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Field trials against *Hoplia philanthus* (Coleoptera: Scarabaeidae) with a combination of an entomopathogenic nematode and the fungus *Metarhizium anisopliae* CLO 53

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Abstract

The white grub, *Hoplia philanthus* Füessly (Coleoptera: Scarabaeidae), is a major pest of turf and ornamental plants in Belgium. Previously, the combination of lethal concentration of the entomopathogenic nematodes *Heterorhabditis megidis* or *Steinernema glaseri* with the entomopathogenic fungus *Metarhizium anisopliae* (strain CLO 53) caused additive or synergistic mortality to third-instar *H. philanthus* in the laboratory and greenhouse. In this present study, we examined this interaction under field conditions and compared a combination of a commercial formulation of *Heterorhabditis bacteriophora* (Nema-green®) and *M. anisopliae*. Controls were *M. anisopliae*, chlorpyrifos (Dursban 5 Granules) and *H. bacteriophora*. Field applications (surface or subsurface) were made against a mixed population of second/third-instar *H. philanthus* at a sport field and lawn infested in the province of West-Flanders. In both trials, the combination of *M. anisopliae* with *H. bacteriophora* at 5×10^{12} conidia/ha $+2.5 \times 10^{9}$ infective juveniles/ha resulted in additive or synergistic effects, causing more than 95% grub mortality when the nematodes was applied 4 weeks after the application of fungus. However, application of nematode, chlorpyrifos or fungus alone provided 39–66%, 42–60% (surface) and 33–76%, 82–100% or 37–65%, (subsurface) control of *H. philanthus*. We concluded that the pathogen combinations we tested are compatible elements of integrated pest management and are likely to improve control of *H. philanthus* larvae and perhaps other insect pests beyond what is expected from single application of the pathogen.

Keywords: Chlorpyrifos; Combined applications; *Hoplia philanthus*; *Heterorhabditis bacteriophora*; *Metarhizium anisopliae*; Scarab beetles; Synergism

1. Introduction

Larvae of *Hoplia philanthus* Füessly (Coleoptera: Scarabaeidae) are important pests of sports turf, lawns, pastures and ornamentals throughout Belgium (Casteels and De Clercq, 1998; Ansari et al., 2003b), resulting in high costs for pest control and for renovating or replacing damaged turf each year. Adults emerge from the soil in the first week of June, feed on the foliage of various plants, and eventually oviposit in the soil (Ansari, 2004). Most grubs reach the second-instar by mid September and may continue feeding until November of the first year. Larvae move downwards into the soil for overwintering before the soil surface freezes. In the second year, most economic damage is caused after the larvae have molted from the second- to the third-instar in June. In late October and early November of the second year, third-instar larvae migrate deeper into soil. In late March of the third year overwintering larvae become active and move towards the soil surface for a brief feeding period and initiate pupation in May. Control of the larvae with soil insecticides is still the primary means for turfgrass managers, growers and homeowners. However,

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these insecticides are rather difficult to apply and are often undesirable because of the public use of the target fields (Vlug, 1989) and the negative impact of these insecticides on the environment.

The entomopathogenic nematodes, *Heterorhabditis bacteriophora* Poinar and *Steinernema glaseri* (Steiner) Wouts, Mracek, Gerdin & Bedding, and the fungi, *Metarhizium anisopliae* (Metsch.) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin have been reported to occur naturally as pathogens of *H. philanthus* (Ansari et al., 2003a, 2004a, 2005). These nematodes are effective at controlling a variety of economically important pest insects including scarab beetles (Kaya and Gaugler, 1993; Shapiro-Ilan et al., 2002; Koppenhöfer et al., 2004). Experiments with entomopathogenic nematodes (Ansari et al., 2003b) and fungi (Ansari et al., 2004a) have been carried out, but susceptibility, especially of the third-instar of *H. philanthus*, has been variable. Previously, we demonstrated synergistic and/or additive effects of a combined application of *Heterorhabditis megidis* Poinar, Jackson & Klein or *S. glaseri* with *M. anisopliae* against third-instar *H. philanthus* under laboratory and greenhouse conditions (Ansari et al., 2004b). This interaction was observed over a lethal concentration of nematodes and *M. anisopliae*, with simultaneous or delayed nematode application. The lethal concentration of *M. anisopliae* is hypothesized to act as a stressor affecting the pest insect. The objectives of the present study were to expand our knowledge on synergism between entomopathogenic nematodes and *M. anisopliae* under field conditions in which we compared *H. bacteriophora*, *M. anisopliae*, Dursban 5G (active ingredient: chlorpyrifos), and a combination of the nematode and fungus.

