

Compatibility among bacterial isolates and their efficacy in the biocontrol of *Meloidogyne exigua* in tomato plants

Compatibilidade entre isolados bacterianos e suas eficácias no biocontrole de *Meloidogyne exigua* em tomateiro

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ABSTRACT - Tomatoes are the second most cultivated vegetable in Brazil. Despite their importance, nematodes of the genus *Meloidogyne* can compromise their productivity. Some bacteria have potential for managing these pathogens. Three experiments were carried out, each repeated once. In experiment 1, the efficiency of 20 bacterial isolates obtained from biofertilizers in the mortality of second-stage juveniles (J2) of *M. exigua* was evaluated. Bacterial isolates B₇₄, B₈₆, B₈₈, and B₁₂₁ were as efficient as Fluensulfona, with mortality probabilities of up to 82%. Experiment 2 was carried out to verify the compatibility between the seven most promising isolates evaluated in experiment 1. There was no antagonistic effect between the isolates, and they were identified as follows: B₇₄: *Bacillus thuringiensis*; B₇₅: *Bacillus wiedmannii*; B₈₄: *Bacillus cereus*; B₈₅: *Micrococcus luteus*; B₈₆: *Bacillus cereus*; B₈₈: *Bacillus thuringiensis* and B₁₂₁: *Brevibacterium epidermidis*. In experiment 3, the effect of the bacterial isolates selected in experiment 2, applied individually or in combination, on the biocontrol of *M. exigua* parasitizing tomato plants was evaluated. All treatments reduced the population of *M. exigua*, with the best results obtained by Fluensulfona and isolate B₁₂₁. There are no reports in the literature on the role of *B. epidermidis* as a biocontrol agent for *M. exigua*. It is concluded that isolates B₇₄, B₈₆, B₈₈, and B₁₂₁ are promising in the biocontrol of *M. exigua* and can be applied together, as there is no antagonistic effect between them.

Keywords: *Bacillus thuringiensis*. *Brevibacterium epidermidis*. Biological control. Bionematicides.

RESUMO - O tomateiro é a segunda hortaliza mais cultivada no Brasil. Apesar de sua importância, nematoides do gênero *Meloidogyne* podem comprometer sua produtividade. Algumas bactérias possuem potencial para o manejo desses patógenos. Foram conduzidos três experimentos, cada um deles repetido uma vez. No experimento 1 foi avaliada a eficiência de 20 isolados bacterianos obtidos a partir de biofertilizantes na mortalidade de juvenis de segundo estágio (J2) de *M. exigua*. Os isolados bacterianos B₇₄, B₈₆, B₈₈ e B₁₂₁ foram tão eficientes quanto o Fluensulfona, com probabilidades de mortalidade de até 82%. O experimento 2 foi conduzido com objetivo de verificar a compatibilidade entre os sete isolados mais promissores avaliados no experimento 1. Não houve efeito antagonístico entre os isolados e eles foram assim identificados: B₇₄: *Bacillus thuringiensis*; B₇₅: *Bacillus wiedmannii*; B₈₄: *Bacillus cereus*; B₈₅: *Micrococcus luteus*; B₈₆: *Bacillus cereus*; B₈₈: *Bacillus thuringiensis* e B₁₂₁: *Brevibacterium epidermidis*. No experimento 3 avaliou-se o efeito dos isolados bacterianos selecionados no experimento 2, aplicados de forma isolada ou combinada, no biocontrole de *M. exigua* parasitando o tomateiro. Todos os tratamentos reduziram a população de *M. exigua* sendo os melhores resultados obtidos por Fluensulfona e o isolado B₁₂₁. Não há relatos na literatura sobre o papel de *B. epidermidis* como agente de biocontrole de *M. exigua*. Conclui-se que os isolados B₇₄, B₈₆, B₈₈ e B₁₂₁ são promissores no biocontrole de *M. exigua* e podem ser aplicados de forma conjunta, pois não há efeito antagonístico entre eles.

Palavras-chave: *Bacillus thuringiensis*. *Brevibacterium epidermidis*. Controle biológico. Bionematicidas.

Conflict of interest: The authors declare no conflict of interest related to the publication of this manuscript.



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INTRODUCTION

Coffee is a shrub of African origin, with particular emphasis on two economically important species: *Coffea arabica* L., native to Ethiopia, and *C. canephora* (Pierre ex A. Froehner), originating from Congo. The national average yield for *C. arabica* for Brazil's 2025 crop season is estimated at 24.9 bags/ha, a 5.1% reduction compared to the 2024 crop (CONAB, 2025).

Several plant-parasitic nematodes can infest coffee plants; however, *Meloidogyne exigua* Goeldi, 1887, is the most widespread species, causing yield reductions that vary, among other factors, according to the physicochemical and biological properties of the coffee rhizosphere (BOTELHO et al., 2019).

Although *M. exigua* is the most widespread root-knot nematode species in areas cultivated with *C. arabica*, the tomato plant serves as a suitable host and has been used in research studies, as well as for the maintenance and multiplication of this pathogen (GOUVEIA et al., 2024).

As *M. exigua* is a soil-dwelling pathogen, its management is complex, requiring the integration of various methods, among the most widely used of which is chemical control. However, chemical nematicides are toxic to both animals and humans and increase production costs; for this reason, researchers worldwide have sought alternative methods for managing this pathogen, such as

biological control agents (ZINGER et al., 2021).

The primary bacteria studied for the biological control of plant-parasitic nematodes are those that inhabit the rhizosphere and possess the capacity to invade internal plant tissues, specifically facultative endophytes and phosphorus-solubilizing bacteria, particularly those belonging to the genus *Bacillus* Ehrenberg (SANTOS et al., 2022). Nevertheless, bacteria such as *Micrococcus* sp. Cohn also demonstrate potential for the biocontrol of plant nematodes (WILLE; GOMES; MOTA, 2019).

Although there are few studies conclusively demonstrating the specific modes of action of bacteria against plant-parasitic nematodes, these bacteria may exert their effects either directly through the action of toxins that impair the hatching or motility of juvenile nematode forms or indirectly, by altering root exudates, thereby hindering the nematodes' ability to locate the roots (HU et al., 2022).

There are biofertilizers that reduce populations of *Meloidogyne* spp. in the field (OSMAN et al., 2021). According to the authors, these products exhibit considerable microbial diversity in their composition, which complicates the understanding of the isolated effects of these microorganisms on nematodes.

Côgo (2020) studied the genetic diversity of the microorganisms present in two biofertilizers: one organic product containing strains of *Bacillus subtilis*, *B. licheniformis*, and *Lactobacillus* sp., and another 100% organic fluid product rich in organic matter (humic and fulvic acids). Based on this screening, it was possible to expand knowledge regarding the biodiversity of the microbiota within these biofertilizers, thereby enabling a better understanding of the genetic diversity of the microbial communities present in these products. According to the author, morphological characterization made it possible to group 218 bacterial isolates obtained from the two biofertilizers into 61 distinct groups; of these, 38 were found in the first biofertilizer, 2 were isolated from the second, and 21 were present in both biofertilizers.

