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RESEARCH ARTICLE

Abiotic factors affecting the infectivity of *Steinernema carpocapsae* (Rhabditida: Steinernematidae) on larvae of *Anastrepha obliqua* (Diptera: Tephritidae)

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The effects of soil depth, soil type and temperature on the activity of the nematode *Steinernema carpocapsae* (Filipjev) were examined using larvae of the West Indian fruit fly, *Anastrepha obliqua* (Macquart). Bioassays involved applying infective juveniles (IJs) to the surface of sterilized sand in PVC tubes previously inoculated with fly larvae of two ages. The 50% lethal concentration (LC_{50}) values estimated for 6-day-old larvae were 9, 20 and 102 IJs/cm^2 in tubes containing 2, 5 and 8 cm depth of sand, respectively, whereas for 8-day-old larvae, LC_{50} values were 16, 40 and 157 IJs/cm^2, respectively. The effect of soil texture on the activity of *S. carpocapsae* was tested by applying the corresponding LC_{50} concentrations of nematodes to sand, sand–clay and loamy–sand soils. For 6-day-old larvae, soil type had a highly significant effect on infection with the highest percentages of infection observed in the sand–clay mixture (60–82% depending on depth) compared to 45–64% infection in sand and 23–30% infection in loamy–sand soil. A very similar pattern was observed in 8-day-old larvae except that infection rates were significantly lower than in younger larvae. There was a significant interaction between soil type and soil depth. The effect of three temperatures (19, 25 and 30°C) on infection was examined in sand–clay soil. The infectivity of *S. carpocapsae* was affected by temperature and soil depth and by the interaction of these two factors. Response surface analysis applied to second order multiple linear regression models indicated that the optimal temperature for infection of larvae of both ages was ~26°C, at a depth of 7.9 cm for 6-day-old larvae and <2 cm for 8-day-old larvae, resulting in a predicted 91.4% infection of 6-day-old larvae and 61.2% infection of 8-day-old larvae. These results suggest that *S. carpocapsae* may have the potential to control fruit fly pests in tropical ecosystems with warm temperatures and high soil moisture levels, although this assertion requires field testing.

Keywords: entomopathogenic nematode; infection; soil depth; soil type; temperature; tephritid fruit fly

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Introduction

The West Indian fruit fly, *Anastrepha obliqua* (Macquart) is a widely distributed pest in the Americas ranging from the southern USA to Brazil (Aluja and Norrbom 2000). In Mexico, this species causes serious economic losses in mango (Aluja et al. 1996). Conventional control methods involve the use of synthetic insecticides in bait formulations targeted at adult flies, or insecticides applied directly to the soil surface in mango orchards for control of pupae and pupating stages of this insect. Soil application of broad-spectrum insecticides has been considered as an important tactic in programs of integrated pest management (Penrose 1993). However, the impact of such treatments on non-target invertebrate soil fauna is an issue of concern (Aluja 1994) and has stimulated a small number of studies on alternative, biorational control measures (Gazit, Rössler, and Glazer 2000; Toledo et al. 2006, 2007).

The nematode *Steinernema carpocapsae* (Filipjev) is a soil-dwelling entomopathogen that can exploit a wide range of insect hosts, principally those in humid environments (Kaya 1985). The infective juvenile (IJ) stages penetrate the host via spiracles, mouth or anus (Triggiani and Poinar 1976; Woodring and Kaya 1988) or directly through the integument (Peters and Ehlers 1994). These nematodes are associated with symbiotic bacteria (*Xenorhabdus* spp.), which inhabit the gut of the IJ stage. Having penetrated the host, the nematodes release the bacteria that cause a generalized septicemia and the death of the insect. The nematode feeds on the proliferating bacteria and two or three cycles of reproduction occur in the host prior to emergence of progeny infective stages (Forst, Dowds, Boemare, and Stackebrandt 1997).

