

## Optimization of Inoculation for In Vivo Production of Entomopathogenic Nematodes

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**Abstract:** Entomopathogenic nematodes are potent biopesticides that can be mass-produced by in vitro or in vivo methods. For in vivo production, consistently high infection rates are critical to efficiency of the process. Our objective was to optimize in vivo inoculation of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* in *Galleria mellonella* and *Tenebrio molitor* by determining effects of inoculation method, nematode concentration, and host density. We found immersing hosts in a nematode suspension to be approximately four times more efficient in time than pipeting inoculum onto the hosts. The number of hosts exhibiting signs of nematode infection increased with nematode concentration and decreased with host density per unit area. This is the first report indicating an effect of host density on inoculation efficiency. We did not detect an effect of nematode inoculum concentration on nematode yield per host or per gram of host. Yield was affected by host density in one of the four nematode-host combinations (*S. carpocapsae* and *T. molitor*). We conclude that optimization of inoculation parameters is a necessary component of developing an in vivo production system for entomopathogenic nematodes.

**Key words:** culture, *Heterorhabditis bacteriophora*, in vivo, production, *Steinernema carpocapsae*.

Entomopathogenic nematodes (*Steinernema* and *Heterorhabditis*) are biopesticides capable of controlling a variety of economically important insect pests (Klein, 1990; Shapiro-Ilan et al., 2002). These nematodes are obligate parasites of insects that kill their hosts with the aid of bacteria carried in the nematode's alimentary canal (Poinar, 1990). The third-stage infective juvenile nematode, the only free-living stage, enters the host via natural openings, i.e., mouth, anus, spiracles (Poinar, 1990), or occasionally through the insect cuticle (Bedding and Molyneux, 1982). The nematodes then release their symbiotic bacteria, which are the primary agents responsible for killing the host within 24 to 72 hours. After the nematodes complete one to three generations within the insect cadaver, infective juveniles exit to find new hosts (Poinar, 1990). These nematodes possess a number of attractive qualities as biocontrol agents including a durable infective stage, host-seeking ability, safety to mammals and other nontarget organisms, and suitability to mass production (Kaya and Gaugler, 1993).

Entomopathogenic nematodes can be mass-produced using in vivo or in vitro (solid or liquid) culture methods (Friedman, 1990; Gaugler and Han, 2002; Shapiro-Ilan and Gaugler, 2002). Although in vitro production has the advantage of economy of scale (Friedman, 1990), in vivo culture is still essential to numerous scientific and industrial interests. Relative to the other methods, in vivo production requires less capital and technical expertise. In vivo production is therefore the

method of choice for laboratory-scale production (e.g., for generating material for field trials) and among the many small nematode-producing companies (cottage industry); in vivo production is also arguably the most appropriate technology for grower cooperatives and for developing countries where labor is less expensive (Gaugler et al., 2000; Gaugler and Han, 2002).

In vivo entomopathogenic nematode production is a two-dimensional process, which translates into a system of shelves and trays (Friedman, 1990). In vivo production has been described by a number of authors (Dutky et al., 1964; Flanders et al., 1996; Kaya and Stock, 1997; Lindegren et al., 1993; Poinar, 1979; Woodring and Kaya, 1988), all of which use the White trap (White, 1927) as a basis for the method. In general, insects are inoculated on a tray or dish lined with an absorbent substrate, and after 2 to 7 days infected cadavers are moved to a harvest dish (White trap). For example, infective juveniles emerge into water from infected cadavers placed on moist filter paper in an inverted petri dish lid, which is held in a larger petri dish containing water. Recently, an alternative approach has been developed (Gaugler and Brown, 2001; Gaugler et al., 2002), which reduces labor by not requiring transfer of infected cadavers from one dish to another, and increases harvest efficiency by not requiring the infective juveniles to migrate to a water trap (instead, the infective juveniles are washed down with an overhead misting system).

The most common and most studied insect host used for entomopathogenic nematode culture is the last instar of the greater wax moth, *Galleria mellonella* (L.), because of its high susceptibility to most nematodes, wide availability (at least in the United States, where it is sold commonly for pet food or fish bait), ease in rearing, and high yields (Shapiro-Ilan and Gaugler, 2002; Woodring and Kaya, 1988). The mealworm, *Tenebrio molitor* L., also has been advocated as a host for nematode production, but few studies have addressed its relative suitability (Blinova and Ivanova, 1987; Shapiro-Ilan and Gaugler, 2002).

