Journal of Invertebrate Pathology 207 (2024) 108213

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Direct effects of *Xenorhabdus* spp. cell-free supernatant on *Meloidogyne incognita* in tomato plants and its impact on entomopathogenic nematodes

M.M. González-Trujillo [a], J. Artal [b], I. Vicente-Díez [a], R. Blanco-Pérez [c], M. Talavera [d], J. Dueñas-Hernani [a], S. Álvarez-Ortega [e], R. Campos-Herrera [a,*]

[a] Departamento de Viticultura. Instituto de las Ciencias de la Vid y del Vino (ICVV: Universidad de La Rioja, CSIC, Gobierno de La Rioja), 26007, Logroño, La Rioja, Spain
[b] ARTAL Smart Agriculture, Pol. Ind. Fuente del Jarro, 46988, Paterna, Valencia, Spain
[c] Departamento de Suelos, Biosistemas y Ecología Agroforestal, Misión Biológica de Galicia (BMG-CSIC), 36143, Pontevedra, Spain
[d] IFAPA Centro Alameda del Obispo, 14004 Córdoba, Spain
[e] Departamento de Biología y Geología, Física y Química Inorgánica, Universidad Rey Juan Carlos, Campus de Móstoles, 28933 Móstoles, Madrid, Spain

ARTICLE INFO

Keywords:
Beneficial soil organisms
Biocontrol
Heterorhabditis bacteriophora
Root-knot nematode
Steinernema feltiae

ABSTRACT

Entomopathogenic *Xenorhabdus* spp. bacteria, symbiont of the nematode *Steinernema* spp., shows potential for mitigating agricultural pests and diseases through bioactive compound production. The plant-parasitic nematode (PPN) *Meloidogyne incognita* affects the yield and quality of numerous crops, causing significant economic losses. We speculate that Cell-Free Supernatants (CFS) from *Xenorhabdus* spp. could reduce the impact of the root-knot nematode (RKN) *M. incognita* without negatively affecting entomopathogenic nematodes (EPNs), which are considered beneficial organisms. This study explored the activity of seven CFS against *M. incognita* (two populations, AL05 and Chipiona) and their possible effects on EPNs. The *in vitro* impact of CFS at 10 %, 40 %, and 90 % concentrations on nematode motility at four and 24 h were tested on the PPN *M. incognita* and two EPNs, *S. feltiae* and *H. bacteriophora.* Additionally, EPN viability and virulence were evaluated at two and five days. On the other hand, tomato plant-mesocosm experiments examined the activity of four CFS on *M. incognita* reproductive capacity and EPN virulence. *In vitro* exposure of *M. incognita* to 90 % concentration of CFS resulted in reductions of activity over 60 % after four hours of expossure in four out of seven CFS. In the *in vitro* evaluation of two species of EPNs, none of the CFS affected the activity across any tested doses after four hours of exposure nor after 24 h. Plant-mesocosm experiments showed that CFS application significantly reduced RKN galls, egg masses, and galling index. However, the virulence of both EPN species decreased 15 days after application, with a significant impact on *S. feltiae*. Overall, these findings suggest that CFS could be used as a bio-tool against *M. incognita* in tomato crops, mitigating its impact on plant growth. However, this study also highlights the necessity of investigating the effects of CFS on non-target organisms.

1. Introduction

Nematodes are a crucial component of soil ecosystems that play a pivotal role in soil food webs by influencing microbial activity and contributing to various functions that enhance soil health (Sánchez-Moreno et al., 2008; Martin and Sprunger, 2022). Among the nematodes, the group entomopathogenic nematodes (EPNs) stands out for their significant role in biological control against a range of arthropod, being the only group of nematodes applied in agricultural soils to artificially increase nematode populations to decrease the pest (Lacey et al., 2015; Salari et al., 2015; Koppenhöfer et al., 2020, Toledo et al., 2023). EPNs are distributed worldwide, except in Antarctica (Hominick, 2002; Kaya et al., 2006; Hazir et al., 2018). EPNs include two families, Steinernematidae and Heterorhabditidae, which are in symbiotic relationships with gram-negative facultative anaerobic bacteria from the *Xenorhabdus* and *Photorhabdus* genera (Enterobacterales: Morganellaceae), respectively (Stock, 2015; Campos-Herrera, 2015; Cevizci et al., 2020). The infective juveniles (IJs) of EPNs are soil-dwelling organisms that actively seek out arthropod hosts by entering through several orifices, such as the mouth, spiracles, anus, or intersegmental membrane, and subsequently release bacteria into the host's hemocoel (Griffin et al., 2005; Stock, 2015; Shapiro-Ilan et al., 2017). These bacteria

* Corresponding author.
E-mail address: raquel.campos@icvv.es (R. Campos-Herrera).

https://doi.org/10.1016/j.jip.2024.108213
Received 19 July 2024; Received in revised form 24 September 2024; Accepted 26 September 2024
Available online 28 September 2024
0022-2011/© 2024 The Author(s). Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

produce toxins that prevent colonization by other organisms and kill the insect host within 24 to 48 h (Dillman et al., 2012; Stock, 2015). EPNs undergo six life stages: egg, four juvenile stages, and adult. While inside the host, they feed on host tissues and can produce one to three generations. Once the nutritional resources are depleted, juveniles restart the symbiotic relationship by capturing bacterial cells and emerge from the host carcass in search of new food sources (Shapiro-Ilan et al., 2017). The infective juvenile (IJ), is the only free-living stage with environmental resistance. It can persist in the soil for long periods without feeding and actively seeking new insect hosts (Mitani et al., 2004; Li et al., 2023a). Hence, most of the life cycle occurs within the host insect (Stuart et al., 2006).

On the other hand, plant-parasitic nematodes (PPNs) pose a significant threat to agriculture, causing significant yield and quality losses in crops worldwide (Koenning et al., 1999; Singh et al., 2015). These losses can amount to an estimated annual sum of up to $173 billion (Elling, 2013). Among the most harmful nematodes are those belonging to the genus *Meloidogyne* (Tylenchida: Heteroderidae), comprising obligate endoparasitic nematodes called Root-Knot Nematodes (RKN). These nematodes alter plant cell growth, infect roots, hinder water and nutrient absorption, and disrupt translocation, causing symptoms like growth retardation, lack of vigor, and wilting (Moens et al., 2009). Initial infections may not exhibit visible symptoms, often mimicking other forms of damage or nutrient deficiencies (Smiley and Nicol, 2009; Chen et al., 2020). RKN species can infect over 2000 plant species. *M. incognita* is globally distributed, affecting a wide range of vegetables and other than food, including fiber, oil, ornamental, and industrial crops (Perry et al., 2009).

Producers have historically relied on chemically synthesized nematicides as the primary control method to address the significant losses caused by PPNs. However, several countries have restricted the use of many nematicides due to their detrimental environmental and human health impacts (Sasanelli et al., 2021). Overall, the extensive application of these synthetic chemical compounds has led to adverse effects on ecosystems (Thomas, 1996), impacting beneficial organisms (Stirling, 2014), affecting the abundance and diversity of free-living nematodes (Waldo et al., 2019; Grabau et al., 2020), contaminating soil and groundwater (Gullino et al., 2003), and posing risks to animals and human health (Jang et al., 2003; Gemmill et al., 2013).

Current integrated pest management (IPM) strategies to control RKNs focus on incorporating multiple methods. These approaches include the introduction of new nematode-resistant cultivars, the use of biological control agents, and the incorporation of by-products derived from various organisms (Ntalli and Caboni, 2012; Mukhtar et al., 2016; Burns et al., 2023). The use of EPNs and their by-products, derived from the symbiotically associated bacteria, has been reported as a potential tool in the management of PPNs such as *Meloidogyne* (Samaliev et al., 2000; Lewis and Grewal, 2004; Kusakabe et al., 2022). While the efficacy of EPNs alone in controlling PPNs might not be as potent as chemical control methods, their presence could influence the foraging behavior of PPNs, inhibiting root penetration and subsequently decreasing their impact on plants (Felicitas et al., 2021; Li et al., 2023b). Furthermore, bacterial by-products have demonstrated significant effectiveness against PPNs, especially those derived from *Xenorhabdus*. *In vitro* studies have shown that exposure to CFS at different concentrations and periods can reduce RKN egg hatching (Samaliev et al., 2000; Sasnarukkit et al., 2002; Sayedain et al., 2019), affect the RKN penetration of second-stage juveniles (J_2) into tomato roots (Sasnarukkit et al., 2002), and reduce the galling index and decrease the number of egg masses while increasing total yield in tomato plants (Kepenekci et al., 2018). While these studies have primarily focused on the effects of CFS against PPNs, few have investigated the adverse effects on non-target organisms. For example, Boina et al. (2008) observed that *Caenorhabditis elegans* was negatively affected by synthetic analog of certain compounds regularly obtained in CFS derived from *Photorhabdus,* although the EPN *H. bacteriophora* was not affected. This result is in contrast to those by Kusakabe et al. (2022) that showed that CFS from another *Photorhabdus* species, *P. luminescens sonorensis* increased the mortality of *M. incognita* across various concentrations and periods, with minimal adverse effects on the non-target organisms *C. elegans* and the EPN *S. carpocapsae, H. sonorensis* and *H. bacteriophora*. It seems a priority to extend such research to assess the broader ecological impacts of using CFS as a potential tool for PPN management.

Therefore, this study aimed to screen CFS from various *Xenorhabdus* spp. for their efficacy in managing *M. incognita* in tomato plants as a model crop while investigating potential non-target effects on beneficial soil organisms like EPNs. Specifically, our objectives were to evaluate (i) the impact of CFS produced by *Xenorhabdus* spp. at different concentrations on *M. incognita*, *S. feltiae*, and *H. bacteriophora* activity, (ii) the impact of CFS on the viability and virulence of the beneficial organisms *S. feltiae* and *H. bacteriophora*, (iii) the capability of *M. incognita* to reproduce on tomato plants after the exposure to CFS *in planta*-mesocosm, and (iv) EPNs virulence after exposure to CFS in the same *in planta*-mesocosm set-up. We hypothesized that applying CFS of various *Xenorhabdus* strains would reduce the impact of *M. incognita* on the plant in a species-specific manner. We also hypothesize that these compounds will not adversely affect other members of the soil biota with beneficial actions, such as EPNs. The tomato was selected as a model plant due to its susceptibility to *M. incognita* infection (Singh and Khurma, 2007) and because it was established as a model for studying plant and fruit physiology (Costa and Heuvelink, 2018).

