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Variations in Immune Response of *Popillia japonica* and *Acheta domesticus* to *Heterorhabditis bacteriophora* and *Steinernema* Species[1]

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Abstract: The infectivities of *Steinernema carpocapsae, S. glaseri, S. scapterisci,* and *Heterorhabditis bacteriophora* to Japanese beetle larvae, *Popillia japonica,* and house cricket adults, *Acheta domesticus,* were compared using external exposure and hemocoelic injection. Only *H. bacteriophora* and *S. glaseri* caused high *P. japonica* mortality after external exposure. When nematodes were injected, *P. japonica* had a strong encapsulation and melanization response to all species except *S. glaseri. Heterorhabditis bacteriophora* and *S. carpocapsae* were able to overcome the immune response, but *S. scapterisci* was not. All species except *S. scapterisci* were able to kill and reproduce within the host. Only *S. scapterisci* and *S. carpocapsae* caused *A. domesticus* mortality after external exposure. When nematodes were injected, *A. domesticus* had a strong immune response to all species except *S. scapterisci. Steinernema carpocapsae* effectively overcame the strong immune response and caused high host mortality, but *S. glaseri* and *H. bacteriophora* did not. *Steinernema scapterisci* caused high host mortality and reproduced, *S. glaseri* and *H. bacteriophora* caused low host mortality but only *S. glaseri* reproduced, and *S. carpocapsae* was able to kill the host but reproduced poorly. Most (ca. 90%) of the *S. carpocapsae* in the hemocoel of *P. japonica* became encapsulated and melanized within 8 hours postinjection. The symbiotic bacterium, *Xenorhabdus nematophilus,* was often released before this encapsulation and melanization.

Key words: Acheta domesticus, encapsulation, entomopathogenic nematode, *Heterorhabditis bacteriophora,* immune response, melanization, *Popillia japonica, Steinernema carpocapsae, S. glaseri, S. scapterisci, Xenorhabdus* spp.

Entomopathogenic nematodes from the families Steinernematidae and Heterorhabditidae are prospective agents for the biological control of soil-inhabiting insect pests (7,8,15). Although field and laboratory experiments have demonstrated that hundreds of insect species are susceptible to nematode infection (22), different nematode species showed different levels of infectivity to particular insects (2,15,19).

Successful infection by entomopathogenic nematodes initially involves host location and penetration into the hemocoel. Nematodes subsequently encounter the host immune system. *Steinernema carpocapsae* can evade the host immune response by not being recognized as foreign in *Galleria mellonella* larvae (4,5), but nematodes become encapsulated in *Culex pipiens* and *Leptinotarsa decemlineata* larvae (25,26). The symbiotic bacteria (*Xenorhabdus* spp.) released by the nematodes not only provide essential nutrients for the nematodes but also play a role in suppression of the host immune response (26). The nematode is also capable of secreting hydrolytic enzyme(s) that destroy host antibacterial protease(s) (10).

Cellular and humoral responses of insects to nematodes were reviewed by Poinar (21). The major cellular responses are encapsulation, in which hemocytes accumulate around the nematode without secondary melanization, and melanotic encapsulation, in which an inner layer of melanin forms around the nematode. In some insects, encapsulation and melanization involve only noncellular components of hemolymph. The host immune response varies somewhat with species of nematode or insect. Sometimes the response is sufficiently effective to kill nematodes (4,18,24), but sometimes it is not a major factor influencing host susceptibility (13).

The effects of insect immune response on the infectivity of different nematode species have not been fully documented.

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The biological control specialist must know when host immune responses are elicited and how these responses influence nematode infectivity. Therefore, we compared the host immune responses elicited by four nematode species in Japanese beetle (*Popillia japonica*) and house cricket (*Acheta domesticus*), and we assessed the impact of these responses on nematode infectivity and reproduction.

Materials and Methods

Nematodes: Four nematode species were tested: *S. glaseri* (NC strain), *S. carpocapsae* (All strain), *S. scapterisci* (Colon strain), and *Heterorhabditis bacteriophora* (HP88 strain). *Steinernema scapterisci* was cultured in *A. domesticus,* the host best suited for nematode reproduction. All other nematodes were cultured in last-instar *Galleria mellonella* (6). Infective juveniles were held at room temperature in deionized water for 3 days after emergence from the host cadaver. All experiments were conducted at 25 C.