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A critical step in *in vivo* nematode production is inoculation. Maximizing the percentage of patent infections will increase production efficiency (Shapiro-Ilan and Gaugler, 2002). A patent infection is the invasion of a pathogen into a host, resulting in signs and symptoms distinct for that particular disease (Lacey and Brooks, 1997). For entomopathogenic nematodes, the most characteristic sign is a change of color in the infected host, i.e., tan for *S. carpocapsae* (Weiser) and reddish for *H. bacteriophora* Poinar (Woodring and Kaya, 1988). Hosts that do not exhibit characteristic color changes (e.g., those that turn black and become putrid) are contaminated with other microbes and should be removed because they may contaminate the rest of the batch (Woodring and Kaya, 1988). Additionally, non-infected live hosts must be removed. The removal of non-patent infections and live hosts can be costly in terms of labor. Therefore, parameters that affect infection levels should be optimized.

Factors that can affect inoculation include method of inoculation, nematode concentration, and host density (Shapiro-Ilan and Gaugler, 2002). Inoculation can be accomplished by applying nematodes (e.g., by pipet) to an absorbent substrate on which insects are added, by immersing the hosts in a nematode suspension or, in some cases, by applying the nematodes to the insect's food (Shapiro-Ilan and Gaugler, 2002). Inoculation methods may differ in labor (time) requirements or in the ability to cause infection. Previously, comparisons of inoculation methods have been minimally addressed in only one study (Blinova and Ivanova, 1987).

The concentration of nematodes exposed to hosts during inoculation clearly has been shown to be positively related to the resulting number of infected insects (e.g., Blinova and Ivanova, 1987; Flanders et al., 1996; Shapiro et al., 1999). However, it has also been suggested that too high a concentration results in increased contamination and decreased nematode infection or yield (Woodring and Kaya, 1988). Furthermore, reports on the effects of nematode concentration on yield have varied (Boff et al., 2000; Elawad et al., 2001; Flanders et al., 1996; Zervos et al., 1991), indicating there is a need to investigate this question further. Selvan et al. (1993) reported that an intermediate number of nematodes inside the host results in maximum yield per host. The number of nematodes entering a host is positively related to the number to which the host is exposed to (Epsky and Capineria, 1993). Therefore, we hypothesized that yield per host will also be maximized at an intermediate nematode inoculum concentration.

Effects of host density per unit area on nematode yield have rarely been addressed. Flanders et al. (1996) did not observe effects of host density on nematode yield. Yet, due to the nematode's requirement for oxygen (Burman and Pye, 1980), one would expect that overcrowding of hosts could lead to reduced production; thus, we hypothesized that yield per host will be

maximized at intermediate host densities (at the greatest density that does not have overriding crowding effects).

Previous studies on inoculation parameters have concentrated only on a single nematode species or a single host and have been limited in scale (i.e., laboratory scale rather than production scale). More in-depth studies on inoculation parameters for *in vivo* production are warranted. Our objective was to optimize inoculation of *S. carpocapsae* and *H. bacteriophora* in *G. mellonella* and *T. molitor* by determining effects of inoculation method, nematode concentration, and host density. We focused on *S. carpocapsae* and *H. bacteriophora* because they are the most widely available and used species of commercially produced nematodes (Gaugler et al., 2000). Our experiments were conducted at a scale we deemed relevant to small-scale commercial or large-scale laboratory production (e.g., for generating field trial material).

## MATERIALS AND METHODS

*Nematodes and insects:* Nematode inocula for all experiments (*S. carpocapsae*, All strain, and *H. bacteriophora*, Hb strain) were reared on last instar *G. mellonella* according to procedures described by Woodring and Kaya (1988). *Galleria mellonella* for nematode culture and experiments were obtained from Sunfish Bait Co. (Webster, WI); *T. molitor* were obtained from Southeastern Insectaries, Inc. (Perry, GA).

*Comparison of inoculation methods:* Time efficiency of application of nematodes by pipet was compared with immersion of hosts in a nematode suspension. For the immersion method, 40 g of *G. mellonella* (ca. 200 insect larvae) were submerged for about 2 seconds in 300 ml of nematode inoculum (8,000 *S. carpocapsae* infective juveniles per ml) in a 500-ml plastic beaker. The hosts were then deposited into a metal sieve to allow excess inoculum to drain, and then onto a perforated metal tray (24.5 × 29.5 × 3.5 cm with 1.6-mm holes leaving 30% open area). These trays were designed for the LOTEK *in vivo* production system (Gaugler et al., 2002). The holes are designed to retain hosts yet permit the passage of harvested nematodes through rinsing (Gaugler et al., 2002). For the pipet method, the insect hosts were placed on metal trays (as used for immersion) lined with paper towels (one sheet thick), and then 11 ml of nematode inoculum was pipeted evenly onto the tray (11 ml was the minimum amount needed to cover the entire surface area). The time required to complete inoculation was recorded for each method with three replicates each. The experiment was organized in a completely randomized design. It may appear obvious that the immersion method would be more time-efficient than pipeting, but the question had to be demonstrated experimentally to justify which method to use in subsequent experiments.

