# EFFECT OF SOIL MOISTURE ON THE PERSISTENCE AND EFFICACY OF *HETERORHABDITIS BACTERIOPHORA* (RHABDITIDA: HETERORHABDITIDAE) AGAINST *ANASTREPHA LUDENS* (DIPTERA: TEPHRITIDAE) LARVAE

JORGE TOLEDO[1,*], JOSÉ E. SÁNCHEZ[1,2], TREVOR WILLIAMS[3], ANAXIMANDRO GÓMEZ[1], PABLO MONTOYA[2,4] AND JORGE E. IBARRA[5]

[1]Departamento de Agricultura, Sociedad y Ambiente, El Colegio de la Frontera Sur, Apartado Postal 36, Tapachula, Chiapas 30700, Mexico

[2]Centro de Biociencias (CenBio). Universidad Autónoma de Chiapas. Campus IV. Tapachula, Chiapas 30700, Mexico

[3]Instituto de Ecología AC, Xalapa, Veracruz 91070, Mexico

[4]Programa Moscafrut (SAGARPA-IICA). Camino a 105 Cacaotales S/N. Metapa de Dominguez, Chiapas 30860, Mexico

[5]Departamento de Biotecnología y Bioquímica, Centro de Investigación y de Estudios Avanzados, IPN. Apartado Postal 629. Irapuato, Guanajuato 36500, Mexico

*Corresponding author; E-mail: jtoledo@ecosur.mx

ABSTRACT

The efficacy of *Heterorhabditis bacteriophora* (Poinar) infective juveniles (IJs) was evaluated against third instar *Anastrepha ludens* (Loew) (Diptera: Tephritidae) under laboratory conditions in a sandy clay soil at various levels of soil moisture. Three experiments were performed in which the efficacy of the IJs against *A. ludens* was estimated, i.e., (a) at 6 different levels of soil moisture, (b) in soil that was allowed to lose moisture over a 15 day period, and (c) in soil with an initial moisture content of 16% and in which moisture loss was partially mitigated by adding water at 5-day intervals. In the first experiment, the greatest *A. ludens* mortality (80%) was observed in soil with 18% moisture (-63.1 bars), although this was not significantly greater than *A. ludens* mortality at 21% moisture (-20.4 bars). At 24% soil moisture (-7.70 bars), percentage of mortality of *A. ludens* declined to about 50%. Likewise insect mortality was substantially lower at soil moisture levels of 15% (-240.1 bars) and 12% (-1,232 bars) and very much lower (about 16%) at 9% soil moisture (-10,147 bars). In the second experiment, as soil moisture declined from 16% to less than 10% over a 15 day period, the infectivity of IJs, as indicated by *A. ludens* larval mortality, progressively declined from more than 55% to less than 10%. In the third experiment, in which moisture loss was partially mitigated by adding water at 5-day intervals, the decline in infectivity of IJs was gradual up to 21 days, but decreased thereafter. We conclude that soil moisture levels must be carefully considered when applying *H. bacteriophora* IJs to control *A. ludens* under field conditions, because soil moisture has a marked effect on the efficacy of IJs for the biological control of this pest.

Key Words: entomopathogenic nematodes, fruit flies, infectivity, microbial control

RESUMEN

La capacidad infectiva de los juveniles infectivos (JIs) del nemátodo *H. bacteriophora* (Poinar) fue evaluada contra larvas de tercer estadio de *Anastrepha ludens* (Loew) (Diptera: Tephritidae), bajo condiciones de laboratorio, utilizando suelo con textura areno-arcillosa con diferentes porcentajes de humedad. El estudio consistió de tres experimentos, donde se midió la efectividad de los juveniles infectivos (JIs): 1) probando seis diferentes niveles de humedad en el suelo; 2) en suelo con pérdida constante de humedad, durante 15 días; y 3) en suelo con 16% de humedad inicial y rehidratación cada cinco días. En el primer experimento se observó la mayor infección en suelo con 18% de humedad (-63.1 bars), aunque esta mortalidad no fue significativamente mayor a 21% de humedad (-20.4 bars). Con un suelo a 24% de humedad (-7.70 bars), el porcentaje de mortalidad de *A. ludens* disminuyó

a cerca de 50%. De la misma forma, la mortalidad larval disminuyó significativamente en suelos con 15% y 12% de humedad (-240.1 y -1,232 bars, respectivamente), decreciendo aún más (16% de mortalidad) a 9% de humedad (-10,147 bars). En el segundo experimento se observó que a medida que la humedad del suelo decreció desde 16% hasta menos del 10% durante un período de 15 días, la infectividad de los JIs, expresada en mortalidad larval de *A. ludens*, disminuyó progresivamente desde más del 55% hasta menos del 10%. En el tercer experimento, la reposición periódica de la humedad permitió incrementar el período de infectividad de los JIs hasta los 21 días post-aplicación, pero después la infectividad de los JIs también fue disminuyendo. En conclusión, la humedad del suelo es un factor importante que debe ser considerado para mantener la infectividad de los JIs de *H. bacteriophora* como agentes de control biológico de dicha plaga.

