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Effect of Application Method on Fitness of Entomopathogenic Nematodes Emerging at Different Times

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Abstract: The entomopathogenic nematode species *Steinernema feltiae* and *Heterorhabditis bacteriophora* were compared for survival and infectivity of infective juveniles (IJ) collected with a standard White trap (i.e., emerging from hosts and accumulating in water) and later applied to sand (treatment A) to IJ allowed to emerge from hosts into sand (treatment C). Percentage IJ survival and infectivity was compared between treatments for *S. feltiae* IJ that emerged between days 1 to 3 and days 4 to 6. For *H. bacteriophora,* percentage IJ survival and infectivity was compared between treatments only for infective juveniles that emerged between days 4 to 6. For *S. feltiae* IJ percentage survival and infectivity decreased with time ($P \leq 0.05$) and was greater ($P \leq 0.05$) for IJ from treatment C than for IJ from treatment A. For *H. bacteriophora* IJ percentage survival decreased ($P \leq 0.05$) and percentage infectivity increased ($P \leq 0.05$) with time. While percent survival was higher ($P \leq 0.05$) for treatment C than for A, percent infectivity was not different between treatments.

Key words: desiccation, *Heterorhabditis bacteriophora,* infectivity, *Steinernema feltiae,* survival.

Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae can provide levels of insect control similar to those of chemical insecticides (Georgis and Hague, 1991). The success of entomopathogenic nematodes as biological control agents is mainly determined by the ability of the third-stage infective juveniles (IJ) to survive in the soil environment and infect an insect host. Inconsistent results in field and laboratory trials using entomopathogenic nematodes are often due to variation in survival and infectivity of IJ. Developing methods to increase IJ survival and infectivity may improve the ability of entomopathogenic nematodes to control insects.

Environmental or biological variables that can be manipulated and have an impact on IJ survival and infectivity are of particular interest because they offer the potential to optimize the use of entomopathogenic nematodes as biological control agents. Nematodes that exit hosts into the natural environment behave differently than nematodes that are suspended in water and later applied to the environment (Shapiro and Glazer, 1996). Infective juvenile survival and infectivity of some heterorhabditid and steinernematid species were higher when nematode-infected cadavers were applied and IJ emerged naturally into sand than when IJ were applied to sand in water suspension (Perez et al., 2003; Shapiro and Lewis, 1999). It was hypothesized that the difference in infectivity was due in part to a chemical substance in the infected host from which the IJ emerged that stimulates infection and that chemical substance would be lost when IJ were suspended in water (Shapiro and Lewis, 1999).

Aqueous suspension is routinely used to store and apply IJ for insect control and also to conduct research experiments. Perez et al. (2003) demonstrated that some heterorhabditid and steinernematid species survived longer and were more infective when IJ emerged directly into sand than when IJ were collected in water and later applied to sand. However, these experiments were conducted only on IJ that had emerged from cadavers during days 1 to 3 of emergence. The IJ may emerge from the insect host for a period of up to 18 days and earlier emerging nematodes can have different biological characteristics than those emerging later (Lewis et al., 1995; Ryder and Griffin, 2003; Stuart et al., 1996). The effect of water suspension on IJ survival and infectivity may be different on nematodes that emerge at various times of the emergence phase. The objective of the present work was to use IJ that emerge from the host at different times during the emergence phase and compare survival and infectivity of nematodes that emerged directly into sand from cadavers (cadaver treatment) to the survival and infectivity of those applied to sand in aqueous suspensions (aqueous treatment). In a previous experiment, we compared *Heterorhabditis bacteriophora* IJ survival and infectivity between the cadaver and aqueous treatments using IJ that emerged during days 1 to 3 of emergence (Perez et al., 2003). In the present work we placed emphasis on IJ that emerged during days 4 to 6 of emergence. For *Steinernema feltiae,* no cadaver vs. aqueous treatment has been previously addressed; therefore, we compared survival and infectivity between the aqueous and cadaver treatments with IJ that emerged during days 1 to 3 and with IJ that emerged during days 4 to 6 of emergence.

Materials and Methods

We compared survival and infectivity between IJ emerging under two treatments. Treatments were emergence of IJ into water from hosts in White traps (White, 1927) (treatment A) and emergence of IJ directly into sand from hosts placed in sand (treatment C). Two experiments were conducted with *S. feltiae* (SN strain) (one using IJ that emerged during days 1 to 3 of emergence and the other using IJ that emerged during days 4 to 6). Nematodes used to initiate experiments

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were cultured in the last instar of the greater wax moth, *Galleria mellonella,* according to procedures described by Kaya and Stock (1997).