*Effects of nematode concentration:* The results of the previous experiment indicated that immersion was more time-efficient than pipeting; and thus, immersion was used in the nematode concentration experiments. Hosts were immersed in 300 ml of inoculum and deposited on perforated metal trays, as described above. Based on preliminary testing, a range of concentrations was used for each nematode-host combination. Concentration regimes for both nematode species for *G. mellonella* were 1,000, 4,000, 8,000, and 12,000 infective juveniles per ml. Concentrations for *T. molitor* were 3,500, 7,000, 14,000, and 21,000 per ml for *S. carpocapsae* and 7,000, 14,000, 21,000, and 28,000 for *H. bacteriophora*. We calculated that approximately  $0.12 \pm 0.01$  and  $38.1 \pm 4.1$  ul of inoculum adhered to each *T. molitor* larva and *G. mellonella* larva, respectively. These quantities were calculated by averaging the amount of inoculum loss after each of four (replicate) immersion inoculations and dividing by the number of insects per inoculation.

The rationale behind choosing these concentration regimes was to include four levels, ranging from a concentration that would fail to produce an acceptable percentage of patent infections to at least one concentration that would produce an acceptable percentage of patent infections. We considered hosts that exhibited the characteristic color changes of patent infections. Each producer will need to decide what percentage of patent infections is optimum economically. For the purpose of this study, we designated 90% patent infections in the hosts to be acceptable.

There were three replicates of each treatment (concentration and nematode-host combination), and each experiment was repeated once (two trials). Experiments testing concentration effects for each nematode-host combination were conducted separately. Following inoculation, the trays from each replicate were suspended above a shallow (ca. 1-cm) layer of water (to create 100% relative humidity) in large, lidded plastic containers ( $76.2 \times 30.5 \times 12.7$  cm) and incubated at approximately 25 °C. After 5 days of incubation, the percentage of patent infections was recorded. Five patent-infected insects from each tray (15 per treatment per trial) were randomly chosen and placed individually on White traps (50-mm petri dish lid inside a 90-mm petri dish); total number of infective juveniles produced per insect was recorded after emergence ceased or became negligible (approximately 2 weeks after initial emergence) (Shapiro et al., 1999). These experiments (and those described subsequently) were organized in randomized complete-block designs (blocked by container).

The immersion method, described above, achieved acceptable ( $\geq 90\%$ ) levels of patent infections for all insect-nematode combinations except *H. bacteriophora* inoculation of *T. molitor*, for which we could not achieve greater than 55% regardless of the nematode concen-

tration tested. Therefore, an alternative inoculation approach was used for *H. bacteriophora* inoculation of *T. molitor*. Insects (2.4 g = 30 insects) were inoculated using the pipet method in plastic trays ( $15 \times 15 \times 3.5$  cm) filled with 1 cm of plaster of paris substrate. Nematode concentrations were 200, 400, and 800 infective juveniles ( $\pm 5\%$ ) per insect applied in a total of 36 ml of water per dish. The trays were then incubated at 25 °C, and percentage of patent infections and yield were determined as described previously.

Flanders et al. (1996) reported that host size has a substantial effect on infective juvenile yield of *H. bacteriophora* (Oswego strain). Thus, determination of yield per insect is unsatisfactory due to variation caused by host size differences. To avoid such criticism we used hosts of similar size—i.e., *G. mellonella* averaged ( $\pm$  sd)  $0.2 \pm 0.04$  g per insect, and *T. molitor* averaged  $0.12 \pm 0.02$  g per insect. Furthermore, we did an additional measurement for *H. bacteriophora* to determine if standardizing yields based on host size would affect our analysis and conclusions. We recorded the mass of each insect (*G. mellonella* and *T. molitor*) and analyzed treatment (concentration) differences in the yield per gram of insect. Because our analysis did not reveal any differences in interpretation of results for *H. bacteriophora* when yield per gram insect was used, we did not repeat the weight measurements for *S. carpocapsae*.

*Effects of host density:* Effects of host density on percentage of patent infections and yield were tested separately for each nematode-host combination. All experiments contained three replicates, were conducted at 25 °C, and were repeated once. Inoculation of *G. mellonella* was accomplished using the immersion method by submerging the insects in 115-ml nematode suspensions with 12,000 and 8,000 infective juveniles per ml for *H. bacteriophora* and *S. carpocapsae*, respectively. Concentrations were chosen based on successful inoculation from results obtained in the previous experiments. The insects were deposited on 150-mm petri dishes, and percentage patent infections were recorded after 6 days. Yields were determined by placing the petri dish (without lid) in covered plastic containers ( $30 \times 13 \times 8$  cm) with ca. 0.5 cm water to create a large White trap, and determining the number of infective juveniles produced as described previously. The *G. mellonella* densities were 0.07, 0.13, 0.20, and 0.27 g of host per  $\text{cm}^2$  (approximately 0.33, 0.66, 1.0, and 1.30 insects per  $\text{cm}^2$ ).

