants were watered every three to four days, and when they were 30 days old, a suspension of *M. enterolobbi* eggs (approximately 1500 eggs per pot) was inoculated using a sterile micropipette following standard inoculation procedures (Naz and Khan 2013).

To evaluate the biological control in greenhouse assays, 400 mL of a) water (negative control), b) selected strain endospores ($1\times10^6$ spores/mL), and c) Verango® (fluopyram, 41.7%, as a positive control, 1 L/ha) were applied by irrigation to twenty plants postinoculation of the *M. enterolobii* eggs. After 30 and 60 days of nematode inoculation, 5 plants from each treatment were picked randomly, and the roots were carefully washed to remove soil remnants. The galling index was measured to evaluate the biological efficacy of the treatments applied according to the aforementioned visual scale proposed by Baker (1978) (Table 1S, Fig. 1S). The egg number per pot was also recorded by counting under an inverted microscope to determine the reproduction factor (RF) of *M. enterolobii* according to Eq. 2.

$$RF = \frac{Final\ egg\ number}{Initial\ egg\ number} \ (Eq.\ 2)$$

## Biological control in open field assays

### Root-knot nematodes

Biological control of the root-knot nematode *M. incognita* was conducted in Calvillo, Aguascalientes, Mexico, in tomato plants (*Solanum licopersicum)* of the Optimax variety during May and June 2022. A randomized complete block design was employed, where 100 mL (per plant) of a) water as a negative control, b) *B. paralicheniformis* TB197 strain endospores ($1 \times 10^8$ spores/g; 4 kg/ha) and 3) Lila Plus® as a positive control (*Purpureocillium lilacinum*, 0.48 kg/ha) were applied by uniform drenching after 4 weeks of tomato crop growth in four replicate plots (1.8 x 4 m) per treatment. At 15 and 30 days after the treatments, 5 plants were extracted from each plot (20 plants per treatment), and the galling index was determined by the visual scale described previously (Table 1S, Fig. 1S). Additionally, to determine the population density of *M. incognita* J2 at the beginning, middle and end of the assay, 100 g of roots of 4 different plants for each treatment were obtained by 4 zigzag samplings and processed for the extraction and quantification of *M. incognita* (J2, five counts visualized at 10X, MOTIC, AE 2000, inverted microscope) as described previously.

### Burrowing nematodes

The biological control of the burrowing nematode *Radopholus similis* was conducted in Cihuatlan, Manzanillo-Mexico in banana (*uss asp.*) of the gran nain cultivar during May and June 2022. A randomized complete block design was employed, where 100 mL of a) water (negative control), b) *B. paralicheniformis* TB197 strain ($1 \times 10^8$ spores/g; 4 kg/ha) and c) Nemacem® (6 L/ha, positive control) were applied by uniform drenching in four replicate plots (1.8 x 4 m) per treatment. At 15 and 30 days of the assay, 5 plants were extracted from each plot (20 plants per treatment), and the necrosis index was determined based on a visual scale of 0 to 5 proposed by Coyne et al. 2007 (Table 2S). To determine the population density of *R. similis* J2 at the beginning, middle and end of the assay, 100 g of roots of 4 different plants for each treatment were obtained by 4 zigzag samplings and processed for the extraction and quantification of *R. similis* J2 as described previously.

## Statistical analysis

The results of all experiments are reported as the mean ± standard deviation. One-way analysis of variance (ANOVA) and Tukey−Kramer (95% confidence limit) tests were performed to establish significant differences among treatments using NCSS software (Number Cruncher Statistical System or Windows, Kaysville, UT, USA, version 7.0).

# Results

### In vitro screening for nematicidal activity from secretomes

A total of 100 thermotolerant bacterial strains were isolated from rhizospheric soil samples collected at the Sonoran Desert, where 43 strains were isolated from "La Primavera", 30 strains were isolated from "Apache", and 27 strains were isolated from "El Pinacate". From all strains, only 27 secretomes showed some nematicidal activity (3−96% mortality *M. incognita* J2 larvae) (Fig. 1). Secretomes from the A106, A136, A137, A138, A144, A21, A22, A44, A51, A58, A62, and A73 strains showed low nematicidal activities (≤ 50%), those from the A12, A31, A39, A52 and A6 strains presented moderate activities (50−70%), nda those from the A101, A124, A131, A132, A133, A134, A82, A95, A74 and A81 strains were highly effective (70−96%). Because the A81 isolate showed the highest and consistent nematicidal activity (96%), it was chosen as the most promising microorganism for PPN control; thus, a comprehensive characterization of this strain, including its molecular identification, *in vivo* effectiveness assays (both endospores and secretome), and chemical characterization of its metabolites, was performed.

# Molecular Identification Of The Selected Sonoran Desert Strain

According to the 16S *rRNA* (Fig. 2) and the housekeeping *gyrA, rpoB, purH*, and *groEL* (Figs. 2-5S) gene sequences, the A81 strain showed 99.27 to 100% identity to *Bacillus paralicheniformis*. The percent identity and the accession numbers of the sequences are listed in Table 2. The A81 strain was deposited at the National Genetic Resources of Mexico, CNRG, and renamed the *Bacillus paralicheniformis* TB197 strain.

