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Virulence comparisons of high-temperature-adapted *Heterorhabditis bacteriophora, Steinernema feltiae* and *S. carpocapsae*

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Summary

Entomopathogenic nematodes (EPNs) are environmentally safe alternative control agents. Nematodes in the Heterorhabditidae and Steinernematidae families are widely used in biological control frameworks, especially for soil-inhabiting insect pests. In this experiment, *Heterorhabditis bacteriophora* (Poinar, 1976)*, Steinernema feltiae* (Filipjev, 1934) and *S. carpocapsae* (Weiser, 1955) adapted at high temperature were assessed in order to detect differences in virulence between adapted and non-adapted populations. All species were exposed to 38 °C for 2 h. After this treatment, live infective juveniles (IJs) were used to infect to last instar *Galleria mellonella* (Linnaeus, 1758). larvae at the following doses: 1, 2, 3, 4 and 5 IJs/larva. The LD_{50} and LD_{90} were obtained for these species. Non-adapted populations of the nematode species were used as controls for this experiment. The results indicated that differences in *S. feltiae* and *S. carpocapsae* virulence between the adapted and non-adapted populations were significant; no significant difference was observed between the adapted and non-adapted *H. bacteriophora* populations.
Keywords: *Heterorhabditis bacteriophora; Steinernema feltiae; S. carpocapsae;* temperature; adaptation; virulence

Introduction

Due to the hazardous effects of agricultural chemicals on humans and the environment, pesticide resistance and low MRL (Maximum Residue Limits) policies on agricultural products, biological control has become one of the most alternative control methods. In the biological control framework, entomopathogenic nematodes (EPNs) from the Steinernematidae and Heterorhabditidae families have been used to control soil-dwelling insect pests for a few decades, especially in Europe and the USA (Shapiro & Gaugler, 2010). EPNs are safe for non-target organisms and the environment (Boemare *et al*., 1996; Ehlers 2003). EPNs can also be mass-produced in liquid culture (Lunau *et al*., 1993; Ehlers *et al*., 1998; Strauch & Ehlers, 1998; Ehlers, 2001) for widespread commercial use. Furthermore, Infective Juveniles (IJs) can resist shear stress; standard pesticide sprayers or irrigation systems can be used for application (Georgis, 1990; Wright *et al*., 2005). The use of EPNs has been growing worldwide, but some limiting factors prevent the use of EPNs in certain regions. These initial factors include heat, desiccation, persistence, and effectiveness (Strauch *et al*., 2000). Heat is one of the major stress factors for EPNs in soil conditions. Generally, the optimal temperature for EPNs is approximately 25 °C; however, some strains can handle temperatures up to 40 °C without any adaptation, or genetic selection (Koppenhöfer, 2000; Ulu & Susurluk, 2014). Several studies have achieved improvement in these characteristics with hybridization (Mukuka *et al*., 2010a). For a commercial EPN strain, higher heat tolerance alone may not be enough. EPNs must survive several environmental conditions and have high effectiveness in order to be a feasible commercial product. Therefore, several characteristics and how these traits are related must be assessed to obtain a superior EPN strain.

The aim of the present study was to determine and compare the effectiveness of 4 strains from the following high-temperature-adapted EPN species: *Heterorhabditis bacteriophora, Steinernema feltiae* and *S. carpocapsae.* Thus, relationship between heat tolerance

and virulence can be examined and data for further hybridization and genetic selection studies can be provided.

Material and Methods

Nematode strains

The following nematode strains used in the study were isolated from cities in Turkey: *Heterorhabditis bacteriophora* (H.b. 17) isolated in Kırklareli, *H. bacteriophora* (H.b. 1138) isolated in Antalya (H.b. 1138), *Steinernema feltiae* (TUR-S3) isolated in Ankara and *S. carpocapsae* (TUR-S4) isolated in Bursa. All strains were identified using a molecular technique (PCR-RFLP) (unpublished data). For soil sampling, ten soil samples (500 g) were taken from each area. Samples were merged in the laboratory to prevent labor loss. To isolate the nematodes, containers (with 100 gr capacities) were filled with soil, and 3 last instar *Galleria mellonella* (Lepidoptera: Pyralidae) larvae were placed on the soil. The containers were incubated at 25 °C for 3 days. Following the incubation, the dead larvae were transferred to a White trap. After two weeks after on a White trap, the nematodes emerged from the cadaver and were collected in a culture flask. The nematodes were then stored at +4 °C.