For experiments, moth larvae were exposed individually to IJ (<15 days old) in 24-well (1.5-cm-diam.) plates and lined with two filter paper discs (Whatman no. 1). The exposure rates were approximately 50 or 100 IJ of *S. feltiae* or *H. bacteriophora,* respectively, per moth larva. Three days after inoculation, the infected moth larvae were divided between the two treatments. Infective juveniles collected from 12 infected wax moth larvae were used for each treatment. For the aqueous treatment (A), 12 nematode-infected wax moth larvae were placed individually in White traps for later collection of IJ from water. Three days after the beginning of IJ emergence, nematodes from the White traps were removed and concentrated with a vacuum pump onto nitrocellulose paper with 0.3-μm openings (Millipore, Bedford, MA). The concentrated nematodes were collected with a spatula from the nitrocellulose paper and applied to petri dishes (150-mm-diam.) filled with moist sand (10% water w/w). The same White traps were used to collect IJ emerging between 4 to 6 days under treatment A and later used in the second experiment. During days 4 to 6 of emergence, IJ were collected from the White traps and applied to petri dishes (150-mm diam.) as described.

For the cadaver treatment (C), 12 nematode-infected wax moth larvae were placed inside petri dishes filled with sand (10% water w/w). Three days after the beginning of IJ emergence, moth larvae were removed from the petri dishes. For the second experiment, the same moth larvae were transferred to another petri dish where IJ emerged for 3 additional days under treatment C. Consequently for each treatment (A or C), the number of IJ in each petri dish filled with sand was the total that emerged from 12 wax moth larvae during 3 days (either from days 1 to 3 of emergence for the first experiment or from days 4 to 6 of emergence for the second experiment. Only one experiment was conducted with *H. bacteriophora* Poinar (Hb strain) using IJ that emerged during days 4 to 6 of emergence as previously described.

Experiments were conducted at ca. 25 °C in desiccators maintained at 75% relative humidity with an NaCl-saturated salt solution (Winston and Bates, 1960). In each experiment, an experimental unit consisted of a petri dish (150-mm-diam.) filled with approximately 220 cm^3 of moist sand (10% water w/w) and contained IJ that had emerged from 12 wax moth larvae during 3 days. Each treatment was replicated four times and each experiment was conducted twice.

The fourth (in the experiments using IJ that emerged during days 1 to 3 of emergence) and the seventh (in experiments using IJ that emerged during days 4 to 6 of emergence) days after IJ emergence from the moth larvae were considered as "day one" in the statistical analyses. Every 3 days thereafter, a 10-cm^3 sand sample was taken from each experimental unit and infectivity and survival of the IJ therein were recorded. Each 10-cm^3 sand sample consisted of several cores taken with a core borer (9-mm-diam.) at a depth of approximately 12 mm. Infectivity was tested by exposing five wax moth larvae for 16 hours to the approximately 10-cm^3-sand sample in a petri dish (60-mm-diam.). The petri dishes were then sealed with parafilm and kept in an incubator at 25 °C. After the 16-hour exposure period, the wax moth larvae were removed from the sand sample, rinsed, and left for 48 hours at ambient temperature before storage at −20 °C. Moth larva cadavers were dissected using the pepsin digest method (Caroli et al., 1996; Mauleon et al., 1993) and the number of infecting nematodes was recorded. After removal of the wax moth larvae from the sand, IJ were extracted from the sand by sedimentation in tap water. Live and dead IJ were counted. Nematodes were considered live if they were naturally moving or responded to probing with a fine needle. Percentage of infecting nematodes (number infecting/number infecting + number alive + number dead) and percentage of live nematodes (number alive + number infecting/number alive + number infecting + number dead) were calculated for each sample and sampling date.

The experimental design was a split-plot design. Whole plot factors were the two treatments (aqueous and cadaver) arranged in a completely randomized design. Sub-plots were the sampling dates (samples taken the first day dishes were placed in the desiccators and every 3 days thereafter). Data were subjected to analysis of variance for a split-plot design (Montgomery, 1991) using SAS (SAS Institute, Cary, NC). For each entomopathogenic nematode species within treatments, mean percentage of live or infecting nematodes (y) were fitted to sampling dates (x) using least squares analysis.