Table 2
Molecular identification and genotyping of *Bacillus paralicheniformis*
TB197 based on 16S *rRNA, gyrA, rpoB, purH* and *groEl* gene sequences.

| Gene | Number of nucleotides | Identity (%) | Accession number |
|---|---|---|---|
| 16S rRNA | 1466 | 100.00 | OP962217 |
| gyrA | 954 | 99.27 | Ongoing |
| rpoB | 2018 | 99.99 | Ongoing |
| purH | 911 | 99.78 | Ongoing |
| groEL | 938 | 99.68 | Ongoing |

# Chemical Characterization Of Secretomes By Thin-layer Chromatography (Tlc)

The secretome of the *B. paralicheniformis* TB197 strain was analyzed by thin-layer chromatography (TLC) employing UV light (to reveal conjugated pi bonds, such as aromatic compounds), ninhydrin (to reveal peptides and free amino groups) and iodine vapors (to reveal unsaturated compounds, particularly fatty acids), as shown in Fig. 3. TLC plates demonstrated the presence of high-polarity compounds with low distance traveled (Rf from 0 to 0.4), active to UV light (Fig. 3a and b), and high reactivity to ninhydrin and iodine vapors (Fig. 3c and d, respectively). On the other hand, at least one compound of medium polarity (Rf near 0.5) was revealed with ninhydrin and iodine vapors (Fig. 3d and e) but was inactive or slightly active to UV light (Fig. 3a and b). Finally, two low-polarity compounds with an Rf of 0.8 and 0.9 were visualized employing UVC and UVA, respectively (Fig. 3a and b), and both were reactive to ninhydrin and iodine vapors (Fig. 3c and d, respectively).

# Secretome Nematicidal Activity In Field Assays

The effect of the secretome of the TB197 strain on the galling index (GI) and the percentage of infestation severity on tomato roots caused by *M. enterolobbi* was evaluated in field experiments and compared with conventional management (employed by the owner of the field and kept confidential) and the application of a chemical nematicide as a positive control (fluopyram, 41.7% w/v). The three treatments suppressed the rate of galling (0) caused by *M. enterolobii* during the 60 days of application. At 90 days of the experiment, no differences were observed (p ≥ 0.05) in the treatments, where the secretome of the TB197 strain (GI: 0.2; Severity (%): 4) and the conventional management (GI: 0.2; severity (%): 4) were applied. In both cases, the progress of the infection was slight, with a GI and severity percentage less than 0.5 and 5%, respectively, while the positive control (fluopyram) completely suppressed the galling and damage caused by *M. enterolobii.*

# Biological Control In Greenhouse Assays

A higher and progressive nematode infestation was observed in the control (water) at the first (30 days) and second evaluation (60 days) (Fig. 4a and d), resulting in a galling index (Table 3) up to 10-fold higher than the application of *B. paralicheniformis* TB197 endospores and the chemical nematicide ($p \geq 0.05$). *Bacillus paralicheniformis* TB197 endospores effectively suppressed the infestation of *M. enterolobii* in tomato roots (Fig. 4b and e) in a similar manner ($p \leq 0.05$) as the chemical nematicide (Fig. 4c and f) at 30 and 60 days of application. The reproduction factor of *M. enterolobii* in tomato roots (Table 3) treated with the chemical nematicide and TB197 endospores were similar between them ($p \leq 0.05$) but 116-fold lower than that of the negative control ($p \geq 0.05$).

Table 3
Effect of treatments on *M. enterolobii* (greenhouse test) infestation of tomato plants measured through the galling index (GI), infestation severity, population density and reproduction factor.

| Treatments | 30 days | | 60 days | | |
|---|---|---|---|---|---|
| | GI | Severity (%) | GI | Severity (%) | Reproduction factor |
| Water | 1.80 ± 0.83[b] | 8.2[b] | 4.2 ± 0.75[b] | 64.4[b] | 7.00 ± 0.006[b] |
| B. paralicheniformis **TB197** endospores | 0.20 ± 0.44[a] | 0.2 [a] | 0.8 ± 0.75[a] | 0.8[a] | 0.06 ± 0.002[a] |
| Fluopyram | 0.00 ± 0.00[a] | 0.0[a] | 0.4 ± 0.49[a] | 0.4[a] | 0.06 ± 0.001[a] |

Experimental data are represented as the mean ± standard deviation for five individual experiments. The different superscript lowercase letters on columns indicate significant differences for each assay according to the Tukey−Kramer test ($p < 0.05$).

# Biological Control In Open-field Assays

As in the greenhouse assay, a higher and rapid infestation of the root-knot nematode (*M. incognita*) was observed in the control (water) during the open-field assay (15 and 30 days), while *Bacillus paralicheniformis* TB197 endospores and Lila-Plus® (*Paecilomyces lilacinus* 3% w/w) effectively reduced the infection parameters evaluated in tomato plants ($p \leq 0.05$) without significant differences between them (Table 4). With both treatments, the GI, severity percentage and population density of *M. incognita* were up to 2-, 5- and 7-fold lower than those of the control, respectively (Table 4). Additionally, based on the reduction in galling severity, the efficacy of TB197 and the positive control in controlling M. ingognita was 83.7 and 81.3%, respectively (Fig. 5b).

