Evaluación de Persistencia e Infección de Nematodos Entomopatógenos, Steinernema feltiae & Heterorhabditis bacteriophora, en menta

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RESUMEN

Los nematodos entomopatógenos se aplican como agentes de control biológico de plagas de insectos que habitan en el suelo, como los gusanos blancos o ciertas larvas, en diversos sistemas agrícolas y pueden ser eficaces solos o como complemento de los insecticidas aplicados al suelo. Sin embargo, no está claro cuánto tiempo estos agentes de control biológico pueden persistir en el suelo y cómo su eficacia contra las plagas de insectos que habitan en el suelo cambia a lo largo del tiempo. El objetivo de este experimento es evaluar la supervivencia (persistencia) y la infectividad de dos especies de nematodos entomopatógenos, ***Steinernema feltiae*** (Sf), y ***Heterorhabditis bacteriophora*** (Hb), solos y en combinación en macetas de menta durante un periodo de dos semanas, recogiendo muestras de suelo y cebándolas con gusanos de la cera bajo condiciones de laboratorio. El tratamiento que consistió únicamente en *S. feltiae* mostró la mejor persistencia e infección de las larvas de cera a lo largo del experimento, teniendo un buen comportamiento en la recuperación de la población tras la adaptación a las condiciones del medio de la maceta, y teniendo un incremento en la infección hasta alcanzar un porcentaje de infección estable al final del experimento, siendo este un comportamiento muy diferente al de H. *bacteriophora* que no estuvo presente al final del experimento en ninguno de los dos tratamientos en los que se aplicó.

PALABRAS CLAVE

Control biológico, Nematodos entomopatógenos, Persistencia, Infección, Galleria mellonella

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OBJETIVO GENERAL

Evaluar la persistencia y la infección de *Steinernema feltiae* & *Heterorhabditis bacteriophora* en macetas de menta bajo condiciones de invernadero.

OBJETIVOS ESPECIFICOS

- Ejecutar el protocolo de reproducción de los nematodos entomopatógenos bajo condiciones de laboratorio.
- Tomar muestras de suelo en las macetas donde los nematodos fueron previamente aplicados.
- Infectar larvas de gusano de cera (*Galleria mellonella*) con nematodos para evaluar las variables seleccionadas.
- Determinar la persistencia e infección de las especies de nematodos entomopatógenos a lo largo de un periodo de dos semanas de evaluación en las macetas de menta bajo invernadero.

Evaluation of the persistence of entomopathogenic nematodes, *Steinernema feltiae* and *Heterorhabditis bacteriophora*, in mint pots

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ABSTRACT

Entomopathogenic nematodes are applied as biological control agents of soil-dwelling insect pests, like white grubs or caterpillars, in a variety of agricultural systems and can be effective alone or as a complement to soil-applied insecticides. However, it is unclear how long these biological control agents may persist in the soil and how their efficacy against soil-dwelling insect pests changes over time. The goal of this experiment was to evaluate the survival (persistence) and infectivity of two species of entomopathogenic nematodes, *Steinernema feltiae (Sf)*, *and Heterorhabditis bacteriophora (Hb)*, alone or in combination in mint pots over a two-week period by collecting soil samples and baiting them with waxworms under laboratory conditions. The treatment that consisted only of *S. feltiae* showed the best persistence over 14 days. With regards to infection of the waxworm larvae by each entomopathogenic nematode species throughout the experiment, we found a significant time by species interaction, such that *H. bacteriophora* showed the highest percent infection up to 7 days after treatment, while *S. feltiae* showed the highest percent infection 14 days after treatment.

