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Research Article

New Insight into the Management of the Tomato Leaf Miner, *Tuta absoluta* (Lepidoptera: Gelechiidae) with Entomopathogenic Nematodes

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Abstract

The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a serious threat to tomato production in the world. Due to serious issues with insecticide resistance, there is a dire need for alternative control methods. Entomopathogenic nematodes (EPN) have potential for the biological control of *T. absoluta*. In the laboratory, we examined the effect of temperature, soil type, and exposure time on the efficacy of the EPN species *Steinernema carpocapsae* (Nematoda: Steinernematidae) and *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae) against last-instar *T. absoluta* larvae. Both species caused high mortality in loamy sand (89%) and coco peat (93%) but not in sandy loam (17%). *H. bacteriophora* caused 92–96% mortality at 19, 25, and 31°C; *S. carpocapsae* caused 89–91% mortality at 25 and 31°C but only 76% at 19°C. Both species caused similar mortality levels after 65-min exposure; thereafter, mortality increased only with *S. carpocapsae* reaching high levels even at a low concentration. Both species infected larvae within leaf galleries. When applied to whole large tomato plants in the greenhouse, both species provided similar control levels (48–51%) at high pest densities. Both species could be incorporated as an effective alternative to synthetic insecticides into *T. absoluta* management programs in greenhouse tomato production.

Key words: *Steinernema carpocapsae*, *Heterorhabditis bacteriophora*, insect pathology, abiotic factor, greenhouse

The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), attacks various solanaceous plants but prefers tomatoes (*Solanum lycopersicum* L.) as a host. Originating in South America where it is widespread (Guillemaud et al. 2015), it was first detected outside of South America in eastern Spain in 2006 and has since rapidly spread to many other European countries, throughout the Mediterranean basin and into the Middle East (Desneux et al. 2010, Desneux et al. 2011, Guillemaud et al. 2015) and sub-Saharan Africa (Sylla et al. 2017). On field- and greenhouse-grown tomatoes, the four larval stages mine leaves, shoots, stems, flowers, and developing fruit, causing severe damage, particularly on young plants, that can result in up to 100% crop loss if not adequately managed (Desneux et al. 2010, Desneux et al. 2011, Tropea Garzia et al. 2012). While mostly residing inside the mines, larvae occasionally exit to start a new mine on different parts of the plant (Fernandez and Montagne 1990a). The mature fourth stage larvae mostly drop to the ground where pupation mainly occurs, but cocoons can also be found attached to all plant parts (Torres et al. 2001). Under optimal condition, the pest can develop through up to 12 generations per year (Biondi et al. 2012a,b; Tropea Garzia et al. 2012; Guillemaud et al. 2015; Biondi et al. 2018).

Current management tactics for *T. absoluta* are mainly based on monitoring with sex pheromone traps and application of synthetic insecticides (Biondi et al. 2018). However, the pest's short development period with multiple generations per year usually requires repeated insecticide applications per season which have detrimental effects on nontarget invertebrates and wildlife (Desneux et al. 2007; Biondi et al. 2013a,b; Biondi et al. 2018). Overuse of insecticides also has repeatedly resulted in the development of insecticide resistance in *T. absoluta* (Siqueira et al. 2001; Lietti et al. 2005; Haddi et al. 2012, 2017; Campos et al. 2014), even to diamides, a new powerful class of insecticides (Roditakis et al. 2015).

An integrated pest management (IPM) strategy, in which natural enemies of the pest and other alternative measures play significant

roles, is required to minimize negative environmental impacts and to reduce insecticide resistance development rates.

Biological control of *T. absoluta* is currently based on parasitoids of eggs (Chailleux et al. 2013a) and larvae (Biondi et al. 2013, Zappalà et al. 2013), and on omnivorous predators (Perdikis and Arvaniti 2016, Salehi et al. 2016, Sylla et al. 2016), employed alone or in combination with parasitoids (Chailleux et al. 2013b, Naseli et al. 2017). Entomopathogens, such as *Metarhizium anisopliae* var. *anisopliae* (Metsch.) Soroki, *Beauveria bassiana* (Balsamo) Vuillemin, and *Bacillus thuringiensis* (Berliner), have shown efficacy versus *T. absoluta* in laboratory and greenhouse tests (Rodriguez et al. 2005, González-Cabrera et al. 2011).

Entomopathogenic nematodes (EPNs) have been used as biological control agents of a great variety of insect pests (Georgis et al. 2006, Lacey and Georgis 2012). There are several products based on EPN species including *Steinernema feltiae* (Filipjev), *Steinernema carpocapsae* (Weiser), and *Heterorhabditis bacteriophora* (Poinar) which are used in IPM programs (Wraight et al. 2017). The EPN species were able to infect all four larval stages of *T. absoluta* inside or outside of leaf galleries in Petri dish experiments (Batalla-Carrera et al. 2010, Van Damme et al. 2016). In laboratory experiments in soil, *S. carpocapsae* and *H. bacteriophora* were highly infective and more infective than *S. feltiae*, to fourth stage larvae, but all three species were ineffective against pupae (Garcia-del-Pino et al. 2013). All three species provided high levels of control of larvae in pot experiments using small tomato plants (Batalla-Carrera et al. 2010).

