Efficacy of Entomopathogenic Nematodes and Entomopathogenic Fungi against Masked Chafer White Grubs, *Cyclocephala* spp. (Coleoptera: Scarabaeidae)

Shaohui Wu

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Dr. Roger R. Youngman, Co-Chair
Dr. Loke T. Kok, Co-Chair
Dr. James M. Goatley
Dr. Douglas G. Pfeiffer
Dr. Sally L. Paulson

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ABSTRACT

Entomopathogenic nematodes (EPN) (*Heterorhabditis bacteriophora* and *H. megidis*) and entomopathogenic fungi (EPF) (*Metarhizium anisopliae* and *Beauveria bassiana*) were evaluated for efficacy against masked chafer white grub, *Cyclocephala* spp., under laboratory and greenhouse conditions, as well as their efficacy against various grub stages in the field. Under both laboratory and greenhouse conditions, additive interactions were found between EPN and EPF in their combined application against *Cyclocephala* spp., except a few observations that showed antagonism or synergism. Significantly greater control occurred from the combination of a nematode and a fungus compared with a fungus alone, but not compared with a nematode alone. The combined effect did not differ significantly for nematode and fungi applied simultaneously or at different times. EPF had no significant impact on EPN infection and production of infective juveniles (IJs) in grub carcasses. Nematodes alone or in combination with fungi were comparable to the insecticide Merit 75 WP (imidacloprid) against 3rd instar *Cyclocephala* spp in the greenhouse. Efficacy of EPF and EPN varied dramatically between field sites and conditions; EPN and EPF applied alone or in combination were less effective than Merit 75 WP in >50% field trials, but some EPN + EPF treatments were more effective than the insecticide in reducing grub numbers. EPN and EPF showed better potential than insecticides for providing extended control of white grubs in the subsequent generation. In addition, the sub-lethal effects of EPF on southern masked chafer, *C. lurida*, were investigated. Neither *M. anisopliae* nor *B. bassiana* had a sub-lethal effect on grub weight gain, adult longevity, oviposition, pupation and eclosion. Finally, interaction between *H. bacteriophora* and *M. anisopliae* was examined to determine the potential of the nematode in improving fungal distribution in soil. *H. bacteriophora* enhanced fungal distribution in sandy loam soil without grass thatch, but not in sandy soil with thatch. In both soil types, soil depths significantly affected nematode and fungal distribution. In water profile, *M. anisopliae* conidia germinated hyphae that attached to sheath of *H. bacteriophora* IJs, which molted to detach from the fungus. IJs mortality and virulence were not affected by the presence of *M. anisopliae*.

DEDICATION

This is dedicated to

my beloved husband Feng Li & daughter Sophie Li,

for their unwavering love and support,

to make my accomplishment possible.

Thank you.

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Abstract

White grubs are among the most widespread and damaging turfgrass pests. Currently, the control of these pests still relies heavily on the broad-scale use of chemical insecticides. However, the impact from long-term use of insecticides on the environment, human health, natural enemies, and insecticide resistance has increased public awareness for a more bio-rational approach to managing turfgrass pests. The desirability of developing environmentally safe alternatives to achieve persistent and sustainable control of the pests have resulted in a surge of scientific and commercial interest in various biological control agents including entomopathogenic nematodes and entomopathogenic fungi. Commercialization of nematode and fungal-based products has thus triggered attempts to use them for inundative white grub control. This review describes the characteristics of the entomopathogenic nematodes and entomopathogenic fungi associated white grubs, discusses their efficacies and factors affecting their performance in suppressing white grub populations, as well as their current status and future prospects.

Keywords: white grub, entomopathogenic nematode, entomopathogenic fungus, turfgrass

Introduction

The larvae of scarab beetles (Coleoptera: Scarabaeidae), commonly called white grubs, are among the most widespread and important turfgrass pests in the United States and elsewhere (Vittum et al., 1999). Each year, Japanese beetle alone causes an estimated loss of $234 million in damage in the nation, among which $78 million are spent for control and $156 million for replacement of damaged turf (Anonymous, 2013). At least nine species of white grub cause turfgrass damage in the United States (Table 1).

I. White Grub Life Cycle & Infestation

Although species vary, the larval stage of these scarabs share the same characteristic of mostly white color, true head capsule and C-shape. The species of white grubs can be identified by observing the raster (hair) pattern on the inner tip of a grub's abdomen. Scarabs have a complete life cycle, comprised of egg, three larval instars, pupa and adult stages. The larval or grub stage is the stage that causes the most damage, with the exception of Japanese beetles, *Popillia japonica* Newman, whose adult stage is also of significant economic importance. Unlike most foliar and thatch pests of turf, the larval stage of all scarabs are obligate root feeders, except for green June beetle, *Cotinis nitida* (L.), which also feeds on

organic matter and causes damage by tunneling. The 3rd instar is often the most damaging stage, because in addition to the relatively large body size, 3rd instars may be present in soil for the longest period of time. The feeding or tunneling behavior of grubs causes the turf to be spongy and easily pulled up. Heavy infestation causes large brown irregular patches (Potter, 1998; Shetlar, 1994).

Table 1. Significant white grub species of turfgrass and their distribution in the United States *.

Scientific name	Common name	Origin	Distribution in U.S.
Popillia japonica Newman	Japanese beetle	Japan	Severe through most eastern states; regional importance in Northeast
Maladera castanea (Arrow)	Asiatic garden beetle	China; Japan	CT, DE, DC, MD, NJ, NY, PA, SC, VA
Ataenius spretulus (Haldeman)	Black turfgrass ataenius	Native to U.S.	41 of 48 contiguous states: most prevalent in midwestern states; substantial in Central Plains & northeastern states.
Rhizotrogus majalis (Razoumowsky)	European chafer	Western & central Europe	NY, NJ, CT, PA, MA, RI, OH, MI, DE
Cotinis nitida (L.)	Green June beetle	Eastern U.S.	Throughout U.S.
Cyclocephala borealis Arrow	Northern masked chafer	Native to U.S.	From NY west to IL; south to KY & MO
C. lurida Bland	Southern masked chafer	Native to U.S.	KY, VA, IN, IL, west to NE & KS, south to TX
Phyllophaga spp.	May or June beetle	Native to U.S.	All contiguous states in U.S.
Anomala orientalis (Waterhouse)	Oriental beetle	Probably Philippine Islands; Japan	CT, HI, MD, MA, NJ, NY, NC, OH, PA, RI, VA

*Data summarized from Brandenburg and Villani (1995).

Most white grub species have one generation per year and overwinter as full-grown third instars. Exceptions to this life cycle include the black turfgrass ataenius, *Ataenius spretulus* (Haldeman), which has one or two generations per year depending on location and weather conditions, and overwinters as adults; and the May or June beetle *Phyllophaga* spp. which may take from one to three years to complete the life cycle. In warmer climates the life cycle of these scarabs can be shorter, for example, the masked chafers *Cyclocephala lurida* Bland and *C. parallela* Casey have two generations each year throughout Florida, but have one generation per year in most other locations in the United States.

II. Management

Due to the protection afforded them by the grass and thatch layers, white grubs are often difficult to control in turf with chemical insecticides or entomopathogenic organisms (Potter and Braman, 1991; Vittum et al., 1999). Integrated pest management for grub control involves chemical, biological, and cultural tactics, as well as other measurements such as mating disruption and trapping. No resistant grass cultivars have been found to provide effective control against white grubs (Koppenhöfer, 2007a). Moreover, reports indicate that the impact of the turfgrass endophyte on grub populations is variable, and grub survival is not necessarily lower in endophytic grass than in an endophyte-free cultivar. Also, the endophyte does not have significant effects in increasing the susceptibility of white grubs to some diseases and parasites (Grewal et al., 1995; Koppenhöfer et al., 2003; Walston et al., 2001).

Less focus has been given to tactics other than chemical and biological control because of their limited efficacy or lack of suitability for curative control. For example, mass trapping can be effective in reducing the population of Japanese beetle adults, but success is limited due to the cost and labor involved. However, an exception can be made for Japanese beetle management or eradication in recently introduced areas (Hamilton et al., 1971; Wawrzynski and Ascerno, 1998). Mating disruption has been tested as a promising management option for *A. orientalis*. For example, with microencapsulated sprayable formulations of (*Z*)-7-tetradecen-2-one, male *A. orientalis* beetles in traps decreased by 90-100% in the treated area during the first 7-10 days after treatment, and the ensuing larval population was reduced by 68-74%, although the effects were not persistent (Koppenhöfer et al., 2005).

Insecticides often play important roles in preventive and curative control when white grubs are abundant (Potter, 1998). Nevertheless, many insecticides are only effective against 1st and 2nd instars, and efficacy is limited when targeting 3rd instars. For maximum impact, insecticides are usually applied no later than July to control grubs before they reach the 3rd instar. However, if an insecticide fails to control a grub population in its earlier stages, 3rd instars can cause a problem. Some insecticides such as diazinon and chlorpyrifos, which were frequently applied for curative grub control, have been removed from use due to implementation of the Food Quality Protection Act. This limits options for the turf managers when grub damage becomes severe in fall.

Currently, one of the more common strategies for managing grubs is the use of a preventive application of a conventional insecticide in late spring or early summer. However, insecticides are usually applied without sampling, and large areas need to be sprayed for good coverage. Although some of the newer insecticides are less toxic to non-target organisms, these insecticides might have an indirect impact on the natural enemies of some turfgrass pests by depriving them of food sources. Areas such as golf course roughs are often left untreated, which can lead to a buildup of white grub numbers that can cause,

indirectly, serious sod-stripping in the fall from skunks and other rodents digging for the grubs. In addition, the impact from long-term use of insecticides on the environment, natural enemies, and insecticide resistance has increased public awareness for a more bio-rational approach in managing turfgrass pests.

Difficulties in achieving long-term and consistent efficacy against white grubs with chemical approaches, and the increasing public concern about the use of insecticides, have given more importance to the development of biological control strategies. Using biological agents against white grubs is not a new idea. For example, about 130 years ago, the entomopathogenic fungus, *Metarhizium anisopliae*, was used for controlling a scarab species (Zimmermann, 2007). Several categories of biological microbes, including bacteria, entomopathogenic fungi and entomopathogenic nematodes, have been tested and proven to be effective against white grubs. In this review, the use of entomopathogenic nematodes and entomopathogenic fungi for the control of various scarab larvae are discussed in detail.

Entomopathogenic Nematodes

I. Taxonomy & Biology

Compared with the studies on entomopathogenic fungi, the history of using entomopathogenic nematodes is relatively short, dating back to the 1930s when Rugolf William Glaser (1888-1947) pioneered research on the culture and field application of entomopathogenic nematodes, including using *Steinernema glaseri* against the Japanese beetle (Gaugler and Kaya, 1990).

Nematodes associated with Scarabaeidae belong to four orders: Rhabditida, Mermithida, Spirurida and Oxyurida. The type of association ranges from commensalism, e.g. in Rhabditidae and Diplogasteridae (Poinar, 1975), to obligate parasitism. Although many of these associations have little to no obvious detrimental effect on the host insects, some nematodes can be entomopathogenic or insecticidal, like Mermithidae, Steinernematidae and Heterorhabditidae, or reduce longevity and reproductive potential like Allantonematidae. Of all insecticidal nematodes, those in the families Heterorhabditidae and Steinernematidae (Rhabditida) have received the most attention from researchers. Currently, more than 85 species of entomopathogenic nematodes have been described (Lewis and Clarke, 2012). Among those, at least 12 steinernematid and six heterorhabditid nematodes are pathogenic to scarab grubs, and 11 of them were found in North America (Table 2).

Table 2. Steinernematidae and Heterorhabditidae associated with Scarabaeidae [*].

Nematode	Host	locality	Reference
Steinernema anomali	*Anomala dubia* Scop.	Europe	

(Kozodoi)			
S. arenaria (Art.)	*Melolontha hippocastani* Fab.	Europe	
S. bothynodera (Kirj. & Puch.)	*Melolontha afflicta* Ballion	Europe	
S. chresima (Steiner in Glaser, McCoy & Girth, 1942)	*Popillia japonica* Newman	North America	
S. carpocapsae (Weiser)	*Popillia japonica* Newman	North America	
S. feltiae (Filipjev)	*Onitis alexis* Klug, *Pentodon algerinum* Herbst	Egypt	
S. georgica (K. & V.)	*Amphimallon solstitialis* (L.)	Europe	
S. glaseri (Steiner)	*Popillia japonica* Newman	North America	
	Strigoderma arboricola (Fab.)	North America	
	Anomala flavipennis Burm.	North America	
S. kushidai Mamiya	*Anomala cuprea* Hope	Japan	
S. scarabaei Stock & Koppenhöfer	*Anomala orientalis* (Waterh.); *Popillia japonica* Newman	North America	(Stock and Koppenhöfer, 2003)
Steinernema sp.	*Adoryphorus couloni* (Burm.)	Australia	
Steinernema sp.	*Scitala sericans* Erich.	Australia	
Heterorhabditis bacteriophora Poinar	*Popillia japonica* Newman, *Cyclocephala hirta* LeConte	North America	
	Phyllophaga sp.	North America	
H. megidis Poinar, Jackson & Klein	*Popillia japonica* Newman	North America	
H. zealandica Poinar	*Heteronychus arator* (F.)	New Zealand	
H. marelatus Liu and Berry	*Popillia japonica* Newman	North America	(Liu and Berry, 1996)
Heterorhabditis sp.	*Popillia japonica* Newman	North America	
Heterorhabditis sp.	*Pericoptus truncatus* (F.)	New Zealand	

*Data updated from Tables 8.4 & 8.5 in Poinar (1992).

Heterorhabditid and steinernematid nematodes are associated with the symbiotic bacteria *Photorhabdus* and *Xenorhabdus*, respectively (Forst et al., 1997). The bacteria are Gram-negative, facultative anaerobic rods in the family Enterobacteriaceae, and are found within the intestine of the infective juvenile (IJ) nematode. The genus *Xenorhabdus* contains 20 species including the type species *X. nematophilus* Thomas and Poinar (1979); *X. beddingii, X. bovienii, X. poinarii* described by Akhurst and Boemare (1988); *X. japonica* corrig. Nishimura et al. (1994), four species (*X. budapestensis, X. ehlersii, X. innexi, X. szentirmaii*) described by Lengyel et al. (2005); *X. indica* described by Somvanshi et al. (2006), and ten species described by Tailliez et al. (2006); *Photorhabdus* contains 3 species, *P. temperata* (subspecies *temperata*), *P. asymbiotica*, and *P. luminescens* which is further divided into 3 subspecies, *luminescens*, *akhurstii* and *laumondii* (Fischer-Le Saux et al., 1999). Both *Xenorhabdus* and *Photorhabdus* have two phenotypic forms: phase I and phase II. Phase I is the cell form normally associated with entomopathogenic nematodes in nature, and which produces significantly greater amounts of antibiotics, exoenzymes and toxins than phase II form which generally occurs in the laboratory when the bacterial cultures are in the stationary stage.

The relationship between the nematode and the symbiotic bacterium is a type of symbiosis where both benefit from the association (i.e. mutualism). The nematode provides protected shelter for the symbiotic bacteria and carries the bacteria into the host. After entering the host, usually via insect body openings (e.g. mouth, anus, spiracles), the nematode penetrates through the gut wall, and regurgitates symbiotic bacteria into the insect hemocoel, and the bacteria secret toxins to rapidly kill the host insect, usually within 48 hours. The bacteria break down the host tissues, and provide food sources for the nematode, which feeds and multiplies on bacterial cells and degrading host tissues. During the process, the bacteria provide the nematode and themselves a protected niche by producing antibiotics that suppress the competition from other microorganisms. The nematodes reproduce for two to three generations, and finally exit the host as IJs after depleting the host nutrients. Due to the different symbiotic bacteria associated with EPN, heterorhabditid nematodes turn the host cadaver red, purple, orange, yellow, brown or sometimes green, whereas steinernematid nematodes turn the insect cadaver tan, ochre, gray or dark gray.

The complete life cycle of insecticidal nematodes consists of egg, four juvenile stages, and adult. The infective juvenile (IJ), also called 'dauer juvenile', is the 3rd stage juvenile nematode ensheathed by the cuticle of 2nd stage. Each IJ develops into a hermaphroditic female in the first generation of *Heterorhabditis*, and amphimictic female or male in *Steinernema* or in the second generation of *Heterorhabditis*. The non-feeding IJ is the only stage of nematode that is present outside the host in nature, and is especially adapted for host-seeking. It may actively search for a potential host, so called

'cruiser', for example *S. glaseri*, *H. bacteriophora* and *H. megidis*, or adopt a 'sit and wait' strategy to ambush the host, namely 'ambusher' like *S. carpocapsae* and *S. scapterisci*. Some nematodes such as *S. riobrave* may exploit both ambusher and cruiser means of host-finding. In general, the cruisers are well adapted to attack immobile subterranean hosts such as white grubs, and the sedentary ambushers are better adapted for surface-mobile hosts like mole crickets. The IJs locate the host insects through CO_2, pH, bacterial symbionts, or host-associated materials, e.g. feces, cuticle (Gaugler et al., 1980; Pye and Burman, 1981; Schmidt and All, 1979).

Entomopathogenic nematodes are safe to plants, vertebrates and other non-target organisms (Akhurst, 1990; Bathon, 1996; Georgis et al., 1991; Poinar, 1989). Although these nematodes are able to infect a broad range of insects in laboratory tests, they have a restricted host range in nature (Gaugler, 1981; Gaugler, 1988; Poinar, 1979). Nematodes naturally dwell in moist, cryptic habitats (soil in most cases), where they are protected from environmental extremes (e.g. desiccation, temperature and ultraviolet), and thus do not normally encounter insects in other habitats, e.g. foliage-feeding lepidopteran larvae in exposed settings.

II. Efficacies

Since R. W. Glaser and his coworkers initiated the era of biological control using entomopathogenic nematodes in the 1930s, significant progress has been achieved in the development of this area. In the past decades, studies have been conducted to test the efficacies of nematode species and isolated strains against various insect pests in different crop systems and habitats. As typical soil-inhabiting pests, white grubs are ranked at the top of the list of target pests by researchers in nematode studies. Efficacies in insect control with insecticidal nematodes have been reviewed by several authors (Begley, 1990; Georgis and Hague, 1991; Georgis et al., 2006; Georgis and Manweiler, 1994; Kaya, 1985; Klein, 1990; Nickle, 1984; Wouts, 1991). Grewal et al. (2005) summarized the studies on the efficacy of entomopathogenic nematodes against white grubs.

Encouraging results have been obtained in greenhouse and laboratory studies on the control of white grubs using beneficial nematodes. For example, in a laboratory study, four strains of *S. glaseri* (NJ21, NJ29, NJ42, and NJ65) achieved 100% larval mortality of western masked chafer *Cyclocephala hirta* 6 days after treatment (Converse and Grewal, 1998). Although the efficacy may vary with insect or nematode species and environmental conditions, some species or strains of nematodes perform better than others against certain insect species, and have potential for pest control in field applications. In the same study, in contrast to the outstanding efficacy of *S. glaseri*, *S. feltiae* (Argentina strain) had a marginal effect on *C. hirta*, causing only 16% mortality; *S. carpocapsae* (All and Mexican strains) and *S. scapterisci* (Colon strain) were nonpathogenic to *C. hirta*, whereas *S. riobrave* caused no larval mortality

at 25°C, but inflicted 45-71% mortality at 30°C (Converse and Grewal, 1998). This indicates that the four strains of *S. glaseri* outperformed other nematode species and strains, and may have better potential for success in controlling this pest in the field. Similarly, in a laboratory and greenhouse study conducted by Koppenhöfer et al. (2006), *S. scarabaei* had the highest virulence against *R. majalis* and *P. japonica* and lowest toward *C. borealis* at the 3rd instar, whereas *H. zealandica* and *H. bacteriophora* were slightly virulent against *R. majalis*, and had similar modest virulence to *P. japonica*, *A. orientalis*, *C. borealis*, and *M. castanea*.

Host susceptibility may vary with nematode/host species as well as strains. Among white grub species of economic importance of turfgrass in the USA, *P. japonica* appears to be most susceptible to the commonly used entomopathogenic nematodes, e.g. *H. bacteriophora* and *S. glaseri*, whereas other species such as *A. orientalis*, *Cyclocephala* spp., *M. castanea*, *R. majalis* and *Phyllophaga* spp. are less susceptible (Cappaert and Koppenhöfer, 2003; Grewal et al., 2002; Grewal et al., 2005; Koppenhöfer et al., 2000a; Koppenhöfer et al., 2002; Koppenhöfer and Fuzy, 2003a; Koppenhöfer and Fuzy, 2003b; Koppenhöfer et al., 2004; Koppenhöfer et al., 2006; Koppenhöfer et al., 2000c; Shapiro-Ilan et al., 2002; Simard et al., 2001). *S. scarabaei* was shown to be more pathogenic than *H. bacteriophora* to *A. orientalis*, *R. majalis* and *P. japonica* (Cappaert and Koppenhöfer, 2003; Koppenhöfer et al., 2004), but not to *C. borealis* (Koppenhöfer et al., 2006). Strain GPS11 of *H. bacteriophora* was more virulent than other strains tested against *A. orientalis*, *C. borealis*, and *P. japonica*, but showed low infectivity against *R. majalis* (Grewal et al., 2002).