Table 4
Effect of treatments on *M. incognita* infestation of tomato plants (field test) measured through the galling index (GI), infestation severity and population density.

| Treatments | 15 days | | | 30 days | | |
|---|---|---|---|---|---|---|
| | GI | Severity (%) | Population density | GI | Severity (%) | Population density |
| Water | 3.2[b] | 21[b] | 80.8 [b] | 3.5[b] | 43[b] | 172.3 [b] |
| B. paralicheniformis **TB197** endospores | 1.4[a] | 5 [a] | 11.5 [a] | 1.6 [a] | 7[a] | 23.8 [a] |
| Lila Plus® (P. lilacinus) | 1.3[a] | 4[a] | 14.5 [a] | 1.7[a] | 8[a] | 28.0 [a] |

Experimental data are represented as the mean ± standard deviation of five individual experiments. The different superscript lowercase letters on columns indicate significant differences for each assay according to the Tukey−Kramer test (p < 0.05).

The *Bacillus paralicheniformis* TB197 endospores also showed consistent nematicidal activity against the burrowing nematode *R. similis* in banana crops. TB197 strain and Nemacem® (aqueous extract from *Tagetes erecta*, alpha terthienyl 10% w/w) effectively reduced the infection caused by *R. similis* (Table 5) without significant differences between them (p ≥ 0.05) during the open-field assay (15 and 30 days). Both reduced necrosis of roots and nematode population density up to 4.4- and 2.6-fold, respectively, compared with the control (water), with which banana crops showed a faster *R. similis* infestation. Additionally, based on the reduction in the necrosis percentage, the efficacy of TB197 and the positive control in controlling R. similis was 81 and 71.5%, respectively (Fig. 5c).

Table 5
Effect of treatments on *Radopholus similis* infestation of banana plants (field test) measured through the necrosis percentage and population density.

| Treatments | 15 days | | 30 days | |
|---|---|---|---|---|
| | Necrosis (%) | Population density | Necrosis (%) | Population density |
| Water | 16.9 [b] | 53.3 [b] | 26.3 [b] | 99.0 [b] |
| B. paralicheniformis **TB197 endospores** | 3.8 [a] | 19.8 [a] | 5.0 [a] | 28.8 [a] |
| Nemacem® (Alpha terthiernyl) | 4.4 [a] | 23.5 [a] | 7.5 [a] | 30.3 [a] |

Experimental data are represented as the mean ± standard deviation of five individual experiments. The different superscript lowercase letters on columns indicate significant differences for each assay according to the Tukey−Kramer test ($p < 0.05$).

## Discussion

Harsh abiotic conditions existing in desert environments (e.g., extreme temperatures and radiation, water scarcity, erosion, low organic matter content, and sedimentation, among others) negatively impact living organism development (especially in plants and microorganisms) (Alsharif et al. 2020). However, several species have developed adaptations to remain metabolically active (Schultz and Soares 2020) and to be able to proliferate under these adverse conditions. Such is the case for the Sonoran Desert, which possesses the greatest biodiversity of flora and fauna among deserts worldwide, although the temperatures in summer can exceed 45−50°C (Zolotokrylin et al. 2020). Desert soils worldwide possess a huge diversity of microorganisms dominated by Actinobacteria (36.8%), Proteobacteria (23.8%), Firmicutes (8.6%) and Acidobacteria (5.5%) (Dean et al. 2020); however, in the case of the Sonoran Desert, there is scarce information about rhizospheric and soil microbiota composition and the biotechnological potential from their microorganisms. Sutto-Ortiz et al. 2017 and Bacame-Valenzuela et al. 2015 reported the isolation of thermotolerant actinomycetes and fungal strains capable of synthesizing enzymes of industrial interest, such as phospholipase A and feruloyl esterases, respectively. Additionally, Camacho-Ruiz et al. 2020 reported the isolation from Sonoran Desert soils of *Streptomyces*, *Bacillus*, *Aspergillus and Aureobasidium* strains able to produce gastrointestinal lipase inhibitors. Moreover, Baqueiro et al. 2019 reported the biotechnological potential of *Bacillus* strains capable of producing stable proteases. Regardless of agricultural applications, some plant growth-promoting rhizobacteria (PGPRs), such as *Azospirilum brasilense* and *Bacillus pumilus*, have been isolated (Galaviz et al. 2018, Bashan et al. 2012), but there are no reports focusing on PPN control. Indeed, this is the first work to explore and demonstrate the potential of microorganisms from the Sonoran Desert for the control of PPNs.

In the current work, from 100 thermotolerant bacteria isolated from rhizospheric soils collected in the Sonoran Desert, the secretomes from 27 of them showed some nematicidal activity *in vitro* (3−96%) against *M. incognita*. Of these strains, 10 produced high mortality in *M. incognita* larvae (70−90%), but the secretome from the TB197 strain produced the highest (> 95%) (Fig. 1). Consequently, strain TB197 was selected for identification and evaluation in greenhouse and open-field tests. Sequence analysis of the 16S *rRNA* gene had the most significant impact on the identification and taxonomic classification of the TB197 strain. Even so, analyses of the housekeeping gene sequences *gyrA*, *rpoB*, *purH*, and *groEL* were also included because previous studies mention difficulties in the differentiation and phylogenetic relationships of certain groups of *Bacillus* based on 16S *rRNA* gene sequences due to their similarities (Table 2). Additionally, the sequencing of housekeeping genes has been shown to be more appropriate than 16S *rRNA* sequencing for discriminating certain species of *Bacillus* (Ozdemir and Arslan 2022). In addition, the use of these housekeeping genes provides a high resolution between species closely related to groups of *Bacillus*, as they are generally more conserved between bacterial species than the 16S *rRNA*

gene (Liu et al. 2013; Franco-sierra et al. 2020). This information allowed the robust identification of the TB197 strain as *Bacillus paralicheniformis*.

