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Original article (Orijinal araştırma)

Physiological reactions of some entomopathogenic nematodes to long-term storage

Bazı entomopatojen nematodların uzun süreli depolamaya fizyolojik tepkileri

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Abstract

Entomopathogenic nematodes (EPNs) are commonly used for pest control. Determining the optimal storage duration for EPNs is crucial for their effective utilization. The aim of this study is to determine the efficacy and reproductive capacities of some EPNs stored for different durations. *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH Hybrid Strain, HBNL, and HB4 isolates, as well as *Steinernema feltiae* Weiser, 1955 (Rhabditida: Steinernematidae) SADIÇ and ST5 isolates, were used in the study. The Infective Juveniles (IJs) stored at 4ºC for 6, 12, 18, and 24 months were assessed for their efficacy and reproductive capacities on last instar larvae of *Galleria mellonella* L., 1758 (Lepidoptera: Pyralidae) at the end of the periods. This study was conducted at Bursa Uludağ University, Plant Protection Department, Nematology Laboratory. The highest mortality rate observed on *G. mellonella* larvae was 86.67% on the *H. bacteriophora* HBH Hybrid Strain stored for 6 months. Similarly, the highest reproductive capacity was determined to be 153 000 IJs/*G. mellonella* larva, also on the *H. bacteriophora* HBH Hybrid Strain stored for 6 months. This study showed significant results in determining the effects of storage durations on the efficacy and reproductive capacity of the EPNs.

Keywords: *Heterorhabditis bacteriophora,* reproductive capacity, *Steinernema feltiae*, storage duration

Öz

Entomopatojen nematodlar (EPN) zararlıların mücadelesinde yaygınlıkla kullanılmaktadır. EPN'lerin özellikle depolama süresinin uzunluğunun belirlenmesi EPN'lerin etkili bir şekilde kullanılması açısından önemlidir. Bu çalışmanın amacı, farklı süreler boyunca depolanmış olan bazı EPN'lerin stok süreleri sonunda etkinlikleri ve üreme kapasitelerinin belirlenmesidir. Çalışmada *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH Hibrit Irkı, HBNL ve HB4 izolatı ve *Steinernema feltiae* Weiser, 1955 (Rhabditida: Steinernematidae) SADIÇ ve ST5 izolatları kullanılmıştır. 4ºC'de 6, 12, 18 ve 24 ay boyunca inkübe edilmiş olan infektif jüvenillerin (IJs) belirtilen süreler sonunda *Galleria mellonella* L., 1758 (Lepidoptera: Pyralidae) son dönem larvaları üzerinde etkinlikleri ve üreme güçleri belirlenmiştir. Bu çalışma 2024 yılında Bursa Uludağ Üniversitesi, Bitki Koruma Bölümü, Nematoloji Laboratuvarında gerçekleştirilmiştir. Sonuç olarak, *G. mellonella* larvaları üzerinde görülen en yüksek ölüm oranı 6 ay depolanmış olan *H. bacteriophora* HBH Hibrit Irkında %86.67 olarak belirlenmiştir. En yüksek üreme kapasitesi de aynı şekilde 6 ay depolanmış olan *H. bacteriophora* HBH Hibrit Irkında 153 000 IJs/ *G. mellonella* larva şeklinde belirlenmiştir. Bu çalışma, EPN'nin depolanma süresinin EPN etkinliği ve üreme kapasitesi üzerindeki etkilerinin belirlenmesi açısından önemli sonuçlar taşımaktadır.

Anahtar sözcükler: *Heterorhabditis bacteriophora,* üreme kapasitesi, *Steinernema feltiae*, depolama süresi

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Introduction

With the recent regulations implemented by the European Union, restrictions have been imposed on the use of pesticides in areas where agricultural production takes place, due to the toxic effects of pesticides on non-target organisms (Jess et al., 2014; Lechelet et al., 2017; Marchand, 2023; Yang et al., 2024). This situation has brought other control methods such as biological control to the forefront in the potential pest managements (Bale et al., 2008; Ulu et al., 2016; Filgueiras et al., 2023).