Studying the microorganisms present in these biofertilizers under controlled conditions is essential for determining which of them are effective in causing mortality in *M. exigua*, and for elucidating the role, whether isolated or combined, that this microbiota plays in the mortality of this pathogen.

The objectives of the present study were to evaluate the efficacy of 20 bacterial isolates obtained from biofertilizers in causing mortality among second-stage juveniles (J2) of *M. exigua*; subsequently, the seven most promising isolates were evaluated for their mutual compatibility; finally, the selected isolates were tested both individually and in combination to assess their potential for the biocontrol of *M. exigua* parasitizing tomato plants.

MATERIALS AND METHODS

The experiments were carried out in a greenhouse at the Nematology Laboratory (NEMATUFES), in partnership with the Agricultural and Environmental Biotechnology Laboratory (BIOTA), situated within the Phytopathology Sector of the Center for Scientific and Technological Development in Phytosanitary Management of Pests and Diseases (NUDEMAFI) at the Federal University of Espírito

Santo (UFES), located in the municipality of Alegre, ES (latitude 20°45' S, longitude 41°48' W, and altitude 250 m).

Experiment 1 (in vitro). Effect of bacterial isolates on *M. exigua* mortality.

The experiment was carried out to evaluate the efficacy of bacterial isolates obtained from biofertilizers against second-stage juveniles (J2) of *M. exigua* using a completely randomized design. The study comprised 22 treatments (20 bacterial isolates + a positive control [Fluensulfone 480 g L⁻¹] + a negative control [sterile water]) with four replicates, each replicate consisting of a 2 mL Eppendorf-type microtube. The experiment was repeated once over time and designated as Assay 1 and Assay 2. The bacterial isolates tested were: B₇₄, B₇₅, B₇₆, B₇₇, B₇₈, B₇₉, B₈₁, B₈₃, B₈₄, B₈₅, B₈₆, B₈₇, B₈₈, B₈₉, B₁₀₇, B₁₀₉, B₁₁₅, B₁₂₀, B₁₂₁, and B₁₂₃.

Obtainment and Preparation of Bacterial Isolates

The bacteria present in two biofertilizers (one organic product containing strains of *Bacillus subtilis*, *B. licheniformis*, and *Lactobacillus* sp., and another 100% organic fluid product rich in organic matter, humic acids, and fulvic acids) were initially isolated by Côgo (2020) and preserved in 15% glycerol at -20 °C.

To reactivate these bacterial isolates, the spread plate technique was employed. Subsequently, bacterial suspensions were prepared; to this end, the bacteria were cultured in a shaker incubator at 180 rpm for 24 hours. The concentration of the suspensions was adjusted using a spectrophotometer to an optical density of OD₆₀₀ = 0.6, which corresponds to approximately 1 × 10⁸ CFU/mL (bacterial cells per mL of suspension).

Obtaining and Preparing the Inoculum of second-stage juveniles (J2) of *M. exigua*

Second-stage juveniles (J2) were obtained from a pure population of *M. exigua* multiplied on coffee plants (*Coffea arabica* L.) cv. 'Catuaí-144' in pots containing an autoclaved substrate composed of soil and sand in a 1:1 ratio (v:v), maintained for 60 days in a greenhouse.

For extraction, the roots were cut into pieces 1 to 2 cm in length and subsequently macerated in a blender for 60 seconds in a 0.5% sodium hypochlorite solution to release the nematodes and eggs, after which the mixture was poured into hatching chambers. The aqueous suspensions were collected using 500-mesh sieves and transferred to beakers. Collections of J2 were performed every 24 hours using a wash bottle; three collections were required to obtain the necessary quantity of J2.

Mortality Assay of *M. exigua* J2

For the effect of the bacterial isolates on nematode mortality, 700 µL of an aqueous suspension containing 32 newly hatched J2 was placed into each Eppendorf-type microtube, along with 700 µL of a culture suspension of each bacterial isolate. After 24 hours of incubation at 28 ± 2°C, the tubes were removed from the growth chamber, and the J2 were quantified using a Peters counting chamber with the aid of an inverted microscope. For the count, the number of

mobile and immobile J_2 was determined first in the control group, and subsequently in the remaining treatments.

To facilitate the visualization of mobile and immobile J_2 , 200 μ L of a 1% (w/v) sodium hydroxide solution (1 N NaOH) was added to the nematode suspension for 3 minutes; following this period, the mobile and immobile forms were quantified. J_2 that appeared straight and immobile were considered dead, while those that were curved and/or mobile were considered alive.

The percentage of J_2 mortality in each treatment was calculated by comparison with the count obtained in the control group, using the formula $ME (\%) = (D/T) \times 100$, where ME represents the percentage of J_2 mortality, D represents the number of dead J_2 , and T represents the total number of J_2 used in the assay.

The seven most promising bacterial isolates from Experiment 1 were selected for use in Experiment 2. However, these isolates were first identified using molecular techniques.

To this end, the colonies underwent DNA extraction using the Wizard Genomic DNA Purification Kit (Promega, Madison, USA), as recommended by the manufacturer. Total DNA concentration was determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific). The 16S rRNA region was amplified via PCR using the RP1/FD2 primers, followed by sequencing.

The PCR products were purified using the PCR ExoSAP-IT™ kit and sequenced on an ABI PRISM 3100 sequencer. The reagents and equipment used for sequencing the DNA fragments were supplied by Applied Biosystems. Consensus sequences were assembled using the SeqAssem software. All sequences were manually verified and adjusted, and ambiguous nucleotide positions were resolved using sequences from both DNA strands. Fragments of approximately 1,400 bp were obtained. For identification purposes, the consensus sequences were queried against the GenBank database to determine their percentage identity.

As not all bacterial isolates were identified at the species level, additional molecular analyses were performed, as described below:

PCR Amplification: This was performed using the HOT FIREPol® EvaGreen® PCR Supermix (5×) kit (Solis BioDyne), following the manufacturer's recommendations, with adjustments to the annealing conditions specific to each primer (Table 1) in a final reaction volume of 20 μ L. Each reaction mixture contained 4 μ L of Supermix, 0.4 μ L of forward primer (10 μ M), 0.4 μ L of reverse primer (10 μ M), 2 μ L of template DNA, and 13.2 μ L of ultrapure water.

The thermal cycling program, including the annealing conditions specific to each primer, is presented in Table 1.

Table 1. Steps regarding the analysis of six bacterial genes by PCR and the respective protocols for each primer set.