Palabras Claves: nemátodos entomopatógenos, *H. bacteriophora*, moscas de la fruta, control microbiano

Entomopathogenic nematodes have great potential for the control of different types of agricultural insect pests (Klein 1990; Georgis 1992). Variations in the infectivity and specificity of infective juveniles (IJs) of these nematodes are influenced by diverse factors, including behavior, physiology, geographic origin and physical factors of the habitat (Doucet et al. 1996; Ehlers & Gerwien 1993; Kaya 1990). In the case of host density, the efficacy of *Heterorhabditis bacteriophora* IJs applied against *A. ludens* under field conditions was affected by larval host density in a sandy-clay soil (Toledo et al. 2006a).

*Heterorhabditis bacteriophora* (Nematode: Heterorhabditidae) is currently used to control a variety of agricultural insect pests (Klein 1990; Georgis 1992), and previous studies have indicated that certain strains have the potential to infect tephritid fruit fly larvae including *Anastrepha ludens* (Loew), *A. obliqua* (MacQuart), *A. serpentina* (Wiedemann) and *A. fraterculus* (Loew) (Toledo et al. 2005a, 2005b, 2006a, 2006b; Barbosa-Negrisoli et al. 2009).

In Mexico, *A. ludens* is the most severe pest of citrus fruits and mango, and it is responsible for direct damage and indirect losses resulting from the severe quarantine restrictions that many countries impose against the importation of fruit from *A. ludens* infested areas (Aluja 1994). The management of pestiferous tephritid populations tends to focus on an integrated approach involving chemical, biological and cultural control practices (Reyes et al. 2000), rather than depending entirely on chemical pesticide-based control measures.

Lezama-Gutiérrez et al. (1996) evaluated different species of nematodes against *A. ludens* in soil with 15% moisture content under laboratory conditions. Fly larvae were moderately to highly susceptible to infection whereas pupae were mostly resistant to infection. However, when adults emerged from pupae and passed through IJ-treated soil, an increased prevalence of infection was observed, indicating that infection could occur during adult emergence (Toledo et al. 2005b). Similarly, *A. suspensa* larvae and adults were also susceptible to several species of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) in sterile petri dishes lined with filter paper under laboratory conditions (Beavers & Calkins 1984).

Soil moisture is of key importance for the survival, displacement and infectivity of IJs targeted at soil dwelling pests (Kung et al. 1991; Molyneux & Bedding 1984). As tephritid fruit flies pupate in soil they could be controlled by application of IJs if soil conditions, particularly moisture, favored the action of these organisms. The main objective of this study was to determine the influence of soil moisture content on the efficacy and persistence of *H. bacteriophora* IJs against *A. ludens* third instars in a sandy-clay soil.

## Material and Methods

### Biological Material

*Anastrepha ludens* larvae were obtained from the mass-rearing facility at the Moscafrut Plant (SAGARPA-IICA) at Metapa de Domínguez, Chiapas, Mexico, where the colony was maintained as described by Domínguez et al. (2010).

The *H. bacteriophora* strain used in this study originated from soil samples taken in Costa Rica (Castillo & Marbán-Mendoza 1996). The nematode was reared in larvae of *Galleria mellonella* L. (Pyralidae) and IJs were collected using White traps (Woodring & Kaya 1998). IJs were counted with a binocular dissecting microscope (10 counts per suspension) and adjusted to a density of 800 IJs/mL sterile distilled water. IJs suspensions were maintained at 10 ± 2 °C for a maximum of 4 wk until required for experiments (Woodring & Kaya 1998). During this period the mortality of the IJs was minimal (< 3.0%). These suspensions were adjusted to the densities required in each of following experiments.

Soil was obtained from the 'Viva México' mango orchard (N 14° 54' 42" -W 92° 20' 05") at 78 m asl between the towns of Tapachula and Huixtla, Chiapas State, Mexico. No insecticide had been applied to this soil during the previous 5 yr. This

soil comprised 74% sand, 14% silt, 12% clay, and 2% organic material with a pH of 6.4 (Toledo et al. 2006a). Soil was sieved (mesh 18), placed in bags and heated to 121 °C for 15 min. After this, it was dried in trays at room temperature, placed in an oven at 100 °C for 24 h, allowed to cool, and sealed in plastic containers until required for experiments.

