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Exploring the effects of entomopathogenic nematode symbiotic bacteria and their cell free filtrates on the tomato leafminer *Tuta absoluta* and its predator *Nesidiocoris tenuis*

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ABSTRACT

The use of biocontrol agents, such as predators and entomopathogenic nematodes, is a promising approach for the effective control of the tomato leafminer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidaean), an oligophagous insect feeding mainly on Solanaceae species and a major pest of field- and greenhouse-grown tomatoes globally. In this context, the effects of two entomopathogenic nematode species *Steinernema carpocapsae* (Weiser) (Rhabditida: Steinernematidae) and *Heterorhabditis bacteriophora* (Poinar) (Rhabditida: Heterorhabditidae), as well as their respective bacterial symbionts, *Xenorhabdus nematophila* and *Photorhabdus luminescens* (Enterobacterales: Morganelaceae), which were applied as bacterial cell suspensions and as crude cell-free liquid filtrates on *T. absoluta* larvae, were investigated. The results showed that of all treatments, the nematodes *S. carpocapsae* and *H. bacteriophora* were the most effective, causing up to 98 % mortality of *T. absoluta* larvae. Regarding bacteria and their filtrates, the bacterium *X. nematophila* was the most effective (69 % mortality in young larvae), while *P. luminescens* and both bacterial filtrates showed similar potency (ca. 48–55 % mortality in young larvae). To achieve a holistic approach of controlling this important pest, the impact of these factors on the beneficial predator *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) was also studied. The results demonstrated that although nematodes and especially *S. carpocapsae*, caused significant mortality on *N. tenuis* (87 %), the bacterial cell suspensions of *X. nematophila* and *P. luminescens* and crude cell-free liquid filtrates had minimum impact on this beneficial predator (~11–30 % mortality).

1. Introduction

Nematodes which belong in Heterorhabditidae and Steinernematidae families are effective biological agents against a wide range of insect pests (Labaude and Griffin, 2018; Koppenhöfer et al., 2020). Nematodes of the genera *Steinernema* and *Heterorhabtitis* cause mortality to insects because of their symbiotic bacteria, which belong to the genera *Xenorhabdus* and *Photorhabdus* respectively. The infective juveniles enter their hosts through natural openings and release their symbiotic bacteria that eventually kill the hosts within 48 h (Kaya and Gaugler, 1993; Grewal et al., 2001; Dillman et al., 2012; Van Zyl and Malan, 2014). These Gram-negative bacteria belong to Enterobacteriaceae family, are anaerobic and are found in the intestinal tract of the infective juvenile (Gulcu et al., 2012; Tarasco et al., 2023). The symbiotic bacteria produce secondary metabolites to compromise their host's immune system, in order to prevent the growth of other microorganisms in the host's body and to support the development of nematodes and cellular communication (Joyce et al., 2008; Shi and Bode, 2018; Cimen et al., 2022). Furthermore, these metabolites, produced by bacteria as part of their ecological interactions, exhibit potent inhibitory effects against various organisms as they have insecticidal, acaricidal, antimicrobial, antibacterial and antifungal activity (Cimen et al., 2022).

The tomato leafminer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is one of the most destructive insects in crops of the Solanaceae family and especially in tomato crops (*Lycopersicon esculentum* L), as it can cause a reduction of production up to 80–100 % (Desneux et al., 2010; Campos- Herrera et al., 2017). This pest, originated in Peru, has spread worldwide due to its high reproduction, its ability to disperse and

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its quick adaptation. Another reason is the lack of effective quarantine measures to effectively prevent entry of this invasive species (Han et al., 2019; Desneux et al., 2022). Larvae after hatching, wander around for few minutes before they begin to feed on the leaf, where they form galleries on stems, leaves, and fruits, but occasionally, larger larvae are found outside mines either temporarily or on their way to pupation (soil or on the surface of a leaf, in a curled-up leaf); this feeding activity results in severe damage that ultimately diminishes crop yield (Tropea Garzia et al., 2012; Urbaneja et al., 2012; Desneux et al., 2010; Gebremariam, 2015).

Control of the leafminer by using chemical pesticides appears to be a challenge, due to development of larvae inside the leaves and fruits and as a result the lack of exposure to insecticides (Birhan, 2018; Guedes et al., 2019). Furthermore, continuous use of insecticides results in development of resistant populations and causes side effects in non-target organisms (Urbaneja et al., 2012; Birhan, 2018). The current practices for integrated control of *T. absoluta* rely on minimal use of synthetic pesticides, adoption of semiochemicals and use of biological control agents by conservation or by augmentation, especially in greenhouse crops (Konan et al., 2023). The omnivore predator *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) feeds on a variety of prey in tomato crop such as whiteflies, mites, thrips, aphids, and eggs/young larvae of lepidopteran pests and is considered a quite successful biological control agent of *T. absoluta*, that is now widely used in Europe, Asia and north Africa and is typically released as adults (Urbaneja et al., 2009; Desneux, 2022).