In agriculture, many strains from the *Bacillus* genus have been employed in biofungicides and biofertilizer formulations due to their biological activities, including antagonism against phytopathogenic agents, such as bacteria, fungi and nematodes; induction of plant systemic resistance against phytopathogens; and promotion of plant growth and development (Villarreal-delgado et al. 2018; Valenzuela-Ruiz et al. 2019). Furthermore, *Bacillus* is one of the three most important genera for PPN control by several mechanisms: direct parasitism, antibiosis, reduction in plant root penetration and competition for essential nutrients (Engelbrecht et al. 2018). Antibiosis has been identified as the main control mechanism of PPN through the production of antibiotics and other secondary metabolites (lipopeptides, siderophores, and endotoxins, among others) and lytic enzymes secreted during growth and the stationary phase (Xiong et al. 2015, Gao et al. 2016, Liu et al. 2020, Engelbrecht et al. 2018). Nevertheless, information on secondary metabolites produced by *B. paralicheniformis* with nematicidal activity is almost nonexistent. Valenzuela-Ruiz et al. (2019) reported that the draft genome of *B. paralicheniformis* TRQ65, isolated from wheat fields in the Yaqui Valley, Mexico, revealed the presence of putative genes related to osmotic and oxidative stress responses and auxin, lipopeptides, siderophores and antibiotic biosynthesis.

For preliminary chemical characterization, extracts obtained by lixiviation of freeze-dried supernatants with methanol were employed. TLC plates from samples obtained by methanolic extraction are shown in Fig. 3, and they show the presence of several fractions with different Rf values. The spots from TLC plates containing the methanolic extract can be visualized by different methods (UV and stains); almost all of them were reactive to ninhydrin and iodine vapors (Fig. 3c and d), but only some of them were active to UVA and/or UVC (Fig. 3a and b) with slight activity for UV light but high activity for iodine vapors and ninhydrin (Fig. 3). The reactivity to ninhydrin suggests the presence of peptide-natural compounds (Dlamini et al. 2020) and probably molecules that contain unsaturated fatty acids due to reaction with iodine vapors (Meyers and Meyers, 2008). In the case of some UV-active spots, the molecules could include aromatic compounds, such as aromatic amino acids.

The information provided by TLC analyses fits with the description of siderophores and cyclic lipopeptides usually produced by *Bacillus* species (Horak et al. 2019) and the presence of putative genes to produce them, according to the draft genome of *B. paralicheniformis* TRQ65 (Valenzuela-Ruiz et al. 2019). In general, lipopeptides contain a hydrophobic fatty acid chain bonded to a hydrophilic cyclic peptide. According to the nature of the cyclic peptide, lipopeptides produced by *Bacillus* species have been classified mainly into three families: surfactin, fengycin, and iturin (Yang et al. 2015; Falardeau et al. 2013). Members from the fengycin and iturin families usually contain aromatic amino acids in their peptide ring, such as tyrosine (Zhao et al. 2017), which would explain the UV activity of some compounds (Fig. 3). Compounds with Rf values between 0 and 0.3 or 0.2 and 0.4 could belong to the fengycin and iturin families, respectively, as mentioned by Geissler et al. (2016). On the other hand, low-molecular-weight metal-chelating compounds of the hydroxamate and catecholate types, which are involved in the

uptake of iron (siderophores) by microorganisms, have been reported as metabolites produced by several *Bacillus* species (Sansinenea and Ortiz 2011). The results of the TLC analysis strongly suggest that secondary metabolites synthesized by the *B. paralicheniformis* TB197 strain could be siderophores and/or cyclic lipopeptides; however, an exhaustive chemical characterization of their secretome is currently underway to verify this hypothesis.

This study demonstrated the potential of the secondary metabolites contained in the secretome from *B. paralicheniformis* TB197 to effectively control infections caused by *M. enterolobbi* in open-field assays. The GI and the infection severity percentage observed in tomato roots were very low and similar to the conventional management performed by the farmer. In this sense, there are few studies evaluating the effectiveness of secretomes of *Bacillus* species for the control of PPN in agricultural fields. For example, Liu et al. (2020b) showed that the application of 16 mL/m$^2$ of various secretomes of *Bacillus* strains in tomato roots resulted in a GI of 1, while the GI reported in the present work by employing the secretome of the TB197 strain was 0.2. Therefore, the secretome of *B. paralicheniformis* TB197 appears to be more effective as a lower concentration (0.8 mL/m$^2$) was applied and obtained better GI results.

However, additional work over the application of secretomes regarding biosafety, doses, application times, optimization of culture medium and culture conditions and the exhaustive characterization of their bioactive metabolites is required. Although the chemical nematicide fluopyram (pyridinyl-ethyl-benzamide) was highly effective in suppressing infection by *Meloidogyne* species, there are reports that this chemical shows a reversible inhibition phenomenon that could lead to possible crop infestation even after application (Watson 2022). In addition, fluopyram is reported to be a recalcitrant compound that modifies the general soil structure and the activity and function of beneficial soil microorganisms (Zhang et al. 2014). Consequently, as an advancement toward sustainable agriculture, the secondary metabolites contained in the secretome of the TB197 strain appear to be a promising alternative to chemical nematicides.

