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The effects of soil moisture, soil texture, and host orientation on the ability of *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) to infect *Galleria mellonella* (Lepidoptera: Pyralidae)

Cover Page Footnote

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The Effects of Soil Moisture, Soil Texture, and Host Orientation on the Ability of *Heterorhabditis bacteriophora* (Rhabditida:Heterorhabditidae) to Infect *Galleria mellonella* (Lepidoptera:Pyralidae)

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Abstract

Entomopathogenic nematodes (EPN) demonstrate potential as a biological control for soil dwelling insects. However, edaphic factors, such as soil moisture and texture impact the ability of EPN to find host. The objectives of this study were to examine the effects of soil texture and moisture on 1) the infection rate of *Galleria mellonella* L. by EPN and; 2) the ability of *Heterorhabditidae bacteriophora* (Poinar) to move through the soil to find a host at different orientations. Soil textures consisted of sand, a sand/silt/peat mixture, and a silt/peat mixture at 50% and 100% moisture. An ANOVA was used to evaluate infection rates and EPN movement. Both soil moisture ($p < 0.05$) and texture ($p < 0.05$) had significant effects on nematodes infection rates of *G. mellonella*. Texture, moisture, and host orientation did not significantly affect ($p > 0.05$) the ability of EPN to find a host. While EPN were able to find a host within a variety of soil types, soils that held more water had higher infection rates than soils that held less water, suggesting that moisture may be a key component in facilitating infection by EPN. By understanding the factors that influence the ability of EPN to find and infect a host, improved bio-control programs using EPN may be developed.

Keywords: Soil moisture, soil texture, host orientation, nematode infection, *Galleria mellonella*

Insect pests are the largest contributor to crop loss and account for 15% reduction in potential crop yields on the world's eight major crops (Yudelman et al. 1998). Losey and Vaughan (2006) estimate these losses to be more than \$18.7 billion dollars annually. Despite an increased use of pesticides, the number of crops lost to pest damage has increased since 1965. This phenomenon has led to an increased interest in identifying biological control agents to add to the arsenal of tools utilized in integrated pest management (Yudelman et al. 1998). Natural enemies, like entomopathogenic nematodes, can control pest populations through parasitism thus regulating the spread and impact of pest species (Hajek 2004). However, in the case of invasive agricultural pest species in novel environments, natural enemies may be absent or scarce (Lockwood et al. 2007). Identifying the potential biological control agents, as well as their presence, abundance, and effectiveness, becomes necessary when considering integrated pest management for invasive agricultural pests (Riudavets

and Castane 1998). With more than 90% of extant insect species having life history stages in the soil, entomopathogenic nematodes show potential for biological control in agricultural systems (Hominick 2002).

Entomopathogenic nematodes (EPN) are found on every continent, except for Antarctica and have been used as biological controls for numerous soil dwelling agricultural pests and invasive species (Peters and Ehlers 1994, Hominick 2002, Lewis et al. 2006, Campos-Herrera et al. 2007). Previous studies have shown that EPN show promise for suppressing Dipteran, Coleopteran, and Lepidopteran agricultural pests (Peters and Ehlers 1994; Lacey and Unruh 1998, Lolla-Luz et al. 2005). EPN infect a host during a free-living infective juvenile (dauer) stage when they crawl into a host via any possible opening such as the mouth, anus, spiracles, and even intersegmental membranes (Bedding and Molyneux 1982, Kaya et al. 1993). Once inside, the infected juveniles release symbiotic enteric bacteria, and the larval host dies via septicemia within 24–48 hours (Molyneux and Bedding 1984, Ansari and Butt 2011). EPN reproduce inside the cadaver for two to three generations before

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leaving the cadaver en masse to seek a new host (Shapiro-Ilan et al. 2006). However, the effectiveness of EPN has varied dramatically from study to study depending on soil characteristics, species, agricultural management, and competition with native EPN (Georgis et al. 2006, Campos-Herrera et al. 2008). One approach to understanding how EPN can be utilized to manage soil dwelling pests is to fully investigate the influence of soil conditions and host orientation on the host seeking success of EPN.