Efficacy and behavior of EPNs are affected by many factors such as soil texture, moisture, temperature, and host depth (Kaya 1990, Shapiro-Ilan et al. 2000). Soil is the natural habitat of EPNs, and it has been shown that in this habitat, some EPN species [*e.g.*, *S. carpocapsae* and *S. scapterisci* (Nguyen and Smart)] use an 'ambush' approach to find hosts near the soil surface, whereas other species [*e.g.*, *H. bacteriophora* and *S. glaseri* (Steiner)] use a 'cruiser' searching approach to find hosts throughout the soil profile (Kaya and Gaugler 1993, Lewis 2002). Soil texture has a profound effect on critical EPN traits like foraging, persistence, and movement (Kaya 1990). Therefore, consideration of the effects of soil factors on EPN activity is a pre-requisite for successful EPN use. Temperature is another important factor that affects EPN success (Griffin 1993). It directly influences host searching, virulence, and survival (Molyneux 1985, Kung and Gaugler 1991).

The objective of this study was to evaluate the effect of factors like infective juvenile (IJ) concentration, substrate type, temperature, and exposure time on EPN efficacy versus last instar *T. absoluta*; identify the possible relations among these factors; and measure the ability of EPNs to control the larvae inside and outside of tomato leaf galleries. Subsequently, greenhouse tests were conducted to prove laboratory assertions and to determine the ability of both EPN species to control *T. absoluta* larvae under experimental conditions similar to commercial greenhouse tomato production.

Materials and Methods

A *T. absoluta* colony was maintained on tomato plants under greenhouse condition. The colony had been established from larvae collected in July 2015 from a commercial tomato greenhouse in Mashhad (Khorasan Razavi, Iran) that used *B. thuringiensis* var *kurstaki* (Biobit, Dipel, Javelin) for the pest's management.

The colony of insect were reared on tomato plant, *Solanum lycopersicum* cv. Ergon Mobil. The larvae used in the laboratory experiments were obtained by placing infested leaves on wire screen to collect last instar larvae after they passed through the screen.

H. bacteriophora HBoj strain (Kamali et al. 2013) and *S. carpocapsae* (commercial strain from Koppert Co., Berkel en Rodenrijs, the Netherlands) were produced in late instar greater wax moth, *Galleria mellonella* L., larvae at 25 ± 1°C. The IJ stage of the nematodes were collected using White traps (Kaya and Stock 1997) and stored in tap water at 12 ± 1°C for less than 2 wk before use. For experiments, the IJs were acclimatized at room temperature (25 ± 2°C) for 1 h and their viability was verified under a stereomicroscope. Wax moth larvae were reared on an artificial diet at 27 ± 1°C, RH 65 ± 5%, and a photoperiod of 16:8 (L:D) h (Birah et al. 2008).

Laboratory experiments were conducted in a growth chamber at 25 ± 1°C, RH 65 ± 5%, and a photoperiod of 12:12 (L:D) h. The experiment examining the effect of temperature on EPN virulence against last instar larvae was performed at 19 ± 1, 25 ± 1, and 31 ± 1°C under aforementioned humidity and photoperiod conditions. Three types of substrate were used to study the effect of substrate on EPN virulence (Table 1). For each substrate, texture (percent sand, silt, clay), pH, and carbon percentage was determined in the Soil Analysis Laboratory of the Ferdowsi University of Mashhad. The substrates were sieved through an 18-mesh size sieve and autoclaved. Both EPN species were tested in a preliminary experiment on the larvae to determine the required concentrations.

Effect of Nematode Concentration

Bioassays were conducted in clear plastic containers (4-cm diameter, 4-cm height, and 12.6-cm^2 soil surface area). Each unit was filled with 20 g of loamy sand soil (Table 1). Soil moisture was adjusted to 7% (w/w). IJs were uniformly applied to the soil surface at 0, 5, 10, 20, and 50 IJs/cm^2 in 1 ml of distilled water. The final soil moisture was 10% (w/w). The containers were then kept at room temperature for 1 h before ten last instar *T. absoluta* larvae per container were placed on the soil surface. There were four replicates for each concentration. The containers were kept for 72 h under controlled conditions in a growth chamber. Then, the larvae were separated from the substrate by gentle sieving and were individually maintained in controlled conditions until adult emergence. Three days later, 25% of the dead larvae were selected randomly and dissected under a stereomicroscope to confirm nematode infection. The experiment was conducted twice.

