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Biocontrol potential of endosymbiotic bacteria of entomopathogenic nematodes against the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

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Abstract

Background: The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is a major pest of tomato plants threatening global tomato production. The control of the pest is becoming increasingly difficult due to the rapid development of resistance to insecticides. Entomopathogenic nematodes (EPNs) of families Steinernematidae and Heterorhabditidae are successful biocontrol agents for many insect pests. Recently, their bacteria, *Xenorhabdus* spp. and *Photorhabdus* spp. have attracted great attention due to their major role in the pathogenicity of EPNs. In the present study, the pathogenicity of eleven EPNs isolates belonging to *Steinernema feltiae* and *Heterorhabditis bacteriophora* species was screened against the 1st/2nd and 3rd/4th instar larvae of *T. absoluta*. The cell-free supernatants and cell suspensions of the symbiotic bacteria from the most efficient isolates were further evaluated for their biocontrol potential in the oral and contact treatments on the larvae of *T. absoluta*.

Results: KBC-4 and MCB-8 isolates of *S. feltiae* showed superior virulence relative to other EPNs species/isolates and induced 90% larval mortality against the 3rd/4th instar of *T. absoluta* larvae, whereas there were no clear differences in the efficacy of EPNs species/isolates against the 1st/2nd instar of *T. absoluta*. The 1st/2nd instar of *T. absoluta* larvae was more susceptible to cell-free supernatants and cell suspension of selected EPNs. The highest mortality (80%) was obtained from *X. bovienii* MCB-8 strain in the contact treatment of supernatants. In contact treatment of cell suspension, higher mortalities were obtained compared to oral treatments. Mortality rates ranged between 30 and 57.5% in the contact treatments of cell suspensions while the highest mortality did not exceed 20% in oral treatments. The antifeedant activity was observed in oral treatments of cell suspension and most of the larvae avoided feeding on treated leaves.

Conclusion: The results indicated that symbiotic bacteria of EPNs had a great potential against *T. absoluta* larvae and contact treatment of cell-free supernatants against early instars of *T. absoluta* can be an ideal application. However, further studies are needed to investigate the field effectiveness of symbiotic bacteria.

Keywords: *Tuta absoluta*, Entomopathogenic nematodes, Symbiotic bacteria, *Xenorhabdus* spp., *Photorhabdus* spp., Potential

Background

The profitability of tomato cultivation dramatically depends on proper management strategies of pests and diseases. Among various insect pests that cause economic losses to tomato crops, the tomato leaf miner,

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Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae), is one of the most destructive pests, with a high dispersal ability (Bhat et al. 2020). Today, *T. absoluta* is one of the major pests of tomato both in the field and greenhouse conditions and threatening world tomato production (Santana et al. 2019). Since its first introduction into Turkey in 2009, it has been reported to cause severe yield losses in key tomato production areas of Turkey (Helvaci 2020). The control of *T. absoluta* is quite challenging due to its high reproduction potential, polyphagous nature and the endophytic feeding habits of larvae (Mohamed et al. 2015). Synthetic insecticides are the primary control method of *T. absoluta* infestations in Turkey (İnak et al. 2021). Frequent use of insecticides leds to the rapid development of insecticide resistance in *T. absoluta* against many chemicals as well as adverse effects on beneficial fauna and human health (Prasannakumar et al. 2021). Most recently, Inak et al. (2021) reported that Turkish *T. absoluta* populations have already developed resistance to a wide range of commonly used insecticides.

Biological control is a great alternative to conventional chemicals and one of the most important components of integrated pest management (IPM) strategies. Among biological control agents, entomopathogens (i.e., fungi, virus, nematodes, and bacteria) stand out due to their capability to cause epizootics in insect populations and provide important protection against invasive species (Lacey et al. 2015). Of entomopathogenic organisms, entomopathogenic nematodes (EPNs) and their symbiotic bacteria have attracted great attention in the last two decades because of their ability to suppress pest populations within a short time and provide environmentally safe control (Koppenhöfer et al. 2020). The infective juveniles (IJs) of the genera *Steinernema* and *Heterorhabditis* of EPNs, free-living third-stage larvae, live in mutualistic relationships with bacteria of the genera; *Xenorhabdus* and *Photorhabdus*, respectively (Bhat et al. 2020). Once IJs penetrate into a host body via natural body openings and thin cuticles, they release their bacterial associates into the host hemolymph (Bode 2009). The bacterial symbionts play a major role in the pathogenicity of IJs by releasing a range of bioactive compounds with insecticidal and immunosuppression activity such as enzymes, killer proteins, and toxins (Tobias et al. 2018). The host generally dies within 24–48 h, following infection due to septicemia (Boemare and Akhurst 2006).

