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Evaluation of local isolates of entomopathogenic nematodes for the management of black cutworm, *Agrotis ipsilon* Hufnagel (Lepidoptera: Noctuidae)

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Abstract

The black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) is considered to be one of the serious polyphagous pests that spend a large part of its life in the soil environment, where many microorganisms live including entomopathogenic nematodes (EPNs) (Rhabditida: Steinernematidae and Heterorhabditidae). EPNs have been long used for suppressing the soil-dwelling insects like cutworms and are successful biological control agent against *A. ipsilon* larvae. In the present study, the efficacy of local EPNs isolates against the fourth larval instar of *A. ipsilon* was evaluated at different concentrations (10, 25, 50, 100 IJs/larva/Petri dish and 25, 50, 100 IJs/cm^2 soil) in two different experiment environments including filter papers in Petri dishes and soil in plastic containers under laboratory conditions at 25 ± 1 °C. Larval mortalities of *A. ipsilon* were recorded first, second, third, and fourth day post inoculation where the mortality rates increased by increasing the concentrations. The maximum mortality rate (100%) was reached within 2 days after inoculation, inoculating the *Heterorhabditis bacteriophora* FLH-4-H and *H. indica* 216-H isolates at concentrations of 50 and 100 IJs/cm^2, in the plastic container experiment. The highest mortality rate (90%) was obtained by *Steinernema carpocapsae* E76-S isolate at a concentration of 100 IJs/larva/Petri on the fourth day after inoculation, in the Petri dish experiment. The lethal concentration values (LC_{50} and LC_{90}) of the *A. ipsilon* larval population were 52 IJs and 129 IJs, respectively, for *S. feltiae* E76-S isolate in the Petri dish tests. In the plastic container experiment, the lowest LC_{50} and LC_{90} values found to be 17 IJs and 23 IJs, respectively, for *H. bacteriophora* FLH-4-H isolate. The results showed that all indigenous EPN isolates had good potentials in the management of *A. ipsilon*.

Keywords: *Agrotis ipsilon*, Entomopathogenic nematodes, *Steinernema*, *Heterorhabditis*, Efficacy

Background

The black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae), is a major pest of over 30 economically important crops in many agricultural regions, which makes it survive nearly in every agroecosystem (Rings et al. 1975). *A. ipsilon* causes serious crop losses due to its wide host range including weeds, hidden lifestyle, feeding behavior, prolonged egg laying, and its ability for long-distance migration (Ya-Zhong 1992; Showers et al. 1993 and Capinera 2001). More than 80% of the losses occur after reaching the fourth instar of larvae, which cuts several plants overnight (Abdel-Gawaad and El-Shazli 1971 and Capinera 2001). Chemical control used against *A. ipsilon* larvae is often not effective and remains inadequate for the control of this pest because of its larval hiding behavior during the daylight hours and the resistance to most of the chemicals (Capinera 2001 and Takeda 2008). Therefore, the negative impact of the chemicals has led researchers to search for new control strategies (Laznik and Trdan 2012).

Biological control may fill the gap left by chemical pesticides and has a great potential in the suppression of agricultural pests. The entomopathogenic nematodes (EPNs), *Steinernema* spp. and *Heterorhabditis* spp. (Rhabditida: Steinernematidae and Heterorhabditidae), are among the

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most successful biological control agents that have distinctive features from other entomopathogens in many ways such as killing the host within 48 h with the help of the bacteria they carry in their intestines, providing long-lasting protection from any further pest infestation by settling into application area, and having infective juveniles (IJs) (a non-feeding stage), which have different foraging strategies (Gaugler 1981; Kaya and Gaugler 1993). EPNs are impactful microorganisms against insect pests in both soil and cryptic habitats. Satisfactory results also have been obtained by foliage applications depending on the new formulation techniques developed (Trdan et al. 2007; Laznik et al. 2010 and Ebssa and Koppenhöfer 2011). Many studies have been conducted in order to evaluate the virulence and control potential of entomopathogenic nematodes (EPNs) species or isolates against *A. ipsilon* larvae (Shamseldean et al. 1994; Mathasoliya et al. 2004; Fetoh et al. 2009; Seal et al. 2010; Ebssa and Koppenhöfer 2011 and Khattab and Azazy 2013).