2. Materials and methods

2.1. Plant material and nematode cultures

Tomato seeds (*Solanum lycopersicum* L.) cv. Moneymaker (Germisem Sementes Lda. Oliveira do Hospital, Portugal), lacking the resistance gene (*Mi*-1.2 gene), were initially cultivated in seedbeds containing a mixture of sterile fine sand and perlite in a 2:1 ratio for four weeks. Upon reaching a height of 10 cm with 3 to 4 true leaves in the fifth week, they were transplanted into 9 × 9 × 9.5 cm pots filled with a mixture of soil from Valdegon (geographical coordinates, 42.466611, −2.292674, Logroño, La Rioja, Spain) (Sand = 56.3 %, Silt = 33.9 %, Clay = 9.8 %, pH = 8.4, OM = 0.41 %, and EC = 0.12 mmhos/cm), and fine pure sand, both autoclaved twice in a 3:1 ratio. All plants were placed in chambers under controlled conditions (16:8 light: darkness photoperiod, 25 °C ± 1 °C, and 60 % RH).

M. incognita populations were obtained from two Spanish localities in Cádiz and Almería (Andalusia, Spain), named “Chipiona” and “AL05”, using those as model for this PPN species (Table 1). Each population was derived as pure culture after culturing one egg mass on tomato cv. Moneymaker plants under controlled conditions (16:8 light: darkness

Table 1
Species of nematodes of the genus *Steinernema* and their symbiont bacteria, *Heterorhabditis bacteriophora* and *Meloidogyne incognita (*Chipiona and AL05 populations).

Nematodes species	Population	ITS region GenBank accession	Associated bacteria	16S rRNA GenBank accession
Steinernema feltiae	AM-25	MG551674	*X. bovieni*	MW574909
	AM-75	MG551675		MW467378
	RM-107	MW480131		MW467374
	VM-25	MW480136		MW574907
	VM-31	MW574912		MW574905
Steinernema affine	VO-53	MW480137	*X. bovieni*	MW467379
Steinernema riojaense	RM-30	MK503133	*X. kozodoii*	MW467375
Heterorhabditis bacteriophora	VM-21	MW480135	–	–
Meloidogyne incognita	Chipiona	OQ305837	–	–
	AL05	PP481898	–	–

photoperiod, 25 °C ± 1 °C, and 60 % RH). Molecular identification of one egg mass (in three individual and independent DNA extractions) using procedures described by Vicente-Díez et al. (2021a) confirmed their identity (Table 1). The *M. incognita* populations were maintained by inoculating 4–6 new tomato plants every two months with eggs extracted from infested tomato roots. All plants were maintained under controlled conditions (16:8 light: darkness photoperiod, 25 ± 1 °C, and 60 % RH).

Nematode eggs were extracted from infected tomato roots by stirring them in a 1 % NaClO solution. The egg suspension was concentrated on a 20 µm sieve and washed with tap water over 50 ml Falcon tubes to be used as inoculum in subsequent experiments. Egg concentration in the suspensions was estimated by counting them in a nematode counting slide under a stereoscope (Motic® SMZ-161 Series Stereo Zoom Microscopes, Barcelona, Spain) (Hussey and Barker, 1973). Juvenile inoculum: For experiments on nematode activity, egg suspensions were placed on Baermann funnels to obtain second-stage juveniles (J_2). Juveniles hatching within 48 h were stored in aqueous suspensions and used as inoculum.

Two EPN populations, *S. feltiae* 107 and *H. bacteriophora* VM-21, were used (Table 1), both isolated from vineyards in La Rioja in previous studies (Blanco-Pérez et al., 2020, 2022). The molecular identification was performed as described by Vicente-Díez et al. (2021a). Briefly, about 500 IJs were mechanically disaggregated using sterile blue pestles. DNA was then extracted using the Speedtools kit (Biotools, Madrid, Spain), analyzed for quality and quantity using a Nanodrop spectrophotometer, and stored at −20 °C until use. The ITS region of the rDNA of each EPN species was amplified using universal primers, including a negative control with mQ water instead of DNA. PCR was verified by 2 % agarose gel electrophoresis, and the resulting bands were cleaned, sequenced, aligned, and compared with sequences in Blast, before submission to Genbank (Vicente-Díez et al., 2021a). These EPN populations were propagated in larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) reared at the Instituto de las Ciencias de la Vid y del Vino (ICVV, Logroño, Spain) using protocols and diet described by Vicente-Díez et al. (2021b). EPN population was multiplied within five final *G. mellonella* instars placed in 5.5 cm diameter Petri dishes with Whatman no. 1 paper (in duplicate). Each dish was inoculated with a 400 µl suspension of infective juveniles (IJs) for each EPN species. The dishes were then kept in the dark at 25 °C ± 1 °C. After 72–96 h, the cadavers were arranged in a star shape to facilitate EPN emergence and were placed in a 9 cm diameter Petri dish with tap water (Woodring and Kaya, 1988). The emergence time for *S. feltiae* was 10 to 12 days, while for *H. bacteriophora* ranged from 15 to 18 days. The IJs were stored in 80–100 ml tap water in 250 ml cell culture flasks within chambers at a temperature of 14 °C in darkness. New cultures and fresh nematodes harvested within 15–20 days maximum were employed in each trial to ensure the reproducibility of the studies.

2.2. Bacteria isolation and production of the cell-free supernatant

The symbiotic bacteria derived from EPN (Table 1) were isolated following procedures described by Vicente-Díez et al. (2021a, 2021b). Briefly, approximately 500 IJs of each EPN population were exposed to 5 % NaClO solution for 2–5 min, washed with distilled water, and disaggregated in a 50:50 (v/v) distilled water and Nutrient Broth (VWR Chemicals, Barcelona, Spain) suspension. Each nematode-bacterium complex suspension (50 µl) was plated in Petri dishes with Nutrient Brothymol Blue Agar (NBTA) prepared with Nutrient Agar (VWR Chemicals, Barcelona, Spain), 2,3,5-Triphenyl tetrazolium chloride (TTC) (VWR, Chemicals, Leuven, Belgium), and Ampicillin (AM) (50 mg/ml) (Thermo Scientific, Kandel, Germany). Plates were incubated for 48 h under controlled conditions (25 ± 2 °C, 20 % RH in the dark). All pure bacteria isolates were stored in darkness at 4 °C and refreshed every week. Additionally, aliquots of each pure culture were stored in glycerol at −80 °C (Vicente-Díez et al., 2021a).

To obtain the CFS of each strain, a single colony from each evaluated bacterium was inoculated in Tryptone Soy Broth (TSB) (PanReac AppliChem, ITW Reagents, Barcelona, Spain) and incubated at 25 ± 2 °C overnight with orbital agitation at 150 rpm (J.P. Selecta s.a.u, Rotabit 3000974, Barcelona, Spain). Subsequently, 1 ml of colony suspension was inoculated in 250 ml of TSB within a 500 ml Erlenmeyer flask. The mixture was then incubated for 72 h on the orbital shaker at 150 rpm at 25 ± 2 °C and under dark conditions. The CFSs were obtained by centrifugation of these cultures for 20 min at 68,905 X g (Thermo Scientific™ Sorvall LYNX 4000 Superspeed Centrifuge, Fisher Scientific SL, Madrid, Spain) at 4 °C. The supernatant was filtered through a 0.22 µm sterile pore filter (Ansari et al., 2003; Sayedain et al., 2019; Vicente-Díez et al., 2021a). The obtained filtrates were cultured on NBTA Petri dishes to confirm the absence of bacteria. A new CFS was generated for each trial to ensure the reproducibility of the studies. The TSB medium was filtrated for the control treatments through a 0.22 µm sterile pore filter to ensure the same conditions.

2.3. Screening of bacterial cell-free supernatant activity against nematodes in liquid suspension

Initially, a screening of CFS activity from seven *Xenorhabdus* spp. associated bacteria with the EPN species *S. feltiae*, *S. affine*, and *S. riojaense* was conducted to determine the toxicity against the J_2 stage of the PPN *M. incognita* (Chipiona and AL05 population) and the IJs of two EPNs species (*S. feltiae* and *H. bacteriophora*) to explore the non-target effects (Table 1).

First, to maintain the nematodes free of contaminations before combination with the treatment, all individuals were exposed for 3 min to a 1 % NaClO solution (commercial bleach). Then, they were washed with autoclaved distilled water for 3 min, repeating this procedure three times. A concentration of approximately 100 nematodes (J_2 for PPN and IJ for EPN per dish, described as "NEM") was prepared to be combined with the treatments. Three sterile Petri dishes with 3.5 cm diameter were used as experiment units, and the nematode-treatment mixture resulted in a final volume of 1500 µL per dish. The treatments were as follows (Table 2): (i) CFS-10 % NEM-bleach, (ii) CFS-40 % NEM-bleach, (iii) CFS-90 % NEM-bleach, (iv) TSB-10 % NEM-bleach, (v) TSB-40 % NEM-bleach, (vi) TSB-90 % NEM-bleach, (vii) control NEM-bleach in water, and an additional control treatment (viii) NEM without bleach in water, with the aim to compare with the NEM-bleach in water to verify that the procedure did not harm the individuals. Nematodes were counted using a counting plate to estimate IJs or J_2 (depending on the target nematode group, EPN or PPN, respectively) per dish after 0, 4, and 24 h, registering live (moving, active) and inactive (paralyzed or dead) nematodes. Each straight nematode was touched with a fine needle three times to test activity. The results of CFS exposure were expressed as the percentage of inactive nematodes (no mobile, paralyzed, or dead) after 4 and 24 h of exposure. After the initial trial (Fig. S1, Table S1), only the four most promising CFS were selected for subsequent trials, with new PPN and

Table 2
Description of the experimental design treatments (code) and their correspondence with the used solution (Cell-Free Supernatant, CFS, Tryptic Soy Broth, TSB, or water), concentration, and the presence of 1% NaClO (bleach) for surface sterilization for plant-parasitic nematodes and entomopathogenic nematodes *in vitro* studies.