The potential of this nematode for control of dipteran pests has been demonstrated in flies from the families Muscidae (Mullens, Meyer, and Cyr 1987), Phoridae (Scheepmaker, Geels, Van Griensven, and Smits 1998), Sciaridae (Grewal and Richardson 1993), Tipulidae (Peters and Ehlers 1994), and various species of tephritid fruit flies (Tephritidae) (Beavers and Calkins 1984; Lindegren and Vail 1986; Lindegren, Wong, and McInnis 1990; Gazit et al. 2000; Yee and Lacey 2003; Lezama-Gutiérrez et al. 2006). However, the efficacy of this nematode as a biocontrol agent of soil-dwelling pests varies markedly according to the host species and host age (Gaugler 1988). Biotic factors such as antagonist organisms and abiotic factors, such as soil texture, moisture, temperature and host depth, are also believed to be influential on the effectiveness of this nematode (Kaya 1990; Shapiro, McCoy, Fares, Obreza, and Dou 2000). The present study examined the effect of soil depth, temperature and soil type on the infectivity of *S. carpocapsae* towards host larvae of two ages in an attempt to determine the conditions under which this entomopathogenic nematode is likely to perform best as a biocontrol agent against *A. obliqua*.

Materials and methods

Larvae of *A. obliqua* were obtained as second and third instars from the Moscafrut (SAGAR-IICA) mass-rearing facility in Metapa, Chiapas, Mexico where they are reared in a semi-synthetic diet (Artiaga-López et al. 2004). A nematode strain initially identified as *S. feltiae* but later found to be *S. carpocapsae* by ITS sequencing, was isolated from a British commercial product of unknown name in 1995 and maintained in the entomopathogen collection of the Centro de Investigaciones y de Estudios Avanzados (CINVESTAV-IPN), in Irapuato, Mexico. The nematode culture was

maintained by propagation on larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) and infective stages were collected using White traps (Woodring and Kaya 1988). Following collection, infective stages were diluted, counted in a hemocytometer and adjusted to a concentration of 800 nematodes/mL and subsequently stored at 12 ± 2°C, for no more than 30 days, until their use in experiments. These suspensions were used to prepare the concentrations required in each of the following experiments. The first two stages of this study were performed at 25 ± 1°C, 70 ± 5% RH and 12 h L:12 h D photoperiod in a bioclimatic chamber. The experiment involving the effect of temperature on the infective capacity of IJs was performed at temperatures of 19 ± 1, 25 ± 1 and 30 ± 1°C under identical conditions of humidity and light cycle. Soils of different types were collected from three sites close to the villages of Jaritas, Viva México, and Huehuetán, all within a 30 km radius of the city of Tapachula, Chiapas, Mexico. Each soil was characterized for composition and pH in the Soil Analysis Laboratory of the Universidad Autónoma de Chiapas, Chiapas, Mexico.

Effect of larval age and soil depth on infection

Bioassays were performed in PVC tubes of 5 cm diameter that varied in height, being 2, 5 or 8 cm tall and with a capacity of 30, 70 and 120 g (dry wt) of sand, respectively. The sand used in this study was sieved through a size 18 mesh, washed with distilled water, and sterilized by autoclaving. Sterilized sand was dried completely in an oven at 95 °C for 24 h. Distilled water was then added to obtain a moisture content of 10% (w/w). The water matric potential of sand samples was determined in triplicate using a calibrated soil equitensiometer (EQ2 Probe, Delta-T Devices, Cambridge, UK) when tensiometer readings reached a steady state, 24–48 h after placing the probe in sand. The water matric potential of sand samples was $-0.875 \pm 0.32 \times 10^{-3}$ kPa. PVC tubes were filled with this sand and 6- or 8-day-old 25 *A. obliqua* larvae were placed on the surface (19.6 cm^2 of surface area). Larvae immediately burrowed into the sand. After a period of 10 min, each concentration of nematodes was applied uniformly to the surface: 0, 6, 13, 25, 51, 76, 102, 127, and 204 nematodes/cm^2 of surface area in a volume of 1 mL. Each concentration of nematodes was prepared by dilution in a total volume of 100 mL water. For each concentration tested there were five replicates of each depth of sand and each age of larva giving $9 \times 5 \times 3 \times 2 = 270$ experimental units in total. After 7 days, fruit fly larvae had pupated and were separated from the sand by gentle sieving. The number of larvae that died due to infection was counted using a binocular dissecting microscope. In order to calculate the 50% lethal concentration (LC_{50}) for each treatment, results were subjected to probit analysis (SAS Institute 1992). No nematode-induced mortality was observed in the controls and no correction for control mortality was applied.

