

Quantification of Invasion of Two Strains of *Steinernema carpocapsae* (Weiser) into Three Lepidopteran Larvae¹

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Abstract: Studies with last instar larvae of the fall armyworm, *Spodoptera frugiperda* (J. E. Smith), the black cutworm, *Agrotis ipsilon* (Hufnagel), and the greater wax moth, *Galleria mellonella* (L.) were used to quantify the invasive ability of two strains (All and Mexican) of *Steinernema carpocapsae* and to determine how factors in the bioassay procedure affect both nematode invasion and host mortality. Nematode invasive ability was variable, with 10–50% of nematodes successfully infecting the host. The percentage of infectives invading the host (invasion efficiency) was positively related to increases in length of host exposure time and number of hosts per arena, negatively related to increases in substrate surface area per host, and not affected by nematode concentration. There was a direct relationship between concentration applied and the number of nematodes invading the host. Mortality was less affected than invasion efficiency by bioassay conditions and appears to be a much less sensitive index of nematode activity than invasive ability.

Key words: *Agrotis ipsilon*, efficacy, entomogenous nematode, *Galleria mellonella*, invasive ability, nematode, *Spodoptera frugiperda*.

The entomogenous nematode, *Steinernema carpocapsae* (Weiser), has great promise for use as a biological control agent, and a number of insect species are susceptible to infective-stage nematodes (infectives) in laboratory trials (18,21). However, efficacy, as indicated by LC₅₀, varies greatly both among different nematode strains tested against a single target insect and among different insects tested with a single nematode strain (1,22). Abiotic factors are well known to affect nematode performance, and suboptimal environmental conditions are deleterious to nematode field efficacy (9). Less is known about the biological basis for differences in the level of host mortality. Dunphy and Webster (4) found that differential virulence between the DD-136 strain and the Mexican strain of *S. carpocapsae* against larvae of the greater wax moth, *Galleria mellonella* (L.), was not due to the virulence of the nematode–symbiotic bacteria complex per se, or to the host internal immune response. They speculated that the difference may be in the abilities of the two strains to invade the host.

The model for the nematode–host interaction is based primarily on infection of greater wax moth larvae by DD-136 strain infectives (23). These nematodes enter the host through natural body openings and then move from the alimentary tract into the hemocoel. A similar invasion route was observed with DD-136 strain in the Formosan termite, *Coptotermes formosanus* Shiraki, although entrance into that host was primarily through the anal opening (7). Both oral and anal openings were used by this nematode to enter larvae of the cutworm *Spodoptera litura* (17). Few studies have quantified the number of nematodes that successfully invade the host, much less determined how variable this is between hosts or under different experimental conditions.

Larvae of the fall armyworm, *Spodoptera frugiperda* (J. E. Smith), and the black cutworm, *Agrotis ipsilon* (Hufnagel), are susceptible to infection by *S. carpocapsae* in both laboratory and field trials (2,8,25). The LC₅₀ estimates from laboratory bioassays with last instar fall armyworm and black cutworm larvae have shown that black cutworm larvae are significantly more resistant to both the Mexican strain and the All strain of *S. carpocapsae* (Epsky, unpubl. data). There is a 10-fold increase in LC₅₀ estimate from the most susceptible host–nematode combination (fall armyworm–*S. carpocapsae* Mexican strain) to the

Received for publication 14 April 1992.

¹ Florida Agricultural Experiment Station Journal Series No. R-02270.

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least susceptible (black cutworm—*S. carpocapsae* All strain). Therefore, these two host–nematode combinations were used to quantify invasive ability of the infectives, to determine how factors in the bioassay procedure affect both nematode invasion and host mortality, and to ascertain if these effects varied in hosts with different levels of susceptibility. For comparative purposes, additional studies were conducted with greater wax moth—*S. carpocapsae* All strain.

MATERIALS AND METHODS

Insects and nematodes

Fall armyworm and greater wax moth larvae were obtained from the Insect Attractants, Behavior and Basic Biology Research Laboratory, USDA, Gainesville, Florida. A colony of the black cutworm was obtained from J. C. Reese, Kansas State University, and cultured (24). *Steinernema carpocapsae* Mexican strain and All strain nematodes were obtained from G. C. Smart, Jr., Dept. of Entomology and Nematology, University of Florida, Gainesville, Florida. These were reared in vivo in greater wax moth larvae (5). Infectives were stored in deionized water and held at 6 C until use within 1 month of collection from the host cadaver. Fall armyworm were tested with *S. carpocapsae* Mexican strain infectives; black cutworm and greater wax moth were tested with *S. carpocapsae* All strain infectives.