2. Materials and methods

2.1. Nematodes

The biocontrol product Nema-green® (e-nema GmbH, Keel, Germany) was used in the field trials. This product contains a preparation of *H. bacteriophora*. This species was chosen because it had shown promise for white grub control (Sulistyanto and Ehlers, 1996) and was recently isolated from naturally infected larvae of *H. philanthus* in Belgium (Ansari et al., 2003a). Before use, the product was stored at 10 °C; it was applied according to the manufacturer instructions.

2.2. Fungus production

Metarhizium anisopliae CLO53 was isolated in 2002 from naturally infected third-instar *H. philanthus* from a private lawn at Lovendegem, Belgium and cultured on Sabouraud Dextrose Agar (SDA) (Becton Dickinson, Benelux N.V., Belgium) according to Ansari et al. (2004a). This isolate was selected because it was demonstrated to have a relatively high virulence to third-instar *H. philanthus* (Ansari et al., 2004a).

To obtain conidia for the field trials, *M. anisopliae* was grown on barley kernels. Two kilograms of slightly crushed barley kernels (still containing the husk) were mixed with 700 ml of tap water and transferred to polypropylene bags (Sekuroka®Entsorgungsbeutel, Carl Roth GmbH, Karlsruhe, Germany). To provide sufficient air, a short stalked, non absorbent cotton plug (3 cm dia.) was inserted at top of the bags upside down and fixed by wrapping with autoclavable tape. Bags were autoclaved at 121 °C for 30 min. After cooling, each bag was inoculated with 30 pieces of SDA (1.5×1.5 cm) cut from petri dishes containing 15-day-old sporulating cultures of *M. anisopliae*. To avoid clumping of the grain/fungus mixtures, the bags were shaken every 3 days during the 3 week incubation period at 25 ± 1 °C. After 21 days, the kernels were fully colonized and stored at 4 °C until required. The number of conidia per gram of kernels was determined using a hemocytometer (Improved Neubauer, 0.1 mm depth). The average production was 1.25×10^8 conidia/g of grain/fungal mixture.

2.3. Insecticide

Chlorpyrifos (Dursban 5% Granules, Dow Agrosciences, B.V., Wilrijk, Belgium) was applied at the recommended rate of 2 kg AI/ha.

2.4. Field trials

Trial 1 was conducted on a naturally infested sport field containing a mixture of second- (48%) and third-instar (52%) *H. philanthus* at Eeklo (province East-Flanders, Belgium) in summer 2003. The lawn consisted mainly of red fescue (*Festuca rubra* L.) with a thatch layer <5 mm and maintained using standard management procedures. The soil was a sandy loam (88.0% sand, 5.6% silt, 6.4% clay with 5.8% organic matter). No natural entomopathogenic nematodes and fungi were detected by baiting soil samples with greater wax moth [*Galleria mellonella* (L.)] larvae. None of the sites had been treated with insecticides during the previous year. One week before treatment, the pre-treatment *H. philanthus* larval density was determined for each plot by taking six $16 \times 16 \times 10$–15 cm soil plugs with a shovel. The living larvae were counted and expressed as number of larvae/m^2. The grubs were then placed back in the soil plug, which was returned to its original place.

The plots, measuring 4×4 m with 1 m buffer, were arranged in a complete randomized block design with five replications per treatment. Treatments were applied on September 2, 2003 at 1400 h (soil temperature at 7 cm depth 20.5 °C; air temperature 25 °C; partial cloudy). The treatments included: (1) *M. anisopliae* (5×10^{12} conidia/ha = 40 kg barley kernels), (2) *H. bacteriophora* (2.5×10^9 IJs/ha), (3) chlorpyrifos (2 kg AI/ha), (4) the combination of *M. anisopliae* with *H. bacteriophora* and (5) untreated control. Treatments were applied both as surface and subsurface application.