Effect of soil type on infection

The effect of three soil types (Table 1) on the activity of *S. carpocapsae* towards *A. obliqua* larvae was tested using the PVC tubes previously described. All soils had been sieved, sterilized and dried as described above, only the soil sand was washed and afterwards was adjusted to a moisture content of 10% (w/w) for sand, and 15% (w/w) for sand–clay and loamy–sand soils. Water matric potential readings were performed in four similar samples of each type of soil as described for the sand

Table 1. Characteristics of the soils used as a substrate for larvae of *Anastrepha obliqua* exposed to infective juvenile stages of *Steinernema carpocapsae*.

Soil type	Sand content (%)	Silt content (%)	Clay content (%)	Organic material (%)	pH
Sand	96.2	0.7	3.1	0.2	6.63
Loamy–sand	80.2	13.7	6.1	11.4	6.28
Sand–clay	74.2	13.7	12.1	2.0	6.36

samples. The water matric potentials of sand, sandy–clay and loamy–sand samples were $-0.875 \pm 0.32 \times 10^{-3}$, -1182 ± 443, and -12532 ± 3792 kPa, respectively. As before, 25 *A. obliqua* larvae, either 6- or 8-days-old, were placed on the surface of each experimental unit, allowed to burrow into the substrate, and 10 min later, nematodes were uniformly applied to the surface in a volume of 1 mL. In each case, the concentration of IJs applied to the soil surface corresponded to the LC_{50} calculated for each depth and larval age combination in the previous experiment. As before, mortality was quantified 7 days later by sieving and observing the percentage of lethal infection of larvae or pupae. The experiment was performed using a trifactorial design and the data were subjected to analysis of variance (ANOVA) with larval age, soil depth and soil texture as factors giving 18 treatment combinations, and means were separated by a Tukey test ($P = 0.05$). Each treatment combination comprised five replicates. None of the results required transformation prior to analysis.

Effect of temperature on infection

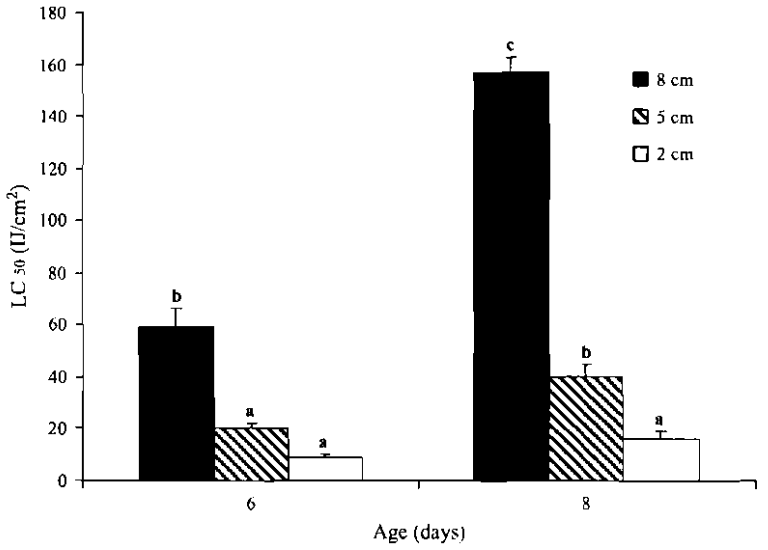
The effect of three different temperatures on the activity of *S. carpocapsae* was evaluated using the sand-clay soil mixture (in this case adjusted to a moisture content of 15%, w/w) tested in the previous experiment which had resulted in the highest infection. As before, PVC tubes were used to produce three depths of soil and 25 *A. obliqua* larvae of 6 or 8 days were placed onto the soil surface, allowed to burrow, and the surface was subsequently inoculated with the LC_{50} concentrations of IJs that corresponded to each soil depth and larval age combination. Experimental units were maintained at 19 ± 1, 25 ± 1 or 30 ± 1°C in bioclimatic chambers. The infectivity of nematodes was determined at 7 days post-inoculation as described previously. Each treatment combination was replicated five times. Percentages of infection results from each age group were normalized by arcsine transformation and were subjected to a bifactorial ANOVA with soil depth and temperature as factors. Means were compared using a least significant difference procedure (SAS Institute 1992). Arcsine transformed percent infection values were also subjected to multiple linear regression against soil depth and temperature that involved fitting a second-order polynomial equation. Response surface analysis was performed to determine whether the fitted surface was an adequate approximation of the true response function. Characterization of response surfaces was performed by canonical analysis to determine points of maximum or minimum response, saddle points or ridges of separate second-order models for larvae of each age (Montgomery 1991).