The present study was carried out to evaluate the control potential of four native EPNs isolates and two different applications against *A. ipsilon* under laboratory conditions.

Materials and methods

Insect culture

Healthy *A. ipsilon* larvae were collected from different corn, sugarbeet, and pumpkin fields, in the Mediterranean and Central Anatolia, Turkey, during May and June of 2017. A laboratory culture consisted of healthy larvae was established and maintained in a growth chamber at 25 ± 1 °C, R.H 60%, and a photoperiod of 16:8 (L: D). Larvae were reared individually on lettuce in separate plastic cups (63 × 80 mm) to avoid cannibalism and the lettuce cleaned with sterile water three times and dried before given to the larvae. Pupae were collected and placed in a glass jar for adult emergence and a cotton pad soaked in 10% honey solution were provided as food to adults and to support egg laying.

Entomopathogenic nematodes isolates

EPN isolates of *Heterorhabditis bacteriophora* FLH-4-H (Kayseri, Felahiye), *H. indica* 216-H (Kahramanmaraş, Afsin), *Steinernema bicornotum* MGZ-4 (Kayseri, Melikgazi), and *S. carpocapsae* E76-S (Kayseri, Tomarza) were obtained between years 2013 and 2017 from soil samples collected from Kayseri and Kahramanmaras provinces in the Central Anatolia and Mediterranean region of Turkey, respectively (Canhilal et al. 2016, 2017). EPN isolates were cultured on the last instar of *Galleria mellonella* L. (Lepidoptera: Pyralidae) at 25 ± 1 °C and R.H. 60% under laboratory conditions. Harvested infective juveniles (IJs) were kept in a sterilized distilled water at 5–9 °C in the fridge until their use in the experiments (Kaya and Stock 1997 and Ehlers 2001). Only 2-week old nematodes were used in the experiments.

Pathogenicity test

The virulence of four indigenous isolates against *A. ipsilon* larvae was evaluated in Petri dish arenas and plastic containers including soil in laboratory experiments. Isolates were tested against the fourth (L4) larval instar, the most susceptible to most EPN species (Ebssa and Koppenhöfer 2012). L4 instar larvae were detected by measuring their head capsule size and larval body length (Capinera 2001 and Gullan and Cranstone 2005). In order to avoid larval cannibalism, only one larva was placed in each Petri dish including two moist filter papers (100 × 15 mm) and plastic containers (63 × 80 mm) including 20 g of sterilized air-dried sand (application surface area 27 cm^2). In order to prevent the larvae from undergoing starvation stress in the two experimental arenas, they were provided by a piece of cleaned lettuce every day (approximately 2 cm^2). The arenas for the two experiments were incubated in the dark at 25 ± 1 °C, R.H. 60%, and only distilled water was applied to the Petri dishes used as a control. Mortality rates at each experiment arena were recorded; first, second, third, and fourth day post treatment (DAT) and all the dead larvae were placed individually onto White traps, and the emergence of IJs from the dead body of the larvae was observed to confirm the nematode infection.

Each Petri dish was provided by 1 ml of distilled water, containing different concentrations of IJs (10, 25, 50, and 100 IJs/larva/Petri), and each nematode concentration was tested against ten *A. ipsilon* larvae and replicated four times. Petri dish experiment for each nematode isolate consisted of 160 Petri dishes (10 larvae × 4 concentrations × 4 replication).

Plastic containers of 180 ml capacity were filled by 20 g of sterilized air-dried sand. Three nematode concentrations were used for the plastic container's arena: 25, 50, and 100 IJs/cm^2 corresponding to 675, 1350, and 2700 IJs per plastic containers or larva, respectively. Plastic container experiment for each nematode isolate consisted of 120 plastic containers (10 larvae × 3 concentrations × 4 replication). The moisture content of each plastic container was 10% (*w*/*w*) after water-suspended nematodes were applied to the containers that included one healthy larva. Then, the cups were sealed with a lid allowing air exchange.