Entomopathogenic nematodes (EPNs) are widely used in agricultural fields for pest control within the scope of biological control (Gaugler, 1988; Ehlers, 1996; Campos-Herrera et al., 2012; Lacey et al., 2015; Baker et al., 2020; Ulu & Erdoğan, 2023). These EPNs, belonging to the families Heterorhabditidae and Steinernematidae of the class Secernentea, are endoparasitic organisms that require a host to complete their life cycle and spend almost their entire lives underground in the soil (Kaya & Koppenhöfer, 1996; Ehlers, 2001; Susurluk, 2008; Dillman & Sternberg, 2012). When their life cycle is examined, the biological stages consist of egg, juvenile 1, juvenile 2, 3 juvenile (Infective Juvenile), juvenile 4, and adults. Additionally, only during the Infective Juvenile (IJ) stage, these organisms possess the ability to infect their hosts (Lewis et al., 2006; Shapiro-Ilan et al., 2006; Susurluk & Ehlers, 2008; Koppenhöfer et al., 2020; Dede et al., 2022). They can locate their hosts by following various volatile compounds emitted by either other nematodes or the hosts themselves (Erdogan et al., 2021; Stevens et al., 2023). Upon encountering their hosts, they enter the host organism through natural openings such as the mouth or anus (Kaya & Koppenhöfer, 1996; Ehlers, 2001; Vashisth et al., 2013; Tarasco et al., 2023; Susurluk & Bütüner, 2024). Once inside the host organism, EPNs release gram-negative bacteria belonging to the Enterobacteriaceae family, with which they live symbiotically, into the host. Following this, the host typically succumbs to septicemia and dies approximately 24-72 hours later. Members of the Heterorhabditidae family carry *Photorhabdus* spp. in a dispersed state within their bodies symbiotically, whereas members of the Steinernematidae family are in a symbiotic relationship with *Xenorhabdus* spp. (Gaugler et al., 1992; Ciche et al., 2006; Ulu & Susurluk, 2014; Şahin et al., 2018; Bütüner et al., 2024; Ulu & Susurluk, 2024).

With the determination of the high efficacy of EPNs in pest control, research efforts have become crucial in identifying the conditions required for the mass production and long-term shelf life of EPNs (Ehlers, 2001; Gaugler, 2002; Sharma et al., 2011; Maru et al., 2016). Various factors such as temperature, humidity, heat, and light play significant roles in determining the shelf life of EPNs. Additionally, studies have suggested that the shelf life of EPNs may have an impact on their physiological characteristics, such as their effectiveness and reproductive capacity on hosts (Ulu & Susurluk, 2014; Susurluk & Ulu, 2015; Bütüner et al., 2023; Bütüner & Susurluk, 2023; Ulu, 2023).

The aim of this study is to determine the reproductive capacity and efficacy of different isolates including *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH Hybrid Strain, HBNL, and HB4 isolates, as well as *Steinernema feltiae* Weiser, 1955 (Rhabditida: Steinernematidae) SADIÇ and ST5 isolates, stored for various durations.

Materials and Methods

Entomopathogenic Nematode Species

In this study, the patented (TPMK Patent No: TR 2013 06141 B) hybrid strain HBH of *H. bacteriophora*, along with two different isolates, HBNL and HB4, and the isolates of *S. feltiae*, SADIÇ and ST5, were used. The hybrid strain and isolates were harvested using last instar larvae (6th instar) of *Galleria mellonella* L., 1758 (Lepidoptera: Pyralidae), then stored at 4ºC for 6, 12, 18, and 24 months until use. Subsequently, the reproductive capacity and efficacy of the EPNs stored for different durations were determined using last instar larvae of *G. mellonella*.