Insects: Last-instar *P. japonica* were field collected. Adult *A. domesticus* were fed on Hartz (Harrison, NJ) Rabbit Pellets for 3 days before use. To avoid injury or cannibalism, all insects were held individually in 35-mm-d petri dishes.

Inoculation: Insects were exposed individually to 100 or 1,000 nematodes in 35-mm-d petri dishes containing 4 g moist sand (10% moisture). The petri dishes were sealed with parafilm to maintain constant moisture. Thirty insects, in three blocks of 10 insects each, were used for each treatment and control. Host mortality was recorded daily for 5 days.

Nematodes used for hemocoel injections were surface sterilized in 1% Hyamine (methylbenzethonium chloride) for 5 minutes and then rinsed with sterile Ringer's saline. Twenty infective juveniles in 4 µl of this saline were injected with a microinjection syringe into the hemocoel of each insect through the base of the first leg. The syringe was inserted parallel to the body to avoid injuring the gut. The injected insects were then divided into two groups. One group (30 insects in three blocks of 10 insects each) was maintained for 5 days to record mortality, and each cadaver was transferred to a White nematode trap (6) to count the emerged infective juveniles. Insects from the second group (30 insects in three blocks of 10 insects each) were dissected 24 hours after injection to determine the proportion of nematodes encapsulated and melanized. The same amount of Ringer's saline was injected into the hemocoel of insects in the control group (30 insects in three blocks of 10 insects each).

Time course of melanization and bacterial release: For observation of the encapsulation and melanization process, 20 infective juveniles of *S. carpocapsae* in 5 µl Ringer's saline were injected into the hemocoel of grubs with the technique described above. The same volume of saline was injected into each grub of the control group. The grubs were fed individually with fresh grass roots and dissected at 0.17, 0.5, 1, 2, 4, 8, 16, and 24 hours postinjection. Five grubs were used for each time interval. Grubs were surface sterilized by rinsing in 75% ethanol before withdrawing hemolymph and dissecting. Nematodes recovered during dissection were grouped as free, encapsulated (entrapped or arrested in insect tissue without melanin), or encapsulated and melanized (melanotic encapsulation in process or complete).

For determination of *X. nematophilus* levels in the hemolymph, 5 µl of hemolymph were withdrawn from each grub before dissection and suspended in 45 µl of sterile Ringer's saline. Three 5-µl subsamples of the saline containing grub hemolymph were streaked on 100-mm-d NBTA plates (Difco Bacto-Nutrient agar 7.5 g, bromothymol blue 0.0125 g, 2,3,5-triphenyl tetrazolium chloride 0.02 g, deionized water 500 ml). The plates were incubated at 25 C for 3 days. Colonies of *X. nematophilus* were counted and the mean number of colony forming units (CFUs) per microliter of hemolymph was calculated. The same method was used to estimate the time of bacterial release after injection of 20

surface-sterilized infective juveniles of *S. glaseri* or *H. bacteriophora* into the hemocoels of *P. japonica.*

Statistical analyses: Data were transformed with arcsine square roots and analyzed using analysis of variance and Tukey's studentized range test ($\alpha = 0.05$) (23). Means are presented with standard errors.

Results

Infectivity: S. glaseri and *H. bacteriophora* were highly infective to *P. japonica* larvae (Fig. 1); 100% grub mortality occurred after external exposure to 1,000 infective juveniles. In contrast, *S. carpocapsae* and *S. scapterisci* had a low level of infectivity. Even at a dosage of 1,000 nematodes per grub, *S. scapterisci* did not cause lethal infections, and *S. carpocapsae* induced only 37% mortality. Mortality was not observed in the control group.

Steinernema glaseri and *H. bacteriophora* were not infective at 100 nematodes per *A. domesticus* adult, a dose at which *S. carpocapsae* and *S. scapterisci* induced 23 and 20% mortality (Fig. 2). A dosage of 1,000 nematodes per host was required for *S. glaseri* and *H. bacteriophora* to reach approximately these levels of mortality. *S. carpocapsae* and *S. scapterisci* induced 60 and 77% mortality at a dosage of 1,000 nematodes. Mortality was not observed in the control group.