Code	Treatment	Concentration (%)	Bleach (1 %)
i	CFS	10	Yes
ii	CFS	40	Yes
iii	CFS	90	Yes
iv	TSB	10	Yes
v	TSB	40	Yes
vi	TSB	90	Yes
vii	water	–	Yes
viii	water	–	No

EPN batches and fresh CFS for each replicate (trial 1 and trial 2).

Additionally, the impact of the four selected CFSs on the EPN virulence in the last instar larvae of *G. mellonella* was assessed following the methodology described by Campos-Herrera et al. (2023a). Briefly, for each treatment, five 5.5 cm diameter Petri dishes were prepared, each with filter paper on both sides moistened with 250 ml of tap water. Then, the IJs exposed to the previously described treatments for 24 h were placed on the filter paper at a final concentration of 50 IJs per dish. Five larvae were included per dich, and trials were incubated in the dark for five days at 22 °C. A control treatment with only water was a negative control to confirm larval survival. Additionally, controls with only CFS application were performed to ensure no impact on the insects. Larval mortality was evaluated after 3 and 5 days. To confirm the death of *G. mellonella* by EPNs, all cadavers were placed in 5.5 cm diameter Petri dishes on filter paper, and the color and the typical symptoms of EPN infection were checked after 72 h. These assays were performed twice at different times, with new EPN batches and freshly prepared CFS for each replicate.

2.4. Evaluation of bacterial cell-free supernatant activity against nematodes in tomato plants

The experiments were conducted using 9 × 9 × 9.5 cm pots (769.5 cm^3), each containing a 10 cm tall plant with 3–4 true leaves, serving as the experimental unit. The experimental design was the same for *M. incognita* than for EPN, so we will describe the treatments containing nematodes (NEM). The treatments (n = 6) included (i) control (only water), (ii) NEM only in water, (iii) NEM with 10 ml of the CFS, and (iv) NEM with 20 ml of CFS. The CFS was applied right after the nematode application to the corresponding treatments on the day of the experiment setup and again after seven days post-inoculation. The PPNs were inoculated at 2000 eggs per pot (Refaei et al., 2007), while the EPNs were applied at 3000 IJs per pot (Schroeder et al., 1996).

Plants were maintained in chambers under controlled conditions (16:8 light: darkness photoperiod, 25 ± 1 °C, and 60 % RH). A randomized split-plot design was employed to ensure consistent conditions across all treatments. Three pots per treatment were retrieved for evaluation at each of the two-time points during the experiment.

The impact of CFS on PPNs was assessed after 30- and 60-days post-inoculation by counting the number of galls and egg masses per plant. Brilliant Blue FCF Erioglaucin dye (AppliChem Panreac ITQ Companies, Barcelona, Spain) was used to stain the egg masses for visualization (Premachandran et al., 1988). Additionally, root damage was evaluated using the scale defined by Bridge and Page (1980) at 60 days post-exposure. For the EPN activity assessment, the impact of CFS was evaluated after 15 and 30 days. The fresh soil (200 g per plant) was placed in closed containers, and ten *G. mellonella* larvae were inoculated. After four days, larval mortality was recorded (Blanco-Pérez et al., 2022). Additionally, late mortality of the alive *G. mellonella* was recorded after 24 h of the larval mortality revision. To confirm the death of *G. mellonella* by EPNs, they were placed in 5.5 cm diameter Petri dishes on filter paper, and the color and the typical symptoms of EPN infection were checked after 72 h. Finally, all plants were dried in an oven at 40 °C for a week, and the dry weight of the aerial and root parts was recorded.

2.5. Statistical analysis

For experiments conducted in liquid suspension, the variables analyzed were the percentage of inactivity (PPNs and EPNs) after 4- and 24 h post-exposure and the virulence of EPNs (larval mortality percentage in *G. mellonella*) after two- and five-days post-exposure. Inactivity was corrected for the treatments evaluated using the modified Abbot formula (PEAm) (using Inactivity values instead of Mortality): corrected inactivity % = [(% Inactivity in a treated dish − Inactivity % in control dish) / (100 – Inactivity % in control dish)] * 100 (Cabello, 1997). In this case, we averaged all the control treatments in each trial because these were not significantly different, to allow the calculation of the corrected inactivity. For the plant experiments, the variables analyzed included the number of galls and egg masses per gram of fresh root (for 30- and 60 days post-exposure) and the galling index (visual scale of 0—10) (Bridge and Page, 1980) after 60 days post-exposure. For the EPN, the larval mortality percentage was evaluated for each time (15 and 30 days). The dry weight of the plant's roots and aerial parts was also measured.

The results were analyzed by verifying the assumption of normality and homogeneity of variances for the generalized linear mixed model (GLMM). In the case of the PPN, the model included the PPN population (Chipiona and AL05) as a random effect. For the infectivity analysis, a logistic regression model was used to validate the dispersion of the data. All statistical analyses were performed using R studio software version 3.4.2 (R Core Team 2023), GLMM tests using the *glmer* function from the lme4 package (Bates et al., 2015). Post-hoc for-significance analyses ($P < 0.05$) were performed using the estimated marginal means (EMMeans) approach with the *emmeans* function (Lenth, 2021).

3. Results

3.1. Screening of bacterial cell-free supernatant activity against nematodes in liquid suspension

In the initial trial, three out of seven CFSs tested failed to make inactive (paralyzed or dead) PPNs and were excluded from further studies (Fig. S1, Table S1). Overall, the four remaining CFSs strongly negatively impacted on *M. incognita*, causing its inactivity after 4 h (Fig. 1A, Table S2). Significant differences were found in the interaction between the treatments and doses evaluated ($P < 0.001$). Notably, all concentrations caused J_2 inactivity, with the 40 % and 90 % concentrations exerting the most significant influence on the evaluated treatments (Fig. 1A, Table S2). Significant differences were only found at the 40 % dose for the *Xb*_VO53 treatment compared to the other CFSs ($P < 0.001$). A marginal difference was found for *Xb*_VO53 at the 90 % concentration ($P = 0.165$). A similar trend was observed for the treatments after 24 h (Fig. S2A, Table S2).

In the evaluation of EPNs, none of the four CFSs affected the activity across any of the tested doses after four hours of exposure (Fig. 1B and C, Table S2) nor after 24 h (Fig. S2B and C, Table S2). In addition, CFS did not affect the virulence against *G. mellonella* larvae at doses of 40 and 90 % after three (Fig. S3A and B, Table S3) or five days (Fig. 2, Table S3) after application for both species, indicating their equal effectiveness. Significant differences were only found concerning the different concentrations *versus* the control for *S. feltiae* ($P < 0.001$) and *H. bacteriophora* ($P < 0.001$) (Fig. 2A and 2B, Table S3). No significant differences were observed between the evaluated doses of 40 % and 90 % ($P = 0.191$) and the interaction with CFS ($P = 0.324$) (Fig. 2A and 2B, Table S3). A similar trend was observed after three days of exposure (Fig. S3A and B, Table S3).

3.2. Evaluation of bacterial cell-free supernatant activity in tomato plants: Impact on plant-parasitic nematodes

Regarding the impact on PPN, measured *in vivo* experiments, the application of CFSs reduced the impact of PPN on plants after 30 days (Fig. S4, Table S4) and after 60 days (Fig. 3, Table S4). While no significant differences were found among the four CFSs for the number of galls ($P = 0.491$), *Xb*_AM75 exhibited the highest reduction in gall number per root at the highest dose (Fig. 3A). The interaction was not significant ($P = 0.671$). Still, the dose significantly affected the reduction in gall production ($P < 0.001$) (Fig. 3A, Table S4). For egg mass production, only the dose was found to affect this parameter ($P < 0.001$, Fig. 3B, Table S4). The CFS that resulted in the most significant reduction was *Xb*_AM75 for the 20 ml dose. There were no significant

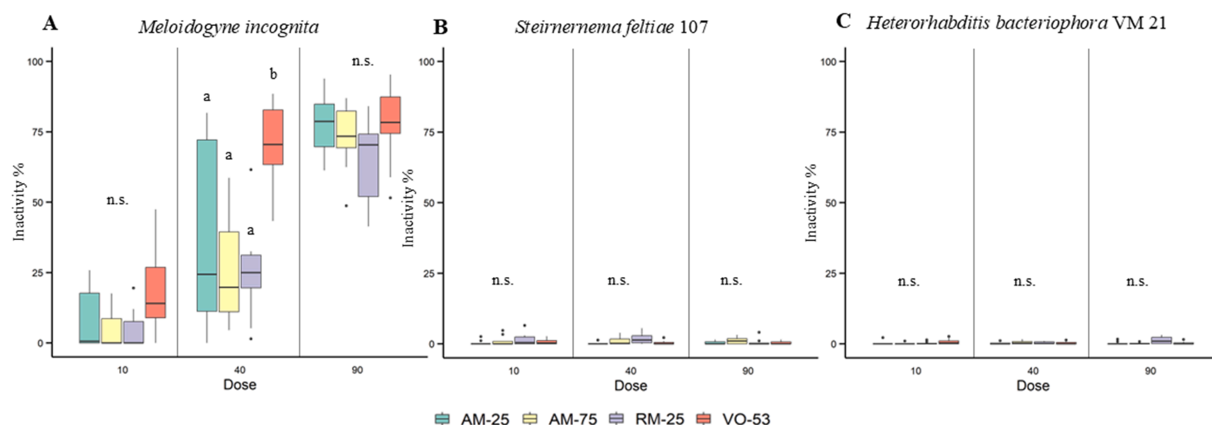

Fig. 1. Effect of four cell-free supernatants (CFS) on nematode inactivity after four-hours exposure under three concentrations. **A.** The plant-parasitic nematode, *Meloidogyne incognita* (AL05 and Chipiona both populations). **B** and **C** represent entomopathogenic nematodes (*Steinernema feltiae* 107 and *Heterorhabditis bacteriophora* VM 21, respectively). Different letters indicate significant differences for GLMM tests at $P < 0.05$; n.s., not significant.