Inoculation of *T. molitor* was accomplished using the pipet method by applying 1 ml of water containing approximately 200 and 800 infective juveniles ( $\pm 5\%$ ) per insect for *S. carpocapsae* and *H. bacteriophora* to a 90-mm petri dish lined with filter paper (Whatman No. 1). Concentrations were chosen based on successful inoculation from results obtained previously. The range of *T. molitor* densities on petri dishes was 0.04, 0.07, 0.13, and 0.20 g of host per  $\text{cm}^2$  (approximately 0.28, 0.57,

1.10, and 1.70 insects per cm<sup>2</sup>). Each of the three replicates per host density-nematode combinations contained three 90-mm petri dishes (total surface area of 171 cm<sup>2</sup>). After 6 days of incubation the percentage of patent infections was determined. Yields were determined as previously described.

In the host-density experiments, yields were determined in each experiment for all treatments that averaged  $\geq 90\%$  patent infections, or for at least two treatments (the two causing the highest percentage of patent infections). The rationale for this limitation was that inoculation parameters that fail to produce high levels of infections would not be worth pursuing for mass in vivo production. Furthermore, when a particular treatment failed to produce a high percentage of infections, then the resulting host density that would be used for yield determination no longer approximated the density originally intended for that treatment. Yet in the cases where only one density tested produced "acceptable" ( $\geq 90\%$ ) patent infections, we included an additional treatment with the next-highest percentage of patent infections for yield determination so that we could have at least two treatments for comparison in the experiment. In such a case, additional patent-infected cadavers from other dishes (not being used) were added to the dishes to ensure that each dish used in yield determination would contain at least 90% of the original number of hosts inoculated.

**Data analysis:** The comparison of time efficiency in inoculation methods was analyzed using a T-test ( $\alpha = 0.05$ ) (SAS Institute, Cary, NC). Statistically significant treatment differences in all other experiments were detected through analysis of variance ( $\alpha = 0.05$ ) and the Student-Newman-Keuls' multiple-range test. Percentage data were arcsine transformed prior to analysis of variance (Southwood, 1978). The relationships between nematode concentration or host density and percentage infection and infective juvenile yield also were analyzed using linear regression ( $\alpha = 0.05$ ) (SAS Institute, Cary, NC).

**RESULTS**

**Inoculation methods:** The immersion method of inoculation was nearly four times more time efficient than pipeting. Average ( $\pm$  sd) times to complete the inoculation of one tray were  $7.7 \pm 0.6$  and  $28.0 \pm 1.7$  seconds for the immersion and pipet method, respectively. The difference between the means was significant according to the T-test ( $P = 0.0001$ ).

**Nematode concentration:** Nematode concentration had a positive effect on the ability to produce patent infections using the immersion method, except for inoculation of *T. molitor* with *H. bacteriophora* ( $P < 0.006$  in all ANOVA tests except *T. molitor* with *H. bacteriophora*, where  $P > 0.05$ ) (Figs. 1–3). When *T. molitor* was inoculated with *H. bacteriophora* using the pipet method,

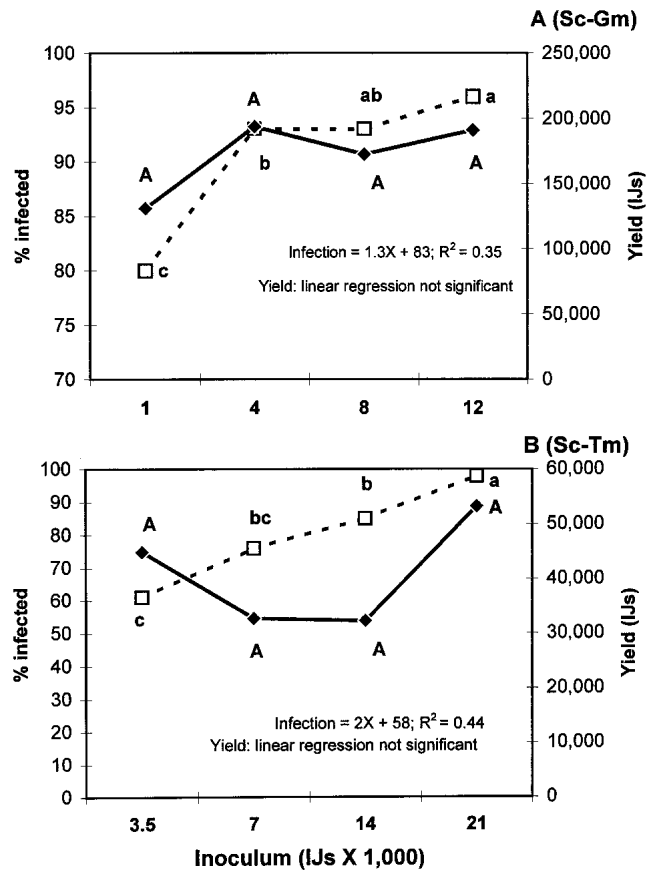


FIG. 1. Effects of *Steinernema carpocapsae* (Sc) inoculum concentration on infection (□) and yield (◆) in A) *Galleria mellonella* (Gm) and B) *Tenebrio molitor* (Tm). IJs = infective juvenile nematodes. Different lower- and uppercase letters denote statistical differences among mean infection rates and yields, respectively ( $\alpha = 0.05$ ).