Recently, endosymbionts of EPNs and their associated bioactive compounds have drawn great interest among researchers due to their control potential against insects and many studies have been conducted to evaluate the pathogenicity of the bacterial cell suspensions and cell-free supernatants of *Xenorhabdus* and *Photorhabdus* spp. against a diversity of insect pests from different orders (Vicente-Díez et al. 2021). Bacterial symbionts of EPNs are normally carried into the host hemocoel by IJs, following penetration into the host body. Although the survival of *Xenorhabdus* and *Photorhabdus* bacteria is considered limited in the absence of their nematode associates, it has been shown that these bacteria can survive in fresh water and soil for 6 days (Abd-Elgawad 2022). To date, there is no report about the efficacy of symbiotic bacteria of EPNs on *T. absoluta*. In addition, earlier studies indicated that the pathogenicity of symbiotic bacteria is a strain-specific feature depending on the differences in toxin complexes produced by different strains, which emphasize the importance of pathogenicity screening studies (Hasan et al. 2019).

Therefore, the present study was conducted to: I) assess the pathogenicity of native EPNs species/strains against larval instars of *T. absoluta*, II) evaluate the virulence of cell suspensions and cell-free supernatants of the most efficacious EPN strains against larval instars of *T. absoluta*, and III) to identify biocontrol potential of native EPNs species/isolates and their respective bacterial strains for the control of *T. absoluta*.

Methods

Entomopathogenic nematodes isolates

Seven isolates of *Steinernema feltiae* (UKK-1, MKB-2, KBC-4, MCB-8, DDKY-11, MAY-12, ATB-13, and DDKB-17) and two isolates of *Heterorhabditis bacteriophora* (UMK-7 and AVB-15), obtained in a previous study, were included in the bioassays (Yuksel and Canhilal 2019) (Table 1). To obtain fresh IJs, EPNs were reared on the last instar *Galleria mellonella* larvae (L.) (Lepidoptera: Pyralidae) under controlled conditions (25 ± 2 °C, 60% RH). The IJs of EPN species were inoculated to Petri plates (9 cm Ø) lined with two filter papers at the concentration of 1000 IJs. Then, ten *G. mellonella* larvae were transferred to each Petri plate. The Petri plates were maintained at 25 ± 2 °C, 60% RH in dark conditions after sealing with parafilm. The dead larvae were placed on modified White traps 48 h. after inoculation (McMullen and Stock 2014). Newly emerged IJs were rinsed with sterile water several times and kept in culture flasks (250 ml) horizontally at 9 °C until their use in the experiments. Only one-week-old IJs were used in the experiments.

Galleria mellonella culture

In order to multiply the IJs of EPNs, the last larval instars of *G. mellonella* were obtained from Izzet Baysal University (Bolu, Turkey) and reared under controlled conditions (30 ± 2 °C, 60% RH). The first and second larval instars of *G. mellonella* were placed into glass jars (1 L) and fed on an artificial diet according to the methodology

Table 1 List of entomopathogenic nematode species/strains used in the experiments

Entomopathogenic nematodes	Strain	Habitat	Coordinates	GenBank accession number
Steinernema feltiae	ÜKK-1	Vegetable	38°33′13 N 34°35′4 E	MG462715
S. feltiae	MKB-2	Fruit orchards	38°30′07 N 34°36′3 E	MG462716
S. feltiae	KBC-4	Pasture	39°36′46 N 34°45′3 E	MG602330
S. feltiae	ÜTP-5	Vegetable	38°30′84 N 34°56′5 E	MG602331
S. feltiae	MCB-8	Vegetable	38°40′40 N 34°29′5 E	MG602334
S. feltiae	DDKY-11	Fruit orchards	38°24′19 N 34°47′4 E	MG602337
S. feltiae	MAY-12	Pasture	38°38′26 N 34°35′2 E	MG602338
S. feltiae	ATB-13	Field crops	38°33′20 N 34°34′2 E	MG602339
S. feltiae	DDKB-17	Vegetable	38°23′29 N 34°45′3 E	MG602343
Heterorhabditis bacteriophora	ÜMK-7	Pasture	38°33′50 N 34°55′1 E	MG602333
H. bacteriophora	AVB-15	Fruit orchards	38°43′43 N 34°52′1 E	MG602341

adopted by Metwally et al. (2012). The jars were sealed with perforated filter papers allowing airflow. The adults were collected after 20–25 days of incubation and placed into separate jars to promote egg-laying. The eggs laid on filter papers were transferred to new jars and this process continued until the required number of larvae was procured.