KEYWORDS

Biological control, Infectivity, *Galleria mellonella*

INTRODUCTION

Entomopathogenic nematodes (EPNs) are beneficial roundworms which have the potential to control several soil-dwelling insect pests that cause significant economic damage in various agricultural crops. Although EPNs are natural members of the soil biota, understanding their ecology and behavior is difficult given their microscopic size and exclusive survival in the soil (Brown & Gange, 1990; Fierer, Strickland, Liptzin, Bradford, & Cleveland, 2009). However, the application of EPNs has become a common integrated pest management (IPM) strategy, due to their ability to attack pest insects in the soil that may be difficult to contact with insecticides, making them a safer and potentially more sustainable pest management strategy. Yet, to maintain pest populations below damaging levels, it is necessary to understand how long EPNs survive and persist under different biotic conditions, which also has implications for their long-term efficacy and the likelihood that farmers will adopt them as a sustainable IPM strategy.

Entomopathogenic nematodes have a simple life cycle, which consists of an egg stage that hatches into the second juvenile (J2) stage, and then each sequent juvenile stage feeds and molts through four juvenile stages (J4 stage), which finally transforms to the adult stage. The infective stage

varies according to the group, but in the case of the species used in the study (steinernematids and heterorhabditids) the third juvenile stage (J3) is the infective juvenile (IJ) stage that has the capacity to attack pest insects (Kaya & Stock, 1997). The life cycle of EPNs from host penetration of IJs to emergence is completed within 6–11 days for Steinernematids and 12–14 days for Heterorhabditids (Kaya, 1999). The ability of EPNs to kill soil-dwelling insects is linked to their association with bacteria in the genera *Xenorhabdus* and *Photorhabdus* in the ventricular portion of its intestine (Boemare, 2002). The infection process occurs after IJs locate a susceptible host and enter through natural openings in the insect body, such as the mouth, anus, or spiracles, or by penetrating the cuticle. Once they enter the body of the host, the IJs begin ingesting hemolymph, which triggers the release of symbionts, either by defecation in Steinernema *spp*., or regurgitation in *Heterorhabditis spp*. (Grewal et al. 2005). The bacteria disseminate quickly and break down the host's tissues, converting the insect cadaver into an effective growth base for EPNs. The bacteria and the degrading tissues of the host cadaver serve as food sources for nematode growth and reproduction (Boemare, 2002). The entomopathogenic nematode–bacteria complex kills the host within 24–48 hours (about 2 days) throughhemolymph poisoning by the bacterial toxins in the insect hemocele , also known as septicimia or toxemia (Forst and Clarke 2002).

Many EPN species have been tested as a complement or alternative to synthetic insecticides to manage soil insect pests in agriculture, but species belonging to the genera *Steinernema* and *Heterorhabditis* in particular, have been proven to be effective as biological control agents of insect pests that are seriously damaging in many crops, such as *Spodoptera litura (*Yan et al., 2020*), Popillia japonica (*Graser & Farrell, 1935*) and Melolontha melolontha (*Skowronek et al., 2020*).* However, despite their known efficacy, there are obstacles to using EPNs in commercial pest control, including limited shelf life (Strauch, Oestergaard, Hollmer, & Ehlers, 2004), and requirements for moist, loose-textured soil environments. Unfavorable environmental conditions can decrease EPN survival and activity as biological control agents once they are released into the field environment (Glazer, 2015). Therefore, knowledge of their persistence in the soil under optimal conditions will allow for the development of release strategies that maximize EPN effectiveness over time and across diverse agricultural environments. Entomopathogenic nematodes can survive unfavorable environmental conditions, such as lack of water, extreme temperatures, lack of oxygen, and osmotic stress. For example, it is already known that EPNs can persist for 2–3 weeks in dry soil (Kaya, 1990; Kung & Gaugler, 1990), but under ideal conditions, individual IJs can survive for months but become visibly lighter as lipid reserves are used up, and eventually die of starvation when they do not have a host to infect (Fitters & Griffin, 2006; Hass, Downes, & Griffin, 2002; Patel et al., 1997).