### The Effect of Soil Moisture in Relation to the Nematode Activity

Soil moisture content was adjusted to 9, 12, 15, 18, 21 and 24% (wt/wt). The matrix potential of the soil was determined using the pressure membrane technique. Soil-water retention curves were used to calculate the matrix potential at each percentage of soil moisture (range, 9-24%) (Brady & Weil 2003). The matrix potentials of the experimental soil were -10,147, -1,232, -240.1, -63.1, -20.4 and -7.70 bars, respectively. Experimental units consisted of a 5-cm-diam PVC cylinder (19.6 $cm^2$ exposed surface area) containing 120 g (dry wt) of soil. To avoid moisture loss each tube was covered with a Petri dish lid (6 cm diam). Twenty five *A. ludens* third instar larvae were placed on the surface of each tube and allowed to burrow into the soil for 10 min. By a pipette 2,350 IJs were then applied in 1 mL of sterile distilled water at a concentration of 120 IJs/$cm^2$ of soil surface and uniformly distributed over the soil surface. Tubes were incubated at 26 ± 1 °C, 70 ± 5% RH, and 12:12 h L:D photoperiod. Seven days after inoculation, the soil from each tube was sieved gently (mesh 18) to separate larvae and pupae. Infection was determined by observation with a binocular dissecting microscope. The experiment was replicated 6 times.

### Persistence of Infectivity over Time

Bioassays were performed using a single concentration of 120 IJs/$cm^2$ of soil surface as described above. This rate was estimated to result in 50% mortality of *A. ludens* third instars larvae in a previous study (Toledo et al. 2006a). Two hundred and fifty PVC tubes were each prepared with 120 g soil adjusted to 16% moisture content (estimated matrix potential -185.0 bar). The soil surface of each tube was then inoculated with IJ's in 1 mL water. Tubes were then placed in a bioclimatic chamber and kept in darkness at 26 ± 1 °C and 70 ± 5% RH.

Each day for 15 days, 5 experimental units were taken at random and 25 *A. ludens* third instar larvae were placed on the soil surface of each tube and allowed to burrow into the soil. These tubes were then returned to the bioclimatic chamber and incubated for a further 7 days, after which soil was sieved and insects were examined for infection. Control experimental units were treated identically but not inoculated with IJs. Simultaneously, each day, a subsample was taken and weighed before being heated. The soil was heated in an oven at 100 °C until constant weight was achieved and the amount of water that was lost by weight was calculated to estimate moisture content.

### Persistence of IJ Efficacy When Soil Moisture Loss Was Partially Mitigated by Adding Water at 5-Day Intervals

To examine the influence of periodic application of water to experimental soil units on the persistence of IJs infectivity, a total of 330 tubes were prepared as described in the previous experiment. Tubes were incubated at 26 ± 1 °C and 70 ± 5% RH and 5 mL of water was applied to each tube at 5 day intervals. As before, IJs were inoculated onto the soil surface of each tube at the beginning of the study and *A. ludens* larvae were applied to randomly selected tubes at daily intervals and incubated for 7 days. As in the previous experiment, moisture content was determined at daily intervals. The experiment lasted 21 days.

### Data Analysis

The results of the first experiment were subjected to analysis of variance (ANOVA), followed by means separation using the Tukey test ($P > 0.05$) (SAS Institute 2003). To describe the relation between the larval mortality (dependent variable) and the loss of soil moisture (independent variable), the results of the second and third experiments were subjected to a semi-logarithmic regression considering the relation between the larval mortality with 2 variables that were soil moisture (in a range of 9 to 16%) and exposure time (in a range of 1 to 15 days for the first experiment and 1 to 21 days for the second experiment). The analysis was performed using JMP Statistical Discovery Software (SAS Institute 2003).