Results

Effect of larval age and soil depth on infection

Larval age (instar) had a marked effect on infection (Figure 1). In all cases, 6-day-old larvae had significantly lower LC_{50} values than 8-day-old larvae. The effect of soil depth, however, was of far greater magnitude. The LC_{50} value calculated for insects in 5 cm of sand was approximately double that of insects in 2 cm of sand, whereas the LC_{50} values for insects in 8 cm of sand was approximately 10-fold greater than the values calculated for the 2 cm treatment. This pattern was the same for both 6- and

A



B

Age of larvae and depth of container (cm)	*N* (replicates)	Slope ± S.E.	Intercept ± S.E.	χ^2
6-day-old larvae				
2	535 (4)	1.27 ± 0.05	2.30 ± 0.19	1.96
5	801 (5)	1.05 ± 0.07	2.28 ± 0.21	3.84
8	650 (4)	0.71 ± 0.11	2.82 ± 0.35	1.15
8-day-old larvae				
2	547 (4)	1.93 ± 0.02	2.28 ± 0.40	3.98
5	750 (5)	1.31 ± 0.25	1.54 ± 0.32	3.52
8	820 (5)	2.35 ± 0.23	0.06 ± 0.009	2.78

Figure 1. Probit analysis of concentration-infection response of early (6-day-old) and late (8-day-old) stadium *Anastrepha obliqua* third instars by infective juveniles of *Steinernema carpocapsae* in sterilized sand of 10% moisture in tubes of 2, 5 and 8 cm depth. (A) Estimated LC_{50} values. Vertical bars indicate SE Columns headed by identical letters do not differ significantly for comparisons within each age group. (B) Table of number of replicates, regression slope, intercept and goodness-of-fit test (χ^2).

8-day-old larvae. Of the few dead larvae observed in the untreated control (1.6%), none were infected with nematodes as confirmed by dissection under microscope.

Effect of soil texture on infection

Soil type had a highly significant effect on infection ($F = 31.6$; df = 2, 72; $P < 0.001$) with the highest percentage of lethal infection observed in the sand–clay mixture (60–82% depending on depth) and far fewer infections in the loamy–sand soil (23–30%) in 6-day-old larvae (Figure 2a). An intermediate percentage of lethal infection was observed in sand. A very similar pattern was detected in the 8-day-old larvae (Figure 2b), except that the percentage of lethal infection was significantly lower than in