The trends from the hemocoel injection of nematodes were similar to those of external exposures, except *S. carpocapsae* was more pathogenic when injected (Fig. 3). *Steinernema carpocapsae, S. glaseri,* and *H. bacteriophora* induced 100% mortality of *P. japonica,* but *S. scapterisci* still did not kill a single grub. In *A. domesticus, S. carpocapsae* and *S. scapterisci* induced 100% mortality, whereas *S. glaseri* and *H. bacteriophora* caused only 33 and 20% host mortality, respectively. Mortality was not observed in the control group.

Encapsulation and melanization: We found substantial differences at 24 hours after injection in host immune response to the different nematode species (Fig. 4). In *P. japonica,* few *S. glaseri* (2.8%) were encapsulated and melanized, whereas nearly all injected infective juveniles of the other three species tested became encapsulated and melanized. In *A. domesticus,* most *S. glaseri* (88%) and *S. carpocapsae* (92%) be-

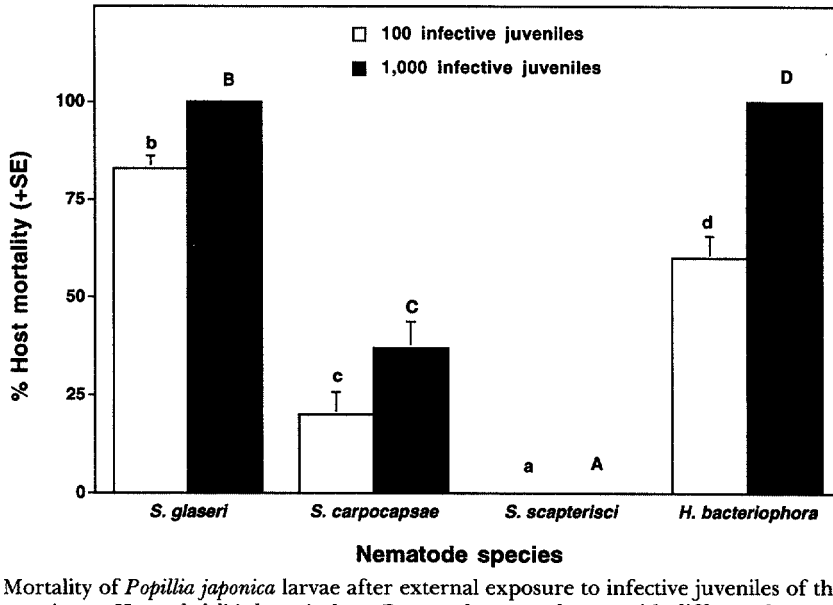

FIG. 1. Mortality of *Popillia japonica* larvae after external exposure to infective juveniles of three different *Steinernema* species or *Heterorhabditis bacteriophora.* Bars at the same dosage with different letters are different ($P < 0.05$) by Tukey's studentized range test. SE = standard error.

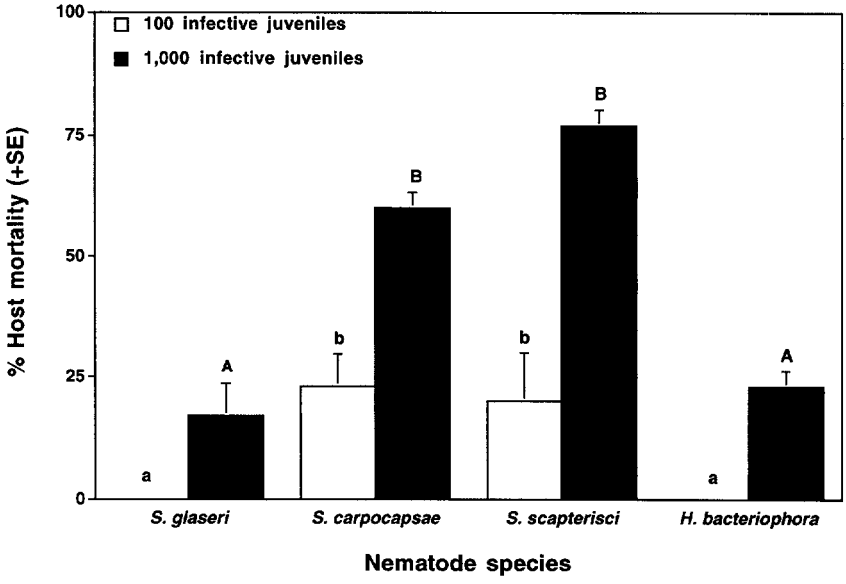

FIG. 2. Mortality of *Acheta domesticus* adults after external exposure to infective juveniles of three *Steinernema* species or *Heterorhabditis bacteriophora*. Bars at the same dosage with different letters are different ($P < 0.05$) by Tukey's studentized range test. SE = standard error.