Results

Analysis showed no interaction between replicated runs in each test. Therefore, results from duplicate tests were combined for final analysis. For *H. bacteriophora* IJ that emerged during days 4 to 6 of emergence, the relationship between percentage of infecting IJ (y) and days in the desiccator (x) was linear with a positive slope for both the aqueous and cadaver treatments (Fig. 1A,B). Treatment A, $r^2 = 0.72$, $P \leq 0.05$ (Fig. 1A); treatment C, $r^2 = 0.65$, $P \leq 0.05$ (Fig. 1B). The regression slopes were not different ($P > 0.05$) between the two treatments. The relationship between percentage survival (y) and days in the desiccator (x) was linear and decreasing for both treatments. Treatment A, $r^2 = 0.65$, $P \leq 0.05$ (Fig. 2A); treatment C, $r^2 = 0.88$, $P \leq 0.05$ (Fig. 2B). The regression slopes were not different ($P > 0.05$) between the two treatments. Percent of infecting IJ averaged over the whole experiment was not different

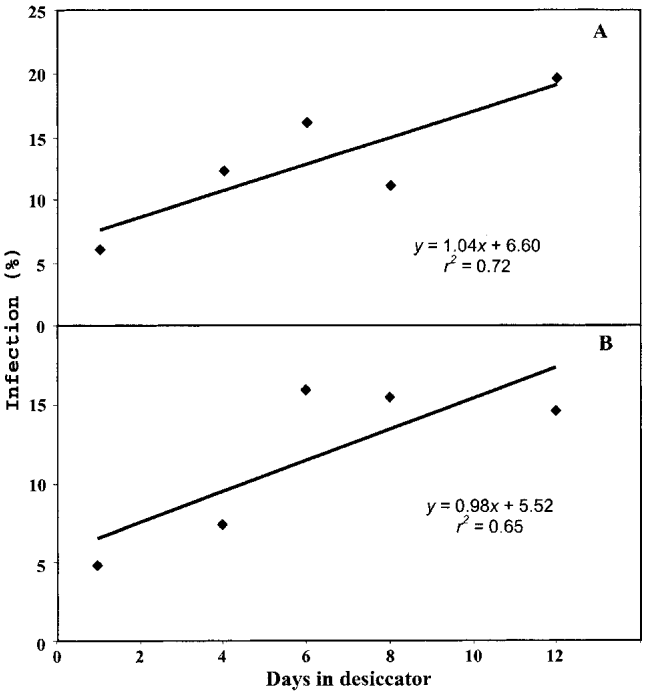

FIG. 1. Percentage of *Heterorhabditis bacteriophora* infective juveniles (IJ) parasitizing *Galleria mellonella* after 16-hour exposure period to IJ stored in sand (10% water) for various times in desiccator at 75% relative humidity. Infective juveniles emerged during days 4 to 6 of emergence. A) Infective juveniles collected from White traps, filtrated, and applied to sand with a spatula. B) Infective juveniles emerging from insect cadavers into sand.

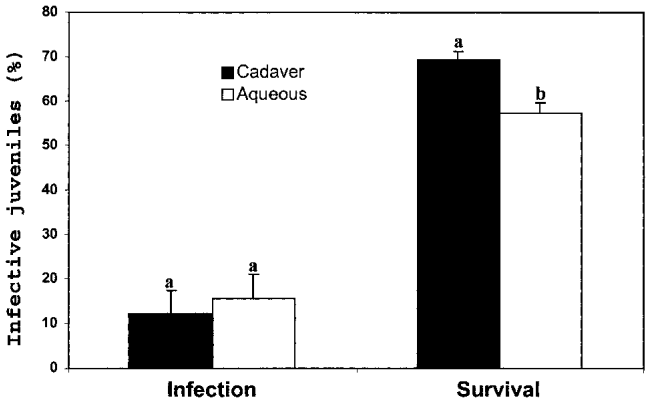

FIG. 3. Mean ± SE of percent survival and infectivity of *Heterorhabditis bacteriophora* infective juveniles (IJ) averaged over all sample dates during the course of the experiment. Infective juveniles emerged during days 4 to 6 of emergence.

($P > 0.05$) between the two treatments (Fig. 3). Percentage survival averaged over the whole experiment was higher ($P \leq 0.05$) for IJ from treatment C than for IJ from treatment A (Fig. 3).