Statistical analysis

Statistical analysis was performed by SPSS (Version 11.0) statistical software package. Data was evaluated without being regulated by the Abbott formula since there was no mortality in control plates (Abbott, 1925). The significance of the main effects was determined by

factorial analysis of variance (ANOVA). Mean values were separated using Tukey's multiple range test ($P < 0.05$). Estimation of the lethal concentration required to kill 50% (LC_{50}) and 90% (LC_{90}) of the pest population for each nematode isolates was calculated using Probit Analysis.

Results and discussion

The pathogenicity of the four local isolates of EPNs was tested against fourth larval instar of *A. ipsilon* under laboratory conditions. The results indicated that all isolates of nematodes tested were effective in controlling *A. ipsilon* and caused significant mortality rates. Mortality rates increased as the time of exposure increased. There was no mortality in control treatments for both experiment arenas.

In the Petri dish test, the mortality rate of *A. ipsilon* larvae was significantly affected by the nematode isolates (N), IJ concentration (C), and their interaction (N × C) for second, third, and fourth days after treatment. Mortality rates were significantly affected by the only nematode isolates for the first day after treatment.

The highest mortality rate (90%) was obtained by E76-S isolate at the highest applied concentration (100IJs/larva/Petri dish) in the Petri dish experiment for all exposure times. E76-S, 216-H, and MGZ-4-S isolates were found to be the most effective isolates at the concentration of 100 IJs/larva for the third and fourth days after treatment. At the lowest concentration, isolate 216-H was recorded as the most virulent isolate among other isolates with 5, 25, 32, and 40% mortality rates at the first, second, third, and fourth days after treatments, respectively. The nematode isolates E76-S and 216-H caused the highest *A. ipsilon* larval mortality (15 and 10%), respectively, at the concentration of 100 IJs/larva/Petri, and no mortality was recorded by other tested isolates at all the application concentrations for the first day after treatment (Table 1).

In the plastic container experiment, the mean mortality rates of *A. ipsilon* larvae were significantly influenced by the nematode isolates (N). IJ concentrations (C) were found to be statistically significant at the second day after treatment, while no significant effect was observed by the IJ concentrations at the other exposure times. Insignificant differences were noted statistically between mortality rates caused by the interaction between the nematode isolates and IJ concentrations (N × C) for all the exposure times.

The results of the plastic container experiment showed that FLH-4-H was the most virulent isolate for all the applied concentrations and exposure times. The highest larval mortality rates were achieved by the FLH-4-H and 216-H isolates, starting with the second day after treatment. All isolates tested showed a great mortality rate at the lowest concentration of 25 IJs/cm^2 in the second day of exposure time and the lowest and highest mortality rates were (80 and 100%) caused by MGZ-4-S and FLH-4-H, respectively. Insignificant differences were found in mortality rates caused by the isolates tested and the concentrations used for the fourth day after treatment (Table 2).

The estimated LC_{50} values of the nematode isolates tested on the fourth instar *A. ipsilon* larvae ranged between 52 and 142 IJs at the Petri dish experiment and between 17 IJs and 24 IJs at the plastic container experiment at the second day after treatment. The lowest LC_{50} and LC_{90} values were achieved by the isolate E76-S (52 IJs) at the Petri dish experiment. In the plastic container experiment, the lowest LC_{50} and LC_{90} values were obtained by using the isolate FLH-4-H (17 IJs).