came encapsulated and melanized, whereas *S. scapterisci* experienced a lower level of immune response (16% melanized). *Heterorhabditis bacteriophora* had high levels of encapsulation and melanization in both of the host species. The encapsulation observed in both hosts was melanotic encapsulation.

Nematode reproduction: In the hemocoel injection experiment, *S. glaseri*, *H. bacteriophora*, and *S. carpocapsae* reproduced successfully in *P. japonica* (Table 1). In *A. do-*

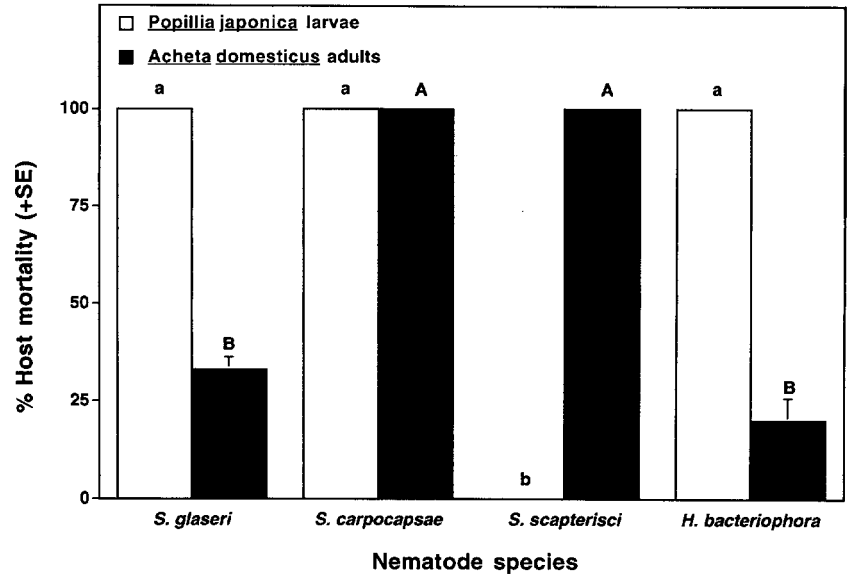

FIG. 3. Mortality of *Popillia japonica* larvae and *Acheta domesticus* adults after injection of 20 surface-sterilized infective juveniles of three *Steinernema* species or *Heterorhabditis bacteriophora*. Bars (in the same host) with the same letters are not different ($P > 0.05$) by Tukey's studentized range test. SE = standard error.