An alternative strategy for controlling *T. absoluta* includes the use of entomopathogenic nematodes (EPNs); specifically, the species *Steinernema feltiae* (Filipjev), *Steinernema carpocapsae* (Weiser) (Rhabditida: Steinernematidae) and *Heterorhabditis bacteriophora* (Poinar) (Rhabditida: Heterorhabditidae) are highly effective against *T. absoluta* (Batalla-Carrera et al., 2010; Van Damme et al., 2016; Husin and Port, 2021). Batalla-Carrera et al., (2010) conducted assays in petri dishes, leaf bioassays in the laboratory and pot experiments in the greenhouse to evaluate the susceptibility of larvae and pupae against the three nematode species. Results indicated that *S. feltiae, S. carpocapsae* and *H. bacteriophora*, were able to successfully kill larvae inside the mines and pupae with no significant differences in mortality percentages among the nematodes' species. Van Damme et al. (2016) and Husin and Port (2021), assessed the efficacy of *S. feltiae, S. carpocapsae* and *H. bacteriophora* against the leafminer in leaf bioassays. Overall, in both studies, Steinernema species caused higher mortality than *H. bacteriophora*. These nematodes can be effective in foliar applications against larvae of *T. absoluta*, due to their ability to penetrate in the mines, created by the larvae. Inside the galleries nematodes find and infect their host and alongside they are being protected from unfavorable environmental conditions (Batalla-Carrera et al., 2010; Van Damme et al., 2016; Husin and Port, 2021).

The efficacy of EPNs in foliar applications for above-ground pests is reduced because UV radiation, low humidity and higher temperatures limit their activity (Kaya and Gaugler, 1993; Lacey and Georgis, 2012; Griffin, 2015). In the case of *T. absoluta*, EPNs can have a limited window of time to enter tunnels and successfully parasitize the *T. absoluta* larvae. However, some limited studies suggest that foliar applications of steinernematid nematodes can provide adequate control of *T. absoluta* at least in laboratory and greenhouse conditions (Moisan et al. 2024). The exploration of symbiotic bacteria derived products (including metabolites) for arthropod control has gained some impetus over the last years. The use of EPNs bacterial symbionts and their products such as metabolites could be an alternative solution to conventional agrochemicals and the base of forming new biopesticides and other new naturally derived green molecules (Eroglu et al., 2019; Cevizci et al., 2020; Campos-Herrera et al., 2021; Vicente-Díez et al., 2021; Cimen et al., 2022; Vicente-Díez et al., 2023). However, these consortia of bacteria and/or their products should have minimal impact on non-target organisms, such as other arthropod biological control agents, that are used regularly (Cevizci et al., 2020).

Taking into consideration the adverse environmental conditions on the behavior and efficacy of EPNs for above ground pests, such as *T. absoluta*, the aim of the present study is to assess the effectiveness of nematodes, their associated bacteria, and their cell free filtrates against larvae of *T. absoluta,* under laboratory conditions. Yüksel (2022) tested the cell-free supernatants and cell suspensions of the symbiotic bacteria of an isolate of *S. feltiae* and *H. bacteriophora* against young and older *T. absoluta* larvae. In addition, it is known that *S. carpocapsae* is more virulent in higher temperatures and lower RH than *S. feltiae* (Husin and Port, 2021) making it more competent for control of *T. absoluta*; therefore, it is of interest to assess the efficacy of *Xenorhabdus* bacteria from *S. carpocapsae* and their products on *T. absoluta*. In this study, a step further is taken by investigating the potency of these bacteria and their metabolites on its non-target predator *N. tenuis*. Several studies have shown a possible adverse effect of EPNs on mirid predators including *N. tenuis* (Garriga et al., 2019; Steenman et al., 2023). Therefore, the assessment of the toxicity of these treatments on this predator is important to warrant the compatibility of multiple biological control agents used in biocontrol consortia against *T. absoluta* (Zappala et al., 2012; Soares et al., 2019; van Lenteren et al., 2020; Steenman et al., 2023). We hypothesize that EPN bacteria and their products are differentially toxic to the targeted insects (pest and natural enemy) than EPN themselves and furthermore, they differentially affect the tested insects. By testing these hypotheses, we aim to explore new avenues to exploit EPN as pest control tools.