Experimental design

In the study, the hybrid strain and isolates were stored in 250 ml culture containers with filter capped, containing 50 ml of Ringer solution (Ringer, 1882) with a capacity of 1 000±20 IJs. The EPNs were maintained at 4ºC to preserve their viability and activity. Last instar larvae (6th instar) of *G. mellonella* were used to determine the reproductive capacity and efficacy of the EPNs. After placing the larvae into 24-well tissue culture plates at a depth of 3-3.5 cm and a diameter of 1.5-2 cm, the plates were covered with 10% moist alluvial soil and inoculated with EPNs.

Determination of efficacy

After placing the larvae into 24-well tissue culture plates at a depth of 3-3.5 cm and a diameter of 1.5-2 cm, the plates were covered with 10% moist alluvial soil, and EPNs stored at 4ºC for 6, 12, 18, and 24 months were inoculated in the form of 20 IJs. The tissue culture plates were then incubated at 25ºC for 3 days after inoculation to ensure accurate results, with a low dose of IJs determined for inoculation. Subsequently, infected and dead larvae were identified to determine the efficacy of EPNs. Moreover, we carefully dissected the dead larvae to determine whether their death was caused by the EPNs or other factors. This process was repeated three times, with 10 larvae used in each repetition. As a control, newly harvested infective juveniles (IJs), aged 3-4-day, and stored at 4ºC, were utilized.

Determination of reproduction capacity

The EPNs were stored at 4ºC for the specified durations (6, 12, 18, and 24 months), and their reproductive capacities on *G. mellonella* were determined. The research was conducted as follows. Initially, EPNs used in the study were inoculated into 24-well tissue culture plates containing *G. mellonella* larvae at the 6th instar stage. Infected *G. mellonella* larvae were then transferred to a White Trap (White, 1927), and after 10-12 days, their reproductive capacity was determined. The reproductive capacity was evaluated using last instar *G. mellonella* larvae with a length of approximately 1.5-2 cm and an average weight of approximately 280±20 mg. The number of IJs obtained was determined as the total number obtained from these larvae. This process was repeated ten times, with one larva used in each repetition. As a control, newly harvested 3-4-day old IJs kept at 4ºC was used.

Statistical analyses

Efficiency and reproductive capacity were analyzed by analysis of variance (ANOVA) using JMP® Pro 16.0.1 software, followed by Student-T test using the least significant difference (LSD) test ($p < 0.05$) to determine mean differences.

Results

Efficiency of *H. bacteriophora* HBH hybrid strain

The highest mortality rate exhibited by the HBH hybrid strain on *G. mellonella* larvae was observed in larvae treated with IJs stored at 4ºC for 6 months. This value was obtained as 86.67%. These values were determined as 33.33, 43.33, and 73.33%, respectively, in individuals treated with IJs stored at 4ºC for 24, 18, and 12 months. In the control group, this rate was obtained as 96.67%. Statistically significant differences we found among the results (Table 1).

Efficiency of *H. bacteriophora* HBNL Isolate

The HBNL isolate demonstrated the highest mortality rate of 83.33% on *G. mellonella* larvae when treated with IJs stored at 4ºC for 6 months. These values were determined as 26.67, 46.67, and 70%, respectively, in individuals treated with IJs stored at 4ºC for 24, 18, and 12 months. In the control group, this rate was obtained as 93.33%. Statistically significant differences were observed among the results (Table 1).

Efficiency of *H. bacteriophora* HB4 Isolate

The HB4 isolate showed the highest mortality rate of 83.33% on *G. mellonella* larvae when treated with IJs stored at 4ºC for 6 months. These values were determined as 23.33, 40, and 70%, respectively, in individuals treated with IJs stored at 4ºC for 24, 18, and 12 months. In the control group, where IJs were applied to larvae, this rate was obtained as 90%. Statistically significant differences were detected among the results (Table 1).

Efficiency of *S. feltiae* SADIÇ Isolate

The SADIÇ isolate exhibited the highest mortality rate of 73.33% on *G. mellonella* larvae when treated with IJs stored at 4ºC for 6 months. These values were determined as 26.67, 43.33, and 66.67%, respectively, in individuals treated with IJs stored at 4ºC for 24, 18, and 12 months. In the control group, where IJs were applied to larvae, this rate was obtained as 83.33%. Statistically significant differences were found among the results (Table 1).