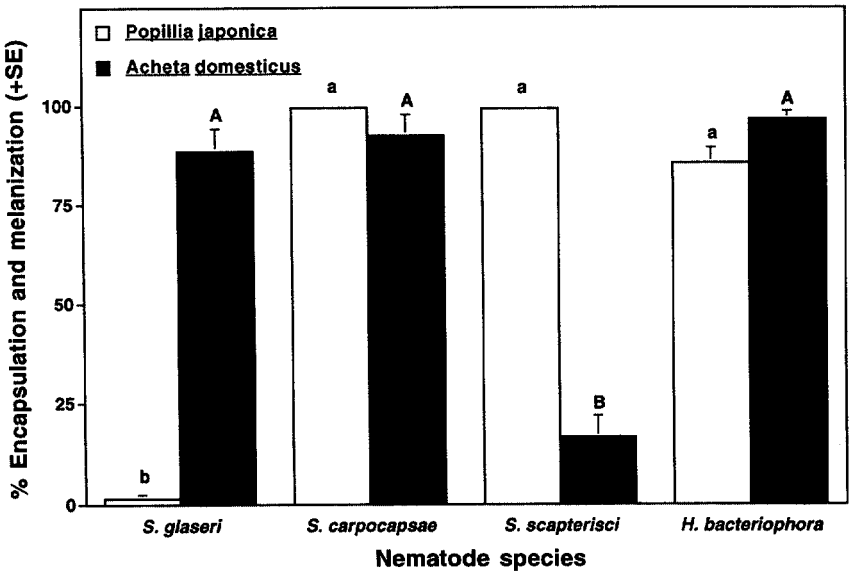

FIG. 4. Percentage of encapsulated and melanized *Steinernema glaseri, S. carpocapsae, S. scapterisci,* and *Heterorhabditis bacteriophora,* 24 hours after injection of 20 surface sterilized infective juveniles into the hemocoel of *Popillia japonica* or *Acheta domesticus.* Bars (in the same host) with the same letters are not different between nematode species ($P > 0.05$) by Tukey's studentized range test. SE = standard error.

mesticus, however, only *S. glaseri* and *S. scapterisci* reproduced in large numbers (12,600 and 13,700 infective juveniles per cadaver). *Steinernema carpocapsae* produced only 7.3% as many infective juveniles in *A. domesticus* as in *P. japonica. Heterorhabditis bacteriophora* did not develop in *A. domesticus* adults.

Time course of encapsulation and melanization: Nearly 90% of hemocoel-injected *S. carpocapsae* were encapsulated within fat body and connective tissue by 0.17 hours; 11% were free, and fewer than 1% were melanized (Fig. 5). Melanization proceeded quickly thereafter, with 90% of the injected nematodes melanized by 8 hours. Concurrently, the number of free nematodes decreased sharply, none being observed after 4 hours.

Bacterial examination: In *P. japonica* hemolymph, few *Xenorhabdus nematophilus* CFUs were found 4 hours after injection of *S. carpocapsae* (ca. 4 CFU/μl hemolymph), but the number increased sharply by 16 hours (1,875 CFU/μl hemolymph) (Fig. 5). By 24 hours, the number reached almost 20,000 CFU/μl hemolymph, and the grubs were dead. Bacteria were not found in the hemolymph of control grubs.

DISCUSSION

Nematodes have two general ways of dealing with the host immune response: evasion, by entering host tissue, molecular mimicry, or antigen sharing, and counteracting (24). Dunphy and Webster (5) proposed that *S. carpocapsae* evaded the immune response of *G. mellonella* by not being recognized as non-self. The unreactive surface of the nematode could be innately

TABLE 1. Infective juvenile production of three *Steinernema* species and *Heterorhabditis bacteriophora* in two hosts (*Popillia japonica* and *Acheta domesticus*) after injection of 20 infective juveniles into hemocoel.

Nematode species	*P. japonica*	*A. domesticus*
S. glaseri	5,182 ± 830	12,640 ± 4,001
S. carpocapsae	22,618 ± 6,045	1,656 ± 1,197
S. scapterisci	0	13,713 ± 4,398
H. bacteriophora	27,400 ± 4,598	0

Number of nematodes emerging per host cadaver is expressed as mean ± standard error.

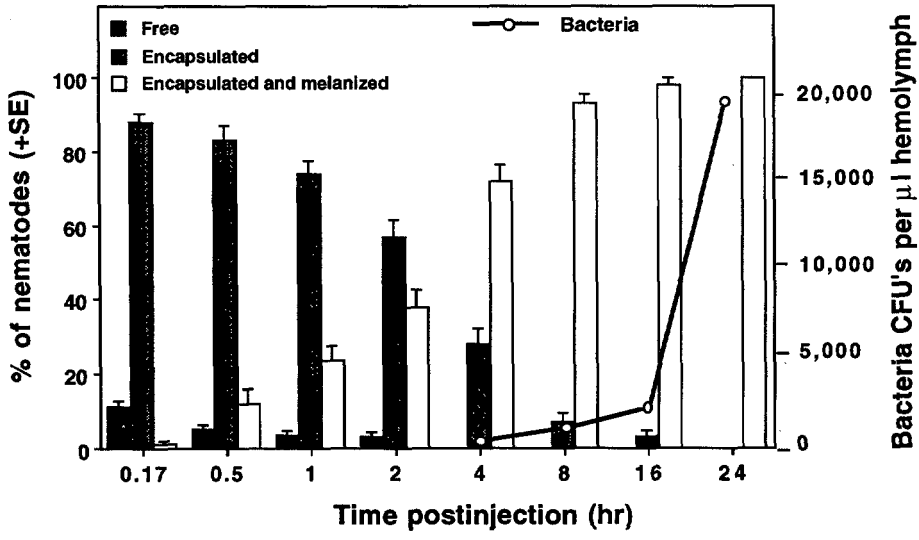

FIG. 5. Time course of encapsulation and melanization of *Steinernema carpocapsae* injected into the hemocoel of *Popillia japonica,* and associated bacterial (*Xenorhabdus nematophilus*) release and multiplication. SE = standard error.