Gene Locus	Primer Name: Sequence 5'-3'	Initial Denaturation	PCR Steps			Final Extension	Amplicon size pb
			Denaturation	Annealing	Extension		
<i>glpF</i>	glpF-F: GCGTTTGTGCTGGTGAAGT glpF-R: CTGCAATCGGAAGGAAGAAG	95°C 5 min	95°C 30s	59°C 30s 30 cycles	72°C 30s	72°C 5min	381 bp
<i>gmk</i>	gmk-F: ATTTAAGTGAGGAAGGGTAGG gmk-R: GCAATGTTCCACCAACCACAA	95°C 5 min	95°C 30s	55°C/56°C 30s 30 cycles	72°C 30s	72°C 5min	504 bp
<i>ilvD</i>	IlvD-F: CGGGGCAAACATTAAGAGAA IlvD-R: GGTCTGTGTCGTTCCATTC	95°C 5 min	95°C 30s	58°C 30s 30 cycles	72°C 30s	72°C 5min	393 bp
<i>Pta</i>	pta-F: GCAGAGCGTTTAGCAAAAGAA pta-R: TGCAATGCGAGTTGCTTCTA	95°C 5 min	95°C 30s	56°C 30s 30 cycles	72°C 30s	72°C 5min	414 bp
<i>pycA</i>	pycA-F: GCGTTAGGTGGAACGAAAG pycA-R: CGCGTCCAAGTTTATGGAAT	95°C 5 min	95°C 30s	57°C 30s 30 cycles	72°C 30s	72°C 5min	363 bp
<i>16S rRNA</i>	341F: CCTACGGGNGGCWGCAG 785R: GACTACHVGGGTATCTAATCC	96°C 2 min	96°C 15 s	50°C 30s 30 cycles	72°C 1 min	72°C 10 min	464 bp

PCR reactions were performed in a Kasvi thermocycler (model K33-20TG).

PCR product purification and agarose gel electrophoresis

The PCR products were purified using the PCR-100 Preps Purification Kit (Ludwig Biotecnologia Ltda.), in accordance with the manufacturer's instructions. The procedure is based on the use of silica adsorption columns and BB, WB, and EB buffers.

Briefly, BB buffer was added to the PCR products at a ratio of 5:1 (BB buffer: sample) corresponding to 175 μ L of BB buffer per 35 μ L of amplified DNA followed by gentle

mixing.

The mixture was then transferred to the adsorption column. Subsequently, the column was subjected to two washes with 600 μ L of WB buffer to remove contaminants and residual ethanol.

The purified DNA was eluted with 50 μ L of EB buffer, applied directly to the column membrane to maximize DNA recovery.

Following purification, the PCR products were analyzed by agarose gel electrophoresis. For each sample, a final volume of 10 μ L was prepared, containing 2 μ L of DNA, 1 μ L of loading dye, and 6 μ L of ultrapure water.

The samples were loaded onto a 1.4% agarose gel

prepared in 1× TAE buffer. The gel was prepared by dissolving 0.84 g of agarose in 60 mL of 1× TAE buffer, followed by the addition of 5 µL of GelRed® nucleic acid gel stain (10,000×) to achieve the final concentration recommended for DNA visualization.

After electrophoresis, the DNA bands were visualized using a UV transilluminator.

Sequencing and Sequence Alignment

For each isolate, three to five housekeeping genes (*glpF*, *ilvD*, *gmk*, *pycA*, and *pta*) were amplified and sequenced, except for isolate B85, for which only the 16S rRNA gene was amplified and sequenced, as indicated in Table 1.

Sequencing was performed using the Sanger method, with both strands (forward and reverse) sequenced for each gene to ensure sequence accuracy. Following sequencing, the raw sequences were edited using BioEdit software, and consensus sequences were assembled using the PREGAP4 and GAP4 tools.

The resulting consensus sequences were subsequently analyzed using BLASTn to assess sequence identity and coverage relative to sequences deposited in the public NCBI (National Center for Biotechnology Information) database.

Statistical Analysis

The J_2 mortality data obtained were analyzed using a generalized linear model (GLM) with a binomial distribution, and means were compared using Tukey's test ($p < 0.05$).

Analyses were performed using R software version 4.1.3 and data from assays 1 and 2 were analyzed jointly. To this end, individual analyses were first conducted, and the Residual Mean Squares (RMS) of the experiments were compared. As the ratio between the largest and smallest RMS was less than 7, a joint analysis of the data was performed.

Experiment 2 (in vitro). Selection and compatibility testing of bacterial isolates antagonistic to *M. exigua*

Experimental Design

Based on the results of the previous experiment, the seven bacterial isolates most effective in causing J_2 mortality were selected to assess their compatibility with one another.

The experimental design employed was a completely randomized design, comprising 9 treatments (7 isolates + positive control + negative control) and four replicates, with each replicate consisting of a single Petri dish.

Compatibility Testing Between Isolates

To evaluate the compatibility among the bacterial isolates, cultures were paired on Kado and Heskett medium using the antibiogram methodology. To this end, five (5) equidistant wells were created; into three of these wells 50 µL of each bacterial isolate adjusted to an optical density $OD_{600} = 0.6$ was added per well. Into the two remaining wells, 50 µL of 2.5% sodium hypochlorite (positive control) and 50 µL of the liquid medium in which the bacteria had been cultured (negative control) were added (Figure 1).

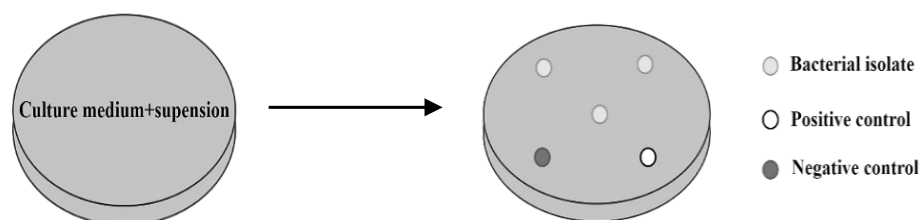


Figure 1. Model for well placement in Petri dishes containing Kado medium for characterizing neutral or antagonistic interactions among seven bacterial isolates (B₈₆, B₈₈, B₁₂₁, B₇₄, B₇₅, B₈₄, and B₈₅) + positive control (2.5% sodium hypochlorite) + negative control (50 µL of the liquid medium in which the bacteria were cultured).

Following incubation at 27 °C for 24 hours in the dark, the presence or absence of inhibition halos was evaluated. All combinations were performed such that each isolate was paired with every other isolate present in the Petri dish or wells.

Experiment 3 (in vivo): Effect of bacterial isolates applied individually or in combination on the population reduction of *M. exigua* (Me) parasitizing tomato plants

The experiment was carried out in a greenhouse at the Center for Agricultural and Engineering Sciences of the Federal University of Espírito Santo (CCAUE-UFES), Alegre – ES, located at an altitude of 250 m, latitude 20° 45' S, and longitude 41° 29' WGr. According to the international Köppen classification, the region's climate is of the "Cwa" type that is, hot and humid tropical with a cold, dry winter. The average annual temperature is 23.1 °C and average annual total precipitation 1,341 mm. During the experimental period,

between December 2022 and January 2023, daily average temperatures ranged between 15°C (minimum) and 32°C (maximum).

The experiment was carried out over a period of 40 days from inoculation to evaluation using a completely randomized design with 11 treatments and 8 replicates. The experiment was repeated once, designated as assays 1 and 2.

The following treatments were tested: T1: negative control (absence of bacteria or Me); T2: negative control (plants inoculated with Me); T3: *Bacillus subtilis* + *B. licheniformis* + *Lactobacillus* sp. + Me; T4: positive control (Fluensulfone) + Me; T5: B₇₄ + Me; T6: B₇₅ + Me; T7: B₈₃ + Me; T8: B₈₄ + Me; T9: B₈₅ + Me; T10: B₈₈ + Me; and T11: B₁₂₁ + Me.