Soil moisture, texture, pH, organic matter, and bulk density may affect the survival and host seeking success of EPN (Gruner et al. 2007). EPN require a thin film of water to navigate through the soil to find a host. The relationship of soil texture and organic matter in determining the porosity and water-holding capacity of a soil is crucial to the survival and efficacy of EPN. If the soil is too dry, there may not be a continuous film of water to allow for nematode movement (Koppenhöfer and Fuzy 2006, Lewis et al. 2006). Concomitantly, interstitial space, the space between soil particles, is essential for providing oxygen and allowing EPN to move through soil (Kung et al. 1990, Koppenhöfer et al. 1995) For example, sand provides interstitial space for movement and oxygen but lacks water retention. On the other hand, clay lacks interstitial space and can easily become anoxic, but has a high capacity for retaining water (Meats 1972, Thien and Gravelle 1997). Therefore, it is possible that a tradeoff between soil texture and soil moisture level may create the ideal substrate for EPN movement and host seeking success. While other studies have examined the effects of different soil textures at the same moisture level, few have compared soil textures at different moisture levels with regards to host seeking success (Molyneux and Bedding 1984, Kung and Gaugler 1991, Koppenhöfer and Fuzy 2006).

Foraging strategies of EPN exist on a continuum from ambush to cruising foragers (Salame and Glazer 2015). *Heterorhabditis bacteriophora* (Poinar) is a cruiser species of EPN that currently can be purchased commercially as biological control agent (Shapiro-Ilan et al. 2002). Compared to other EPN species, *H. bacteriophora* is better adapted to finding more sedentary insects that live deep in the soil profile where the soil is moist (Campbell et al. 1996, Salame and Glazer 2015). However, Heterorhabditid populations have also demonstrated poor desiccation tolerance when compared to Steinernematids, the second major family of EPN (Surrey and Wharton 1995, Liu and Glazer 2000). To escape desiccation in drier soils, *H. bacteriophora* has been found to migrate deeper into the soil (Salame and

Glazer 2015). This downward movement toward soil moisture could impact the ability *H. bacteriophora* to successfully find a host, depending on the soil texture, moisture, and host orientation.

The purpose of this study was to identify the optimum conditions of soil texture, soil moisture, time, and host orientation in which *H. bacteriophora* and its associated enteric bacteria (*Photorhabdus* spp.) causes the greatest mortality on *Galleria mellonella* L. (Lepidoptera: Pyralidae). This study was part of a larger study examining the potential use of EPN for regulating an invasive agricultural pest, *Tipula paludosa* Meigan. Since the invasive pest could not be obtained, *G. mellonella* was used as a model for control of invasive agricultural pests. *Galleria mellonella* have been used as a host in numerous EPN studies since they are easily raised in captivity and are very susceptible to dauers (Woodring and Kaya 1988). There were two experiments conducted to address the purpose of this study. The first experiment examined the effects of soil texture and moisture on EPN location and infection of *G. Mellonella*, and the second experiment examined the effects of soil texture, moisture, time, and host orientation on the rate EPN move through the soil and infect a host.

Methods

Experimental Design. Two laboratory experiments were conducted to understand the role of soil texture, soil moisture, and host orientation on host infection by *H. bacteriophora*. In both experiments, three different soil textures at two soil moisture levels were used to form a total of six soil treatments. For the three soil texture treatments, soil was collected near a stream using a shovel in Millersville, PA on 19 February 2013. Play sand was purchased at a local hardware store. The sand and soil were transported to the laboratory where both were dried in an oven at 93°C, ground through a two-millimeter sieve, and autoclaved for 40 minutes at 121°C. Soil texture was determined by using the hydrometer method as described by California Department of Pesticide Regulation (2005). Fifty grams of soil were mixed with 250 mL of distilled water and 100 mL of sodium hexametaphosphate and allowed to soak for 24 hours. The soil mixture was homogenated with a “blend” setting on a blender (Hamilton Beach Blendmaster®, Glen Allen, Virginia) for one minute before being poured into a 1000mL sedimentation cylinder. Distilled water was added to bring the volume to one liter. The homegenated soil was mixed vigorously with a plunger for one minute and a