These studies provide a good basis for the field application and eventual commercialization of beneficial nematodes for public use. Although compared with laboratory findings, field efficacy may be less impressive and inconsistent due to various limitations, which will be discussed in detail later, satisfactory results from field applications have been achieved. As shown in Table 3, nematode species/strains providing good control of *P. japonica* include *S. scarabaei*, *H. bacteriophora* GPS11 & TF strain, and *H. zealandica* X1; *S. scarabaei*, *H. zealandica* X1 and *H. bacteriophora* GPS11 appear to be the most promising nematode species/strains against *C. borealis*. *S. scarabaei* appears to be the only nematode species that provides high control efficacy for *A. orientalis*, *M. castanea*, and *R. majalis* in the field.

Table 3. Field efficacy of *Steinernema* spp. and *Heterorhabditis* spp. against various white grubs in turf [*].

Insect host	Nematode species (strain)	Control	Reference
Popillia japonica	*S. carpocapsae*	55% 39 DAT [a]	
		2-19%	

	55%	
S. carpocapsae (All)	41% in 1989; 66-81% in 1991	(Alm et al., 1992)
	7-50% 34 DAT; 84-90% 280 DAT; 0% 386 DAT	(Klein and Georgis, 1992b)
S. glaseri	8-15% reduction 1st yr, significant reduction subsequent yr	
	Colonization unsuccessful	
	2-31% 14 DAT	
S. glaseri (Biosys*2)	39-71% in Aug. 1991; 0-66% in Sep. 1991	(Alm et al., 1992)
S. glaseri (NC)	62%	(Koppenhöfer et al., 2000a)
S. glaseri (MB)	6-58%	(Grewal et al., 2004)
S. glaseri (NJ)	20%	(Grewal et al., 2004)
S. kushidai	62-80% 14-21 DAT	(Koppenhöfer et al., 2000c)
S. kraussei (UK)	30%	(Grewal et al., 2004)
S. feltiae (Biosys*27)	25% in 1989; 10-33% in 1990	(Alm et al., 1992)
S. feltiae (Biosys*980)	13-42%	(Alm et al., 1992)
S. scarabaei	Fall: 54% 21 DAT Spring: ≤66% 41 DAT	(Cappaert and Koppenhöfer, 2003)
	100% 14-21 DAT	(Koppenhöfer and Fuzy, 2003b)
H. marelatus	49% 41 DAT	(Cappaert and Koppenhöfer, 2003)
H. indica	No significant control	(Koppenhöfer et al., 2000a)
H. zealandica (X1)	73-98%	(Grewal et al., 2004)
H. bacteriophora	73%	

		64% 47 DAT	
		No significant control	(Cappaert and Koppenhöfer, 2003)
	H. bacteriophora (TF)	65-78% 14-21 DAT	(Koppenhöfer et al., 2000c)
		40-85%; 90.8%	(Koppenhöfer et al., 2000a)
		74-93% 14-21 DAT at 2.5 $\times 10^9$ IJ/ha; 33-40% 14-21 DAT at 10^9 IJ/ha	(Koppenhöfer and Fuzy, 2003b)
		56-70% 22 DAT	(Koppenhöfer et al., 2002)
	H. bacteriophora (NC)	Fall: 35-60% 34 DAT; 84-96% 280 DAT; 93-99% 386 DAT	(Klein and Georgis, 1992b)
		Spring: 35-68% 28 DAT; 39-67% 138 DAT	
	H. bacteriophora (HP88)	Spring: 100% 28 DAT; 93-97% 138 DAT	(Klein and Georgis, 1992b)
		>70%	
		30%	(Alm et al., 1992)
		52%	(Grewal et al., 2004)
	H. bacteriophora (GPS11)	34-97%	(Grewal et al., 2004)
Cyclocephala borealis	*S. carpocapsae*	<40%	
	S. scarabaei	58-84% 21 DAT	(Koppenhöfer and Fuzy, 2003b)
	S. glaseri (MB)	No significant control	(Grewal et al., 2004)
	S. kraussei (UK)	50%	(Grewal et al., 2004)
	H. bacteriophora (HP88)	>70%	
		36%	(Grewal et al., 2004)
	H. bacteriophora (TF)	6-20% 21 DAT	(Koppenhöfer and Fuzy, 2003b)

	H. bacteriophora (GPS11)	47-83%	(Grewal et al., 2004)
	H. zealandica (X1)	72-96%	(Grewal et al., 2004)
Cyclocephala hirta	*S. feltiae*	65% 28 DAT	
	S. glaseri (NC)	45% 14-21 DAT	(Koppenhöfer et al., 2000c)
		No significant control	(Koppenhöfer et al., 2000a)
	S. kushidai	35-50% 14-21 DAT	(Koppenhöfer et al., 2000c)
	H. bacteriophora (HP88)	47% 28 DAT	
	H. bacteriophora	No significant control	(Koppenhöfer et al., 2000c) (Koppenhöfer et al., 2000a)
	H. bacteriophora (NC1)	34-45% 24-28 DAT	(Koppenhöfer et al., 1999)
Cyclocephala pasadenae	*H. bacteriophora* (NC1)	No significant control	(Koppenhöfer et al., 1999)
Ataenius spretuIus	*S. carpocapsae* (All)	46-94%	(Alm et al., 1992)
	S. glaseri (Biosys*2)	14%	(Alm et al., 1992)
Anomala orientalis	*S. carpocapsae* (All)	No significant control	(Alm et al., 1992)
	S. feltiae	No significant control	(Alm et al., 1992)
	S. scarabaei	80-94% 14 DAT, 93-97% 21 DAT; 43-85% 21 DAT at 0.4-2.5×10^9 IJ/ha	(Koppenhöfer and Fuzy, 2003b)
	S. glaseri (Dongrae)	64% 21 DAT	(Koppenhöfer et al., 1999)
	H. bacteriophora (HP88)	No significant control	(Alm et al., 1992)
	H. bacteriophora (TF)	0-7% 14 DAT, 40-53% 21 DAT; 11-45% 21 DAT at 1-2.5×10^9 IJ/ha	(Koppenhöfer and Fuzy, 2003b)
		40-43% 22 DAT	(Koppenhöfer et al., 2002)
Rhizotrogus majalis	*S. scarabaei*	Fall: 74.7-88% 21 DAT Spring: 56-59% 41	(Cappaert and Koppenhöfer, 2003)

		DAT	
	H. marelatus	36% 41 DAT	(Cappaert and Koppenhöfer, 2003)
	H. bacteriophora	No significant control	(Cappaert and Koppenhöfer, 2003)
Maladera castanea	*S. scarabaei*	71-86% 14-21 DAT	(Koppenhöfer and Fuzy, 2003a)
	H. bacteriophora (TF)	12-33% 14-21 DAT	(Koppenhöfer and Fuzy, 2003a)
Hoplia philanthus	*H. bacteriophora*	39-66% surface; 33-76% subsurface	(Ansari et al., 2006)
White grubs[b]	*S. glaseri* (MB)	54-74%	(Grewal et al., 2004)

[a] DAT = Days after treatment.

[b] White grubs---mixed populations of *Popillia japonica* and *Cyclocephala borealis.*

[*]Data updated from Table 1 in Klein (1990).

III. Factors Affecting Efficacy

Compared with laboratory success, only limited field studies have shown satisfactory efficacies in white grub control. Unlike studies in the laboratory with optimal conditions and precluding interference from other organisms, field efficacies of entomopathogenic nematodes may be affected by various factors, both biotic and abiotic, such as predators, competitors, environmental extremes, explained in detail below. By understanding these factors and exploiting relative strategies to minimize or avoid deleterious impacts, performance of insecticidal nematodes in field applications might be significantly improved.

Biotic factors

Host suitability is a critical biotic factor that affects pathogenicity, and matching the insect with the proper nematode species and strains is a key factor in successful control. As mentioned earlier, although many insecticidal nematodes possess a broad host range in the laboratory with environmental conditions optimal for infection, they are only able to infect a limited spectrum of insects in the field due to ecological barriers, physiological adaptations and environmental restrictions. Using nematodes that ambush at or near the soil surface to suppress sedentary subterranean insects such as white grubs would result in control failure in field application, since it is very likely that the nematode would never encounter the target. *S. carpocapsae* is very effective against various caterpillars, but has poor performance against white grubs. In this case, nematode behavior and habitat adaptation may explain the

failure toward white grubs. Other factors such as physiological adaptation and host suitability also need to be considered. Spiracles are the main route of entry for *S. carpocapsae* when attacking caterpillars, but this approach precludes invasion in white grubs whose spiracles are covered with sieve plates (Forschler and Gardner, 1991a). In addition, *S. carpocapsae* encounters immediate encapsulation and quick melanization from the Japanese beetle, whereas *S. glaseri* invasion only elicits a weak immune response that is quickly overcome by the nematode released anti-immune proteins (Wang et al., 1995). This indicates that *S. glaseri* is a better match than *S. carpocapsae* for the control of Japanese beetle, and this has been proven by extensive laboratory and field tests.

In addition to host matching, nematode age (age of IJs) and host developmental stage also play significant roles in the successful use of insecticidal nematodes for pest control. Because IJs do not feed while host-searching, which is an energy-consuming process especially for cruisers, the older IJs have less reserves for finding a host, which negatively affects infectivity. For example, in a laboratory study, IJs with depleted lipid reserves could kill *Tenebrio molitor* larvae when placed in contact with the host, but were not infective when host search was required (Vanninen, 1990). Nematode efficacy may also vary with the developmental stage of white grubs, depending on white grub and nematode species. The early instars are generally more susceptible to nematodes, and susceptibility may decline as grubs grow larger. For example, *H. bacteriophora* efficacy decreased from 1st over 2nd to 3rd instar and also from small 3rd instar to large 3rd instar in *A. orientalis*, but did not differ significantly between *P. japonica* larval stages; the 2nd instar of *A. orientalis* was more susceptible than the 3rd instar to *H. bacteriophora*, but not to *S. glaseri,* or *S. scarabaei* which was equally lethal to all white grub instars (Koppenhöfer and Fuzy, 2004). Thus, the selection of nematode species for targeting grub species and developing stages needs to be considered for maximizing efficacy in application.

Intra-specific and inter-specific competition between insecticidal nematodes and competition with entomopathogenic microbes may also affect nematode performance in insect control in natural systems. Intra-specific competition might be incurred if too many IJs of the same species are present in a single host, and hence negatively affecting nematode fitness, progeny survival and recycling. As for inter-specific competition, the heterorhabditid and steinernematid species are incompatible, and generally cannot coexist in the same host, due to the difference in their mutualistic bacteria (Alatorre-Rosas and Kaya, 1990; Alatorre-Rosas and Kaya, 1991). According to Alatorre-Rosas and Kaya (1990), no advantage is gained by using two nematode species in inundative application for suppression of a single soil pest. Competition or incompatibility might also occur when entomopathogenic nematodes are applied with other insecticidal microbes against the same pest. For example, with the antibiotics produced by the symbiotic bacteria, the nematodes outcompete the entomopathogenic fungus *Beauveria*

bassiana if applied simultaneously, and the host must be infected one to several days before nematode exclusion occurs for the fungus to be successful (Barbercheck and Kaya, 1990).

Other biotic factors affecting control efficacy and nematode survival include nematophagous fungi such as *Hirsutella rhossiliensis*, protozoan parasites, and predators (e.g. predatory nematodes, mites, protozoans, turbellarians, tardigrades, oligochaetes and insects) (Kaya and Koppenhöfer, 1996).

Abiotic factors

Compared with biotic factors, abiotic factors also play a significant role in the success of using insecticidal nematodes to control insect pests. Environmental conditions such as ultraviolet light, desiccation, soil moisture, soil temperature, soil texture, pH, thatch build-up, and agrichemical compatibility, greatly affect control efficacy in field applications. Georgis and Gaugler (1991) reported that thatch thickness was negatively correlated with nematode efficacy. Also, entomopathogenic nematodes and their symbiotic bacteria are very sensitive to sunlight, especially ultraviolet radiation (Fujiie and Yokoyama, 1998; Gaugler et al., 1992). For example, as short as 4 minutes exposure to medium-wave ultraviolet light caused significant loss of pathogenicity of *H. bacteriophora*; the infectivity of *Steinernema carpocapsae* was significantly inactivated after 6 minutes exposure. Hence, it is preferred to deliver the nematodes to the field in the early morning or evening to minimize or avoid ultraviolet exposure.

Hydration is essential for nematode survival and insect infection. Nematodes perish from rapid desiccation, which is a major limiting factor that prevents the foliar spray of nematodes for insect control in exposed locations. To enhance efficacy, insecticidal nematodes are normally applied in soil or other cryptic habitats with adequate moisture to maintain nematode activities. Moisture not only prevents nematodes from desiccation, but also provides a film of water for nematodes to disperse and find the host. If nematodes desiccate slowly, they might still be able to survive in the soil for a longer period of time by entering a noninfectious state similar to anhydrobiosis. However, the infectivity and virulence of nematodes may decline, as nematodes are inactivated by low soil moisture, and are prevented from normal movement and host infection. The insecticidal activity of *S. kushidai* against the white grub *Anomala cuprea* in turfgrass was found to increase as soil moisture increased from 10 to 40% (Fujiie et al., 1996). Similarly, Grant and Villani (2003) found that nematode virulence increased with soil moisture content, and virulence of nematodes in low moisture conditions could be restored by rehydrating the soil. Soil moisture is critical for nematode mobility, establishment, infectivity and persistence (Koppenhöfer et al., 1997; Koppenhöfer and Fuzy, 2007; Koppenhöfer et al., 1995), but saturated soil may inhibit nematode movement and decrease their survival by creating an anaerobic condition (Molyneux and Bedding, 1984). In field applications, watering is generally required prior to and

immediately after the delivery of entomopathogenic nematodes unless spraying occurs in rain, and the soil should be kept moist for at least one wk to allow nematodes to take effect.

Other than soil moisture, soil temperature is also critical to nematode mobility and pathogenicity in insect control. Both high and low temperature extremes are detrimental to most insecticidal nematodes, by inactivating or diminishing their infectivity, and threatening survival at extremes, with the exception of a few species and strains that are more heat or cold tolerant than the others. For example, the exsheathed infective juveniles of *H. zealandica* do not survive below -6 ^{o}C (Wharton and Surry, 1994), while *S. feltiae* is more freeze tolerant and can maintain infectivity at soil temperatures below 10 ^{o}C (Brown and Gaugler, 1996; Chen et al., 2003). Temperatures higher than 30 ^{o}C will greatly reduce nematode survival, although some species such as *S. riobrave* remain effective at killing insects at soil temperatures above 35 ^{o}C (Lacey and Unruh, 1998). Normally in field conditions, efficacy declines rapidly when temperature drops below 16 ^{o}C, under which the ability of nematodes in host-finding and infection diminishes. For example, significantly higher mortality of green June beetle was caused at 25 ^{o}C than at 12 ^{o}C by *S. carpocapsae* and *H. bacteriophora* (Townsend et al., 1998). If not killed, insecticidal nematodes may respond to cold temperatures by entering a noninfectious or "diapause" state (Fan and Hominick, 1991), and storage of nematodes at low temperatures prior to application may reduce the control efficacy (Kaya and Gaugler, 1993).

Soil texture and pH also affect nematode performance in the control of insect pests. Clay soils have small pore spaces that limit nematode movement and survival, since nematodes require a film of water to intake oxygen and disperse in the soil for host-finding. In general, nematode efficacy declines as the proportion of clay increases in the soil. Researchers found that nematode migration, survival, pathogenicity and host-finding ability decreased in soil with more clay content or small pore sizes (Choo and Kaya, 1991; Hsiao and All, 1996; Kung et al., 1990b). Compared with soil texture, soil pH has relatively less effect on nematode survival and pathogenicity, except at extreme pH values. Soil pH between 4 and 8 has little or no impact on nematode survival (Kaya, 1990). However, soil salinity and acidity beyond this range might have negative effects on the survival and infectivity of insecticidal nematodes. For example, all infective juveniles of *S. carpocapsae* died when the pH value was adjusted to 2 in a phosphate buffer, whereas 70% survival rate was maintained after 10 d at pH 4-12 (Cheng and Hou, 1997); Kung et al. (1990a) reported that steinernematid nematodes survived poorly in soils with a pH of 10. Moreover, the infectivity of *H. bacteriophora*, *H. zealandica*, *S. glaseri* and *S. scarabaei* was the lowest in acidic sand soil with a pH of 3.9 in a laboratory experiment (Koppenhöfer and Fuzy, 2006).

Entomopathogenic nematodes are compatible with many pesticides and compounds, including insecticides, fungicides, herbicides, soil amendments, fertilizers, adjuvants and surfactants, plant-extracts,

and bio-rationals like *Bacillus thuringiensis* (Bt) (Barbarossa et al., 1996; Baur et al., 1997; Baweja and Sehgal, 1997; Bednarek and Gaugler, 1997; Gordon et al., 1996; Nishimatsu and Jackson, 1998; Scheepmaker et al., 1998; Schroeder and Sieburth, 1997; Shamseldean and Ismail, 1997; Shapiro et al., 1996; Stark, 1996). Specifically, increased efficacy or synergism was found by combined use of an insecticide and nematode, e.g. imidacloprid plus nematode (Koppenhöfer et al., 2000a; Koppenhöfer et al., 2002; Koppenhöfer et al., 2000b; Koppenhöfer and Kaya, 1998), as well as nematode plus Bt in white grub control (Koppenhöfer et al., 1999; Koppenhöfer and Kaya, 1997). However, there are still some chemicals that cannot be mixed with nematodes in field application, and need to be applied separately, or at 1 to 2 wk intervals between applications. Georgis and Poinar (1994) summarized a list of commonly used chemical compounds that can be applied with *S. carpocapsae* in turf and ornamentals. Usually, suppliers provide a list of chemicals that can or cannot be mixed with their nematode products (e.g. http://www.nemasyspro.com/pdf/Compatibility.pdf).

IV. Commercialization & Future Prospects

Entomopathogenic nematodes have been commercialized since the 1980s. Presently, there are at least four species of nematodes commercially available for white grub control: *S. glaseri*; *H. bacteriophora*; *H. megidis*; *H. marelatus* (http://oardc.osu.edu/nematodes/nematode_suppliers.htm).

Entomopathogenic nematodes possess many advantages. Different from other insecticidal microbes, nematode products have been exempted from registration in the United States by the Environmental Protection Agency (Gorsuch, 1982). They are safe on non-target organisms, but can be highly insecticidal to their target hosts. Also, they can be applied with standard spray equipment, are as easy to use as conventional insecticides, and are compatible with many chemical insecticides as mentioned. In addition, most of these nematode agents can be mass-produced in vitro. Noticeably, all these characteristics have triggered the rapid development and commercialization of nematodes.

Although considerable efforts in research and development have been made in the past few decades, the use of nematodes in white grub management is still limited. Despite the merits of entomopathogenic nematodes, they are far less competitive in the market than conventional insecticides that are generally cheaper with much longer shelf-life time. High costs to manufacturers and end-users, short shelf-life, and unstable field efficacies are among the major disadvantages that limit the development and large-scale application of nematode products. Despite the cost savings from registration exemption, it is still expensive to produce and market insecticidal nematodes, given the high costs and techniques involved in mass-production and formulation, and the low market share of clientele. Most infective juveniles can only live for several weeks to a few months at most. Their viability, infectivity and virulence decrease in storage. Nematodes with high control efficacies in the laboratory might not maintain the same control

success in the field. Also, nematodes proven successful in some cases may fail in other situations due to the various factors described above. Some nematode species such as *Steinernema scarabaei* are highly effective against a wide range of white grub species (Koppenhöfer and Fuzy, 2003b; Koppenhöfer et al., 2004), but attempts of mass production in vitro have been unsuccessful.

Further advancements are anticipated in improving mass-production techniques and lowering the manufacturing costs (e.g. improvement in fermentation and media formulation processes to increase the yield and quality), and in developing more advanced carriers and techniques in formulation to lengthen the IJs shelf-life. Also, genetic improvement may be considered in increasing nematode performance and efficacies in the field; for example, selection against scarab larvae resulted in a 72-fold increase in host-finding capability (Gaugler and Campbell, 1991). Since the symbiotic bacteria *Photorhabdus* and *Xenorhabdus* are highly insecticidal against certain groups of insect pests, the potential of insecticidal toxins isolated from these bacteria as novel insecticidal proteins for insect control is also under investigation (ffrench-Constant et al., 2010). Overall, the future use of entomopathogenic nematodes is promising, given all the advantages they possess, as well as the increasing desirability of any virulent microbial pathogen to help mitigate the environment and resistance pressure of chemical insecticides.

Entomopathogenic Fungi

I. History & Taxonomy

Fungi causing diseases in insects was first recognized by Bassi, who demonstrated in 1835 that the muscardine fungus, *Beauveria bassiana*, could cause disease in silkworms (Steinhaus, 1975). The first attempt to use entomopathogenic fungi against scarabs was in 1879 when Metschnikoff used *Metarhizium anisopliae* in the control of the wheat chafer, *Anisoplia austriaca* in Russia. Since then, many attempts have been made to use naturally occurring fungi against insect pests including soil-inhabiting pests like scarab larvae. However, early attempts were soon overshadowed by the development of chemical insecticides, and use of entomopathogenic fungi for controlling insect pests did not resurface until the 1970s as public awareness of pesticide hazards increased. With many chemical pesticides being withdrawn, the desire of using environmentally safe biological agents increased, and this has prompted research and development of entomopathogenic fungi in pest control.