A chemical analysis of the substrate was performed, and the following characteristics were observed: pH (H₂O): 4.24; P: 6.9 mg/dm³; Na: 35.9 mg/dm³; K: 105.9 mg/dm³; Ca: 0.31 cmol_c/dm³; Mg: 0.22 cmol_c/dm³; Al: 0.22 cmol_c/dm³; H+Al: 3.12 cmol_c/dm³; SB: 0.95 cmol_c/dm³; T: 1.17 cmol_c/

dm³; CEC7.0: 4.07 cmol_c/dm³; V: 23.43%; m: 18.73%. Corrections were made following technical recommendations for fertilization and liming specific to the crop.

A pure population of *M. exigua* was obtained as described in Experiment 1.

Four-week-old tomato seedlings (cv. Santa Clara) exhibiting a uniform growth pattern were selected and carefully removed from the substrate to avoid damaging their root systems. Tomato plants were chosen for this study as they serve as a standard reference for susceptibility to *M. exigua*.

To bacterize the tomato seedlings, bacterial isolates were subcultured using an inoculation loop, putting them onto Petri dishes containing Kado medium; these dishes were then placed in a growth chamber at 28°C. After 24 hours, bacterial suspensions were prepared by culturing the bacteria in a rotary shaker for 24 hours.

Prior to transplanting, the seedlings were bacterized by immersing their root systems for 10 minutes in each bacterial suspension, adjusted to an optical density of OD₆₀₀ = 0.3. Seven days later, the plants were inoculated with an initial population (Pi) of 1,000 *M. exigua* J₂ juveniles. For inoculation, an aqueous suspension containing the inoculum was deposited into four holes made with a glass rod in the rhizosphere region of the tomato seedlings; following inoculation, the holes were covered with sterile washed sand.

The chemical nematicide (Fluensulfone) was applied once, at a dosage of 480 g/L, using the drench method as a continuous stream around the base of the stem on both sides of the plant.

The substrate consisted of soil and sand in a 2:1 ratio (v/v), which was previously solarized and contained in plastic pots with a capacity of 1,000 cm³.

Cultural practices, as well as the management of pests and diseases affecting the aerial parts, were carried out in accordance with crop-specific recommendations. Irrigation was managed to maintain soil moisture at approximately 80% of field capacity.

At the conclusion of the experiment, the following evaluations were performed: shoot dry mass (SDM), final population (Fp), and foliar analysis for the following nutrients: N, P, K, Ca, Mg, Fe, Mn, and Zn.

To determine SDM (g), the aerial parts of the plants were separated from the root systems, dried in a forced-air circulation oven at 65°C for 72 hours, and weighed using a

precision balance.

The final population (Fp) was quantified using the technique described by Boneti and Ferraz (1981), and counted in Peters chambers under an optical microscope.

For the nutritional analysis, leaves from four plants per treatment were collected separately, placed in paper bags, and dried in an oven at 65°C until a constant mass was achieved, following the methodology adopted by Salles et al. (2021). Subsequently, the samples were ground in a Wiley mill and submitted to a laboratory for analysis.

The total concentrations of the nutrients listed below were determined as follows: nitrogen (N; g/kg) - sulfuric acid digestion followed by determination via atomic absorption spectrophotometry; phosphorus (P; g/kg) - nitro-perchloric acid digestion followed by determination via spectrophotometry; and potassium (K; g/kg) - nitro-perchloric acid digestion followed by determination via flame photometry. Calcium (Ca; g/kg) and magnesium (Mg; g/kg) - nitro-perchloric digestion with determination by atomic absorption spectrophotometry; iron (Fe; mg/kg), manganese (Mn; mg/kg), and zinc (Zn; mg/kg) - nitro-perchloric digestion with determination by atomic absorption spectrophotometry.

Statistical Analysis

The data were subjected to statistical analysis using the R software.

A non-parametric test (Kruskal-Wallis) equivalent to the DIC was performed for the variables Fp and SDM.

RESULTS AND DISCUSSION

Experiment 1. Effect of isolated bacteria on *M. exigua* mortality

Data from assays 1 and 2 were analyzed jointly.

Several bacterial isolates showed promise regarding *M. exigua* mortality, with the best results obtained using isolates B₈₆, B₁₂₁, B₇₄, B₈₈, B₈₅, B₈₄, and B₇₅. It was observed that isolates B₈₆, B₁₂₁, B₇₄, and B₈₈ were as effective as the positive control (Fluensulfone 480 g L⁻¹), with nematode mortality rates ranging between 75% and 82%, whereas the positive control (Fluensulfone 480 g L⁻¹) resulted in 89% mortality (Figure 2).

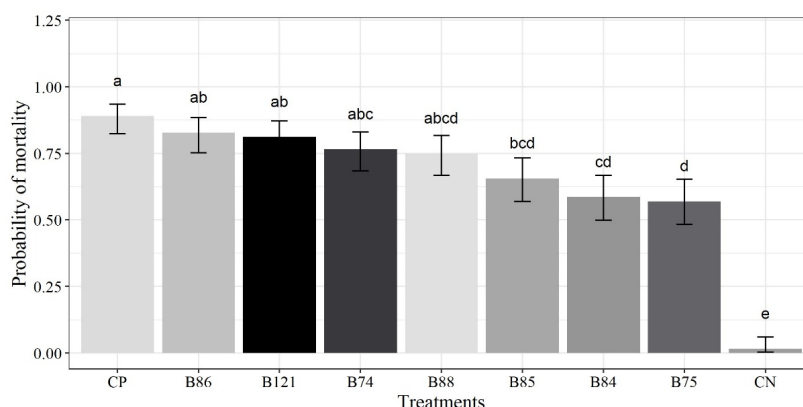


Figure 2. Probability of mortality of *M. exigua* subjected to treatment with different bacterial isolates (B₈₆, B₁₂₁, B₇₄, B₈₈, B₈₅, B₈₄ and B₇₅) for 24 hours. PC: Positive control (Fluensulfone 480 g L⁻¹); NC: Negative control (sterile water).

Isolates B₈₈, B₈₅, B₈₄, and B₇₅ were the least effective and did not differ from one another (Figure 2).

After the efficacy of these seven isolates against *M. exigua* mortality was confirmed, their molecular identification was performed. The bacterial isolates were identified as follows: B₇₄, B₇₅, B₈₄, B₈₆ (*Bacillus* spp.); B₈₈ (*B.*

thuringiensis Berliner); B₈₅ (*Micrococcus* sp.); and B₁₂₁ (*Brevibacterium epidermidis* Collins) (Table 2).

Multilocus sequence analysis was performed for the species-level taxonomic identification of isolates B₇₄, B₇₅, B₈₄, and B₈₆ using housekeeping genes (Table 3).

Table 2. Molecular identification of seven bacterial isolates used in *M. exigua* mortality tests.