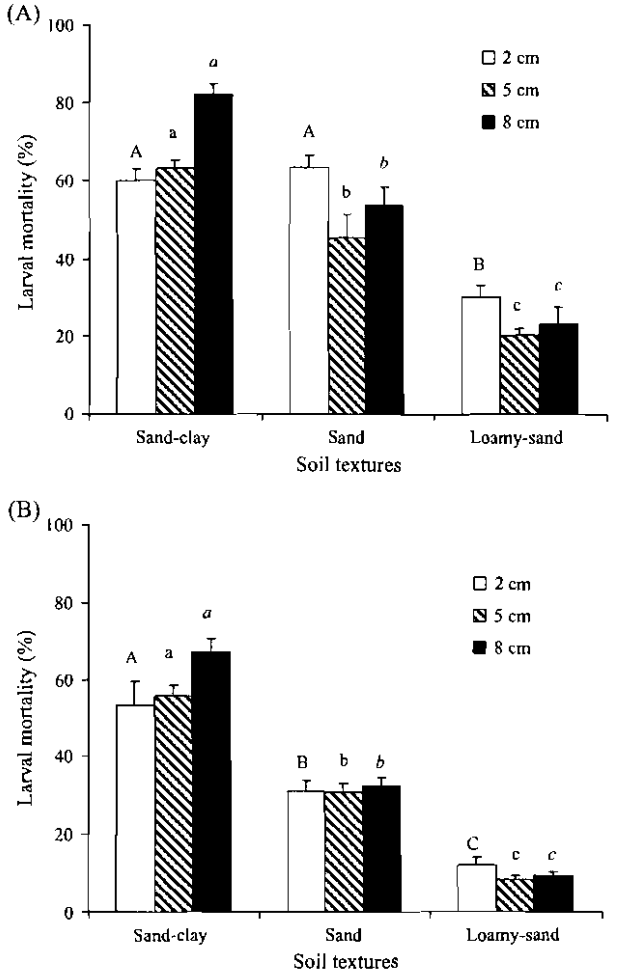


Figure 2. Mortality of (A) 6-day-old and (B) 8-day-old *Anastrepha obliqua* larvae infected by *Steinernema carpocapsae* in three types of sterilized soil in tubes of 2, 5 and 8 cm depth. Vertical bars indicate SE Columns headed by identical letters do not differ significantly for comparisons within each soil type (Tukey test, $P > 0.05$).

6-day-old larvae despite the fact that each had been inoculated with their respective LC_{50} concentrations of nematodes ($F=49.2$; df $=1$, 72; $P<0.001$). There was also a significant interaction between soil type and soil depth ($F=8.42$; df $=4$, 72; $P<0.001$); infection tended to increase with soil depth in sand–clay soil but decrease with soil depth in loamy–sand soil.

Effect of temperature on infection

Temperature had a marked positive effect on infection in 6-day-old larvae, being highest at 30°C and lowest at 19°C ($F=52.6$; df $=2$, 36; $P<0.001$) (Figure 3a). Infection was also affected by soil depth ($F=113.1$; df $=2$, 36; $P<0.001$) and by the interaction of these two factors ($F=8.05$; df $=4$, 36; $P<0.001$). Surface response analysis indicated that, for 6-day-old larvae, the optimal temperature for infection was 26.1°C, at a soil depth of 7.9 cm, that resulted in a predicted maximum infection rate of 91.4% (Figure 3a).

Compared to 6-day-old larvae, infection was markedly lower in 8-day-old larvae and required separate analyses to be performed. Temperature had a significant effect on infection of 8-day-old larvae being highest at 25°C at all soil depths tested ($F=123.3$; df $=2$, 36; $P<0.001$) (Figure 3b). At 19°C the percentage of lethal infection was particularly low (19–26% depending on soil depth). There was no significant effect of soil depth on infection ($F=0.84$; df $=2$, 36; $P=0.44$) and no significant interaction between temperature and soil depth in 8-day-old larvae ($F=2.46$; df $=4$, 36; $P=0.063$). Response surface analysis indicated that the optimum temperature for infection in 8-day-old larvae was 25.9°C at a depth of less than 2 cm, resulting in a predicted 61.2% infection, although the estimated maximum point was outside of the range of experimental values (Figure 3b).