To date, at least 90 genera and over 700 species of entomopathogenic fungi have been described (Goettel et al., 2010). The role of entomopathogenic fungi in insect control has been reviewed in detail by many authors (Boucias and Pendland, 1998; Butt et al., 2001; Carruthers and Soper, 1987; Evans, 1989; Ferron, 1985; Ferron et al., 1991; Glare and Milner, 1991; Goettel et al., 2010; Hajek, 1997; Hajek and St. Leger, 1994; McCoy et al., 1988; Roberts and Hajek, 1992; Samson et al., 1988; Tanada and Kaya,

1993; Upadhyay, 2003; Wraight and Carruthers, 1999; Wraight et al., 2007), and information on fungal pathogens of scarabs is covered by Glare (1992).

The entomopathogenic fungi described as pathogenic to scarabs belong to four major classes: Ascomycetes, Coelomycetes, Deuteromycetes, and Zygomycetes, as listed in Table 4. Among those, the Deuteromycetes, or Fungi Imperfecti, is an artificial grouping of species known only by an asexual stage. Of approximately 200 species found pathogenic to insects, 10 species mostly in the genera *Beauveria*, *Metarhizium*, *Paecilomyces*, *Hirsutella* are associated with scarabs. In Ascomycetes, there is only one genus, *Cordyceps*, with pathogenic capabilities against scarabs. Anamorphs of *Cordyceps* species have been classified among the entomopathogenic Deuteromycetes genera including *Hirsutella*, *Paecilomyces* and *Verticillium*. Although it was reported to have 11 species being described from scarabs, many reports of this genus infecting scarabs have not been classified to species level, and very little is known about most species. In Zygomycetes, approximately 200 species in the order Entomophthorales have been reported as entomopathogenic, and among those only one species, *Pandora brahminae* (=*Entomophthora brahminae*) has been recorded on Scarabaeidae. Coelomycetes include only one species, *Entoderma colletosporium*, known to be pathogenic to Japanese beetle, *P. japonica*, at the larval stage (Hanula et al., 1991).

Table 4. Fungal species specifically recorded as pathogenic to Scarabaeidae [*].

Classes	Fungal species
Ascomycetes	*Cordyceps aphodii* Mathieson
	C. barnesii Thw.
	C. brittlebankii Mclennan & Cookson
	C. geotrupis Teng
	C. melolonthae (Tul.) Sacc.
	C. michiganensis Mains
	C. neovolkiana Kobayasi
	C. ravenelii Berkley & Curtis
	C. superficialis (Peck) Sacc.
	C. stylophora Berk. & Br.
Coelomycetes	*Entoderma colletosporium* Hanula, Andreadis & Blackwell
Deuteromycetes	*Akanthomyces angustispora* Mains

	Beauveria amorpha (Hoehnel) Samson & Evans
	B. bassiana (Balsamo) Vuillemin
	B. brongniartii (Saccardo) Petch
	Hirsutella stylophora Mains
	Metarhizium anisopliae (Metschnikoff) Sorokin
	var. *anisopliae* Tulloch
	var. *majus* (Johnston) Tulloch
	Paecilomyces canadensis (Vuillemin) Brown & Smith
	P. cicadae (Miquel) Samson
	P. fumosoroseus (Wize) Brown & Smith
	Penicillium sp.
	Lecanicillium (=*Verticillium*) *lecanii* (Zimm.) Viegas
Zygomycetes	*Pandora brahminae* (Bose & Mehta) Humber

*Table reprinted with permission of Glare (1992).

II. Pathogenicity & Safety

For most entomopathogenic fungi, the route of infection is direct penetration of the cuticle wall, not via ingestion. Spores or conidia adhere to the cuticle of susceptible hosts, and germinate hyphae tubes to penetrate the body wall directly, probably with the aid of both enzymatic degradation (e.g. chitinases and proteases) and mechanical pressure. External penetration may occur at any site of the host body, but mainly occurs at the inter-segmental joints. Infection via the alimentary canal is rare. After penetration, the fungus then proliferates in the insect hemocoel to fill the host with hyphae, or mycelia, and kills the host eventually, which may take weeks and even months in white grubs. After death of the host, hyphae outgrow and sporulate on the cadaver at favorable conditions, usually starting at the inter-segmental joints, producing spores or conidia to start a new infection cycle.

Entomopathogenic fungi kill the host by a variety of means, such as starvation, nutrient depletion, or body obstruction by the hyphae. Some fungi may produce insecticidal toxins, such as destruxins, which are cyclic peptide toxins secreted by *Metarhizium* spp. (Roberts, 1981), *Aschersonia* sp. (Krasnoff et al., 1996), and *Beauveria felina* (Kim et al., 2002), to assist in pathogenesis. Currently, more than 35 different destruxins have been described (Liu et al., 2004). In addition to toxicity at high doses, some destruxins may reduce growth and reproduction of the host (Brousseau et al., 1996), act as repellent or

antifeedant (Amiri et al., 1999; Robert and Riba, 1989; Thomsen and Eilenberg, 2000), or be linked to increased host range (Amiri-Besheli et al., 2000). Synthetic analogs of destruxins have been proven to be toxic to lepidopteran insects, and their potential as novel pesticides has been considered (Thomsen and Eilenberg, 2000).

Entomopathogenic fungi may be highly virulent against target hosts, varying with situations. *M. anisopliae* isolate MM was reported to cause up to 88.6% mortality of the barley chafer grub, *Coptognathus curtipennis*, at 10^8 conidia (g soil)$^{-1}$ concentration after 3-4 wk exposure under laboratory conditions (Anbesse et al., 2008). Also, *M. anisopliae* isolate DAT F-001 direct-drilled into uncultivated pasture soil reduced the redheaded cockchafer, *Adoryphorus couloni*, in one generation by 94% in autumn and 50% in spring, and prospects for sustained control of the pest are good because the fungus can survive in the soil for at least three years under local conditions in Australia (Rath, 1992). In another case study, *M. anisopliae* did not cause significant mortality of 3rd instar white grub, *Hoplia philanthus*, in the greenhouse at the rate of 2×10^4-2×10^5 conidia (g soil)$^{-1}$ (13.8-28% compared with 8% in control) (Ansari et al., 2004), but caused 37-65% mortality from sub-surface application in the field (Ansari et al., 2006). In Europe, the fungus *B. brongniartii* has been applied for approximately 100 years to control white grubs and adults of the European cockchafer, *Melolontha* spp. (Zimmermann, 1992).

Specificity and pathogenicity of entomopathogenic fungi not only vary between genera and species, but may also differ with strains or types as well as host developmental stages. Among several hundred isolates of *M. anisopliae* being tested, isolate FI 147 and FI 153 were highly pathogenic to a range of sugar-cane white grub species including *Lepidiota frenchi* and *L. consobrina*, and isolate FI 114 was especially effective against *Antitrogus parvulus* in Australia (Milner, 1992). Strains of *M. anisopliae* pathogenic to eggs, larvae and pupae of *Rhopaea verreauxi* were different from strains infecting adults (Milner, 1989). *C. aphodii* was virulent against pasture cockchafer grubs at younger instars, but rarely infects pupae (Coles, 1980; Mathieson, 1949). Also, the 1st instar stage of white grub *Holotrichia* sp. was more susceptible than the 2nd and 3rd instar grubs to *B. bassiana* (Mohi-ud-din et al., 2007).

Many insecticidal fungi, like *Metarhizium* and *Beauveria*, are generally considered to possess a broad host range, while some others like *Cordyceps* are more host-specific (Kobayasi, 1941; McEwen, 1963). Veen (1968) listed 204 insect species naturally infected by *M. anisopliae*, with over 70 scarab species included; *B. bassiana* was reported to possess a host range that covers over 700 species of arthropods (Goettel et al., 1990a). However, *C. aphodii* is pathogenic to only two scarab species in one genus, *Aphodius tasmaniae* (=*A. howitti*) in New Zealand (Helson, 1965), and *A. tasmaniae* and *A. ambigus* in Australia (Coles, 1980), in addition to a Carabidae, *Hypharpax* sp..

Most entomopathogenic fungi do not pose a threat to human and other vertebrates (Saik et al., 1990; Siegel and Shadduck, 1990), with the exception of a few species such as *Conidiobolus coronatus*, *Aspergillus flavus*, *Paecilomyces lilacinus*, which are pathogenic to vertebrates and thus are not considered for commercialization as microbial pesticides. However, such pathogenicity may vary with strains, e.g. the strain P 251 of *P. lilacinus*, isolated in the Philippines, has been successfully developed as a safe bio-nematicide (Copping, 2001). Before being registered as a microbial control agent, any entomopathogenic fungus must undergo a series of stringent tests for potential harmful effects on mammals and other vertebrates (Laird et al., 1990; Siegel, 1997).

A few pathogenic cases have been reported, e.g. in laboratory assays of *B. bassiana* to silverside fish embryos and fry (Genthner and Middaugh, 1992), *M. anisopliae* to grass shrimp embryos (Genthner et al., 1997) and silverside fish embryos and fry (Genthner and Middaugh, 1995), and deep tissue infection of an immunosuppressed female by a *Beauveria* sp. (Henke et al., 2002). However, there have been no reports of infections in vertebrates directly resulting from the use of commercial strains of entomopathogenic fungi in the field (Goettel et al., 2001; Vestergaard et al., 2003). Although metabolites produced by some entomopathogenic fungi may be toxic or carcinogenic to vertebrates (Strasser et al., 2000; Vey et al., 2001), significant level of exposure to those metabolites would be required to cause hazard to vertebrates. Strasser et al. (2000) speculated that the use of entomopathogenic fungi as microbial control agents should pose no obvious risk to humans, as toxin levels should never rise up to harmful levels in the environment, and most of them will not grow at 37 ^{o}C.

III. Environmental Constraints

Many entomopathogenic fungi achieving encouraging control results under controlled laboratory conditions show unreliable and unstable efficacies against white grubs in the field. In nature, the soil ecosystem involves a very complex interaction between various environmental components and the fungal agent as well as the host insect, affecting the overall performance of the fungi. As with other entomopathogens, there are a variety of environmental factors, both biotic and abiotic, that affect the survival, pathogenicity and persistence of fungi in soil.

Temperature is an essential abiotic factor that has significant influence on field efficacies by affecting fungal germination, growth and viability. Although most entomopathogenic fungi may tolerate a wide range of temperatures, the optimum temperatures for infection, growth and sporulation are usually restricted to 20-30 ^{o}C, varying with species and strains. *M. anisopliae* stay active at a wider range of temperatures, between 15 to 35 ^{o}C, with optimum at 25-30 ^{o}C for germination and growth (Alves et al., 1984; Ekesi et al., 1999; Hywel-Jones and Gillespie, 1990; Milner et al., 2003; Müller-Kögler, 1965; Roberts and Campbell, 1977; Walstad et al., 1970; Welling et al., 1994). Some cold-active or heat

tolerant isolates were also found to be able to grow outside the range. For example, *M. anisopliae* DAT F-001 was able to germinate at 2 to 25 ^{o}C, and infect the host scarab, *Adoryphorus couloni*, at 10 ^{o}C or at a fluctuating temperature of 15/5 ^{o}C (Rath et al., 1995a). Both *B. brongniartii* and *B. bassiana* remain pathogenic below 15 ^{o}C (Glare, 1992). Although *M. anisopliae* has a much shorter shelf-life and lower temperature for storage prior to application than *B. bassiana*, its conidia may remain infective in soil for over one year (Latch and Falloon, 1976; Milner and Lutton, 1976; Müller-Kögler and Stein, 1976; Rath, 1992; Samuels and Pinnock, 1988), longer than the period *Beauveria* conidia can survive (Müller-Kögler and Stein, 1970).

Besides temperature, moisture and humidity are also critical to the field performance of entomopathogenic fungi against scarab grubs. Conidia or spores of many fungal species may only germinate at R.H. 90% or above (Milner, 1989; Zimmermann, 1986), although it is rarely a limitation in the soil environment unless the fungi are exposed on the surface. Moisture is also essential for fungal germination and sporulation. In many cases, high moisture is desirable for the effective use of fungal agents, whereas dry weather conditions have been considered responsible for control failures. In addition to rainfall or hydration, soil type or texture may have an influence on soil moisture and spore movement, and hence affect the fungal fate, infectivity and persistence. Ferron (1971b) reported that *B. brongniartii* remained infective in sandy loam soil for one year but lost infectivity in turf after six months, while Coles (1980) found that soil type was relatively unimportant in the infection of *A. tasmaniae* by *C. aphodii*.

Other abiotic factors, including solar radiation, inorganic matter, pH, aeration, and pesticides cannot be disregarded in the successful use of entomopathogenic fungi in scarab control. Solar radiation from ultraviolet A and B lights can be damaging or even lethal to fungal infective propagules (Braga et al., 2001a), although susceptibility may differ with species and strains (Braga et al., 2001b; Fargues et al., 1996; Morley-Davies et al., 1995). Also, compatibility of fungal agents with pesticides needs to be taken into account. Compatibility or synergism has been reported between the entomopathogenic fungi and synthetic pesticides. Such reports include compatibility of *M. anisopliae* with common pesticides for white grub control in sugar-cane fields (Samuels and Pinnock, 1988); and synergism between *B. brongniartii* (*B. tenella*) and parathion or trichloronate (organophosphates) against *M. melolontha* (Ferron, 1971a). Entomopathogenic fungi are compatible with many agrochemicals including most insecticides, but not with fungicides (McCoy et al., 1988). Fungicides may have detrimental effects on fungal germination or vegetative growth in the laboratory; however, the impact might be mitigated in field environments when applications were made asynchronously (Jaros-Su et al., 1999).

Similar to abiotic factors, biotic factors also play a crucial role in the field success of fungal applications in scarab control, although fewer studies have been reported in this area. Biotic factors include the level

of organic matter, plant root system, microflora and fauna populations. Conidia or spores survival in soil may be improved by the presence of fertilizers and organic matter (Dutky, 1959; Oliveira et al., 1981). Parasitism by other organisms such as fungi and bacteria is one of the major factors that debilitate the control success of entomopathogenic fungi in the field. Reports include fungal parasitism in pseudosclerotia of *Cordyceps aphodii* (Coles, 1980), and fungistatis in *B. bassiana* probably caused by *Penicillium urticae* (Lingg and Donaldson, 1981). Also, compatibility of insecticidal fungi with other entomopathogens used for scarab control in field applications needs to be considered. Additive or synergistic interactions have been reported between entomopathogenic fungi and other microbial agents, e.g. synergism between *B. brongniartii* and entomopoxvirus, rickettsia or *Bacillus (Paenibacillus) popilliae* against *M. melolontha* (Ferron and Hurpin, 1974; Ferron et al., 1969; Hurpin and Robert, 1972).

IV. Commercialization & Future Prospects

Currently, the development of entomopathogenic fungi as microbial insecticides for scarabs is still below its potential. Although over 700 entomopathogenic fungal species have been described, only a few species have been commercialized worldwide, mostly from the Deuteromycetes (Alves et al., 2003; Copping, 2001; Shah and Goettel, 1999; Wraight et al., 2001). Among those, very few fungal species are available as mycoinsecticides for scarab control. These include *B. bassiana* marketed as BotaniGard and Mycotrol in the USA; *B. brongniartii* for the control of European cockchafer *Melolontha melolontha* L. and some other grub species in Europe; and *M. anisopliae* for red-headed cockchafer *Adoryphorus coulonii* (Burmeister), and sugar-cane white grubs *Tomarus* spp. in Australia. In the USA, *B. bassiana* is the only commercially available fungal agent labeled for white grub control in turf. Due to the stringent regulatory policies on importing and exporting microbial agents, *B. brongniartii* has not been marketed in the US yet. Although *M. anisopliae* is available in the US, it has not been registered for scarab control in turf.

Commercialization of fungal agents as mycoinsecticides may be affected by several factors, such as high costs associated with registration, host range and safety testing, formulation techniques, low to moderate efficacies or instability in field performance, slow speed of kill, storage requirements, and potential market share. For example, when applied as a surface application in turfgrass, *M. anisopliae* strain F 52 in oil formulation only causes marginal control of masked chafer grubs, because much of the active material remains in the thatch, lacking sufficient contact with the target pests in the soil to have an impact (Wu et al. unpublished data). This might be one of the factors that hindered the registration of this product for masked chafer control besides the high input to output cost ratio.

Moderate or unstable efficacy is among the major factors that have hindered the commercialization and registration of entomopathogenic fungi as microbial insecticides. Efficacies of fungal agents may be

improved by strain selection, genetic modification (St. Leger and Screen, 2001), formulation (Wraight et al., 2001), and application strategies (Bateman and Chapple, 2001). Because natural variation between strains of fungal species exists, strains are selected for better potential in commercialization based on virulence, host range, environmental persistence, or some other important variable. In the process of strain selection, both biotechnological and genetic techniques may be adopted. Studies on genetic modification are limited so far. The most advanced research has been on *M. anisopliae* and *B. bassiana*, focused on transformation systems (Bernier et al., 1989; Goettel et al., 1990b; Inglis et al., 1999; Sandhu et al., 2001; St. Leger et al., 1995), strain improvement (St. Leger, 2001; St. Leger et al., 1996), cuticle degrading protease (St. Leger et al., 1992) and chitinase genes (Bogo et al., 1998; Screen et al., 2001). For example, the overexpression of the *pr1* gene by insertion of multiple copies resulted in increased speed of kill of a host insect, despite poor sporulation ability (St. Leger, 2001). Improvement in formulation can extend the shelf stability of fungal propagules. Both formulation and application technology can improve field efficacies by increasing host contact with the infective propagules.

Currently, the market share of entomopathogenic fungi is still below the full potential, due to high costs, limited products, inadequate field efficacies, and insufficient public awareness. For development of entomopathogenic fungi as mycoinsecticides, further commitment is needed for improving methods of production, formulation, and application, as well as developing new fungal species and new strains for commercialization. Inevitably, with increasing public concerns about environmental sustainability, the use of entomopathogenic fungi will play an increasingly important role in the management of white grubs.

Conclusion

As discussed above, although entomopathogenic nematodes and fungi may provide good control of white grubs in certain cases, the control efficacies are not always consistent or satisfactory in field applications due to environmental limitations. To improve their efficacies, all environmental constraints, both biotic and abiotic, need to be considered during field application. Despite low market share and limited products being commercialized, the development of entomopathogenic fungi and entomopathogenic nematodes as microbial pesticides has promising potential for the future. For both microbials, further efforts to improve production and formulation techniques, lower costs, extend the shelf life, improve field efficacies and persistence, develop new products with new species and strains, and improve application strategies are necessary.

The potential of improving control efficacies by combining entomopathogenic nematodes with fungi, or with other types of control agents has shown encouraging results. For example, when the nematode *S. carpocapsae* was applied with the fungus *B. brongniartii*, a significant increase in grub mortality was observed over the application of the fungus alone (Choo et al., 2002). Also, the combined application of

H. bacteriophora and *M. anisopliae* isolate MM at the concentration of 380 IJ/grub and 1.7×10^7 conidia (g soil)−1, respectively, increased larval mortality of the barley chafer grub, *Coptognathus curtipennis* Faimaire in an additive and synergistic manner (Anbesse et al., 2008). A similar result occurred from the combination of *M. anisopliae* CLO 53 and nematode *H. megidis* or *S. glaseri* against 3rd instar *Hoplia philanthus* Füessly under laboratory and greenhouse conditions (Ansari et al., 2004), and from the combined use of *M. anisopliae* CLO 53 and *H. bacteriophora* in the field (Ansari et al., 2006). In addition, additive or synergistic effects in grub control have also been reported in combining nematodes with bacterium Bt (Koppenhöfer et al., 1999; Koppenhöfer and Kaya, 1997), nematodes with insecticides (Koppenhöfer et al., 2002; Koppenhöfer et al., 2000b; Koppenhöfer and Kaya, 1998), and fungus *M. anisopliae* with bacterium *Serratia entomophila* (Glare, 1994).

The desire for developing safe use of microbial agents, including entomopathogenic nematodes and entomopathogenic fungi, for scarab control is increasing, given the economic impact of white grubs in turfgrass and other cropping systems, and environmental pressure rising from the large-scale use of chemical insecticides. This increases the potential demand for more concerted efforts in development and use of these nematodes and fungi as mycoinsecticides for integrated pest management of white grubs.

References

Akhurst, R. J., 1990. Safety to nontarget invertebrates of nematodes of economically important pests. In: M. Laird, et al., (Eds.), Safety of Microbial Insecticides. CRC Press, Boca Raton, FL, pp. 234-238.

Akhurst, R. J., Boemare, N. E., 1988. A numerical taxonomic study of the genus *Xenorhabdus* (Enterobacteriaceae) and proposed elevation of the subspecies of *X. nematophilus* to species. J. Gen. Microbiol. 134**,** 1835–1845.

Alatorre-Rosas, R., Kaya, H. K., 1990. Interspecific competition between entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema* for an insect host in sand. J. Invertebr. Pathol. 55**,** 179-188.

Alatorre-Rosas, R., Kaya, H. K., 1991. Interaction between two entomopathogenic nematode species in the same host. J. Invertebr. Pathol. 57**,** 1-6.

Alm, S. R., Yeh, T., Hanula, J. L., Georgis, R., 1992. Biological control of Japanese, oriental, and black turfgrass ataenius beetle (Coleoptera: Scarabaeidae) larvae with entomopathogenic nematodes (Nematoda: Steinernematidae, Heterorhabditidae). J. Econ. Entomol. 85**,** 1660-1665.

