Entomologia Experimentalis et Applicata **94:** 283–293, 2000.

Synergism of imidacloprid and entomopathogenic nematodes against white grubs: the mechanism

Albrecht M. Koppenhöfer[1,*], Parwinder S. Grewal[2] & Harry K. Kaya[1]
[1]*Department of Nematology, University of California, Davis, CA 95616-8668, USA (E-mail: koppenhofer@aesop.rutgers.edu);* [2]*Department of Entomology, Ohio State University, OARDC, Wooster, OH 44691-4096, USA;* **Present address: Department of Entomology, Rutgers University, New Brunswick, NJ 08901, USA*

Accepted: December 9, 1999

Key words: Japanese beetle, northern masked chafer, *Cyclocephala hirta*, *Steinernema*, *Heterorhabditis*, integrated pest management, host attachment, behavior, defense

Abstract

Entomopathogenic nematodes and the chloronicotinyl insecticide, imidacloprid, interact synergistically on the mortality of third-instar white grubs (Coleoptera: Scarabaeidae). The degree of interaction, however, varies with nematode species, being synergistic for *Steinernema glaseri* (Steiner) and *Heterorhabditis bacteriophora* Poinar, but only additive for *Steinernema kushidai* Mamiya. The mechanism of the interaction between imidacloprid and these three entomopathogenic nematodes was studied in the laboratory. In vials with soil and grass, mortality, speed of kill, and nematode establishment were negatively affected by imidacloprid with *S. kushidai* but positively affected with *S. glaseri* and *H. bacteriophora*. In all other experiments, imidacloprid had a similar effect for all three nematode species on various factors important for the successful nematode infection in white grubs. Nematode attraction to grubs was not affected by imidacloprid treatment of the grubs. Establishment of intra-hemocoelically injected nematodes was always higher in imidacloprid-treated grubs but the differences were small and in most cases not significant. The major factor responsible for synergistic interactions between imidacloprid and entomopathogenic nematodes appears to be the general disruption of normal nerve function due to imidacloprid resulting in drastically reduced activity of the grubs. This sluggishness facilitates host attachment of infective juvenile nematodes. Grooming and evasive behavior in response to nematode attack was also reduced in imidacloprid-treated grubs. The degree to which different white grub species responded to entomopathogenic nematode attack varied considerably. Untreated *Popillia japonica* Newman (Coleoptera: Scarabaeidae) grubs were the most responsive to nematode attack among the species tested. Untreated *Cyclocephala borealis* Arrow (Coleoptera: Scarabaeidae) grubs showed a weaker grooming and no evasion response, and untreated *C. hirta* LeConte (Coleoptera: Scarabaeidae) grubs showed no significant response. Chewing/biting behavior was significantly increased in the presence of nematodes in untreated *P. japonica* and *C. borealis* but not in *C. hirta* and imidacloprid-treated *P. japonica* and *C. borealis*. Our observations, however, did not provide an explanation for the lack of synergism between imidacloprid and *S. kushidai*.

Introduction

Entomopathogenic nematodes offer an environmentally safe alternative to chemical insecticides in the management of white grubs, the root-feeding larvae of scarabaeid beetles pestiferous in turfgrass and pastures. Because the level of control by these nematodes is often inconsistent and unsatisfactory (Georgis & Gaugler, 1991; Klein, 1993), chemical insecticides are still the first choice of turfgrass managers for white grub control. Several studies have shown that the efficacy of entomopathogenic nematodes to curatively control white grubs can be improved if they are integrated with other pathogens but these combina-

tions have limitations. For example, the combination of nematodes and *Bacillus popilliae* Dutky (Thurston et al., 1993, 1994) is feasible only for long-term control in high economic threshold situations whereas the combination of nematodes and *Bacillus thuringiensis* Berliner Buibui strain (Koppenhöfer & Kaya, 1997; Koppenhöfer et al., 1999) is feasible only for scarab species that are sufficiently susceptible to this bacterium.

A more efficient combination with wider applicability should be that of nematodes and the chloronicotinyl insecticide, imidacloprid (Koppenhöfer & Kaya, 1998; A. M. Koppenhöfer, I. Brown, R. Gaugler, P. S. Grewal, H. K. Kaya & M.G. Klein, unpubl.). Currently, imidacloprid is one of the most popular insecticides for preventative white grub control because of its high efficacy, relatively low vertebrate toxicity, low application rates, and long systemic persistence (Schroeder & Flattum, 1984; Elbert et al., 1991). As a broad-spectrum insecticide imidacloprid has the potential to disrupt any existing natural control of turfgrass insect pests by predatory or parasitic insects, however, the effect on beneficial invertebrates appears to be relatively small (Kunkel et al., 1999). Because its efficacy declines with advancing white grub development (Potter, 1998), imidacloprid is applied in a preventative approach, the optimum period for application being during the month preceding egg hatch until the time when grubs are beginning to hatch (Potter, 1998). However, white grub outbreaks are difficult to predict because they tend to be localized and sporadic and the eggs and first instars are difficult to sample for. As a result imidacloprid is applied over large areas although often only small fractions of lawns may require grub control. The combination of imidacloprid and nematodes would allow curative treatments against older white grub stages, and because these stages are easier to detect, treatments could be limited to infested areas only, reducing cost and environmental impact.

Koppenhöfer & Kaya (1998) and A. M. Koppenhöfer, I. Brown, R. Gaugler, P. S. Grewal, H. K. Kaya & M.G. Klein, (unpubl.) showed that combined applications of the scarab-adapted entomopathogenic nematodes *Steinernema glaseri* (Steiner) or *Heterorhabditis bacteriophora* Poinar and imidacloprid resulted in synergistic mortality of third-instar white grubs. This interaction was observed over a range of imidacloprid rates, with simultaneous or delayed nematode application, and for five scarab species with different degrees of nematode susceptibility. The degree of interaction, however, was usually greater for *S. glaseri* than for *H. bacteriophora*. In addition, combination of imidacloprid with the scarab-specific nematode *Steinernema kushidai* Mamiya, which is highly efficient and persistent for white grub control (Koppenhöfer et al., 2000), resulted only in additive grub mortality.

The objective of the present study was to elucidate the mechanism of interaction between entomopathogenic nematodes and imidacloprid. In order to successfully infect a host, the infective juvenile stage nematodes have to locate a potential host, attach to its cuticle, penetrate, and establish in the host's body cavity. However, during their coevolution with soil pathogens such as entomopathogenic nematodes, white grubs have developed a series of behavioral, morphological, and physiological barriers to infection.