Table 1. Characteristics of substrates used in infectivity assays of *H. bacteriophora* and *S. carpocapsae* on last-instar larvae of *T. absoluta*

Soil type	Source locality	Sand (%)	Silt (%)	Clay (%)	pH	Carbon (%)
Loamy sand	I: Mashhad, Khorasan Razavi, Iran	82	8	10	7.94	0.838
Sandy loam	II: Mashhad, Khorasan Razavi, Iran	60	26	14	5.63	0.614
Coco peat	III: Research Greenhouse Unit of FUM	—	—	—	8.58	51.67

Note: FUM, Ferdowsi University of Mashhad.

Effect of Substrate Type

The effect of three substrate types (Table 1) on virulence of *H. bacteriophora* and *S. carpocapsae* against the last instar larvae was determined using the same methodology as in the previous section. The soil moisture was adjusted to 12% for the loamy sand, 17% for the sandy loam and 25% for coco peat. IJs were applied to the soil surface at 0, 5, and 20 IJs/cm^2. Each treatment had four replicates. The experiment was performed twice.

Effect of Temperature

The effect of temperature on virulence of *H. bacteriophora* and *S. carpocapsae* was determined using the same methods as in the section on nematode concentration except that experimental units were kept at 19 ± 1, 25 ± 1, and 31 ± 1°C. The IJs were applied at 0, 5, and 20 IJs/cm^2. Each treatment had four replicates and the experiment was performed twice.

Effect of Exposure Time

Because IJs activity and survival after foliar applications at relative humidity levels below 99% is very short, the IJs must be able to penetrate a host quickly (Navaneethan et al. 2010). Hence, this experiment examined the time required for IJs to infect *T. absoluta* larvae. IJs were applied uniformly unto a double layer of filter paper in Petri dishes (5.5-cm diameter) at 0, 5, and 20 IJs/cm^2 in 2 ml of distilled water. Then, 10 last instar larvae were placed in each Petri dish for 65, 240, and 480 min. Subsequently, they were rinsed and moved to another Petri dish with moist filter paper without IJs and incubated for another 72 h. All dead larvae were dissected to confirm nematode infection. For each EPN species, IJ concentration and exposure time, there were four replicates. The assay was conducted twice.

Leaf Bioassay

Tomato leaves infested with *T. absoluta* larvae were collected from the Research Greenhouse Unit of FUM. There were between one and six larvae per leaf with an average of two per leaf. Leaves were randomly assigned to treatments. Each leaf was sprayed with 1 ml of suspensions of *H. bacteriophora* or *S. carpocapsae* adjusted at 150 IJs/ml resulting in a concentration of approximately 5 IJs/cm^2 on the leaves. A single treated leaf was placed in Petri dishes lined with wet filter paper, sealed, and kept on trays in an incubator. Larval mortality (inside or outside of the leaf) was determined after 72 h. There were 23 replicates per EPN species and the experiment was performed twice.

Greenhouse Tests

Tomato plants ('Ergon Mobil' variety) were grown from seed in peat-based growing medium. They were first grown in a growth chamber (190 × 85 × 81 cm; 25 ± 1°C, photoperiod of 11:13 (L:D) h until they reached the two- to three-leaf stage. Then, they were transplanted into a greenhouse. The plants were kept there until they reached the target size for the experiments (approximately 80-cm diameter and 2-m height). The plants were kept pest free in large cages under semifield conditions; no insecticides were used during the plant development period. The plants were allowed to be naturally infested by the resident *T. absoluta* population in the greenhouse.

In the first greenhouse experiment, experimental arenas consisted of individual *T. absoluta*-infested leaves (approximately 8-cm length × 4-cm width, 32-cm^2 leaf surface) on tomato plants. Infected leaves were marked with a label on the tomato plants. *S. carpocapsae* and *H. bacteriophora* were sprayed at two rates (20 and 50 IJs/cm^2) in 10 ml of distilled water onto the upper and lower leaf surface using a manual sprayer (Keshtzarsanat Co., Karaj, Iran) until the whole target surface was covered. Control treatments were sprayed with 10-ml distilled water only. Each treatment had four replicates. After treatment, the leaves were enclosed with a thin plastic bag, sealed, and kept in the greenhouse (25 ± 5°C, RH 60 ± 10%). After 3 d, mortality was determined by dissecting the leaves and the dead larvae (within and outside of the leaf galleries). The experiment was conducted twice.

The second experiment was conducted using the entire surface of the mature tomato plants. The surface area of each tomato plant was estimated to calculate the appropriate amount of IJs to be applied. *S. carpocapsae* and *H. bacteriophora* were extracted directly from commercial formulations (Koppert Biological Systems). EPN suspension (320 IJs/ml) was applied using a conventional air blast-sprayer (Matabi, Kima 12) at a rate of 50 IJs/cm^2 (on average 1.6 × 10^6 IJs/plant). Control plants were sprayed with water only. There were six plants per treatment. After treatment, the plants were covered with mosquito netting and kept in the greenhouse (22 ± 2°C). The relative humidity kept at 65 ± 5% RH for the first 240 min after treatment greenhouse with a misting system to provide enough time along with suitable condition for IJs to find and infect the target pest. To evaluate EPN efficacy, the number of adult *T. absoluta* emerging per plant over 15 d after treatment was determined with TUA-Optima (lure) (Russell IPM, United Kingdom), a new sexual pheromone lure, on each plant within the mosquito netting. The sticky rolls of traps incorporated the lure into the adhesive layer. The adhesive trap was replaced every 3 d.

