Potential efficacy of Iranian isolates of *Heterorhabditis bacteriophora* and *Steinernema feltiae* on *Pieris brassicae* (Lepidoptera:Pieridae)

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Summary. In a regional survey, native entomopathogenic nematodes, *Heterothabditis bacteriophora* and *Steinernema feltiae* isolates, collected from soil samples in Kurdistan province in Iran were applied against the 4th and 5th instars of cabbage butterfly larvae. The experiments were performed on both filter paper and cabbage leaves. Bioassay experiments on filter paper were conducted at two temperatures, 25°C and 30°C. LC_{50} values for *H. bacteriophora* on filter paper at 25°C and 30°C against the 4th instars were 85.4 and 66.7 IJ per insect, respectively; however, these values for *S. feltiae* were 96.2 and 66.0 IJ per insect. LC_{50} values for *H. bacteriophora* and *S. feltiae* were defined as 44.2 and 54.2 IJ per cabbage butterfly 4th instars, respectively.

Key words: biological control, Brassicaceae, entomopathogenic nematode, LC_{50}, temperature.

The cabbage butterfly, *Pieris brassicae* L. (Lepidoptera: Pieridae), is one of the most important and destructive cabbage pests in the world (Cartea *et al.*, 2009). The 4th and 5th instars can cause significant economic damage to cabbage, Brussels sprouts, cauliflower, kohlrabi and turnips (Karowe & Schoonhoven, 1992). The *P. brassicae* butterfly lays large yellow eggs in batches on cabbage leaves. Larvae typically go through five instars before pupation. Newly emerged larvae only consume epidermis and parenchyma layers of leaves, in contrast to 4th and 5th instars, which are larger and consume the entire leaf with the exception of the midrib. Last instars of *P. brassicae* move long distances in search of a suitable pupation site; a process called wandering. Pupation lasts for 10 to 15 days. Between two and four generations are possible each growing season in moderate climates (Feltwell, 1982).

Currently the most reliable management of this pest is provided by chemical insecticides. However, hazards and residues of insecticides along with potential for resistance development encouraged development of alternative control measures. Entomopathogenic nematodes (EPN) are commercially available and showed promise for controlling this pest. EPN belonging to the families of Heterorhabditidae and Steinernematidae are obligate parasites of insects (Kaya & Gaugler, 1993). The non-feeding infective juveniles (IJ) actively seek out insect hosts and penetrate the insect body usually *via* natural openings. These IJ invade the haemocoel and the symbiotic bacteria are released from the nematode gut and cause septicemia which kills the host (Akhurst, 1983; Lewis *et al.*, 1993; Forst & Clarke, 2002). No adverse effects of EPN have been proven on non-target insects, *viz.*, predators and parasitoids (Mbata & Shapiro-Ilan, 2010).

EPN efficacy has been tested against many pest species (Athanassiou *et al.*, 2008; Girling *et al.*, 2010; Shapiro-Ilan *et al.*, 2013). Efficacy varies among insect species/EPN species combinations due to variable pathogenicity (Grewal *et al.*, 2005).

Susceptibility to heat and desiccation in large scale field applications is major disadvantage of EPN, which limits their use on foliar environment (Georgis, 1992; Strauch *et al.*, 2000). However, pests have been successfully controlled by EPN in above ground when they resided in protected locations on plants (Begley, 1990). The structure of cabbage plants may offer an opportunity to apply

EPN to relatively protected conditions because of the structure of the external leaves, which protect the inner ones from sunlight and also keep high relative humidity.

The aims of this study were: *i*) to evaluate the susceptibility of 4th and 5th instars of *P. brassicae* larvae to native isolates of EPN; *ii*) to evaluate the effects of temperature on the pathogenicity of IJ; and *iii*) to determine the impact of low concentrations of IJ on the viability of subsequent stages under laboratory conditions.

MATERIALS AND METHODS

Entomopathogenic nematode isolates. The isolates of *H. bacteriophora* and *S. feltiae* were recovered from soil samples collected from alfalfa field and grasslands in Kurdistan province, Iran. The last instars of *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae, reared on an artificial medium were used for isolating nematodes from the soil samples.

The infected *G. mellonella* larvae were moved to a White trap (Kaya & Stock, 1997) to obtain infective juveniles from the cadavers. The isolated EPN were stored in tap water at 12°C.