The first step in the infection process of entomopathogenic nematodes, detection of a potential host, may be made more difficult through the white grubs' tendency to release CO_2 in bursts rather than continuously. CO_2 is an important volatile host cue for entomopathogenic nematodes (Lewis et al., 1993). Nematodes that have located a white grub and attached to its cuticle, can be effectively eliminated by the grub's aggressive grooming behaviors. These behaviors include rubbing with an abrasive raster situated on the ventral end of the abdomen or brushing with legs or mouthparts (Gaugler et al., 1994). In addition, white grubs evade nematode attack by moving away from the nematodes (Schroeder et al., 1993; Gaugler et al., 1994). Both behaviors have been demonstrated for grubs of the Japanese beetle, *Popillia japonica* Newman.

Nematode penetration into insect hosts generally can occur (1) directly through thin parts of the cuticle (only common in *Heterorhabditis* spp.) or (2) through tracheae via the spiracles or (3) through the midgut epithelium via mouth or anus. In white grubs, the spiracles are covered with sieve plates impenetrable to nematodes. Nematode penetration through the midgut epithelium is delayed by a dense peritrophic membrane (Forschler & Gardner, 1991). This delay increases the chances of inactivating gut fluids (Wang et al., 1995) and/or food passage removing nematodes from the vulnerable alimentary tract. Nematodes that have penetrated into the grubs' hemocoel still have to face a strong immune response, melanotic encapsulation, that can effectively eliminate invaders (Wang et al., 1994, 1995).

Because imidacloprid acts on the cholinergic receptors in the postsynaptic membranes, disrupting

normal nerve function (Bai et al., 1991), we hypothesized that the major factor responsible for the synergistic interaction would be the breakdown of white grub behavioral defenses that they display in response to nematode attack. However, negative effects on any of the described defense mechanisms may contribute to the synergistic interaction of entomopathogenic nematodes and imidacloprid. In a series of experiments, we tested the effect of exposure to imidacloprid on the white grub defensive mechanisms.

Material and methods

General methods. Field-collected third-instar white grubs were used in all experiments. *Cyclocephala hirta* LeConte was collected at the Woodbridge Golf and Country Club, Woodbridge, California, USA; *P. japonica* and *Cyclocephala borealis* Arrow were collected at the Lyons Den Golf Course, Canal Fulton, Ohio, USA. Grubs were kept at 10 °C for 4–10 weeks in a mixture of organic compost and loamy sand with grass seeds provided as food. *H. bacteriophora* NC1 strain and *S. glaseri* NC strain were cultured in the last instar of the greater wax moth, *Galleria mellonella* L. The emerging infective juveniles (IJs) were harvested from White traps and stored in sterilized deionized water at 10 °C (Kaya & Stock, 1997) for 4–21 days before use. *S. kushidai* was cultured in third-instar *C. hirta*, harvested as described above and stored at 15 °C. Imidacloprid (Miles Inc., Kansas City, MO, USA) was obtained as a wettable powder with 75% active ingredient (AI) (Merit® 75 WP). A loamy sand (87% sand, 7% silt, 6% clay, 0.3% organic matter, pH 6.9) autoclaved 3 months before use and prepared at 12% (w/w) (-6 kPa water potential) was used in the experiments. The experiments were conducted at room temperature (20–24 °C)

For all experiments except the one examining grub evasive behavior, the grubs were exposed to imidacloprid in polystyrene snap cap vials (25 mm inner diameter $\times$ 47 mm height; 4.9 cm^2) before use in experiments. The bottoms and lids of these vials were perforated with holes (1 mm diam) for drainage (four holes) and aeration (15 holes), respectively. The vials were filled with dry sterilized soil to a height of 4 cm (20 cm^3 soil), seeded with rye grass, and watered as needed. After 10 days, the germinated grass was cut and one grub per vial was placed on the soil surface. Grubs that did not dig into the soil overnight were replaced with new ones. The grubs were allowed to acclimatize at room temperature for 2–4 days before each vial was treated with 1.5 ml of water containing 0 or 10 μg AI imidacloprid (200 g AI ha^{-1}). The grubs were left in the treated vials for 2 days before being used in experiments.

Establishment of interaction under laboratory conditions. In the first experiment, we confirmed that the imidacloprid-nematode interaction previously observed in greenhouse pot trials (Koppenhöfer & Kaya, 1998) also occurred under laboratory conditions. In addition, the laboratory set-up allowed us to quantify the effect of imidacloprid on the nematodes' speed of kill and number establishing in the hosts. Vials with grubs that had been exposed to water or imidacloprid (see above) were treated with 1 ml water containing no nematodes; *S. kushidai* at rates of 62, 125, or 250 IJs/vial; *S. glaseri* at rates of 125 or 250 IJs/vial; or *H. bacteriophora* at rates of 250 or 500 IJs/vial. The vials were checked daily for 14 days. Dead grubs were recovered, rinsed in water, incubated at room temperature for 2 days, dissected and digested in a pepsin solution (Mauleon et al., 1993), and the number of nematodes established per grub determined. The experiment was conducted three times with ten to 16 replicate vials per treatment and trial using *C. hirta*.

Effect of imidacloprid on nematode attraction to grubs. To determine whether imidacloprid affects the attraction of IJs to grubs, we quantified IJ migration through soil columns to grubs exposed to water or imidacloprid (see above). The soil columns consisted of two polyvinyl segments (30 mm inner diameter; 7.1 cm^2) filled to a total height of 5 cm with soil. The lower segment (1 cm height) was separated from the upper segment (5 cm height) by a metal mesh (1 mm openings). A grub was introduced into the lower segment and kept for 24 h to allow for a possible gradient of nematode attractants to build up. Then, 500 IJs of *S. kushidai*, *S. glaseri*, or *H. bacteriophora* in 1 ml of deionized water were applied to surface of the soil column. After 24 h for *S. glaseri* and *H. bacteriophora*, and 48 h for the slower dispersing *S. kushidai*, the soil from each segment was rinsed separately into a petri dish. The grubs were also rinsed and the rinse-off kept with the soil from the lower segment. The nematodes were extracted from the petri dish contents with a decant-and-sieve method. Briefly, the petri dishes were washed separately into a 1-litre beaker that was filled with tap water and vigorously stirred to suspend the soil. After allowing the soil to settle

for 30 s, the water was decanted through a coarse sieve (No. 100 mesh, 150 μm opening) and a fine sieve (No. 635 mesh, 20 μm opening). The contents of the fine sieve were washed into a petri dish and the number of IJs in the dish counted with a dissecting microscope. For *S. glaseri* and *H. bacteriophora*, there were five replicates each without grubs and seven replicates each with untreated or treated grubs. For *S. kushidai*, there were ten replicates without and 16 replicates each with untreated and treated grubs. The experiment was conducted twice using *C. hirta*.