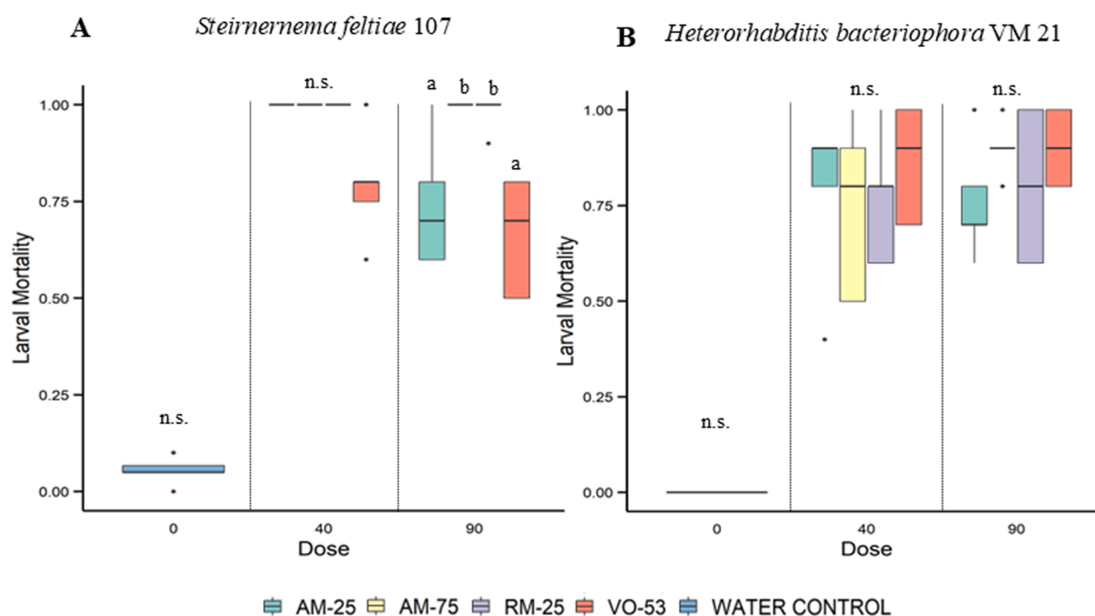

Fig. 2. Effect of four cell-free supernatant (CFS) on entomopathogenic nematode exposure for different concentrations after five days post-exposure. **A.** Mortality of *Galleria mellonella* larvae by *Steinernema feltiae* 107. **B.** Mortality of *G. mellonella* larvae by *Heterorhabditis bacteriophora* VM 21. Different letters indicate significant differences for Logistic regresion model at $P < 0.05$; n.s., not significant.

differences in egg mass production among the CFSs ($P = 0.232$, Fig. 3B), and no significant interaction between dose and CFSs was observed ($P = 0.515$, Fig. 3B). A similar trend was observed for the number of galls and egg masses after 30 days (Fig. S4A and B, Table S4).

Analysis of the galling index revealed that all four CFS reduced the proportion of galls as the dose increased (Fig. 3C, Table S4). Significant differences were found both between CFSs ($P = 0.013$) and between the doses ($P < 0.001$), with no significant interaction ($P = 0.055$). At the 10 ml dose, one difference was found among the CFS, with *Xb*_RM25 having a lower index than *Xb*_AM75 ($P = 0.032$). For the 20 ml dose, no significant differences were found among the CFSs. However, *Xb*_VO53 exhibited the greatest reduction in the galling index (Fig. 3C).

After 60 days post-exposure, only the dose had a significant effect ($P < 0.001$) on the dry weight of the aerial part of the plants, increasing the dry weight with increasing dose evaluated (10 and 20 ml) against the control ($P < 0.001$) (Fig. 3D, Table S4). However, no significant differences were found among the CFSs at any of the doses evaluated, although there was a tendency to increase plant dry weight with the CFS *Xb*_RM25 and *Xb*_VO53. CFSs did not affect the dry weight of roots ($P = 0.329$), but the dose had a significant effect on root dry weight ($P < 0.001$). No significant differences were found in the interaction of CFS and dose ($P = 0.086$) (Fig. 3E, Table S4). A similar trend was observed for the plants after 30 days post-exposure (Fig. S4A and B, Table S4).

3.3. Evaluation of bacterial cell-free supernatant activity in tomato plants: Impact on entomopathogenic nematodes

The results found in the *in vivo* experiments showed that the application of the four CFSs resulted in a reduction in virulence against *G. mellonella* larvae for both species of EPNs after 15 days of application (Fig. 4, Table S5). However, that trend was not maintained after 30 days because the overall EPN virulence was reduced (Fig S5, Table S5). After 15 days of exposure, a more pronounced reduction was observed in *S. feltiae* ($P < 0.001$). Notably, *Xb*_RM25 had the greatest negative effect in both populations (*S. feltiae* and *H. bacteriophora*), followed by *Xb*_VO53, while CFS *Xb*_AM75 exhibited the least effect on both populations. Significant effects of dose were observed for *S. feltiae* ($P < 0.001$) but not on *H. bacteriophora* ($P = 0.021$). Furthermore, the

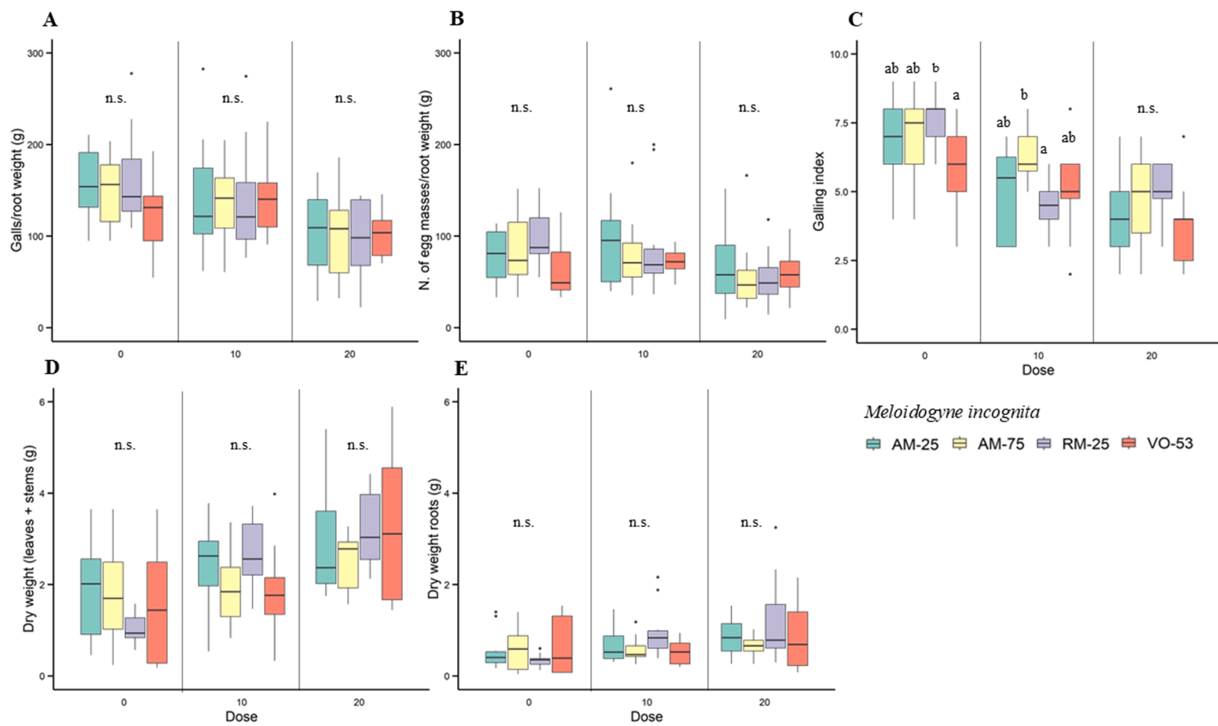

Fig. 3. Effect of four cell-free supernatant (CFS) on plant-parasitic nematode, *Meloidogyne incognita* (AL05 and Chipiona both populations) after 60-day exposure. **A.** Number of galls/root weight (g). **B.** Number of egg masses/root weight (g). **C.** Gall index scale. **D.** Aerial (leave and stem) dry weight (g), and **E.** Root dry weight (g). Different letters indicate significant differences for GLMM tests at $P < 0.05$; n.s., not significant.

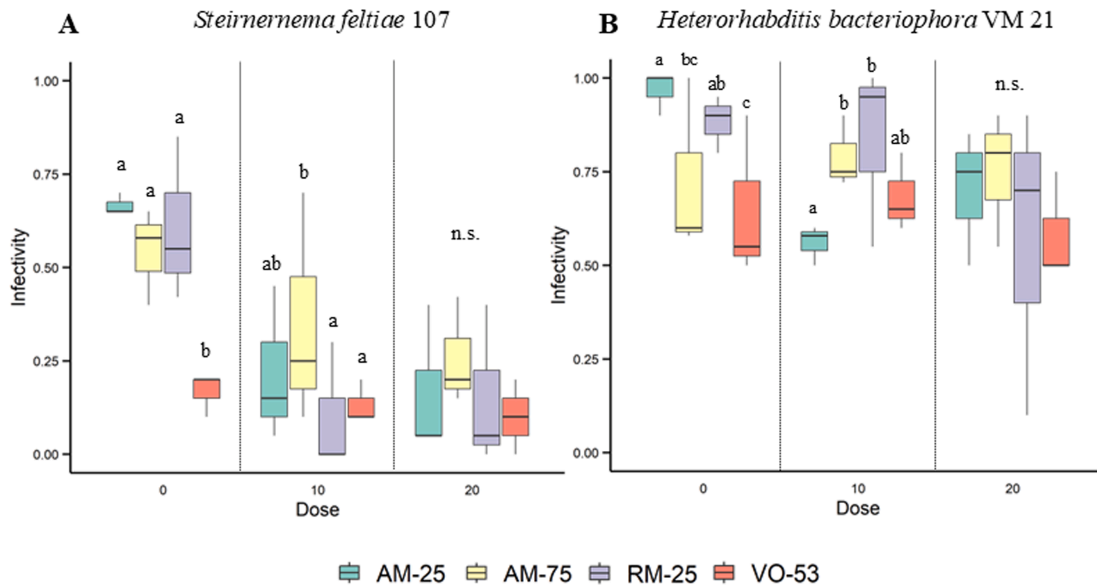

Fig. 4. Effect of four cell-free supernatant (CFS) on entomopathogenic nematode exposure for different concentrations after 15 days post-exposure. **A.** Infectivity of *Steinernema feltiae* 107 in *Galleria mellonella* larvae. **B.** Infectivity of *Heterorhabditis bacteriophora* VM 21 in *G. mellonella* larvae. Different letters indicate significant differences for Logistic regression model at $P < 0.05$; n.s., not significant.

interaction between dose and CFS showed a significant effect on both *S. feltiae* ($P = 0.005$) and *H. bacteriophora* ($P < 0.001$) (Fig. 4A and 4B).