2. Materials and methods

2.1. Rearing of EPNs

The nematodes *S. carpocapsae* and *H. bacteriophora* were obtained by Bio-Insecta (Thessaloniki, Greece). Nematodes were reared *in vitro* on the last instar larvae *Galleria mellonella* L. according to the method of Woodring and Kaya (1998). Newly produced nematodes were collected and preserved in containers at 10 °C (Kaya and Stock, 1997). All treatments tested were derived from no more than 2-week-old nematodes.

2.2. Experimental insects

A culture of *T. absoluta*, originated from Aristotle University Farm, was initiated in the Laboratory of Entomology, School of Agriculture, Faculty of Agriculture, Forestry and Natural Environment, Aristotle University of Thessaloniki. The insects were reared in mesh cages (60 × 60 × 60 cm) at 25 °C, and adults, 1st/2nd and 3rd/4th instars of *T. absoluta* were kept in different cages. One-month old potted tomato seedlings (var. Ace 55 VF), in peat–perlite mixture (4:1 ratio), were placed in cages with adults, to achieve oviposition on the leaves and were supplied with 10 % (w/v) honey water solution. After 24–48 h of oviposition, the plants were transferred to the cages of the juvenile stages for the eggs to hatch and were kept there until growth to the desired stage, 1st/2nd or 3rd/4th instar for each experiment. *Nesidiocoris tenuis* were provided by Bio-Insecta (Thessaloniki, Greece) at the adult stage of the age of between two and four days old.

2.3. Isolation culture conditions and identification of Xenorhabdus and Photorhabdus

The bacterial symbionts *X. nematophila* and *P. luminescens* were isolated from the dead larvae of *G. mellonella* previously infected with EPNs. The *G. mellonella* cadavers were then disinfected with 95–100 % ethanol. The hemolymph containing each bacterium was obtained using a sterile needle and then streaked on plates of nutrient bromothymol blue-triphenyl tetrazolium chloride agar (NBTA for 1 L: 8 gr nutrient agar, 25 mg Bromothymol Blue and 40 mg 2,3,5 triphenyl tetrazolium chloride). The bacterial strains were initially identified macroscopically,

according to Thanwisai et al. (2012): the colonies of *Xenorhabdus* species are dark blue and catalase negative while those of *Photorhabdus* are dark green on NBTA and catalase positive. The bacterial strains' single colonies were obtained using the serial dilution method (Ben-David and Davidson, 2014), maintained on NBTA medium in the dark at 28 °C and sub-cultured every 20 days.

The DNA of *Xenorhabdus* and *Photorhabdus* was extracted using dNEAT Bacterial Genomic DNA Isolation Kit (Labbox Labware, S.L., Barcelona, Spain), according to the manufacturer's protocol. Two genes were targeted for sequence analysis: a partial region of recA (890 bp) and a partial region of 16SrRNA (1500 bp). The recA region of all single colonies was amplified with primers recA_F/recA_R (Yimthin et al., 2021) and the amplification of the 16S region was performed using the primers 16SP1_F/16SP2_R (Lalramnghaki et al., 2017). Amplification conditions for primers recA_F/recA_R were as follows: 98 °C for 30 s; followed by 35 cycles of 10 s at 98 °C, 30 s at 55 °C, and 40 s at 72 °C; and final extension 2 min at 72 °C. Regarding primers 16SP1_F/16SP2_R, amplification conditions were as follows: 98 °C for 30 s; followed by 35 cycles of 10 s at 98 °C, 30 s at 65 °C, and 40 s at 72 °C; and final extension 2 min at 72 °C. PCR products were separated by electrophoresis in 1.5 % agarose gel in TAE buffer (TAE; Tris acetate EDTA) and visualized with MIDORIGreen advance (Nippon Genetics, Düren, Germany) under UV light. Purification of PCR products was performed using the Monarch PCR and DNA Clean Up Kit (New England Biolabs, Boston, USA) according to the manufacturer's protocol. All DNA sequences were edited using Geneious Prime® 2022.1.1 (Biomatters Ltd., Auckland, New Zealand) software, and all obtained sequences were compared with sequences in the National Center for Biotechnology Information database using Blast software. Following the Blast software search, the isolate of *Xenorhabdus* (GenBank OR782825 and OR791744) showed sequence 99 % similarity to *X. nematophila* for both targeted genes. The isolate of *Photorhabdus* (GenBank OR782824 and OR791746) was recognized as *P. luminescens* with 99 % similarity for both genes that were sequenced (16S and RecA) (Table 1).