Effect of imidacloprid on grub grooming behavior. To determine the effect of imidacloprid on grub grooming behavior in response to nematode attack, grubs exposed to imidacloprid or water (see above) were individually placed in petri dishes (60 $\times$ 15 mm) lined with one filter paper. The dishes had been treated 1 h earlier with 0.25 ml water containing 0 or 5000 IJs. The grubs were allowed to acclimate for 30 min. Then their behavior was observed for 5 min and the number of times they rubbed (raster abrading against body), brushed (legs or mouth parts swept across body), or performed chewing/biting motions between being motionless (in 'C'-shape or raised on legs) was noted. This experiment was performed with *C. hirta*, *C. borealis*, and *P. japonica*. Treatments were grubs exposed to water only treated with (1) water only, (2) *H. bacteriophora*, or (3) *S. glaseri*, and grubs exposed to imidacloprid treated with (4) water only, (5) *H. bacteriophora*, or (6) *S. glaseri*. There were two trials with five replicates per treatment for each scarab species.

Effect of imidacloprid on grub evasive behavior. The effect of imidacloprid on grub evasive behavior in response to IJs was determined in soil observation chambers (Gaugler et al., 1994). The chambers consisted of two 12 $\times$ 12 cm sheets of fiberglass with rubber tubing (10 mm diam) as a spacer on the bottom and the sides. The chambers were filled with soil, and grass was allowed to grow for 7–10 days. One grub was added and allowed to acclimate for one day before the chambers were treated with water or imidacloprid (200 g AI ha^{-1}). After 2 days, 40 μl of sterilized deionized water containing 0 or 2000 IJs were added to the side of the grub's soil cell using a long 18-gauge syringe. To avoid clogging of the syringe with soil, the entry channel for the syringe was pre-bored with a similar size wooden stick. The position of the grub was marked on the outside of the chamber every 20 min for 100 min.

The experiment was conducted with *C. hirta*, *C. borealis*, and *P. japonica*. The treatments were the same as in the grooming behavior experiment. Each treatment had five replicates. Three trials were conducted with *C. hirta* and one trial each was conducted with *C. borealis* and *P. japonica*.

Effect of imidacloprid on nematode attachment to grubs. Two experiments were conducted to determine the effect of imidacloprid on IJ attachment to grubs using procedures modified from Gaugler et al. (1994). Third-instar *C. hirta* that had been exposed to water or imidacloprid (see above) were placed in petri dishes (60 $\times$ 15 mm) filled with moist soil. The grubs were allowed to acclimatize for 1 h before the soil surface was treated with nematode suspension. At the end of the exposure period, the grubs were recovered and rinsed with water in a petri dish, and the number of nematodes in the rinse-off counted. In the first experiment, the grubs were exposed to 1000 IJs for 150 min (*S. glaseri* or *H. bacteriophora*) or 200 min (*S. kushidai*). In the second experiment, grubs were exposed to 500 *S. glaseri* for 100 (trial 1) or 150 (trial 2) min. In the second experiment, the grubs were also unrestrained or restrained in different size cages made from fine plastic mesh folded around the grubs and closed with metal staples. The large cages (approximately 40 $\times$ 20 $\times$ 5 mm) allowed the grubs to turn and move to some extent. The small cages (approximately 20 $\times$ 13 $\times$ 4 mm) almost completely inhibited grub movement with the staples applied as close as possible to the grubs without causing injury. In both experiments, there were seven replicates per treatment in trial 1 and eight replicates per treatment in trial 2.

Effect of imidacloprid on nematode establishment in grub hemocoel. Two experiments were conducted to test the effect of imidacloprid on the establishment of nematodes in the grub hemocoel. The IJs used were not surface-sterilized to avoid changes in the nematodes' cuticle. Instead, they were washed five times in sterilized deionized water (final dilution 3 $\times$ 10^6). The washed IJs were injected into the grubs in 10 μl of sterilized deionized water using a sterilized hypodermic syringe (25 gauge). After injection, the grubs were placed back into the vials in which they had been exposed to imidacloprid or water. The mortality was checked daily for 10 days. Dead grubs were incubated for 2 days and dissected to determine the number of nematodes that had established in them. In the first

experiment, IJs were injected directly into the grubs' hemocoel. The rates were 5, 10 or 20 IJs for *S. glaseri* and *H. bacteriophora* and 10 or 20 IJs for *S. kushidai*. In the second experiment, IJs were injected orally by introducing a blunt syringe through the mouth into the oesophagus until it was visible in the thorax behind the head capsule. The rates were 5 or 20 IJs for *S. glaseri*, and 20 IJs for *S. kushidai* and *H. bacteriophora*. Both experiments were conducted using *C. hirta* in two trials with ten replicates per treatment and trial.

Statistics. In the experiment establishing the interaction under laboratory conditions, synergistic, additive or antagonistic interactions between nematodes and imidacloprid were determined using a chi-square test (Finney, 1964; McVay et al., 1977; Koppenhöfer & Kaya, 1998). Analysis of variance (PROC GLM) and means separation with Tukey's test (SAS Institute, 1996) were applied on data from the experiments determining the effect of imidacloprid on nematode attraction to grubs (by nematode species), grub grooming and evasive behavior (by scarab species), and nematode attachment to grubs (first experiment by nematode species and sampling time; second experiment by sampling time). *T* test was applied on data from the experiment establishing the nematode-imidacloprid interaction in the snap cap vials (by nematode species and dose), and the experiments determining the effect of imidacloprid on nematode establishment in the grub after intra-hemocoelic or oral injection (by nematode species). Data are presented as means ± standard error of the mean. Differences among means are considered significant at $P < 0.05$.