## Results

### The Effect of Soil Moisture on IJ Efficacy

Soil moisture had a significant effect on IJ infectivity ($F = 19.48$; df = 5, 29; $P < 0.001$). Soil moistures between 12% (-1,232 bars) and 21% (-20.4 bars) resulted in over 50% of mortality of *A. ludens* when IJs were applied at a concentration of 120 IJs/$cm^2$, with the highest mortality observed in the 18% moisture (-63.1 bars) treatment (Fig. 1). More extreme values of soil moisture, both dryness (9%; -10,147 bars) or wetness (24%; -7.70 bars), resulted in less than 50% mortality of

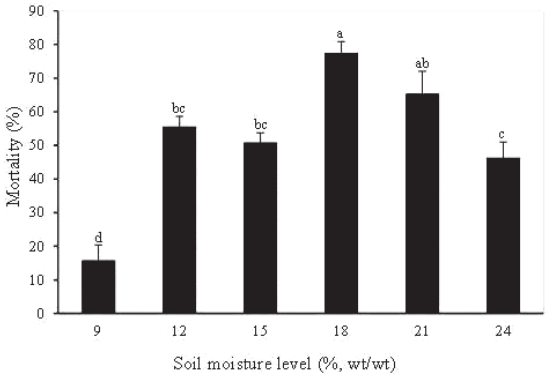


Fig. 1. The effect of the different soil moisture levels on the efficacy of *Heterorhabditis bacteriophora* against *Anastrepha ludens* larvae in a sandy clay soil. ($n$ = 150 larvae/ treatment). The error bars represent the standard error. The matrix potentials of the experimental soil with moisture levels of 9, 12, 15, 18, 21 and 24% were -10,147, -1,232, -240.1, -63.1, -20.4 and -7.70 bars, respectively,

*A. ludens*; an effect that was particularly evident in the 9% moisture treatment.

### IJ Infectivity in Soil over Time

Mortality of *A. ludens* larvae was highest (56.8–56.0%) in larvae exposed to inoculated soil at 2 and 3 days post-inoculation (Fig. 2). Larval mortality decreased significantly over time ($r^2$ = 0.785; $F$ = 45.59; df = 1, 13; $P$ < 0.001). After 3 days post-inoculation, mortality values began to decline and remained below 10% in larvae exposed to contaminated soil. By the end of the experiment, soil moisture had fallen from 16% to a final value of 9.9%.

### Persistence of IJ Efficacy When Soil Moisture Loss Was Partially Mitigated by Adding Water at 5-Day Intervals

The mortality value was highest (70.4%) in *A. ludens* larvae exposed to inoculated soil at 1 day post-inoculation (Fig. 3). The prevalence of mortality in the insects declined significantly over the 21 days of the study ($r^2$ = 0.866; $F$ = 122.60; df = 1, 19; $P$ < 0.001). Despite not greatly affecting overall soil moisture levels, periodic addition of 5 ml water to the soil surface increased of IJ-induced mortality in *A. ludens* by 10-20% at most time points, compared to mortality values observed in the previous experiment. Soil moisture levels over the course of the experiment varied from 16% at day 1 to 7.9% moisture at day 20 post-inoculation.

## Discussion

The efficacy of *H. bacteriophora* IJs against *A. ludens* third instar larvae in a sandy-clay soil was dependent on soil moisture. Soil moisture levels of 18-21% (-63.1 to -20.4 bars) resulted in the greatest efficacy, in terms of prevalence of infection; significantly fewer infections occurred at higher or lower moisture values.

Several isolates of *H. bacteriophora* have been shown to produce high mortalities in fruit fly larvae (Barbosa-Negrisoli et al. 2009; Malan & Manrakhan 2009; Toledo et al. 2005a; 2005b; 2006b). For this to occur, IJs have to move through the soil to locate potential hosts (Kung et al. 1991). Such movement can be hindered by soil physicochemical characteristics including texture, soil pore size relative to nematode length, pH, and moisture. These same factors are therefore likely to influence the success of the nematode as a biological control agent in field conditions (Portillo-Aguilar et al. 1999). Soils that differ in moisture

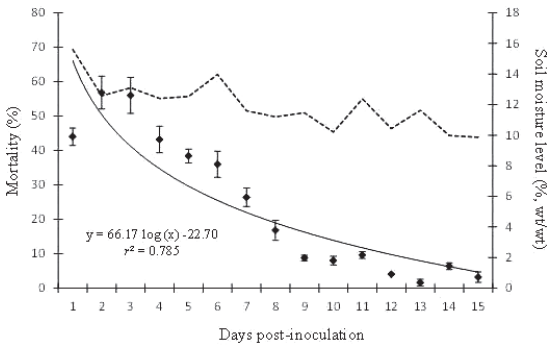


Fig. 2. Mortality of *Anastrepha ludens* larvae caused by *Heterorhabditis bacteriophora* IJs in a sandy clay soil undergoing the loss of moisture. The bars represent the standard error of mean mortality. (—) Represents larval mortality and (----) represents the soil moisture level.