Bioassay procedure

Last instar larvae were used for all studies. Larvae were exposed to nematodes in petri dish bioassays (30). Arenas used for this study included small (35 × 10 mm), medium (60 × 15 mm), and large (100 × 15 mm) petri dishes with two pieces of filter paper (Whatman #1; 2.5, 5.5, 9.0 cm d, respectively). Infective nematodes were added in 0.2, 1.0 and 2.0 ml deionized water, respectively. Unless otherwise stated, individual larvae were tested in medium arenas and groups of larvae were tested in large arenas. The insects were added to the bioassay arenas 1 hour after the infectives. Arenas were enclosed in large plastic

bags to minimize loss of moisture and held at 25 C. Mortality among control insects was rare because larvae had completed most of their feeding. Therefore, separate controls for mortality were not used. Number of nematodes invading the host was determined by dissection of hosts 24–96 hours after initial exposure to infectives (14). The exact time period until host dissection varied among the different experiments. Invasion efficiency, that is, the percentage of the infective stage nematodes that successfully invaded the host and, in dissections made after host death, completed transformation to the free-living stage (summed from all hosts in trials with multiple hosts per arena), was calculated from the number of nematodes recovered from dissection divided by the number of nematodes per treatment, multiplied by 100. Accuracy of the dissection procedure was checked with a two-step sampling procedure in preliminary experiments. Some sampled hosts were rinsed and dissected immediately; others were rinsed and moved to nematode-free arenas and dissected 24–48 hours after host death. Comparison of number of internal nematodes early and late in the infection cycle indicated that essentially all infectives that invaded the host successfully initiated development in the hosts. Therefore, number of nematodes recovered from dissection was assumed to equal number of nematodes that invaded the host.

Effects of number of hosts per arena: The LC₅₀ and invasion efficiency were determined for one and three fall armyworm per arena. The substrate surface area per host was kept approximately equal to maintain a constant host density. Infective concentration treatments were 0, 10, 20, and 40 infectives per host, and there were 160 larvae tested (40 replicates of one larva per infective concentration [40 × 1 × 4 larvae]) and 468 larvae tested (39 replicates of three larvae per infective concentration [39 × 3 × 4 larvae]). Mortality was recorded after 48 hours, and cadavers of larvae from the 20- and 40-infective treatments were dissected after 72 hours.

Black cutworm larvae are cannibalistic, so greater wax moth larvae were used to determine the effect of multiple hosts per arena for studies with the All strain infectives. The LC_{50} was determined for 1 and 10 greater wax moth per arena. The latter follows the standard LC_{50} bioassay procedure (30) and represents an increase in host density per arena. A total of 65 larvae (one larva per arena) were exposed to concentrations of 1, 10, and 50 infectives per host (30, 20, and 15 larvae per infective concentration, respectively), and 60 larvae (10 larvae per arena) were exposed to concentrations of 1, 5, and 10 infectives (20 larvae per infective concentration). Mortality was recorded after 48 hours. Effect of greater wax moth host density on invasion efficiency was tested in medium arenas. Treatments were 1 infective versus 1 larva, 10 infectives versus 1 larva, 50 infectives versus 1 larva, and 50 infectives versus 5 larvae. There were 60, 10, 10, and 10 replicates, respectively, for a total of 130 larvae tested [$60 \times 1 + 10 \times 1 + 10 \times 1 + 10 \times 5$ larvae]. These treatment levels allowed comparisons between a constant number of nematodes per host (10-on-1 versus 50-on-5), between a constant number of nematodes per bioassay arena (50-on-1 versus 50-on-5), and between a varying number of nematodes per host or arena. Preliminary experiments indicated that a concurrent decrease in substrate surface area per host is partially responsible for the effect observed when host density is increased. Therefore, a one infective versus one larva treatment was tested in a small arena to determine the effect of a decrease in substrate surface area per host, alone. There were 25 replicates, and mortality was recorded after 48 and 72 hours. The one-on-one treatments evaluated the invasive ability of individual nematodes (19), and invasion efficiency was considered equal to percentage mortality. Invasion efficiency was determined from dissection of cadavers 24 hours after death for larvae from the 10- and 50-nematode treatments.