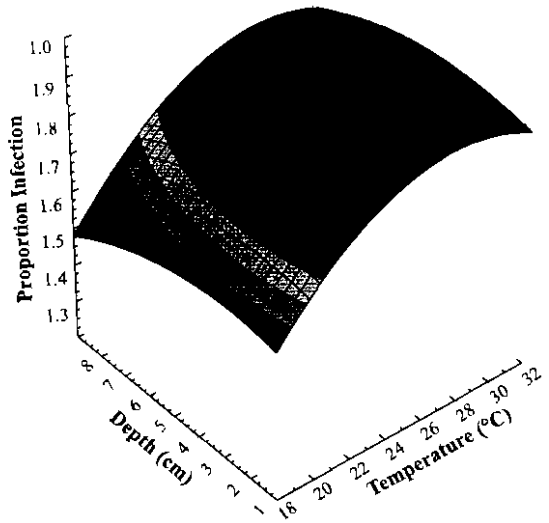
Discussion

Laboratory studies that mimicked a range of environmental conditions demonstrated the ability of *S. carpocapsae* to infect *A. obliqua* larvae. A marked difference was detected in the percentages of lethal infection of 6- and 8-day-old larvae that presumably reflects the duration of the period for which the host was susceptible to infection. Eight-day-old larvae pupate in 24 h and, following pupation, host penetration by the nematode infective stages becomes severely restricted or impossible (Woodring and Kaya 1988). In contrast, 6-day-old larvae were susceptible to nematode penetration for a period of approximately 72 h resulting in the elevated percentage of lethal infection that we observed. Moreover, young larvae are more active than conspecifics that are about to pupate and so the frequency of contact between nematode and host may have been greater for 6-day-old larvae.

Under field conditions, however, contact between soil-dwelling nematodes and 6-day-old larvae would be unlikely as *A. obliqua* larvae do not usually leave the fruit to pupate until the late third instar, at approximately 8-days-old. For a given concentration of nematodes, infection rates were substantially greater when host larvae were placed in 2 cm of sand compared to greater depths, suggesting that *A. obliqua* larvae have a probabilistic refuge from infection at greater soil depths or that nematode displacement efficiency during host search is negatively correlated with soil depth. Clearly, depth is directly related to the volume of soil in which IJs are

$z = -1.3843 + 0.1608\,x + 0.0097\,y + 0.0007\,xy - 0.0029\,x^2 - 0.0036\,y^2$

(A)



$z = -4.50519 + 0.41335\,x - 0.01291\,y - 0.00243\,xy - 0.0078\,x^2$

(B)

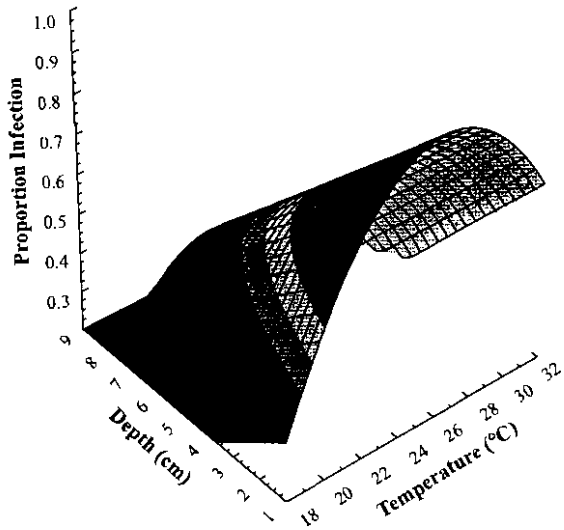


Figure 3. Multiple linear regression of temperature and soil depth on aresine transformed percentage of infection of (A) 6-day-old and (B) 8-day-old *Anastrepha obliqua* larvae by *Steinernema carpocapsae* in sterilized sand–clay soil at 19–30°C in tubes of 2–8 cm depth. Each second-order model fitted was subjected to response surface analysis (see text for details).

distributed, such that increasing depths will result in greater dilution of IJ stages. However, under field conditions, application rates for IJs are calculated based on soil surface area, in line with the methodology of the present study, mainly because the depth-distribution of hosts in the soil is usually poorly understood (Montoya, Flores, and Toledo 2008), and it is difficult to determine without laborious and time-consuming sampling procedures (Hodgson, Sivinski, Quintero, and Aluja 1998). In this respect, 8-day-old larvae may have been able to burrow deeper in the 8 cm tubes than 6-day-old larvae and therefore avoid infection; we currently have no information on the distribution of infected and non-infected insects in our experimental laboratory system and this is an issue that merits further study.

Nematode replication within the host was confirmed by direct observation in all experiments. However, in contrast to the high mortality of infective stages reported by Lindegren and Vail (1986), we observed large quantities of IJs emerging from infected hosts except in cases where the infected host managed to pupate and infective stages became trapped within the host puparium which acted as a barrier to the emergence of IJs.