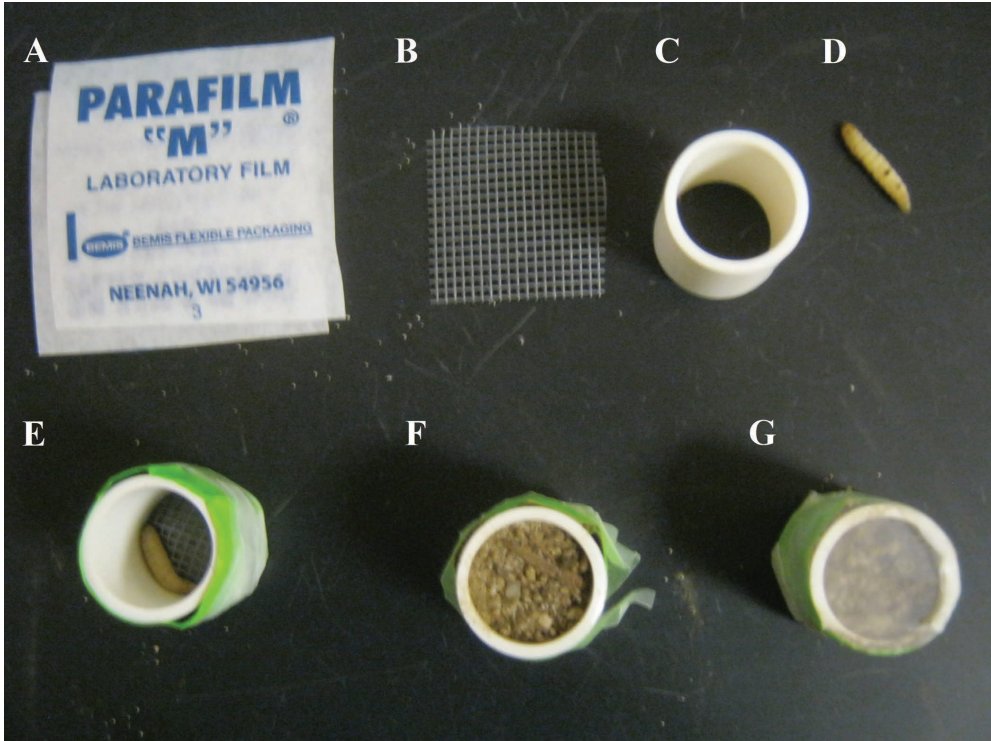


Figure 1. Assembly of experimental chambers A) Parafilm®, B) fiberglass screening, C) piece of PVC, D) *Galleria mellonella* larva, E) PVC portion containing *G. mellonella*, F) PVC portions containing *G. mellonella* and soil, G) assembled chamber.

hydrometer reading was taken 30 seconds, 60 seconds, 90 minutes, and 24 hours after mixing. Using a set of equations as described by the California Department of Regulation in their standard operating procedure for using a soil hydrometer, the soil texture was determined to be a silty loam, 64% silt, 26% clay, 10% sand (California Department of Regulation 2005). Sphagnum peat moss, a common horticultural potting media, was added to the silty loam as proxy for organic matter in a 1:1 ratio to create a silt/peat soil mixture (Ansari and Butt 2011). Several other studies have also used peat as a medium when examining the efficacy of EPNs (Lola-Luz et al. 2005, Lola-Luz and Downes 2007, Ansari et al. 2008, Ansari et al. 2010, Ansari and Butt 2011). For a second soil texture, sand, silty loam, and peat were added in a 1:1:1 ratio to create a sand/silt/peat soil mixture. Finally, the third soil texture was 100% sand obtained from a garden store.