Statistical Analysis

Insect mortality was control-corrected (Abbott 1925) and square-root transformed when required to meet assumptions of normality and homogeneity of variances. In all experiments, control-corrected mortality was subjected to multifactorial analysis of variance (ANOVA) and means separation by LSD test (SAS Institute 2002–2003). For the leaf bioassay and evaluation of EPNs in the greenhouse conditions, mortality of larvae within or outside of the tomato leaf were analyzed by two-way analyses of covariance (ANCOVA) in which EPN species were considered the main factors and the total number of tomato leaf miner larvae was entered as a covariate. Larval mortality data from the second greenhouse test were subjected to one-way ANOVA and completely randomized design followed by Tukey's multiple-comparison test ($P < 0.05$).

Results

Effect of Nematode Concentration

In the untreated control, 80 ± 3% of the released larvae emerged as adults. Control-corrected mortality increased with IJ concentration ($F = 22.38$; $P < 0.01$; df = 3, 48) and was higher for *H. bacteriophora* (93.9 ± 1.7%) than for *S. carpocapsae* (89.3 ± 0.3%) ($F = 5.51$; $P < 0.05$; df = 1, 48); there was no significant IJ concentration * EPN species interaction. The regression analysis of the data showed that mortality of last-instar *T. absoluta* larvae significantly increased with IJ concentration for *S. carpocapsae* ($R^2 = 0.231$; $P < 0.05$; Fig. 1a) and *H. bacteriophora* ($R^2 = 0.179$; $P < 0.05$; Fig. 1b). Maximum mortality (99.1 ± 0.03) was achieved when they were treated with *S. carpocapsae* and *H. bacteriophora* at concentrations 20 and 50 IJs/cm^2, respectively.

Effect of Substrate Type

In the untreated control, 85 ± 6% of the released larvae emerged as adults. Control-corrected mortality increased with IJ concentration

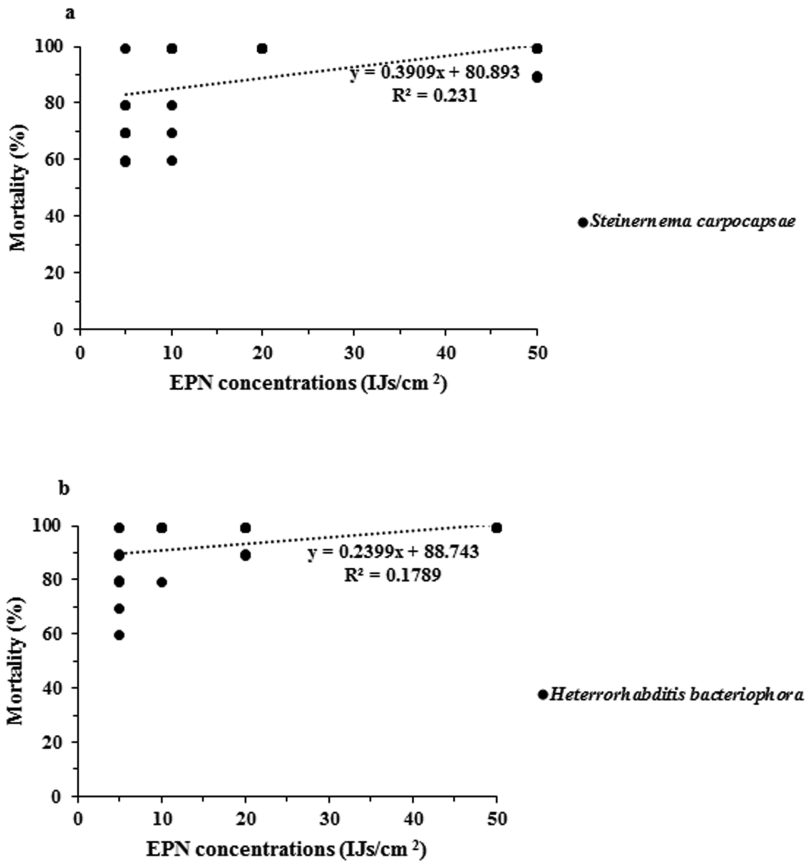

Fig. 1. Linear regression of mean percentage mortality (±SE) of last-instar larvae of *T. absoluta* caused by the EPN species *S. carpocapsae* (a) and *H. bacteriophora* (b) at concentrations of 5, 10, 20, and 50 IJ/cm^2.