Isolates	Molecular Identification	Reference Accession	% Identity
B ₇₄	<i>Bacillus</i> sp.	OP393694.1	99.80%
B ₇₅	<i>Bacillus</i> sp.	MT197315.1	99.22%
B ₈₄	<i>Bacillus</i> sp.	MG786930.1	99.86%
B ₈₅	<i>Micrococcus</i> sp.	MH777900.1	99.71%
B ₈₆	<i>Bacillus</i> sp.	MN733016.1	99.44%
B ₈₈	<i>Bacillus thuringiensis</i>	CP116325.1	99.86%
B ₁₂₁	<i>Brevibacterium epidermidis</i>	KJ575060.1	99.57%

Table 3. Identification of bacterial isolates at the species level based on BLASTn analysis of housekeeping genes.

Isolated	Genetic locus	Encoded protein	Consultation Coverage	% Identity	Species	GenBank Access
B74	<i>glpF</i>	Glycerol uptake facilitator protein	100%	99.81%	<i>Bacillus thuringiensis</i>	CP021061.1
					<i>Bacillus cereus</i>	CP063158.1
	<i>ilvD</i>	Dihydroxyacid dehydratase	99%	99.46%	<i>Bacillus cereus</i>	CP063158.1
	<i>gmk</i>	Guanylate kinase (putative)	100%	99.17%	<i>Bacillus thuringiensis</i>	CP160237.1
	<i>Pta</i>	Phosphate acetyltransferase	100%	99.47%	<i>Bacillus thuringiensis</i> *	CP110109.1
B75	<i>pycA</i>	Pyruvate carboxylase	99%	99.45%	<i>Bacillus cereus</i>	CP063158.1
					<i>Bacillus thuringiensis</i>	CP012099.1
					<i>Bacillus cereus</i>	CP012483.1
	<i>glpF</i>	Glycerol uptake facilitator protein	99%	99.27%	<i>Bacillus thuringiensis</i>	CP083134.1
	<i>ilvD</i>	Dihydroxyacid dehydratase	99%	99.46%	<i>Bacillus wiedmannii</i> *	CP036073.1
B84	<i>gmk</i>	Guanylate kinase (putative)	99%	99.33%	<i>Bacillus wiedmannii</i> *	CP036070.1
					<i>Bacillus cereus</i>	CP032365.1
	<i>Pta</i>	Phosphate acetyltransferase	100%	99.47%	<i>Bacillus cereus</i>	CP123058.1
	<i>pycA</i>	Pyruvate carboxylase	99%	99.45%	<i>Bacillus thuringiensis</i>	CP032365.1
					<i>Bacillus cereus</i>	CP123058.1
B85	<i>glpF</i>	Glycerol uptake facilitator protein	100%	99.81%	<i>Bacillus thuringiensis</i>	CP035727.2
	<i>ilvD</i>	Dihydroxyacid dehydratase	100%	99.46%	<i>Bacillus thuringiensis</i>	CP036073.1
	<i>gmk</i>	Guanylate kinase (putative)	99%	99%	<i>Bacillus cereus</i> *	CP020383.1
	<i>pycA</i>	Pyruvate carboxylase	99%	99.63%	<i>Bacillus thuringiensis</i>	CP132200.1
					<i>Bacillus cereus</i>	CP024655.1
B86	<i>glpF</i>	Glycerol uptake facilitator protein	100%	99.09%	<i>Bacillus cereus</i> *	CP029468.1
	<i>Pta</i>	Phosphate acetyltransferase	100%	99.82%	<i>Bacillus cereus</i> *	CP012483.1
	<i>pycA</i>	Pyruvate carboxylase	99%	99.45%	<i>Bacillus thuringiensis</i>	CP083134.1
					<i>Bacillus cereus</i> *	CP020383.1
					<i>Bacillus cereus</i>	CP185782.1
B88	<i>glpF</i>	Glycerol uptake facilitator protein	100%	99.09%	<i>Bacillus thuringiensis</i>	CP074714.1
	<i>Pta</i>	Phosphate acetyltransferase	100%	99.82%	<i>Bacillus thuringiensis</i>	CP074714.1
	<i>pycA</i>	Pyruvate carboxylase	99%	99.45%	<i>Bacillus cereus</i>	CP029468.1
					<i>Bacillus thuringiensis</i>	CP074714.1
					<i>Bacillus thuringiensis</i>	CP074714.1

* The asterisks indicate the most consistent taxonomic position of the bacterial isolates based on multilocus sequence analysis.

Isolates B₇₄, B₇₅, B₈₄, and B₈₆ were identified using the *glpF*, *ilvD*, *gmK*, *pta*, and *pycA* genes, allowing for their classification within the *Bacillus cereus* sensu lato group.

As expected for this genetically complex group, several loci exhibited limited discriminatory power due to high interspecific similarity. However, consistent with previous reports (PRIEST et al., 2004), the *gmK* locus provided additional taxonomic resolution, corroborating the differential affiliation at the species level among the isolates.

Specifically, isolate B₇₄ was identified as *Bacillus thuringiensis*, isolate B₇₅ as *Bacillus wiedmannii*, and isolates B₈₄ and B₈₆ as *Bacillus cereus*, based on multilocus sequence analysis and the highest sequence identity observed at the discriminatory loci.

In contrast, isolate B₈₅ was taxonomically distinct from the *Bacillus* group. Its identification based on sequence analysis of the 16S rRNA gene, which serves as the standard and well-established molecular marker for identification (DEVOS, 2012) revealed a unique and high sequence identity (99.33%) with *Micrococcus luteus*, thereby enabling its unequivocal assignment at the species level.

Experiment 2. Selection and compatibility testing among bacterial isolates antagonistic to *M. exigua*

There was no antagonistic effect among the combinations of all bacterial isolates studied (Figure 3).