2.4. Preparation of bacterial cell suspensions and cell-free liquid filtrate

Single 48-h old colonies of *X. nematophila* and *P. luminescens* respectively, were aseptically transferred into 40 ml of Tryptone Soy Broth (TSB) in 50 ml falcon tubes (Neogen, Lansing, USA). The liquid cultures were incubated on a rotary shaker at 180 rpm, in darkness, at 28 °C and the optical density (625 nm) after 96 h ranged between 2.5 and 3.5, using a Nanophotometer P300 (Implen, USA). Both *X. nematophila* and *P. luminescens* bacterial cell suspensions were obtained by centrifugation at 4000 rpm, for 10 min. The pellet was then resuspended in tap water at a concentration of approximately 1×10^8 cfu, ($O.D._{625}$ value 0.7). The supernatant was recentrifuged at 8000 rpm for 10 min and then filter sterilized using a 0.45 and then a 0.20 μm syringe filter (LLG Labware, Mechenheim, Germany). Fresh suspensions were prepared each time before bioassays and both the suspensions, and the cell free supernatants were streaked on NBTA to prove viability and absence of cells respectively.

2.5. Pathogenicity bioassays

The experiments to assess the effectiveness of nematodes, bacteria, and their filtrates against *T. absoluta* were conducted in 9 cm (diameter) Petri dishes. Young tomato leaves form the shoot apex (var. Mountain Fresh) (≈ 7 cm) were collected from the Aristotle University Farm and were washed and refrigerated 24 h prior to the bioassays. An inverted tomato leaf, as eggs are laid on the underside of leaves (Desneux et al., 2010), was placed in a petri dish with filter paper with the leave's edge in contact with wet cotton to prevent bursting. The experiments were conducted separately for young (1st /2nd instar) larvae and old (3rd/4th instar) larvae. For each growth stage there were 30 replicates per treatment.

For nematode bioassays, 2 ml containing ≈1000 nematodes were pipetted onto each leaf and then 4 larvae were transferred with a brush to each leaf. The same procedure was followed for bioassays with bacterial cell suspensions of *P. luminescens* and *X. nematophila* and their crude cell-free liquid filtrates. To evaluate the efficacy of the crude, cell-free liquid filtrates, 2 ml were sprayed on both sides of each leaf, and four larvae were added. Petri dishes containing leaves sprayed with 2 ml of TSB medium and leaves sprayed with water, were used as control treatments. Four larvae were added to each control leaf. All plates were sealed with parafilm, and larval mortality was monitored after 48 h and 96 h at a room temperature of 25 ± 1 °C in the dark. For each type of larvae, the experimental set consisted of 10 replicates per treatment. A total of 3 experimental sets were performed, so there were in total 30 replicates per treatment.

The same setup was followed to assess the mortality of predator *N. tenuis* caused by nematodes, bacterial cell suspensions and crude cell-free liquid filtrates. As control treatments were used petri dishes sprayed with TSB and water like in the setup above. In order to ensure the vitality of *N. tenuis, Artemia* cysts (Agrobio®, Almeria, Spain) were added ad libitum to the petri dish. Subsequently, four adults *N. tenuis* of young age (~2–4 days old) were transferred in the petri dish and their mortality was monitored for 48 h at room temperature (25 ± 1 °C), to assess the effect in the early adult age. The exposure time of 48 h for assessing mortality of *T. absoluta* larvae was used to cross-compare treatment toxicity with this on *N. tenuis*. Typically, *N. tenuis* are released as young adults and their lifespan is short under food limitation (Urbaneja et al., 2005). A total of 3 experimental sets were performed, so there were in total 30 replicates per treatment.

2.6. Statistical analysis

The observed mortality in the respective control treatments, both in the experiment with *T. absoluta* and *N. tenuis*, did not exceed 15 % and therefore, the Abbott's formula was used (Abbott 1925). The correction was made based on the respective control of each treatment. The effects of different treatments (nematodes, their respective symbiotic bacteria and their filtrates) and larval stage on insect mortality were explored with logistic regression assuming quasi-binomial distributed errors. Post hoc tests were performed using a Least Square Difference Test at a = 0.05 among estimated marginal means of different treatments. In the case of *T. absoluta*, Post-hoc tests were conducted separately for 1st /2nd instar larvae and 3rd/4th instar larvae and for different time intervals (48 and 96 h). All statistical analyses were performed with the SPSS v 23.0 software (SPSS Inc., Chicago, IL).

Table 1
Symbiotic hosts, isolation data and accession numbers for the isolated symbiotic bacteria of entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabtitis bacteriophora*.