In the case of the plant impact, after 15 days, there were no significant differences between CFSs regarding the aerial part dry weight of plants for *S. feltiae* (Fig. 5A and B, Table S6). No differences were observed in either *S. feltiae* for CFS ($P = 0.096$), doses ($P = 0.082$), and interaction ($P = 0.866$) for *S. feltiae,* but differences were observed in CFS ($P = 0.012$), doses ($P = 0.032$) in *H. bacteriophora,* without having effect in the interactions ($P = 0.639$). However, *Xb*_AM75 showed the most significant increase for *S. feltiae* and *Xb*_RM25 for *H. bacteriophora*. No effect on dose and interaction was found in any population (Fig. 5A and 5B). Similarly, significant differences were observed in root dry weight for CFSs ($P = 0.037$ and $P = 0.008$), without having an effect in dose ($P = 0.302$ and $P = 0.282$), and their interaction ($P = 0.360$ and $P = 0.184$) (Fig. 5C and D, Table S6). However, *Xb*_AM75 showed a slight increase in root dry weight for *S. feltiae,* and *Xb*_RM25 exhibited a similar effect for *H. bacteriophora* (Fig. 5C and Fig. 5D). A similar pattern was observed for the plant weights after 30 days (Fig. S6, Table S6).

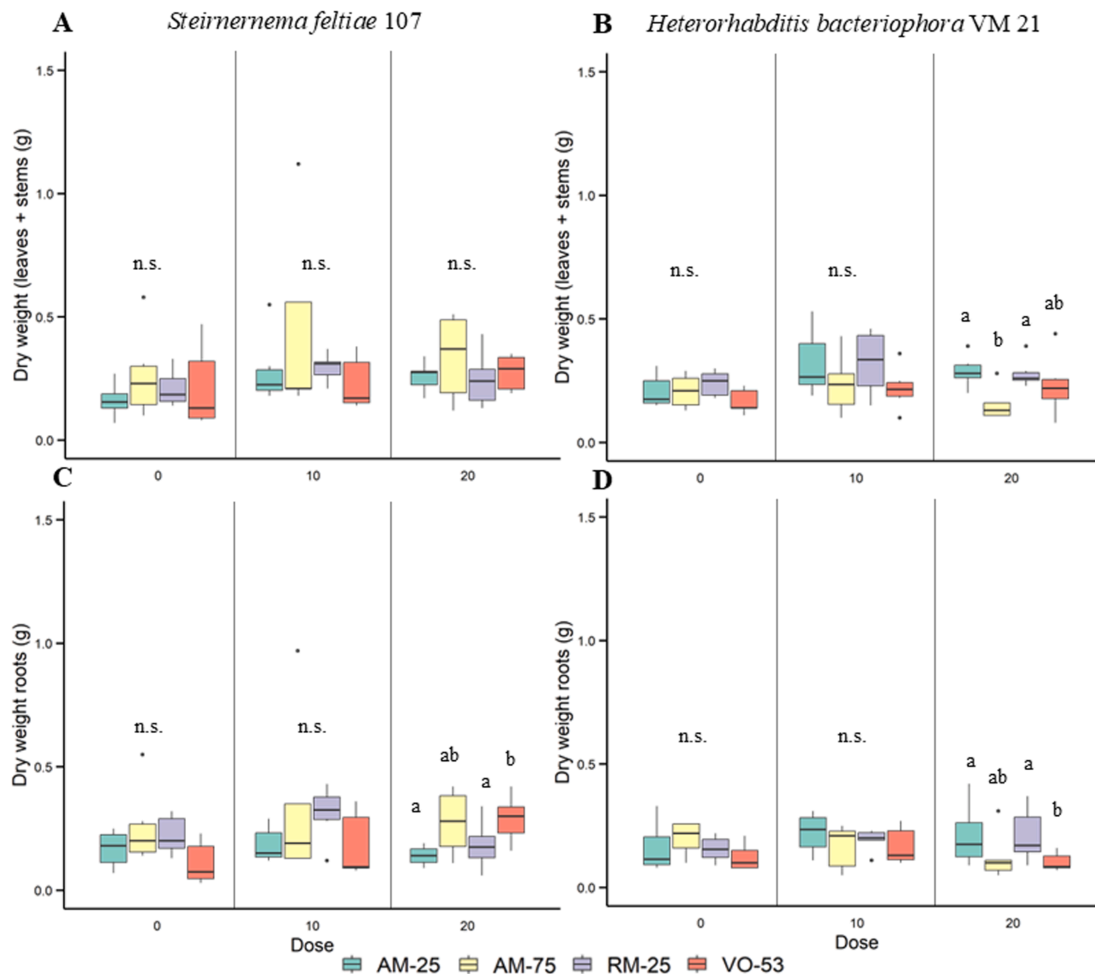

Fig. 5. Effect of four cell-free supernatant (CFS) on plant impact, exposure for different concentrations after 15 days post-exposure. **A.** Impact in tomato plant (*Steinernema feltiae* 107). **B.** Impact in tomato plant (*Heterorhabditis bacteriophora* VM 21). Different letters indicate significant differences for GLMM tests at $P < 0.05$; n.s., not significant.

4. Discussion

4.1. Bacterial cell-free supernatant activity against nematodes in liquid suspension

This study demonstrates the potential of CFS produced from different strains of *Xenorhabdus* to reduce the impact of the root-knot nematode *M. incognita* on tomato plants. It has been described that metabolite and toxins production may differ considerably among different bacterial strains and species of EPNs (Engel et al., 2017; Tobias et al., 2017). Although CFS produced by *Xenorhabdus* spp. shows promise as an efficient biocontrol agent for PPNs, there is a lack of research on its effects on beneficial soil organisms, such as EPNs.

In laboratory experiments, Kusakabe et al. (2022) found that CFS produced by *P. luminescens sonorensis* selectively affected two species of PPNs, *M. incognita,* and *Tylenchulus semipenetrans*, while having minimal impact on non-target-organisms such as *C. elegans*, *S. carpocapsae*, *H. bacteriophora* and *H. sonorensis in vitro*. Similarly, Orozco et al. (2016) reported that extracts from the *P. luminescens* strain TT01 had nematicidal effects against *M. incognita* juveniles (J_2), with a lower impact on *C. elegans* and no effect in *S. carpocapsae*. Also, Boina et al. (2008) observed that a synthetic analog of a compound found among the metabolites derived from *Photorhabdus* sp. showed nematicidal activity against J_2-*M. incognita* and adults of *C. elegans*, but not against the IJs of *H. bacteriophora.* In agreement with these previous observations, our screening experiments of bacterial CFS activity against nematodes in liquid suspension corroborate these findings. We observed no adverse effects on the survival and infectivity of *Steinernema feltiae* and *Heterorhabditis bacteriophora*, but a significant impact on *M. incognita*.

Similarly, our results on the liquid suspension approach against *M. incognita* agreed with previous studies. For example, Sayedain et al. (2019) reported that using CFS from *X. bovienii* resulted in mortality rates of 67.7 % for J_2 and 100 % egg-hatching inhibition for *M. javanica* after 24 h and ten days of exposure, respectively. Similarly, Srivastava and Chaubey (2022) observed that exposure of J_2 of *M. incognita* at 24 h and to different concentrations of CFS from *Xenorhabdus* spp. and *Photorhabdus* spp. reduced mortality by 90 % and 80 %, respectively, at the highest concentration (90 %). El-Deen et al. (2014) showed that CFS from *P. luminescens*, *X. budapestensis*, and *X. szentirmaii* against *M. incognita* effectively generated 24-hour mortality rates of 91 %, 94 %, and 100 %, respectively, at the highest concentration (80 %). Therefore, our studies aligned with previous reports on the potential of the CFS tested from the native *X. bovienii* strain to reduce the activity of the J_2 of PPN *M. incognita*.

Concerning the mechanisms, certain studies suggest that the inactivity effect on PPNs could be likely due to secondary metabolites such as indole, stilbene derivatives, and ammonia, which exhibit selective nematicidal activity (Pérez and Lewis, 2002; Boina et al., 2008; El-Deen et al., 2014). Kusakabe et al. (2022) further identified that the indole compounds *trans*-cinnamic acid (t_CA) and (4E)-5-phenylpent-4-enoic acid (PPA) have selective nematicidal effects on PPNs. Samaliev et al. (2000) reported that exposure to *X. nematophilus* and *P. oryzihabitans*

and their CFS caused disorientation and convulsive movements in J_2 of *M. javanica*. However, these investigations have been conducted *in vitro*. In our *in vitro* experiments on evaluating bacterial CFS activity against PPN, we did not obtain significant differences among the four CFS, with few exceptions. However, the doses evaluated did have effects on *M. incognita*. Multiple studies have shown that applying CFS at different concentrations affects PPNs (Samaliev et al., 2000; Sasnarukkit et al., 2002; Sayedain et al., 2019). Then, the fine-tuning of the strain selection and concentration arises as a critical factor in establishing the potential of a CFS to control the PPN *M. incognita.*

4.2. Bacterial cell-free supernatant activity in tomato plants: Impact on plant-parasitic nematodes

In experimental approaches that included plants and soil, the effect of certain chemicals can be buffered. Early studies have already demonstrated the effectiveness of CFS from *Xenorhabdus* spp. and *Photorhabdus* spp. against J_2 of *M. incognita* (Grewal et al., 1997). From the perspective of PPN management, the application of the highest dose resulted in a more robust and consistent reduction of the reproductive parameters of *M. incognita* in the plant, hence its possible overall damage, as shown in the gall index. These results agreed with previous observations. For example, Kepenekci et al. (2016) reported that applying CFS from *X. bovienii* reduced the number of *M. incognita* egg masses in tomato plants (SC-2121 variety). Similarly, Aatif et al. (2021) showed that metabolites from *Xenorhabdus* spp. significantly reduced the number of females and galls. Sasnarukkit et al. (2002) observed that exposing tomato roots (cv. seedatip) to CFS of *Xenorhabdus* spp. for 2 h reduced the penetration of *M. incognita* after 30 days. Kepenekci et al. (2018) tested CFS produced by *X. bovienii* against *M. incognita* and *M. javanica* on tomato plants (Care F1 and Ilgin F1 cultivars). They noted a reduction in the galling index and increased total yield. Other approaches, such as dipping roots on the metabolites, are also promising. For example, El-Deen et al. (2019) demonstrated that dipping roots of grapevine seedlings (var. Taify) in bacterial filtrates of *X. budapestensis* DSM 16342 and *X. szentirmaii* DSM 16338 significantly reduced *M. incognita* infestation four months after application, impacting on number of galls and egg masses, eggs/g root, and plant lengths and weights. In our study, we only evaluated the fitness of *M. incognita* in the plant, so it still remains unknown if the CFS affected the egg hatching of the J_2 viability or mobility. However, although the mechanism for reducing the impact of *M. incognita* in the plant remains to be unraveled, these findings are consistent with previous results regarding the reproductive parameters of *M. incognita* on tomato plants, and its potential to be included in possible IPM or organic production system to decrease the level of damage in the crop. In any case, further studies on semifield experiments and commercial exploitation are warranted to determine the possible effect of the type of soil, cultivar and PPN species and pathotype in the possible control of this biotic threat.