Effect of host exposure period: Individual

fall armyworms were exposed to treatments of 50 and 100 infectives, with 35 larvae per infective concentration (a total of 70 larvae tested), and individual black cutworms were tested with single treatment of 100 infectives, with 160 larvae tested. Mortality was recorded daily. Ten to 20 larvae were sampled after host exposure periods of 24, 48, 72, and 96 hours. Sampled larvae were rinsed and dissected immediately. At each sampling time, 10 additional black cutworms were rinsed and moved to nematode-free arenas. These larvae and the remaining unsampled black cutworm (host exposure time ≥ 120 hours) were dissected 24 hours after death. Black cutworm that were still alive after 10 days were not dissected.

Effect of concentration: Individual fall armyworm and black cutworm larvae were exposed to treatments of 10, 50, 100, 250, and 500 infectives. There were 100 fall armyworm tested (30, 20, 10, 10, and 10 per concentration, respectively) and 90 black cutworm tested (20, 20, 30, 10, and 10 per concentration, respectively). Mortality was recorded after 48 hours and cadavers were dissected after 72 hours.

Statistical analysis

PCPOLO (26) probit analysis was used to estimate LC_{50} and slope and to compare statistically the effect of multiple hosts per arena on nematode efficacy. Invasion efficiency was analyzed by factorial analysis of variance (ANOVA) with Proc GLM (27) to determine the significance of the main effects and to test for interaction between main effects. Results from significant ANOVAs were followed by Duncan's (3) multiple-range test ($P = 0.05$) for all tests, except for the study on host density effects in the greater wax moth. Although there were three factors tested (concentration, number of hosts per arena, and substrate surface area), all factors were not tested at all levels. Therefore, one-way ANOVA was run on the five treatments (31). Tukey's test ($P = 0.05$) was used for one-at-a-time, pair-wise comparisons between all treatments. For the single-factor exper-

iments on effect of concentration, factorial analysis was followed by regression analysis using Proc REG (27) to test the adequacy of a linear model to describe the relationship between concentration and number of nematodes invading the host.

RESULTS AND DISCUSSION

Effect of number of hosts per arena: Average percentage mortality appeared slightly higher for fall armyworm tested in groups of three than for fall armyworm tested individually, for each concentration tested (10 nematodes per host, 66.7% versus 52.0%; 20 nematodes per host, 74.4% versus 72.5%; and 40 nematodes per host, 89.7% versus 80.0%). However, there were no significant differences in either LC_{50} estimate or slope obtained from probit analysis. The LC_{50} estimate (95% CL) for fall armyworm tested in groups of three larvae was 5.2 (1.0–9.2) and for larvae tested individually was 8.3 (3.6–12.4). Both had slopes of 1.3 ± 0.5 . Similarly, the LC_{50} estimate and slope were not affected by greater wax moth host density. The LC_{50} estimate (95% CL) for greater wax moth tested individually was 4.0 (1.8–9.1) and for greater wax moth tested in groups of 10 was 3.2 (1.7–5.8). Slopes were 1.5 ± 0.3 and 1.2 ± 0.3 , respectively. Molyneux et al. (20) cite unpublished data that stress the importance of testing one individual per bioassay unit, because the number of hosts per container affects the level of parasitization. No further details were given. In our studies, LC_{50} estimates were increased slightly, but not significantly, by a decrease in number of hosts per arena.

Invasion efficiency was significantly affected by the presence of multiple fall armyworm in the arena ($F = 37.5$; $df = 1, 32$; $P < 0.01$) and increased from $14 \pm 2\%$ to $37 \pm 3\%$ as the number of hosts in the arena increased from one to three. Nematode concentration did not affect invasion efficiency ($F = 0.01$; $df = 1, 32$; $P = 0.99$), and there was no interaction between the effects of concentration and number of hosts per arena at the levels tested ($F = 1.62$; $df = 1, 32$; $P = 0.21$).

Invasion efficiency was also significantly affected in the greater wax moth trials by the concentration–host density (or substrate surface area) treatment ($F = 6.67$; $df = 4, 26$; $P < 0.01$) (Fig. 1). All greater wax moth mortality occurred by 48 hours in the treatments with 10 or 50 infectives, but mortality increased between 48 and 72 hours in the one-on-one treatments. After 48 hours, there was 9% and 20% mortality in the one-on-one treatment in medium and small arenas, respectively. However, results of the mean comparison test remained the same whether using the data from 48 or 72 hours. The invasion efficiency in the 50-on-5 treatment was significantly higher than invasion efficiencies in the 1-on-1 in medium arena, 10-on-1 and 50-on-1 treatments.