Tuta absoluta culture

A laboratory culture of *T. absoluta* was initiated with the populations collected from different tomato production areas of Antalya province. The healthy larvae were selected to establish the laboratory culture of *T. absoluta.* The larvae and pupae were transferred to tomato seedlings grown in cages ($60 \times 60 \times 60$ cm) under controlled conditions (25 ± 1 °C, 65 ± 5% RH, and 16L: 8D h photoperiod). Tomato seedlings (Karahıdır cultivar) were obtained from the sales office of Erciyes University. The 1st/2nd and 3rd/4th instar larvae feeding within galleries were collected and grouped by measuring body length (Bajracharya and Bhat 2018).

Entomopathogenic nematodes pathogenicity bioassays

Nine isolates of *S. feltiae* and two isolates of *H. bacteriophora* were tested against the $1^{st}/2^{nd}$ and $3^{rd}/4^{th}$ instar larvae of *T. absoluta*. Pathogenicity screening studies were conducted in Petri plates (9 cm Ø) containing 25 g autoclaved field soil (sandy loam) having a moisture content of 10% (w/w). Infective juveniles of each EPN species/strain were applied uniformly to each Petri plate at the concentration of 200 IJs ml^{-1} tap water. Then, one healthy larva of 1st/2nd or 3rd/4th instar was added to each Petri plate. The plates were sealed with parafilm and incubated in the dark (25 °C and 60% RH). The mortality rates were recorded on the 24, 48, and 72 h. after treatment. Only 1 ml of tap water was applied to control treatments. Dead larvae were individually dissected under a stereomicroscope to check the presence of nematodes. Each treatment had four replicates with 10 larvae of each 1st/2nd and 3rd/4th instar per replication. All the bioassays were repeated using different batches of nematodes and insects.

Symbiotic bacteria isolation

Based on the mortality rates obtained 72 h. after treatment, the most efficient EPN species, KBÇ-4 and MCB-8 isolate of *S. feltiae* and AVB-15 isolate of *H. bacteriophora*, were selected for further evaluation of the cell-free supernatants and cell suspensions of their symbiotic bacteria on the larval instars of *T. absoluta*. The symbiotic bacteria of EPNs were identified in a previous project (Project No: 120O041) and the cell lines obtained in this study were used in the experiments. Briefly, the bacterium was isolated using approximately 500 freshly emerged IJs of each isolate. After being surface-sterilized in a sterile Ringer's solution comprising NaClO (10% w/vol) for 10 min, the IJs were washed several times with sterile Ringer's solution and crushed in 1 ml of sterile phosphate-buffered saline. Then, 50 µl of cell suspension solution was seeded onto nutrient bromothymol blue triphenyl tetrazolium chloride agar (NBTA medium) [37 g nutrient agar (Criterion, USA); 0.025 g bromothymol blue (Labchem, England); 0.004 g triphenyl tetrazolium chloride (Sigma chemical, USA); 1000 ml distilled water]. Petri plates were left for incubation at 25 °C, 40% RH in the dark for 48 h. After incubation period, circular and blue-greenish colored colonies (Primary variants) were re-streaked onto the NBTA plates until the pure bacterial colonies were obtained. After 48 h. incubation

period, a loop of pure bacterial colony of each isolate were selected on the basis of morphology, bioluminescence, and pigmentation and transferred into 100 ml Luria–Bertani (LB) broth in a 250-ml Erlenmeyer flask. Then, the flasks were incubated in a rotary incubator at 150 rpm for 144 h (28 °C, 20% RH in the dark) (Boemare and Akhurst 2006). Subsequently, the bacterial culture in the broth suspensions was kept into 50 ml Falcon tubes and centrifuged at 20,000 rpm for 15 min at 4 °C. The supernatant solutions in Falcon tubes were filtered twice through a 0.22 µm Millipore filter (Sigma–Aldrich) to separate the bacterial cells. The filtered cell-free supernatant solutions were streaked onto NBTA agar to check the presence of bacterial cells. The bacterial pellets remaining at the bottom of Falcon tubes were rinsed with sterile distilled water and centrifuged at 20.000 rpm for 15 min at 4 °C. The cell concentration of the pellets was measured using a spectrophotometer (OD600, 600 nm) and adjusted to 4×10^8 cells ml^{-1} colony-forming units (CFU) using sterile water.