To determine soil moisture, a 15cm long PVC pipe with a 5cm diameter was filled vertically with a known weight of dried soil; 450 grams for sand, 250 grams for sand/peat/silty loam, and 150 grams

for peat/silty loam (Fig. 1). On one end of the pipe, a cotton cloth was secured to keep the soil within the tube and distilled water was added until water was seen dripping from the tube. The soil was allowed to drain for 24 hours before weighing to determine the water volume by weight. The amount of water the soil contained after 24 hours was used to determine field capacity or the amount of water the soil can hold after any excess water has been drained (Thien and Graveel 1997). Henceforth, in this study we define soil at field capacity to be 100% saturation. For the following experiments, the necessary amount of water was added to each soil texture type to create two moisture levels: 100% and 50% saturation.

Forty-five mL of soil was placed in 100 mm×15 mm petri dishes and replicated three times for each soil texture and moisture level. EPN purchased from ARBICO Organics were introduced into the petri dishes by applying 2 mL of water containing approximately 45,000 individuals of *H. bacteriophora* to the surface of the soil. The resulting dauer density was approximately 1,000 dauers per cm³ of soil. To estimate the

number of dauers, a solution of EPN was placed into distilled water and stirred on a stirring plate. Two microliters of the solution were subsampled and the nematodes were counted under a dissecting microscope. This step was repeated an additional two times and the three counts of nematodes were averaged to estimate the concentration of the nematodes in the solution. After EPN application, 10 sixth instar *G. mellonella* were placed with forceps into the petri dishes and replicated three times for every soil treatment. *G. mellonella* in Petri dishes were checked daily for nematode infection and infected individuals were removed. Cadavers infected by *H. bacteriophora* were identified by the red color and flaccid body caused by *H. bacteriophora*'s symbiotic enteric bacteria (Koppenhöfer 2008). Any *G. mellonella* larvae that died of causes other than *H. bacteriophora* infection were removed and replaced by live *G. mellonella* larvae.

A second laboratory experiment was conducted to determine the rate that EPN moved through the soil types to find a host at different orientations, soil moistures and soil textures. Each treatment of host orientation, soil moisture, soil texture and time there was replicated five times. The three soil types described above were mixed with the distilled water to create the two saturation levels (50% and 100%) and placed into PVC tubes like those created by Ansari and Butt (2011). PVC pipe (approximately 1.25 cm in diameter) was cut into two-centimeter long segments. At one end of the PVC segment, 1.5 mm×1.5 mm fiberglass screening was installed to keep the *G. mellonella* larva from moving through the length of the tube (Fig. 1). Each end was sealed with a double layer of perforated Parafilm® to prevent the nematodes from escaping but to allow for air exchange (Ansari and Butt 2011). The PVC tube was filled with approximately 10 cm³ of moistened soil and placed at approximate 23° C for 24 hours to allow a gradient of host cues (i.e., carbon dioxide) to accumulate (Lewis 2002). *Heterorhabditis bacteriophora* are mobile active cruisers with a strong response to host cues (Kaya and Gaugler 1993). *Heterorhabditis bacteriophora* infective juveniles were mixed in distilled water to create a concentration of 5 infective juveniles/μL. One hundred microliters of the nematode solution (approximately 500 nematodes) was applied to the soil surface with a 1000 μL micropipette. The approximate density of injective juveniles was 50 dauers per cm³ of soil. The tubes were allowed to sit for 24, 72, and 96 hours to allow for nematode movement. Tubes were placed in three different orientations; horizontal, vertical with the *G. mellonella* larvae on top, and vertical with the *G. mellonella* larvae on the bottom. After

each time period, *G. mellonella* were checked for infection. *Galleria mellonella* larvae that were still alive at the end of the time period were rinsed with distilled water and quarantined for three days to confirm whether or not the larvae had been infected by EPN.

Statistical Analysis. The statistical significance of the two experiments was determined by two separate ANOVAs in Minitab (Minitab 17 Statistical Software 2010). Differences were considered significant at $p \leq 0.05$.