Although bacterial metabolites look promising for PPN control, the use of cells or endospores of *Bacillus* species is the most common way to formulate commercial products (Engelbrecht et al. 2018; Zhou et al. 2016), as living cells, in addition to antibiotic metabolite production, combine several mechanisms, such as nematode–host recognition interference, competition for nutrients, promotion of plant growth and priming of the induced systemic resistance (Chinheya et al. 2017, Ramírez-Cariño et al. 2020, Engelbrecht et al. 2018). In our study, the ability of *B. paralicheniformis* TB197 to control PPN was evaluated for the first time. In the greenhouse assay, both the TB197 strain and fluopyram had a control efficacy of *M. incognita* greater than 98% based on the reduction in galling severity (Fig. 5a).

On the other hand, several studies mention that success of control at the greenhouse level does not guarantee bacterial performance at the field level (Wang et al. 2021; Abdel-Salam et al. 2018; Zhao et al. 2020; Tian et al. 2007), usually because strains are affected by unfavorable factors, such as climatic changes, physical and chemical properties of soil and the competition of native microorganisms (Wang et al. 2021). However, our results show the consistent ability of *B. paralicheniformis* TB197 to control

PPN even at the field level. One assay was performed against root-knot PPN comparing the bacterial effectiveness with a probed commercial biopesticide LilaPlus® (*P. lilacinum*). Both treatments were highly effective and showed consistent results for the control and reduction of the severity of *M. incognita* infection (~ 84% compared with the control, Fig. 5b). Several studies and patents report *Bacillus* cells or endospores as biological control agents, particularly against root-knot nematodes from the *Meloidogyne* genus; however, none of them surpassed the effectiveness reported here for *B. paralicheniformis* TB197. For example, the percent reduction in tGI in field assays through the use of *Bacillus altitudinis* AMCC1040 (Wang et al. 2021), B. *subtilis* Bs-1, *B. cereus* Bc-cm103 (Cao et al. 2019), B. *aryabhattai* KMT-4 (Yin et al. 2021), B. *velezensis* Bv-25 (Antil et al. 2021), B. *marisflavi* CRB2 (Tian et al. 2022), B. *subtilis* CRB7 and *B. methylotrophicus* (Gowda et al. 2022) was < 70%.

A second assay was performed against the burrowing nematode *R. similis*, a migratory, polyphagous endoparasite with a wide global distribution found mainly in tropical and subtropical regions that infects banana crops. The control effectiveness of the TB197 strain was similar to that of Nemacem®, an effective commercial botanical extract from *Tagetes erecta*, employed as a positive control. In both cases, the percentage of necrosis of roots and the nematode population density were reduced by 81.0 and 71.5% (Fig. 5c), respectively, compared with the control (water). The main active metabolite from the botanical extract (Nemacem®) is alpha terthienyl, a well-known molecule acting on the central and peripheral nervous systems of nematodes, causing their immobilization and subsequent death (Plantoria 2022). Although this extract is considered an environmentally friendly alternative, its half-life is very short ( 4 h, according to the National Institute of Ecology and Climate Change of Mexico, (http://www2.inecc.gob.mx/sistemas/plaguicidas/pdf/Alfa%20terthienil.pdf)); this would be a disadvantage compared with bacterial-based nematicides, which, in the case of molecules synthetized by the *Bacillus* genus, are stable at extreme soil pH, temperature and salinity conditions for more than 25 days (Raaijmakers et al. 2010, Engelbrecht et al. 2018).

Our results demonstrate that both the bioactive metabolites contained in the secretome of the *B. paralicheniformis* TB197 strain and their endospores can effectively control PPN infections, are an ecological alternative to chemically synthesized nematicides, and can compete successfully with commercial botanical and microbiological bionematicides.

## Conclusion

Plant-associated bacteria from the Sonoran Desert showed potential for PPN control. In particular, the *Bacillus paralicheniformis* TB197 strain showed a high capacity for PPN control in *in vitro*, greenhouse and agricultural field tests. Moreover, the TB197 strain successfully proliferated on several rhizospheric soils under different climate and soil conditions and crops, indicating high adaptability to colonize environments different from its isolation niche. The effective reduction of root infection by PPN and the damages that they produce, through the application of the secretome or the endospores of TB197 strain, are comparable with those obtained using commercial chemically synthesized and natural nematicides;

this supports *B. paralicheniformis* TB197 as a potential alternative for the development of commercial bionematicidal formulations.

# Declarations

### Declaration of competing interests.

The authors declare that they have no conflicts of interest.

### Author Contributions Statement.

All authors contributed to the study conception and design. ACF performed the secretome field and the greenhouse assays, VCJ obtained the secretomes for the *in vitro* nematicidal assays, FDT performed the molecular characterization, and ECQ performed the chemical characterization of the secretome, analyzed the data and wrote the original manuscript. AAT and VCJ supervised the research and reviewed the manuscript. All authors read and approved the manuscript.

### Data availability.

*The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.*

### Ethical statement.

This article does not contain any studies with human participants or animals performed by any of the authors.