Symbiotic bacteria pathogenicity bioassays

Toxicity of cell-free supernatants and cell-suspension solutions of symbiotic bacteria was tested by oral and contact applications against larval instar of *T. absoluta* in Petri dish arenas (Ø 9 cm) lined with two filter papers under laboratory conditions (60 ± 5% RH, 25 ± 1 °C/14 h light, and 14 ± 1 °C/10 h darkness). In contact efficacy bioassays of cell-free supernatants, one larva at the 1st/2nd or 3rd/4th instar stage was placed into Petri dishes and 1 ml cell-free supernatant solution was applied directly onto each larva using a mini spray bottle (50 ml) (Ø 0.5 mm nozzle). A piece of pesticide-free tomato leaf (1 cm^2) was provided for the larvae as food. For the oral efficacy studies of cell-free supernatants, 0.5 ml cell-free supernatant solution was sprayed onto both sides of tomato leaves. Then, one leaf treated with cell-free supernatants was placed in a Petri dish for each larva to feed. Control treatments were performed by spraying nutrient broth only. The same procedure was followed in the contact and oral efficacy of cell-suspension (1 ml of cell suspension containing 4×10^8 cells) bioassays, except for control treatments. In the cell-suspension bioassays, only 1 ml of sterile water was applied to leaves. Petri dishes were sealed with parafilm and maintained under controlled conditions. Mortality rates were recorded daily for three days after treatment. Each treatment consisted of four replicates and 10 larvae were used for each replicate. The bioassays were conducted twice under the same conditions. Only two weeks-old cell-free supernatants and cell-suspensions were included in the bioassays, and they were stored at 9 °C until the bioassays (Additional file 1).

Statistical analyses

No correction was made in the mortality rates since all larvae in the control groups were alive during the experiments. Mortality data were arcsine transformed and analyzed using IBM SPSS statistics version 20.0 for Windows (SPSS Inc., Chicago, IL, USA). The significance of the main effects was subjected to factorial analysis of variance (ANOVA) and the mean differences were grouped using Tukey's multiple range tests ($P \leq 0.05$).

Results

Entomopathogenic nematodes pathogenicity bioassays

The pathogenicity screening studies revealed that the mortality rates of *T. absoluta* larvae were significantly influenced by all main factors [Nematode species (N), larval stage (L), and exposure time (t)] and their associated interactions (Table 2). The mortality rates varied between 47.5–62.5 and 70–90% for the 1st/2nd and 3rd/4th larval instars after 72 h. exposure to IJs, respectively. In general, the mortality rates tended to increase with increasing exposure time. Although mortality rates were similar for the 1st/2nd instar larvae 72 h. after treatment, the highest mortality rate (62.5%) was obtained from *H. bacteriophora* AVB-15 isolate. *S. feltiae* UTP-5 and *S. feltiae* MCB-8 induced the highest mortality (90%) in the $3^{rd}/4^{th}$ instar larvae of *T. absoluta* (Table 3).

Symbiotic bacteria pathogenicity bioassays

Cell-free supernatant and cell suspension of symbiotic bacteria of selected EPN species/isolates were tested for their contact and oral bioefficacy against the 1st/2nd and

Table 2 Repeated Measures ANOVA parameters for the main effects and associated interactions for mortality rates of the larvae of *Tuta absoluta* in Petri dish bioassay of entomopathogenic nematodes

Sources*	df	*F*-value	*P*-value
Nematod (*N*)	10	320.227	**0.000**
Larval stage (*L*)	1	672.470	**0.000**
*N*L*	10	2.451	**0.015**
Error[1]	66		
Exposure time (*t*)	2	1.653.122	**0.000**
*L*t*	2	34.172	**0.000**
*N*t*	20	4.335	**0.000**
*N*L*t*	20	2.519	**0.001**
Error[2]	132		

Bold values indicate statistical significance at level alpha = 0.05

*Tukey ($P \leq 0.05$)

Table 3 Mortality rates (%) of 1st/2nd and 3rd/4th instars of *Tuta absoluta* larvae 24, 48, and 72 h after application of different entomopathogenic nematode species/strains at the concentrations of 200 infective juveniles (IJs)/Petri