Results

In the first laboratory experiment examining the effects of soil texture and moisture on infection rate of *G. mellonella* by *H. bacteriophora*, a general linear model showed that there was a significant difference between soil texture and soil moisture on the number of *G. mellonella* larvae infected by *H. bacteriophora*. After six days, *G. mellonella* larvae in silt/peat soil had a suffered higher mortality ($F_s = 15.50$; $df = 2$; $P < 0.05$) than larvae in sand/silt/peat soils or pure sand soils. Soil moisture also had a significant effect ($F_s = 5.54$; $df = 1$; $P < 0.05$) effect *G. mellonella* mortality. Soils that were at 100% moisture had more infected *G. mellonella* larvae than sites with 50 % soil moisture (Fig. 2). There was no significant interaction between soil moisture and soil texture ($F_s = 1.50$; $df = 2$; $P > 0.05$).

In the second experiment evaluating the effects of time, host orientation, soil texture and moisture, a general linear model showed that only time had a significant ($F_s = 4.64$; $df = 2$; $P < 0.05$) effect on the number of *G. mellonella* killed by *H. bacteriophora*. At 24 hours, infective juveniles were able to move the length of the tube (2 cm) to find and infect the 17% of the *G. mellonella* larvae, at 72 hours 33% of the larvae were infected, and at 96 hours 78% of the larvae were infected (Fig. 3). Variations in host orientation ($F_s = 0.33$; $df = 2$; $P > 0.05$), soil texture ($F_s = 1.77$; $df = 2$; $P > 0.05$), and soil moisture ($F_s = 0.43$; $df = 1$; $P > 0.05$) did not have significant effects on the movement of EPN through the soil to infect a host.

Discussion

In order for EPN to move through the soil and find a host, they require oxygen and a thin film of water (Ansari and Butt 2011). While sand may provide greater interstitial space for EPN to move than silt, sand has a lower water holding capacity than silt or clay soils (Saxton and Rawls 2006). In our first experiment, the addition of peat moss, which is characterized by large pieces of organic debris, may have increased both

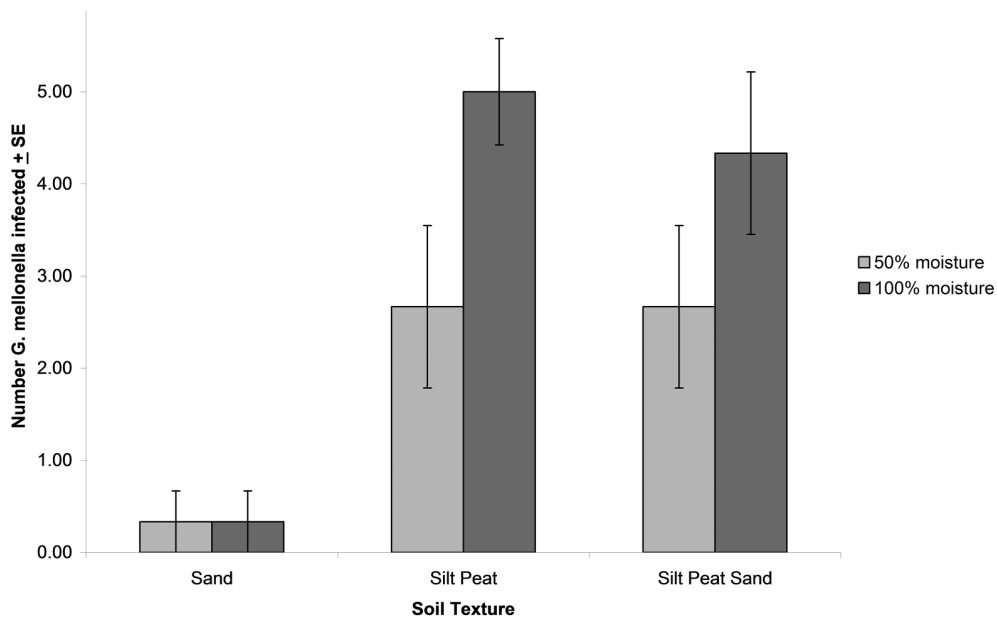


Figure 2. Average number of *Galleria mellonella* larvae infected by *Heterorhabditis bacteriophora* after six days across three types of soil mixture.