Study System

One important, soil-dwelling insect pest that may be targeted with EPNs is *Maladera castanea* (Arrow) (Coleoptera: Scarabaeidae), better known as the Asiatic Garden Beetle (AGB). Asiatic garden beetle attacks over 100 species of plants such as cut-flowers plants (roses, aster, chrysanthemum, dahlia), ailanthus, and some fruits and vegetables crops such as peach, cherry, common carrot, red pepper, and radish (Hawley & Hallock, 1936). It causes economic damage during both the adult and larval stages. As adults, they feed on leaves, occasionally causing severe

damage when present in large numbers. In the immature (larval) stage, AGB feeds on roots, causing delayed development in ornamentals, turf, gardens, sweet potatoes, soybeans, corn (Skelley 2013), and other field crops, including peppermint, particularly in north central Indiana.

Peppermint is grown as a perennial crop that remains in the field for 3-5 years in Indiana, as part of a rotation with corn and soybean (Gumz, 2007). Mint production in the United States is concentrated around the Pacific Northwest and Midwest, and in 2020, Indiana producers harvested 2,270 ha of peppermint (USDA-NASS 2021). Indiana ranks 3rd and 4th in the nation for spearmint and peppermint oil production, respectively, so this crop contributes significantly to the state's economy (USDA-NASS 2020). Because AGB has emerged as a novel pest of mint in the state, farmers need alternative and sustainable strategies to manage this damaging pest.

The objective of this study was to evaluate the efficacy and persistence of *S. feltiae* (Sf) and *H. bacteriophora* (Hb), alone and in combination when applied to peppermint grown in the greenhouse in sandy soil over a two-week period. This project aims to enhance our understanding of the persistence of EPNs as a potential biological control strategy for AGB in sandy soils where mint crops are grown in Indiana. We predicted that the combined treatment of both Sf + Hb EPNs would provide better infection than either of the individual EPN treatments alone. The results of this study will help provide research-based recommendations about EPNs as biological control agents for pests like AGB and potentially help farmers reduce dependence on chemical control methods.

Materials and Methods

Peppermint plants

This experiment was conducted in the Entomology Environmental Laboratory (EEL) greenhouse at Purdue University in West Lafayette. For the experiments, 1-liter pots (15x15x11cm) were filled with a 2:1 sand-soil mixture to a height of 8 cm and planted with a single 10 cm (about 3.94 in) stolon of peppermint (*Mentha piperita*) var. 'Black Mitcham'. All pots were watered as needed until the end of the experiment.

The EPN species used in this study were *Steinernema feltiae* (Sf) and *Heterorhabditis bacteriophora* (Hb). Both species were cycled through waxworms, *Galleria mellonella* (L), three times before their use in experiments to gain robust and healthy infective juveniles (IJs). A Galleria based non-white trap rearing system (Testa and Shields 2017) was used to produce IJs for application.

Experimental treatments

This experiment was conducted using a randomized complete block design with four treatments: 1) an untreated check, 2) *Sf* alone 3) *Hb* alone, and 4) an equal mixture of *Sf* + *Hb*. Each treatment consisted of 8 replicates randomly placed inside the mesh insect cages, differentiated by colored pot tags. The application rate of each EPN species was based on the manufacturer's recommendation of 50 million IJs per acre. For the experimental pots for each treatment, 255 IJs were applied.

Mint pots of each treatment were assigned to mesh insect cagesand each mint pot was placed in a circular plastic watering tray (radius 20,5 cm; capacity 900 ml) to avoid potential cross-contamination of EPN species from other treatments via watering.

Either an EPN treatment or water control was applied to the soil surface of every mint pot on November 6, 2024 in dilution with 100 ml of water. Then, the soil from all experimental mint pots was baited for EPNs 1 day, 7 days, 10 days, and 14 days after application. At each sampling date, 3 soil samples were collected from each replicate mint pot (n=8) using a metal soil core sampler in each treatment and combined in a single-gallon-sized Ziploc bag. The three subsamples of soil for each treatment were baited for EPNs by placing 5 *G. mellonella* larvae as indicator hosts in each bag and allowing EPNs four days at room temperature in the dark to infect waxworm larvae. After four days, dead *G. mellonella* were examined for nematode infection by observing the condition and color of the cadaver (Poinar 1984). Cadaver coloration between *S. feltiae* and *H. bacteriophor*a are dark brown and brick red, respectively (Shields et al. 2021). All waxworm cadavers were then placed in White's traps Petri dishes (White 1927) and observed for IJ emergence after 4 days.