In surface applications, nematodes suspended in 10 l of water were applied per plot directly to the turf using a sprinkling can. In subsurface applications, a sod cutter set to a depth of 5 cm was used to cut 10 strips of sod, each 0.4 m wide × 4.2 m long prior to nematode application. On the day of application, the sod strips were rolled up, nematodes were applied directly to the soil and the sod was immediately put back.

Chlorpyrifos granules were applied as surface and subsurface as described above, while *M. anisopliae* was applied as subsurface treatment only by spreading fungus colonized barley kernels on the soil. Directly after application of the insecticide or fungus, the plots were irrigated with 10 l of water. Untreated control plots (surface only) were treated with 10 l water only. All plots received regular overhead irrigation (10 mm) at 2-week intervals during 7 weeks.

In a prior study with *H. philanthus*, synergism between a stressor, *M. anisopliae*, and *H. megidis* or *S. glaseri* had been observed if larvae had been exposed for at least 3 or 4 weeks to the stressor (Ansari et al., 2004b). At the onset of the current study we expected a similar interaction between *M. anisopliae* and *H. bacteriophora*. Therefore, 4 weeks after the application of *M. anisopliae*, *H. bacteriophora* was applied on October 1, at 1700 h (soil temperature at 7 cm depth 18.1 °C; air temperature 20 °C; cloudy) in the fungal-treated plots. The larval density after treatment was determined by the same method as for the pre-treatment larval counts, 3 weeks after nematode (combination plots) or 7 weeks after fungus, nematode or chlorpyrifos application. During the experimental period, average soil temperature at 5 cm depth, air temperature and average rainfall were recorded. In order to determine long-term effects of applied treatments, a second observation was made after 1 year on 13 September 2004.

A second trial done at Lokeren (province East-Flanders, Belgium) was evaluated as described for trial 1 but with following exceptions. The lawn was covered with a mixture of perennial ryegrass (*Lolium perenne* L.) and Kentucky bluegrass (*Poa pratensis* L.) with a thatch layer <5 mm. The soil was sandy (92.4% sand, 5.7% silt, 1.9% clay, and 4.9% organic matter). Pre-treatment larval sampling showed that a mixture of second- (20%) and third-instar (70%) larvae of *H. philanthus* was present. Approximately 10% of the remaining population in the plots consisted of wireworms, *Agriotes* spp. (Coleoptera: Elateridae), June beetle, *Phyllopertha horticola* L. and the European cockchafer or May beetle, *Melolontha melolontha* L. (Coleoptera: Scarabaeidae). The treatments were similar to those in trial 1 and applied on 31 August 2004 at 1000 h (soil temperature at 7 cm depth 18.2 °C; air temperature 21.4 °C; cloudy, raining). Four weeks after the application of *M. anisopliae*, *H. bacteriophora* was applied on September 29 at 1700 h (soil temperature at 7 cm depth 17.1 °C; air temperature 22 °C; partial cloudy) in the fungal-treated plots. The larval density after treatment was determined 3 weeks after nematode or 7 weeks after fungus or chlorpyrifos application. A second observation was made after 1 year on 10 August 2005.

2.5. Data analysis

Synergistic, additive, or antagonistic interactions between agents in the combination treatments were determined using χ^2 tests (Finney, 1964; McVay et al., 1977). Grub mortality was calculated by subtracting the post-treatment larval count from the pre-treatment larval count in a treatment plot and correcting for control mortality (Abbott, 1925). The expected additive proportional mortality M_E for the nematode-*M. anisopliae* combinations was calculated by $M_E = M_N + M_M (1 - M_N)$, where M_N and M_M are the observed proportional mortalities caused by nematodes and *M. anisopliae* alone, respectively. Results from a χ^2 test, with $\chi^2 = (M_{NM} - M_E)^2/M_E$, where M_{NM} is the observed mortality for the nematode-*M. anisopliae* combination, were compared to the χ^2 table value for 1 df. If the calculated χ^2 values exceeded the table value, there would be reason to suspect a non-additive effect, i.e., synergistic/antagonistic between the two agents (Finney, 1964). If the differences $M_{MN} - M_E = D$ had a positive value, an interaction was then considered synergistic, and if D had a negative value, an interaction was considered antagonistic. The effect of the various treatments on *H. philanthus* was analyzed using two-way analysis of variance; means were separated with Tukey's test (SAS, 1999). Differences among means in all experiments were considered significant at $P < 0.05$. Means ± SE are presented.