The pathogenicity of the four local Turkish EPN isolates, tested against fourth instar *A. ipsilon* larvae under laboratory conditions, showed that all of them caused significant mortality rates at both Petri dish and plastic container experiment arenas. In Petri dish experiment, *S. carpocapsae* E76-S isolate performed a more efficient control of the *A. ipsilon* larvae at 25, 50, and 100 IJs/larva/Petri concentrations for all the exposure times, except at the first day. There was a positive relation between percent mortality and nematode concentration in both experiment arenas, which is in line with the studies of (Shoeb et al. 2006; Bélair et al. 2013; Mahmoud et al. 2016). In the previous Petri dish studies, *S. carpocapsae* was found more virulent than *H. bacteriophora* against the *A. ipsilon* larvae (Bélair et al. 2013 and Mahmoud et al. 2016).

In the Petri dish study, the commercial formulation of *S. carpocapsae* (CSc) and some indigenous Canadian isolates were tested against the fifth instar of *A. ipsilon* larvae. CSc was found to be more effective than the other isolates tested (Bélair et al. 2013). CSc caused 37 and 48% mortality rates at the concentration of 50 IJs/larva/Petri, after second and third days of exposure times, respectively, while the highest mortality rate among the indigenous isolates was 33%, which is quite low compared to the obtained results. In the present study, mortality rates were found as 60 and 75% at the same concentration for the second and third days of exposure times, respectively. In the same study, the highest mortality rate for indigenous isolates (94%) and CSc (98%) was reached at 250 and 1000 IJs/larva/Petri concentrations after third day exposure time, respectively. The highest mortality rate for *S. carpocapsae* Turkish isolate E76-S (85%) was reached at the concentration of 100 IJs/larva/Petri after the third exposure time. Considering the obtaining of high mortality with low concentration, the virulence of Turkish indigenous isolate E76-S against *A. ipsilon* was very high as compared to results in the present study.

Table 1 Percentage mortality (mean ± SD) of *Agrotis ipsilon* larvae exposed to different isolates of entomopathogenic nematodes, at four different concentrations of infective juveniles (IJs) in Petri dish at 25±1 °C

EPNs[a]	Mortality rates (%) (mean ± SD) of *Agrotis ipsilon* larvae*															
	1st day				2nd day				3rd day				4th day			
	10 IJs/larva	25 IJs/larva	50 IJs/larva	100 IJs/larva	10 IJs/larva	25 IJs/larva	50 IJs/larva	100 IJs/larva	10 IJs/larva	25 IJs/larva	50 IJs/larva	100 IJs/larva	10 IJs/larva	25 IJs/larva	50 IJs/larva	100 IJs/larva
FLH-4-H	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa	15 ± 6Ba	25 ± 6Bb	27 ± 9Ab	38 ± 8Ac	20 ± 0Ba	25 ± 9Aa	40 ± 8Ab	45 ± 9Ab	20 ± 7Aa	35 ± 11ABb	40 ± 14Ab	52 ± 11Ac
216-H	5 ± 1Ba	7 ± 2Ba	10 ± 3Ba	10 ± 0Ba	25 ± 5Ba	30 ± 7Ba	52 ± 9Bb	55 ± 10Abb	32 ± 5Ca	52 ± 9Bb	70 ± 8Bc	72 ± 9Bc	40 ± 9Ba	52 ± 9BCab	70 ± 8BCbc	87 ± 9Bc
E76-S	0 ± 0Aa	5 ± 1Ba	7 ± 3Ba	15 ± 4Bb	20 ± 8Ba	57 ± 11Cb	60 ± 9Bb	67 ± 12Bc	27 ± 11Ca	72 ± 12Cb	75 ± 7Bb	85 ± 5Bb	35 ± 11Ba	70 ± 12Cb	82 ± 5Cb	90 ± 8Bb
MGZ-4-S	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa	0 ± 0Aa	2 ± 1Aa	10 ± 5Aab	29 ± 5Ab	62 ± 8Abc	10 ± 4Aa	15 ± 4Aa	52 ± 6ABb	80 ± 2Bc	12 ± 7Aa	20 ± 8Aa	60 ± 8Bb	87 ± 5Bc