EPNs*	Larval stages	Moratality rates (%) after treatment		
		24 h	48 h	72 h
S. f. ÜKK-1	1st/2nd	2.5 A[a]a[b]	17.5 Ab	50.0 Ac
S. f. MKB-2		2.5 Aa	17.5 Ab	52.5 Ac
S. f. KBC-4		5.0 Aa	20.0 Ab	55.0 Ac
S. f. UTP-5		2.5 Aa	35.0 Bb	47.5 Ac
S. f. MCB-8		2.5 Aa	25.0 ABb	57.5 Ac
S. f. DDKY-11		12.5 Ba	27.5 ABb	52.5 Ac
S. f. MAY-12		2.5 Aa	27.5 ABb	50.0 Ac
S. f. ATB-13		2.5 Aa	35.0 Bb	52.5 Ac
S. f. DDKB-17		0.0 Aa	12.5 Aa	47.5 Ac
H. b. UMK-7		0.0 Aa	30.0 ABb	52.5 Ac
H. b. AVB-15		2.5 Aa	35.0 Bb	62.5 Ac
S. f. ÜKK-1	3rd/4th	17.5 ABa	42.5 Ab	75.0 Ac
S. f. MKB-2		27.5 Ba	45.0 Ab	77.5 ABc
S. f. KBC-4		0.0 Aa	57.5 Bb	90.0 Bb
S. f. UTP-5		22.5 ABa	57.5 Bb	70.0 Ac
S. f. MCB-8		22.5 ABa	40.0 Aa	90.0 Bc
S. f. DDKY-11		10.0 ABa	60.0 Bb	87.5 Bc
S. f. MAY-12		12.5 ABa	50.0 ABb	75.0 Ac
S. f. ATB-13		12.5 ABa	55.0 ABb	75.0 Ac
S. f. DDKB-17		10.0 ABa	50.0 ABb	80.0 ABc
H. b. UMK-7		25.0 Ba	60.0 Bb	75.0 Ab
H. b. AVB-15		25.0 Ba	62.5 Bb	85.0 ABc

*UKK-1, MKB-2, KBC-4, UTP-5, MCB-8, DDKY-11, MAY-12, ATB-13, DDKB-17: *Steinernema feltiae*; UMK-7 and AVB-15: *Heterorhabditis bacteriophora*.
[a]Different capital letters show statistically significant differences among entomopathogenic nematode species/strains for each exposure time levels.
[b]Different lowercase letters show statistically significant differences among the exposure time levels for each entomopathogenic nematode species ($P \leq 0.05$, Tukey)

3rd/4th instars of *T. absoluta* larvae (Table 4). Analysis of data showed that all main factors had significant effects on the mortality rates of *T. absoluta* larvae in contact and oral toxicity bioassays of cell-free supernatants (Table 5).

The results indicated that the 1st/2nd larval instars of *T. absoluta* were more susceptible to cell-free supernatants and cell suspension applications in both contact and oral bioassays than the 3rd/4th instars. The mortality rates were also higher in contact treatments of cell-free supernatants and cell suspension of all symbiotic bacteria. The larval mortalities generally showed a significant increase depending on the increasing of exposure time and the highest mortalities were obtained after 72 h. exposure. The contact treatment of cell-free supernatant of *X. bovienii* MCB-8 strain yielded the highest mortality (80 and 55%) after 72 h. exposure against the 1st/2nd and 3rd/4th larval instars of *T. absoluta*. In oral treatments, none of the cell-free supernatants of bacteria species/strains was able to provide larval mortality over 50% against *T. absoluta* (Table 6). Additionally, *X. bovienii* MCB-8 and *P. luminescens* subsp. *kayaii* caused higher mortalities than *X. bovienii* KBÇ-4 strain in contact and oral efficacy bioassays against *T. absoluta* larvae (Additional file 1).

In the contact treatment of cell suspensions, all main factors and their interactions significantly affected the mortality rates of *T. absoluta* larvae. However, in the oral treatment of cell suspensions, the effect of cell suspensions of symbiotic bacteria was non-significant on the larvae of *T. absoluta,* while other main factors caused significant differences in the mortality rates (Table 7). Contact treatment of cell suspensions induced higher mortalities than the oral one. The 1st/2nd larval instars showed a higher susceptibility to cell suspensions. In the contact treatment, *X. bovienii* MCB-8 and *P. luminescens* subsp. *kayaii* AVB-15 provided mortalities over 50%, 72 h. after treatment. *P. luminescens* subsp. *kayaii* AVB-15 was the most efficient strain causing 42.5 and 57.5% mortalities 48 and 72 h. after treatment, respectively. No mortalities occurred in *T. absoluta* larvae after 24 h. exposure to cell suspension in the oral treatment. The highest efficacy (20%) against the 1st/2nd larval instars was obtained from *X. bovienii* KCS-4 strain 72 h. after treatment. In the oral treatment, cell suspensions failed to provide a mortality over 10% against the 1st/ 2nd larval instars (Table 8). Little feeding behavior was observed also in all larval instars in the oral treatment of cell suspensions during the experiment.