4.3. Bacterial cell-free supernatant activity in tomato plants: Impact on entomopathogenic nematodes

Despite numerous laboratory studies on the persistence of EPNs under different abiotic conditions, extrapolating the findings to semi-natural and natural systems remains challenging (Stuart et al., 2015). Our research found that the results obtained under *in vitro* conditions differed from those obtained in the plant-mesocosm system. Studies on the survival of EPNs reveal a complex interaction between these organisms and the soil. In our evaluation of bacterial CFS activity against EPNs in the semi-natural system, we observed a reduction in the virulence of *S. feltiae* 107. This reduction is likely due to the buffering effect of the soil. The complex and multifaceted nature of the soil ecosystem, with its diverse compounds, secondary metabolites, and toxins produced by microorganisms, plays a significant role in these dynamics (Schulz-Bohm et al., 2015; Gols et al., 2023). As Lewis et al. (2015) suggested, biotic and abiotic factors may influence IJ survival. Alteration of normal soil conditions, such as changes in pH, moisture, salinity, ultraviolet (UV) light, oxygen, temperature, soil texture, and structure, as well as anthropogenic interventions, can significantly impact the survival, persistence, and EPN potential virulence (Koppenhöfer et al., 2007; Susurluk and Ehlers, 2008; Lacey and Georgis, 2012; Lacey et al., 2015; Stuart et al., 2015; Karthik Raja et al., 2021). Indeed, EPNs can respond to different stimuli such as temperature, CO_2, and chemical compounds (e.g., produced by hosts or metabolites and toxins from other soil organisms) (Liu and Glazer, 2000; Grewal, 2000). However, our studies indicate that exposure to CFS from the bacterium *X. bovienii* may condition their survival or virulence to kill the host insect.

Regarding EPN survival, studies by Shapiro-Ilan et al. (2006) indicate that while *Steinernema* and *Heterorhabditis* species can endure pesticides and chemical fertilizers, they are highly susceptible to nematicides present in agroecosystems. For *in vitro* studies, Campos-Herrera et al. (2023a) showed that exposure of IJs of steirnernematids and heterorhabditids to commercial adjuvants did not affect the viability of EPNs but did reduce the infectivity after two days of *S. feltiae* 107, *S. feltiae* Koppert and *H. bacteriophora* VM-21 exposed during 24 h to these chemicals. Additionally, exposure to copper and sulfur fungicides negatively affected the viability and virulence of *S. feltiae* without affecting *S. carpocapsae* (Campos-Herrera et al., 2023b). Our results showed that the presence of CFS in the plant-mesocosm system, as a potential nematicidal product, negatively affected EPN, particularly steinernematids. These studies serve as a basis for understanding how pesticides, fertilizers, and bio-products can affect beneficial soil species such as EPNs. Brown and Gaugler (1997) mention that the survival of EPNs varies among species and depends on the environmental conditions in which the host insect is located. Timper and Kaya, (1989, 1992) and Timper et al. (1991) found that endoparasitic nematophagous fungi exhibited higher infection in steinernematids compared to heterorhabditids. Their research observed that the conidia of the fungi *Hirsutella rhossiliensis* or *Drechmeria coniospora* were not infective to sheathed heterorhabditids. However, these fungi displayed high infectivity towards unsheathed heterorhabditids and steinernematids, which are typically unsheathed. They showed that infective heterorhabditids tend to retain the second instar cuticle, which envelops them as a protective sheath that prevents spore germ tubes from attaching. The maintenance of the second stage cuticle by *Heterorhabditis* can support our observations linked to the virulence, where we observed higher mortality of *G. mellonella* larvae by the species *H. bacteriophora* VM-21 compared to *S. feltiae* 107. However, the virulence effect in our experiments was lost after four weeks in both control and soil with the application of CFS. Nonetheless, studies report that EPNs can remain active in the soil for over eight weeks (Kaya and Gaugler, 1993; Shapiro-Ilan et al., 2006). Therefore, the differential impact of the CFS on the virulence observed by the EPN evaluated could be due to the particular population used, any specific compounds derived from the bacteria selected, the interaction with the soil, or a combination of those. In any case, further studies are warranted to unravel the impact of these CFS on beneficial soil organisms and how the ecological functions displayed can be altered or modulated.

5. Conclusions

This study confirms the efficacy of CFS produced by *X. bovienii* as a promising biocontrol alternative. It demonstrated the significant inactivity of *M. incognita in vitro* and the inhibitory effect on reproduction parameters in the plant-mesocosm system. Among the challenges to obtaining better nematicides is the identification of the compounds present in CFS. Studies by Abebew et al. (2022) identified compounds present in the CFS of *Xenorhabdus*, including Fabclavins, Rhabdopeptides, and Xenocoumacins and emphasize that the separation of natural compounds with nematicidal activity from other components is key to the development of more effective and selective nematicides. However,

further research is needed to explore the impact of CFS on other beneficial soil organisms, such as EPNs, and, more importantly, on the soil function they display. Likewise, there is a critical need for research aimed at developing valuable and sustainable bio-tools for managing harmful organisms while promoting the conservation of beneficial soil fauna (Lewis and Papavizas, 1991; Tariq et al., 2020; Upadhyay et al., 2021). These efforts should include integrated and ecological practices within the framework of the European Green Deal (Apitz et al., 2006). Such approaches aim to reduce dependence on synthetic chemical, fostering more sustainable and resilient agriculture systems.

CRediT authorship contribution statement

M.M. González-Trujillo: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **J. Artal:** Writing – review & editing, Resources, Conceptualization. **I. Vicente-Díez:** Writing – review & editing, Methodology. **R. Blanco-Pérez:** Writing – review & editing, Software, Formal analysis, Data curation. **M. Talavera:** Writing – review & editing, Methodology, Conceptualization. **J. Dueñas-Hernani:** Writing – review & editing, Methodology. **S. Álvarez-Ortega:** Writing – review & editing, Supervision, Conceptualization. **R. Campos-Herrera:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Acknowledgments

We want to thank Company ARTAL Smart Agriculture for the collaboration provided and the financing in the projects (20201484) and (20216507). The authors also thank Elisabet Vaquero Jiménez and Javier Manzanares for their help and support during the development of this research. M.M. González-Trujillo and I. Vicente-Díez were supported by predoctoral contracts (FPI-CAR 2022 and FPI-UR 2020, respectively). Rubén Blanco-Pérez is supported by the funded by MCIN/AEI/10.13039/501100011033 and by "European Union NextGenerationEU/PRTR" (reference JDC2022-048978-I). Jorge Dueñas-Hernani is supported by the Programa Investigo from the Government of La Rioja and the by "European Union NextGenerationEU/PRTR". M.M. González-Trujillo also thanks CSIC for her support with the fellowship "Introduction to Research" reference JAEINT20_EX_0939.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2024.108213.