nematode concentration had a positive effect on the ability to produce patent infections ( $P = 0.002$ ) (Fig. 2B). Using the immersion method and the nematode concentrations tested,  $\geq 90\%$  patent infections were observed with 4,000 to 12,000 infective juveniles per ml for inoculation of *G. mellonella* with *S. carpocapsae* or *H. bacteriophora* (Figs. 1A and 2A), and for inoculation of *T. molitor* with *S. carpocapsae* only 21,000 infective juveniles per ml produced  $\geq 90\%$  patent infections (Fig. 1B). None of the *H. bacteriophora* concentrations tested produced  $\geq 90\%$  patent infections using the immersion method (Fig. 3), but two concentrations (400 and 800 infective juveniles per insect) produced  $\geq 90\%$  patent infections using the pipet method (Fig. 2B). Most of the insects that died after inoculation were patent infections, i.e., an average ( $\pm$  se) of  $3.5 \pm 0.6$ ,  $5.8 \pm 0.65$ ,  $4.0 \pm 1.4$ , and  $5.6 \pm 1.2\%$  of the insects that died were not patent infections for *H. bacteriophora* inoculation of *G. mellonella*, *S. carpocapsae* inoculation of *T. molitor*, and *H. bacteriophora* inoculation of *T. molitor*, respectively. Regression analysis confirmed the ANOVA results: a linear relationship was detected between infection and inocula levels for all nematode concentration tests

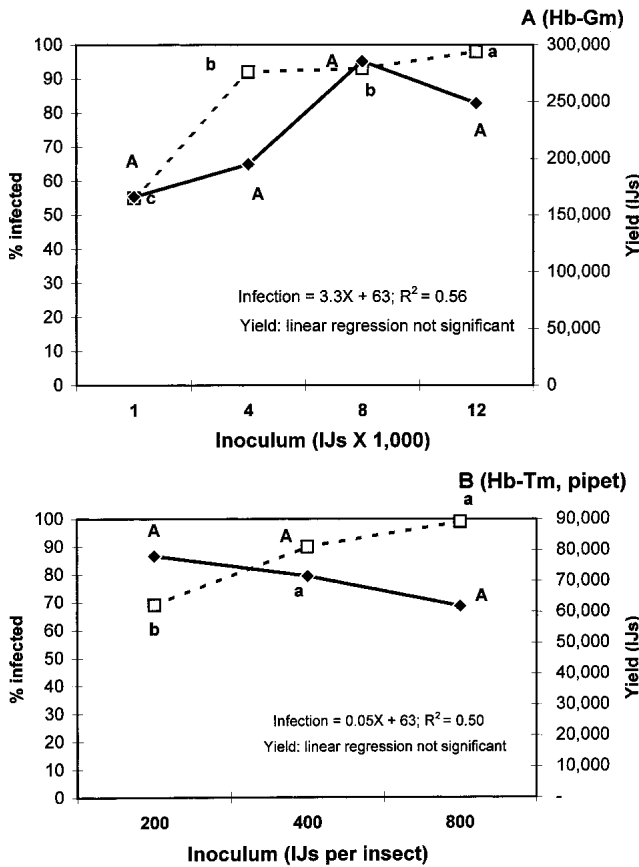


FIG. 2. Effects of *Heterorhabditis bacteriophora* (Hb) inoculum concentration on infection (□) and yield (◆) in A) *Galleria mellonella* (Gm) using the immersion inoculation method and B) *Tenebrio molitor* (Tm) using the pipet inoculation method. IJs = infective juvenile nematodes. Different lower- and uppercase letters denote statistical differences among mean infection rates and yields, respectively ( $\alpha = 0.05$ ).