(F = 12.4; P < 0.01; df = 1, 72), was significantly higher in loamy sand (88.9 ± 2.4%), and coco peat substrate (92.6 ± 1.7%) than in sandy loam (16.8 ± 2.4%; F = 402.26; P < 0.01; df = 2, 72) but did not differ significantly between *H. bacteriophora* (66.6 ± 5.5) and *S. carpocapsae* (65.8 ± 5.3); there were no significant interactions between any of these factors.

Effect of Temperature

In the untreated controls, 68.8 ± 5.2, 80.0 ± 3.8, and 66.3 ± 5.0% of the released larvae emerged as adults at 19, 25, and 31°C, respectively. Control-corrected mortality of *T. absoluta* was significantly affected by temperature (F = 7.05; P < 0.01; df = 2, 72), EPN species (F = 24.16; P < 0.01; df = 1, 72), and IJ concentration (F = 22.15; P < 0.01; df = 1, 72). There was a significant temperature * EPN species interaction (F = 3.81; P < 0.05; df = 2, 72), but there were no significant interactions between IJ concentration and temperature or EPN species. When data were combined across IJ concentration, *H. bacteriophora* was not affected by temperature whereas mortality caused by *S. carpocapsae* was significantly lower at 19 ± 1°C than at 25 ± 1 and 31 ± 1°C and *H. bacteriophora* caused greater mortality than *S. carpocapsae* at 19 ± 1 and 25 ± 1°C but not at 31 ± 1°C (Fig. 2).

Effect of Exposure Time

In the untreated control, 73.8 ± 3.2% of the released larvae emerged as adults. Control-corrected mortality was significantly affected by exposure time (F = 20.12; P < 0.01; df = 2, 72), EPN species (F = 76.8; P < 0.01; df = 1, 72), and IJ concentration (F = 130.6; P < 0.01; df = 1, 72), but there were significant interactions for exposure time * EPN species (F = 12.72; P < 0.01; df = 2, 72), exposure time * IJ concentration (F = 3. 49; P < 0.01; df = 2,72), EPN species * IJ concentration (F = 9.95; P < 0.01; df = 1, 72), and exposure time * EPN species * IJ concentration (F = 4.97; P < 0.01; df = 2, 72). When data were analyzed with exposure time * EPN species * IJ concentration combinations as treatments, mortality caused by *H. bacteriophora* did not change with exposure time at either IJ rate, whereas *S. carpocapsae*-caused mortality increased significantly at both IJ rates from 65 to 240 min exposure but did not increase from 240 and 480 min at both rates. Mortality did not differ between species after 65 min at either rate, after 240 min was significantly higher for *S. carpocapsae* than *H. bacteriophora* at both rates, and after 480 min was higher for *S. carpocapsae* than *H. bacteriophora* at the lower but not at the higher rate. Mortality was significantly higher at 20 than at 5 IJs/cm^2 for *H. bacteriophora* after each exposure time and for *S. carpocapsae* after 65 and 240 min but not after 480 min (Fig. 3).

Leaf Bioassay

Inside the galleries, mortality of *T. absoluta* larvae was significantly higher for *S. carpocapsae* than *H. bacteriophora* (F = 5.09; P < 0.05; df = 1, 89); mortality was not affected by the covariate total numbers of larvae (F = 2.62; P > 0.05; df = 1, 89). Outside the galleries, mortality did not differ between EPN species but was significantly affected by total number of larvae (F = 36.98; P < 0.01; df = 1, 89; Fig. 4).

Greenhouse Tests

In the first experiment, where EPNs were applied directly to tomato leaves, and the leaves were covered with thin plastic cover, larval

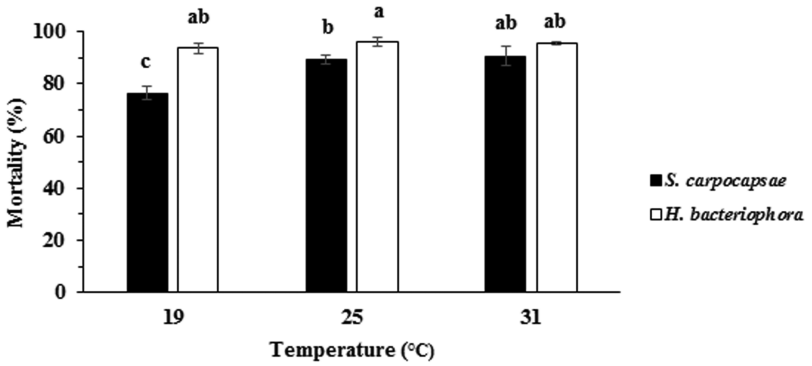

Fig. 2. Effect of temperature on mortality (±SE) of last-instar larvae of *T. absoluta* exposed to two concentrations (5 and 20 IJs/cm²) of *S. carpocapsae* and *H. bacteriophora*. Different letters indicate significant differences among interactive treatments (three-way ANOVA and Fisher's protected LSD at $P < 0.05$).