interstitial space and water holding capacity (Portillo-Aguilar et al. 1999). Soils rich in organic matter retain more moisture compared to soils lower in organic matter (Saxton and Rawls 2006). The soils containing peat (peat/silty and sand/peat/silty loam) had significantly higher *G. mellonella* infection rates than pure sand. Unlike most other studies that have shown that EPN efficacy was higher in sandier soils (Kung et al. 1990, Campos-Herrera et al. 2008), this study demonstrated that EPN were more easily infected in soils with higher clay and organic material contents. Similar to our findings, Toledo et al. (2009) found improved efficacy of EPN in sand-clay soils rather than in pure sand. Our study also found that soils with 100% moisture had greater *G. mellonella* larvae infection rate than soils at 50% moisture, which may suggest that soil moisture may be more important than soil texture alone. Molyneux and Bedding (1984) also demonstrated that moisture to be the most important factor in their study comparing soil textures and moisture. However, tradeoffs between interstitial space and soil moisture may still exist. A soil with high organic matter, such as peat, and high field capacity may have more interstitial space required for successful host encounters for EPN. Peat moss also lowers bulk density (a measure of compaction) to allow for larger

soil pores to facilitate the movement of *H. bacteriophora* through the soil. Future work will involve the measurement of interstitial spaces within different soil types.

Given the results of our first experiment, we would have expected to see a significant difference in soil moisture and texture on the ability of nematodes to find a host; instead soil moisture and texture were not significant. One explanation for these contradicting results could be the difference in EPN concentration between the two experiments. In the first experiment, there were 1,000 dauers/cm³, whereas, in the second experiment there were 50 dauers/cm³. A second explanation for the difference between the two experiments is the effect of time. EPN were given six days to find a host in the first experiment, while the maximum time in the second experiment was four days. It could be that the lower density of EPN and the shorter time frame of experiment masked difference in soil texture and soil moisture.

A third explanation for the difference between the two experiments could be soil compaction (bulk density). In the first experiment, the soil was scooped into the petri dish and very little compaction occurred. However, in the second experiment, the soil had to be stuffed into the PVC tube. The process of filling the tube could have led to a higher bulk density which lowered intersti-

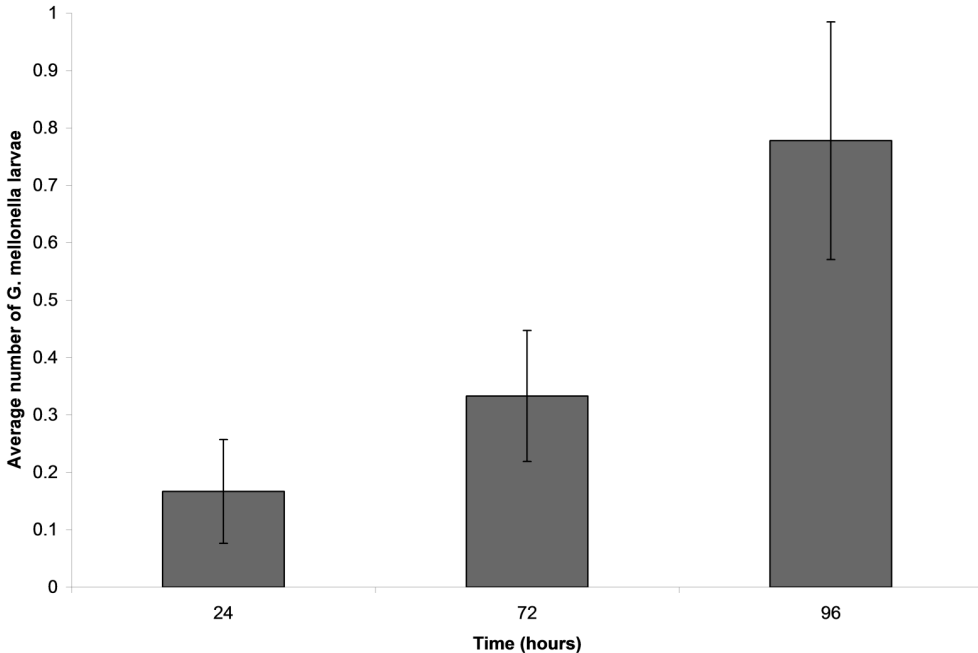


Figure 3. Average number of *Galleria mellonella* killed when nematodes traveled 2 cm to find a host at three different time intervals (24, 72, 96h).