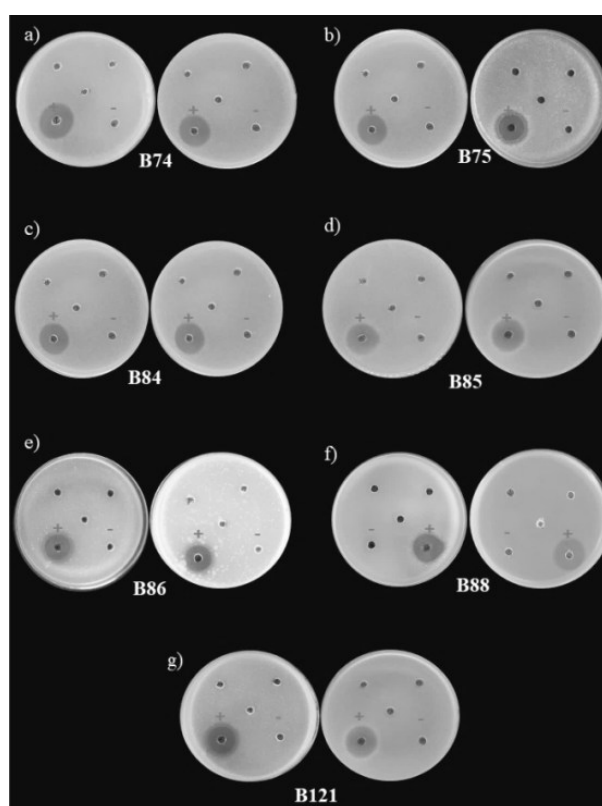


Figure 3. Analysis of compatibility between bacterial isolates using the antibiogram method: a) B₇₄: B₈₆ + B₈₈ + B₁₂₁; B₇₅ + B₈₄ + B₈₅; b) B₇₅: B₇₄ + B₈₄ + B₈₅; B₈₆ + B₈₈ + B₁₂₁; c) B₈₄: B₇₄ + B₇₅ + B₈₅; B₈₆ + B₈₈ + B₁₂₁; d) B₈₅: B₇₄ + B₇₅ + B₈₄; B₈₆ + B₈₈ + B₁₂₁; e) B₈₆: B₇₄ + B₇₅ + B₈₄; B₈₅ + B₈₈ + B₁₂₁; f) B₈₈: B₇₄ + B₇₅ + B₈₄; B₈₅ + B₈₆ + B₁₂₁; g) B₁₂₁: B₇₄ + B₇₅ + B₈₄; B₈₅ + B₈₆ + B₈₄. The positive (+) and negative (-) signs represent the controls. *B₇₄: *Bacillus thuringiensis*; B₇₅: *Bacillus wiedmannii*; B₈₄: *Bacillus cereus*; B₈₅: *Micrococcus luteus*; B₈₆: *Bacillus cereus*; B₈₈: *Bacillus thuringiensis* and B₁₂₁: *Brevibacterium epidermidis*.

Upon examining the results, it is evident that these bacteria possess the potential to be used in combination for the management of plant-parasitic nematodes, as they do not exhibit antagonistic effects toward one another.

Experiment 3 (in vivo): Effect of bacterial isolates applied individually or in combination on the population reduction of *M. exigua*

For the variables Fp and SDM, and for the nutritional assessment in Experiment 1, the normality test (Shapiro-Wilk) indicated that the residuals were not normal at a 5% significance level ($p = 0.005001165$); similarly, the test for

homogeneity of variance, at a 5% significance level, indicated that the variances were not homogeneous.

For Experiment 2, the same pattern was observed for Fp and for the nutritional assessment, for which a non-parametric test was also performed. Regarding SDM, according to the Shapiro-Wilk test at a 5% significance level, the residuals were considered normal; furthermore, regarding the test for homogeneity of variance ($p = 0.08654942$), Bartlett's test at a 5% significance level indicated that the variances were considered homogeneous.

The data on the Fp of *M. exigua* and SDM of plants from assays 1 and 2 are presented in Figure 4.

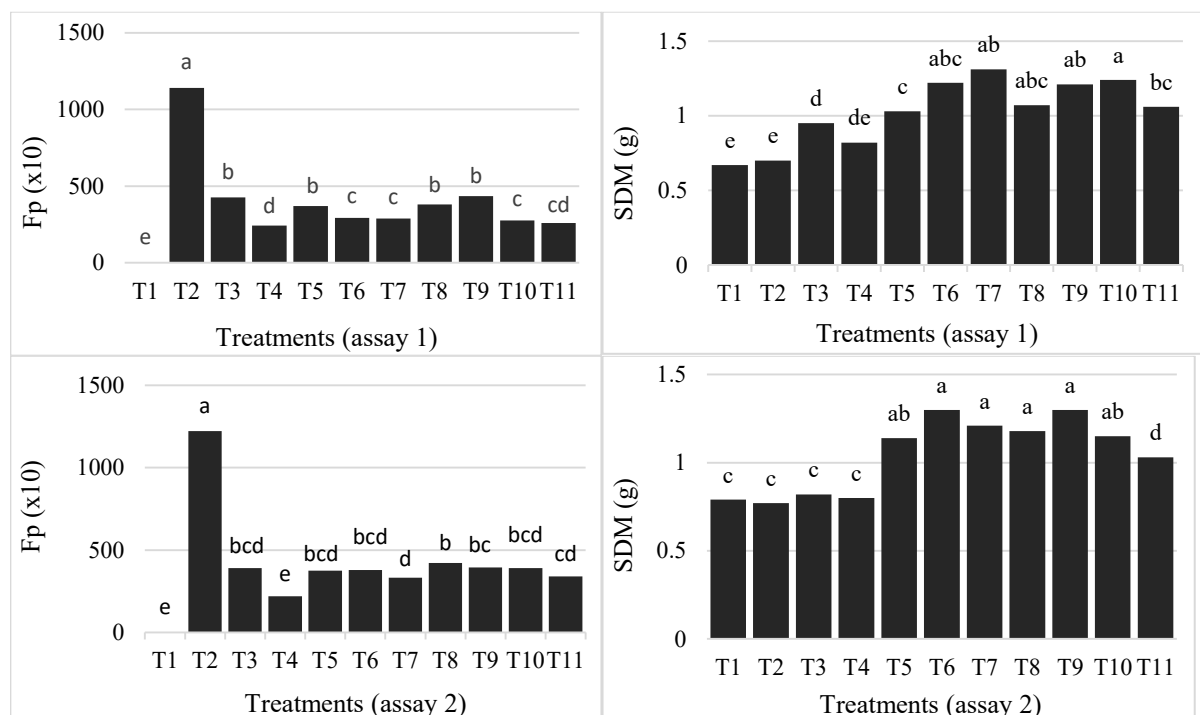


Figure 4. Final population (Fp) of *Meloidogyne exigua* (Me) and shoot dry mass weight (SDM) of tomato plants (cv. Santa Clara) grown in a greenhouse and subjected to the following treatments: T1: negative control (absence of bacteria or Me); T2: negative control (plants inoculated with Me); T3: *Bacillus subtilis* + *B. licheniformis* + *Lactobacillus* sp. + Me; T4: positive control (Fluensulfone) + Me; T5: B₇₄ + Me; T6: B₇₅ + Me; T7: B₈₃ + Me; T8: B₈₄ + Me; T9: B₈₅ + Me; T10: B₈₈ + Me; T11: B₁₂₁ + Me. The data refer to assays 1 and 2.

The *M. exigua* population was reduced in both assays by all treatments, with the best results obtained by T4 (Fluensulfone) and T11 (B₁₂₁), which did not differ significantly from each other (Figure 4). Treatments T6 (B₇₅), T7 (B₈₃), and T10 (B₈₈) also yielded good results in assays 1 and 2 (Figure 4).

In relation to SDM in both assays (1 and 2), T5: B₇₄ + Me, T6: B₇₅ + Me, T7: B₈₃ + Me, T8: B₈₄ + Me, T9: B₈₅ + Me, T10: B₈₈ + Me and T11: B₁₂₁ + Me presented the best results. In assay 1, T3 was also promising (Figure 4).

The concentration of P in tomato leaves is presented in Figure 5.