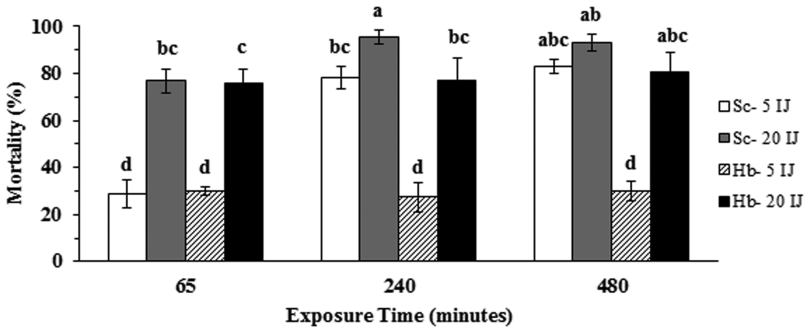

Fig. 3. Effect of exposure duration on mortality (±SE) of last-instar larvae of *T. absoluta* exposed to two concentrations (5 and 20 IJs/cm²) of *S. carpocapsae* (Sc) and *H. bacteriophora* (Hb). Larvae were exposed for 72 h at 25°C on filter paper. Different letters indicate significant differences between interactive treatments (three-way ANOVA and Fisher's protected LSD at $P < 0.05$).

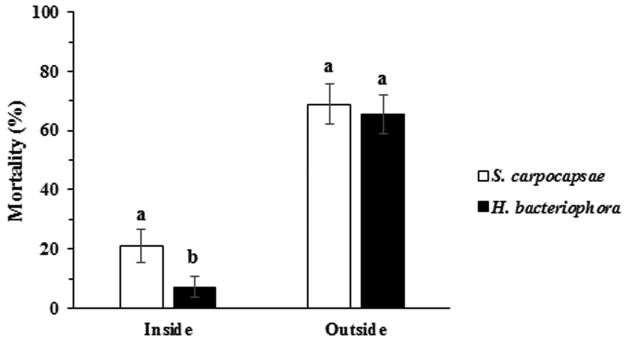

Fig. 4. Mortality (±SE) of *T. absoluta* larvae found inside and outside of tomato leaf galleries after exposure to 5 IJs/cm² of *S. carpocapsae* and *H. bacteriophora*. Larvae were exposed for 72 h at 25°C in Petri dishes. The different letter for each leaf location (inside vs outside) showing significant different (two-way ANCOVA and Fisher's protected LSD at $P < 0.05$).

mortality was significantly influenced by the total numbers of leaf miner larvae per leaf which was analyzed as a covariates ($F = 12.79$; $P < 0.01$; df = 27, 1). There were no significant interactions between EPN species and IJ concentration. Both EPN species were effective against *T. absoluta* larvae, but *S. carpocapsae* (48.1 ± 6.4%) caused significantly higher mortality than *H. bacteriophora* (18.0 ± 2.0%; $F = 25.58$; $P < 0.01$; df = 1, 27) and mortality was significantly higher at 50 IJs/cm² (40.6 ± 7.5%) than at 20 IJs/cm² (25.5 ± 3.3%) ($F = 4.65$; $P < 0.05$; df = 1, 27) with the greatest mortality caused by the high rate of *S. carpocapsae* (65.75 ± 7.4%; Table 2; Fig. 5).

In the second experiment, very high numbers of adult *T. absoluta* emerged from the untreated control tomato plant. Adult emergence

Table 2. Overall infection levels of *T. absoluta* larvae infesting tomato leaves treated with *S. carpocapsae* and *H. bacteriophora* (20 and 50 IJs/cm²) in the greenhouse

Nematode species (Concentration: IJ/cm²)	Overall infection ± SE (%)	Total larvae
S. carpocapsae		
10	30.47 ± 15.09 b*	124
50	65.75 ± 21.16 a	87
H. bacteriophora		
10	20.56 ± 3.21 b	174
50	14.17 ± 2.20 ab	85

*Means with different letter indicate significant differences for each treatment ($P < 0.05$)

from the EPN-treated tomato plants was significantly reduced by around 50% by *S. carpocapsae* and *H. bacteriophora* with no significant difference between the two species.