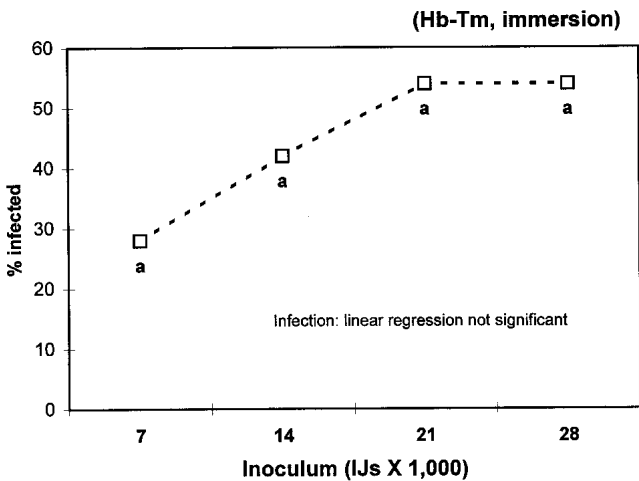


FIG. 3. Effects of *H. bacteriophora* (Hb) inoculum concentration on infection (□) in *Tenebrio molitor* (Tm) using the immersion inoculation method. IJs = infective juvenile nematodes. Different lower- and uppercase letters denote statistical differences among mean infection rates ( $\alpha = 0.05$ ).

except inoculation of *T. molitor* with *H. bacteriophora* ( $P < 0.002$  in all regression analyses except *T. molitor* with *H. bacteriophora*, where  $P > 0.05$ ) (Figs. 1–3).

Despite significant effects on infection, we did not detect an effect of nematode concentration on yield ( $P > 0.05$ ) (Figs. 1–2). Yield differences for *H. bacteriophora* also were not significantly affected by nematode concentration when yield per gram insect was analyzed: Yields ( $\pm$  se) in *G. mellonella* were  $727,049 \pm 27,874$ ,  $923,728 \pm 24,576$ ,  $1,245,584 \pm 413,638$ , and  $1,103,704 \pm 122,412$  per gram for concentrations of 1,000, 4,000, 8,000, and 12,000 infective juveniles per ml, respectively; yields in *T. molitor* were  $942,338 \pm 139,351$ ,  $804,302 \pm 80,607$ , and  $886,277 \pm 264,055$  infective juveniles per gram for concentration of 200, 400, and 800 infective juveniles per insect, respectively.

**Host density:** Analysis of variance and regression analysis indicated that the ability to produce patent infections decreases as host density increases in all nematode-host combinations tested ( $P < 0.0014$  in all ANOVA and regression tests) (Figs. 4 and 5). Within the range of host densities tested, patent infections were  $\geq 90\%$  at only one concentration (0.07 g per host) for *S. carpocapsae* inoculation of *G. mellonella* (Fig. 4A), 0.04–0.13 g per host for *S. carpocapsae* inoculation of *T. molitor* (Fig. 4B), 0.07 and 0.13 g per host for *H. bacteriophora* inoculation of *G. mellonella* (Fig. 5A), and only 0.04 g per host for *H. bacteriophora* inoculation of *T. molitor* (Fig. 5B). In all nematode-host combinations, few or no insects survived after inoculation (i.e., almost all insects died regardless of whether they exhibited patent infections or not). All insects were killed

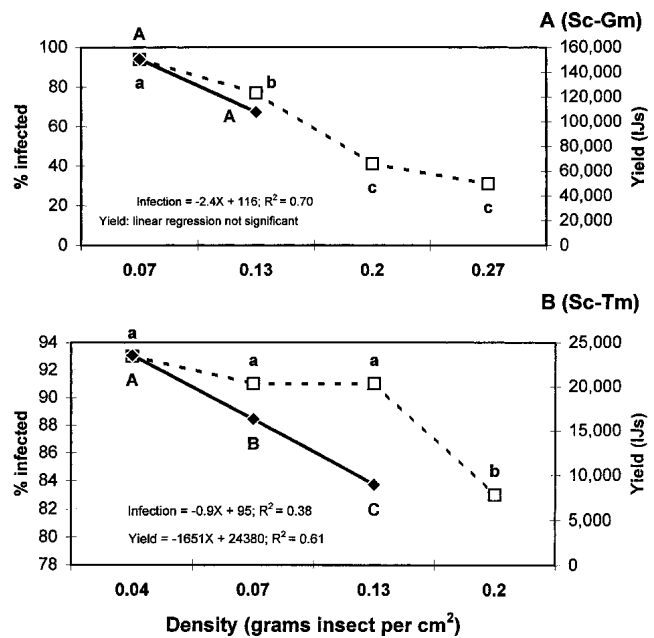


FIG. 4. Effects of A) *Galleria mellonella* (Gm) and B) *Tenebrio molitor* (Tm), host density on *Steinernema carpocapsae* (Sc) infection (□) and yield (◆). IJs = infective juvenile nematodes. Different lower- and uppercase letters denote statistical differences among mean infection rates and yields, respectively ( $\alpha = 0.05$ ).