References

Aatif, H.M., Ijaz, M., Zeeshan Mansha, M., Ikram, K., Raheel, M., Muhammad, C., Hanif, S., Abbas, H.T., 2021. Impact of shelflife on pathogenicity of entomopathogenic bacteria and their metabolites against *Meloidogyne incognita* in egg plant. Fresen. Environ. Bull. 30, 9322–9327.
Abebew, D., Sayedain, F.S., Bode, E., Bode, H.B., 2022. Uncovering nematicidal natural products from Xenorhabdus bacteria. J. Agric. Food Chem. 70 (2), 498–506.
Ansari, M.A., Tirry, L., Moens, M., 2003. Entomopathogenic nematodes and their symbiotic bacteria for the biological control of *Hoplia philanthus* (Coleoptera: Scarabaeidae). Biol. Control 28, 111–117.
Apitz, S.E., Elliott, M., Fountain, M., Galloway, T.S., 2006. European environmental management: Moving to an ecosystem approach. Integr. Environ. Assess. Manag. 2, 80–85.
Bates, D., Mächler, M., Bolker, B.M., Walker, S.C., 2015. Fitting Linear Mixed-Effects Models Using lme4. J. Stat. Softw. 67, 1–48.
Blanco-Pérez, R., Sáenz-Romo, M.G., Vicente-Díez, I., Ibáñez-Pascul, S., Martínez-Villar, E., Marco-Mancebón, V.S., Pérez-Moreno, I., Campos-Herrera, R., 2020. Impact of vineyard ground cover management on the occurrence and activity of entomopathogenic nematodes and associated soil organisms. Agr Ecosyst Environ 301, 107028.
Blanco-Pérez, R., Vicente-Díez, I., Ramos-Sáez de Ojer, J.L., Marco-Mancebón, V.S., Pérez-Moreno, I., Campos-Herrera, R., 2022. Organic viticulture enhanced the activity of native entomopathogenic nematodes in DOCa Rioja soils (North of Spain). Agr Ecosyst Environ 332, 107931.
Boina, D.R., Lewis, E.E., Bloomquist, J.R., 2008. Nematicidal activity of anion transport blockers against *Meloidogyne incognita*, *Caenorhabditis elegans* and *Heterorhabditis bacteriophora*. Pest Manag. Sci. 64, 646–653.
Bridge, J., Page, S.L.J., 1980. Estimation of root-knot nematode infestation levels on roots using a rating chart. Tropical Pest Management 26, 296–298.
Brown, I.M., Gaugler, R., 1997. Temperature and humidity influence emergence and survival of entomopathogenic nematodes. Nematologica 43, 363–375.
Burns, A.R., Baker, R.J., Kitner, M., Knox, J., Cooke, B., Volpatti, J.R., Vaidya, A.S., Puumala, E., Palmeira, B.M., Redman, E.M., Snider, J., Marwah, S., Chung, S.W., MacDonald, M.H., Tiefenbach, J., Hu, C., Xiao, Q., Finney, C.A.M., Krause, H.M., MacParland, S.A., Stagljar, I., Gilleard, J.S., Cowen, L.E., Meyer, S.L.F., Cutler, S.R., Dowling, J.J., Lautens, M., Zasada, I., Roy, P.J., 2023. Selective control of parasitic nematodes using bioactivated nematicides. Nature 618, 102–109.
Cabello, T., 1997. Análisis de eficacia de productos fitosanitarios. Phytoma n. 92, 30–40.
Campos-Herrera, 2015. Nematode pathogenesis of insects and other pests. In: Campos-Herrera, R. (Ed.), Ecology and Applied Technologies for Sustainable Plant and Crop Protection. Springer International Publishing Switzerland.
Campos-Herrera, R., Carpentero, E., Puelles, M., Sáezde Ojer, J.L.R., Pérez, R.B., 2023a. Entomopathogenic nematode compatibility with vineyard fungicides. J. Nematol. 55.
Campos-Herrera, R., González-Trujillo, M. del M., Vicente-Díez, I., Carpentero, E., Puelles, M., Vaquero, E., Cepulyte, R., 2023b. Exploring entomopathogenic nematodes for the management of *Lobesia botrana* (Lepidoptera: Tortricidae) in vineyards: Fine-tuning of application, target area, and timing. Crop Protection 174.
Cevizci, D., Ulug, D., Cimen, H., Touray, M., Hazir, S., Cakmak, I., 2020. Mode of entry of secondary metabolites of the bacteria *Xenorhabdus szentirmaii* and *X. nematophila* into *Tetranychus urticae*, and their toxicity to the predatory mites *Phytoseiulus persimilis* and *Neoseiulus californicus*. J. Invertebr. Pathol. 174.
Chen, J., Li, Q.X., Song, B., 2020. Chemical nematicides: recent research progress and outlook. J. Agric. Food Chem. 68, 12175–12188.
Costa, J.M., Heuvelink, E., 2018. The global tomato industry. Classification and taxonomy. CAB International. Tomatoes, 2nd edition 1–26.
Dillman, A.R., Chaston, J.M., Adams, B.J., Ciche, T.A., Goodrich-Blair, H., Stock, S.P., Sternberg, P.W., 2012. An entomopathogenic nematode by any other name. PLoS Pathog. 8 (3), 1002527.
El-Deen, A.N., Alghamdi, A., Al-Barty, A., Darwish, H., Samra, B., Alotaibi, S., Bogami, B. A., 2019. Entomopathogenic bacteria, *Xenorhabdus*: An alternative biocontrol agent for integrated management of root-knot nematode on grapevine. Journal of Pure and Applied Microbiology 13, 1499–1508.
El-Deen, N., Fodor, A., El-Barty, A.F., 2014. Nematicidal activity of entomopathogenic bacteria against root-knot nematodes. *Meloidogyne Incognita* in-Vitro, International Journal of Advanced Research 2 (6), 708–713.
Elling, A., 2013. Major Emerging Problems with Minor *Meloidogyne* Species. Review Phytopathology. The American Phytopathological. Society Vol. 103, 11, 1092–1102.
Engel, Y., Windhorst, C., Lu, X., Goodrich-Blair, H., Bode, H.B., 2017. The global regulators lrp, leuo, and hexa control secondary metabolism in entomopathogenic bacteria. Front. Microbiol. 8, 209.
Felicitas, E.F.A., Caoili, B.L., Latina, R.A., 2021. Antagonistic effect of *Steinernema abbasi* and *Heterorhabditis indica* Philippine isolates on root penetration and development of *Meloidogyne incognita* in tomato. Biocontrol Sci. Tech. 31, 865–876.
Gemmill, A., Gunier, R., Bradman, A., Eskenazi, B., Harley, K., 2013. Residential proximity to methyl bromide use and birth outcomes in an agricultural population in California. Environ. Health Perspect. 121 (6), 737–743.
Gols, R., van Geem, M., Bullock, J.M., Martens, H.J., Wagenaar, R., van der Putten, W.H., Harvey, J.A., 2023. Communities of nematodes, bacteria and fungi differ among soils of different wild cabbage populations. Eur. J. Soil Biol. 117, 103512.
Grabau, Z.J., Mauldin, M.D., Habteweld, A., Carter, E.T., 2020. Nematicide efficacy at managing Meloidogyne arenaria and non-target effects on free-living nematodes in peanut production. J. Nematol. 52, 1–10.
Grewal, P.S., 2000. Enhanced ambient storage stability of an entomopathogenic nematode through anhydrobiosis. Pest Manag. Sci. 56, 401–406.
Grewal, P.S., Martin, W.R., Miller, R.W., Lewis, E.E., 1997. Suppression of plant-parasitic nematode populations in turfgrass by application of entomopathogenic nematodes. Biocontrol Sci. Tech. 7, 393–400.
Griffin, C.T., Boemare, N.E., Lewis, E.E., 2005. Biology and behaviour. In: Nematodes as Biocontrol Agents. CABI Publishing, UK, pp. 47–64.
Gullino, M., Camponogara, A., Gasparrini, G., Rizzo, V., Clini, C., Garibaldi, A., 2003. Replacing methyl bromide for soil disinfestation. The Italian experience and implications for other countries. Plant Dis. 87 (9), 1012–1021.
Hazir, S., Shapiro-Ilan, D.I., Bock, C.H., Leite, L.G., 2018. Thermo-stability, dose effects and shelf-life of antifungal metabolite-containing supernatants produced by *Xenorhabdus szentirmaii*. Eur. J. Plant Pathol. 150, 297–306.
Hominick, W.M., 2002. Biogeography., in Entomopathogenic Nematology. CABI Publishing UK, 115–143.
Hussey, R.S., Barker, K.R., 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. Plant Disease Reporter 57, 1025–1028.
Jang, W., Youngentob, S., Schwob, J., 2003. Globose basal cells are required for reconstitution of olfactory epithelium after methyl bromide lesion. J Comp Neurol 460 (1), 123–140.