Discussion

Our observations clearly indicate the good potential of *S. carpocapsae* and *H. bacteriophora* for the control of *T. absoluta* larvae in greenhouse-grown tomatoes. Despite very high pest densities (ca. 377 individual per plant), both EPN species reduced the emergence of adult *T. absoluta* by around 50% after foliar applications targeting larvae at a rate of 50 IJs/cm². For foliar applications, *S. carpocapsae* may be superior to *H. bacteriophora* if high RH can be provided for several hours after application through misting or

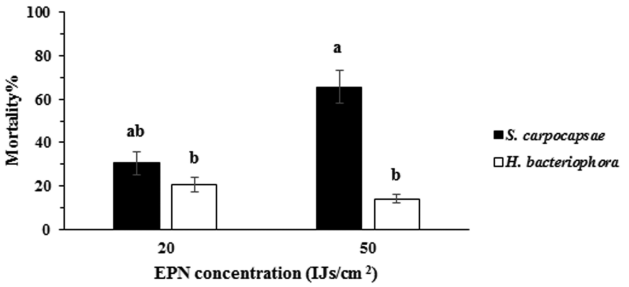

Fig. 5. Mortality (±SE) of *T. absoluta* larvae exposed to 20 and 50 IJs/cm² of *S. carpocapsae* and *H. bacteriophora* larvae in the greenhouse. Different letters indicate significant differences between treatments (two-way ANOVA and Fisher's protected LSD at $P < 0.05$).

other mechanisms (Schroer and Ehlers 2005). Significant increases in efficacy could be achievable by the addition of adjuvants to foliar sprays (Van Damme et al. 2016). Due to the short generation time and overlap of all life stages, successful management of *T. absoluta* will require targeting several life stages, in particular larvae in and on the foliage and fourth instar larvae entering the substrate for pupation. Our observations confirm the excellent potential of EPNs for substrate applications and delineate optimal conditions therefore.

Effective foliar applications are the most important component in *T. absoluta* management as they directly suppress the damage caused by the feeding larvae. Our observations combined with those of Batalla-Carrera et al. (2010) showed that EPNs are able to find and parasitize *T. absoluta* larvae inside leaf mines. The higher mortality rates observed inside the leaves by Batalla-Carrera et al. (2010) (77–92%) can be ascribed to the use of a 12 times higher IJ rate (60 IJs/cm²). Since there is typically a mix of different larval stages present and IJs survival on the foliage is limited to a few hours, it is important to understand the range of stages that can be affected by EPN applications. In leaf disc experiments in Petri dishes (Van Damme et al. 2016), control rates of first, second, third, and fourth instar larvae were about 36, 33, 86, and 84% with *H. bacteriophora*, 41, 75, 92, and 95% with *S. carpocapsae*, and 59, 82, 91, and 78% with *S. feltiae*. They observed a high-mortality rate of fourth stage larvae even within the mines, and this in contrast with the data obtained in the present study

In our greenhouse experiment, in which large tomato plants were infested naturally with *T. absoluta* larvae, *S. carpocapsae* and *H. bacteriophora* provided around 50% control at a rate of 50 IJs/cm². Batalla-Carrera et al. (2010) observed larval mortalities at 4 d after the last application of 87% for *S. carpocapsae* and 95% for *H. bacteriophora* and *S. feltiae* on artificially infested tomato plants in the greenhouse. Batalla-Carrera et al. (2010) applied 15,000 IJs in 15 ml per plant applied twice 1 day apart but do not specify plant size (1-liter pots) and IJ application rate per cm². However, given the small amount of IJs applied, it appears unlikely that amount of IJs used by Batalla-Carrera et al. (2010) resulted in a rate higher than 50 IJs/cm². The higher control rates observed in the Batalla-Carrera et al. (2010) study compared to ours are more likely related to presence of different larval stages and use of an adjuvant that should have improved efficacy (Van Damme et al. 2016). At the time of application, the larval stages in the Batalla-Carrera et al. (2010) greenhouse study must have been composed primarily of third- and fourth-stage larvae because the plants had been artificially infested with first through third instars 5–7 d before EPN application; in our study, the full range of larvae was present. Thus, our greenhouse experiment included also stages that were less susceptible to *S. carpocapsae* and especially *H. bacteriophora*, whereas the Batalla-Carrera et al. (2010) study likely only included the most susceptible stages. Another reason for differences in control rates might be related to the use of the adjuvant Addit (Koppert) in the Batalla-Carrera et al. (2010) study but not in ours. Based on a leaf disc study (Van Damme et al. 2016), adjuvants including Addit improve EPN performance against *T. absoluta* in foliar applications.

For foliar applications, it is extremely important to give the applied IJs enough time to penetrate into the leaf mines since the majority of *T. absoluta* larvae at any given time are inside the mines. Once inside the mines, the IJs should be well protected from desiccation. IJ survival time on the leaf surface can be increased by keeping the RH high to delay evaporative loss of the spray solution and desiccation of any IJs outside of spray droplets. Batalla-Carrera et al. (2010) observed that when *T. absoluta* larvae were exposed for different lengths of time to EPNs, mortality increased for all three EPN species from 23 to 53% with 1-h exposure to 63 to 86% with 3-h exposure; this study, however, was conducted in soil giving it limited relevance for foliar applications. In our study, in which larvae were exposed on filter paper, mortality did not further increase beyond 1-h exposure for *H. bacteriophora* but significantly increased from 1- to 4-h exposure with *S. carpocapsae*. It is, however, possible that mortality would already have leveled off somewhere between 1- and 4-h exposure. Future studies need to examine in detail and under more realistic greenhouse conditions how long the RH needs to be kept high for optimal EPN performance and how this would be affected by the use of adjuvants.