Preparation of *P. brassicae*. The eggs of cabbage butterflies were collected from the cabbage fields of Urmia (West Azerbaijan province, Iran). Newly hatched larvae were fed with fresh cabbage leaves grown in a research plot located at the Iranian Research Institute of Plant Protection, Tehran, Iran, until the 4th and 5th instars larvae appeared.

Bioassays. Newly emerged 4th and 5th instars *P. brassicae* larvae were used for bioassay tests. Two isolates of *H. bacteriophora* and *S. feltiae* were used against larvae on filter paper and cabbage leaf bioassays to determine the lethal concentrations of EPN. Concentration ranges for all experiments (including bioassay on filter paper and on cabbage leaf) were selected based on preliminary tests for EPN and instars, and based on the preliminary results, logarithmic concentrations for the experiments were determined.

Also, LT_{50} experiments were carried out for *H. bacteriophora* and *S. feltiae* on both larval instars on filter paper, and tests on the effects of low concentrations of EPN on larval development were also conducted. The experiment was repeated three times and before each experiment, the IJ were acclimatised at room temperature (23-28°C) for 2 h.

Effects of lethal concentrations at different temperatures. Filter paper bioassays were conducted in Petri dishes (8 cm diam.) containing a layer of ashless filter paper. Experiments were performed at two temperatures, 25±2°C and 30±2°C. At 25±2°C, 4th instars larvae were exposed to 20, 35, 63, 112 and 200 IJ per insect of *H. bacterophora*, and 30, 48, 77, 124 and 200 IJ per insect for *S. feltiae*. To assay the LC_{50} values of *H. bacteriophora* on 5th instars larvae, the concentrations were 20, 35, 55, 90 and 150 IJ per insect and 20, 35, 63, 112 and 200 IJ per insect for *S. feltiae*. At 30±2°C, 4th instars larvae were exposed to 15, 25, 42, 71 and 120 IJ per insect of *H. bacteriophora* and *S. feltiae*. For 5th instars, concentrations were 10, 17, 30, 52 and 90 IJ per insect. All experiments were carried out in three replications.

All concentrations were prepared in a final volume of 1 ml of distilled water and applied to the surface of the filter paper. Control treatments received only distilled water. Thereafter, fifteen 4th and 5th instars larvae were placed into each 8 cm diam. Petri dish and a piece of cabbage leaf was added as food source and every 24 h the leaf was replaced by new one. For each concentration, three replicates were performed. After 2 days, the numbers of dead and living insects were recorded. Dead insects were dissected under a stereo microscope to confirm nematode infection.

Table 1. LC_{50} values (IJ per insect) estimated for *Heterorhabditis bacteriophora* and *Steinernema feltiae* on 4th *Pieris brassicae* instars larvae on filter paper at two temperatures

EPN species	Temperature	LC_{50}	χ^{2a}	ρ^b	Intercept (a)	Slope (b)±SE[c]
H. bacteriophora	25	85.44 (59.73-138.57)	2.60	0.46	1.65	1.74±0.27
	30	66.70 (51.90-95.23)	2.67	0.44	2.02	1.63±0.29
S. feltiae	25	96.23 (81.05-114.04)	0.55	0.91	0.38	2.33±0.49
	30	65.95 (51.89-91.83)	0.29	0.56	1.89	1.70±0.29

[a]: Pearson χ^2 of the slope; [b]: *P*-values represent the probability of the slope; [c]: Standard error.

Table 2. LC_{50} values (IJ per insect) estimated for *Heterorhabditis bacteriophora* and *Steinernema feltiae* on 5th *Pieris brassicae* instars larvae on filter paper at two temperatures

EPN species	Temperature	LC_{50}	χ^{2a}	ρ^{b}	Intercept (a)	Slope (b)±SE[c]
H. bacteriophora	25	54.77 (45.36-66.13)	1.10	0.78	1.15	2.22±0.32
	30	43.48 (31.56-68.38)	2.37	0.50	1.97	1.85±0.28
S. feltiae	25	68.01 (46.46-103.45)	3.46	0.32	1.26	2.05±0.28
	30	55.04 (45.00-71.95)	0.61	0.89	2.22	1.60±0.28

[a]: Pearson χ^2 of the slope; [b]: *P*-values represent the probability of the slope; [c]: Standard error.