Table 1. Dates that entomopathogenic nematodes (EPN), *H. bacteriophora* and *S. feltiae*, were applied to mint pots and soil was baited with waxworm larvae to detect infectivity. DPT = Days post treatment.

EPN Application	1 DPT	7 DPT	10 DPT	14 DPT
Nov. 6, 2023	Nov. 7, 2023	Nov. 13, 2023	Nov. 16, 2023	Nov. 20, 2023

Statistical Analysis

The persistence of EPNs in each experimental treatment (untreated, *Sf* alone, *Hb* alone, and the *Sf* + *Hb* mix) was expressed as the presence of dead waxworms in each replicate soil sample at each time point following EPN inoculation.

EPN infectivity was expressed as the percentage of infected waxworms in each replicate soil sample of each treatment. Significant differences in the percent infection of waxworms between treatments were compared using repeated measures analysis of variance (ANOVA) in SAS.

Results

EPN persistence: Throughout the experiment, EPNs were only detected in treatments inoculated with EPNs; no EPNs were detected in the soil of untreated (control) mint pots.

EPNs were detected until the end of the experiment, but the pattern differed depending on EPN treatment.

Steinernema feltiae: Bioassay results for ***Sf*** indicated that this species was present and persistent in soil samples until 14 days post treatment (DPT). By the final date of the experiment, it was present in two of the three replicate soil samples

.***Heterorhabditis bacteriophora:*** Bioassay results for **Hb** indicated that this species was present and persistent in soil samples until 7 DPT. From 10 DPT to 14 DPT of the experiment, *H. bacteriophora* was not detected in any of the soil samples.

S***teinernema feltiae x Heterorhabditis bacteriophora:*** Bioassay results for the combined EPN treatment indicated that EPNs were present in the soil samples across all 14 days of the study; however, the presence of each species differed over time. *H. bacteriophora* was only present and persistent until 7 DPT, while *S. feltiae* was present and persistent until 14 DPT.

Table 2. Persistence of entomopathogenic nematodes species in soil of mint pots over a 14-day period in the greenhouse.

Days Post Treatment (DPT)	1 DPT			7 DPT			10 DPT			14 DPT		
Treatment	**1 REP**	**2 REP**	**3 REP**	**1 REP**	**2 REP**	**3 REP**	**1 REP**	**2 REP**	**3 REP**	**1 REP**	**1 REP**	**1 REP**
Control	0	0	0	0	0	0	0	0	0	0	0	0
Sf	1	1	1	1	1	1	0	1	1	1	1	0
Hb	1	1	1	1	1	1	0	0	0	0	0	0
Sf x Hb	1 (Sf)	1(Sf/Hb)	1	1(Sf)	1(Sf)	1(Hb)	1(Sf)	0	0	0	0	1(Sf)

Presence represented for 1 and Absence represented for 0

Sf: Steinernema feltiae
Hb: Heterorhabditis bacteriophora
DPT: Days Post Treatment
Rep: Replicate

EPN infectivity: Throughout the experiment, no EPN infectivity was detected in the soil of untreated (control) mint pots. The percent infectivity of waxworms by each EPN treatment varied with time (days post –treatment) ($F_{9,8}=4.56$, $P=0.02$).

Steinernema feltiae: Infectivity by S. *feltiae* was evident throughout all 14 days of the experiment. The average percent infection at 1 DPT was 40%, and decreased to 20% 7 DPT, but it tended to increase again for 10 DPT and 14 DPT to 60% of infection of the waxworms in the White traps that were kept in the laboratory.