Isolate information					Accession numbers	
Bacterial species	Isolate number	Isolation source	Symbiotic host	Date	16S	RecA
Xenorhabdus nematophila	XN1	*Galleria mellonella* larvae	*Steinernema carpocapsae*	June 2023	OR782825	OR791744
Photorhabdus luminescens	PL1	*Galleria mellonella* larvae	*Heterorhabditis bacteriophora*	June 2023	OR782824	OR791746

3. Results

3.1. Tuta absoluta pathogenicity bioassays

The effect of treatment (nematodes, their respective symbiont bacteria and their filtrates) significantly influenced larval mortality (mortality at 96 h: $F_{5,347} = 47.372$, $P<0.001$). In addition, the 1st/2nd instars were more susceptible than the 3rd/4th instars to different treatments (mortality at 96 h: $F_{1,347} = 3.927$, $P<0.05$). Furthermore, the interaction between treatment and larval stage was also significant (mortality at 96 h: $F_{1,347} = 5.766$, $P<0.001$). The mortality rates increased with the extension of exposure time (tables S1 and S2 supplementary material). Treatments with both nematode species caused equally the highest mortality in both young and older larvae by 96 h (see also supplementary material tables S1 and S2). Across all treatments, the nematodes *S. carpocapsae* and *H. bacteriophora* were reported to exhibit the highest mortality rate of 87 % and 98 % in young and older larvae respectively (Figs. 1 and 2). For 1st /2nd instar larvae, bacterial suspensions of *X. nematophila* caused higher mortality (69 %) than bacterial suspensions of *P. luminescens* (52 %), whereas bacterial filtrates of both species had equal potency raging between 48–55 % (Fig. 1). Regarding 3rd/4th instar larvae, bacterial cell suspensions of *P. luminescens* and *X. nematophila* and crude cell-free liquid filtrates caused not significantly different mortality rates, ranging from 42 to 51 % (Fig. 2).

3.2. Nesidiocoris tenuis pathogenicity bioassays

The effects of different treatments (nematodes, their respective symbiont bacteria and their filtrates) significantly influenced adult *N. tenuis* mortality ($F_{5,174} = 28.983$, $P<0.001$). The mortality of *N. tenuis* caused by *S. carpocapsae* was the highest among all treatments (87 %) and significantly higher than the one caused by *H. bacteriophora* (36 %) (Fig. 2). The mortality caused by bacterial suspensions of *X. nematophila* and *P. luminescens* and crude cell-free liquid filtrates of *P. luminescens* were similar and relatively low (11–16 %), however the mortality caused by the crude cell-free liquid filtrates of bacterium *X. nematophila* was higher than the other non-nematode treatments (Fig. 3).

4. Discussion

There is an urgent need for the development of innovative and sustainable ways to control *T. absoluta*. Recently, there has been a growing interest in the symbiotic bacteria associated with entomopathogenic nematodes as well as their secondary metabolites, and in numerous occasions their potential for controlling important pests such as mosquitoes *Aedes* sp. (Yooyangket et al., 2018; Subkrasae et al., 2022) and the moths *Earias vittella* (Adithya et al., 2020), *Ectomyelois ceratoniae* (Alotaibi et al., 2021), *Lobesia botrana* (Vicente-Díez et al., 2021), *Tuta absoluta* (Yüksel, 2022), *Pieris brassicae* (Tomar et al., 2023) has been demonstrated. Different studies demonstrate the efficacy of EPNs against larvae of *T. absoluta*, however less attention has been given to the screening of the efficacy of bacterial cell suspensions of EPNs and their crude cell-free liquid filtrates combining also the effect of those treatments with its key predator *N. tenuis*.

The results of this study showed that both EPN species, *S. carpocapsae* and *H. bacteriophora*, where highly effective at a dose of 500 IJs/ml causing up to 87 % mortality of young *T. absoluta* larvae; Moisan et al. (2024) under laboratory conditions reported that *S. carpocapsae and S. feltiae* caused about 60 % mortality of *T. absoluta* 2nd instar larvae for a two-fold dose from that of our study (1 million IJs/L). In our study, mortality rates of *T. absoluta* larvae caused by *H. bacteriophora* and *S. carpocapsae* did not differ significantly. According to other studies, EPNs are highly effective regardless of the applied dose (Batalla- Carrera et al., 2010; Moisan et al., 2024), but higher concentrations of EPNs can lead to higher mortality rates, as the load of the symbiotic bacteria is higher (Eleftherianos et al., 2010; Mutegi et al., 2017). These bacteria, *Photorhabdus* spp. and *Xenorhabdus* spp., secrete molecules that have toxic activity in insect haemocytes (Eleftherianos et al., 2010). Thus, higher titers of these molecules accelerate insect mortality. This study showed that *S. carpocapsae* and *H. bacteriophora* could kill their host within 48 h, a trait that was previously recorded by Van Damme et al. (2016). According to other experiments, where similar dosages of EPNs were applied to test the pathogenicity variety of EPN species against *T. absoluta*, *S. carpocapsae* was found to be more effective than *H. bacteriophora* (Van Damme et al., 2016; Husin and Port, 2021).