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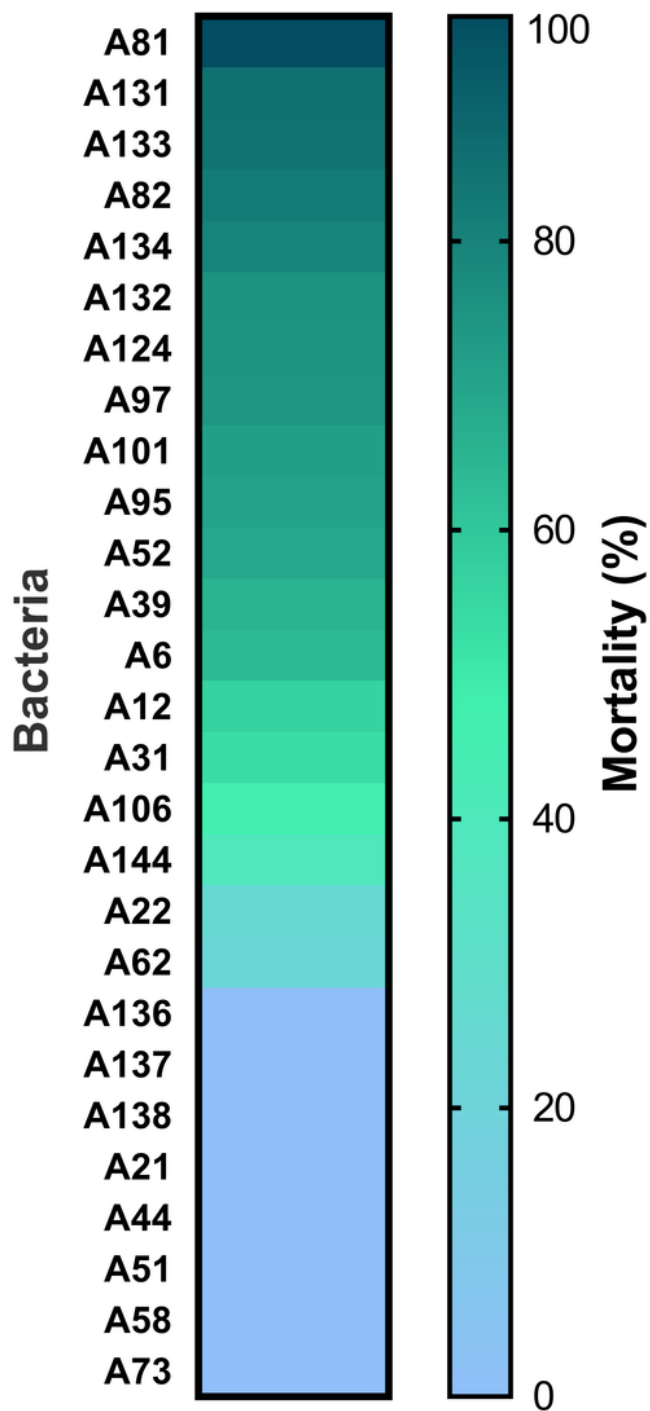
# Figures

**Figure 1**

Toxicity of secretomes from bacteria isolated from rhizospheric soil samples from the Sonoran Desert against *M. incognita*. Color bars represent the means of three replicates.
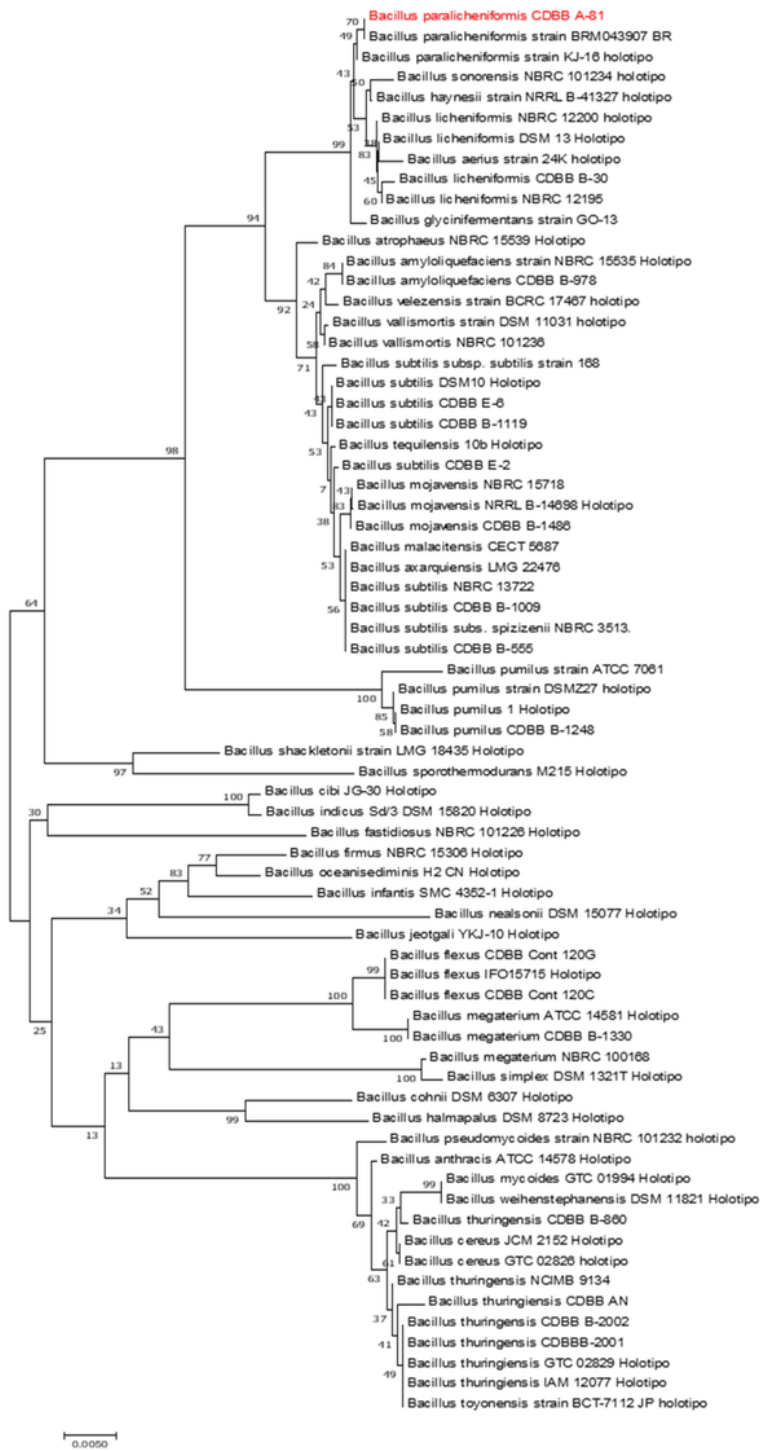
**Figure 2**