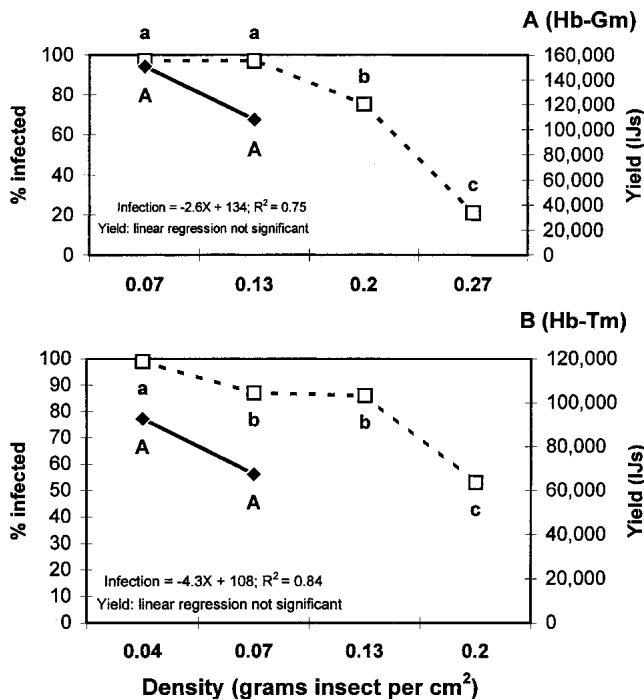


FIG. 5. Effects of A) *Galleria mellonella* (Gm) and B) *Tenebrio molitor* (Tm), host density on *Heterorhabditis bacteriophora* (Hb) infection (□) and yield (◆). IJs = infective juvenile nematodes. Different lower- and uppercase letters denote statistical differences among mean infection rates and yields, respectively ( $\alpha = 0.05$ ).

when *G. mellonella* was inoculated with either nematode, and an average ( $\pm$  se) of  $0.1 \pm 0.1$  and  $4.9 \pm 0.6$  were still alive after inoculation of *T. molitor* with *H. bacteriophora* and *S. carpocapsae*, respectively. A significant effect of host density on infective juvenile yield was detected in only one nematode-host combination, i.e., *S. carpocapsae* inoculation of *T. molitor*, in which the infective juvenile yield decreased as host density increased ( $P = 0.0001$ ) (Fig. 4B).

#### DISCUSSION

We determined that the immersion method is more time-efficient than the pipet method. Inoculation by immersion onto the perforated trays offers the additional advantage of not requiring labor to transfer fragile cadavers from an inoculation tray to a harvest tray (Gaugler et al., 2002). We did not compare inoculation by feeding with other methods because removal of the infected insects from food would require significant additional labor for mass production, and because we were able to obtain acceptable infection levels with more efficient methods.

The immersion method produced acceptable levels of patent infections for all host-nematode combinations except *H. bacteriophora* in *T. molitor*. This may have been caused by an inability of the nematodes to infect or adhere to the host in aqueous suspension. It is unlikely the nematodes could have entered the host while in aqueous suspension because immersion was brief (ca. 2

seconds) and, perhaps, the nematodes have difficulty penetrating the host without the aid of a substrate to gain leverage. Thus, the bulk of successful infections using the immersion approach most likely occur after the insects are removed from the aqueous suspension and placed on the inoculation tray. Conceivably, the smooth cuticle and relatively small surface area on *T. molitor* does not allow sufficient adhesion of nematodes to be carried from the suspension to the tray. It is doubtful if immersion in higher concentrations of *H. bacteriophora* would achieve acceptable infection levels because our data indicate that the maximum infection was already reached at lower concentrations of 21,000 infective juveniles per ml (indicating saturation of adhesion to the host is reached at that level). Furthermore, even if higher concentrations were beneficial in increasing infection rate, the economics of using such high concentrations must be considered.

Our finding that nematode concentration is positively related to infection is widely confirmed in the literature (Blinova and Ivanova, 1987; Flanders et al., 1996). Woodring and Kaya (1988) and Kaya and Stock (1997) state too high a nematode concentration may lead to contamination due to introduction of foreign bacteria. Bonifassi et al. (1999) demonstrated that infective juveniles may carry non-symbiotic bacteria under their external cuticle; thus, it is reasonable to assume that large numbers of invading infective juveniles may carry along contaminating bacteria into the host, which could decrease ability to produce patent infections and or reduce yields due to competition. Within the range of infective juvenile concentrations we tested, however, we did not observe any reduction of patent infections or yield as concentration increased. If we had tested higher concentrations perhaps we would have observed such an effect, but testing higher concentrations was not necessary because acceptable levels of infection were achieved using the range we applied.

Our finding that nematode concentration did not affect infective juvenile yield (in any nematode-host combination) is consistent with the findings of Flanders et al. (1996) using *H. bacteriophora* (Oswego) and *G. mellonella*, and Elawad et al. (2001) using *S. abbasi* and *G. mellonella*. Our finding (for *H. bacteriophora*) was the same, whether on a per-insect or per-gram basis. Contrarily, other authors report that intermediate concentrations of infective juveniles produce optimum yields in *G. mellonella*—e.g., Boff et al. (2000) using *H. megidis* and Zervos et al. (1991) using *H. bacteriophora* and *S. glaseri*. Similarly, Selvan et al. (1993) demonstrated that an optimum number of infective juveniles invading the host maximizes reproduction. Again, we may have observed effects on yield had we tested higher nematode concentrations, but higher concentrations were not called for to achieve acceptable infection.