It appears, therefore, that the environmental conditions employed in the present study were suitable for the multiplication and dissemination of nematode infective stages. Such conditions of temperature and moisture content are likely to be similar to those found in the soil beneath the tree canopy in mango orchards in the warm lowland tropical regions of Mexico (Yahia, Ornelas-Paz, and Ariza-Flores 2006), where daytime air temperatures are usually 30–35°C and under-canopy, soil temperatures usually fluctuate between 27 and 29°C during the fruiting period (J. Toledo, unpublished data).

Unexpectedly, nematode performance was higher in sand–clay soil than in sand alone. It is often suggested that nematodes disperse more readily in sandy soils than in other types of soil and that soil structure can have a significant influence on the degree of control of soil-dwelling insects achieved by application of nematodes (Georgis and Poinar 1983). Hence moisture content, or perhaps more importantly the free water present around and between each soil particle, is an important factor affecting the performance of IJs. Although the reason for the higher infection in sand–clay soil compared to sand alone is not immediately apparent, it may be related to the moisture-retaining properties of the clay component that in turn improved nematode survival and displacement during the experimental period (Moyle and Kaya 1981). The dispersal capacity of *S. carpocapsae* is well recognized (Lewis, Campbell, Griffin, Kaya, and Peters 2006); infective stages can disperse up to 14 cm horizontally and up to 12 cm vertically when soil conditions are suitable and host-related stimuli are present (Georgis and Poinar 1983). Soil moisture affects the depth at which larvae pupate and the survival of immature tephritids (Hodgson et al. 1998; Aluja, Sivinski, Rull, and Hodgson 2005; Montoya et al. 2008), although there are conflicting reports on the relationship between soil moisture and depth of pupation in some species (Jackson, Long, and Klungness 1998; Hou, Xie, and Zhang 2006).

Lindegren et al. (1990) previously reported a 90% reduction in adult emergence of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) following application of a high concentration of *S. feltiae* (500 IJs/cm^2) in a vermiculite substrate. The density of infective stages required to achieve a similar percentage of lethal infection in sand–clay soil observed in the present study was markedly reduced compared to the values reported by Lindegren et al. (1990), albeit with a different methodology. In

contrast, the performance of *S. carpocapsae* in loamy–sand was poor. The low efficiency IJs in loamy–sand soil is unlikely to be related to the presence of antagonistic organisms or harmful microbial metabolites (Kaya 1990), as all soils were washed and sterilized prior to use. However, there may have been some effect of the high content of organic material in the loamy–sand soil that could have impeded the movement of IJs in this type of soil. Comparable with the findings of the present study, infection of *A. ludens* (Loew) by *S. carpocapsae* Weiser and *S. riobrave* Cabanillas, Poinar and Roulston were highest in sandy–loam soil compared to loam or clay soils (Lezama-Gutiérrez et al. 2006), indicating that the sand component of soil directly or indirectly favors the activity of steinernematids.

Temperature had a clear effect on the activity of *S. carpocapsae* being highest at 25–30°C and lowest at 19°C, presumably because cool temperatures reduced the metabolic rate and consequently the mobility of the nematode. These results compare favourably with those of Yee and Lacey (2003) who observed maximum infection rates by *S. feltiae* at 25–27°C in *Rhagoletis indifferens* Curran larvae exposed to IJs, whereas others have noted that temperatures approaching or exceeding 30°C may be detrimental to IJ efficacy and survival in different host species (Gray and Johnson 1983).

The commercial applications of entomopathogenic nematodes as control agents for a diversity of insect pests is due to a number of unique characteristics including their ability to kill quickly, host search and location behaviour, relatively simple mass-production processes, combined with their excellent environmental safety record and the fact that they can be applied using conventional technology (Kaya 1985; Poinar 1990). The environmental conditions of mango production appear to be compatible with the characteristics required by this nematode to attain high levels of infection in *A. obliqua*, and may also have potential in the control of other tropical fruit tree pests such as citrus root weevils, *Pachnaeus litus* (Germer) and *Diaprepes abbreviatus* L. (Bullock, Pelosi, and Killer 1999). The results of the present study lead us to predict that *S. carpocapsae* may be a useful agent for control of fruit fly pests, especially in tropical ecosystems with warm temperatures and high levels of soil moisture, although this assertion requires confirmation in field tests that are currently in progress.

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