Table 4 The symbiotic bacteria species/strains of selected entomopathogenic species/isolates used in the experiments

Entomopathogenic nematodes	Strain	Symbiotic bacteria	Accession number
S. feltiae	KBC-4	*Xenorhabdus bovienii*	MZ688379
S. feltiae	MCB-8	*Xenorhabdus bovienii*	MW403818
Heterorhabditis bacteriophora	AVB-15	*Photorhabdus luminescens* subsp. *kayaii*	MG602333

Table 5 Repeated Measures ANOVA parameters for the main effects and associated interactions for mortality rates of the larvae of *Tuta absoluta* in contact and oral efficacy bioassay of cell-free supernatants of symbiotic bacteria

Sources*	Contact			Oral		
	df	***F*-value**	***P*-value**	**df**	***F*-value**	***P*-value**
Supernatant (*S*)	2	22.976	**0.000**	2	12.738	**0.000**
Larval Stage (*L*)	1	70.829	**0.000**	1	60.246	**0.000**
*S*L*	2	2.780	0.089	2	1.820	0.191
Error[1]	18			18		
Exposure Time (*t*)	2	507.112	**0.000**	2	160.810	**0.000**
*L*t*	2	6.205	**0.005**	2	13.397	**0.000**
*S*t*	4	2.815	**0.039**	4	1.371	0.263
*S*L*t*	4	2.519	**0.001**	4	0.595	0.669
Error[2]	36			36		

Bold values indicate statistical significance at level alpha = 0.05

*Tukey ($P \leq 0.05$)

Table 6 The mortality rates of *Tuta absoluta* larvae in contact and oral efficacy bioassay of cell-free supernatants of symbiotic bacteria

Symbiotic bacteria	Larval stages	Moratality rates (%) after treatment		
		24 h	**48 h**	**72 h**
Contact				
X. b. MCB-8	1st/2nd	5.0A[a]	35.0AB[b]	80.0A[c]
P. l. AVB-15		15.0B[a]	45.0B[b]	65.0B[c]
X. b. KBC-4		2.5A[a]	25.0A[b]	55.0B[c]
X. b. MCB-8	3rd/4th	0.0A[a]	30.0A[b]	55.0A[c]
P. l. AVB-15		5.0A[a]	22.5AB[b]	47.5A[b]
X. b. KBC-4		0.0A[a]	12.5B[b]	45.0A[b]
Oral				
X. b. MCB-8	1st/2nd	5.0A[a]	27.5A[b]	40.0A[b]
P. l. AVB-15		2.5A[a]	32.5A[b]	40.0A[b]
X. b. KBC-4		0.0A[a]	20.0A[b]	27.5B[b]
X. b. MCB-8	3rd/4th	0.0A[a]	12.5A[b]	22.5A[b]
P. l. AVB-15		5.0A[a]	17.5A[b]	22.5A[b]
X. b. KBC-4		0.0A[a]	10.0A[b]	17.5A[b]

*KBC-4 and MCB-8: *Xenorhabdus bovienii,* AVB-15: *Photorhabdus luminescens* subsp. *kayaii*. [a]Different capital letters show statistically significant differences among symbiotic bacteria species/strains for each exposure time levels. [b]Different lowercase letters show statistically significant differences among the exposure time levels for each entomopathogenic nematode species ($P \leq 0.05$, Tukey)

Discussion

Recently, symbiotic bacteria of EPNs have garnered significant attention due to their biocontrol potential against a variety of insect pests. Many studies have been conducted to isolate and evaluate their pathogenicity against economically important pests (Muhammad et al. 2022). Numerous studies have demonstrated the pathogenicity of EPNs on the larvae of *T. absoluta*. However, there is no study examining the insecticidal effect of cell-free supernatants of their symbiotic bacteria on *T. absoluta,* while there is only one study conducted to test the efficacy of cell suspension of symbiotic bacteria (Ngugi 2021).

The present results revealed that the pathogenicity of tested EPNs varied according to EPN species/isolates, different larval instars of *T. absoluta*, and exposure time. AVB-15 and UMK-7 isolate of *H. bacteriophora* generally induced higher mortalities in the 1st/2nd instars larvae of *T. absoluta* after 72 h of exposure. This might be explained by the morphological difference in the IJs of *Steinernema* and *Heterorhabditis* species. Unlike *Steinernema* spp., the IJs of the *Heterorhabditis* genus bear a dorsal tooth and can penetrate into their host body through the thin cuticles of host insects (Griffin et al. 2005). The variation in the pathogenicity of EPN species/isolates has been demonstrated in earlier studies. Van Damme et al. (2016) reported that, the last instar larvae was more susceptible to EPNs tested and the highest mortalities were 97 and 60% for the last and first instar larvae, respectively. In the same study, *S. feltiae* and *S. carpocapsae* performed better than *H. bacteriophora*. In another study carried out by Gözel et al. (2009), different EPNs species including *S. feltiae*, *S. affine*, *S. carpocapsae*, and *H. bacteriophora* were tested on the last larval instars of *T. absoluta* at 30 IJs/larva and 75, 70, 62, and 70% larval mortality rates were obtained, respectively. The mortality rates in these studies were positively affected by the exposure time and nematode species which is in line with the present study.