For *S. feltiae* IJ that emerged during days 1 to 3 of emergence, the relationship between percentage infecting IJ (y) and days in the desiccator (x) was linear with a negative slope for both the aqueous and cadaver treatments (Fig. 4A,B). For treatment A, $r^2 = 0.83$, $P \leq$

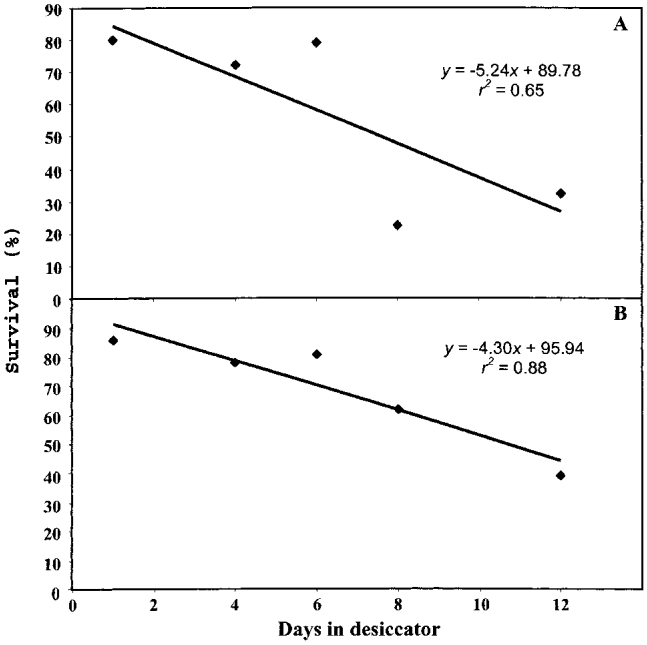

FIG. 2. Percentage survival of *Heterorhabditis bacteriophora* infective juveniles (IJ) in sand (10% water) over time at 75% relative humidity. Infective juveniles emerged during days 4 to 6 of emergence. A) Infective juveniles collected from White traps, filtrated, and applied to sand with a spatula. B) Infective juveniles emerging from insect cadavers into sand.

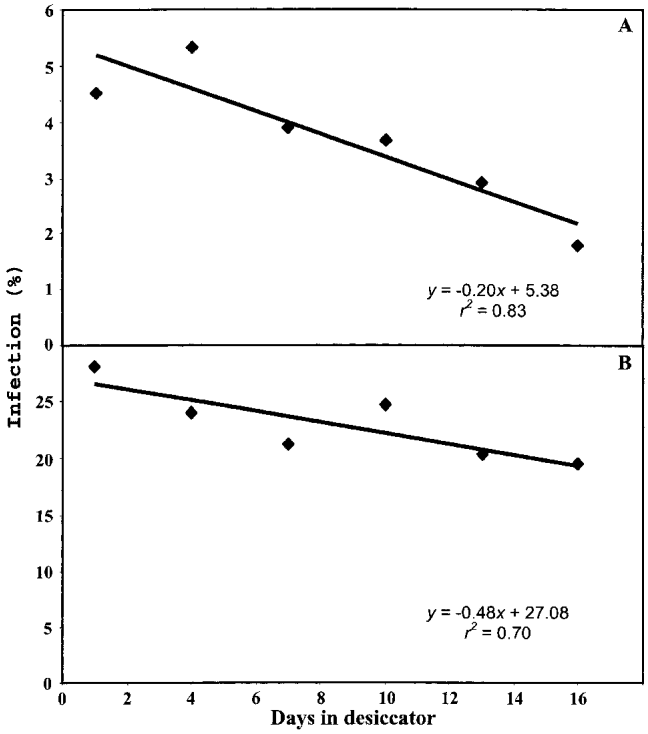

FIG. 4. Percentage of *Steinernema feltiae* infective juveniles (IJ) parasitizing *Galleria mellonella* after 16-hour exposure period to IJ stored in sand (10% water) for various times in desiccator at 75% relative humidity. Infective juveniles emerged during days 1 to 3 of emergence. A) Infective juveniles collected from White traps, filtrated, and applied to sand with a spatula. B) Infective juveniles emerging from insect cadavers into sand.