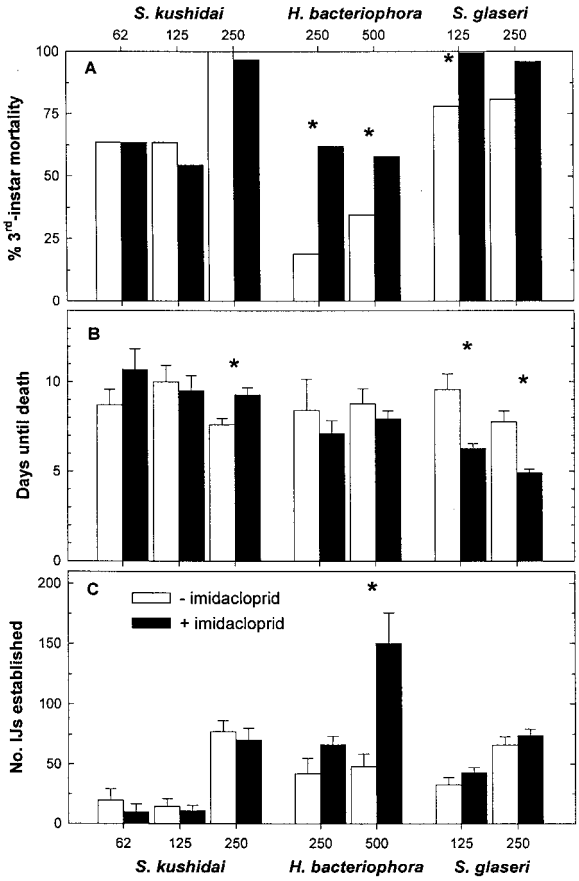

Figure 1. Effect of imidacloprid on (A) mortality after 14 days, (B) days until death, and (C) establishment of entomopathogenic nematode infective juveniles in third-instar *Cyclocephala hirta* exposed to different rates of *Steinernema kushidai*, *Heterorhabditis bacteriophora*, or *S. glaseri*. Grubs were exposed to imidacloprid (200 g AI ha^{-1}) for 2 days in soil with grass before nematodes were added. Asterisks indicate significant difference within nematode species and nematode rate. Mortality in control (4.8%) and imidacloprid only (9.5%) treatments are not shown.

Results

Establishment of interaction under laboratory conditions. In our vial studies, combination of imidacloprid and entomopathogenic nematodes had a similar effect on third-instar *C. hirta* mortality as we had previously observed in greenhouse pot trials. Control mortality (4.8%) and mortality in the imidacloprid only treatments (9.5%) were low. The effect of imidacloprid on nematode infection of grubs showed different trends among the three nematode species. For *S. kushidai*, the general trend suggested a limited negative effect of imidacloprid on grub mortality, speed of kill, and number of IJs establishing in the grubs (Figure 1A–C), but only at one nematode rate (250 IJs) and only for speed of kill was this effect significant ($t = 3.1$; df = 11.0; $P = 0.003$). For *H. bacteriophora*, imidacloprid had a synergistic effect on grub mortality ($\chi^2 \geq 12.65$; df = 1; $P < 0.001$). Speed of kill, and nematode establishment were also enhanced, however, only nematode establishment was significantly increased at one nematode rate (500 IJs: $t = 4.7$; df = 18.3; $P = 0.003$) (Figure 1A–C). For *S. glaseri*, imidacloprid had a synergistic effect on grub mortality ($\chi^2 \geq 4.0$; df = 1; $P < 0.05$). Speed of kill was significantly increased in the combination treatments ($t \geq 0.3.5$; df = 18.0; $P < 0.01$), but nematode establishment was not significantly higher (Figure 1A–C).

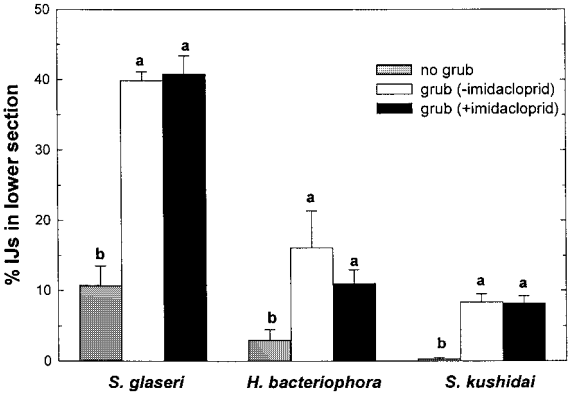

Figure 2. Effect of third-instar *Cyclocephala hirta* treatment with imidacloprid on attractiveness to entomopathogenic nematode. Grubs were confined in the lower section (1 cm height) of a soil column (5 cm total height) for 24 h before the soil surface was treated with 500 infective juveniles (IJs). After 24 h for *Steinernema glaseri* and *Heterorhabditis bacteriophora* and after 48 h for *S. kushidai*, the IJs in the two sections were extracted and counted. Bars with same letter are not significantly different within species.

Effect of imidacloprid on nematode attraction to grubs. Imidacloprid exposure did not affect the attractiveness of grubs to entomopathogenic nematodes as quantified by IJ migration through soil columns. Although the presence of grubs significantly increased the percentage of IJs extracted from the lower section of the columns for *S. glaseri* ($F = 49.1$; $df = 2, 16$; $P < 0.001$), *H. bacteriophora* ($F = 4.9$; $df = 2, 16$; $P < 0.05$), and *S. kushidai* ($F = 49.1$; $df = 2, 39$; $P < 0.001$), differences between treatments with imidacloprid-treated grubs and treatments with non-treated grubs were not significant (Figure 2).