Alves, S. B., Pereira, R. M., Lopes, R. B., Tamai, M. A., 2003. Use of entomopathogenic fungi in Latin America. In: R. K. Upadhyay, (Ed.), Advances in Microbial Control of Insect Pests. Kluwer, New York, pp. 193-211.

Alves, S. B., Risco, S. H., Almeida, L. C., 1984. Influence of photoperiod and temperature on the development and sporulation of *Metarhizium anisopliae* (Metsch.) Sorok. J. Appl. Entomol. 97**,** 127-129.

Amiri-Besheli, B., Khambay, B., Cameron, S., Deadman, M., Butt, T. M., 2000. Inter- and intra-specific variation in destruxin production by the insect pathogenic *Metarhizium*, and its significance to pathogenesis. Mycol. Res. 104**,** 447-452.

Amiri, B., Ibrahim, L., Butt, T. M., 1999. Antifeedant properties of destruxins and their use with the entomogenous fungus *Metarhizium anisopliae* for improved control of crucifer pests. Biocontrol Sci. Technol. 9**,** 487-498.

Anbesse, S. A., Adge, B. J., Gebru, W. M., 2008. Laboratory screening for virulent entomopathogenic nematodes (*Heterorhabditis bacteriophora* and *Steinernema yirgalemense*) and fungi (*Metarhizium anisopliae* and *Beauveria bassiana*) and assessment of possible synergistic effects of combined use against grubs of the barley chafer *Coptognathus curtipennis*. Nematology. 10**,** 701-709.

Anonymous, 2013. American-lawns. Grubs. http://www.american-lawns.com/insects/grubs.html April 03.

Ansari, M. A., Shah, F. A., Tirry, L., Moens, M., 2006. Field trials against *Hoplia philanthus* (Coleoptera: Scarabaeidae) with a combination of an entomopathogenic nematode and the fungus *Metarhizium anisopliae* CLO 53. Biol. Control. 39**,** 453-459.

Ansari, M. A., Tirry, L., Moens, M., 2004. Interaction between *Metarhizium anisopliae* CLO 53 and entomopathogenic nematodes for the control of *Hoplia philanthus*. Biol. Control. 31**,** 172–180.

Barbarossa, B., Frulla, R., Grazzi, G., Rovesti, L., 1996. Compatibility of *Steinernema carpocapsae* with pesticides. Bulletin of the International Organization for Biological and Integrated Control of Noxious Animals and Plants. 19**,** 136-139.

Barbercheck, M. E., Kaya, H. K., 1990. Interactions between *Beauveria bassiana* and the entomogenous nematodes, *Steinernema feltiae* and *Heterorhabditis heliothidis*. J. Invertebr. Pathol. 55**,** 225-234.

Bateman, R., Chapple, A., 2001. The spray application of mycopesticide formulations. In: T. Butt, et al., (Eds.), Fungal Biocontrol Agents. CABI Publishing, Wallingford, pp. 289-309.

Bathon, H., 1996. Impact of entomopathogenic nematodes on non-target hosts. Biocontrol Sci. Technol. 6**,** 421-434.

Baur, M. E., Kaya, H. K., Gaugler, R., Tabashnik, B., 1997. Effects of adjuvants on entomopathogenic nematode persistence and efficacy agaisnt *Plutella xylostella*. Biocontrol Sci. Technol. 7**,** 513-525.

Baweja, V., Sehgal, S. S., 1997. Potential of *Heterorhabditis bacteriophora* Poinar (Nematoda, Heterorhabditidae) in parasitizing *Spodoptera litura* Fabricius in response to malathion treatment. Acta parasitol. 42**,** 168-172.

Bednarek, A., Gaugler, R., 1997. Compatibility of soil amendments with entomopathogenic nematodes. J. Nematol. 29**,** 220-227.

Begley, J. W., 1990. Efficacy against insects in habitats other than soil. In: R. Gaugler, H. K. Kaya, (Eds.), Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, FL, pp. 215-231.

Bernier, L., Cooper, R. M., Charnley, A. K., Clarkson, J. M., 1989. Transformation of the entomopathogenic fungus *Metarhizium anisopliae* to benomyl resistance. FEMS Microbiol. Lett. 60**,** 261-266.

Bogo, M. R., Rota, C. A., Pinto, H. J., Ocampos, M., Correa, C. T., Vainstein, M. H., Schrank, A., 1998. A chitinase encoding gene (chit1 gene) from the entomopathogen *Metarhizium anisopliae*: isolation and characterization of genomic and full-length cDNA. Curr. Microbiol. 37**,** 221-225.

Boucias, D. G., Pendland, J. C., 1998. Principles of Insect Pathology. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Braga, G. U. L., Flint, S. D., Miller, C. D., Anderson, A. J., Roberts, D. W., 2001a. Both solar UVA and UVB radiation impair conidial curability and delay germination in the entomopathogenic fungus *Metarhizium anisopliae*. Photochem. Photobiol. 74**,** 734-739.

Braga, G. U. L., Flint, S. D., Miller, C. D., Anderson, A. J., Roberts, D. W., 2001b. Variability in response to UV-B among species and strains of *Metarhizium* isolated from sites at latitude from 61°N to 54°S. J. Invertebr. Pathol. 78**,** 98-108.

Brandenburg, R. L., Villani, M. G., 1995. Handbook of Turfgrass Insect Pests. Entomological Society of America, Lanham, MD.

Brousseau, C., Charpentier, G., Bellonick, S., 1996. Susceptibility of spruce budworm, *Choristoneura fumiferana* Clemens, to destruxins, cyclodepsipeptidic mycotoxins of *Metarhizium anisopliae*. J. Invertebr. Pathol. 68**,** 180-182.

Brown, I. M., Gaugler, R., 1996. Cold tolerance of Steinernematid and Heterorhabditid nematodes. J. Therm. Biol. 21**,** 115-121.

Butt, T. M., Jackson, C. W., Magan, N., 2001. Fungi as Biological Agents. CABI Publishing, Wallingford, UK.

Cappaert, D. L., Koppenhöfer, A. M., 2003. *Steinernema scarabaei*, an entomopathogenic nematode for control of the European chafer. Biol. Control. 28**,** 379-386.

Carruthers, R. I., Soper, R. S., 1987. Fungal diseases. In: J. R. Fuxa, Y. Tanada, (Eds.), Epizootiology of Insect Diseases. John Wiley and Sons, New York, pp. 357-416.

Chen, S., Li, J., Han, X., Moens, M., 2003. Effect of temperature on the pathogenicity of entomopathogenic nematodes (*Steinernema* and *Heterorhabditis* spp.) to *Delia radicum*. Biol. Control. 48**,** 713-724.

Cheng, C. C., Hou, R. F., 1997. Effects of environmental factors on survival of the entomopathogenic nematode, *Steinernema carpocapsae*. Chin. J. Entomol. 17**,** 120-131.

Choo, H. Y., Kaya, H. K., 1991. Influence of soil texture and presence of roots on host finding by *Heterorhabditis bacteriophora*. J. Invertebr. Pathol. 58**,** 279-280.

Choo, H. Y., Kaya, H. K., Huh, J., Lee, D. W., Kim, H. H., Lee, S. M., Choo, Y. M., 2002. Entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis bacteriophora*) and a fungus *Beauveria brongniartii* for biological control of the white grubs, *Ectinohoplia rufipes* and *Exomala orientalis*, in Korean golf courses. BioControl. 47**,** 177-192.

Coles, R. B., The biology of *Cordyceps aphodii* (Sphaeriales: Clavicipitaceae). In: T. K. Crosby, R. P. Pottinger, Eds.), Proceedings of the 2nd Australasian Conference on Grassland Invertebrate Ecology, Palmerston North, New Zealand, 1980, pp. 207-212.

Converse, V., Grewal, P. S., 1998. Virulence of entomopathogenic nematodes to the western masked chafer *Cyclocephala hirta* (Coleoptera: Scarabaeidae). J. Econ. Entomol. 91**,** 428-432.

Copping, L. G., 2001. The Biopesticide Manual. British Crop Protection Council, Farnham.

Dutky, S. R., 1959. Insect microbiology. Adv. Appl. Microbiol. 1**,** 175-200.

Ekesi, S., Maniania, N. K., Ampong-Nyarko, K., 1999. Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti*. Biocontrol Sci. Technol. 9**,** 177-185.

Evans, H. C., 1989. Mycopathogens of insects of epigeal and aerial habitats. In: N. Wilding, et al., (Eds.), Insect-fungus Interactions. Academic Press, London, UK, pp. 205-238.

Fan, X., Hominick, W. M., 1991. Effects of low storage temperature on survival and infectivity of two *Steinernema* species (Nematoda: Steinernematidae). Revue Nématol. 14**,** 407-412.

Fargues, J., Goettel, M. S., Smits, N., Ouedraogo, A., Vidal, C., Lacey, L. A., Lomer, C. J., Rougier, M., 1996. Variability in susceptibility to simulated sunlight of conidia among isolates of entomopathogenic Hyphomycetes. Mycopathologia. 135**,** 171-181.

Ferron, P., 1971a. Modification of the development of *Beauveria tenella* mycosis in *Melolontha melolontha* larvae, by means of reduced doses of organophorus insecticides. Entomol. Exp. Appl. 14**,** 457-466.

Ferron, P., 1971b. Problèmes poses par la mise au point d'un procédé de lutte microbiologique contre *Melolontha melolontha* au moyen de la mycose à *Beauveria brongniartii* (Delacr.) Siemaszko. Phytiatrie-Phytopharmacie. 10**,** 159-168.

Ferron, P., 1985. Fungal control. In: G. A. Kerkut, L. I. Gilbert, (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology. Pergamon, Oxford, pp. 313-346.

Ferron, P., Fargues, J., Riba, G., 1991. Fungi as microbial insecticides against pests. In: D. K. Arora, et al., (Eds.), Handbook of Applied Mycology, Vol. 2: Humans, Animals, and Insects. Dekker, New York, pp. 665-706.

Ferron, P., Hurpin, B., 1974. Effects of simultaneous or successive contamination of *Beauveria tenella* and by *Entomopoxvirus melolonthae* of *Melolontha melolontha* larvae (Col., Scarabaeidae). Annales. Societe Entomologique de France. 10**,** 771-779.

Ferron, P., Hurpin, B., Robert, P. H., 1969. Sensibilisation des larves de *Melolontha melolontha* L. à la mycose *Beauveria tenella* par une infection préalableà *Bacillus popilliae*. Entomophaga. 14**,** 429-437.

ffrench-Constant, R. H., Waterfield, N., Daborn, P., 2010. Insecticidal toxins from *Photorhabdus* and *Xenorhabdus*. In: L. I. Gilbert, S. S. Gill, (Eds.), Insect Control: Biological and Synthetic Agents. Academic Press, Oxford, U.K., pp. 313-327.

Fischer-Le Saux, M., Viallard, V., Brunel, B., Normand, P., Boemare, N. E., 1999. Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. *temperata* subsp. nov. and *P. asymbiotica* sp. nov. Int. J. Syst. Bacteriol. 49**,** 1645-1656.

Forschler, B., Gardner, W., 1991. Parasitism of *Phyllophaga hirticula* by *Heterorhabditis heliothidis* and *Steinernema carpocapsae*. J. Invertebr. Pathol. 58**,** 396-407.

Forst, S., Dowds, B., Boemare, N., Stackebrandt, E., 1997. *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. Annu. Rev. Microbiol. 51**,** 47-72.

Fujiie, A., Takata, Y., Tachibana, M., Yokoyama, T., 1996. Insecticidal activity of an entomopathogenic nematode, *Steinernema kushidai* (Nematoda: Steinernematidae) against *Anomala cuprea* (Coleoptera: Scarabaeidae) larvae under different soil moisture conditions. Appl. Entomol. Zool. 31**,** 453-455.

Fujiie, A., Yokoyama, T., 1998. Effects of ultraviolet light on the entomopathogenic nematode, *Steinernema kushidai* and its symbiotic bacterium, *Xenorhabdus japonicus*. Appl. Entomol. Zool. 33**,** 263-269.

Gaugler, R., 1981. Biological control potential of neoaplectanid nematodes. J. Nematol. 13**,** 241-249.

Gaugler, R., 1988. Ecological considerations in the biological control of soil-inhabiting insect pests with entomopathogenic nematodes. Agric., Ecosyst. Environ. 24**,** 351-360.

Gaugler, R., Bednarek, A., Campbell, J. F., 1992. Ultraviolet inactivation of heterorhabditid and steinernematid nematodes. J. Invertebr. Pathol. 59**,** 155-160.

Gaugler, R., Campbell, J. F., 1991. Selection for enhanced host-finding of scarab larvae (Coleoptera: Scarabaeidae) in an entomopathogenic nematode. Environ. Entomol. 20**,** 700-706.

Gaugler, R., Kaya, H. K., 1990. Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, FL.

Gaugler, R., LeBeck, L. M., Nakagaki, B., Boush, G. M., 1980. Orientation of the entomogenous nematode *Neoaplectana carpocapsae* to carbon dioxide. Environ. Entomol. 9**,** 649-652.

Genthner, F. J., Foss, S. S., Gals, P. S., 1997. Virulence of *Metarhizium anisopliae* to embryos of the grass shrimp *Palaemonetes pugio*. J. Invertebr. Pathol. 69**,** 157-164.

Genthner, F. J., Middaugh, D. P., 1992. Effects of *Beauveria bassiana* on embryos of the Inland Silverside fish (*Menidia beryllina*). Appl. Environ. Microbiol. 58**,** 2840-2845.

Genthner, F. J., Middaugh, D. P., 1995. Nontarget testing of an insect control fungus: effects of *Metarhizium anisopliae* on developing embryos of the inland silverside fish *Menida beryllina*. Dis. Aquat. Org. 22**,** 163-171.

Georgis, R., Gaugler, R., 1991. Predictability in biological control using entomopathogenic nematodes. J. Econ. Entomol. 84**,** 713-720.

Georgis, R., Hague, N. G. M., 1991. Nematodes as biological insecticides. Pesticide Outlook. 2**,** 29-32.

Georgis, R., Kaya, H. K., Gaugler, R., 1991. Effect of steinernematid and heterorhabditid nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) on nontarget arthropods. Environ. Entomol. 20**,** 815-822.

Georgis, R., Koppenhöfer, A. M., Lacey, L. A., Bélair, G., Duncan, L. W., Grewal, P. S., Samish, M., Tan, L., Torr, P., van Tol, R. W. H. M., 2006. Successes and failures in the use of parasitic nematodes for pest control. Biol. Control. 38**,** 103-123.

Georgis, R., Manweiler, S. A., 1994. Entomopathogenic nematodes: A developing biological control technology. Agric. Zool. Rev. 6**,** 63-94.

Georgis, R., Poinar, G. O., Jr., 1994. Nematodes as bioinsecticides in turf and ornamentals. In: A. R. Leslie, (Ed.), Handbook of Integrated Pest Management for Turf and Ornamentals. CRC Press, Boca Raton, FL, pp. 477-489.

Glare, T. R., 1992. Fungal pathogens of scarabs. In: T. A. Jackson, T. R. Glare, (Eds.), Use of Pathogens in Scarab Pest Management. Intercept, Andover, UK, pp. 63-77.

Glare, T. R., 1994. Stage-dependent synergism using *Metarhizium anisopliae* and *Serratia entomophila* against *Costelytra zealandica*. Biocontrol Sci. Technol. 4**,** 321–329.

Glare, T. R., Milner, R. J., 1991. Ecology of entomopathogenic fungi. In: D. K. Arora, et al., (Eds.), Handbook of Applied Mycology, Vol. 2: Humans, Animals, and Insects. Marcel Dekker, New York, pp. 547-612.

Goettel, M. S., Eilenberg, J., Glare, T., 2010. Entomopathogenic fungi and their role in regulation of insect populations. In: L. I. Gilbert, S. S. Gill, (Eds.), Insect Control: Biological and Synthetic Agents. Academic Press, Oxford, UK, pp. 387-431.

Goettel, M. S., Hajek, A. E., Siegel, J. P., Evans, H. C., 2001. Safety of fungal biocontrol agents. In: T. Butt, et al., (Eds.), Fungal Biocontrol Agents - Progress, Problems and Potential. CABI Press, Wallingford, pp. 347-375.

Goettel, M. S., Poprawski, T. J., Vandenberg, J. D., Li, Z., Roberts, D. W., 1990a. Safety to nontarget invertebrates of fungal biocontrol agents. In: M. Laird, et al., (Eds.), Safety of Microbial Insecticides. CRC Press, Boca Raton, FL, pp. 209-231.

Goettel, M. S., St. Leger, R. J., Bhairi, S., Jung, M. K., Oakley, B. R., Roberts, D. W., Staples, R. C., 1990b. Pathogenicity and growth of *Metarhizium anisopliae* stably transformed to benomyl resistance. Curr. Genet. 17**,** 129-132.

Gordon, R., Chippett, J., Tilley, J., 1996. Effects of two carbamates on infective juveniles of *Steinernema carpocapsae* All strain and *Steinernema feltiae* Umeå strain. J. Nematol. 28**,** 310-317.

Gorsuch, A. M., 1982. Regulations for the enforcement of the Federal Insecticide, Fungicide, and Rodenticide Act exemption from regulation of certain biological control agents. Federal Register. 47**,** 23928-30.

Grant, J. A., Villani, M. G., 2003. Soil moisture effects on entomopathogenic nematodes. Environ. Entomol. 32**,** 80-87.

Grewal, P. S., Grewal, S. K., Malik, V. S., Klein, M. G., 2002. Differences in susceptibility of introduced and native white grub species to entomopathogenic nematodes from various geographic localities. Biol. Control. 24**,** 230–237.

Grewal, P. S., Koppenhöfer, A. M., Choo, H. Y., 2005. Lawn, turfgrass and pasture applications. In: P. S. Grewal, et al., (Eds.), Nematodes as Biocontrol Agents. CABI Publishing, Wallingford, UK, pp. 115-146.

Grewal, P. S., Power, K. T., Grewal, S. K., Suggars, A., Haupricht, S., 2004. Enhanced consistency in biological control of white grubs (Coleoptera: Scarabaeidae) with new strains of entomopathogenic nematodes. Biol. Control. 30**,** 73–82.

Grewal, S. K., Grewal, P. S., Gaugler, R., 1995. Endophytes of fescue grasses enhance susceptibility of *Popillia japonica* larvae to an entomopathogenic nematode. Entomol. Exp. Appl. 74**,** 219-224.

Hajek, A. E., 1997. Ecology of terrestrial fungal entomopathogens. Adv. Microb. Ecol. 15**,** 193-249.

Hajek, A. E., St. Leger, R. J., 1994. Interactions between fungal pathogens and insect host. Annu. Rev. Entomol. 39**,** 293-322.

Hamilton, D. W., Schwartz, P. H., Townsend, B. G., Jester, C. W., 1971. Traps reduce an isolated infestation of Japanese beetle. J. Econ. Entomol. 64**,** 150-153.

Hanula, J. L., Andreadis, T. G., Blackwell, M., 1991. *Entoderma colletosporium* gen. and sp. nov., a unique cuticular fungus infecting Japanese beetle, *Popillia japonica* (Coleoptera: Scarabaeidae). J. Invertebr. Pathol. 58**,** 327-334.

Helson, G. A. H., 1965. Entomogenous micro-organisms of New Zealand. Trans. R. Soc. N.Z. (Zool.). 6**,** 1-6.

Henke, M. O., de Hoog, G. S., Gross, U., Zimmermann, G., Kraemer, D., Weig, M., 2002. Human deep tissue infection with an entomopathogenic *Beauveria* species. J. Clin. Microbiol. 40**,** 2698-2702.

Hsiao, W. F., All, J. N., 1996. Effects of temperature and placement site on the dispersal of the entomopathogenic nematode, *Steinernema carpocapsae* in four soils. Chin. J. Entomol. 16**,** 95-106.

Hurpin, B., Robert, P. H., 1972. Comparison of the activity of certain pathogens of the cockchafer *Melolontha melolontha* in plots of natural meadowland. J. Invertebr. Pathol. 19**,** 291-298.

Hywel-Jones, N. L., Gillespie, A. T., 1990. Effect of temperature on spore germination in *Metarhizium anisopliae* and *Beauveria bassiana*. Mycol. Res. 94**,** 389-392.

Inglis, P. W., Tigano, M. S., Valadares-Inglis, M. C., 1999. Transformation of the entomopathogenic fungi, *Paecilomyces fumosoroseus* and *Paecilomyces lilacinus* (Deuteromycotina: Hyphomycetes) to benomyl resistance. Genet. Mol. Biol. 22**,** 119-123.

Jaros-Su, J., Groden, E., Zhang, J., 1999. Effects of selected fungicides and the timing of fungicide application in *Beauveria bassiana*-induced mortality of the Colorado potato beetle (Coleoptera: Chrysomelidae). Biol. Control. 15**,** 259-269.

Kaya, H. K., 1985. Entomogenous nematodes for insect control in IPM systems. In: M. A. Hoy, D. C. Herzog, (Eds.), Biological Control in Agricultural IPM Systems. Academic Press, Orlando, FL, pp. 283-302.

Kaya, H. K., 1990. Soil ecology. In: R. Gaugler, H. K. Kaya, (Eds.), Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, FL, pp. 93-115.

Kaya, H. K., Gaugler, R., 1993. Entomopathogenic nematodes. Annu. Rev. Entomol. 38**,** 181-206.