tial space resulting in poorer efficacy of the nematodes. A study by Portillo-Aguilar et al. (1999) found that EPN in silty clay loam soil at a high bulk density were less effective at moving through 8 cm of soil to infect *G. mellonella* larvae. Wallace (1958) found that nematodes moved best when the size of the pores (interstitial space) found within the soil was approximately equal to the size of their body. Soils with higher bulk density may have pores that are smaller than the size of the EPN. While we did not measure bulk density, we speculate that the process of packing the soil into the tubes for the second experiment reduced the size of the interstitial space relative to experiment and prevented *H. bacteriophora* dauers (diameter 25 μ m) from moving freely through the soil (Portillo-Aguilar et al. 1999, Klingen and Haukeland 2006). We speculate that the difference between the two experiments may provide new information as to how minor changes in the environmental conditions can influence the success of entomopathogenic nematodes to move through the soil to find a host. Koppenhöfer and Fuzy (2006) also found that it was difficult to make conclusions when comparing different soils due to the conglomeration of parameters within each soil type. While previous studies have attempted to address one soil parameter at a

time, a more holistic approach in evaluating the effects of soil texture, moisture, bulk density, pH, and temperature is needed determine the interactions between these parameters and their impact on each EPN species' efficacy.

In order for *H. bacteriophora* to function as an effective biocontrol agent for agricultural pests, it is important that they are able to quickly find, kill, and reproduce inside the host (Peters and Ehlers 1994). The second laboratory experiment indicated that within 24 hours it was possible for *H. bacteriophora* infective juveniles to travel two centimeters to find and infect a host. However, our study found at 48 and 96 hours, significantly more of the *G. mellonella* hosts had been infected by *H. bacteriophora* than at 24 hours. Hence, as more time passed, a higher rate of *G. mellonella* infection by *H. bacteriophora* occurred. While *H. bacteriophora* were able to reach *G. mellonella* larvae 2 cm within 24 hours, a larger proportion of the *G. mellonella* were infected after 96 hours. Several factors may be contributing to this difference, a 96 hour interval did provide more time for host cues to accumulate for the EPN to detect and pursue, it may also allow for more EPN to enter the host and more time for the bacteria to kill the host via septicemia. Previous research has

indicated that small amounts of nitrogen (<0.16mg) released from *H. bacteriophora*-infected hosts during the early stages of infection can attract conspecific dauers to the host. However, when nitrogen is released at higher levels (>0.16mg), it leads to repulsion of nematodes away from the infected host (Shapiro et al. 2000). Our finding emphasizes the importance of applying EPN in the appropriate quantities and during the ideal environmental conditions (i.e., moisture, temperature, pH, bulk density, and texture) to control pest hosts. Given the right parameters, we found that *H. bacteriophora* nematodes were able to survive for 96 hours to infect a host at least 2 cm away (in any direction vertical or horizontal).

We hypothesized that orientation of EPN in respect to the host could influence their ability to find a host. Previous studies have examined the ability of EPN to move vertically or horizontally to find a host crawling on the soil surface (Toledo et al. 2009, Ansari and Butt 2011), but none compared the effects of host orientation on EPN infections. However, this study found that there was no significant difference in the infection rate when EPN move horizontally, vertically (downwards), and vertically (upwards) to find a host. These results suggest *H. bacteriophora* were able to seek out their host in any direction within the soil.

To be an effective biological control agent, it is imperative to know the environmental conditions wherein *H. bacteriophora* are most successful in finding and infecting the host. Our results are consistent with other studies that suggest EPN are most effective at finding and infecting hosts in soils that are high in water holding capacity, such as soils with high organic material and clay content (Molyneux and Bedding 1984). Further studies, in the field and laboratory, are needed to more closely evaluate the complex interactions that maybe occurring across multiple soil parameters to impact the efficacy of *H. bacteriophora*.

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