Effect of imidacloprid on grub grooming behavior. In all three scarab species, the activity of imidacloprid-treated grubs was generally lower than that of untreated grubs (Figure 3). However, the degree of grooming behavior and how it was affected by the presence of nematodes varied considerably among scarab species. Level of activity in general and responsiveness to nematode attack of grubs not treated with imidacloprid were highest in *P. japonica*, about 50% lower in *C. borealis*, and extremely low in *C. hirta*. For *P. japonica*, rubbing (raster abrading against body) frequency in grubs not treated with imidacloprid was significantly higher only in the presence of *H. bacteriophora* ($F = 2.8$; $df = 5, 52$; $P < 0.05$) (not in the presence of *S. glaseri*) and the frequency was reduced to the control level by imidacloprid treatment. Brushing (legs or mouth parts swept across body) ($F = 6.8$; $df = 5, 52$; $P < 0.001$) and chewing ($F = 15.7$; $df = 5, 52$; $P < 0.001$) occurred significantly more often in grubs not treated with imidacloprid in the presence of nematodes and this response was reduced by 42–70% after imidacloprid treatment. In *C. borealis*, frequency of rubbing did not differ significantly among treatments. Brushing ($F = 2.8$; $df = 5, 53$; $P < 0.05$) and chewing ($F = 13.8$; $df = 3, 16$; $P < 0.001$) of grubs not treated with imidacloprid increased in the presence of nematodes but significantly so only with *S. glaseri*. Both behaviors were reduced by > 50% after imidacloprid-treatment for both nematode species, but the reduction was statistically significant only for chewing. In *C. hirta*, rubbing and brushing were observed only in grubs not treated with imidacloprid in the presence of nematodes, but at a very low frequency. Chewing frequency, also very low, was higher in grubs not treated with imidacloprid, but differences between means were not significant.

Effect of imidacloprid on grub evasive behavior. The effect of nematodes and/or imidacloprid on grub movement was similar after every observation period (data not shown) but most pronounced after 60 min (Figure 4). Generally, movement of grubs treated with imidacloprid was lower than that of grubs not treated with imidacloprid, whether nematodes were present or not. Due to the high variation and the lower number of replications in the other grub species, the reduction was significant only for *C. hirta* ($F = 16.6$; $df = 5, 84$; $P < 0.001$). However, untreated *C. hirta* and *C. borealis* did not increase their activity in the presence of either nematode species, and *P. japonica* only responded to the presence of *S. glaseri* ($F = 4.2$; $df = 5, 23$; $P < 0.01$).

Effect of imidacloprid on nematode attachment to grubs. In the first nematode attachment experiment, significantly more IJs of all three nematode species attached to the imidacloprid-treated grubs than to grubs not treated with imidacloprid after 150 min ($F \geq 5.3$; $df = 1, 12$; $P < 0.05$) and 200 min ($F \geq 7.0$; $df = 1, 14$; $P < 0.02$) (Figure 5). The same trend was observed in the second experiment for *S. glaseri* (no cage treatment) after 100 min ($F = 13.85$; $df = 5, 36$; $P < 0.001$) and 150 min ($F = 4.85$; $df = 5, 42$; $P < 0.01$) (Figure 6). In the grubs not treated with imidacloprid, nematode attachment increased as the mobility of the grubs was increasingly restrained. Imidacloprid treatment increased IJ attachment to unrestrained grubs, this effect was weaker for grubs partially restrained

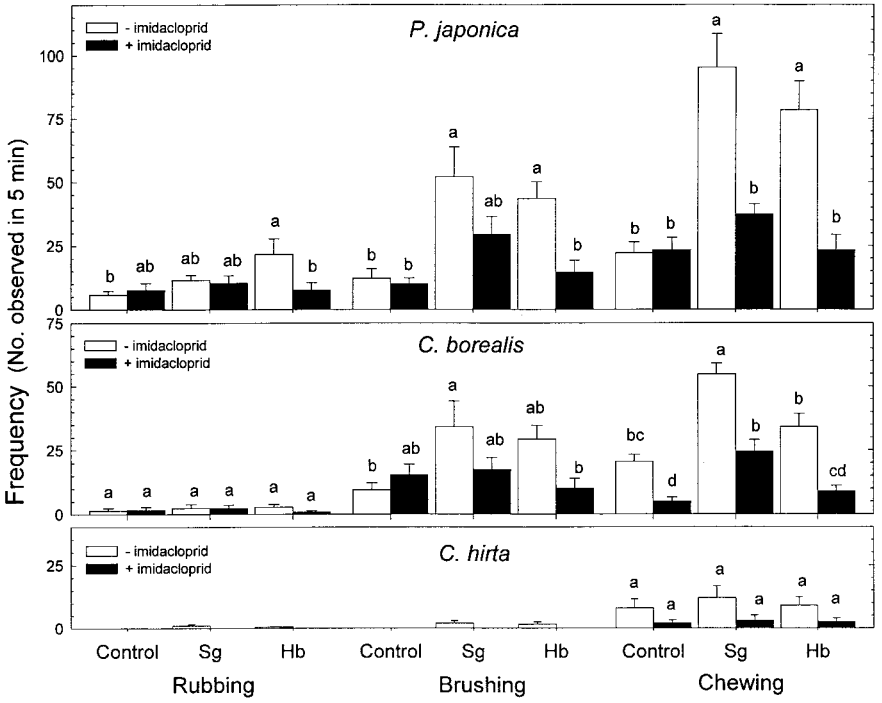

Figure 3. Effect of imidacloprid treatment on third-instar *Popillia japonica*, *Cyclocephala borealis*, and *C. hirta* grooming behavior in response to nematode attack. Frequency (number of times observed during a 5-min observation period) of behaviors displayed during petri dish exposure to *Heterorhabditis bacteriophora* (Hb) or *Steinernema glaseri* (Sg). Bars with same letter are not significantly different within each behavior.

in large cages, and no difference was observed when grubs were completely restrained in small cages.

Effect of imidacloprid on nematode establishment in grub hemocoel. When IJs were injected directly into the grubs' hemocoel, the general trend was for imidacloprid-treated grubs to have a higher number of established nematodes. Due to high variation, this effect was only significant for *S. glaseri* at the rate of 20 IJs ($t = 3.2$; df = 16, 0; P = 0.005). When IJs were injected orally, the only significant increase in nematode establishment in imidacloprid-treated grubs was observed for *S. glaseri* at the rate of 20 IJs ($t = 3.6$; df = 22, 0; P = 0.001). In both experiments, 0–20% of the grubs survived nematode injection without any obvious trends in mortality among treatments. Speed of kill was not significantly affected by imidacloprid treatment.

Discussion

The major factor responsible for synergistic interactions between imidacloprid and entomopathogenic nematodes against white grubs appears to be a general reduction of activity in imidacloprid-treated grubs. This sluggishness facilitates host attachment of infective juvenile nematodes and subsequent penetration. Our experiment with cage-restrained grubs clearly shows a negative correlation between grub mobility and IJ attachment. The experiments studying grooming and evasive behavior in response to nematode attack also demonstrate this reduced activity in imidacloprid-treated grubs. The degree to which different white grub species respond to entomopathogenic nematode attack, however, varies considerably. *P. japonica* was the most responsive among the species tested although the response was not as strong as reported by Gaugler et al. (1994). The grooming response of *C. borealis* was weaker, and *C. hirta* rarely showed grooming behavior even after direct placement of IJs on their cuticle. Neither *Cyclocephala* species responded with evasive behavior to nematode attack.