0.05 (Fig. 4A); for treatment C, $r^2 = 0.70$, $P \leq 0.05$ (Fig. 4B). The regression slopes were not different ($P > 0.05$) between the two treatments. The relationship between percentage survival (y) and days in the desiccator (x) was linear with a negative slope for both treatments (Fig. 5A,B). For treatment A, $r^2 = 0.72$, $P \leq 0.05$ (Fig. 5A); for treatment C, $r^2 = 0.61$, $P \leq 0.05$ (Fig. 5B). The regression slopes were not different ($P > 0.05$) between the two treatments. Percentage surviving and infecting IJ averaged over the whole experiment were higher ($P \leq 0.05$) for IJ from treatment C than for IJ from treatment A (Fig. 6).

For *S. feltiae* IJ that emerged during days 4 to 6 of emergence, the relationship between percentage of infecting IJ (y) and days in the desiccator (x) was linear with a negative slope for the aqueous and cadaver treatments (Fig. 7A,B). For treatment A, $r^2 = 0.81$, $P \leq 0.05$ (Fig. 7A); for treatment C, $r^2 = 0.72$, $P \leq 0.05$ (Fig. 7B). The regression slopes were not different ($P > 0.05$) between the two treatments. The relationship between percentage survival (y) and days in the desiccator (x) was linear with a negative slope for both treatments (Fig. 8A,B). For treatment A, $r^2 = 0.85$, $P \leq 0.05$ (Fig. 8A); for treatment C, $r^2 = 0.76$, $P \leq 0.05$ (Fig. 8B). The regression slopes were not different ($P > 0.05$) between the two treatments. Percentage surviving and infecting IJ averaged over the whole experiment were higher ($P \leq 0.05$) for IJ from treatment C than for IJ from treatment A (Fig. 9).

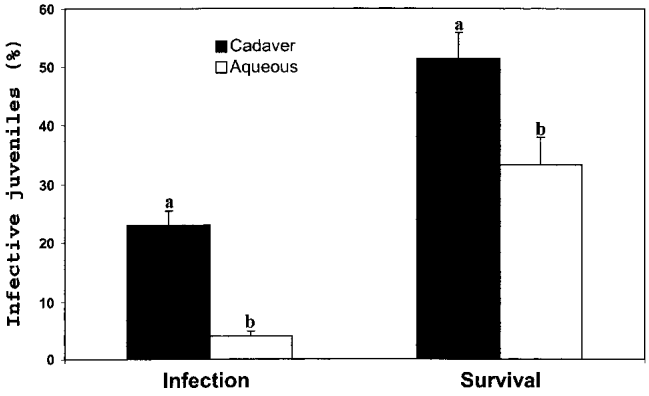

FIG. 6. Mean ± SE of percent survival and infectivity of *Steinernema feltiae* infective juveniles (IJ) averaged over all sample dates during the course of the experiment. Infective juveniles emerged during days 1 to 3 of emergence.

DISCUSSION

We report evidence that application of IJ using the aqueous treatment can affect their survival and infectivity. Percentage survival of *H. bacteriophora* IJ that emerged during days 4 to 6 of emergence was greater for IJ that emerged from the cadaver into sand than for IJ that were collected in water, filtrated, and later applied to sand. However, there was no difference between their infection rates. In a previous work that compared the same treatments but using IJ that had emerged during days 1 to 3 of emergence, we found no

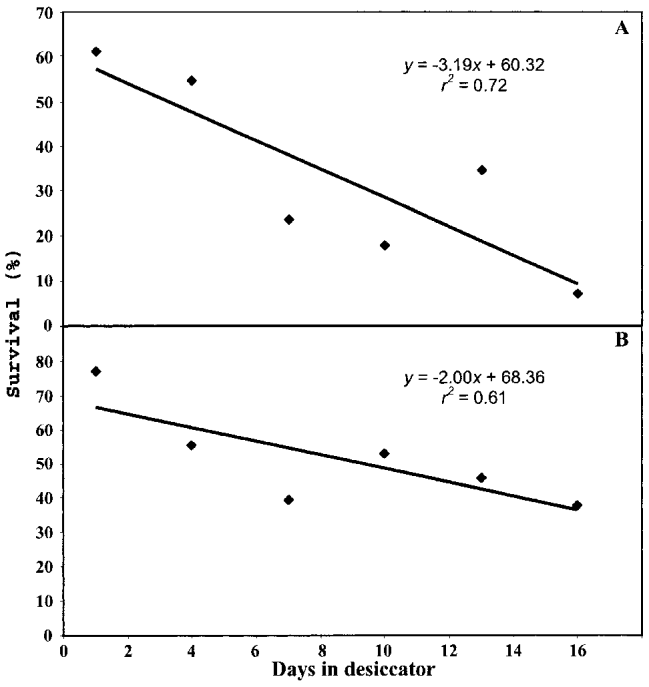

FIG. 5. Percentage survival of *Steinernema feltiae* infective juveniles (IJ) in sand (10% water) over time at 75% relative humidity. Infective juveniles emerged during days 1 to 3 of emergence. A) Infective juveniles collected from White traps, filtrated, and applied to sand with a spatula. B) Infective juveniles emerging from insect cadavers into sand.