Karthik Raja, R., Arun, A., Touray, M., Hazal Gulsen, S., Cimen, H., Gulcu, B., Hazir, C., Aiswarya, D., Ulug, D., Cakmak, I., Kaya, H.K., Hazir, S., 2021. Antagonists and defense mechanisms of entomopathogenic nematodes and their mutualistic bacteria. Biol. Control 52, 104452.
Kaya, H.K., Aguillera, M.M., Alumai, A., Choo, H.Y., de la Torre, M., Fodor, A., Ganguly, S., Hazir, S., Lakatos, T., Pye, A., Wilson, M., Yamanaka, S., Yang, H., Ehlers, R.U., 2006. Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. Biol. Control 38, 134–155.
Kaya, H., Gaugler, R., 1993. Entomopathogenic nematodes. Annu. Rev. Entomol. 181–206.
Kepenekci, I., Hazir, S., Lewis, E.E., 2016. Evaluation of entomopathogenic nematodes and the supernatants of the in vitro culture medium of their mutualistic bacteria for the control of the root-knot nematodes *Meloidogyne incognita* and *M. arenaria*. Pest Manag. Sci. 72, 327–334.
Kepenekci, I., Hazir, S., Oksal, E., Lewis, E.E., 2018. Application methods of *Steinernema feltiae*, *Xenorhabdus bovienii* and *Purpureocillium lilacinum* to control root-knot nematodes in greenhouse tomato systems. Crop Prot. 108, 31–38.
Koenning, S.R., Overstreet, C., Noling, J.W., Donald, P.A., Becker, J.O., Fortnum, B.A., 1999. Survey of crop losses in response to phytoparasitic nematodes in the United States for 1994. Supplement to the Journal of Nematology 31 (4S), 587–618.
Koppenhöfer, A.M., Grewal, P.S., Fuzy, E.M., 2007. Differences in penetration routes and establishment rates of four entomopathogenic nematode species into four white grub species. J. Invertebr. Pathol. 94, 184–195.
Koppenhöfer, A.M., Kostromytska, O.S., Wu, S., 2020. Optimizing the use of entomopathogenic nematodes for the management of *Listronotus maculicollis* (Coleoptera: Curculionidae): Split applications and combinations with imidacloprid. Crop Prot. 137, 105229.
Kusakabe, A., Wang, C., Xu, Y.-M., Molnár, I., Stock, S.P., Gralnick, J.A., 2022. Selective toxicity of secondary metabolites from the entomopathogenic bacterium *Photorhabdus luminescens sonorensis* against selected plant parasitic nematodes of the Tylenchina suborder. Microbiology. Spectrum 10 Issue 1.
Lacey, L.A., Georgis, R., 2012. Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. J. Nematol. 44 (2), 218–225.
Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: Back to the future. J. Invertebr. Pathol. 132, 1–41.
Lewis, E.E., Grewal, P.S., 2004. Interactions with plant-parasitic nematodes, Grewal, Ehlers, and Shapiro-Ilan, (ed). CABI, Nematodes as Biocontrol Agents, pp. 349–362.
Lewis, E.E., Hazir, S., Hodson, A., Gulcu, B., 2015. In: Trophic Relationships of Entomopathogenic Nematodes in Agricultural Habitats. in Nematode Pathogenesis of Insects and Other Pests. Springer International Publishing Switzerland, pp. 139–163.
Lewis, J.A., Papavizas, G.C., 1991. Biocontrol of plant diseases: the approach for tomorrow. Crop Prot. 10, 95–105.
Li, J., Zhao, J., Liao, X., Yi, Q., Zhang, W., Lin, H., Liu, K., Peng, P., Wang, K., 2023a. Long-term returning agricultural residues increases soil microbe-nematode network complexity and ecosystem multifunctionality. Geoderma 430, 116340.
Li, J., Li, Y., Wei, X., Cui, Y., Gu, X., Li, X., Yoshiga, T., Abd-Elgawad, M.M., Shapiro-Ilan, D., Ruan, W., Rasmann, S., 2023b. Direct antagonistic effect of entomopathogenic nematodes and their symbiotic bacteria on root-knot nematodes migration toward tomato roots. Plant and Soil 484, 441–455.
Liu, Q.Z., Glazer, I., 2000. Desiccation Survival of Entomopathogenic Nematodes of the Genus *Heterorhabditis*. Phytoparasitica 28 (4), 331–340.
Martin, T., Sprunger, C.D., 2022. Soil food web structure and function in annual row-crop systems: How can nematode communities infer soil health? Appl. Soil Ecol. 178, 104553.
Mitani, D.K., Kaya, H.K., Goodrich-Blair, H., 2004. Comparative study of the entomopathogenic nematode, *Steinernema carpocapsae*, reared on mutant and wild-type *Xenorhabdus nematophila*. Biol. Control 29, 382–391.
Moens, M., Perry, R., Starr, J. L., 2009. *Meloidogyne* species a diverse group of novel and important plant parasites. In Root-knot Nematodes (eds R.N. Perry, M. Moens and J. L. Starr). CAB International. 117.
Mukhtar, T., Arooj, M., Ashfaq, M., Gulzar, A., 2016. Resistance evaluation and host status of selected green gram germplasm against *Meloidogyne incognita*. Crop Prot. 92, 198–202.
Ntalli, N.G., Caboni, P., 2012. Botanical nematicides: A review. J. Agric. Food Chem. 60, 9929–9940.
Orozco, R.A., Molnár, I., Bode, H., Stock, S.P., 2016. Bioprospecting for secondary metabolites in the entomopathogenic bacterium *Photorhabdus luminescens* subsp. *sonorensis*. J. Invertebr. Pathol. 141, 45–52.
Pérez, E.E., Lewis, E.E., 2002. Use of Entomopathogenic Nematodes to Suppress *Meloidogyne incognita* on Greenhouse Tomatoes. J. Nematol. 34 (2), 171–174.
Perry, R.N., Moens, M., Starr, J.L., 2009. Root-Knot Nematodes. CAB International, Wallingford, Oxfordshire, UK, p. 488.
Premachandran, D., Von Mende, N., Hussey, R.S., Mcclure, M.A., 1988. A method for staining nematode secretions and structures. J. Nematol. 20 (1), 70–78.
R Core Team, 2023. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria https://www.R-project.org/.
Refaei, A.R., El-Nagar, M.E., Salem, M.M., 2007. The role of eggs inoculum level of *Meloidogyne incognita* on their reproduction and host reaction. Afr. J. Agric. Res. 2 (4), 159–163.
Salari, E., Karimi, J., Sadeghi-Nameghi, H., Hosseini, M., 2015. Efficacy of two entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* for control of the leopard moth borer *Zeuzera pyrina* (Lepidoptera: Cossidae) larvae under laboratory conditions. Biocontrol Sci. Tech. 25, 260–275.
Samaliev, H.Y., Andreoglou, F.I., Elawad, S.A., Hague, N.G.M., Gowen, S.R., 2000. The nematicidal effects of the bacteria *Pseudomonas oryzihabitans* and *Xenorhabdus nematophilus* on the root-knot nematode *Meloidogyne javanica*. Nematology 2 (5), 507–514.
Sánchez-Moreno, S., Smukler, S., Ferris, H., O'Geen, A.T., Jackson, L.E., 2008. Nematode diversity, food web condition, and chemical and physical properties in different soil habitats of an organic farm. Biol. Fertil. Soils 44, 727–744.
Sasanelli, N., Konrat, A., Migunova, V., Toderas, I., Iurcu-Straistaru, E., Rusu, S., Bivol, A., Andoni, C., Veronico, P., 2021. Review on control methods against plant parasitic nematodes applied in southern member states (C zone) of the European Union. Agriculture 11, 602.
Sasnarukkit, A., Gaugler, R., Sontirat, S., 2002. Effects of entomopathogenic nematode *Steinernema siamkayai* and its bacterial symbiont on *Meloidogyne incognita*. Int. J. 12 (2), 169–174.
Sayedain, F.S., Ahmadzadeh, M., Talaei-Hassanloui, R., Olia, M., Bode, H.B., 2019. Nematicidal effect of cell-free culture filtrates of EPN-symbiotic bacteria on *Meloidogyne javanica*. Biological Control of Pest & Plant Diseases 8, 17–26.
Schroeder, P.C., Ferguson, C.S., Shelton, A.M., Wilsey, W.T., Hoffmann, M.P., Petzoldt, C., 1996. Greenhouse and field evaluations of entomopathogenic nematodes (nematoda: Heterorhabditidae and Steinernematidae) for control of cabbage maggot (Diptera: Anthomyiidae) on cabbage. J. Econ. Entomol. 89, 5.
Schulz-Bohm, K., Zweers, H., de Boer, W., Garbeva, P., 2015. A fragrant neighborhood: Volatile mediated bacterial interactions in soil. Front. Microbiol. 6, 1212.
Shapiro-Ilan, D.I., Gouge, D.H., Piggott, S.J., Fife, J.P., 2006. Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. Biol. Control 38, 124–133.
Shapiro-Ilan, D., Hazir, S., Glazer, I., 2017. Basic and applied research. In Microbial Control of Insect and Mite Pests. Elsevier. 91–105.
Singh, S.K., Khurma, U.R., 2007. Susceptibility of six tomato cultivars to the root-knot nematode, *Meloidogyne incognita*. The South Pacific Journal of Natural and Applied Sciences 13, 73–77.
Singh, S., Singh, B., Singh, A.P., 2015. Nematodes: a threat to sustainability of agriculture. Procedia Environ. Sci. 29, 215–216.
Smiley, R., Nicol, J., 2009. Nematodes which challenge global wheat production. Wiley-Blackwell. Section II Making of a Wheat Crop. 171–187.
Srivastava, S., Chaubey, A.K., 2022. In vitro study on the nematicidal activity of entomopathogenic bacteria against the root knot nematode *Meloidogyne incognita*. Journal of Applied and Natural Science 14, 1–8.
Stirling, G., 2014. Nematodes, mites and collembola as predators of nematodes and the role of generalist predators. In: Stirling, G. (Ed.), Biological Control of Plant Parasitic Nematodes: Soil Ecosystem Management in Sustainable Agriculture. CABI, Oxfordshire, pp. 157–192.
Stock, P., 2015. In: Diversity, Biology and Evolutionary Relationships. in Nematode Pathogenesis of Insects and Other Pests. Springer International Publishing Switzerland, pp. 3–27.
Stuart, R.J., Barbercheck, M.E., Grewal, P.S., Taylor, R.A.J., Hoy, C.W., 2006. Population biology of entomopathogenic nematodes: concepts, issues, and models. Biol. Control 38, 80–102.
Stuart, R.J., Barbercheck, M.E., Grewal, P.S., 2015. In: Entomopathogenic Nematodes in the Soil Environment: Distributions, Interactions and the Influence of Biotic and Abiotic Factors. Springer International Publishing Switzerland, pp. 97–137.
Susurluk, A., Ehlers, R.U., 2008. Sustainable control of black vine weevil larvae, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) with Heterorhabditis bacteriophora in strawberry. Biocontrol Sci. Tech. 18, 635–640.
Tariq, M., Khan, A., Asif, M., Khan, F., Ansari, T., Shariq, M., Siddiqui, M.A., 2020. Biological control: a sustainable and practical approach for plant disease management. Acta Agriculturae Scandinavica. Section b. Soil & Plant Science 70, 507–524.
Thomas, W., 1996. Methyl bromide: effective pest management tool and environmental threat. Supplement to Journal of Nematology 28 (4S), 586–589.
Timper, P., Kaya, H.K., 1989. Role of the Second-Stage Cuticle of Entomogenous Nematodes in Preventing Infection by Nematophagous Fungi. J. Invertebr. Pathol. 54, 314–321.
Timper, P., Kaya, H.K., 1992. Impact of a Nematode-parasitic Fungus on the Effectiveness of Entomopathogenic Nematodes. J. Nematol. 24 (1), 1–8.
Timper, P., Kaya, H.K., Jaffee, B.A., 1991. Survival of Entomogenous Nematodes in Soil Infested with the Nematode-Parasitic Fungus *Hirsutella rhossiliensis* (Deuteromycotina: Hyphomycetes). Biol. Control 1, 42–50.
Tobias, N.J., Wolff, H., Djahanschiri, B., Grundmann, F., Kronenwerth, M., Shi, Y.M., Simonyi, S., Grün, P., Shapiro-Ilan, D., Pidot, S.J., Stinear, T.P., Ebersberger, I., Bode, H.B., 2017. Natural product diversity associated with the nematode symbionts *Photorhabdus* and *Xenorhabdus*. Nat. Microbiol. 2, 1676–1685.
Toledo, J., Morán-Aceves, B.M., Ibarra, J.E., Liedo, P., 2023. Can Entomopathogenic Nematodes and Their Symbiotic Bacteria Suppress Fruit Fly Pests? A Review. Microorganisms 11, 1682.
Upadhyay, A., Hadiya, J., Gharde, S., 2021. Biocontrol: an effective tool for agricultural insect pests management. The Pharma Innovation 10, 284–288.
Vicente-Díez, I., Blanco-Pérez, R., Chelkha, M., Puelles, M., Pou, A., Campos-Herrera, R., 2021a. Exploring the use of entomopathogenic nematodes and the natural products derived from their symbiotic bacteria to control the grapevine Moth, *Lobesia botrana* (Lepidoptera: Tortricidae). Insects 12, 1033.
Vicente-Díez, I., Blanco-Pérez, R., González-Trujillo, M.D.M., Pou, A., Campos-Herrera, R., 2021b. Insecticidal effect of entomopathogenic nematodes and the cell-

free supernatant from their symbiotic bacteria against *Philaenus spumarius* (Hemiptera: Aphrophoridae) nymphs. Insects 12, 448.

Waldo, B.D., Grabau, Z.J., Mengistu, T.M., Crow, W.T., 2019. Nematicide effects on non-target nematodes in bermudagrass. J. Nematol. 51.

Woodring, J., Kaya, H., 1988. Steinernematid and Heterorhabditid Nematodes: A Handbook of Biology and Techniques. Arkansas Agricultural Experiment Station 331.