In this study, the efficacy of bacterial cell suspensions of EPNs and their crude cell-free liquid filtrates on different larval stages of *T. absoluta* was also investigated. Our results are in accordance with Yüksel (2022), who also investigated the insecticidal effect of *Xenorhabdus* and *Photorhabdus* bacterial cell suspensions and crude cell-free liquid filtrates in 1st/2nd and 3rd/4th larval stages. In both studies, 1st/2nd larval instars were more susceptible to the applications than 3rd/4th larval stages, and this susceptibility can be explained by the feeding habits of the insect. The mortality in the 1st/2nd larval instars is higher probably because these stages are exposed for a longer period to the bacterial cells and filtrates due to the time spent scratching the leaf

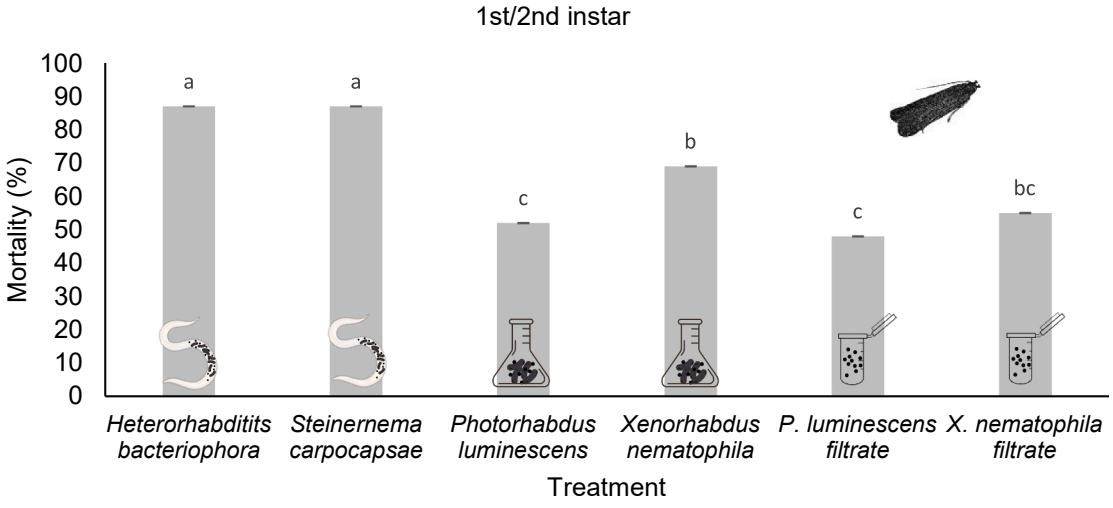

Fig. 1. Mortality rate of *T. absoluta* (average ± S.E.) subjected to six treatments, EPNs, EPNs' symbiotic bacteria and their cell-free filtrates, for 1st/2nd instar after 96 h. Mean values of treatments with the same letter(s) do not differ statistically significantly according to Least Square Difference Test, ($P\leq0.05$). Mortality was corrected using Abbott's formula.