Table 1. Lethal temperatures of non-adapted and adapted populations of the strains (°C)

Strains	Non-adapted populations		Adapted populations	
	LT_{50}	LT_{90}	LT_{50}	LT_{90}
H.b. 17	38.1	40.1	39	41.3
H.b. 1138	37.4	40.4	37.7	40
Tur S3	36.7	39	35.6	37.3
Tur S4	35.5	38.7	35.7	38.2

Effectiveness

High-temperature-adapted IJs from each of the 4 strains were inoculated on last instar *G. mellonella* larva at a dose of 1, 2, 3, 4 or 5 IJs/larva. All doses were applied with a micro-injector. Inoculated larvae were put in each well of a 24-well plate. After the inoculation, plates were sealed with parafilm and incubated at 25 °C for 4 days. The plates were opened at the end of the incubation period, and the dead larvae were dissected under a stereomicroscope to determine whether larvae were killed by the nematode.

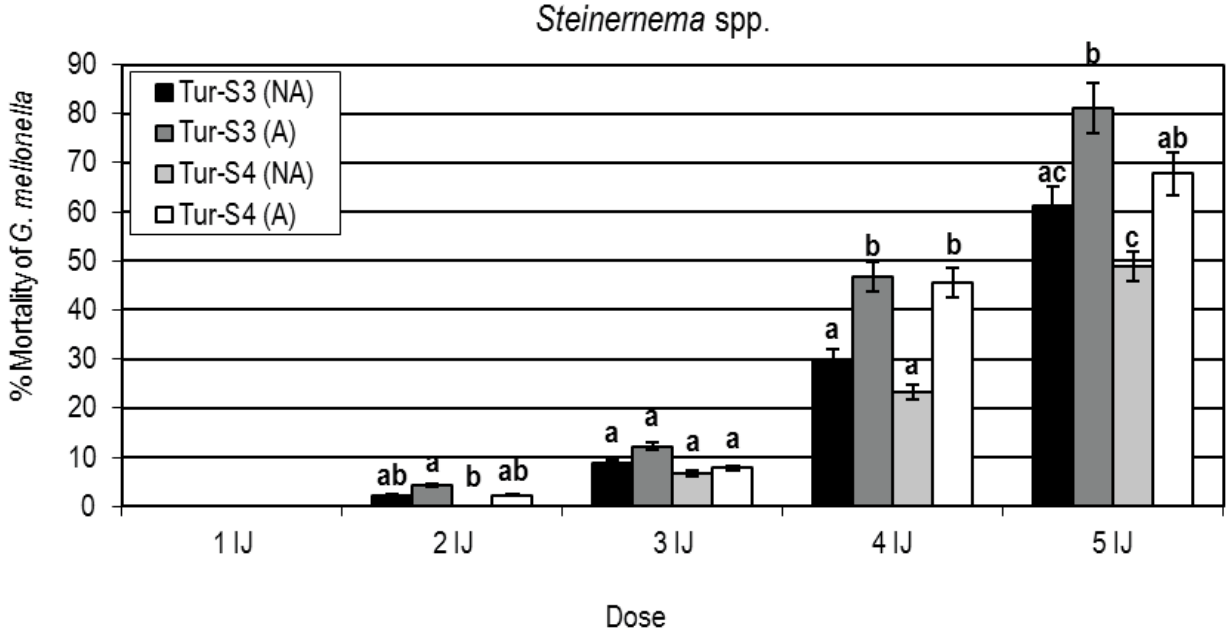

Fig. 1. Effectiveness to *G. mellonella* larvae comparison between heat adapted and non-heat adapted populations of *Steinernema feltiae* (TUR-S3) and *Steinernema carpocapsae* (TUR-S4) (NA = Non-Adapted, A = Adapted). For each dose, means followed by the same letter are not statistically different ($p > 0.05$)

Heat adaptation

Isolated native strains were reproduced on *G. mellonella* as described by Kaya and Stock (1997). Heat adaptation experiments were conducted in 24-well tissue culture plates. For each well, 500 IJs of the desired strain were aliquoted with Ringer solution. Infective juveniles were exposed to 38 °C for 2 h for heat adaptation before the effectiveness experiments were carried out. After the heat exposure, dead individuals were separated from living ones under a stereomicroscope. Live individuals were collected into a flask. The heat adaptation experiments were replicated 5 times. After dissection, the mortality ratios of the strains were calculated. Effectiveness experiments were replicated 5 times using 30 larvae for each replicate.