Kaya, H. K., Koppenhöfer, A. M., 1996. Effects of microbial and other antagonistic organism and competition on entomopathogenic nematodes. Biocontrol Sci. Technol. 6**,** 357-371.

Kim, H. S., Jung, M. H., Ahn, S., Lee, C. W., Kim, S. N., Okk, J. H., 2002. Structure elucidation of a new cyclic hexadepsipeptide from *Beauveria felina*. J. Antibiot. 55**,** 598-601.

Klein, M. G., 1990. Efficacy against soil-inhabiting insect pests. In: R. Gaugler, H. K. Kaya, (Eds.), Entomopathogenic Nematodes in Biological Control. CRC press, Boca Raton, FL, pp. 195-214.

Klein, M. G., Georgis, R., 1992. Persistence of control of Japanese beetle (Coleoptera: Scarabaeidae) larvae with steinernematid and heterorhabditid nematodes. J. Econ. Entomol. 85**,** 727-730.

Kobayasi, Y., 1941. The genus *Cordyceps* and its allies. Science Reports. Tokyo Bunrika Daigaku. Section B. 5**,** 53-260.

Koppenhöfer, A. M., 2007. Integrated pest management of white grubs. In: M. Pessarakli, (Ed.), Handbook of Turfgrass Management and Physiology. CRC Press, Boca Raton, FL, pp. 315-328.

Koppenhöfer, A. M., Baur, M. E., Stock, S. P., Choo, H. Y., Chinnasri, B., Kaya, H. K., 1997. Survival of entomopathogenic nematodes within host cadaverss in dry soil. Appl. Soil Ecol. 6**,** 231-240.

Koppenhöfer, A. M., Brown, I. M., Gaugler, R., Grewal, P. S., Kaya, H. K., Klein, M. G., 2000a. Synergism of entomopathogenic nematodes and imidacloprid against white grubs: greenhouse and field evaluation. Biol. Control. 19**,** 245-251.

Koppenhöfer, A. M., Choo, H. Y., Kaya, H. K., Lee, D. W., Gelernter, W. D., 1999. Increased field and greenhouse efficacy against scarab grubs with a combination of an entomopathogenic nematode and *Bacillus thuringiensis*. Biol. Control. 14**,** 37-44.

Koppenhöfer, A. M., Cowles, R. S., Cowles, E. A., Fuzy, E. M., Baumgartner, L., 2002. Comparison of neonicotinoid insecticides as synergists for entomopathogenic nematodes. Biol. Control. 24**,** 90-97.

Koppenhöfer, A. M., Cowles, R. S., Fuzy, E. M., 2003. Effects of turfgrass endophytes (Clavicipitaceae: Ascomycetes) on white grub (Coleoptera: Scarabaeidae) larval development and field populations. Environ. Entomol. 32**,** 895-906.

Koppenhöfer, A. M., Fuzy, E. M., 2003a. Biological and chemical control of the Asiatic garden beetle, *Maladera castanea* (Coleoptera: Scarabaeidae). J. Econ. Entomol. 96**,** 1076–1082.

Koppenhöfer, A. M., Fuzy, E. M., 2003b. *Steinernema scarabaei* for the control of white grubs. Biol. Control. 28**,** 47-59.

Koppenhöfer, A. M., Fuzy, E. M., 2004. Effect of white grub developmental stage on susceptibility to entomopathogenic nematodes. J. Econ. Entomol. 97**,** 1842-1849.

Koppenhöfer, A. M., Fuzy, E. M., 2006. Effect of soil type on infectivity and persistence of the entomopathogenic nematodes *Steinernema scarabaei*, *Steinernema glaseri*, *Heterorhabditis zealandica*, and *Heterorhabditis bacteriophora*. J. Invertebr. Pathol. 92**,** 11-22.

Koppenhöfer, A. M., Fuzy, E. M., 2007. Soil moisture effects on infectivity and persistence of the entomopathogenic nematodes *Steinernema scarabaei*, *Steinernema glaseri*, *Heterorhabditis zealandica*, and *Heterorhabditis bacteriophora*. Appl. Soil Ecol. 35**,** 128-139.

Koppenhöfer, A. M., Fuzy, E. M., Crocker, R., Gelernter, W., Polavarapu, S., 2004. Pathogenicity of *Heterorhabditis bacteriophora*, *Steinernema glaseri*, and *S. scarabaei* (Rhabditida: Heterorhabditidae, Steinernematidae) against 12 white grub species (Coleoptera: Scarabaeidae). Biocontrol Sci. Technol. 14**,** 87-92.

Koppenhöfer, A. M., Grewal, P. S., Fuzy, E. M., 2006. Virulence of the entomopathogenic nematodes *Heterorhabditis bacteriophora*, *Heterorhabditis zealandica*, and *Steinernema scarabaei* against five white grub species (Coleoptera: Scarabaeidae) of economic importance in turfgrass in North America. Biol. Control. 38**,** 397-404.

Koppenhöfer, A. M., Grewal, P. S., Kaya, H. K., 2000b. Synergism of imidacloprid and entomopathogenic nematodes against white grubs: the mechanism. Entomol. Exp. Appl. 94**,** 283-293.

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

Koppenhöfer, A. M., Kaya, H. K., 1998. Synergism of imidacloprid and an entomopathogenic nematode: A novel approach to white grub (Coleoptera: Scarabaeidae) control in turfgrass. J. Econ. Entomol. 91**,** 618-623.

Koppenhöfer, A. M., Kaya, H. K., Taormino, S. P., 1995. Infectivity of entomopathogenic nematodes (Rhabditida: Steinernematidae) at different soil depths and moistures. J. Invertebr. Pathol. 65**,** 193-199.

Koppenhöfer, A. M., Polavarapu, S., Fuzy, E. M., Zhang, A., Ketner, K., Larsen, T., 2005. Mating disruption of Oriental beetle (Coleoptera: Scarabaeidae) in turfgrass using microencapsulated formulations of sex pheromone components. Environ. Entomol. 34**,** 1408-1417.

Koppenhöfer, A. M., Wilson, M., Brown, I., Kaya, H. K., Gaugler, R., 2000c. Biological control agents for white grubs (Coleoptera: Scarabaeidae) in anticipation of the establishment of the Japanese beetle in California. J. Econ. Entomol. 93**,** 71-80.

Krasnoff, S. B., Gibson, D. M., Belofsky, G. N., Gloer, K. B., Gloer, J. B., 1996. New destruxins from the entomopathogenic fungus *Aschersonia* sp. J. Nat. Prod. 59**,** 485-489.

Kung, S.-P., Gaugler, R., Kaya, H. K., 1990a. Influence of soil pH and oxygen on persistence of *Steinernema* spp. J. Nematol. 22**,** 440-445.

Kung, S.-P., Gaugler, R., Kaya, H. K., 1990b. Soil type and entomopathogenic nematode persistence. J. Invertebr. Pathol. 55**,** 401-406.

Lacey, L. A., Unruh, T. R., 1998. Entomopathogenic nematodes for control of codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae): Effect of nematode species, concentration, temperature, and humidity. Biol. Control. 13**,** 190-197.

Laird, M., Lacey, L. A., Davidson, E. W., 1990. Safety of Microbial Insecticides. CRC Press, Boca Raton, FL.

Latch, C. C. M., Falloon, R. E., 1976. Studies on the use of *Metarhizium anisopliae* to control *Oryctes rhinoceros*. Entomophaga. 21**,** 39-48.

Lengyel, K., Lang, E., Fodor, A., Szállás, E., Schumann, P., Stackebrandt, E., 2005. Description of four novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus budapestensis* sp. nov., *Xenorhabdus ehlersii* sp. nov., *Xenorhabdus innexi* sp. nov., and *Xenorhabdus szentirmaii* sp. nov. Syst. Appl. Microbiol. 28**,** 115–122.

Lewis, E. E., Clarke, D. J., 2012. Nematode parasites and entomopathogens. In: F. Vega, H. K. Kaya, (Eds.), Insect Pathology. Elsevier, Amsterdam, The Netherlands, pp. 395–424.

Lingg, A. J., Donaldson, M. D., 1981. Biotic and abiotic factors affecting stability of *Beauveria bassiana* conidia in soil. J. Invertebr. Pathol. 38**,** 191-200.

Liu, C. M., Huang, S. S., Tzeng, Y. M., 2004. Analysis of destruxins produced from *Metarhizium anisopliae* by capillary electrophoresis. J. Chromatogr. Sci. 42**,** 140-144.

Liu, J., Berry, R. E., 1996. *Heterorhabditis marelatus* n. sp. (Rhabditida: Heterorhabditidae) from Oregon. J. Invertebr. Pathol. 67**,** 48-54.

Mathieson, J., 1949. *Cordyceps aphodii*, a new species, on pasture cockchafer grubs. Trans. Br. Mycol. Soc. 32**,** 113-136.

McCoy, C. W., Samson, R. A., Boucias, D. G., 1988. Entomogenous fungi. In: C. M. Ignoffo, (Ed.), CRC Handbook of Natural Pesticides. Microbial Insecticides, Part A. Entomogenous Protozoa and Fungi, Volume 5. CRC Press, Boca Raton, FL, pp. 151-236.

McEwen, F. L., 1963. *Cordyceps* infections. In: E. A. Steinhaus, (Ed.), Insect Pathology. Academic Press, New York, pp. 205-227.

Milner, R. J., Ecological considerations on the use of *Metarhizium* for control of soil-dwelling pests. In: L. N. Robertson, P. G. Allsopp, Eds.), Proceedings of a Soil-Invertebrate Workshop. QDPI Indooroopilly, Queensland, Australia, 1989, pp. 10-13.

Milner, R. J., 1992. The selection of strains of *Metarhizium anisopliae* for control of Australian sugar-cane white grubs. In: T. A. Jackson, T. R. Glare, (Eds.), Use of Pathogens in Scarab Pest Management Intercept, Andover, UK, pp. 209-215.

Milner, R. J., Lozano, L. B., Driver, F., Hunter, D., 2003. A comparative study of two Mexican isolates with an Australian isolate of *Metarhizium anisopliae* var. *acridum* - strain characterisation, temperature profile and virulence for wingless grasshopper, *Phaulacridium vittatum*. BioControl. 48**,** 335-348.

Milner, R. J., Lutton, G. G., *Metarhizium anisopliae*: survival of conidia in soil. Proceedings of the 1st International Colloquium of Invertebrate Pathology, Kingston, Canada, 1976, pp. 428-429.

Mohi-ud-din, S., Zaki, F. A., Munshi, N. A., Jan, A., Wani, R. A., 2007. Comparative susceptibility of different grub stages to the local strain of *Beauveria bassiana*. J. Entomol. 4**,** 56-59.

Molyneux, A. S., Bedding, R. A., 1984. Influence of soil texture and moisture on the infectivity of *Heterorhabditis* sp. D1 and *Steinernema glaseri* for larvae of the sheep blowfly, *Lucilia cuprina*. Nematologica. 30**,** 358-365.

Morley-Davies, J., Moore, D., Prior, C., 1995. Screening of *Metarhizium* and *Beauveria* spp. conidia with exposure to simulated sunlight and a range of temperatures. Mycol. Res. 100**,** 31-38.

Müller-Kögler, E., 1965. Pilzkrankheiten bei Insekten. Anwendung zur biologischen Scha¨dlingsbeka¨mpfung und Grundlagen der Insektenmykologie. Berlin: P. Parey. p 444.

Müller-Kögler, E., Stein, W., 1970. Gewachshausversuche mit *Beauveria bassiana* (Bals.) Vuillemin zur Infektion von *Sitona lineatus* L. (Coleopt. Curcul.) im Boden. Zeitschrift für Angewandt Entomologie. 65**,** 59-76.

Müller-Kögler, E., Stein, W., 1976. Gewachshausversuche mit *Metarhizium anisopliae* (Metsch.) Sorok. zur Infektion von *Sitona lineatus* L. im Boden. Pflanzenschutzberichte. 83**,** 96-108.

Nickle, W. R., 1984. Plant and Insect Nematodes. Marcel Dekker, New York.

Nishimatsu, T., Jackson, J. J., 1998. Interaction of insecticides, entomopathogenic nematodes, and larvae of the western corn rootworm (Coleoptera: Chrysomelidae). J. Econ. Entomol. 91**,** 410-418.

Nishimura, Y., Hagiwara, A., Suzuki, T., Yamanaka, S., 1994. *Xenorhabdus japonicus* sp. nov. associated with the nematode *Steinernema kushidai*. World J. Microbiol. Biotechnol. 10**,** 207–210.

Oliveira, D. P., Chaves, B. M., Loures, E. G., 1981. Estudo comparativo da sobrevivencia de *Metarhizium anisopliae* (Metsch.) Sorokin em diferentes tipos de solo. Revista Theobroma. 11**,** 233-239.

Poinar, G. O., Jr., 1975. Entomogenous nematodes. A manual and host list of insect-nematode associations. E.J. Brill, Leiden, 317 pp.

Poinar, G. O., Jr., 1979. Nematodes for Biological Control of Insects. CRC Press, Boca Raton, FL.

Poinar, G. O., Jr., 1989. Non-insect hosts for the entomogenous rhabditoid nematodes *Neoaplectana* (Steinernematidae) and *Heterorhabditis* (Heterorhabditidae). Revue Nématol. 12**,** 423-428.

Poinar, G. O., Jr., 1992. Nematodes associated with Scarabaeidae. In: T. A. Jackson, T. R. Glare, (Eds.), Use of Pathogens in Scarab Pest Management. Intercept, Andover, UK, pp. 93-109.

Potter, D. A., 1998. Managing white grubs in turf. Destructive Turfgrass Insects: Biology, Diagnosis and Control. Ann Arbor Press, Chelsea, MI, pp. 129-164.

Potter, D. A., Braman, S. K., 1991. Ecology and management of turfgrass insects. Annu. Rev. Entomol. 36**,** 383-406.

Pye, A., Burman, M., 1981. *Neoaplectana carpocapsae*: nematode accumulations in chemical and bacterial gradients. Exp. Parasitol. 51**,** 13-20.

Rath, A. C., 1992. *Metarhizium anisopliae* for control of the Tasmanian pasture scarab *Adoryphorus couloni*. In: T. A. Jackson, T. R. Glare, (Eds.), Use of Pathogens in Scarab Pest Management. Intercept, Andover, pp. 217-227.

Rath, A. C., Anderson, G. C., Worledge, D., Koen, T. B., 1995. The effect of low temperatures on the virulence of *Metarhizium anisopliae* (DAT F-001) to the subterranean scarab, *Adoryphorus couloni*. J. Invertebr. Pathol. 65**,** 186-192.

Robert, P. H., Riba, G., 1989. Toxic and repulsive effect of spray *per os* and systemic application of destruxin E to aphids. Mycopathologia. 108**,** 170-183.

Roberts, D. W., 1981. Toxins of entomopathogenic fungi In: H. D. Burges, (Ed.), Microbial Control of Pests and Plant Diseases 1970-1980. Academic Press, London, pp. 441-464.

Roberts, D. W., Campbell, A. S., 1977. Stability of entomopathogenic fungi. Misc. Publ. Entomol. Soc. Am. 10**,** 19-76.

Roberts, D. W., Hajek, A. E., 1992. Entomopathogenic fungi as bioinsecticides. In: G. F. Leatham, (Ed.), Frontiers in Industrial Mycology. Chapman and Hall, New York, pp. 144-159.

Saik, J. E., Lacey, L. A., Lacey, C. M., 1990. Safety of microbial insecticides to vertebrates - domestic animals and wildlife. In: M. Laird, et al., (Eds.), Safety of Microbial Insecticides. CRC Press, Boca Raton, FL, pp. 115-132.

Samson, R. A., Evans, H. C., Latgé, J. P., 1988. Atlas of Entomopathogenic Fungi. Springer-Verlag, Berlin, Germany.

Samuels, K. D. Z., Pinnock, D. E., Control of Australian soldier fly and scarabaeid larvae with *Metarhizium anisopliae*. Abstracts of the 11th Annual Meeting of the Society for Invertebrate Pathology, University of California, San Diego, 1988, pp. 32.

Sandhu, S. S., Kinghorn, J. R., Rajak, R. C., Unkles, S. E., 2001. Transformation system of *Beauveria bassiana* and *Metarhizium anisopliae* using nitrate reductase gene of *Aspergillus nidulans*. Indian J. Exp. Biol. 39**,** 650-653.

Scheepmaker, J. W. A., Geels, F. P., Smits, P. H., van Griensven, L. J. L. D., 1998. Influence of *Steinernema feltiae* and diflubenzuron on yield and economics of the cultivated mushroom *Agaricus bisporus* in Dutch mushroom culture. Biocontrol Sci. Technol. 8**,** 269-275.

Schmidt, J., All, J. N., 1979. Attraction of *Neoaplectana carpocapsae* (Nematoda: Steinernematidae) to common excretory products of insects. Environ. Entomol. 8**,** 55-61.

Schroeder, W. J., Sieburth, P. J., 1997. Impact of surfactants on control of the root weevil *Diaprepes abbreviatus* larvae with *Steinernema riobravis*. J. Nematol. 29**,** 216-219.

Screen, S. E., Hu, G., St. Leger, R. J., 2001. Transformants of *Metarhizium anisopliae* sf. *anisopliae* overexpressing chitinase from *Metarhizium anisopliae* sf. *acridum* show early induction of native chitinase but are not altered in pathogenicity to *Manduca sexta*. J. Invertebr. Pathol. 78**,** 260-266.

Shah, P. A., Goettel, M. S., 1999. Directory of Microbial Control Products and Services. Society for Invertebrate Pathology, Gainesville.

Shamseldean, M. M. M., Ismail, A. A., 1997. Effect of the nematode *Heterorhabditis bacteriophora* and the bacterium *Bacillus thuringiensis* as integreated biocontrol agents of the black cutworm. Anzeiger fuer Schädlingskunde Pflanzenschutz Umweltschutz. 70**,** 77-79.

Shapiro-Ilan, D. I., Gouge, D. H., Koppenhöfer, A. M., 2002. Factors affecting commercial success: case studies in cotton, turf and citrus. In: R. Gaugler, (Ed.), Entomopathogenic Nematology. CABI Publishing, Wallingford, UK, pp. 333–355.

Shapiro, D. I., Tylka, G. L., Lewis, L. C., 1996. Effects of fertilizers on virulence of *Steinernema carpocapsae*. Appl. Soil Ecol. 3**,** 27-34.

Shetlar, D. J., 1994. Turfgrass Insect and mite management. In: T. L. Watschke, et al., (Eds.), Managing Turfgrass Pests. CRC Press, Boca Raton, FL, pp. 280-302.

Siegel, J. P., 1997. Testing the pathogenicity and infectivity of entomopathogens to mammals. In: L. A. Lacey, (Ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, CA, pp. 325-336.

Siegel, J. P., Shadduck, J. A., 1990. Safety of microbial insecticides to vertebrates - humans. In: M. Laird, et al., (Eds.), Safety of Microbial Insecticides. CRC Press, Boca Raton, FL, pp. 101-114.

Simard, L., Bélair, G., Brodeur, J., 2001. Susceptibility of the European chafer (Coleoptera: Scarabaeidae) to entomopathogenic nematodes (Rhabditida: Steinernematidae, Heterorhabditidae). J. Nematol. 33**,** 297–301.

Somvanshi, V. S., Lang, E., Ganguly, S., Swiderski, J., Saxena, A. K., Stackebrandt, E., 2006. A novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus indica* sp. nov., symbiotically associated with entomopathogenic nematode *Steinernema thermophilum* Ganguly and Singh, 2000. Syst. Appl. Microbiol. 29**,** 519–525.

St. Leger, R., Screen, S., 2001. Prospects for strain improvement of fungal pathogens of insects and weeds. In: T. M. Butt, et al., (Eds.), Fungi as Biocontrol Agents. CABI Publishing, Wallingford, pp. 219-237.

St. Leger, R. J., 2001. Notification of intent to release a transgenic strain of *Metarhizium anisopliae* (document submitted by the University of Maryland as required by FIFRA) www.epa.gov/pesticides/biopesticides/other-docs/release_notification.htm (16/08/01).

St. Leger, R. J., Frank, D. C., Roberts, D. W., Staples, R. C., 1992. Molecular cloning and regulatory analysis of the cuticle-degrading-protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. Eur. J. Biochem. 204**,** 991-1001.

St. Leger, R. J., Joshi, L., Bidochka, M. J., Roberts, D. W., 1996. Construction of an improved mycoinsecticide overexpressing a toxic protease. Proc. Natl. Acad. Sci. USA. 93**,** 6349-6354.

St. Leger, R. J., Shimizu, S., Joshi, L., Bidochka, M. J., Roberts, D. W., 1995. Co-transformation of *Metarhizium anisopliae* by electroporation or using the gene gun to produce stable GUS transformants. FEMS Microbiol. Lett. 131**,** 289-294.

Stark, J. D., 1996. Entomopathogenic nematodes (Rhabditida: Steinernematidae): Toxicity of neem. J. Econ. Entomol. 89**,** 68-73.

Steinhaus, E. A., 1975. Disease in a Minor Chord. Ohio State University Press, Columbus, OH.

Stock, S. P., Koppenhöfer, A. M., 2003. *Steinernema scarabaei* n. sp. (Rhabditida: Steinernematidae), a natural pathogen of scarab beetle larvae (Coleoptera: Scarabaeidae) from New Jersey, USA. Nematology. 5**,** 191-204.