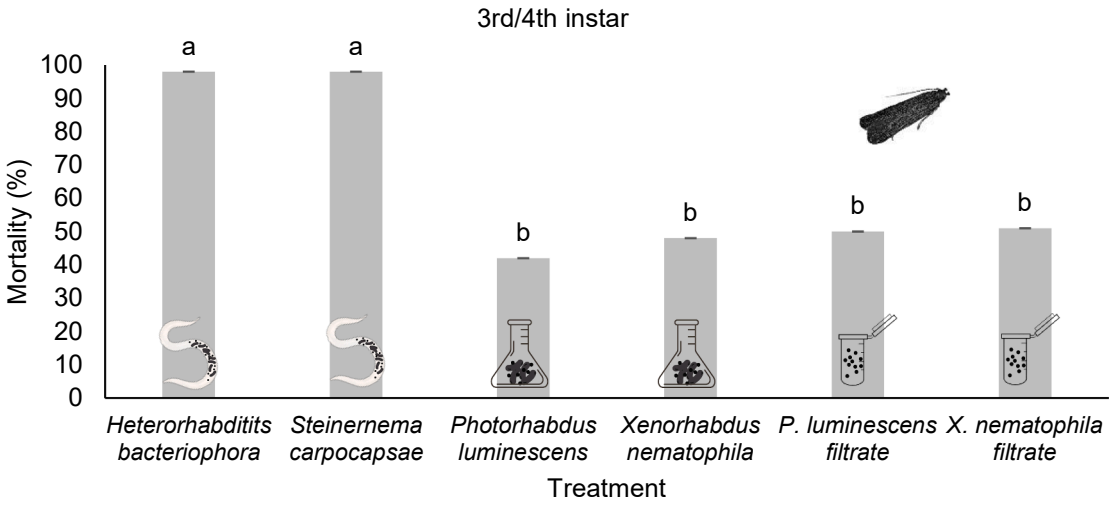

Fig. 2. Mortality rate of *T. absoluta* (average ± S.E.) subjected to six treatments, EPNs, EPNs' symbiotic bacteria and their cell-free filtrates, for 3rd/4th instar after 96 h. Mean values of treatments with the same letter(s) do not differ statistically significantly according to Least Square Difference Test, ($P \leq 0.05$). Mortality was corrected using Abbott's formula.

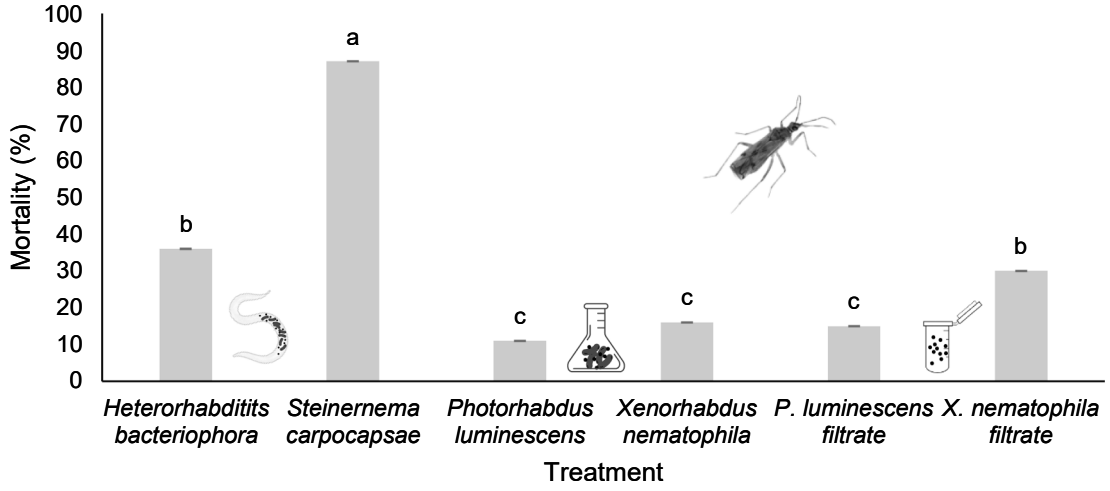

Fig. 3. Mortality rates of the predator *N. tenuis* (average ± S.E.) after exposure to EPNs, EPNs' symbiotic bacteria and their cell-free filtrates for 48 h. Mean values of treatments with the same letter(s) do not differ statistically significantly according to Least Square Difference Test, ($P \leq 0.05$). Mortality was corrected using Abbott's formula.

before penetrating (Giustolin et al., 2001). It could also be attributed to the fact that these instars are significantly smaller in size, and their relative intake is higher as compared to 3rd/4th instars, since the dosage does not change. Moreover, 3rd/4th larval stages have greater biomass, so probably the ingestion of bacterial cells and filtrates were insufficient to induce high mortality (Giustolin et al., 2001).

Overall, bacterial cell suspensions and their respective cell-free filtrates were equally potent against *T. absoluta* larvae, although bacterial suspensions of *X. nematophila* caused higher mortality than that of *P. luminescens* at the 1st/2nd larval stages. The mortality rate of 1st/2nd instar by the bacterial cell suspensions of *X. nematophila* was slightly higher than the one reported in Yüksel (2022). Other studies conducted with the same genera of bacteria against other pests showed similar results (Yooyangket et al., 2018; Adithya et al., 2020; Alotaibi et al., 2021; Subkrasae et al., 2022; Tomar et al., 2023). Yooyangket et al. (2018) and Subkrasae et al. (2022) studied the effects of *Xenorhabdus* and *Photorhabdus* bacteria and their bacterial extracts against *Aedes aegypti* and *Aedes albopictus*; bacterial cells of *Xenorhabdus* spp. were more effective against the two *Aedes sp*. larvae in contrast with *Photorhabdus* bacteria and its bacterial cell extracts. Additionally, *Xenorhabdus* bacteria were found to be effective against other moth pests in several studies (Alotaibi et al., 2021; Adithya et al., 2020; Tomar et al., 2023). Adithya et al. (2020) investigated the effects of *Xenorhabdus* and *Photorhabdus* bacteria and their cell supernatants against *Earias vittella* larvae and reported that *Xenorhabdus* bacteria were more effective than *Photorhabdus* at 24 h., whereas at 72 h both bacteria and their cell supernatants caused similar mortality rates to the larvae. Furthermore, *Xenorhabdus* bacteria caused higher mortality than *Photorhabdus* and bacterial cell free supernatants were more effective than bacterial cell suspensions for larvae of *Ectomyelois ceratoniae* (Alotaibi et al., 2021). Finally, Tomar et al. (2023) showed that bacterial suspensions of *Xenorhabdus* can control *Pieris brassicae* larvae efficiently.