The increase in invasion efficiency observed could be explained by an increase in number of hosts alone (e.g., fall armyworm trials), and (or) by the decrease in substrate surface area per host (e.g., greater wax moth trials). Kondo and Ishibashi (15) found that invasion efficiency decreased as depth of soil in the bioassay arena increased. This increase in substrate volume would increase the distance the in-

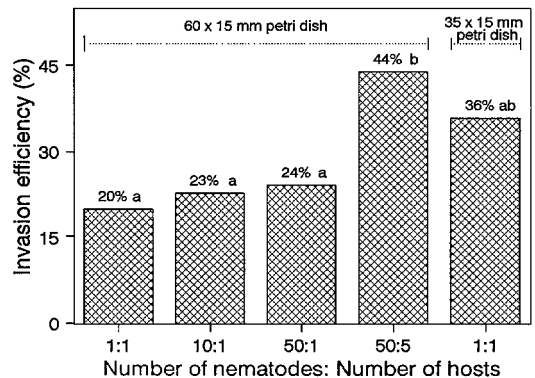


FIG. 1. Comparison of dose (1, 10, or 50 nematodes), host density (one or five per bioassay arena) and substrate surface area treatments on the percentage of applied *Steinernema carpocapsae* All strain nematodes that established in greater wax moth larvae exposed to infectives in petri dish bioassays. Means followed by the same letter are not significantly different (Tukey's test, $P = 0.05$). Data are from individuals that died within 48 hours (dose = 10 or 50 nematodes) or 72 hours (dose = 1 nematode) of exposure to infectives.

fective would have to traverse to locate the host. *Steinernema carpocapsae* infectives have demonstrated poor host-finding ability (10) and may remain inactive until stimulated by the presence of a host (13). Host products have been shown to mediate host-finding activity (28,29). Thus, an increase in the concentration of host cues from multiple hosts in a bioassay or by a decrease in substrate surface area/volume per host may stimulate an increase in host-finding and invasion.

Effect of host exposure period: Length of host exposure period had no effect on mortality for either fall armyworm or black cutworm larvae. All the fall armyworm larvae were dead within 48 hours, and 57% of the black cutworm larvae were dead within 10 days, but percentage mortality for each species was consistent among all exposure periods tested. Time until host death has long been recognized as an important aspect of LC_{50} bioassays. Although maximum host mortality usually occurs within 48 hours in larvae of the greater wax moth, it may take longer in other hosts. Capinera et al. (2) found that high levels of black cutworm mortality occurred within 24 hours of contact with the Kapow strain of *S. carpocapsae*, but not until 72 hours with two other nematodes tested. Estimates of LC_{50} made at 48 hours are often higher than measurements made after 96 hours (28). However, the low mortality in black cutworm after 48 hours was not overcome by lengthening the period of exposure to infectives.

Nematode invasion efficiency was significantly affected by fall armyworm exposure period ($F = 16.66$; $df = 3, 62$; $P < 0.01$), with an increase in invasion as exposure period increased (Table 1). There was no effect due to concentration ($F = 1.9$; $df = 1, 62$; $P = 0.17$), nor was there an interaction between concentration and exposure time ($F = 2.12$; $df = 3, 62$; $P = 0.11$). Invasion efficiency into black cutworm ranged from an average of $2.2\% \pm 1.0$ after 24 hours to $6.3\% \pm 1.9$ after 96 hours, but the differences were not significant ($F = 1.36$; $df = 4, 21$; $P = 0.28$). Black cut-

TABLE 1. Effect of host exposure period on invasion efficiency of *Steinernema carpocapsae* Mexican strain infectives in a petri dish bioassay with fall armyworm larvae.

Host exposure period (hours)	n	Invasion efficiency (%)
24	20	5.7 ± 1.2 a
48	20	18.0 ± 2.4 b
72	20	24.2 ± 2.6 bc
96	10	26.3 ± 3.0 c

Means \pm standard error followed by the same letter are not significantly different ($P = 0.05$) by Duncan's multiple range test.

worms that were dead by 48 hours averaged approximately five internal nematodes, larvae that died between 48 to 96 hours averaged one internal nematode, and few individuals that died after 96 hours contained nematodes.

Within the first 24 hours of contact, Kondo and Ishibashi (16) found a positive correlation between the length of time *S. litura* larvae were exposed to *S. carpocapsae* DD-136 strain and both the host mortality and the number of nematodes invading the host. Similar increases were found within the first 12 hours of contact with larvae of the lepidopteran *Spodoptera litoralis* Boisduval and for both *S. carpocapsae* All strain and Mexican strain infectives (11). For the fall armyworm and black cutworm larvae examined in our study, infectives were found in the host by 24 hours and most host mortality occurred by 48 hours. Number of nematodes per host increased from 24 to 72 hours, and leveled off after 72 hours. Although greater numbers of Mexican strain infectives invaded fall armyworm over time, the period of maximum invasion was similar for both host-nematode combinations.