Similar effects of imidacloprid on insect behavior and synergistic interactions resulting from it have been observed with entomopathogenic fungi. Thus, Boucias et al. (1996) observed that imidacloprid increased the susceptibility of the termite, *Reticulitermes flavipes* (Kollar), to entomopathogenic fungi and ascribed this

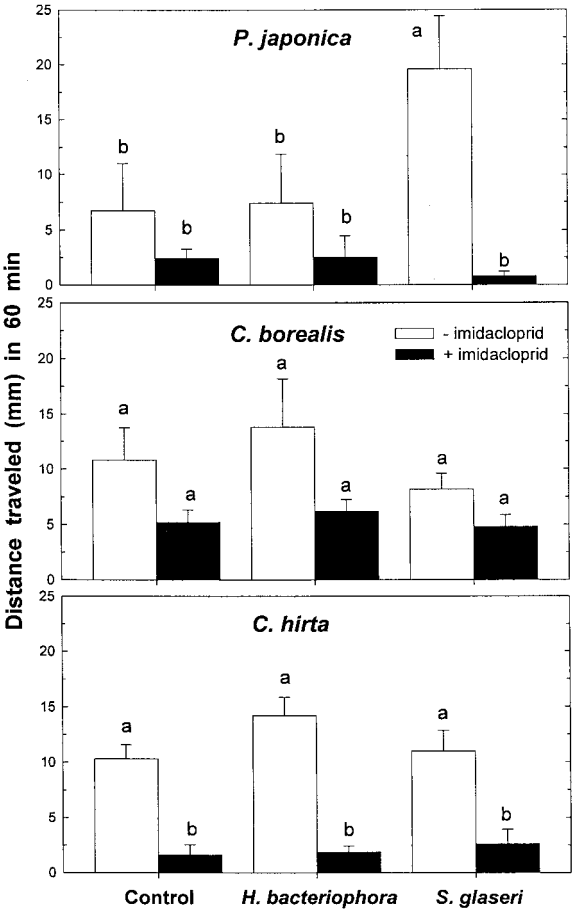

Figure 4. Effect of imidacloprid treatment on third-instar *P. japonica*, *Cyclocephala borealis*, and *C. hirta* evasive behavior in response to nematode attack. Distance traveled in 60 min following introduction of 2000 infective juvenile *Heterorhabditis bacteriophora* or *Steinernema glaseri*. Bars with same letter are not significantly different within species.

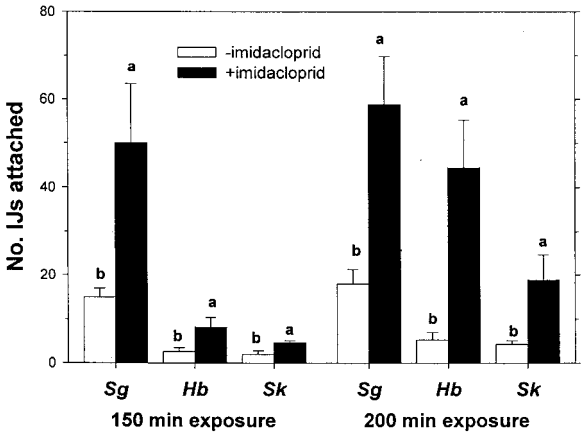

Figure 5. Effect of imidacloprid treatment of third-instar *Cyclocephala hirta* on host attachment of infective juvenile nematodes (IJs). The grubs were placed in petri dishes filled with soil that were then surface-treated with 1000 IJs of *Steinernema kushidai*, *Heterorhabditis bacteriophora*, or *S. glaseri*. After 150 or 200 min the grubs were rinsed with water and the number of IJs attached counted. Bars with same letter are not significantly different within sampling time and species.

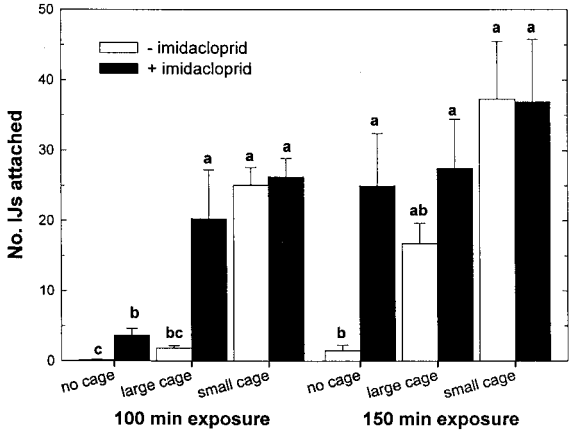

Figure 6. Effect of imidacloprid treatment of third-instar *Cyclocephala hirta* on host attachment of infective juveniles *Steinernema glaseri* (IJs). Unrestrained grubs or grubs restrained in large or small cages were placed in petri dishes filled with soil that were then surface-treated with 1000 IJs. After 100 or 150 min the grubs were rinsed with water and the number of IJs attached counted. Bars with same letter are not significantly different within sampling time.

effect to altered grooming behavior. Quintela & McCoy (1997, 1998) observed increased attachment of entomopathogenic fungal conidia on the cuticle of the citrus root weevil, *Diaprepes abbreviatus* (L.), and suggested that imidacloprid reduced larval movement in soil thereby reducing their ability to void their cuticle of fungal conidia.