secreted by the nematode or acquired from host materials during penetration (4). Our study suggests that *S. glaseri* in *P. japonica* and *S. scapterisci* in *A. domesticus* also evade the immune response. This evasion, however, is not effective against all insects. Our data show that the two nematode species avoid the immune response in hosts similar to those with which they appear to be associated under natural conditions: *S. glaseri* with scarab larvae (9,22) and *S. scapterisci* with mole crickets, *Scapteriscus* spp. (20). The mechanism for this avoidance is unclear and may differ among nematode species.

Heterorhabditis bacteriophora elicited a strong immune response in both *P. japonica* and *A. domesticus.* The host immune response was counteracted in *P. japonica,* and the nematode established and reproduced. In the house cricket, *H. bacteriophora* died before producing progeny. Variations in the virulence of the symbiotic bacteria or speed of host encapsulation could account for this difference.

In previous studies, *H. bacteriophora* and *S. glaseri* were shown to have high efficacy against *P. japonica* (15,27). Our data and other evidence suggest that *H. bacteriophora* may use a different approach to deal with the *P. japonica* immune response. First, the bacterium *Photorhabdus luminescens,* associated with *H. bacteriophora,* is very virulent to *P. japonica.* By injecting different species of bacteria into *P. japonica,* Yeh et al. (27) demonstrated that *P. luminescens* was so virulent that two cells caused about 95% mortality of the host. *Xenorhabdus poinarii,* associated with *S. glaseri,* was not virulent even after 20,000 cells were injected. Similarly, neither *S. glaseri* nor *X. poinarii* alone has been shown to be pathogenic to *G. mellonella* (1). Second, *H. bacteriophora* releases its bacteria earlier than *S. glaseri* in *P. japonica. Xenorhabdus poinarii* was detected in *P. japonica* hemolymph 8 hours after injection of *S. glaseri,* and *P. luminescens* was detected as early as 30 minutes after injection of *H. bacteriophora* (data not shown). We propose that *H. bacteriophora* may rely on the rapid destruction of the host immune response by its symbiotic bacterium, *P. luminescens,* to establish in *P. japonica.*

Steinernema carpocapsae induced low *P. japonica* mortality in external exposures, but caused 100% host mortality and reproduced in the cadaver after hemocoelic injection. This result is consistent with laboratory bioassays and field trials demonstrating poor infectivity of *S. carpocapsae* to

P. japonica (14,15). The reasons for this low infectivity have been attributed to unsuitable host-finding strategy (11,12,16,17) and poor host penetration (3). Our data also suggest that these barriers to infection are most important, because *P. japonica* is a suitable host if sufficient infective juveniles penetrate into the hemocoel.

Steinernema carpocapsae elicited a strong melanotic encapsulation response in both *P. japonica* and *A. domesticus,* but both hosts were killed by nematode injection. Host mortality despite nematode encapsulation may result from the release of the symbiotic bacteria prior to complete encapsulation. Low levels of *X. nematophilus* were detected in the hemocoel of *P. japonica* at 4–8 hours postinjection, before the time at which all injected nematodes were completely encapsulated and melanized (16 hours postinjection). This result is consistent with the idea that time of bacterial release and speed of encapsulation and melanization are important factors in determining whether the infection is successful (26). Comparison of the time of bacterial release by nematodes and the time of complete encapsulation in various hosts is needed to assess the significance of these factors.

Although entomopathogenic nematodes have a broad potential host range, increasing evidence suggests that under natural conditions their host range is much narrower. Therefore, it is important to elucidate the interactions among the nematode, symbiotic bacteria, and host. Efforts to date to determine the most effective nematode species for a particular target pest have been largely trial and error experiments. When selecting a particular entomopathogenic nematode for field application, screening for ability to overcome or evade the host defense response may provide an important first step in narrowing the list of suitable nematode species.

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