In our study, there was a tendency that suspensions of bacterial cells of *X. nematophila* are more potent than their respective crude cell-free liquid filtrates, which is more pronounced at 48 h (see S1 and S2 tables). The higher effectiveness of bacterial cells could be attributed to the presence of living bacterial cells in the suspensions, potentially allowing them to multiply inside the larvae and consistently generate bioactive compounds with larvicidal properties (Subkrasae et al., 2022). The insecticidal and immunosuppressive compounds produced by

Xenorhabdus spp. are secondary metabolites like benzylideneacetone, iodinine, xenorhabdins, and xenocoumacins, and primary metabolites, like alkaline protease (Bode, 2009). *Photorhabdus* spp. produce compounds like anthraquinone pigments, rhabduscin, isopropylstilbenes, transcinnamic acid and mevalagmapeptides (Cimen et al., 2022). Regarding, the observed increased mortality after 96 h compared to 48 h (tables S1 and S2 supplementary material), this can be explained by the time it takes for these bacteria to establish infection, multiply, and produce toxins that ultimately lead to the insect's death. It is known that *Xenorhabdus* spp. and *Photorhabdus* spp. enter in haemolymph of the larva, reproduce and secrete antimicrobial compounds (Owuama, 2001). Regarding cell-free liquid filtrates produced by *Xenorhabdus* sp. and *Photorhabdus* sp., constant feeding activity is possibly required for the toxin to accumulate and reach lethal concentrations inside the larva.

To the best of our knowledge, this study represents the first assessment of the effects bacteria *X. nematophila* and *P. luminescens* and their respective cell-free filtrates have on predator *N. tenuis*. The effect of nematodes alone on the predator have been evaluated before by Garriga et al. (2019). Their results showed that *N. tenuis* is not so susceptible and has an increased survival rate after application of *H. bacteriophora*, but *S. carpocapsae* was characterized as moderately detrimental for adults of the predator (Garriga et al., 2019). The results of this study corroborate the findings of Garriga et al. (2019) with the difference that the mortality percentages observed here were higher, about 31–35 %. According to the International Organization for Biological Control (IOBC) standardized classification for the impact of pesticides on natural enemies on laboratory bioassays, the crude cell-free liquid filtrates of *X. nematophila* and nematodes *H. bacteriophora* were slightly harmful on *N. tenuis* (IOBC scale: 30–79 %), whereas the two bacteria species and crude cell-free liquid filtrates of *P. luminescens* were harmless (IOBC scale: < 30 %) (Hassan, 1992; Sterk et al., 1999). The impact of these EPNs derived treatments on *N. tenuis* might be even lower if we also consider their possible lower persistence in field conditions but this requires further experimental validation. It is also worth mentioning that EPNs' bacterial metabolites show higher toxicity on spider mites than predatory mites (Cevizci et al., 2020).

In conclusion, both nematode species caused the highest mortality on *T. absoluta*, whereas *N. tenuis* was relatively tolerant to *H. bacteriophora*. The symbiotic bacteria of EPNs and their cell-free filtrates were more toxic to *T. absoluta* larvae, than to *N. tenuis* adults. Specifically, bacterial cells of *X. nematophila* were the most effective against young *T. absoluta* larvae, whereas they were marginally harmful to *N. tenuis* adults. It is worth mentioning that in our study nematodes themselves were more effective that their symbiotic bacteria and their products. Nematodes can search actively in the phyllosphere and enter tunnels to parasitize *T. absoluta* larvae but this requires optimal conditions which can be met in laboratory setting or at the best case at highly regulated greenhouse conditions. On the other hand, products of nematodes (bacteria and their secreted metabolites) can form the basis of developing new "green" pesticides that have a higher impact on target pests compared to beneficial arthropods, allowing their adoption in IPM programs. Future research will determine how different doses of these bacteria and their derived products affect their persistence and their potency in more natural conditions.

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CRediT authorship contribution statement

Nathalie Kamou: Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Investigation, Formal analysis. **Ariadni Papafoti:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Vasileia Chatzaki:** Writing – review & editing, Resources, Methodology, Investigation. **Apostolos Kapranas:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2024.108181.

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