At first sight the findings of enhanced nematode attachment rate in one experiment and significant increase in nematode establishment rate in nematode-killed grubs only in one nematode treatment may appear to be contradicting. Imidacloprid treatment obviously increases the percentage of grubs succumbing to infection by *S. glaseri* and *H. bacteriophora*. However, once the first nematodes have successfully established an infection in the hemocoel of grubs not treated with imidacloprid, these grubs will also become less active and finally die. Wang et al. (1995) showed that nematodes continue penetrating into Japanese beetle grub for at least 72 h. Under the conditions existing in the experimental vials (small soil volume, high nematode concentration, few restricting factors on nematodes survival), this would have to lead to similar

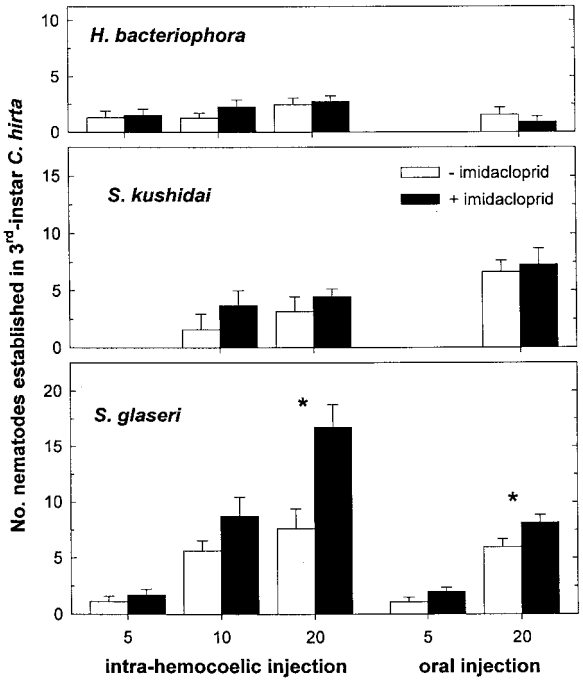

Figure 7. Effect of imidacloprid treatment of third-instar *Cyclocephala hirta* on establishment of intra-hemocoelically or orally injected infective juveniles (IJs) of the entomopathogenic nematodes *Steinernema glaseri*, *Heterorhabditis bacteriophora*, or *S. kushidai*. Asterisks indicate significant difference within nematode species and rate.

numbers of nematodes penetrating, whether they were exposed to imidacloprid or not.

Other factors may also contribute to the synergistic interaction of imidacloprid and entomopathogenic nematodes although to a lesser extent. Establishment of intra-hemocoelically injected nematodes tended to be higher in imidacloprid-treated grubs but the difference was usually small and only significant at one rate of *S. glaseri*. Because oral injection did not change these trends significantly, changes in the speed of food passage due to the nerve-toxic action of imidacloprid are unlikely.

Nematode attraction to grubs was not affected by imidacloprid treatment of the grubs. However, providing food for the grubs might have modified the outcome of the attraction experiment. We are not aware of any observations of increased feeding activity of imidacloprid-treated grub, and our own observations as well as the generally reduced activity of the grubs rather suggest a decrease in feeding activity. Recent research has shown that the feeding activity of grubs enhanced attraction of *H. bacteriophora* but not of *S. glaseri* (Wang & Gaugler, 1998). Therefore, reduced feeding of imidacloprid-treated grubs could contribute to the weaker interaction with *H. bacteriophora* than with *S. glaseri*.

Hypothetically, exposure of the nematodes to imidacloprid could increase the activity and thereby host-finding of the nematodes. However, in another study (Koppenhöfer, unpublished), IJ establishment of the three nematode species tested in this study into healthy grubs was not affected by their previous exposure to various concentrations of imidacloprid. Attraction of the nematodes to imidacloprid was not tested but should not play a significant role because the synergistic interaction occurs after soil applications that create a rather uniform horizontal distribution of imidacloprid. If imidacloprid were a nematode-attractant, it would have a confusing effect.

It is obvious that the interactions between entomopathogenic nematodes and white grubs are affected by nematode and grub species. Our study shows that behavioral responses vary greatly between white grub species and are also affected by nematode species. Despite the weaker or non-existing defensive behaviors, *Cyclocephala* grubs seem to be less susceptible to entomopathogenic nematodes than grubs of the Japanese beetle. Whether a denser and less penetrable peritrophic membrane or a stronger immune response are responsible for this higher resistance, has not been studied yet. Wang et al. (1994, 1995) and Wang & Gaugler (1998) showed that the physiology and immunology of Japanese beetle grub-nematodes interactions varies considerably with nematode species, and it is likely that they would also be affected by white grub species. In addition, different white grub species may also have different degrees of susceptibility to imidacloprid.

Unfortunately, our data cannot explain the different degree of interaction between imidacloprid and the three entomopathogenic nematode species tested (A.M. Koppenhöfer, I. Brown, R. Gaugler, P. S. Grewal, H. K. Kaya & M. G. Klein, unpubl.); i.e., synergistic interaction with *S. glaseri* and *H. bacteriophora*, but no interaction or even limited antagonism with *S. kushidai*. Thus, imidacloprid had similar effects on attraction, attachment, and establishment after injection for all three nematode species. Nevertheless, the first experiment in the vials showed that mortality, speed of kill, and nematode establishment were negatively affected by imidacloprid for *S. kushidai* but positively affected in *S. glaseri* and *H. bacteriophora*. It is unlikely that the symbiotic bacteria of *S. kushidai*, *Xenorhabdus japonicus*, may be nega-

tively affected by imidacloprid because grub mortality and nematode establishment after intra-hemocoelic injection of *S. kushidai* was not negatively affected. The explanation for the lack of synergism between *S. kushidai* and imidacloprid may rather be found in the behavioral ecology of this nematode. Despite abundant evidence for the superior performance of this nematode against various white grub species (Kushida et al., 1987; Ogura, 1993; Koppenhöfer et al., 2000), little is known about its ecology. Comparative studies on entomopathogenic nematode foraging behavior (J. F. Campbell, E. E. Lewis & H. K. Kaya, unpubl.) and our soil column experiment suggest that this nematode may have evolved different adaptations to white grubs as hosts than the highly active cruisers *H. bacteriophora* and *S. glaseri*.

Acknowledgements

We appreciate the technical assistance of Edith Barbour, Peter Kwan, Kevin Power, Kevin McClure, and Eric Ligenfelter. This is New Jersey Agricultural Experiment Station Publication No. D-12277-08-99 supported by state funds and Regional Research Funds.

References

Bai, D., S. C. R. Lummis, W. Leicht, H. Breer & B. Satelle, 1991. Actions of imidacloprid and related nitromethylene on cholinergic receptors of an identified insect motor neuron. Pesticide Science 33: 197–204.

Boucias, D. G., C. Stokes, G. Storey & J. Pendland, 1996. Effect of imidacloprid on the termite, *Reticulitermes flavipes* and its interaction with insect pathogens. Pflanzenschutz-Nachrichten Bayer 49: 103–150.