Efficiency of *S. feltiae* ST5 Isolate

The highest mortality rate observed on *G. mellonella* larvae with the ST5 isolate was determined as 70% in larvae treated with IJs stored at 4ºC for 6 months. These values were determined as 26.67, 40, and 63.33%, respectively, in individuals treated with IJs stored at 4ºC for 24, 18, and 12 months. In the control group, where IJs were applied to larvae, this rate was obtained as 83.33%. Statistically significant differences were seen among the results (Table 1).

Table 1 Mortality of *Galleria mellonella* larvae that were treated with EPN was analyzed separately

EPN Species	Time (Month)	Mortality Rate ± S.E.*	F (df); p
Heterorhabditis bacteriophora HBH Hybrid Strain	Control	96.67 ± 3.33 a	F (4, 10) = 42.18; p <0.0001
	6	86.67 ± 3.33 a	
	12	73.33 ± 3.33 b	
	18	43.33 ± 6.67 c	
	24	33.33 ± 3.33 c	
Heterorhabditis bacteriophora HBNL Isolate	Control	93.33 ± 3.33 a	F (4, 10) = 33.35; p <0.0001
	6	83.33 ± 3.33 ab	
	12	70.00 ± 5.77 b	
	18	46.67 ± 6.67 c	
	24	26.67 ± 3.33 d	
Heterorhabditis bacteriophora HB4 Isolate	Control	90.00 ± 5.77 a	F (4, 10) = 20; p <0.0001
	6	83.33 ± 3.33 ab	
	12	70.00 ± 5.77 b	
	18	40.00 ± 10.00 c	
	24	23.33 ± 3.33 c	
Steinernema feltiae SADIÇ Isolate	Control	83.33 ± 3.33 a	F (4, 10) = 14.20; p =0.0004
	6	73.33 ± 6.67 a	
	12	66.67 ± 8.82 a	
	18	43.33 ± 6.67 b	
	24	26.67 ± 3.33 b	
Steinernema feltiae ST5 Isolate	Control	83.33 ± 3.33 a	F (4, 10) = 15.83; p =0.0003
	6	70.00 ± 5.77 ab	
	12	63.33 ± 8.82 b	
	18	40.00 ± 5.77 c	
	24	26.67 ± 3.33 c	

* Means in columns followed by the same letters are not significantly different.

The reproductive capacity of *H. bacteriophora* HBH hybrid strain

The IJs of the HBH hybrid strain were stored at 4ºC for the specified months. Subsequently, the reproductive capacities of the individuals were evaluated on last instar *G. mellonella* larvae. According to the results obtained, the highest reproductive capacity was observed in individuals stored at 4ºC for 6 months. This value was determined as 146 500 IJs per *G. mellonella* larva. These values were determined as 51 500, 52 500, and 107 000 IJs respectively, for IJs stored at 4ºC for 24, 18, and 12 months. When examining the reproductive capacity of the infective juveniles (IJs) in the control group, which consisted of newly harvested IJs aged 3-4 days and stored at 4ºC, this value was determined as 153 000 IJs. Statistically significant differences were obtained among the results (Table 2).

Table 2 The reproductive capacity of EPNs were obtained from the incubation of IJs at the specified months