In another experiment, leaf disks were prepared and placed into 8 cm diam. Petri dishes, covering the bottom of the dish completely and cabbage leaf was prepared as food and replaced every 24 h. The concentration of *H. bacteriophora* and *S. feltiae* for the 4th larvae instars were 10, 18, 36, 52, 100 IJ per insect, and amounts for the 5th larval instars were 5, 10, 20, 40, 80 IJ per insect; each concentration was replicated three times. All concentrations were prepared with 1 ml distilled water and 1 µl Tween20 for better distribution were added to each concentration. Control treatments received only distilled water and Tween20. After applying the EPN to the leaf disk, 15 larvae were placed in each Petri dish. The dishes were incubated at 25°C. After two days, the numbers of dead and live insects were recorded. Dead insects were dissected under a stereo microscope to confirm nematode infection. This experiment was done to evaluate EPN efficacy on the natural surface in comparison with filter paper.

Effect of low concentrations of EPN on larval development. The test was carried out at 5 concentrations (10, 20, 30, 40 and 50 IJ per insect), which were calculated based on results from the lethal concentration experiments. All experiments were conducted in 8 cm diam. Petri dishes layered with filter paper and prepared cabbage leaves as food (leaves were replaced every 24 h). The experiment was conducted with 4th instars only. Early emerged 4th instars larvae were exposed to nematodes; 24 h after treatment, treated larvae were transferred to clean 8 cm diam. Petri dishes layered with wet filter paper.

In control treatment only water was added. Each treatment was replicated three times and fifteen larvae were used in each replication. Also, Petri dishes were incubated at 25°C. The numbers of dead and infected larvae survived long enough to reach to the 5th instars were enumerated. The pupal stage was counted until butterflies emerged in control treatment. Also the weights of the pupae were measured.

LT_{50} experiments. These experiments were conducted on filter paper disks placed in 8 cm diam. Petri dishes. Cabbage leaves were prepared as food. Two concentrations of 120 and 150 IJ per insect were spread on the filter paper disk, and then the larvae were transferred to the dishes. Mortality was recorded five times (12, 24, 36, 48, and 60 h). Each experiment was replicated three times. Each replication consisted of 15 larvae; control was treated only with distilled water.

Statistical analyses. To calculate LC_{50} and LT_{50} values, probit analysis was carried out. SPSS software, version 19 was used for all statistical analyses (SPSS Inc., 2010). To compare LC_{50} values, lethal dose ratios were used at the 0.05 level (Robertson *et al.*, 2007).

RESULTS

Effects of lethal concentrations at different temperatures. Temperature affected the virulence of the EPN against the larval stages of cabbage butterfly. At 25°C, LC_{50} of *H. bacteriophora* and *S. feltiae* on 4th instars were 85.4 and 96.2, respectively, and these values at 30°C were 66.7 and 66.0 IJ for *H. bacteriophora* and *S. feltiae*, respectively.

The LC_{50} for the 5th instars at 25°C were different, 54.8 and 68.0 for *H. bacteriophora* and *S. feltiae*, respectively. These values at 30°C for *H. bacteriophora* and *S. feltiae* were 43.5 and 55.0 IJ per insect, respectively, in filter paper conditions (Tables 1 & 2). The mortality was always associated with nematode concentration and subsequently their penetration.

LC_{50} values of *H. bacteriophora* and *S. feltiae* on the 4th instars larvae were 44.2 and 54.2 IJ, respectively (Table 3), while LC_{50} values on the 5th larvae were decreased to 26.0 and 34.2 for IJ of *H. bacteriophora* and *S. feltiae*, respectively, in leaf assays (Table 4).

Analytical comparison showed significant differences between LC_{50} from the paper assay (Tables 1 & 2) and the leaf assay (Tables 3 & 4). The treatments carried out by *H. bacteriophora* on 4th instars larvae on the leaf assay (44.2 IJ) showed a significant difference compared to the filter paper assay at both temperatures (85.4 IJ and 66.7 IJ). Application of *H. bacteriophora* on 5th instars on filter paper and cabbage leaf was also significantly different. LC_{50} values were similarly significant in experiments with *S. feltiae* on both instars larvae. Also, the analytical comparisons showed that the values resulted from both EPN were significantly different when they were used on different larval stages.

Effect of low concentrations of EPN on larval development. A low concentration of both nematode species caused some mortality but some larvae survived long enough to reach the 5th instars or even the pupal stage (Table 5). No adults emerged in any treatments.