3. Results

Overall, we observed significant effects of observation time at 7 weeks vs. 1 year ($F = 25.5$; $df = 3, 84$; $P < 0.001$) and treatments ($F = 75.2$; $df = 6, 84$; $P < 0.001$). The interaction between observation time and treatments was not significant ($F = 2.5$; $df = 18, 84$; $P > 0.087$). Application methods (surface and subsurface) had no effect on grub mortality.

In trial 1, 7 weeks after observation with *H. philanthus*, the average temperature during the experimental period was 21.5 °C in the air and 18.4 °C in the soil at 7 cm depth. A rainfall of 35.5 mm was registered during the observation period. Grub mortality differed significantly among treatments ($F = 18.9$; $df = 6, 28$; $P < 0.001$); however, the combination treatment (fungus + nematode) did not cause significantly higher mortality than the subsurface applied chlorpyrifos alone (Fig. 1). The interaction between *H. bacteriophora* and *M. anisopliae* was synergistic ($\chi^2 = 40$; $df = 1$; $P = 0.001$). One year after the treatments, grub mortality differed significantly among treatments ($F = 18.7$; $df = 6, 28$; $P < 0.001$). Interaction between *H. bacteriophora* with *M. anisopliae* was additive ($\chi^2 = 0.993$; $df = 1$; $P = 0.319$). The mortality after the combined application of the fungus and nematode was not increased significantly compared to subsurface applied *H. bacteriophora* or chlorpyrifos alone (Fig. 1).

During the 7 weeks following the treatments in trial 2, the air temperature averaged 19.3 °C and the soil temperature