Strasser, H., Vey, A., Butt, T., 2000. Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium*, *Tolypocladium* and *Beauveria* species? Biocontrol Sci. Technol. 10**,** 717-735.

Tailliez, P., Pagès, S., Ginibre, N., Boemare, N., 2006. New insight into diversity in the genus *Xenorhabdus*, including the description of ten novel species. Int. J. Syst. Evol. Microbiol. 56**,** 2805–2818.

Tanada, Y., Kaya, H. K., 1993. Insect Pathology. Academic Press, San Diego, CA.

Thomas, G. M., Poinar, G. O., Jr, 1979. *Xenorhabdus* gen. nov., a genus of entomopathogenic nematophilic bacteria of the family Enterobacteriaceae. Int. J. Syst. Bacteriol. 29**,** 352–360.

Thomsen, L., Eilenberg, J., 2000. Time-concentration mortality of *Pieris brassicae* (Lepidoptera: Pieridae) and *Agrotis segetum* (Lepidoptera: Noctuidae) larvae from different destruxins. Environ. Entomol. 29**,** 1041-1047.

Townsend, M. L., Johnson, D. T., Steinkraus, D. C., 1998. Laboratory studies of the interactions of environmental conditions on the susceptibility of green June beetle (Coleoptera: Scarabaeidae) grubs to entomopathogenic nematodes. J. Entomol. Sci. 33**,** 40-48.

Upadhyay, R. K., 2003. Advances in Microbial Control of Insect Pests. Kluwer Academic Press, New York.

Vanninen, I., Depletion of endogenous lipid reserves in *Steinernema feltiae* and *Heterorhabditis bacteriophora* and effect on infectivity. Proceedings of the 5th International Colloquium on Invertebrate Pathology and Microbial Control, Adelaide, Australia, 1990, pp. 232.

Veen, K. H., 1968. Recherches sur la maladie, due à *Metarhizium anisopliae* chez le criquet pèlerin. Mededelingen. Landbuowhogeschool Wageningen, Nederland. 68**,** 1-77.

Vestergaard, S., Cherry, A., Keller, S., Goettel, M., 2003. Hyphomycete fungi as microbial control agents. In: H. M. T. Hokkanen, A. E. Hajek, (Eds.), Environmental Impacts of Microbial Insecticides. Kluwer, Dordrecht, pp. 35-62.

Vey, A., Hoagland, R. E., Butt, T. M., 2001. Toxic metabolites of fungal biocontrol agents. In: T. Butt, et al., (Eds.), Fungal Biocontrol Agents. CABI Publishing, Wallingford, pp. 311-346.

Vittum, P. J., Villani, M. G., Tashiro, H., 1999. Turfgrass Insects of the United States and Canada. Cornell University Press, New York.

Walstad, J. D., Anderson, R. F., Stambaugh, W. J., 1970. Effects of environmental conditions on two species of muscardine fungi (*Beauveria bassiana* and *Metarhizium anisopliae*) J. Invertebr. Pathol. 16**,** 221-226.

Walston, A. T., Held, D. W., Mason, N. R., Potter, D. A., 2001. Absence of interaction between endophytic perennial ryegrass and susceptibility of Japanese beetle (Coleoptera: Scarabaeidae) grubs to *Paenibacillus popilliae* Dutky. J. Entomol. Sci. 36**,** 105-108.

Wang, Y., Campbell, J., Gaugler, R., 1995. Infection of entomopathogenic nematodes *Steinernema glaseri* and *Heterorhabditis bacteriophora* against *Popillia japonica* larvae. J. Invertebr. Pathol. 66**,** 178-184.

Wawrzynski, R. P., Ascerno, M. E., 1998. Mass trapping for Japanese beetle (Coleoptera: Scarabaeidae) suppression in isolated areas. J. Arboric. 24**,** 303-307.

Welling, M., Nachtigall, G., Zimmermann, G., 1994. *Metarhizium* spp. isolates from Madagascar: Morphology and effect of high temperature on growth and infectivity to the migratory locust, *Locusta migratoria*. Entomophaga. 39**,** 351-361.

Wharton, D. A., Surry, M. R., 1994. Cold tolerance mechanisms of the infective larvae of the insect parasitic nematode *Heterorhabditis zealandica* Poinar. CryoLetters. 15**,** 353-360.

Wouts, W. M., 1991. *Steinernema* (*Neoaplectana*) and *Heterorhabditis* species. In: W. R. Nickle, (Ed.), Manual of Agricultural Nematology. Marcel Dekker, New York, pp. 855-897.

Wraight, S. P., Carruthers, R. I., 1999. Production, delivery, and use of mycoinsecticides for control of insect pests of field crops. In: F. R. Hall, J. J. Menn, (Eds.), Biopesticides: Use and Delivery. Humana Press, Totowa, NJ, pp. 233-269.

Wraight, S. P., Inglis, G. D., Goettel, M. S., 2007. Fungi. In: L. A. Lacey, H. K. Kaya, (Eds.), Field Manual of Techniques in Invertebrate Pathology. Springer, Dordrecht, The Netherlands, pp. 223-248.

Wraight, S. P., Jackson, M. A., de Lock, S. L., 2001. Production, stabilization and formulation of fungal biocontrol agents. In: T. M. Butt, et al., (Eds.), Fungi as Biocontrol Agents. CABI Publishing, Wallingford, pp. 253-287.

Zimmermann, G., 1986. Insect pathogenic fungi as pest control agents. In: J. M. Franz, (Ed.), Progress in Zoology: Biological Plant and Health Protection. Gustav Fischer Verlag, New York, pp. 217-231.

Zimmermann, G., 1992. Use of the fungus, *Beauveria brongniartii*, for the control of European cockchafers, *Melolontha* spp., in Europe. In: T. A. Jackson, T. R. Glare, (Eds.), Use of Pathogens in Scarab Pest Management. Intercept, Andover, UK, pp. 199-208.

Zimmermann, G., 2007. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. Biocontrol Sci. Technol. 17**,** 879-920.

CHAPTER 2

Bioassay of Selected Entomopathogenic Nematodes and Entomopathogenic Fungi against 3rd Instar Masked Chafer White Grubs, *Cyclocephala* spp. (Coleoptera: Scarabaeidae)

Abstract

Virulence was tested on four entomopathogenic nematode (EPN) species (two *Heterorhabditis* spp. and two *Steinernema* spp.) against 3rd instar masked chafer white grubs, *Cyclocephala* spp. at two rates. *Heterorhabditis bacteriophora* was most virulent, followed by *H. megidis*; the two *Steinernema* spp. were less virulent, and *S. riobrave* did not cause any infection in the insects. The rates of 2.5 and 5 billion IJs/ha were equally effective. In addition, bioassays were conducted on overwintered and pre-diapausing 3rd instar *Cyclocephala* spp. with *H. bacteriophora* and *H. megidis*, and two entomopathogenic fungi (EPF), *Metarhizium anisopliae* strain F52 and *Beauveria bassiana* strain GHA. The LC_{25} and LC_{50} were 0.58 and 2.1 billion IJs/ha for *H. bacteriophora*; 0.48 and 2.2 billion IJs/ha for *H. megidis*, respectively, against pre-diapausing 3rd instars 2 wk after treatment. No LC values were obtained for either EPN species against overwintered grubs. The LC_{25} and LC_{50} were $10^{7.2}$ conidia/g soil and $10^{7.5}$conidia/g soil, respectively, for pre-diapausing 3rd instar masked chafers exposed to *M. anisopliae* for 4 wk. Bioassay tests on *M. anisopliae* against overwintered masked chafer grubs in spring 2009 and 2010 did not show any rate-dependent response. Similarly, *B. bassiana* was not rate-dependent against overwintered *Cyclocephala* spp.

Keywords: white grub, *Cyclocephala* spp., entomopathogenic nematode, entomopathogenic fungus, bioassay

Introduction

White grubs (Coleoptera: Scarabaeidae) are the most widespread and destructive turf pests in America. In Virginia, Japanese beetle, *Popillia japonica* Newman, and masked chafers, *Cyclocephala* spp., are the most important grub species in turf (Dimock, 2004). Our field survey results indicate that, in Blacksburg and Bristol, VA, masked chafers have surpassed the Japanese beetle to become the major grub species (Wu et al. unpublished data). Currently, the most prevalent method to control masked chafers is an application of a conventional insecticide, e.g. imidacloprid, on a preventative basis in early summer to target grubs at their earlier stages. If the attempts fail, the third instars would very likely cause an outbreak late in the season. In addition, impacts on the environment, human health and natural enemies from the long term use of conventional insecticides, have increased public awareness for a more bio-rational approach in managing white grubs.

Entomopathogenic nematodes and entomopathogenic fungi appear to be environmentally safe IPM compatible alternatives to conventional insecticides. Several EPN species, i.e. *Heterorhabditis bacteriophora* Poinar, *H. zealandica* Poinar, *Steinernema scarabaei* Stock & Koppenhöfer, and *S. glaseri* (Steiner) may have potential against southern (*C. lurida* Bland) and northern (*C. borealis* Arrow) masked chafer grubs, although they are generally less susceptible than *P. japonica* (Koppenhöfer et al., 2004; Koppenhöfer et al., 2006). In the current study, four EPN species were tested for their virulence against 3rd instar masked chafers, and the most pathogenic species were selected for laboratory bioassays, along with two EPF species, *Metarhizium anisopliae* (Metschn.) Sorokin, and *Beauveria bassiana* (Balsamo) Vuillemin.

Materials and Methods

1. Bioassay of EPN on 3rd instar masked chafers

1.1. Selection for virulence of EPN species against 3rd instar masked chafers in Fall 2009

Four EPN species (*H. bacteriophora*, *H. megidis* Poinar, Jackson & Klein, *S. feltiae* Filipjev, and *S. riobrave* Cabanillas, Poinar & Raulston) obtained from Becker Underwood Co. (Ames, Iowa) were used to test their virulence against 3rd instar masked chafers. Two rates (2.5 and 5 billion infective juveniles (IJs)/ha) were used for each species. Third-instar masked chafers were collected from Blacksburg Country Club (Blacksburg, VA). Grubs were treated individually in 30 ml cups filled with 25 g soil (soil surface area: 12.15 cm^2). All the treatments were replicated three times, with 10 grubs per replicate. Grubs that did not enter soil within 12 h were replaced.

Before application, nematodes were transferred from 4 ^oC to room temperature for 12 h for acclimation. A solution of 20 µl was used to count the number of nematode IJs, which was repeated five times to confirm IJs concentration. In each cup, soil was moistened with 3.5 ml distilled water first. Then 1 ml IJs solution was pipetted onto the soil surface by drenching, which was followed by another 1 ml water to wash nematodes into the soil. The final soil moisture was adjusted to 22% (v/w). Soil applied was loamy sand soil, comprising of 84.1% sand, 9.5% silt and 6.4% clay, with 4.0% organic matter and pH of 4.9. Before use, soil was covered with a black cloth to be solarized in the greenhouse for one month.

Cups were placed in trays and covered with moist towel paper to maintain the soil moisture. Approximately 0.3 g perennial ryegrass (*Lolium perenne* L.) seeds were added on the soil surface to germinate and provide food for grubs. Grub mortality was assessed weekly for 4 wk. Dead grubs were transferred to individual petri dishes (dia. 10 cm) lined with moist filter paper for further observation. If grubs were killed by EPNs, nematodes seen moving inside grub carcasses under microscope observation

(6x) in 2-3 wk, finally exiting grub carcasses as IJs in another 1-2 wk. The experiment was conducted in an incubator at the diurnal cycle of LD 13:11 (light 13: dark 11), 20 ^{o}C, with an average R. H. of 90%.

1.2. Bioassay of selected EPN species against overwintered masked chafer grubs in Spring 2010

EPN species selected from 1.1 were used for bioassays against overwintered masked chafer grubs. Five rates 0, 25, 50, 100, 200 and 400 IJs/cup with a cup surface area of 12.15 cm^2, equal to 0.21, 0.42, 0.83, 1.67, 3.33 billion IJs/ha, were used for each species, in addition to an untreated water control. All treatments were replicated three times, with 10 grubs per replicate. Soil was moistened with 2.5 ml water first before the introduction of white grubs. Grubs that did not enter soil within 4 h were replaced. Nematode IJs were pipetted to the soil surface in 0.5 ml water suspension, followed by 1.5 ml distilled water to wash the IJs into soil pore spaces. The final moisture was adjusted to 18% (v/w). Cups were placed in trays and covered with lids to maintain the moisture. There were 15 tiny holes punctured on each lid with a thumb tack to allow for air exchange. IJs solutions were shaken well before piping each time. Before application, IJs were counted per 100 µl solution, and five samples were taken to determine IJs concentration. Experimental conditions and soil used were the same as in Experiment 1.1. Data were collected weekly for 6 wk.

1.3. Bioassays of selected EPN species against pre-diapausing 3rd instar masked chafers in Fall 2010

Bioassays of selected EPN species were conducted to confirm the LC values against pre-diapausing 3rd instar masked chafers in fall. Experimental procedures and rates applied were similar to Experiment 1.2, except that the EPNs used in this bioassay were cultured in full grown larvae of the greater wax moth, *Galleria mellonella* (L.), and were collected from the White trap (Kaya and Stock, 1997) within 5 d. Data were collected weekly for 4 wk.

2. Bioassay of *M. anisopliae* strain F-52 on 3rd instar masked chafers

2.1. Bioassay of *M. anisopliae* F-52 against overwintered masked chafer grubs in Spring 2009

Overwintered masked chafer grubs were collected from Tazewell Country Club (Pounding Mill, VA). *M. anisopliae* strain F-52 (4.0×10^9 conidia/ml) in oil emulsifiable formulation was obtained from Novozymes Biologicals, Inc. (Salem, VA). Four rates (10^4, 10^5, 10^6 and 10^7 conidia/g soil, equal to 0.515, 5.15, 51.5 and 515 L/ha) plus an untreated water control were used, in four replicates with 15 grubs per replicate. *M. anisopliae* was applied in 3.9 ml solution by drenching soil surface, and the final moisture was adjusted to 15% (v/w). Cups were placed in trays, and covered with lids to maintain the moisture. There were 15 holes punctured on each lid with a thumb tack to allow for air exchange. This experiment was carried out at room temperature of 24.3 ^{o}C and R.H. of 49.8% on average. Laboratory planted grass roots were added as food source. Mortality was assessed 4 wk after treatment. Soil was a

loamy sand texture, comprising of 77.5% sand, 16.5% silt, 6.0% clay, and 1.9% organic matter with a pH of 5.5.

2.2. Bioassay of *M. anisopliae* F-52 against pre-diapausing 3rd instar masked chafers in Fall 2009

Metarhizium anisopliae F-52 used was in an oil emulsifiable formulation, containing 5.0×10^9 conidia/ml, with a germination rate of 60%. Pre-diapausing 3rd instar masked chafers were collected from the Blacksburg Country Club. Five rates (10^4, 10^5, 10^6, 10^7 and 10^8 conidia/g soil, equal to 0.412, 4.12, 41.2, 412 and 4120 L/ha) plus an untreated water control were used, in three replicates with 15 grubs per replicate. *M. anisopliae* was applied to the soil surface by drenching. The final soil moisture was adjusted to 22% (v/w). Experimental procedures, conditions and soil used were the same as in Experiment 1.1. Mortality was assessed every 2 wk for 8 wk. Dead grubs were transferred to individual petri dishes (dia. 10 cm) lined with moist filter paper to observe growth of *M. anisopliae*. *M. anisopliae*-infected grubs first harden and were covered with white mycelium, which sporulated and turned green eventually.

2.3. Bioassay of *M. anisopliae* against overwintered masked chafer grubs in Spring 2010

Metarhizium anisopliae used was the same material as in Experiment 2.2. Six rates (0.8, 1.6, 3.2, 6.4, 12.8, 25.6 L/ha) either close to or included in the range recommended for field use plus a water control were used. All treatments were replicated three times, with 10 grubs per replicate. Experimental conditions, procedures and soil used were the same as in Experiment 1.2. *M. anisopliae* was delivered to the soil surface by drenching. The final soil moisture was adjusted to 18% (v/w). Mortality was assessed every other wk for 10 wk. Similar to Experiment 2.2, dead grubs were transferred to individual petri dishes to observe growth of *M. anisopliae.*

3. Bioassay of *B. bassiana* against overwintered masked chafer grubs in Spring 2010

Beauveria bassiana strain GHA used is a commercially available product branded as BotaniGard ES from Laverlam International Co. (Butte, MT), labeled for white grub control in turf. *B. bassiana* was formulated in emulsifiable suspension, containing 2.1×10^{10} viable spores/ml. The field recommended rate of BotaniGard ES is 6.4 to 25.6 L/ha for turf use. Seven rates (1.6, 3.2, 6.4, 12.8, 25.6, 51.2, 102.4 L/ha) plus an untreated water control were used. Four replicates and 10 grubs per replicate were used for each treatment. The final soil moisture was adjusted to 18% v/w. Experimental conditions, procedures and soil used were the same as in Experiment 1.2. Grub mortality was assessed every other wk for 10 wk. Surface of *B. bassiana*-infected grubs were covered with white muscardine.

Data Analysis

Software JMP 10.0 (SAS, Cary, NC) was used to test for significant differences among treatments at α=0.05. Software Polo Plus version 1.0 was used for probit analysis of bioassay results.

Results

1. Bioassay of EPN on 3rd instar masked chafers

1.1. Selection for virulence of EPN species against 3rd instar masked chafers in Fall 2009

Two-way ANOVA was used to test effects of EPN treatments, time after treatment, and their interactions. There were significant differences among various EPN species in both mortality (F =23.45, d.f.= 8, *P*<0.0001) and infection rate (F=68.18, d.f.= 8, *P*<0.0001) (Fig. 1 & 2). Among EPN species applied, *H. bacteriophora* was most effective, causing 80% grub mortality on average in 4 wk for both rates; followed by *H. megidis*. The two *Steinernema* spp. were less virulent; *S. riobrave* did not cause any infection in the insects. In addition, the rate of 5 billion IJs/ha had no significantly different effect from 2.5 billion IJs/ha in either grub mortality or infection rate within 4 wk, although in the 1st wk the higher rate caused slightly lower mortality and infection than the lower rate (Fig. 1 & 2).

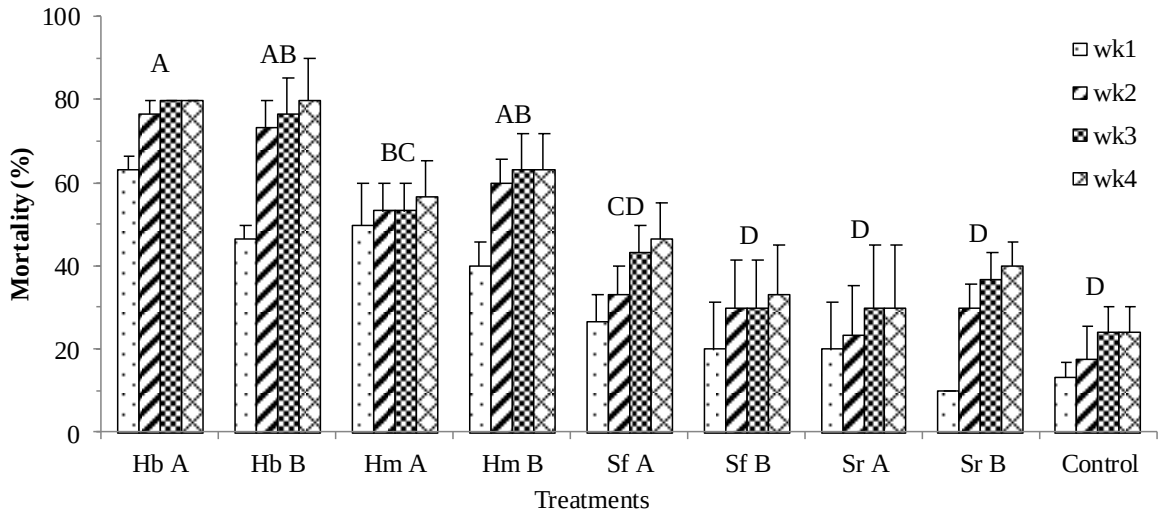

Fig. 1. Mortality of 3rd instar masked chafers treated with nematode *Heterorhabditis bacteriophora* (Hb), *H. megidis* (Hm), *Steinernema feltiae* (Sf) and *S. riobrave* (Sr) at two rates, A (2.5 billion IJs/ha) and B (5 billion IJs/ha) within 4 wk. Different letters indicate significant differences between treatments (Tukey's HSD, α=0.05).

A significant increase in grub mortality over time after treatment (F=8.73, d.f.=3, *P*<0.0001) occurred from 1st wk to 2nd wk; after that no significant changes were found. EPN infection rate did not change significantly within 4 wk (infection: F = 2.53, d.f. = 3, *P* = 0.064), with most infection occurring in the 1st wk. No significant interactions were found between EPN species and time after treatment in either

mortality (F=0.31, d.f.=24, *P*=0.999) or infection rate (F=0.5, d.f.=24, *P*=0.971). Also, in treatments with *H. bacteriophora* or *H. megidis*, most deaths were caused by EPN applied (Fig. 1 & 2). The results indicate that *H. bacteriophora* and *H. megidis* are rapid and efficient killers of 3rd instar masked chafers.