In the lowest concentration (10 IJ per insect) 86.7 and 91.1% of hosts survived through pupation in larvae treated with *H. bacteriophora* and *S. feltiae*, respectively. In the highest concentration (40 IJ per insect), these values were 24.4 and 40.0% in *H. bacteriophora* and *S. feltiae*, respectively. Although most treated larvae died at the 5th instar stage, some larvae reached the pupal stage but were smaller, as measured by weight. In comparison with untreated pupae, the mean percent weight reduction of pupae was 25.5%. No pupae developed successfully to an eclosed adult, possibly due to damage to vital organs. However, pupae in control treatment developed successfully to eclosed adults.

LT_{50} experiments. LT_{50} values of *H. bacteriophora* and *S. feltiae* treated on the 4th and 5th instars larvae of *P. brassicae* are shown in Table 6. The lowest level was obtained for *H. bacteriophora* in 5th instars and the highest was for *S. feltiae* (Table 6).

Table 3. LC_{50} values (IJ per insect) estimated for *Heterorhabditis bacteriophora* and *Steinernema feltiae* on 4th instars larvae of *Pieris brassicae* on cabbage leaf

EPN species	LC_{50}	χ^{2a}	ρ^b	Intercept (a)	Slope (b)±SE[c]
H. bacteriophora	44.21 (33.66-57.30)	1.45	0.89	1.13	2.35±0.46
S. feltiae	54.20 (42.89-66.09)	0.98	0.81	0.2	2.77±0.61

[a]: Pearson χ^2 of the slope; [b]: *P*-values represent the probability of the slope; [c]: Standard error.

Table 4. LC_{50} values (IJ per larva) estimated for *Heterorhabditis bacteriophora* and *Steinernema feltiae* on 5th instars larvae of *Pieris brassicae* on cabbage leaf

EPN species	LC_{50}	χ^{2a}	ρ^b	Intercept (a)	Slope (b)±SE[c]
H. bacteriophora	25.96 (18.62-34.61)	1.55	0.67	1.47	2.50±0.40
S. feltiae	34.17 (24.58-47.58)	1.41	0.70	1.60	2.22±0.40

[a]: Pearson χ^2 of the slope; [b]: P-values represent the probability of the slope; [c]: Standard error.

Table 5. Mortality (%) of *Pieris brassicae* in developmental stages after treatment with different low nematode concentrations (IJ per insect)

	Heterorhabditis bacteriophora					***Steinernema feltiae***				
	10	**15**	**20**	**30**	**40**	**10**	**15**	**20**	**30**	**40**
4th Larva	4.4	6.7	15.5	24.4	31.1	2.2	4.4	6.7	11.1	20
5th Larva	8.9	17.8	28.9	37.8	44.4	6.7	15.5	20	33.3	40
Pupae	86.7	75.5	55.5	37.8	24.4	91.1	80	73.3	55.5	40

There was no eclosion to adults in any concentrations.

Table 6. LT_{50} values (h) calculated for two concentrations of *Heterorhabditis bacteriophora* and *Steinernema feltiae* on 4th and 5th instars *Pieris brassicae* instars larvae

EPN species	Life stage	IJ per Insect	
		120	150
		LT_{50} (h)	LT_{50} (h)
H. bacteriophora	4th	39.44 (34.28-45.51)	37.40 (29.24-45.74)
	5th	32.48 (28.54-36.33)	28.92 (24.54-32.99)
S. feltiae	4th	42.35 (36.13-51.09)	39.52 (30.68-49.47)
	5th	40.40 (33.38-48.24)	30.81 (21.98-38.54)

DISCUSSION

In this study 4th and 5th instars were selected because they are most active due to their wandering behaviour, and this may increase the possibility of infection. The advantage of using cabbage leaf in experiments was correspondence of trial conditions to field conditions. Cabbage plants have succulent leaves and the compact structure of the plant can preserve high relative humidity in the inner layers providing the required moisture and protection from sunlight to extend EPN survival. In addition to target insect, the type of host plant seems to be important in biological control of foliage-feeding insects using EPN.