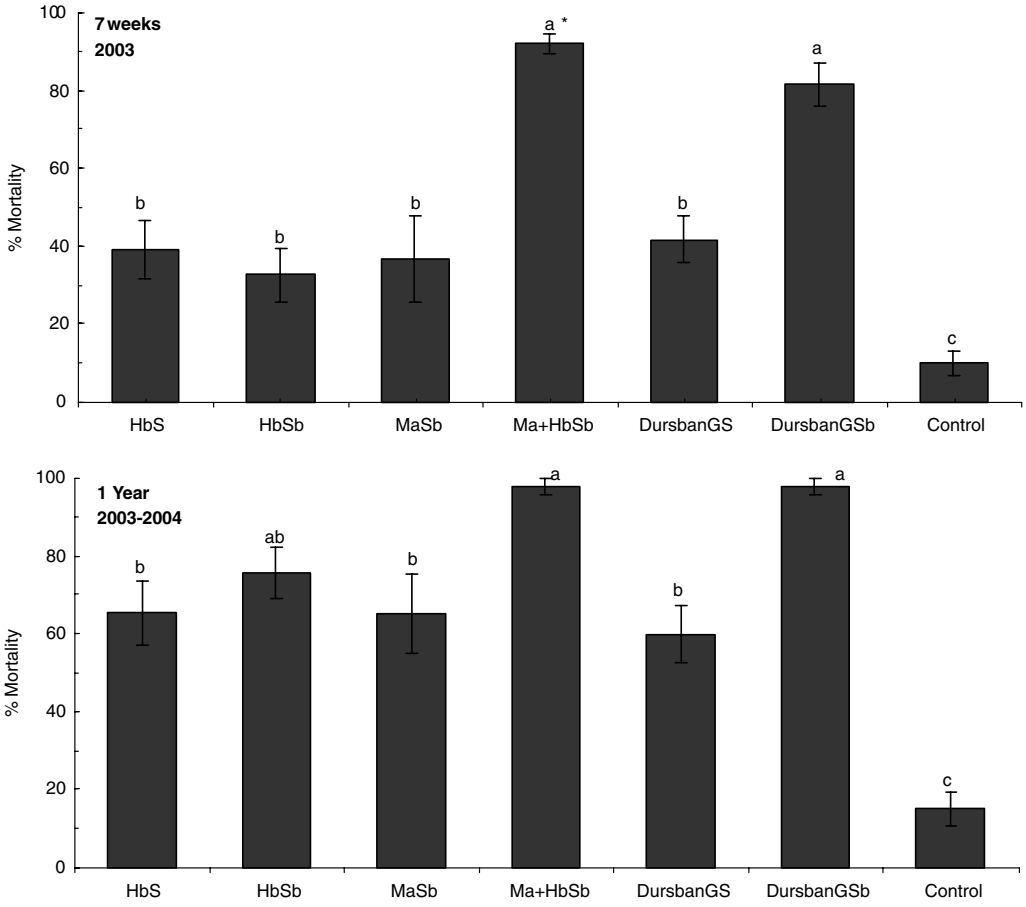

Fig. 1. Effects of treatment with *Metarhizium anisopliae* CLO 53 (Ma) 5×10^{12} conidia/ha, *Heterorhabditis bacteriophora* [2.5×10^{9} infective juveniles/ha (Hb)], chlorpyrifos (Dursban 5G) 2 kg (AI)/ha or the combination of nematode and fungus on mortality of second- and third-instar *Hoplia philanthus* (means of five replicates + SE) (Eeklo). Nematodes were applied 4 weeks after Ma, and plots were sampled 3 weeks after nematode or 7 weeks after Ma or Dursban application. All treatments were applied surface (S) or subsurface (Sb). Means with same letters are not significantly different ($P < 0.05$). An '$*$' indicates significant synergistic interactions between Hb and Ma.

at 7 cm deep averaged 18.5 °C; a rainfall of 48.6 mm was observed. Grub mortality differed significantly among treatments ($F = 77.0$; $df = 6, 28$; $P < 0.001$). The combination treatment did not cause significantly higher mortality than the subsurface applied chlorpyrifos alone (Fig. 2). The interaction between *M. anisopliae* with *H. bacteriophora* was synergistic ($\chi^2 = 13$; $df = 1$; $P = 0.001$). One year after the start of trial 2, the grub mortality differed significantly among treatments ($F = 20.4$; $df = 6, 28$; $P < 0.001$). Interaction between *H. bacteriophora* with *M. anisopliae* was additive ($\chi^2 = 0.889$; $df = 1$; $P = 0.269$). The mortality in the combination treatment had not significantly increased compared to subsurface applied nematodes, fungus or chlorpyrifos alone (Fig. 2).

4. Discussion

Our study confirms the additive or synergistic interactions between *M. anisopliae* and entomopathogenic nematodes, which previously had been observed in laboratory and greenhouse conditions (Ansari et al., 2004b) and expands this information to natural populations of *H. philanthus* and another nematode species under field conditions. Similar to our findings, a few studies have also indicated additive or synergistic effects between entomopathogenic nematodes and *M. anisopliae* against the white grub, *Holotrichia consanguinea* (Coleoptera: Scarabaeidae) (Yadav et al., 2004) and the pecan weevil, *Curculio caryae* Horn (Coleoptera: Curculionidae) (Shapiro-Ilan et al., 2004). Our results show that the degree of interaction varies in time; the strongest interaction was observed close to the application. Ansari et al. (2004b) suggested that a major factor responsible for a synergistic interaction is the weakening of the grubs by a fungal infection so that they are not able to feed or utilize food normally, resulting in their reduced activity. Weakening results in sluggishness that in turn facilitates host attachment by entomopathogenic nematodes and subsequent penetration. In both trials at the 1-year observation, it was not possible to determine whether synergism occurred. The reason for the lack of synergism after 1 year was that the individual treatments had already caused high mortality, which did not leave enough room for a further significant improvement. A possible explanation for the higher mortality might be the production of