Similar to our studies, El Aimani et al. (2021) evaluated the pathogenicity of different *H. bacteriophora* and *S. feltiae* isolates against the 4th larval instar of *T. absoluta* in 24-well plates and the mortality rates varied

Table 7 Repeated Measures ANOVA parameters for the main effects and associated interactions for mortality rates of the larvae of *Tuta absoluta* in contact and oral efficacy bioassay of cell-suspensions of symbiotic bacteria

Sources*	Contact			Oral		
	df	*F*-value	*P*-value	df	*F*-value	*P*-value
Cell Suspension (*C*)	2	14.510	**0.000**	2	0.781	0.473
Larval Stage (*L*)	1	102.918	**0.000**	1	12.500	**0.002**
*C*L*	2	5.571	**0.013**	2	0.219	0.806
Error[1]	18			18		
Exposure Time (*t*)	2	642.405	**0.000**	2	45.538	**0.000**
*L*t*	2	21.000	**0.000**	2	8.615	**0.001**
*C*t*	4	5.230	**0.002**	4	0.654	0.628
*C*L*t*	4	3.365	**0.019**	4	0.192	0.941
Error[2]	36			36		

Bold values indicate statistical significance at level alpha = 0.05

*Tukey ($P \leq 0.05$)

Table 8 The mortality rates of *Tuta absoluta* larvae in contact and oral efficacy bioassay of cell suspension of symbiotic bacteria

Symbiotic bacteria	Larval stages	Moratality rates (%) after treatment		
		24 h	48 h	72 h
Contact				
X. b. MCB-8	1st/2nd	5.0A^{a}	35.0A^{b}	55.0A^{c}
P. l. AVB-15		2.5A^{a}	42.5A^{b}	57.5A^{b}
X. b. KBC-4		0.0A^{a}	32.5A^{b}	42.5B^{b}
X. b. MCB-8	3rd/4th	0.0A^{a}	25.0A^{b}	45.0A^{c}
P. l. AVB-15		0.0A^{a}	22.5A^{b}	30.0B^{b}
X. b. KBC-4		0.0A^{a}	17.5A^{b}	30.0B^{b}
Oral				
X. b. MCB-8	1st/2nd	0.0A^{a}	10.0A^{ab}	15.0A^{b}
P. l. AVB-15		0.0A^{a}	7.5A^{a}	15.0A^{b}
X. b. KBC-4		0.0A^{a}	12.5A^{b}	20.0A^{b}
X. b. MCB-8	3rd/4th	0.0A^{a}	2.5A^{a}	5.0A^{a}
P. l. AVB-15		0.0A^{a}	2.5A^{a}	7.5A^{a}
X. b. KBC-4		0.0A^{a}	5.0A^{a}	7.5A^{a}

*KBC-4 and MCB-8: *Xenorhabdus bovienii*. AVB-15: *Photorhabdus luminescens* subsp. *kayaii*. [a]Different capital letters show statistically significant differences among symbiotic bacteria species/strains for each exposure time levels. [b]Different lowercase letters show statistically significant differences among the exposure time levels for each entomopathogenic nematode species ($P \leq 0.05$, Tukey)

between 40 and 60% for *H. bacteriophora* isolates. The higher mortalities were recorded at *S. feltiae* isolates ranging between 60 and 80% in the same study. Differences in larval mortality among these studies can be explained by the differences in the pathogenicity of EPNs species, host preference of EPNs species/isolates, IJs concentrations, and application environment along with other environmental factors. In the present study, the mortality rates were significantly higher in 3rd/4th larval instars than in 1st/2nd instars larvae in pathogenicity screening bioassay of EPNs. Host size plays an important role in the pathogenicity of EPNs since IJs mostly use natural body openings for the penetration (Bastidas et al. 2014).