Heterorhabditis bacteriophora: Infectivity by *H. bacteriophora* was evident until 7 DPT. At the first date, the percentage of infection in the treatment was of 60%, which decreased considerably to 20% in 7 DPT. After 7 DPT, it did not show infection again in any treatment until 14 DPT.

S***teinernema feltiae x Heterorhabditis bacteriophora:*** Infectivity in the combined EPN treatment was evident across all 14 days of the study, but similarly to the results of EPN persistence, infection was different between each EPN species. *S. feltiae* was evident 14 DPT, while *H. bacteriophora* was evident only until 7 DPT.

The significant differences between treatments occurred between 7 DPT and 10 DPT. However, significative differences were presented comparing the initial infection of *H. bacteriophora* with the infection obtained by *S. feltiae* on the second sampling ($t=4.85$; $df=8$; $P=0.0396$)

Since the second sampling, the infectivity of Hb was significantly different from that obtained initially 1 DPT (t=5.35; df=8; P=0.0228), and the same for the results obtained the third day of sampling (t=5.43; df=8; P=0.0210). In addition, the infectivity of Hb at the end of the experiment is statistically different when compared to at the beginning. (t=5.54; df=8; P=0.0184).

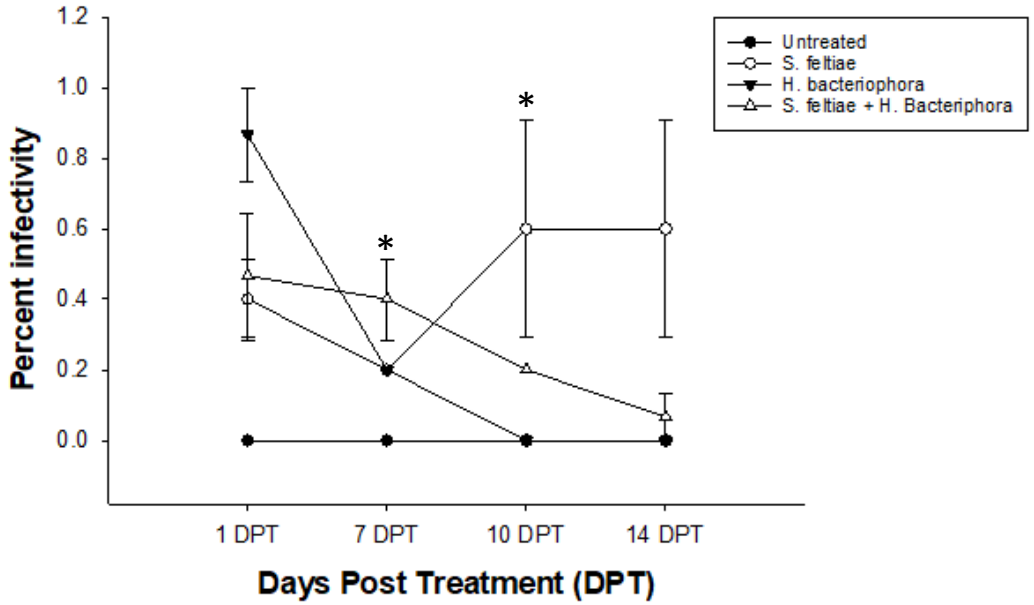

Fig 1. Percent infection of waxworm larvae in four entomopathogenic nematode treatments in the greenhouse after 14 days. The experimental treatments were: 1) untreated control, 2) *S. feltiae* alone, 3) *H. bacteriophora* alone, and 4) combined treatment of *S. feltiae* and *H. bacteriophora*. Asterisks denote significant differences between treatments on each date at α = 0.05 level. (F9,8=4,56 P=0,02)

Discussion

The focus of this study was evaluating the persistence and efficacy (infectivity) of EPNs over time, without insect hosts in the soil.