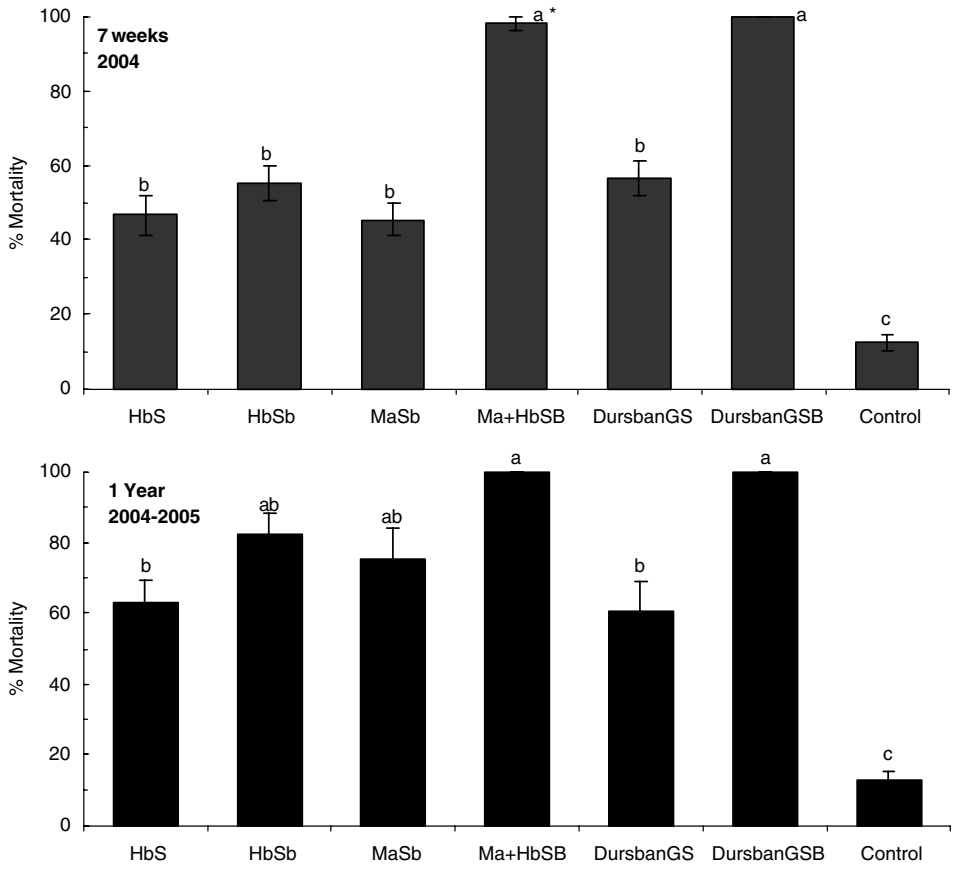

Fig. 2. Effects of treatment with *Metarhizium anisopliae* CLO 53 (Ma) 1×10^{13} conidia/ha, *Heterorhabditis bacteriophora* [2.5×10^{9} infective juveniles/ha (Hb)], chlorpyrifos (Dursban 5G) 2 kg (AI)/ha or their combination on mortality of second- and third-instar *Hoplia philanthus* (means of five replicates + SE) (Lokeren). Nematodes were applied 4 weeks after Ma, and plots were sampled 3 weeks after nematode or 7 weeks after Ma or Dursban application. All treatments were applied surface (S) or subsurface (Sb). Means with same letters are not significantly different ($P < 0.05$). An '$*$' indicates significant synergistic interactions between Hb and Ma.

progeny by both *H. bacteriophora* and *M. anisopliae* which may have caused additional mortality and could have further reduced the grub populations. This type of long-term effect is demonstrated by studies in which the control of grubs has been excellent. For example, Klein and Georgis (1992) reported that *H. bacteriophora* (NC strain) recycled in turf after inundative releases that resulted in prolonged suppression of Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae), 1 year after application. In addition to entomopathogenic nematodes, Rath et al. (1995) demonstrated the excellent soil persistence of *M. anisopliae* strain DAT F-001, that had been drilled below the soil surface against the scarab, *Adoryphorus couloni* Burmeister (Coleoptera: Scarabaeidae). The conidia persisted for 6 months and the number of colony-forming units increased dramatically because of recycling on host cadavers.