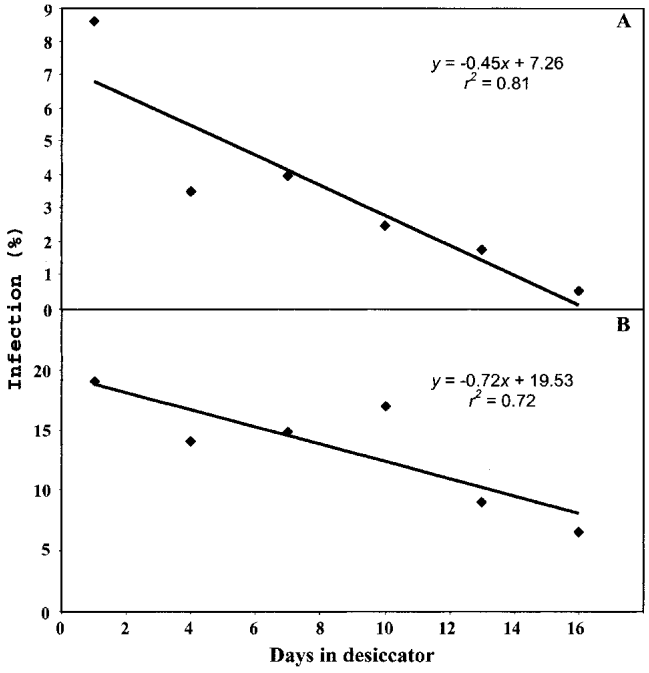

FIG. 7. Percent of *Steinernema feltiae* infective juveniles (IJ) parasitizing *Galleria mellonella* after 16-hour exposure period to IJ stored in sand (10% water) for various times in desiccator at 75% relative humidity. Infective juveniles emerged during days 4 to 6 of emergence. A) Infective juveniles collected from White traps, filtrated, and applied to sand with a spatula. B) Infective juveniles emerging from insect cadavers into sand.

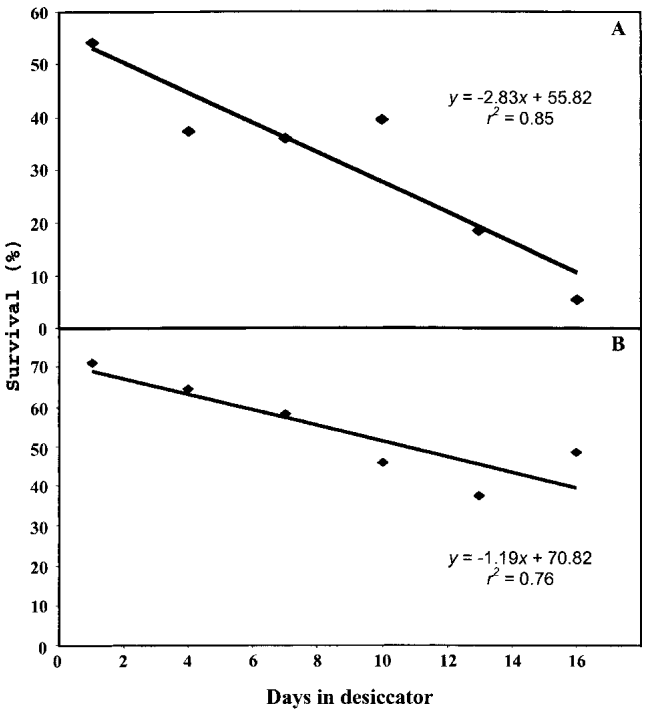

FIG. 8. Percentage survival of *Steinernema feltiae* infective juveniles (IJ) in sand (10% water) over time at 75% relative humidity. Infective juveniles emerged during days 4 to 6 of emergence. A) Infective juveniles collected from White traps, filtrated, and applied to sand with a spatula. B) Infective juveniles emerging from insect cadavers into sand.