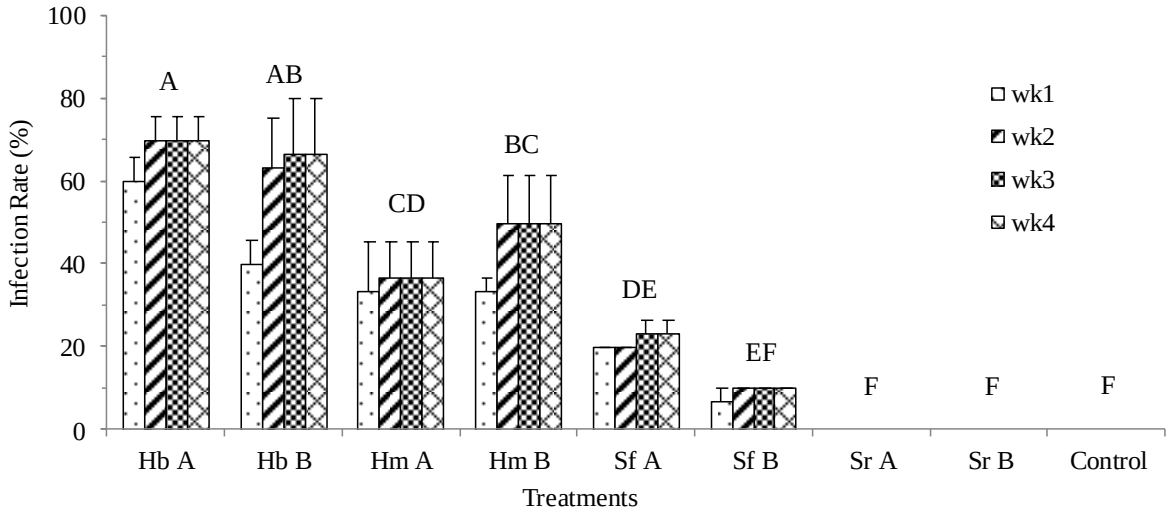

Fig. 2. Infection rate of nematode *Heterorhabditis bacteriophora* (Hb), *H. megidis* (Hm), *Steinernema feltiae* (Sf) and *S. riobrave* (Sr) at two rates, A (2.5 billion IJs/ha) and B (5 billion IJs/ha) on 3rd instar masked chafers within 4 wk. Different letters indicate significant differences between treatments (Tukey's HSD, α=0.05).

1.2. Bioassay of selected EPN species against overwintered masked chafer grubs in Spring 2010

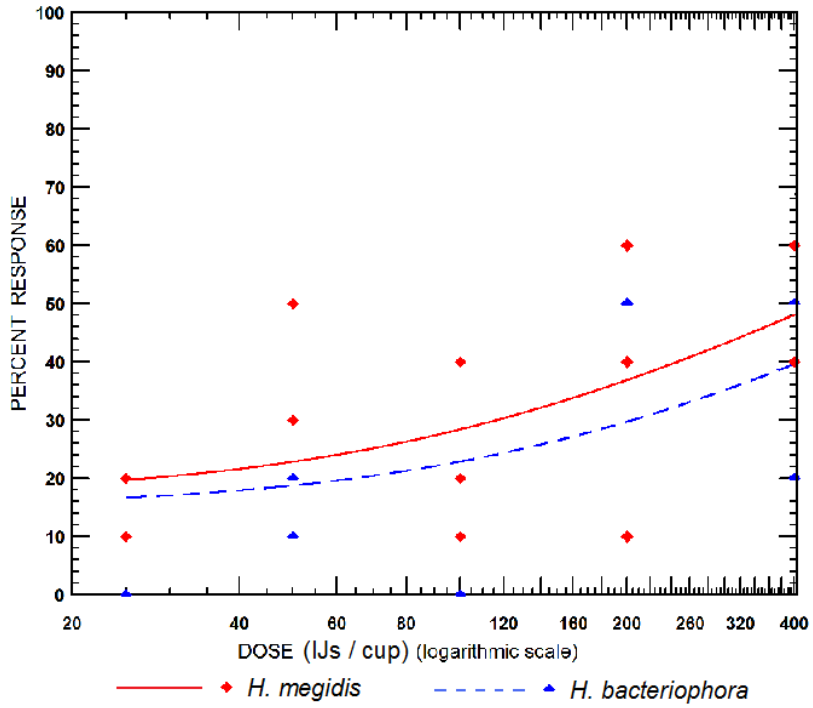

Fig. 3. Mortality of overwintered masked chafer grubs 2 wk after treatment with *Heterorhabditis bacteriophora* and *H. megidis* in different doses.

Heterorhabditis bacteriophora and *H. megidis* were selected for bioassay on overwintered masked chafer grubs. No LC values were obtained for either EPN species 2 wk after treatment (Fig. 3).

1.3. Bioassay of selected EPN species against pre-diapausing 3rd instar masked chafers in Fall 2010

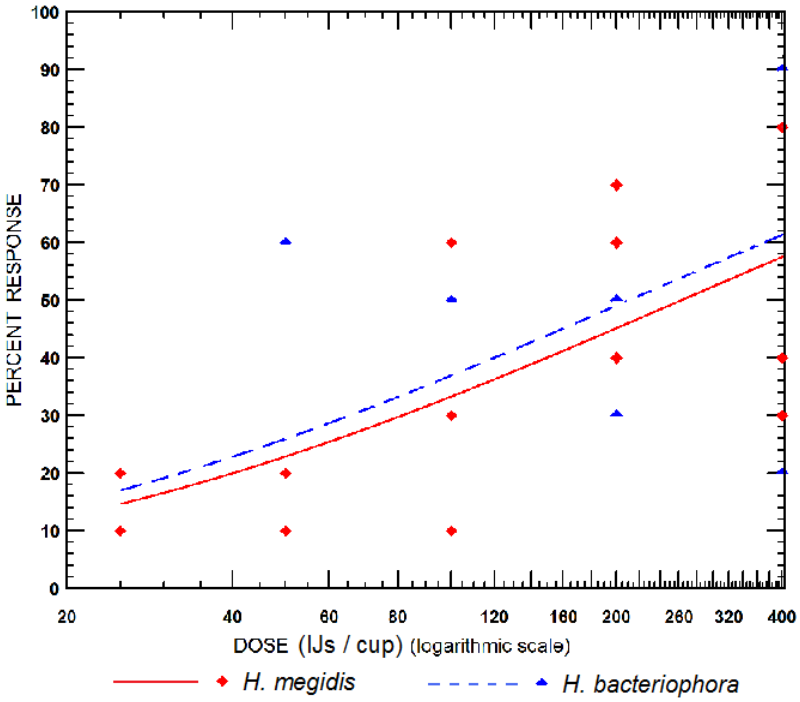

Fig. 4. Mortality of pre-diapausing 3rd instar masked chafers 2 wk after treatment with *Heterorhabditis bacteriophora* and *H. megidis* in different doses.

Analyzing data from each replicate, the LC_{25} and LC_{50} values were 58 (95% fiducial limits: 12-107) IJs/cup (0.48 billion IJs/ha) and 267 (95% fiducial limits: 142-1672) IJs/cup (2.2 billion IJs/ha) for *H. megidis*, respectively, with a slope (±SEM) of 1.02 (±0.26). There were no calculated LC values for *H. bacteriophora*.

When data from three replicates were combined for analyses, the LC_{25} and LC_{50} values were 69 (95% fiducial limits: 18-125) IJs/cup (equal to 0.58 billion IJs/ha) and 251 (95% fiducial limits: 140-647) IJs/cup (equal to 2.1 billion IJs/ha) for *H. bacteriophora*, respectively, with a slope (±SEM) of 1.2 (±0.34). No LC values were obtained for *H. megidis* (Fig. 4).

2. Bioassay of *M. anisopliae* strain F-52 on 3rd instar masked chafers

2.1. Bioassay of *M. anisopliae* F-52 against overwintered masked chafer grubs in Spring 2009

No LC values were obtained 4 wk after *M. anisopliae* application against overwintered grubs (Fig. 5).

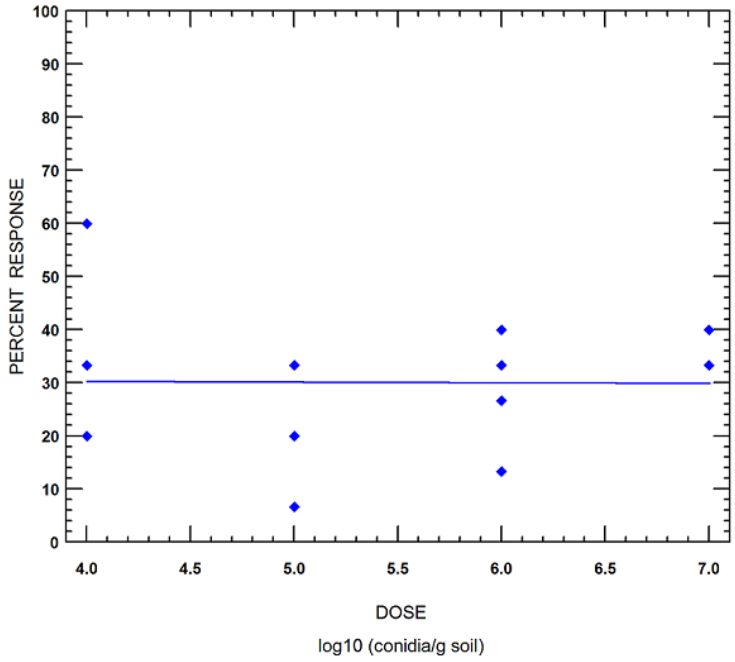

Fig. 5. Mortality of overwintered masked chafer grubs 4 wk after treatment with *Metarhizium anisopliae* in different doses.

2.2. Bioassay of *M. anisopliae* F-52 against pre-diapausing 3rd instar masked chafers in Fall 2009

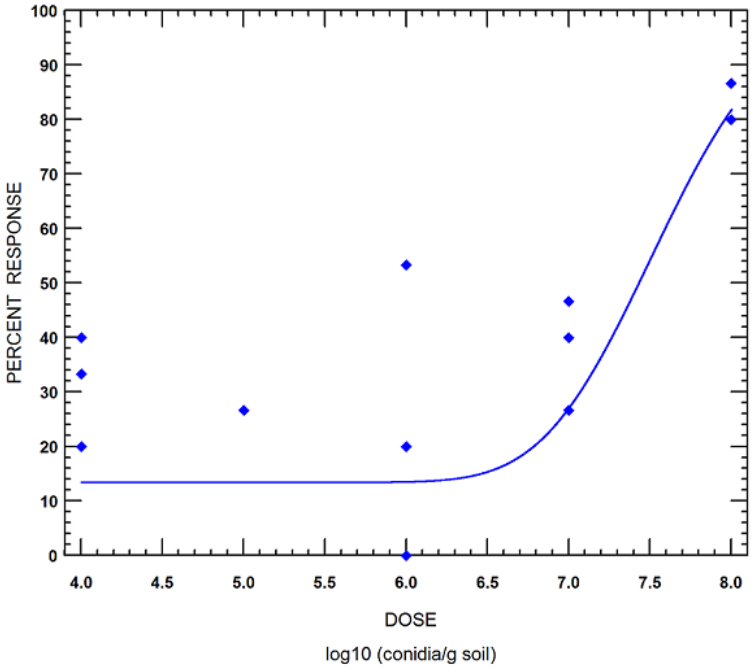

Fig. 6. Mortality of pre-diapausing 3rd instar masked chafers 4 wk after treatment with *Metarhizium anisopliae* in different doses.

Mortality of the pre-diapausing 3rd instar grubs was rate-dependent. The LC_{25} and LC_{50} values were $10^{7.2}$ (95% fiducial limits: $10^{6.1}$-$10^{7.5}$) conidia/g soil and $10^{7.5}$ (95% fiducial limits: $10^{6.9}$-$10^{7.9}$) conidia/g soil 4 wk after application, respectively, with a slope (±SEM) of 31.16 (±8.47) (Fig. 6).

2.3. Bioassay of *M. anisopliae* against overwintered masked chafer grubs in Spring 2010

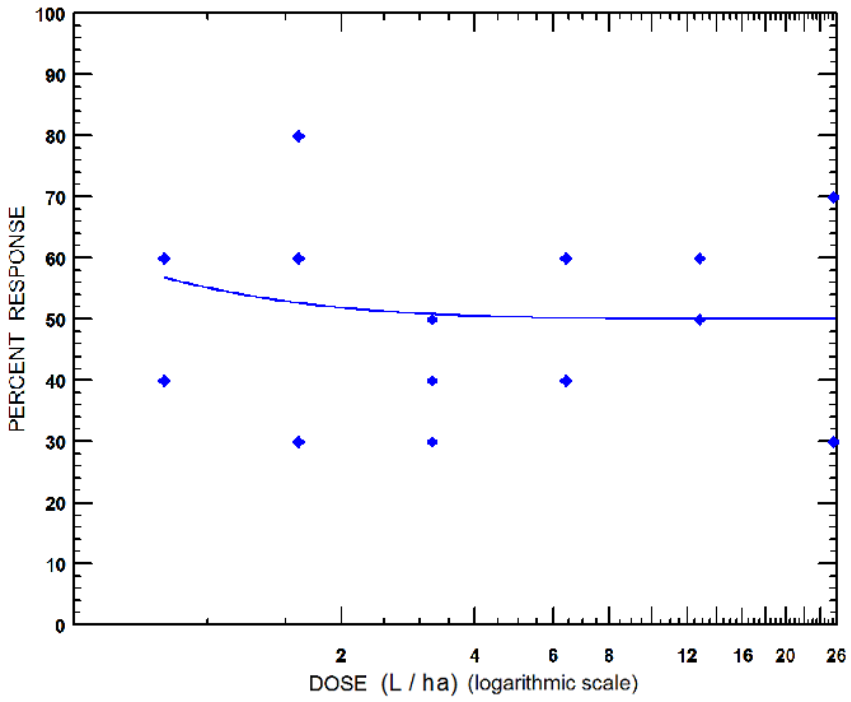

Fig. 7. Mortality of overwintered masked chafer grubs 10 wk after treatment with *Metarhizium anisopliae* in different doses.

No rate-dependent responses showed for *M. anisopliae* against overwintered masked chafer grubs exposed to the six field recommended rates for 10 wk (Fig. 7).

3. Bioassay of *B. bassiana* against overwintered masked chafer grubs in Spring 2010

No LC values were obtained for *B. bassiana* against overwintered masked chafer grubs exposed to the seven field recommended rates for 6 or 10 wk (Fig. 8).

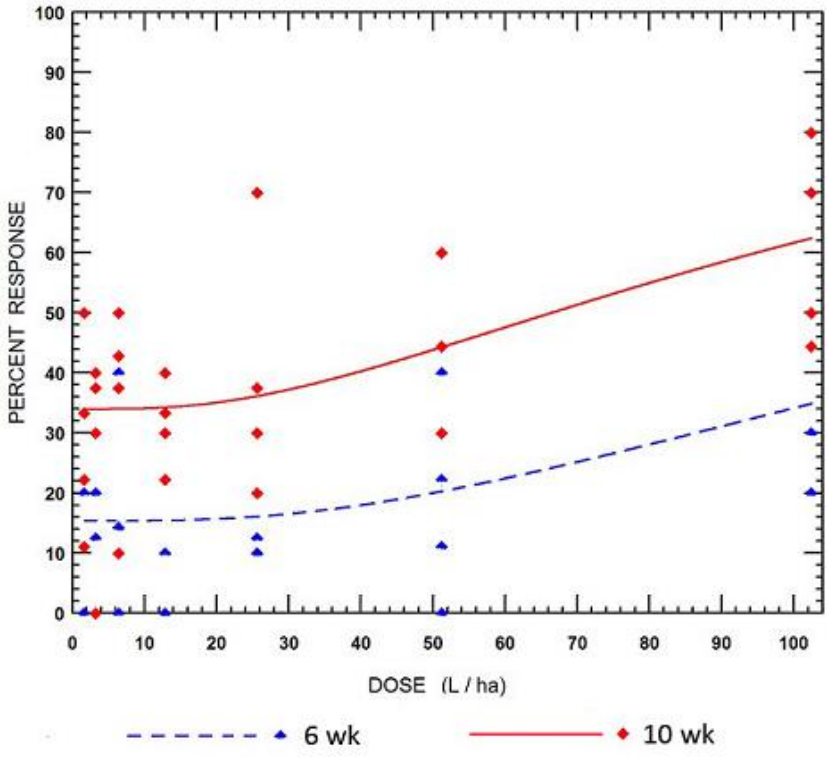

Fig. 8. Mortality of overwintered masked chafer grubs 6 or 10 wk after treatment with *Beauveria bassiana* in different doses.

Discussion

Among the four EPN species, *H. bacteriophora* was most virulent against 3rd instar masked chafers, followed by *H. megidis*; *Steinernema* spp. were less virulent, and *S. riobrave* did not cause any infection in the insects. In addition, the rates of 2.5 and 5 billion IJs/ha were equally effective within 4 wk under laboratory conditions. However, in the 1st wk the higher rate caused slightly lower grub mortality and infection than the lower rate, indicating intra-specific competition that affected overall efficacy at the high rate (Fig. 1 & 2). *H. bacteriophora* and *H. megidis* were selected for bioassay tests. No LC values were given to either EPN species 2 wk after treatment on overwintered grubs (Fig. 3). For the test on pre-diapausing 3rd instar grubs in fall, when data from three replicates were combined for analyses, the LC_{25} and LC_{50} values were 69 IJs/cup (equal to 0.58 billion IJs/ha) and 251 IJs/cup (equal to 2.1 billion IJs/ha) for *H. bacteriophora*, respectively; no LC values were obtained for *H. megidis*. When data were analyzed by replicate, the LC_{25} and LC_{50} were 58 IJs/cup (0.48 billion IJs/ha) and 267 IJs/cup (2.2 billion IJs/ha) for *H. megidis*, respectively; there were no calculated LC values for *H. bacteriophora* (Fig. 4).

Bioassay tests on the efficacy of *M. anisopliae* against overwintered masked chafer grubs in spring 2009 did not show a rate-dependent response (Fig. 5). However, mortality of the pre-diapausing 3rd instar grubs was rate-dependent. The LC_{25} and LC_{50} values were $10^{7.2}$ conidia/g soil and $10^{7.5}$conidia/g soil 4 wk after application, respectively (Fig. 6). The treatment with the highest rate, 10^8 conidia/g soil (equal to 4120 L/ha with 5.0×10^9 conidia/ml), had only one grub infected with *M. anisopliae*, although the mean mortality was 84.5% 4 wk after treatment. A possible explanation is that the oil emulsifiable formulation might have affected grub fitness and facilitated the speed of kill. Unfortunately, a blank control with the formulation alone was not included in the experiment. As some of these rates were too high for field application, a third bioassay was carried out with six rates close to or within the range for field recommended use. There were no rate-dependent responses for *M. anisopliae* against overwintered masked chafer grubs for 10 wk (Fig. 7). Similarly, the bioassay of *B. bassiana* also did not show any rate-dependent response for 6 or 10 wk (Fig. 8).

Overall, both EPN and EPF species tested caused some mortality of masked chafer grubs, but some grubs remained unaffected. When used in combination, this may present an opportunity for either agent to suppress the grub populations.

References

Dimock, W. J., Spatial factors affecting white grub presence and abundance in golf course turf. Virginia Polytechnic Institute and State University, Blacksburg, VA, 2004.

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

Koppenhöfer, A. M., Fuzy, E. M., Crocker, R., Gelernter, W., Polavarapu, S., 2004. Pathogenicity of *Heterorhabditis bacteriophora*, *Steinernema glaseri*, and *S. scarabaei* (Rhabditida: Heterorhabditidae, Steinernematidae) against 12 white grub species (Coleoptera: Scarabaeidae). Biocontrol Sci. Technol. 14**,** 87-92.

Koppenhöfer, A. M., Grewal, P. S., Fuzy, E. M., 2006. Virulence of the entomopathogenic nematodes *Heterorhabditis bacteriophora, Heterorhabditis zealandica,* and *Steinernema scarabaei* against five white grub species (Coleoptera: Scarabaeidae) of economic importance in turfgrass in North America. Biol. Control. 38**,** 397-404.

CHAPTER 3

Interaction between Entomopathogenic Nematodes and Entomopathogenic Fungi against Masked Chafer White Grubs, Cyclocephala spp. (Coleoptera: Scarabaeidae), at Different Developmental Stages

Abstract

Interactions between an entomopathogenic nematode (EPN) (*Heterorhabditis bacteriophora* or *H. megidis*) and an entomopathogenic fungus (EPF) (*Beauveria bassiana* or *Metarhizium anisopliae*) were evaluated for efficacy against masked chafer white grubs, *Cyclocephala* spp., at the 2nd instar, pre-wintering 3rd instar and overwintered stages. Nematodes and fungi were either applied alone or in combination, with nematodes added to the fungi at different time intervals. *H. bacteriophora* caused significantly higher mortality than *H. megidis* when applied alone, or in combination with *B. bassiana* ES at 0 or 2 wk, but not for the 4 wk interval. When applied alone, *B. bassiana* ES & WP and *M. anisopliae* did not have a significant effect in reducing grub populations at various stages, except for the use of *M. anisopliae* against overwintered grubs. Significantly greater control occurred from the combination of a nematode and a fungus compared with a fungus alone, but not compared with a nematode alone. Additive interactions were found between EPN and EPF in most treatments against grubs at various stages, with the exception of a few observations that showed synergism or antagonism. The combined effect did not differ significantly for nematode and fungal applications made simultaneously or at different time intervals. Temperature had a significant impact on the performance of *H. bacteriophora* and *M. anisopliae*, and grub mortality was enhanced significantly as temperature increased. EPF had no significant impact on the EPN infection and IJs production in grub carcasses. Overall, no significant difference was observed among developmental stages for the interaction of EPN and EPF on the grub. The low efficacy of fungal application might account for the lack of a stronger interactive effect in *Cyclocephala* spp.