Although the period of evaluation of EPNs was short relative to the survival and longevity of EPNs under ideal conditions agricultural systems, we found that EPNs are capable of persisting and infecting potential pest insects relatively quickly, so that economic losses might be reduced in crops that may be infested with soil-insect pests. More specifically, *S. feltiae* appeared to be the most effective and persistence EPN species in soil types where mint crops are grown, suggesting it might be a suitable biological control agent for larval AGB, which remain in the soil to depths of 15–30 cm.

Importantly, we only made a single EPN application to all treatments in a standardized manner, and it is possible that repeated sampling of the soil from the pots without re-application of EPNs may have contributed to the reduction in EPN detection and infectivity over time and across treatments

It is possible that we did not detect *H. bacteriophora* throughout the duration of the experiment, even though it was present. Such as that presented by Schroeder et al (1996) has the tendency to move deeper in the soil profile, even deeper than 26 cm within 7 days after application, and in the absence of susceptible hosts, it accumulated at the bottom of the pot. Therefore, future studies may use a different soil sampling methodology, or bait the soil directly in the pot to obtain more accurate results.

Some of the significative differences found between the treatments could be explained by the different origin of each species used in the treatment, in relation to the commercial brand that must be of high quality, and also, that transportation process must comply with adequate compliance with marketing protocols, in addition, these must be passed through a process of adaptation to the new environmental conditions. The quality of the initial generation could affect the subsequent behavior of the populations of each species; however, more information is required about the initial impact on the rest of the populations.

Since the reproduction process of entomopathogenic nematodes, *S. feltiae* showed high activity inside the bodies of waxworms, and the concentration of juveniles per microliter of water was higher compared to *H. bacteriophora*. The importance of choosing a good population before inoculation to the soil guarantees adequate results.

Another interesting study would be to evaluate the influence of variables that influence the survival of EPNs in the soil, such as percent humidity or percent moisture, since mobility under aquatic conditions may differ between EPN species, altering population growth and subsequent potential as a biological control agent.

The changes in the infection percentages of each nematode species were related to the changes in persistence of all treatments, but also, according to the ability that each specie has to move once in the soil after been applied. *H. bacteriophora* tends to have a slower long–distance dispersal after been applied in the soil, also, they have an aggregation pattern that is typical in the natural populations (Campbell et al., 1998; Wilson, Lewis, Yoder, & Gaugler, 2003). This could be the reason to explain why the infectivity 1 DPT of this treatment reached high values comparing to the others treatments, but it may also be the explanation why infection was drastically reduced in subsequent sampling, since by extracting the soil the first time, a large part of the individuals present could be removed, making population recovery more difficult. However, as previously mentioned, this could have moved to the last profile of the pot along the time.

On the other hand, population declines must be considered due to the adaptation of species to the applied environment and the death of individuals due to unfavorable conditions for population reproduction.

When analyzing the treatment that consisted of the combination of the two species of nematodes, it can be said that, since it was a single biological objective, it was evident that the control was predominant by a single species of nematode, *S. feltiae*, since it has been proven that *Steinernema* individuals has the ability to work faster in terms of movement and development than individuals that belong to the genus *Heterorhabditis* (Bal, Michael, & Grewal, 2014; Bal, Taylor, & Grewal, 2014). In this way, although both species could be present in the body of the waxworm larva,

because these two do not compete for space within the host, the symptoms shown of infection may have the predominance of the first EPN species that arrived at the body or the EPN species with the fastest development (life cycle) to kill the waxworm host.

The higher persistence and infectivity of *S. feltiae* in mint pots suggests that this EPN species has potential as a biological control agent for soil insect pests, such as *Maladera castanea*.

Conclusion

Entomopathogenic nematodes are a viable option for biological control of soil-dwelling pest insects in mint production and are able to persist in the soil over short time periods, even without insect hosts. However, more environmental variables, such as soil moisture and soil texture, must be considered to guarantee that they are able to persist and provide adequate biological control services.

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