*Mean values followed by different uppercase letters in the same column and mean values followed by different lowercase letters in the same line are statistically different according to Tukey's test ($P \leq 0.05$)
[a]FLH-4-H: *H. bacteriophora*, E76-S: *S. carpocapsae*, 216-H: *H. indica*, MGZ-4-S: *S. bicornotum*

Table 2 Percentage mortality (mean ± SD) of *Agrotis ipsilon* larvae exposed to different isolates of entomopathogenic nematodes, at three different concentrations of infective juveniles (IJs) in plastic containers at 25 ± 1 °C

EPNs[a]	Mortality rates (%) (mean ± SD) of *Agrotis ipsilon* larvae*											
	1st day			2nd day			3rd day			4th day		
	25 IJs/cm^2	50 IJs/cm^2	100 IJs/cm^2	25 IJs/cm^2	50 IJs/cm^2	100 IJs/cm^2	25 IJs/cm^2	50 IJs/cm^2	100 IJs/cm^2	25 IJs/cm^2	50 IJs/cm^2	100 IJs/cm^2
FLH-4-H	8 ± 5Aa	18 ± 9Ba	20 ± 8Ba	95 ± 10Aa	100 ± 0Ba	100 ± 0Aa	100 ± 0Ba	100 ± 0Aa	100 ± 0Aa	100 ± 0Aa	100 ± 0Aa	100 ± 0Aa
216-H	3 ± 5Aa	5 ± 10Aa	8 ± 9Aa	90 ± 8Aa	100 ± 0Bb	100 ± 0Ab	100 ± 0Ba	100 ± 0Aa	100 ± 0Aa	97 ± 5Aa	100 ± 0Aa	100 ± 0Aa
E76-S	8 ± 5Aa	8 ± 5Aa	10 ± 8Aa	83 ± 9Aa	83 ± 12Aa	90 ± 11Aa	90 ± 8Aa	90 ± 11Aa	97 ± 5Aa	93 ± 5Aa	98 ± 5Aa	98 ± 5Aa

*Mean values followed by different uppercase letters in the same column and mean values followed by different lowercase letters in the same line are statistically different according to Tukey's test ($P \leq 0.05$)
[a]FLH-4-H: *H. bacteriophora*, E76-S: *S. carpocapsae*, 216-H: *H. indica*, MGZ-4-S: *S. bicornotum*

In another Petri dish study, EPNs were applied against the last instar larvae of *A. ipsilon* at seven concentrations (0, 20, 40, 80, and 160 IJs/ml) under laboratory conditions at 25 ± 2 °C (Mahmoud et al. 2016). In this study, the maximum mortality rates for *S. carpocapsae* (100%) and for *H. bacteriophora* (90%) were reached at 80 IJs/ml and at 160 IJs/ml concentrations at the third day after treatment, respectively. In the present study, the highest mortality rates for *S. carpocapsae* (85%) and for *H. bacteriophora* (45%) at the third day after treatment were reached at 100 IJs/larva/Petri concentration. Shoeb et al. (2006) investigated the efficacy of *S. abbasi* and *H. bacteriophora* against the fourth instar larvae of *A. ipsilon* in Petri dishes in the laboratory study at 27 ± 1 °C. They reported that *H. bacteriophora* caused 49, 53, and 73% mortality rates, respectively, at 25, 50, and 100 IJs/larva in the second day after the treatment. Fetoh et al. (2009) evaluated the effectiveness of *S. carpocapsae* and *H. bacteriophora* against fourth instar *A. ipsilon* larvae at concentrations of 25, 50, 100 IJs/ml in Petri dish under laboratory conditions, and higher mortality rates were found compared to ours. Mortality rates, at the second day after treatment, were found as 70, 85, and 100% for *S. carpocapsae* and 80, 90, and 100% for *H. bacteriophora* at concentrations of 25, 50, and 100 IJs/ml, respectively. In this study, mortality rates for the second day after treatment were 57, 60, and 65% for *S. carpocapsae* and 25, 27, and 38% for *H. bacteriophora* at 25, 50, and 100 IJs/ml concentrations, respectively. The reason of these differences in mortality rates can be attributed to the number of larvae used for the experiment. Shoeb et al. (2006), Fetoh et al. (2009), and Mahmoud et al. (2016) used a total of 15, 25, and 20 larvae in their study, respectively, whereas there were a total of 40 larvae for each concentration that belongs to the isolates in our study. The low number of samples can be misleading and does not represent the population exactly. In their study, small sample size might have led to high mortality rates. Another reason may be due to the different stages of the larvae used that the studies showed that fourth instar *A. ipsilon* larvae are the most susceptible stage to most EPN species (Ebssa and Koppenhöfer 2012). Mortality rates also might be affected by the foraging strategies of EPNs. *S. carpocapsae* is considered to be more effective than other species in controlling moving insects due to foraging behavior, and Petri dish might have created an environment that is more favorable to *S. carpocapsae* (Campbell and Gaugler 1993).