Phylogenetic tree of the representative TB197 isolate and *Bacillus* species from NCBI GenBank based on the 16S *rRNA* gene. The sequence was constructed using the neighbor-joining method with 1000 replicates. The bar indicates 0.005 substitutions per nucleotide position.
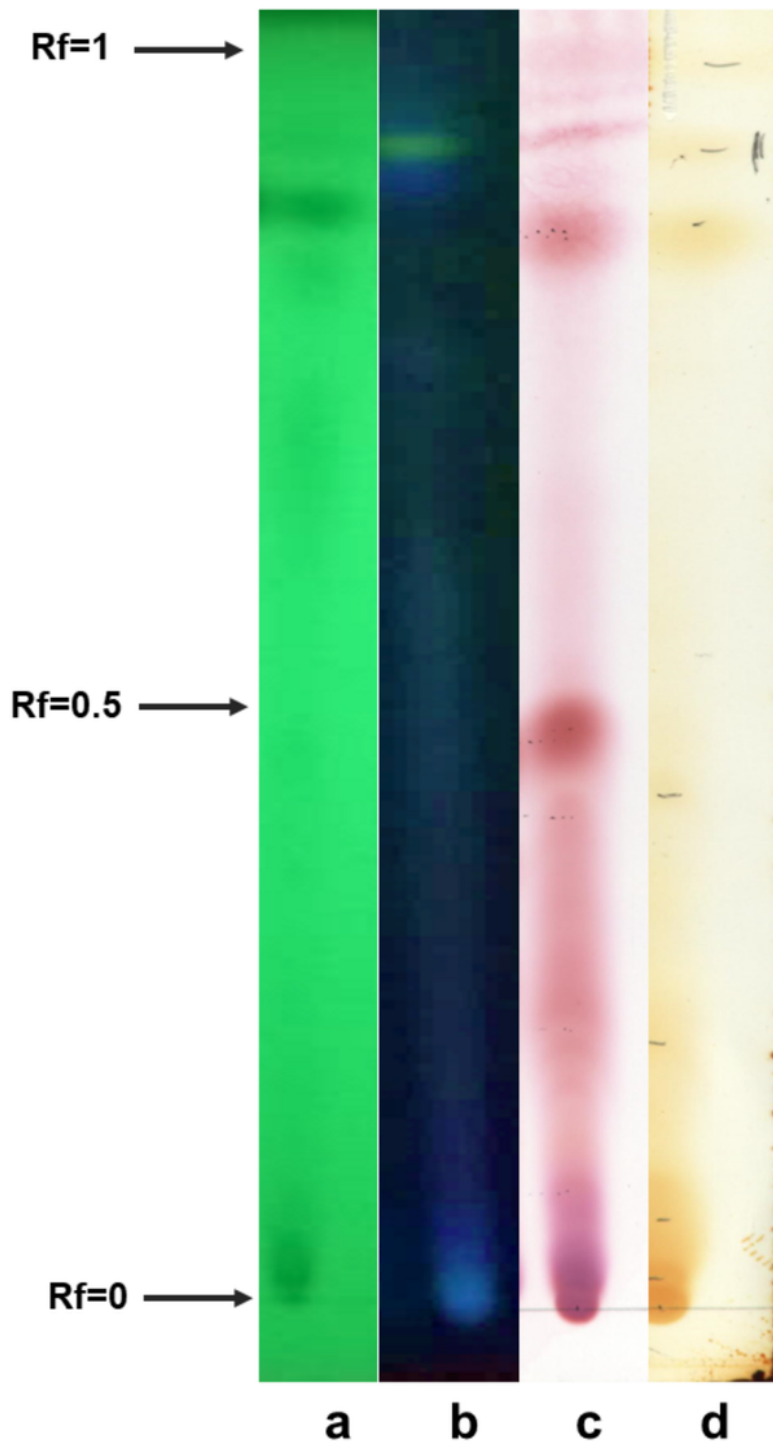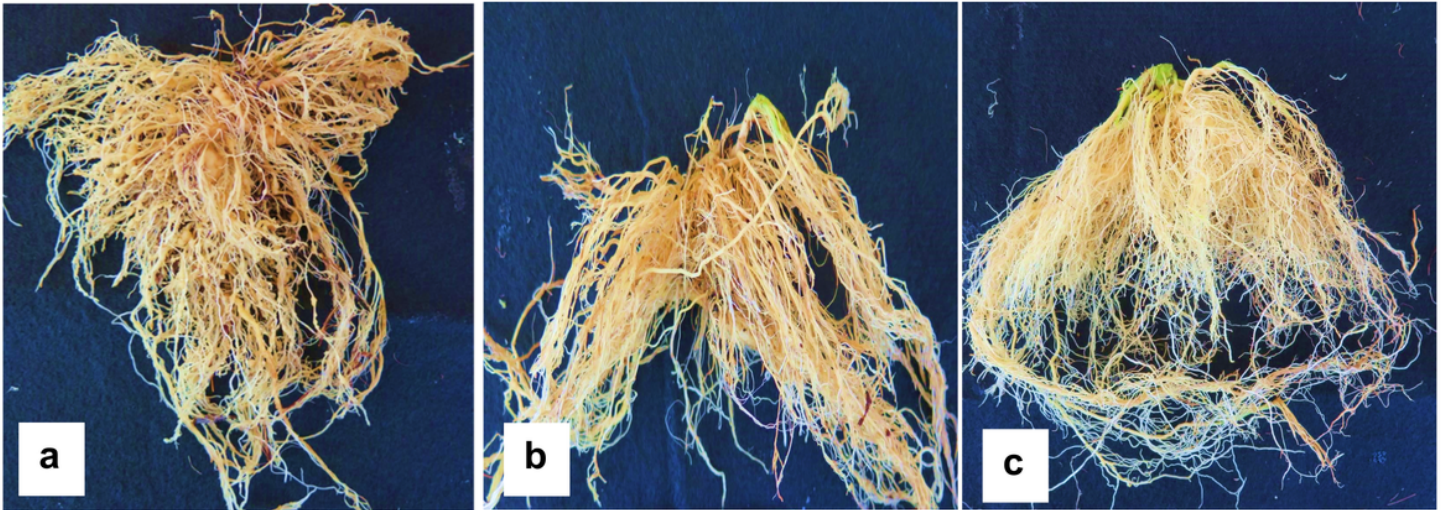
**Figure 3**