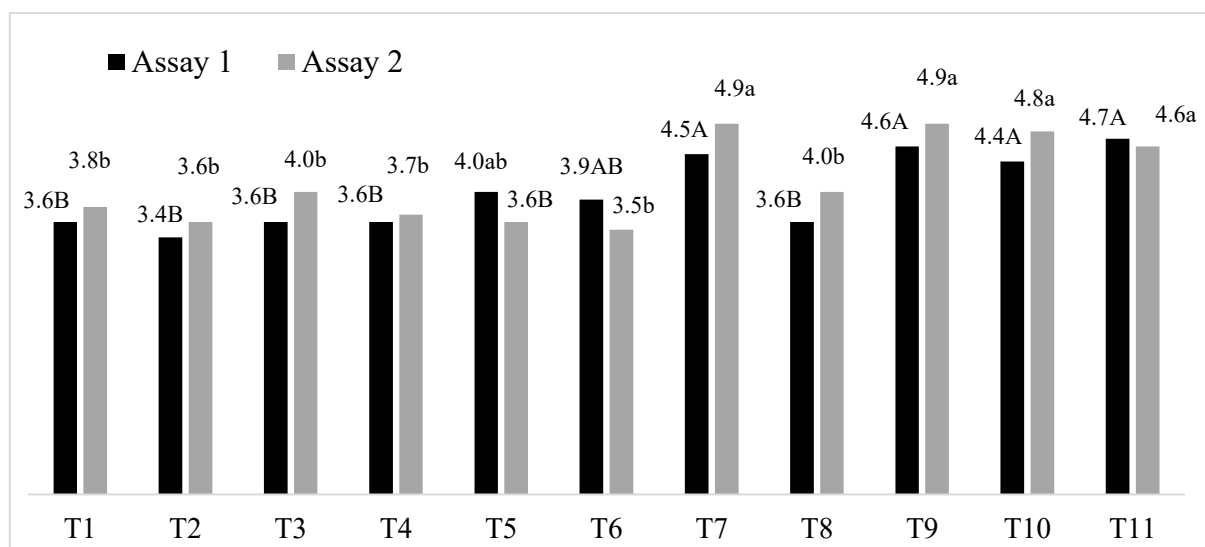


Figure 5. Phosphorus (P) concentration (g/kg) in leaves of tomato plants (cv. Santa Clara) parasitized by *Meloidogyne exigua* (Me) in two assays (Assay 1 and 2) and subjected to the following treatments: T1: Negative control (absence of bacteria or *Meloidogyne exigua* [Me]); T2: Negative control (plants inoculated with Me); T3: *Bacillus subtilis* + *B. licheniformis* + *Lactobacillus* sp. + Me; T4: Positive control (Fluensulfone) + Me; T5: B₇₄ (*Bacillus thuringiensis*) + Me; T6: B₇₅ (*Bacillus wiedmannii*) + Me; T7: B₈₃ + Me; T8: B₈₄ (*Bacillus cereus*) + Me; T9: B₈₅ (*Micrococcus luteus*) + Me; T10: B₈₈ (*Bacillus thuringiensis*) + Me; and T11: B₁₂₁ (*Brevibacterium epidermidis*) + Me. Uppercase letters indicate comparisons of means among treatments in Assay 1, and lowercase letters indicate comparisons among treatments in Assay 2.

As observed in experiments 1 and 3, second-stage juveniles (J_2) of *M. exigua* were affected by the bacterial isolates, likely due to substances such as proteases, chitinases, collagenases, lipases, and enzyme complexes, which can impact different stages of the *M. exigua* life cycle (RADHAKRISHNAN; HASHEM; ABD_ALLAH, 2017). It is worth noting that the *M. exigua* life cycle comprises five phases: first-stage juvenile (J_1), J_2 , J_3 , J_4 , and adult female or male. The J_2 is the infective form; consequently, physiological and/or mechanical damage occurs in plants parasitized by *M. exigua* only when the J_2 successfully penetrate the roots of these plants. Therefore, any control measure that interrupts this or any other stage of the nematode's life cycle is of crucial importance for disease management. Notably, the J_2 stage is one of the most susceptible to control methods, primarily because it is exposed within the soil.

Some authors have reported the toxic effects of substances produced by bacteria on the J_2 of *Meloidogyne* spp. Gattoni, Park and Lawrence (2023), for instance, stated that *Bacillus* spp. increased the mortality of *M. incognita* J_2 by 62.2%, a result attributed to the presence of metabolites. According to Zhang et al. (2022), unlike chemical nematicides, bacterial isolates do not cause environmental damage; rather, they can be safely applied to manage plant-parasitic nematodes through the production of volatile molecules.

The isolate B₈₅ (*Micrococcus luteus* Schroeter) demonstrated promise in inducing mortality in *M. exigua*. According to Wille, Gomes, and Mota (2019), *M. luteus* produces at least one compound associated with the biocontrol of *Meloidogyne* spp. with pathogen mortality levels ranging between 78% and 100%, and inhibition of juvenile hatching between 73% and 100%.

Topalovic et al. (2019) observed between 40 and 200 colony-forming units (CFUs) of the *Micrococcus* sp. isolate K5 adhering to *M. hapla* Chitwood J_2 , affecting juvenile mortality, motility, and hatching. The authors observed a reduction in the invasion of tomato roots by the J_2 s and suggested a beneficial role for *Micrococcus* sp. in soil suppressiveness against *M. hapla*.

Isolate B₁₂₁ (*B. epidermidis*) also yielded promising results regarding the mortality of *M. exigua* J_2 , with a probability of mortality exceeding 75%. There are no reports in the literature regarding the role of this bacterium as a biocontrol agent for *M. exigua*, which underscores the importance of further studies to investigate, in greater depth, its true potential for the management of *Meloidogyne* spp. at the field level.

The effectiveness of bacterial isolates B₇₄ (*Bacillus thuringiensis*), B₈₄ (*Bacillus cereus*), B₈₅ (*Micrococcus luteus*), B₈₆, B₈₈ (*B. thuringiensis*), and B₁₂₁ (*Brevibacterium epidermidis*) in causing the mortality of *M. exigua* J_2 , as observed in Experiment 1 of our study, helps explain the promising results obtained by Cogo (2020) in research conducted in commercial arabica coffee plantations. In addition to their effects on J_2 , metabolites produced by bacteria can induce alterations in the shape, size, or content of the feeding sites established by nematodes within the root system; this leads to poor feeding among the females, thereby impairing their reproduction (CHOUDHARY; PRAKASH; JOHRI, 2007).

It is plausible that a given bacterial isolate exerts not merely a single mode of action against *Meloidogyne* spp., but

rather a combination of events that collectively account for its effectiveness. According to Maulidia et al. (2020), bacteria of the genera *Bacillus*, *Pseudomonas*, *Arthrobacter*, and *Serratia* exhibit nematocidal activity and can cause mortality of *Meloidogyne* sp. of up to 95.46%.

As observed in Experiment 2, the majority of the bacterial isolates belong to the genus *Bacillus*. According to Maulidia et al. (2020), *B. thuringiensis* was the bacterium that most effectively reduced the number of galls and *Meloidogyne* sp. individuals in both roots and soil; furthermore, its primary metabolic products included cholest-5-en-3-ol (3 β)-carbonochloridate.

The compatibility observed among the isolates in Experiment 2 suggests the potential for their combined application in the field for the management of *M. exigua*. According to Migunova and Sasanelli (2021), the combined use of microorganisms for nematode management is a desirable practice, as these antagonists may exhibit distinct modes of action. When utilized in combination, these mechanisms can enhance nematode management efficacy through the production of antibiotics and/or toxins that inhibit juvenile hatching and motility, in addition to modifying root exudates, thereby hindering the nematodes' ability to locate and subsequently infect the roots.