Temperature plays a critical role in the rate, at which EPN infect their hosts (Hazir *et al.*, 2001). Nevertheless, excessive temperatures are deleterious to infection, reproduction and nematodes survival (Gray & Johnson, 1983). Remarkably, in this study both species reacted well to increasing temperature from 25°C to 30°C. Similarly, in research conducted by Toledo *et al.* (2009) *S. feltiae* caused the highest mortality to larvae of *Rhagoletis indifferens* Curran (Diptera: Tephritidae) at 25±2°C and higher temperature had a positive effect on mortality of *Anestrepha oblique* Macquart (Diptera: Tephritidae) larvae. The temperature of 30°C is nearly the required temperature for growing cabbage and development of *P. brassicae* in field conditions.

Treating the 4th larvae instars with low concentration caused the death of some larvae; however, other larvae moulted to the next instars and even pupa, but no adult emerged. Possibly, this phenomenon is due to damage to vital organs of pupae by IJ during the infection process, which caused a decline in pest population in the next generations and showed the efficacy of EPN even at low concentration. Also, results of low concentrations show that these EPN are efficient in control of *P. brassicae* even in low concentrations, which is very important in integrated pest management programmes. Weight reduction of infected pupae may be another reason for all pupae dying before eclosion. No emergence of adults demonstrates the marked effectiveness of these EPN in control of the next generation.

Mortality effect of EPN on *Pieris rapae* was investigated by Finney & Bennett (1984). They used high concentrations of *H. bacteriophora* on larval stages to get 100% mortality; the difference between concentrations was substantial, in contrast to our results, possibly because of biological and ecological differences of the insect species and the assays conditions.

LT_{50} values suggest that with increasing concentration of both nematode species from 120 to 150 IJ per insect the required time to 50.0% mortality of the population was dramatically decreased. Our observations showed quicker mortality of the 5th instars larvae than the 4th instars larvae that reflects greater susceptibility of the 5th instars, which agrees with our LC_{50} experiments. Overall, LT_{50} values caused by *H. bacteriophora* were less than those by *S. feltiae*; as a result mortality in larvae treated with *H. bacteriophora* was faster than those infected with *S. feltiae*.

Wu & Chow (1989) reported 75.0% to 97.5% mortality of larvae of *P. rapae* 3 days after exposure 5,000 to 40,000 IJ of *S. feltiae* per insect, whereas mortality occurred at much lower concentrations in the present study. These differences can be attributed to greater virulence of Iranian EPN isolates and also biological and physiological differences between the target insects.

Pal & Prasad (2012) reported a higher lethal concentration, 347 IJ per insect of *Heterorhabditidis indica* to the 4th instars of *P. brassicae* larvae compared to our results, those biological and ecological differences among nematode isolates and species are assumed.

Heterorhabditis tyserae caused 55.0-100% mortality on *P. rapae* at concentrations of 5-100 IJ

per insect (Saleh, 1995). This finding is in close agreement with ours. Although Pal & Prasad (2012), Saleh (1995) and Wu & Chow (1989) showed lethality of different species of EPN on *P. brassicae* and *P. rapae*, the impact of *H. bacteriophora* and *S. feltiae* against larval stages of *P. brassicae* was investigated for the first time in the present study.

This study demonstrated the feasibility of using EPN for control of *P. brassicae* in laboratory conditions. Further work is necessary to investigate the efficacy of these biological agents to control *P. brassicae* in field conditions. Additionally, the capability of using these nematodes in combination with biopesticides and agrochemicals in cabbage production can be scheduled for further research.

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A. Abdolmaleki, Z. Tanha Maafi, H Rafiee Dastjerdi and E. Lewis. Потенциальная эффективность применения иранских изолятов *Heterorhabditis bacteriophora* и *Steinernema feltiae* против *Pieris brassicae* (Lepidoptera: Pieridae).

Резюме. В рамках регионального исследования, местные изоляты энтомопатогенных нематод видов *Heterothabditis bacteriophora* и *Steinernema feltiae*, полученные из почвенных проб, собранных в провинции Курдистан Исламской республики Иран, тестировали как агентов борьбы с личинками 4-го и 5-го возрастов капустной белянки. Эксперименты проводили как на подложке из фильтровальной бумаги, так и на листьях капусты. Тестирование на фильтровальной бумаге проводили при температурах 25°C и 30°C. Определенные в ходе экспериментов значения LC_{50} для *H. bacteriophora* на фильтровальной бумаге при 25°C и 30°C против личинок 4-го возраста составляли соответственно 85.4 и 66.7 инвазионных личинок на насекомое, тогда как для *S. feltiae* эти показатели составляли 96.2 и 66.0 на насекомое.