Thin-layer chromatogram of the *B. paralicheniformis* TB197 secretome visualized with a) UVA light, 254 nm; b) UVC light, 365 nm; c) ninhydrin (0.1%); and d) iodine vapors. Rf: retard factor.

**30 days**

**60 days**

**Figure 4**

Gall formation on tomato roots caused by *M. enterolobii* in a greenhouse assay after 60 days of treatment. a) and d), negative control (water); b) and e), *B. paralicheniformis* TB197 endospores; c) and f) positive control (fluopyram).
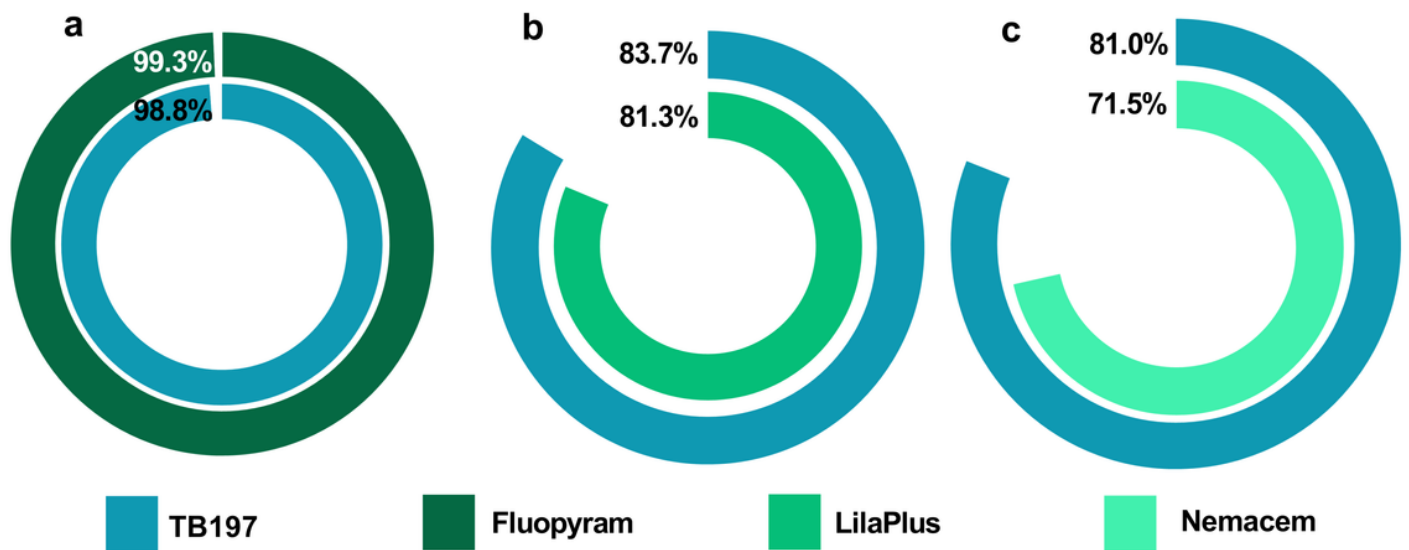
**Figure 5**

Control efficacy on phytopathogenic nematodes using endospores of the *B. paralicheniformis* TB197 strain and commercial nematicides. a) Reduction in the galling severity (%) produced by *M. enterolobbi* (greenhouse test); b) reduction in the galling severity (%) produced by *M. incognita* (field test) and c) reduction in the necrosis percentage produced by *R. similis*(field test). The reduction percentages are based on the negative control (water).

## Supplementary Files

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- Supplementaryinformation.pdf