EPN Species	Time (Month)	Reproductive Capacity IJs/ *G. mellonella* Larva ± S.E.*	F (df); p
Heterorhabditis bacteriophora HBH Hybrid Strain	Control	153 000 ± 4 027.68 a	F (4, 45) = 78.10; p <0.0001
	6	146 500 ± 4 475.24 a	
	12	107 000 ± 8 793.94 b	
	18	52 500 ± 4 297.93 c	
	24	51 500 ± 4 657.73 c	
Heterorhabditis bacteriophora HBNL Isolate	Control	149 000 ± 4 459.69 a	F (4, 45) = 90.24; p <0.0001
	6	142 500 ± 7 001.98 a	
	12	103 500 ± 5 002.77 b	
	18	51 500 ± 3 655.28 c	
	24	47 500 ± 4 669.64 c	
Heterorhabditis bacteriophora HB4 Isolate	Control	143 500 ± 3 419.71 a	F (4, 45) = 119.24; p <0.0001
	6	118 500 ± 4 412.73 b	
	12	64 500 ± 4 044.89 c	
	18	53 500 ± 5 273.10 c	
	24	36 000 ± 3 480.10 d	
Steinernema feltiae SADIÇ Isolate	Control	137 500 ± 3 670.45 a	F (4, 45) = 145.53; p <0.0001
	6	127 500 ± 6 247.22 a	
	12	69 000 ± 4 000.00 b	
	18	38 000 ± 3 511.88 c	
	24	27 500 ± 2 608.74 c	
Steinernema feltiae ST5 Isolate	Control	142 000 ± 3 091.21 a	F (4, 45) = 148.66; p <0.0001
	6	132 500 ± 4 549.11 a	
	12	79 000 ± 6 046.12 b	
	18	48 000 ± 3 958.11 c	
	24	22 500 ± 2 910.71 d	

* Means in columns followed by the same letters are not significantly different.

The reproductive capacity of *H. bacteriophora* HBNL isolate

According to the results obtained for the individuals of the HBNL isolate, the highest reproductive capacity was observed in individuals stored at 4ºC for 6 months. This value was determined as 142 500 IJs per *G. mellonella* larva. These values were determined as 47 500, 51 500, and 103 500 IJs, respectively, for IJs stored at 4ºC for 24, 18, and 12 months. When the reproductive capacity obtained from the IJs in the control group was examined, this value was determined as 149 000 IJs. Statistically significant differences were obtained among the results (Table 2).

The reproductive capacity of *H. bacteriophora* HB4 isolate

For the individuals of the HB4 isolate, the highest reproductive capacity was observed in individuals stored at 4ºC for 6 months. This value was determined as 118 500 IJs per *G. mellonella* larva. These values were determined as 36 000, 53 500, and 64 500 IJs, respectively, for IJs stored at 4ºC for 24, 18, and 12 months. When the reproductive capacity obtained from the IJs in the control group was examined, this value was determined as 143 500 IJs. Statistically significant differences were obtained among the results (Table 2).

The reproductive capacity of *S. feltiae* SADIÇ isolate

For the individuals of the SADIÇ isolate, the highest reproductive capacity was observed in individuals stored at 4ºC for 6 months. This value was determined as 127 500 IJs per *G. mellonella* larva. These values were determined as 27 500, 38 000, and 69 000 IJs, respectively, for IJs stored at 4ºC for 24, 18, and 12 months. When the reproductive capacity obtained from the IJs in the control group was examined, this value was determined as 137 500 IJs. Statistically significant differences were seen among the results (Table 2).

The reproductive capacity of *S. feltiae* ST5 isolate

For the individuals of the ST5 isolate, the highest reproductive capacity was observed in individuals stored at 4ºC for 6 months. This value was determined as 132 500 IJs per *G. mellonella* larva. These values were determined as 22 500, 48 000, and 79 000 IJs, respectively, for IJs stored at 4ºC for 24, 18, and 12 months. When the reproductive capacity obtained from the IJs in the control group was examined, this value was determined as 142 000 IJs. Statistically significant differences were observed among the results (Table 2).