The effect of surface or subsurface applications of nematodes did not change during the year following the applications. Nematodes applied at the soil surface are killed by combination of desiccation and ultra-violet light damage (Smits, 1996; Wilson and Gaugler, 2004). In view of this, one might expect that a subsurface application would yield higher grub mortality. Unexpectedly, we did not observe that. The effect of desiccation and ultra-violet light might have been negligible in our experiments because the rolled up turf in plots where nematodes were applied subsurface was replaced immediately after nematode application. We believe that the post-application irrigation would wash the majority of surface-applied nematodes into the thatch (an area with low ultra-violet light penetration) within 1 h after application. We did not study nematode persistence; however, in field situations natural enemies such as predatory mites prey on nematodes and are capable of reducing populations (Smart, 1995). Further studies, including long-term field trials, are necessary to examine the complex biotic interactions affecting populations and persistence of artificially introduced entomopathogenic nematodes.

Compared with the surface application of chlorpyrifos (42–60%), the grub control obtained with subsurface application at 3–5 cm depth was higher (82–100%), even after irrigation of the surface applied insecticide. These results confirm earlier work that demonstrated that chlorpyrifos applied at a depth of 15–20 cm below the soil surface results in a good control of the cane grub, *Dermolepida albohirtum* Waterhouse (Coleoptera: Scarabaeidae) (Chandler et al., 1993). However, chlorpyrifos binds strongly to organic matter in the soil. The more thatch, the less movement

(Allsopp and Chandler, 1989). Hence, the placement of chlorpyrifos is critical for grub control and optimum placement will vary with the target species. Ansari (2004) reported that *H. philanthus* larvae feed below the thatch layer most of the year and concluded that a shallow placement of insecticides would be much more effective than deep placement.

Our results indicate that the synergistic interaction between the fungus and the nematode can be achieved at rates below the recommended field rates of 6.6×10^{13} conidia/ha and 5.0 billion IJs/ha (Wilson et al., 2003; Logan et al., 2000). Lower fungus and nematode rates not only are effective, they also increase the economic feasibility of this *H. philanthus* control strategy. The combination of fungus and nematode holds a potential only if *M. anisopliae* is apply 4 weeks before *H. bacteriophora*. In these conditions, *M. anisopliae* can be more effective by causing a degree of direct mortality of insect larvae and stressing the remaining grub instars sufficiently to increase their susceptibility to nematodes. Steinhaus (1958) was the first to indicate that stressed insects generally seem to be more susceptibility to pathogens.

Whether the combination of nematodes and fungi will be used in practice will depend on the efficacy of the combination, its cost and competition with chemical insecticides. Obviously, by its synergistic effect, the combination has already a better effect than the individual components. In addition, the combination should also be cheaper than either single component alone at the same efficacy level. For example, nematodes and fungus synergize effectively against third-instar *H. philanthus* (Ansari et al., 2004b). We have fully explored this combination and think that for the combination to be effective one has to use 50% of the full rate of both components or even less. However, this is more expensive as similarly effective organophosphate and carbamate insecticides. The cost of normal rates of chlorpyrifos, imidacloprid or halofenozid are between 250 and 350 USD/ha, *M. anisopliae* between 400 and 600 USD/ha, and *H. bacteriophora* (Nema-green®) between 500 and 1000 USD/ha. As a consequence, to compete with chemicals alone, one would have to reduce both fungus and nematode concentrations to about 1/4 rate and still get more than 80% control. Additional studies on combined application of both agents at reduced rate is under investigation. For small scale applications in environmentally sensitive areas or on home lawns, the price would be much less and issue than for large scale application such as sport turf.

Finally, the advantage of this combination is the absence of broad-spectrum chemical insecticides and the ability of the biocontrol agents to recycle even in combination (Ansari, unpubl.). Under favorable conditions, this may provide control lasting more than one season.

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