Statistical analysis

For the heat adaptation experiments, LT_{50} and LT_{90} values were calculated by Probit analysis using BioStat 2010 software. To determine the difference between the effectiveness of the strains, ANOVA was performed at a 5 % confidence level using JMP 7 software. Comparisons between the effectiveness of the strains

were done using the Least Significant Differences (LSD) method at a 5 % confidence level.

Results

Heat adaptation

Without adaptation, Hb 17 was the most tolerant to heat of the four 4 strains according to both LT_{50} and LT_{90}; Tur S4 was the least tolerant strain (Table 1). With adaptation, all strains developed some tolerance to heat (Table 2). Hb 17 was still the most tolerant

Table 2. Statistical analysis of the effectiveness of non-adapted and adapted populations of used species

Dose	H.b. 17 and H.b. 1138	Tur S3 and Tur S4
1 IJ	-	$F = 4.00$; $df = 3,8$; $P = 0.519$
2 IJ	-	$F = 7.33$; $df = 3,8$; $P = 0.011$*
3 IJ	$F = 0.56$; $df = 3,8$; $P = 0.658$	$F = 0.78$; $df = 3,8$; $P = 0.538$
4 IJ	$F = 2.11$; $df = 3,8$; $P = 0.177$	$F = 7.87$; $df = 3,8$; $P = 0.009$*
5 IJ	$F = 0.95$; $df = 3,8$; $P = 0.960$	$F = 10.09$; $df = 3,8$; $P = 0.043$*

* Statistically significant at $p < 0.05$

no significant differences between the adapted and non-adapted IJs were observed (Fig. 2) (Table 2).

Discussion

Entomopathogenic nematodes (EPNs) are one of the best biological alternatives to chemical agents against soil-dwelling insect pests (Ehlers, 2001). More than 80 species of EPNs have been identified; more than 11 species have been commercialized (Kaya & Koppenhöfer, 1999). Shapiro-Ilan and Gaugler (2010) have listed the more the 25 commercial EPN producers worldwide. There are also several formulation types that contain different media and have different storage durations (Grewal, 2000).

Recent studies have generally focused on the effectiveness of EPNs on different insect pests and the improvement of several characteristics with genetic studies, such as selective breeding or hybridization (Mukuka *et al*., 2010a, 2010c; Nimkingrat *et al*., 2013; Susurluk *et al*., 2013; Ulu & Susurluk, 2014). At present, one of the major obstacles in EPN commercialization is the high application cost due to short shelf life and high production expenses. Developing a superior EPN strain can be an alternative way to reduce application costs indirectly.

Anbesse *et al*. (2013) tried to improve the beneficial traits of entomopathogenic nematodes with genetic selection. Prior to these