In this study, the efficacy of *H. bacteriophora* HBH Hybrid Strain, HBNL and HB4 isolates, as well as *S. feltiae* SADIÇ and ST5 isolates, was examined on *G. mellonella* larvae following storage for varying durations. The results revealed a decrease in efficacy of EPNs with prolonged storage periods. For instance, the highest mortality rate exhibited by *H. bacteriophora* HBH Hybrid Strain on *G. mellonella* larvae was observed at 96.67% when applied with IJs stored for 6 months, whereas this rate decreased to 33.33% when applied with IJs stored for 24 months. Furthermore, the reproductive abilities of the specified EPN isolates were determined after storage for the indicated months. Similarly, an increase in storage duration led to a decrease in reproductive capacity for all EPN isolates and the hybrid strain. Likewise, the highest reproductive rate for *H. bacteriophora* HBH Hybrid Strain was determined to be 153 000 individuals in specimens stored for 6 months, whereas this value decreased to 51 500 individuals in specimens stored for 24 months. These findings underscore the potential of the EPN isolates used in the study to maintain their efficacy and reproductive capacities over time.

Discussion

This study tested the effectiveness and reproductive capacity of *H. bacteriophora* HBH Hybrid Strain, HBNL and HB4 isolates, as well as *S. feltiae* SADIÇ and ST5 isolates, on *G. mellonella* larvae after varied storage periods. The findings demonstrated that EPN effectiveness decreased with longer storage.

After being stored under indicated conditions for specified months, the efficacy, and reproductive capacities of EPNs were investigated on *G. mellonella* larvae. Additionally, the use of different isolates in this study further strengthens the findings. For instance, in the study conducted by Boff et al. (2000), isolates belonging to the species *Heterorhabditis megidis* Poinar, Jackson & Klein, 1987 (Rhabditida: Heterorhabditidae) were stored for up to 70 days at different temperatures, followed by an evaluation of the reproductive capacity and efficacy of these isolates. Upon examination of the results, it was observed that an increase in storage duration led to a decrease in the reproductive capacity and efficacy of the isolates. Similarly, in their study, Sharmila & Subramanian (2016) stored isolates belonging to the species of *Heterorhabditis* and *Steinernema* at different temperatures for 100 days, followed by an assessment of the isolates' efficacy on *Corcyra cephalonica* (Stainton, 1866) (Lepidoptera: Pyralidae) larvae. The results indicated a decrease in efficacy with an increase in storage duration. In the study conducted by Katti et al. (2006), isolates belonging to the species *Oscheius* sp. Andrássy, 1976 (Nematoda: Rhabditidae) and *Steinernema thermophilum* Sudershan & Singh, 2000 (Rhabditida: Steinernematidae) were maintained at room temperature for durations ranging from 5 to 150 days. Subsequently, the efficacy of these isolates was assessed on *G. mellonella* and *C. cephalonica* larvae. The results indicated a decrease in efficacy of the isolates with an increase in storage duration. Thus, there is consistency between the results obtained from the present study and those from previous studies.

In another study conducted by Bütüner et al. (2023), various EPN isolates were stored at 4, 15, 25, and 35ºC for 7, 14, and 21 days. Subsequently, their efficacy was determined on *Tenebrio molitor* L., 1758 (Coleoptera: Tenebrionidae). The results indicated a decrease in efficacy of the isolates with an increase in storage duration for each temperature. Similarly, in the study by Akı et al. (2023), isolates belonging to *Heterorhabditis* and *Steinernema* species were stored in distilled water, tap water, and Ringer's solution at different temperatures and durations. Examination of the results revealed a decrease in efficacy of the isolates with an increase in storage duration. Likewise, in the study conducted by Bütüner & Susurluk (2023), different EPN isolates were stored for 7, 14, and 21 days at 15, 25, and 35ºC, and their reproductive capacities were evaluated. The results indicated a decrease in reproductive capacity with an increase in storage duration for all temperatures. Thus, there is consistency between the results of the current study and previous studies.

According to the results obtained from the study, it has been revealed that long-term storage has adverse effects on the efficacy and reproductive capacities of IJs. While studies in this field generally cover different temperatures, this study examines the effects of long-term storage of EPN isolates at the recommended temperature on the efficacy and reproductive capacities of EPNs. Thus, this study will serve as a reference for future studies or ongoing research in this area.

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