Migunova and Sasanelli (2021) further note that while biological control agents used in isolation may not always provide satisfactory levels of management, the integration of antagonists exhibiting diverse modes of action combined with other management tactics can lead to sustainable strategies for controlling *Meloidogyne* spp., with reduced or eliminated reliance on chemical nematicides. Due to their high toxicity to humans and the environment, as well as their high cost, many chemical nematicides have already been withdrawn from the market. Consequently, microbial and natural products are gaining prominence and represent potential candidates to serve as replacements for chemical agents (ZINGER et al., 2021).

In both assays of Experiment 3, the *M. exigua* population was reduced by all treatments, with the best results obtained by T4 (Fluensulfone) and T11 (B₁₂₁ – *Brevibacterium epidermidis*), which did not differ significantly from one another. Treatments T6 (B₇₅ – *Bacillus wiedmannii*), T7 (B₈₃), and T10 (B₈₈ – *Bacillus thuringiensis*) also yielded good results in assays 1 and 2 (Figures 4 and 5). It is worth noting that these isolates hold potential for inclusion in future biopesticide formulations, as they exhibit no mutual incompatibility. The market for products based on microorganisms antagonistic to nematodes is expanding rapidly, and their technological attributes such as persistence, transportability, storage stability, and shelf life have improved significantly (MIGUNOVA; SASANELLI, 2021). According to these authors, formulations containing two or more bacterial species for the management of *Meloidogyne* spp. are commonly available on the global market.

Despite the practical importance of producing bionematicides that incorporate multiple antagonistic microorganisms with distinct modes of action, most existing commercial formulations are based on a single strain. The primary targets of these products are *Meloidogyne* spp. and *Heterodera* spp.; notably, products based on *Bacillus* spp., *Pseudomonas* spp., and *Serratia* spp. have been identified as the most promising candidates, as they not only suppress nematodes but also stimulate plant growth (XIANG;

LAWRENCE; DONALD, 2018).

The anticipated practical outcome of utilizing microorganisms antagonistic to *Meloidogyne* spp. in field settings is a reduction in the pathogen's population density, coupled with the stimulation of acquired systemic resistance in plants. Chemical pesticides are detrimental to agroecosystems, whereas bacteria constitute important natural components of these ecosystems. The growing need to increase agricultural production and reduce environmental contamination points to a promising future for the use of bacteria in the management of *Meloidogyne* spp. the leading genus of plant-parasitic nematodes worldwide. The multiple 'in situ' modes of action exhibited by bacteria make them a safe and sustainable tool for the management of these pathogens (WOLFGANG et al., 2019).

In Experiment 3, regarding shoot dry mass (SDM) in both assays (1 and 2), treatments T5 ($B_{74} + Me$), T6 ($B_{75} + Me$), T7 ($B_{83} + Me$), T8 ($B_{84} + Me$), T9 ($B_{85} + Me$), T10 ($B_{88} + Me$), and T11 ($B_{121} + Me$) yielded the best results. Treatment T3 (*Bacillus subtilis* + *B. licheniformis* + *Lactobacillus* sp.) also showed promise in increasing dry matter weight in Assay 1 (Figures 4 and 5).

The increase in dry matter accumulation in tomato plants, facilitated by bacterial isolates, is of fundamental importance for ensuring more vigorous initial plant growth, resulting in higher productivity (LINO et al., 2025).

Regarding the nutritional analysis of tomato leaves in Experiment 3, the treatments had no significant effect on the levels of N, K, Ca, Mg, Fe, Mn, or Zn. Conversely, regarding P, treatments T7 (B_{83}), T9 (*Micrococcus luteus*), T10 (*Bacillus thuringiensis*), and T11 (*Brevibacterium epidermidis*) increased the levels of this nutrient relative to the other treatments in Assays 1 and 2; however, they did not differ significantly from one another (Table 2).

Treatments T7, T9, T10, and T11: B_{121} elevated P levels to a range of 4.4 to 4.9 g/kg. According to Poirier, Jaskolowski and Clúa (2022), P is a crucial nutrient for plant metabolism, as it acts directly in cellular energy transfer processes (adenosine triphosphate ATP synthesis), respiration, and photosynthesis, and serves as a structural component of chromosomal nucleic acids, as well as of many coenzymes, phosphoproteins, and phospholipids. Furthermore, P enhances plant water-use efficiency as well as the uptake of other nutrients, thereby fostering robust vegetative development, flowering, and fruiting (HE et al., 2019).

Phosphorus (P) participates in numerous metabolic processes, enhancing plant vigor and reducing the likelihood of phytopathogen occurrence. According to Orr and Nelson (2018), the application of P increased tomato resistance to *Fusarium oxysporum* Link ex Grey the causal agent of Fusarium wilt.

Regarding nematodes that can afflict tomato crops, a reduction in *Meloidogyne* spp. populations has been observed following the application of increasing doses of P to the soil. This is attributed to the fact that this element enhances protein synthesis and cellular activity within plant tissues, thereby ensuring greater plant resistance to the pathogen. P can induce biochemical changes such as increased levels of vitamin C, plant oils, polyphenols, peroxidases, and ammonia, which render the environment more hostile to nematodes, consequently limiting their fecundity and reproduction (MERESA; MATTHYS; KYNDT, 2024).

Phosphorus is a limiting nutrient for plant growth;

although the total P content in most soils is relatively high, the amount of P available to plants is often very low, particularly in weathered tropical soils. Consequently, P must be supplemented in most agricultural soils through the application of chemical fertilizers. On the other hand, it is estimated that a large proportion of the P added to the soil and not removed by crops (>70%) remains in the soil in forms unavailable to plants (PAVINATO et al., 2020). These insoluble P compounds in the soil can be solubilized by organic acids, phosphatases, and chelating agents produced by microorganisms, such as phosphate-solubilizing bacteria (CHEN et al., 2006), notably bacteria belonging to the genera *Bacillus*, *Pseudomonas*, *Azotobacter*, and *Burkholderia* (KALAYU, 2019).

Considering the information presented above, it is plausible that T7 (B_{83}), T9 (*Micrococcus luteus*), T10 (*Bacillus thuringiensis*), and T11 (*Brevibacterium epidermidis*), given that they increased P levels in the plants, are producers of compounds capable of solubilizing this element; however, additional studies are required to confirm this hypothesis.

Future prospects for rhizobacteria-based nematicide formulations are promising, driven by the growth of the bio-inputs market and the quest for sustainable solutions in agriculture. One of the key trends involves the development of multi-species products, a development that underscores the significance of the results obtained in our research.

CONCLUSIONS

Isolates B_{121} (*Brevibacterium epidermidis*), B_{74} (*Bacillus thuringiensis*), B_{86} (*Bacillus cereus*), and B_{88} (*Bacillus thuringiensis*) show potential as biological control agents against *M. exigua* and can be used in combination, as they exhibit no antagonistic effects toward one another. However, field assays are still necessary to validate these results. Isolates B_{75} and B_{84} were identified as *Bacillus wiedmannii* and *Bacillus cereus*, respectively, while B_{85} was identified as *Micrococcus luteus*.

In the 'in vivo' experiment, the *M. exigua* population was reduced by all treatments, with the best results obtained using Fluensulfone and *Brevibacterium epidermidis*.

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