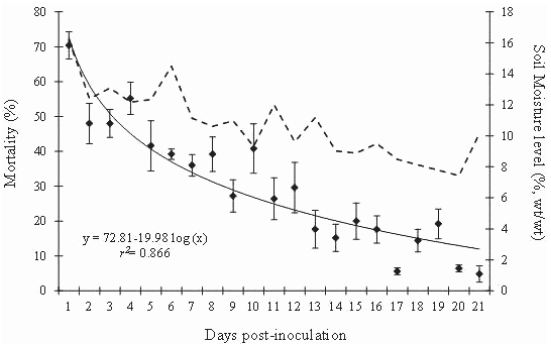


Fig. 3. Mortality of *Anastrepha ludens* larvae caused by *Heterorhabditis bacteriophora* IJs in a sandy clay soil with an initial moisture content of 16% and in which water loss was partially mitigated by adding water at 5-day intervals. The bars represent the standard error of mean mortality. (—) Represents larval mortality and (----) represents the soil moisture level.

content, above all the free water that remains available on the external surface of soil particles, significantly influence the survival and infective capacity of IJs (Barbercheck & Kaya 1991; Molyneux & Bedding 1984; Koppenhöfer et al. 1995; Fujiie et al. 1996). For example, in green june beetle larvae (*Cotinis nitida* L.; Scarabaeidae), mortality caused by *H. bacteriophora* and *S. carpocapsae* IJs was greater in soils with high moisture levels (30%) compared with soils with 10% moisture content (Townsend et al. 1998).

IJ infection of the *A. ludens* host larvae was verified in pupae in which IJs were observed through the puparium or via dissection. Insects from the control treatment were never observed to contain IJs, natural mortality was low (3%) and 88% of adults emerged from pupae.

Larvae of *A. ludens* are susceptible to infection by entomopathogenic nematodes such as *S. riobravis* and *S. carpocapse* IJs that can cause up to 90% mortality. The NC strain of *H. bacteriophora* caused up to 83% mortality whereas treatment with *S. feltiae* and *S. carpocapse* (Tecomán strain) resulted in up to 81% and 76.0% of mortality of *A. ludens*. Similarly, *H. bacteriophora* and *S. glaseri* caused 53% mortality in *A. ludens* third instar larvae at 25 ºC in a sandy-textured soil with 15% moisture content (Lezama-Gutiérrez et al. 1996).

The duration over which IJs retained their infectivity in the present study was similar to that observed in a study on *H. bacteriophora* against *A. obliqua* larvae that were applied to a sandy-clay soil with 16% moisture content, although the infectivity of IJs diminished markedly in the *A. obliqua* study when soil moisture content was 10% or less (Toledo et al. 2005b). Therefore, soil moisture content appears to be critical for IJ survival and movement through the soil matrix in search of hosts (Kung et al. 1991; Womersley 1990; 1993; Smith 1999; Klein 1990).

Additionally, soil compression can directly influence the biology of insects that pass at least one stage of their lives in the soil. In the case of tephritid fruit flies, the depth to which final instar larvae burrow into the soil will have a clear influence on related variables such as temperature, moisture and also their exposure to biotic variables such as predation, parasitism, pathogens and survival during adult emergence (Jackson et al. 1998).

In our study, IJ dispersal was not differentially limited by the soil texture because we used the same soil for all experiments, a soil type that favored infection by IJs (Toledo et al. 2005b). However, additional factors such as host behavior and activity could also limit the capacity of IJs to localize and infect host larvae (Doucet et al. 1996).

Interestingly, *H. bacteriophora* IJs remained efficacious for longer and infected more *A. ludens* larvae when the soil was rehydrated at intervals of 5 days. This occurred despite the fact that periodic applications of 5 mL of water did not greatly influence the mean moisture content of the soil in each experimental container. This may reflect the importance of soil moisture in the upper part of the experimental containers through which *A. ludens* larvae had to burrow, and the ability of IJs to move efficiently through soil with high moisture content. Movement of IJs is known to be influenced by moisture (Koppenhöfer et al. 1995; Fujiie et al. 1996), temperature (Kung et al. 1991), texture and soil density (Barbercheck & Kaya 1991; Portillo-Aguilar et al. 1999).

In this study we confirmed that infection by IJs was favored at intermediate moisture levels in a sandy-clay texture soil. Periodic dampening of soil, such as occurs during rainfall, resulted in increased infection and extended the duration of IJ infectivity. The results of these laboratory experiments require validation in field tests in areas in which *A. ludens* is a serious pest of fruit crops, such as the mango and citrus growing regions of Mexico.

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