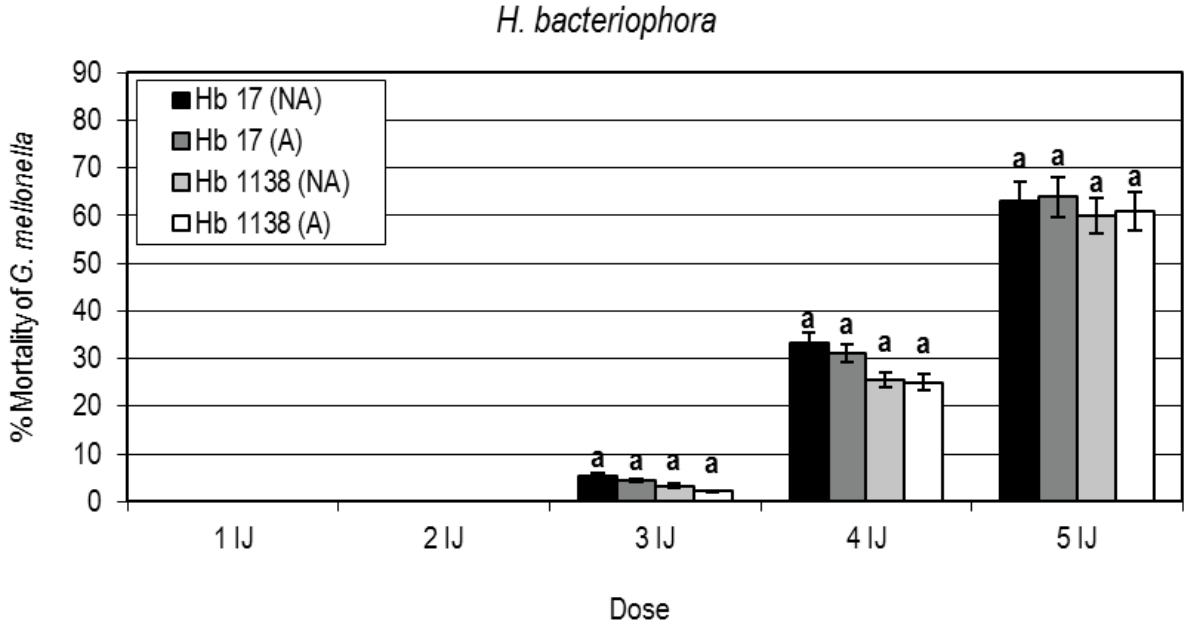

Fig. 2. Effectiveness to *G. mellonella* larvae comparison between heat adapted and non-heat adapted populations of *H. bacteriophora* (NA = Non-Adapted, A = Adapted). For each dose, means followed by the same letter are not statistically different ($p > 0.05$)

strain to heat after heat adaptation. The mortality of the larvae of *G. mellonella* increased with the dose, and significant differences were found between the adapted and non-adapted strains of *Steinernema feltiae* (TUR-S3) and *S. carpocapsae* (TUR-S4). At doses of 2, 4 and 5 IJ/larva the heat-adapted populations of the *Steinernema* species were more effective than the non-adapted populations (Fig. 1). For the *Heterorhabditis bacteriophora* strains, trials, EPN cultures were reared in vivo and in vitro for 7 cycles with exposure to heat. Afterward, the EPNs reproduced in vivo and in vitro for 15 cycles without any selection pressure. In order to determine the effectiveness of the EPN cultures, 1200 IJ from each culture were applied to the center of glass petri dishes in 1 ml of water, and 40 mealworms (*Tenebrio molitor*) were added. The results of this study indicated that the mean mortality of the

commercial EN01 strain (70 %) and different subcultures of the D4 strain (in vivo and in vitro cultures reared under selection pressure) (> 70 %) were significantly greater than the untreated controls. It was also found that the mortality rate of the culture reared in vivo for 15 cycles without selection pressure was not significantly different from untreated cultures. These findings are in accordance with the present study, which its results showed that the heat treated populations of *Steinernema* spp. had greater mortality than untreated populations.

A similar study was performed by Mukuka *et al*. (2010b). The aim of this study was to determine the effect of temperature and desiccation stress on the infectivity of hybrid strains. Strains were exposed to 40 °C for heat tolerance and 0.85 a_w for desiccation tolerance prior to inoculation on *G. mellonella* at a dose of 5 IJ per larva. The results showed that exposure to desiccation significantly reduced the mortality of the hybrids (below 25 %). Untreated hybrids had a mean infectivity of approximately 54 %. In contrast, the heat-tolerant hybrids had higher infectivity, with a mean mortality of 75.4 %. The effect of low temperature on infectivity was also investigated; however, no relation was found between these traits. Even though the populations that exposured to desiccation had lower mortality; the populations that exposured to heat had greater effectiveness than untreated populations, which obtained similarly as in the present study.

Another study by Mukuka *et al*. (2010c) analyzed the fitness of heat- and desiccation-tolerant hybrid strains of the species *Heterorhabditis bacteriophora*. The virulence of the hybrids was calculated by counting the dead-alive *G. mellonella* larvae that were inoculated with 2 IJs each of a hybrid strain. As in the present study, tests were performed in 24 well-plates with sterile sand (with water at 10 % w/w). It was found that virulence in the two heat-tolerant hybrid strains HH1 (75.7 %) and HA1 (71.3 %) were significantly greater than the commercial hybrid strain EN01 (51.5 %), which showed the lowest heat tolerance. Their results are not similar with the present study, which treated and untreated populations of *H. bacteriophora* had no significant differences in virulence. However, the present results for *Steinernema* spp. in the study are in harmony with their findings.

This study plays a key role in the use of improved EPNs by hybridization in biological control systems. The results of the present study are expected to provide important support for sustainable biocontrol experiments using EPNs. However, further studies should investigate beneficial traits, such as heat, desiccation, and effectiveness.

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