Although Petri dish experiment is a quick method to assess the effectiveness of EPNs, this technique can give false results due to the inability in simulating the field conditions. In order to determine the effectiveness of EPNs isolates in the soil, plastic container experiments have been conducted. Mortality rates have been found high in the soil environment, which is the natural habitat of EPNs, and the low mortality rate at the lowest concentration (25 IJs/cm^2) after second day exposure time was 80% (MGZ-4-S), while the maximum mortality rate (100%) was achieved at concentrations of 50 and 100 IJs/cm^2 (FLH-4-H and 216-H) after second day exposure time. Ebssa and Koppenhöfer (2012) evaluated the commercial formulations of *S. carpocapsae* and *H. bacteriophora* on different instars of *A. ipsilon* larvae in plastic cups of 30 ml capacity, filled with soil, and the mortality rates at the second day exposure time were found 30 and 83% for *H. bacteriophora* and 18 and 55% for *S. carpocapsae* at concentrations of 30 and 100 IJs/larva, respectively. In the present study, mortality rates at the second day exposure time were 95 and 100% for *H. bacteriophora* and 82 and 95% for *S. carpocapsae* at concentrations of 25 and 100 IJs/cm^2, respectively. Mortality rates, especially at concentration 25 IJs/cm^2, in our study was much higher than this study, most probably due to the different concentrations used for per larva. In another study, Hussaini et al. (2005), the potential of different EPNs on the last instar of *A. ipsilon* larvae was assessed in plastic containers, filled with soil at 20 IJs/ml and 180 IJs/ml at 25 ± 1 °C. *H. indica* isolates showed more virulence than *S. carpocapsae* isolate and caused 100% mortality at the fourth day of exposure time and *S. carpocapsae* isolate was found as the least effective isolate against the last instar of *A. ipsilon* larvae, which is in line with our study. The maximum mortality rate (100%) was reached on the fourth day after inoculation in this study. It was reached at the second day after inoculation in our study which means that these isolates were more pathogenic (Campbell and Gaugler 1993).

Conclusion

The study indicated that all the local isolates tested induced great mortality on the *A. ipsilon* larvae and gave promising results. Although heterorhabditid isolates appeared to be more virulent than steinernematid ones, generally, there were insignificant differences statistically between the EPN isolates in the plastic container experiment, and all the isolates caused higher mortality rates than at the Petri dish experiment. This study also showed that although Petri dish experiment is a quick method, it could give misleading results; therefore, it is necessary to conduct the experiments in both Petri dish and soil environments to evaluate EPNs effectiveness correctly.

Abbreviations

BCW: The black cutworm; C: Nematode concentration; DAT: Days after treatment; EPNs: Entomopathogenic nematodes; IJs: Infective Juveniles; L4: The fourth larval instars; LC_{50}: The lethal concentration required to kill 50% of the pest population; LC_{90}: The lethal concentration required to kill 90% of the pest population; N: Nematode species; *w/w*: Weight per weight

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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.

Authors' contributions
EY and RC have planned the outline of this research and designed the methodology together. EY carried out the laboratory studies and drafted the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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