difference in *H. bacteriophora* IJ survival rate (Perez et al., 2003). Shapiro and Lewis (1999) tested the same treatments at 100% RH on IJ that emerged between days 1 to 3 of emergence and found that infectivity was higher for IJ that emerged from the insect cadaver into sand than for IJ that were applied to sand in water suspension. The difference between the present and previous findings (Perez et al., 2003; Shapiro and Lewis, 1999) may be due to differences in IJ emergence time or differences in percentage RH. Percentage of *H. bacteriophora* IJ infecting *G. mellonella* increased over time

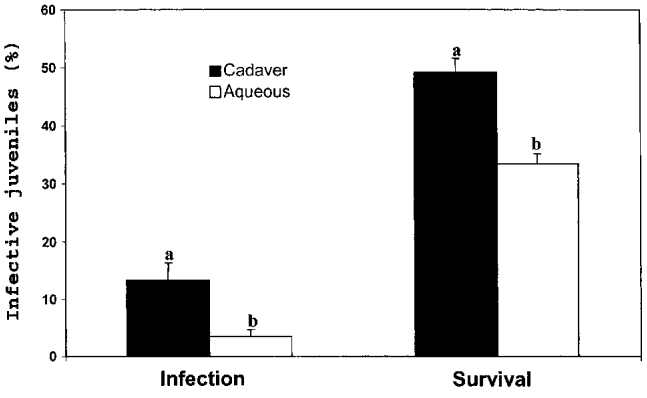

FIG. 9. Mean ± SE of percent survival and infectivity of *Steinernema feltiae* infective juveniles (IJ) averaged over all sample dates during the course of the experiment. Infective juveniles emerged during days 4 to 6 of emergence.

throughout the experiment. This finding agrees with a previous report that *Heterorhabditis megidis* infectivity increases as IJ age (Griffin, 1996). We reported a similar finding in a previous study using *H. bacteriophora* IJ that emerged during days 1 to 3 of emergence; however, in that study infectivity reached a maximum around day 13 after the beginning of the experiment and later declined (Perez et al., 2003). We might have seen a decline in infectivity if we had continued the experiment for a period longer than 13 days. Or it could be that late emerging IJ delay the peak in infectivity longer than early emerging ones. This behavior would maximize the probability of successful infection and reproduction in cases where competition for an uninfected host is high (Campbell et al., 1999; Ryder and Griffin, 2003).

Percentage survival and infection of *S. feltiae* IJ was greater for IJ from the cadaver treatment than for IJ from the aqueous treatment for both early- and late-emerging IJ. Similar results were obtained previously using *S. riobrave* and *S. carpocapsae* IJ, using IJ that emerged during days 1 to 3 of emergence (Perez et al., 2003). Our present and previous findings (Perez et al., 2003) suggest that application of IJ inside their host cadaver could result in a percentage increase of IJ that survive and infect during a given time period. Previous work by Shapiro and Lewis (1999) indicated that a chemical substance in the infected host from which the IJ emerged would stimulate infection. Another reason for a decrease in percentage infection and survival of IJ applied in water suspension could be due to the osmotic stress that nematodes experience in water during the 3 days of collection (Lewis et al., 1995). Infective juveniles emerging from the host directly into sand may enter a sheltered environment of moist sand immediately after emergence, whereas IJ from the aqueous treatment are filtered and placed on the sand surface exposed to 75% RH. Additionally, IJ from the aqueous treatment are subjected to manipulations (e.g., filtration and collection with a spatula) that the IJ from the cadaver treatment are not (Shapiro and Lewis, 1999).

This study is one of several that examine the effects of water storage and collection of entomopathogenic nematode IJ on their behavior, ecology, and biological control potential. Shapiro and Glazer (1996) showed that IJ that enter the soil directly from the cadavers have greater dispersal than IJ applied in water suspension. Entomopathogenic nematodes can persist inside the host cadaver under adverse dry soil conditions and freezing temperatures (Koppenhöfer et al., 1997; Lewis and Shapiro-Ilan, 2002). These findings suggest that there are advantages in the application of entomopathogenic nematodes inside the insect host. Also, nematodes that emerge as a result of recycling from infected hosts may be expected to be superior biocontrol agents to those that were originally applied (Shapiro and Lewis, 1999). Indeed, superior efficacy of the "applied cadaver approach" was demonstrated in

greenhouse experiments using *Heterorhabditis* spp. (Shapiro-Ilan et al., 2003).

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