In vitro entomopathogenic nematode production also indicates a mixture of effects of nematode inocu-

lum level on yield. In solid culture, depending on nematode species or strain, some studies reported an optimum inoculation concentration (Han et al., 1992), whereas others reported no effect of concentration (Han et al., 1993). Similarly, in liquid culture, yields were reported to be dependent on inoculum concentration for *H. bacteriophora* (Han, 1996) but not *H. indica* (Ehlers et al., 2000).

Host density had a substantial impact on the ability to produce patent infections in all host nematode concentrations and on yield of *S. carpocapsae* in *T. molitor*. Contrarily, Flanders et al. (1996) reported no significant effects of host density on infection or yield of *H. bacteriophora* in *G. mellonella*. The discrepancy is likely due to our testing of higher host densities. The highest density tested by Flanders et al. (1996) was approximately 0.51 *G. mellonella* per cm<sup>2</sup>; we observed an effect only at densities higher than 0.66 insects per cm<sup>2</sup> (= 0.13 g per cm<sup>2</sup>).

Entomopathogenic nematodes need sufficient aeration to develop (Burman and Pye, 1980; Friedman, 1990). The host-density effects were likely due, at least in part, to competition for oxygen as host density increased. This crowding/oxygen deprivation effect may have been exacerbated by ammonia given off by infected cadavers in the early stages of pathogenesis (Shapiro et al., 2000); ammonia has been shown to be detrimental to nematodes (Grewal et al., 1999; Rodríguez-Kábana, 1986; Shapiro et al., 1996). Our findings indicate there is an upper threshold or carrying capacity for host density.

Interestingly, we observed similar yields of *H. bacteriophora* in each of *G. mellonella* and *T. molitor* when yield was measured on a per-gram-of host basis. In choosing which host to use for mass production, however, it is the infective juvenile yield per cost of insects that is the definitive factor. In a crude analysis, Blinova and Ivanova (1987) reported that production of *S. carpocapsae* would be more cost efficient using *T. molitor* as a host relative to *G. mellonella*. If we conduct a similar analysis, using the maximum yields from our experiments, then we observe that *H. bacteriophora* produces approximately 2 to 3.5 times as many infective juveniles in *G. mellonella* compared with *T. molitor*, and *S. carpocapsae* produces between three to six times as many infective juveniles in *G. mellonella* compared with *T. molitor*. The current cost per insect in the United States is approximately between four and five times greater for *G. mellonella* than *T. molitor* (e.g., 0.012 vs. 0.0025 USD, H&T Alternative Controls LLC. and Sunfish Bait Co., pers. comm.). Thus, the economic efficiency of *H. bacteriophora* production in *T. molitor* appears to be slightly advantageous relative to production in *G. mellonella*, whereas production efficiency appears to be equal in the two hosts for *S. carpocapsae*. Actual production efficiencies for each host under full-scale, in vivo production also may de-

pend on other factors such as handling time per insect or tray, and culture time (duration).

When infective juvenile yield is not affected by nematode concentration, it makes sense to choose the lowest inoculum concentration that produces acceptable infection levels as optimum. Thus, our optimum concentrations were 4,000 infective juveniles per ml for immersing *G. mellonella* (with either nematode), 21,000 infective juveniles per ml for inoculating *T. molitor* with *S. carpocapsae* (using immersion), and pipeting 400 infective juveniles per insect for inoculating *T. molitor* with *H. bacteriophora*. In the cases where yield is not affected by density, it makes sense to choose the highest density that produces acceptable infection levels as optimum (to maximize the yield per tray). Thus, our optimum host densities were 0.07, 0.13, and 0.04 g host per cm<sup>2</sup> for inoculation of *G. mellonella* with *S. carpocapsae*, *G. mellonella* with *H. bacteriophora*, and *T. molitor* with *H. bacteriophora*, respectively. In cases such as *T. molitor* inoculation with *S. carpocapsae*, where yield is affected by host density, the optimum density must be obtained by analyzing the cost and benefits of increasing density. Factors that must be included in this analysis include yield and cost per insect as well as the cost of labor to handle each tray during a production cycle.

In summary, we demonstrated that inoculation method, inoculum concentration, and host density are important factors for in vivo production of entomopathogenic nematodes. Other factors such as temperature and humidity also may affect yield (Grewal et al., 1994; Woodring and Kaya, 1988) and thus should be optimized. The optimum nematode concentrations and host densities reported herein are likely to differ based on variations in trays, ventilation, and nematode species or strains and should be optimized for each particular production system.

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