Elbert, A., B. Becker, J. Hartwig & C. Erdelen, 1991. Imidacloprid – a new systemic insecticide. Pflanzenschutz-Nachrichten Bayer 44: 113–136.

Finney, D. J., 1964. Probit Analysis. Cambridge University Press, London.

Forschler, B. & W. Gardner, 1991. Parasitism of *Phyllophaga hirticula* (Coleoptera: Scarabaeidae) by *Heterorhabditis heliothidis* and *Steinernema carpocapsae*. Journal of Invertebrate Pathology 58: 396–407.

Gaugler, R., Y. Wang & J. F. Campbell, 1994. Aggressive and evasive behaviors in *Popillia japonica* (Coleoptera: Scarabaeidae) larvae: defenses against entomopathogenic nematode attack. Journal of Invertebrate Pathology 64: 193–199.

Georgis, R., & R. Gaugler, 1991. Predictability in biological control using entomopathogenic nematodes. Journal of Economic Entomology 84: 713–720.

Kaya, H. K. & S. P. Stock, 1997. Techniques in insect nematology. In: L. Lacey (ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, pp. 281–324.

Klein, M. G., 1993. Biological control of scarabs with entomopathogenic nematodes. In: R. Bedding, R Akhurst & H. Kaya (eds.), Nematodes and the Biological Control of Insect Pests. CSIRO, East Melbourne, Australia, pp. 49–58.

Koppenhöfer, A. M. & H. K. Kaya, 1997. Additive and synergistic interaction between entomopathogenic nematodes and *Bacillus thuringiensis* for scarab grub control. Biological Control 8: 131–137.

Koppenhöfer, A. M. & H. K. Kaya, 1998. Synergism of imidacloprid and entomopathogenic nematodes: A novel approach to white grub control in turfgrass. Journal of Economic Entomology 91: 618–523.

Koppenhöfer, A. M., H. Y. Choo, H. K. Kaya, D. W. Lee & W. D. Gelernter, 1999. Increased field and greenhouse efficacy with combinations of an entomopathogenic nematode and *Bacillus thuringiensis* against scarab grubs. Biological Control 14: 37–44.

Koppenhöfer, A. M, M. Wilson, I. Brown, H. K. Kaya & R. Gaugler, 2000. Biological control agents for white grubs (Coleoptera: Scarabaeidae) in anticipation of the establishment of the Japanese Beetle in California. Journal of Economic Entomology 93: In press.

Kunkel, B. A., D. W. Held & D. A. Potter, 1999. Impact of halofenozide, imidacloprid, and bendiocarb on beneficial invertebrates and predatory activity in turfgrass. Journal of Economic Entomology 92: 922–930.

Kushida, T., Y. Mamiya & J. Mitsuhashi, 1987. Pathogenicity of newly detected *Steinernema* sp. (Nematoda) to scarabaeid larvae injurious to tree seedlings. Japanese Journal of Applied Entomology and Zoology 31: 144–149.

Lewis, E. E., R. Gaugler & R. Harrison, 1993. Response of cruiser and ambusher entomopathogenic nematodes (Steinernematidae) to host volatile cues. Canadian Journal of Zoology 71: 765–769.

Mauleon, H., S. Briand, C. Laumond & E. Bonifassi, 1993. Utilisation d'enzyme digestives pour l'étude du parasitisme des Steinernematidae et Heterorhabditidae envers les larves d'insectes. Fundamental and Applied Nematology 16: 185–186.

McVay, J. R., R. T. Gudauskas & J. D. Harper, 1977. Effects of *Bacillus thuringiensis* nuclear-polyhedrosis virus mixtures on *Trichoplusia ni* larvae. Journal of Invertebrate Pathology 29: 367–372.

Ogura, N., 1993. Control of scarabaeid grubs with an entomogenous nematode, *Steinernema kushidai*. Japanese Agricultural Research Quarterly 27: 49–54.

Potter, D. A., 1998. Destructive turfgrass insect: biology, diagnosis, and control. Ann Arbor Press, Chelsea.

Quintela, E. D. & C. W. McCoy, 1997. Pathogenicity enhancement of *Metarhizium anisopliae* and *Beauveria bassiana* to first instars of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) with sublethal doses of imidacloprid. Environmental Entomology 26: 1173–1182.

Quintela, E. D. & C. W. McCoy, 1998. Synergistic effect of two entomopathogenic fungi and imidacloprid on the behavior and survival of larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in soil. Journal of Economic Entomology 91: 110–122.

SAS Institute, 1996. SAS 6.11 for Windows. SAS Institute, Cary, NC.

Schroeder, M. E. & R. F. Flattum, 1984. The mode of action and neurotoxic properties of the nitromethylene heterocycle insecticides. Pesticide Biochemistry and Physiology 2: 148–160.

Schroeder, P. C., M. G. Villani, C. S. Ferguson, J. P. Nyrop & E. J. Shields, 1993. Behavioral interactions between Japanese beetle (Coleoptera: Scarabaeidae) grubs and an entomopathogenic ne-

matode (Nematoda: Heterorhabditidae) within turf microcosms. Environmental Entomology 22: 595–600.

Thurston, G. S., H. K. Kaya, T. M. Burlando & R. E. Harrison, 1993. Milky disease bacterium as a stressor to increase susceptibility of scarabaeid larvae to an entomopathogenic nematode. Journal of Invertebrate Pathology 61: 167–172.

Thurston, G. S., H. K. Kaya & R. Gaugler, 1994. Characterizing the enhanced susceptibility of milky disease-infected scarabaeid grubs to entomopathogenic nematodes. Biological Control 4: 67–73.

Wang, Y. & R. Gaugler, 1998. Host and penetration site location by entomopathogenic nematodes against Japanese beetle larvae. Journal of Invertebrate Pathology 72: 313–318.

Wang, Y., R. Gaugler & L. Cui, 1994. Variations in immune response of *Popillia japonica* and *Acheta domesticus* to *Heterorhabditis bacteriophora* and *Steinernema* species. Journal of Nematology 26: 11–18.

Wang, Y., J. F. Campbell & R. Gaugler, 1995. Infection of entomopathogenic nematodes *Steinernema glaseri* and *Heterorhabditis bacteriophora* against *Popillia japonica* (Coleoptera: Scarabaeidae) larvae. Journal of Invertebrate Pathology 66, 178–184.