Soil/substrate applications should be used to complement foliar applications for optimal *T. absoluta* management. Our observations in soil arenas show that fourth stage larvae in the soil are highly susceptible to *S. carpocapsae* and *H. bacteriophora* (on average 89–94% mortality), which corroborates previous observations. Thus, Garcia-del-Pino et al. (2013) using 50 IJs/cm² observed 100, 97, and 52% mortality with *S. carpocapsae* (strain B14), *H. bacteriophora* (strain DG46), and *S. feltiae* (strain B114), respectively, in boxes with 1 kg of sandy clay loam. Batalla-Carrera et al. (2010) exposed a mix of third (note: not normally found in soil) and fourth instar *T. absoluta* in Petri dishes with sand to the same three EPN strains and observed 86 and 87% mortality with *S. carpocapsae*, 79 and 100% with *H. bacteriophora*, and 100 and 100% with *S. feltiae* at 25 and 50 IJs/cm², respectively. In our study, *S. carpocapsae* and *H. bacteriophora* produced >90% mortality of fourth instars with rates as low as 10 IJs/cm². Once the *T. absoluta* larvae have pupated, EPNs seem to have very limited effect (Batalla-Carrera et al. 2010, Garcia-del-Pino et al. 2013). However, Garcia-del-Pino et al. (2013) found that adults emerging from soil after completing pupation appear to be quite susceptible to *S. carpocapsae* but are not susceptible to *S. feltiae* (*H. bacteriophora* was not tested).

Given the high potential of EPN soil applications for *T. absoluta* management, understanding the effect of different substrates typically used for tomato production is crucial. It is well known that soil type and texture can affect EPN infectivity and persistence (Kaya 1990), although effects can vary with EPN species (Koppenhöfer and Fuzy 2006). In many studies, infectivity tended to decrease in finer textured soil (e.g., Campos-Herrera and Gutiérrez 2009, Hassani-Kakhki et al. 2012). However, in our study both EPN species caused greater mortality in loamy sand (89%) than in sandy loam (17%); such a great difference was not expected based on the limited differences in texture, pH, and carbon content. Excellent performance of EPN in highly organic substrate as in our study (coco peat, 93%) has been observed for some EPN species including *H. bacteriophora* in other studies (e.g., Koppenhöfer and Fuzy 2006). Our findings about EPN performance in different soil types are important as the

appropriate substrates for tomato production is light soil like loamy sand in traditional systems while in hydroponic culture systems, the substrate is better to be coco peat or similar to it.

Temperature did not seem to be a limiting factor for *S. carpocapsae* and *H. bacteriophora* in our study in soil except to a small extent for *S. carpocapsae* at 19°C. In leaf disc assays, both species caused much lower mortality at 19°C than at 25°C after 72 h, whereas *S. feltiae* was unaffected by temperature; however, when already infected but still alive larvae were included in the mortality count, the effect of temperature was similarly small as in our study (Van Damme et al. 2016). The wide range of greenhouse temperatures under which these EPN species can provide good to excellent control of *T. absoluta* larvae further strengthens their potential for the pest's management.

Overall, the environmental conditions of greenhouse tomato production appear to be compatible with the requirements for these EPNs to achieve high levels of *T. absoluta* infection. Our greenhouse tests confirmed the high susceptibility of *T. absoluta* larvae to *H. bacteriophora* and *S. carpocapsae* with around 50% control for both species. However, the extraordinarily high population density of the pest during the greenhouse experiments likely limited EPN efficacy. Considering the high level of relative humidity needed by EPNs for spray applications, the use of adjuvants like Penterra and Silwet L-77 (Portman et al. 2016) is highly recommended. Application of EPN and other biocontrol agents should be done before pest densities reach such high levels. The results of the present study show that both *H. bacteriophora* and *S. carpocapsae* have good potential as alternatives to synthetic insecticides and could be incorporated into *T. absoluta* management programs for greenhouse-grown tomatoes both as foliar and soil applications. At the same time, they also have potential to control other greenhouse pests such as *Trialeurodes vaporariorum* (West.) (Hemiptera: Aleyrodidae) and *Frankliniella occidentalis* (Pergande) (Ebssa et al. 2004, Rezaei et al. 2015), and the greenhouse production system has potential for combined use of microbials and arthropod natural enemies (Gonzalez et al. 2016). Some additional greenhouse studies will be necessary to optimize the efficacy of EPNs for *T. absoluta* with regards to application volumes, use of adjuvants, timing of applications regarding the presence of different pest stages, and the importance of foliar versus soil applications.

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