To our knowledge, this is the first study evaluating the control potential of cell-free supernatants obtained from *Xenorhabdus* and *Photorhabdus* species on different larval instars of *T. absoluta*. The results revealed that the 1st/2nd instar larvae were more susceptible to cell-free supernatants than the 3rd/4th instar larvae of *T. absoluta* and the highest mortality rates generally were achieved in the contact treatments of supernatants. The differences in the susceptibility of larvae can be explained by the variation in the immune responses of insects depending on the developmental stage (Feldhaar and Gross 2008). Humoral immune system secretions such as Phenoloxidase activity are one of the first immune reactions of insects in response to microbial infections (González-Santoyo et al. 2012). The differences in the ability of different larval stages of *T. absoluta* to produce phenoloxidase molecules along with other immune secretions may have limited the toxicity of the cell suspensions and cell-free supernatants. In the oral treatment of the cell suspensions and cell-free supernatants, larval mortalities were generally lower than in contact treatments. Earlier studies showed that the secondary metabolites produced by symbiotic bacteria may act as a direct toxicant, while others might act as antifeedant (Hemalatha et al. 2018). In the present study, feeding inhibition of cell-free supernatants and cell suspension were also observed in the 3rd/4th instar larvae of *T. absoluta*. This might be another reason behind the low mortality rates in the oral treatments.

In a previous study, contact efficacy of cell suspension of *Xenorhabdus* sp. showed high insecticidal activity

against the 2nd larval instar of *T. absoluta*, and 76% mortality was achieved 48 h. after treatment (Ngugi 2021). However, in the present study, the mortalities did not exceed 43% after 48 h. treatment. The differences in the mortality rates can be attributed to the experimental design such as the bacterial cell concentrations sprayed on larvae and the number of larvae used for the experiment (Shahina et al. 2010). Ngugi (2021) used a total of 25 larvae in the experiment whereas there were a total of 40 larvae used in the present study. The small sample size might have led to high mortality rates. Another reason might be due to the different stages of the larvae used. In the present study, the 1st and 2nd instar larvae were tested together since successive larval instars of *T. absoluta* are generally found in mixed groups in field conditions. Another factor affecting the mortality rates may be the variations in the chemical composition of bacterial species/strains. In general, different mortality rates were obtained when *T. absoluta* larvae were exposed to the cell-free supernatant and cell suspension of different strains (KBC-4 and MCB-8) of *X. bovienii*, *Xenorhabdus* and *Photorhabdus* species/strains produce different toxin complexes, enzymes, and lipopolysaccharides with a varying degree of pathogenicity and immunosuppression ability (Hasan et al. 2019). This might also have led to variations in the mortalities.

The results of this study suggest that cell suspension and cell-free supernatants of tested strains of *Xenorhabdus* and *Photorhabdus* bacteria were able to induce mortality in the larvae of *T. absoluta*. However, the contact application of cell-free supernatants provided higher mortalities, which suggests that contact applications of cell-free supernatants on the young instars larvae may be a better option in the control of *T. absoluta,* since older larval instars had a tendency to avoid feeding on the leaves treated with the cell-free supernatants and cell suspension *Xenorhabdus* and *Photorhabdus* bacteria.

Conclusion

The results of this study demonstrated that all EPNs species/isolates were pathogenic to different instars of *T. absoluta* larvae. Of tested EPNs species/isolates, *H. bacteriophora* AVB-15 achieved the highest mortality rate against the 1st/2nd instar larvae. However, MCB-8 and KBC-4 of *S. feltiae* were the most efficacious isolates against older instar larvae of *T. absoluta*. Evaluation of the cell-free supernatant and cell suspension of symbiotic bacteria of EPNs showed that the contact and oral treatments of the cell suspensions and secondary metabolites of selected EPNs species/isolates were lethal to different larval instars of *T. absoluta* with varying degrees of pathogenicity. The 1st/2nd larvae of *T. absoluta* were more susceptible to symbiotic bacteria than the 3rd/4th larval instars. Contact treatments of symbiotic bacteria generally resulted in less survival in the larvae of *T. absoluta*. Our study also indicated that leaf application of symbiotic bacteria showed antifeedant activity, particularly on the 3rd/4th larval instars. Overall, this study demonstrated the great potential of *H. bacteriophora* AVB-15, MCB-8 and KBC-4 isolates of *S. feltiae*, and the cell-free supernatant of *X. bovienii* MCB-8 against *T. absoluta* larvae to develop novel biopesticides for integrated management programs.

Abbreviations

EPNs: Entomopathogenic nematodes; IJs: Infective juveniles; Sf: *Steinernema feltiae*; Hb: *Heterorhabditis bacteriophora*; Xb: *Xenorhabdus bovienii*; Pl: *Photorhabdus luminescens* Subsp. *kayaii*.

Supplementary Information

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Additional file 1: Supplementary figures.

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Author contributions

EY designed the study, performed the laboratory work, and wrote the paper. The author read and approved the final manuscript.

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Availability of data and materials

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

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

This study does not contain any individual person's data.

Competing interests

The authors have no competing interests.

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