Effect of concentration: Percentage mortality was affected by nematode concentration, and increased from 36.7% at the 10-nematode concentration to 90% at the 250-nematode concentration after 48 hours in the fall armyworm, and from 10% at the 10-nematode concentration to 90% at the 250-nematode concentration after 48 hours in the black cutworm. Nematode

concentration, however, did not significantly affect invasion efficiency in the fall armyworm ($F = 1.75$; $df = 4, 42$; $P = 0.17$) or the black cutworm ($F = 0.54$; $df = 4, 67$; $P = 0.70$). Average invasion efficiency per concentration varied greatly, and ranged from $8.6\% \pm 2.4$ at the 100-nematode concentration to $16.2\% \pm 2.4$ at the 500-nematode concentration in the fall armyworm, and from $14.8\% \pm 1.5$ at the 500-nematode concentration to $21.3\% \pm 3.4$ at the 250-nematode concentration in the black cutworm. There was a direct relationship between number of nematodes applied and number invading the host, and it was best fit by a simple linear regression model (Fig. 2). After square-root transformation of the number of invading nematodes (to stabilize the variance), the fit of the data from the fall armyworm improved ($y = 1.14 + 0.016x$; $r^2 = 0.819$), but the fit of data from the black cutworm was unchanged. A direct relationship was also

observed in the greater wax moth larvae (12).

Although there were no significant differences among measurements of invasion efficiency determined at different concentrations, there was a great deal of variability in average percentage invasion that may make comparisons between different host and nematode combinations difficult. Hominick and Reid (12) suggested that a "dose/establishment bioassay," which uses the slope of the regression line to indicate the proportion of infectives that invade the host, be used for comparative studies. Results of our study support his suggestion and show that nematode invasion into a host is very sensitive to bioassay conditions, probably more so than host mortality. Therefore, care should be taken in choosing the bioassay conditions for comparative studies so that differences obtained result from the host-parasite interaction, not bioassay effects.

In summary, nematode invasive ability was generally poor, with 10–50% of applied nematodes successfully infecting the host. Number of infectives invading the host was significantly affected by the bioassay conditions. Invasion efficiency was positively related to increases in length of host exposure period and number of hosts per arena and negatively related to increases in substrate surface area per host. Changes in the bioassay conditions had less effect on mortality; mortality appears to be a relatively insensitive index of nematode activity.

The invasive abilities reported in this paper were made under what should be optimal conditions for infection. In such laboratory trials, there were few of the environmental constraints that limit efficacy in field applications. Clearly, other factors of the host or the nematode population are present that limit successful attack, even under optimal laboratory conditions. One possibility is that some infectives enter a quiescent phase upon emergence from the host cadaver (13) and may not enter an active host-seeking phase for several months (6). Further studies are needed to

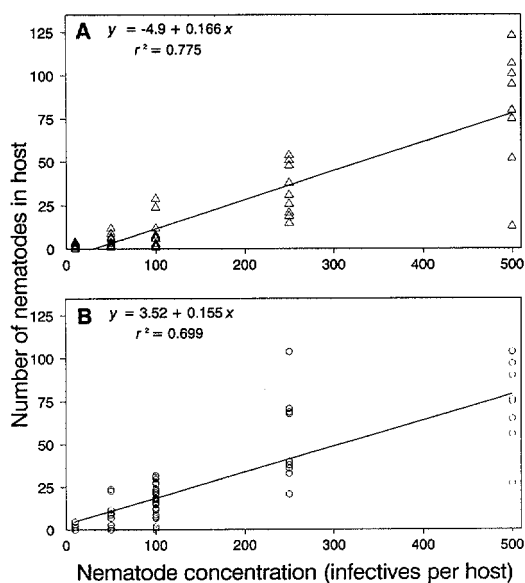


FIG. 2. Scatterplot and linear regression of nematode concentration and the number of A) *Steinernema carpocapsae* Mexican strain nematodes that established in individual fall armyworm larvae exposed to infectives in petri dish bioassays ($n = 59$) and B) *Steinernema carpocapsae* All strain nematodes that established in individual black cutworm larvae exposed to infectives in petri dish bioassays ($n = 72$). Data are from individuals that died within 48 hours of exposure to infectives.

understand factors that limit both host susceptibility and nematode invasion. An understanding of the invasive ability of the nematode and the bioassay operational constraints may provide the key to differentiating these factors.

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