Keywords: white grub, *Cyclocephala* spp., entomopathogenic nematode, entomopathogenic fungus, interaction

Introduction

In Virginia, Japanese beetle (*Popillia japonica* Newman) and masked chafers (*Cyclocephala* spp.) are the most important white grub species in turfgrass (Dimock, 2004). Field survey results indicate that in recent years masked chafer grubs, mainly southern masked chafer (*C. lurida* Bland), account for more than 80% of the white grub numbers in some areas (i.e. Blacksburg, Bristol) in the state (Wu et al. unpublished data). Thus, successful control of *Cyclocephala* spp. may play a significant role in turfgrass

management in Virginia. Being native species in the U.S., the *Cyclocephala* spp. did not receive as much attention from researchers and growers as the Japanese beetle. Also, options in the management of *Cyclocephala* spp. are more limited.

Currently, the most prevalent method to control masked chafers is the preventative application of insecticides, e.g. imidacloprid or clothianidin, which can effectively control damage in the earliest life stages, while efficacy declines as grubs grow larger. Grubs in the 3rd-instar stage are most difficult to control, and can cause severe damage from late August to September. Several older carbamate and organophosphate insecticides, e.g. trichlorfon, carbaryl, diazinon, are effective for curative treatment of grubs. Given their impact on the environment, however, many of these products are no longer registered by Environmental Protection Agency (EPA) for grub control in turf. The impact from long-term use of insecticides on the environment and on non-target organisms has increased public awareness for a more bio-rational approach in managing white grubs.

EPN and EPF appear to be environmentally safe and integrated pest management (IPM) compatible alternatives to conventional insecticides. Several EPN species, i.e. *Heterorhabditis bacteriophora* Poinar, *H. zealandica* Poinar, *Steinernema scarabaei* Stock & Koppenhöfer, *S. glaseri* (Steiner) may have potential against southern (*C. lurida*) and northern (*C. borealis* Arrow) masked chafer grubs, although they are generally less susceptible than *P. japonica* (Koppenhöfer et al., 2004; Koppenhöfer et al., 2006). However, their field efficacies are often inconsistent and unsatisfactory (Georgis and Gaugler, 1991; Klein, 1993), due to various biotic (Kaya, 2002; Kaya and Koppenhöfer, 1996) and abiotic factors (Glazer, 2002; Kaya, 1990; Smits, 1996). EPF green muscardine fungus *Metarhizium anisopliae* (Metschn.) Sorokin, and white muscardine fungus *Beauveria bassiana* (Balsamo) Vuillemin are pathogenic to white grubs (Glare, 1992), but their performances are also constrained by environmental conditions (Wraight et al., 2007).

The combined application of EPN and EPF may achieve a higher level of control against white grubs. When applied together, they may act independently and cause an additive effect, or interact with each other in a synergistic or antagonistic way (Jaques and Morris, 1981). Additive or synergistic interaction has been reported in the combined application of *H. bacteriophora* and *M. anisopliae* isolate MM against the barley chafer grub, *Coptognathus curtipennis* Faimaire (Anbesse et al., 2008). In addition, such effects were also found in the interaction between *M. anisopliae* CLO 53 and *H. megidis* Poinar, Jackson & Klein or *S. glaseri* against the 3rd instar *Hoplia philanthus* Füessly under laboratory and greenhouse conditions (Ansari et al., 2004), and between *M. anisopliae* CLO 53 and *H. bacteriophora* in the field (Ansari et al., 2006). Choo et al. (2002) also reported that the combination of *S. carpocapsae* (Weiser)

with *B. brongniartii* (Saccardo) Petch resulted in a significant increase in the mortality of *Exomala orientalis* (Waterhouse) grub over the application of the fungus alone.

The objective of this study was to evaluate the interaction of an EPF and an EPN against masked chafer grubs at different developmental stages. It was hypothesized that additive or synergistic interactions would be achieved from the combined use of the two types of pathogens and thus improve the overall efficacy in the management of these pests.

Materials and Methods

1. Interaction of EPN and EPF against pre-wintering 3rd instar masked chafers

1.1. Interaction of *B. bassiana* ES and *H. bacteriophora* / *H. megidis* on 3rd instar masked chafers in Fall 2010

Grubs treated in this experiment were 3rd instar masked chafers collected from Tazewell County Country Club (Pounding Mill, VA) in fall 2010. EPF *B. bassiana* strain GHA in emulsifiable formulation (*B. bassiana* ES containing 2.1×10^{10} viable spores/ml), and EPN *H. bacteriophora* and *H. megidis* (Becker Underwood) were used at the rate of 25.6 L/ha, 2.1 and 2.1 billion infective juveniles (IJs)/ha (LC 50 rate of *H. bacteriophora* in 2 wk), respectively. *B. bassiana* ES is a commercially available product labeled as BotaniGard ES from Laverlam International Co. (Butte, MT). Different time intervals were used to determine the best duration between applying the two agents. Initially the experiment was designed to apply both EPN species at 0, 2, 4, or 6 wk interval, but *H. bacteriophora* was not available at 6 wk and was thus applied 8 wk after the fungus instead. There were 12 treatments in total: untreated water control, *B. bassiana* ES alone, *H. megidis* alone, *H. megidis* plus *B. bassiana* ES with *H. megidis* applied 0, 2, 4, or 6 wk after *B. bassiana* ES, *H. bacteriophora* alone, and *H. bacteriophora* plus *B. bassiana* ES with *H. bacteriophora* applied 0, 2, 4, or 8 wk after *B. bassiana* ES. Three replicates and 15 grubs per replicate were used for all treatments. The experiment was carried out in an incubator at 20 ^{o}C and LD 13:11 (light 13h: dark 11h, the photoperiod in mid-April and early-September when 3rd instars were active in Blacksburg, VA).

Grubs were treated individually in 30 ml cups filled with 25g soil (soil surface area: 12.15 cm^2). Approximately 0.3g perennial ryegrass (*Lolium perenne* L.) seeds were added on the soil surface and germinated to provide food for grubs. Soil in individual cups was moistened with 2.5 ml water before introduction of grubs. Grubs that did not enter the soil within 4 h were replaced. Cups were placed in trays, and covered with lids to maintain the moisture. There were 15 holes punctured on each lid with a thumb tack to allow for air exchange. The final soil moisture was adjusted at 18 % v/w. When EPNs were applied 2, 4 or 6 wk after *B. bassiana* ES, they were delivered in 0.5 ml water suspension, followed

with 0.5 ml distilled water to wash the EPNs into soil pores. Other treatments received 1 ml water to compensate for water loss from seed germination, turfgrass growth and water loss through the lid holes by evaporation. Soil used was a loamy sand texture composed of 84.1% sand, 9.5% silt and 6.4% clay, with 4.0% organic matter and a pH of 4.9. Before application, the soil was covered with a clear plastic cloth for solarizing in the greenhouse for at least one month in summer.

All EPNs applied in this and following experiments were cultured with full grown wax moth larvae, *Galleria mellonella* (L.), and collected with White traps (Kaya and Stock, 1997) within 5 d. Results were assessed weekly for treatments with EPNs applied, and every other wk for other treatments for 8 wk after the start of the experiment. Dead grubs were transferred to petri dishes (dia. 60 mm) for further observation. EPN-infected grubs would turn brown or red. Under the microscope (6x), nematodes were seen moving inside the grub carcass within 2-3 wk after death. Grubs being confirmed with EPN infection were transferred to the White trap individually. IJs exiting from the dead grubs were collected. Collected IJs were transferred to a flask and adjusted to the volume of 50 ml. An amount of 100 µl was taken from the well-shaken suspension to count the number of IJs. At least five samples were taken to count the amount of IJs exiting from each grub carcass.

1.2. Interaction of *M. anisopliae* / *B. bassiana* ES and *H. bacteriophora* on 3rd instar masked chafers in Fall 2011

Grubs collected from Virginia Tech Turfgrass Research Center (Blacksburg, VA) around September 01, 2011 were surface-sterilized with 0.5% sodium hydrochloride to remove external contaminants (Lacey and Brooks, 1997). They were then stored individually in egg cells filled with solarized soil for at least 3 d before use. *B. bassiana* ES and *H. bacteriophora* were used at the same rates as in 1.1. *M. anisopliae* strain F-52 (5.5×10^9 conidia/g) in oil emulsifiable formulation, used at the rate of 6.4 L/ha, was provided by Dr. Jarrod E. Leland [Novozymes Biologicals, Inc. (Salem, VA)] for this and subsequent experiments.

Treatments included: water control; *M. anisopliae* alone; *B. bassiana* ES alone; *H. bacteriophora* applied alone 0, 2, or 4 wk after the start of treatment; *M. anisopliae* plus *H. bacteriophora* with *H. bacteriophora* added 0, 2, or 4 wk after *M. anisopliae*; *B. bassiana* ES plus *H. bacteriophora* with *H. bacteriophora* added 0, 2, or 4 wk after *B. bassiana* ES. Experimental procedures were similar to Experiment 1.1. Soil used was a sandy loam texture, and was composed of 73.8% sand, 18.1% silt, 8.1% clay and 1.2% organic matter with a pH of 5.8.

1.3. Effect of temperature on the efficacy of *M. anisopliae* and *H. bacteriophora* on 3rd instars

Grubs treated in this experiment were 3rd instar pre-wintering masked chafers collected from the same site as in 1.2, and were surface-sterilized before use. Three temperatures (12, 20, 28 ^{o}C) and four treatments (control, *M. anisopliae* alone, *H. bacteriophora* alone, *M. anisopliae* plus *H. bacteriophora* applied simultaneously) were used. The rates of *M. anisopliae* and *H. bacteriophora* used were the same as in Experiment 1.2. The experiment was carried out in 3 incubators at the photoperiod of LD 13:11, and R.H. of 84.3%, 84.9%, and 76% for 12, 20 and 28 ^{o}C, respectively. Other experimental procedures and soil used were the same as in Experiment 1.2.

2. Interaction of *M. anisopliae* / *B. bassiana* and *H. bacteriophora* on overwintered masked chafers

Overwintered 3rd instar masked chafers were collected from the Virginia Tech Turfgrass Research Center, and were surface-sterilized before use. *H. bacteriophora*, *B. bassiana* ES and *M. anisopliae* were applied at the same rates as in 1.2. *B. bassiana* strain GHA in wettable powder (WP) formulation (BotaniGard WP from Laverlam International Co., containing 4.4×10^{10} viable spores/g) was used at the rate of 12.3 kg/ha. Treatments included: water control; *M. anisopliae* alone; *B. bassiana* ES alone; *B. bassiana* WP alone; *H. bacteriophora* alone 0 or 4 wk after the start of treatment; *M. anisopliae* plus *H. bacteriophora* applied simultaneously; *H. bacteriophora* added 4 wk after *M. anisopliae*; *H. bacteriophora* added 4 wk after *B. bassiana* ES; *H. bacteriophora* added 4 wk after *B. bassiana* WP. Fungi in various treatments were applied at the same time. There were three replicates per treatment, and 15 grubs per replicate. Soil used was the same as in Experiment 1.1; experimental conditions and procedures were similar to 1.2. Grub mortality was checked every other wk for an 8 wk period.

3. Interaction of *M. anisopliae* / *B. bassiana* and *H. bacteriophora* on 2nd instar masked chafers

Grubs used in this experiment were 2nd instar masked chafers collected from the Virginia Tech golf course (Blacksburg, VA) using a sod cutter, and were surface-sterilized before use. *H. bacteriophora* IJs were applied at 0.58 billion IJs/ha, which was the LC25 rate for 2 wk observation from the bioassay on 3rd instar masked chafers. *M. anisopliae* and *B. bassiana* ES were used at the same rates as in Experiment 1.2. The fungus and nematode were either applied alone, or in combination with the nematode added 0, 2, or 4 wk after the fungus. Treatments, experimental conditions and procedures were the same as in 1.2. Soil used was a sandy loam texture, and was composed of 74.1% sand, 19.6% silt, 6.3% clay and 3.2% organic matter with a pH of 5.2.

Data Analysis

A X^2 test was used to test the interaction of EPF and EPN. Before analysis, all mortality data were corrected for control mortality (Abbott, 1925). The method of determining the type of interaction

(synergistic, additive, or antagonistic) was first described by Finney (1964), and then modified by McVay et al. (1977). The expected additive proportional mortality M_E for the EPN / EPF combinations was calculated by $M_E = M_N + M_F (1-M_N)$, where M_N and M_F are the observed proportional mortalities relatively caused by EPN and EPF alone. A X^2 test was then carried out using the formula $X^2 = (M_{NF} - M_E)^2 / M_E$, where M_{NF} represents the observed mortality for the EPN / EPF combination. The calculated value from the X^2 test was then compared with the X^2 table value for 1 degree of freedom. If calculated values are greater than the table value ($X^2_{1, 0.05} = 3.84$), non-additive effects, e.g. synergistic or antagonistic, could be suspected between the two agents (Finney, 1964). If the differences $M_{NF} - M_E = D$ had a positive value, the interaction was considered synergistic, and an antagonistic interaction was considered if D was negative.

In addition, software JMP 10.0 (SAS, Cary, NC) was used to test for significant differences among treatments with Analysis of Variance (ANOVA), except that the *t*-test was used for analyzing the impact of *M. anisopliae* on *H. bacteriophora* IJs production and infectivity in overwintered grubs. Differences between means were considered significant when $P \leq 0.05$. Tukey's HSD was used for multiple comparisons between treatments at $\alpha=0.05$.

Results

1. Interaction of EPN and EPF against pre-wintering 3rd instar masked chafers

1.1. Interaction of *B. bassiana* ES and *H. bacteriophora* / *H. megidis* on 3rd instar masked chafers in Fall 2010

Additive interactions were found in most treatments and observations between *H. megidis* or *H. bacteriophora* and *B. bassiana* ES, and synergism was shown in the 6 wk observation for *H. bacteriophora* combined with the fungus and applied simultaneously. However, an antagonistic effect was detected at 4 wk observation after nematode application for *H. megidis* added 2 or 4 wk after *B. bassiana* ES, in both 2 and 4 wk observations for *H. megidis* added 6 wk after the fungus, and for the treatment with *H. bacteriophora* added to *B. bassiana* ES at 4 wk interval (Table 1). The antagonism in later weeks was probably due to the high mortality in the untreated control (Fig. 2), as data used for analysis on interaction type were corrected for control mortality.

In addition, there was no significant difference in the production of infective juveniles from grub carcasses among treatments with *H. megidis* applied alone or in combination with *B. bassiana* ES (P=0.892) (Table 2). This indicated that adding *B. bassiana* ES did not significantly affect the IJs production. Also, no significant difference was detected in the rate of infection with *H. megidis*

(*P*=0.075), although infection in the treatment with *H. megidis* added 4 wk after *B. bassiana* ES tended to be lower than in other treatments.

Table 1. Interaction of *Beauveria bassiana* ES and *Heterorhabditis bacteriophora* or *H. megidis* against pre-wintering 3rd instar masked chafers under 20 °C and LD 13:11.

EPN species	Intervals[a]	Wk[b]	Observed mortality (%)[c]	Expected Mortality (%)[d]	X^2	Type of Interaction
H. megidis	0 wk	2	39.1	40.6	0.06	additive
H. megidis	0 wk	4	40.0	41.7	0.07	additive
H. megidis	0 wk	6	40.0	35.6	0.56	additive
H. megidis	2 wk	2	31.4	36.0	0.59	additive
H. megidis	2 wk	4	26.7	42	5.60	antagonistic
H. megidis	4 wk	2	26.7	36.3	2.57	additive
H. megidis	4 wk	4	12.5	57.3	34.98	antagonistic
H. megidis	6 wk	2	25.0	53.1	14.85	antagonistic
H. megidis	6 wk	4	16.7	53.0	24.90	antagonistic
H. bacteriophora	0 wk	2	51.2	58.2	0.84	additive
H. bacteriophora	0 wk	4	74.3	69.4	0.34	additive
H. bacteriophora	0 wk	6	83.3	64.6	5.46	synergistic
H. bacteriophora	2 wk	2	45.7	55.0	1.57	additive
H. bacteriophora	2 wk	4	73.3	69.6	0.20	additive
H. bacteriophora	4 wk	2	13.3	55.2	31.78	antagonistic
H. bacteriophora	4 wk	4	37.5	77.6	20.71	antagonistic
H. bacteriophora	8 wk	2	78.3	63.7	3.35	additive
H. bacteriophora	8 wk	4	92.5	84.3	0.80	additive

a. Wk intervals between the application of *B. bassiana* ES and *H. bacteriophora* or *H. megidis*;

b. Wk after the application of nematodes;

c. Observed mortality was corrected for control mortality with Abbott's formula (Abbott 1925);

d. Expected mortality $M_E = M_N + M_F (1-M_N)$, where M_N and M_F are the observed proportional mortalities relatively caused by nematodes and *B. bassiana* ES alone.

Table 2. Rate of infection with *Heterorhabditis megidis* and IJs production in 3rd instar masked chafers with or without adding *Beauveria bassiana* ES in 12 wk after fungal application under 20 °C and LD 13:11.

Treatment [a]	No. grubs	Mean IJs (±SEM) [b]	Infection (±SEM)% [c]
H. megidis alone	9	22537 (±4783)	22.2 (±8.9)
B. bassiana ES +0 wk+ *H. megidis*	11	16930 (±3610)	33.3 (±3.9)
B. bassiana ES +2 wk+ *H. megidis*	9	18011 (±4418)	28.9 (±2.2)
B. bassiana ES +4 wk+ *H. megidis*	3	11281 (±3480)	8.9 (±2.2)
B. bassiana ES +6 wk+ *H. megidis*	7	18453(±4086)	20(±6.7)

a. *H. megidis* applied alone, or added 0, 2, 4 or 6 wk after *B. bassiana* ES.

b. Data were log transformed for normal distribution before analysis of variance (F=0.28, d.f.=4, *P*=0.892);

c. No significant difference was found in infection rate (F=2.95, d.f.=4, *P*=0.075).

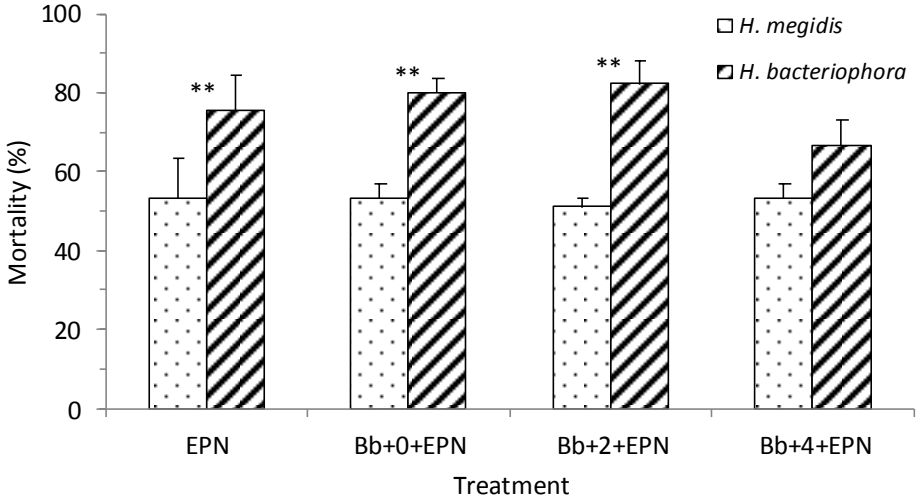

Fig. 1. Mortality of 3rd instar masked chafers within 4 wk after treatment with entomopathogenic nematodes (EPN) (*Heterorhabditis megidis* or *H. bacteriophora*) alone, or added to *Beauveria bassiana* ES at 0 (Bb+0+EPN), 2 (Bb+2+EPN), or 4 wk (Bb+4+EPN) interval (Mean±SEM). ** indicates significant difference between *H. megidis* and *H. bacteriophora* under each treatment (α=0.05).

Efficacy of the two EPN species differed significantly from each other in 4 wk after application (F=28.01, d.f.=1, *P*<0.0001) (Fig. 1). *H. bacteriophora* caused higher mortality than *H. megidis* when they were applied alone (F=6.35, d.f.=1, *P*=0.023), applied with *B. bassiana* ES simultaneously (F=9.15, d.f.=1, *P*=0.008), or added 2 wk after *B. bassiana* ES (F=12.45, d.f.=1, *P*=0.003), but not when they were

added 4 wk after *B. bassiana* ES (F=2.29, d.f.=1, *P*=0.15). There were no significant differences in grub mortality for EPN applied alone, or added 0, 2, or 4 wk after *B. bassiana* ES (F=0.51, d.f.=3, *P*=0.682). This indicates that, compared with EPN alone, adding *B. bassiana* ES to EPN did not significantly improve the control effect for fungal exposure within 8 wk.

A possible explanation might be the low efficacy of *B. bassiana* ES. This was verified by the fact that there was no significant difference in grub mortality between *B. bassiana* ES and the untreated control within 6 wk after treatment, although a difference appeared in the observations made at 8 wk (F=6.0, d.f.=1, *P*=0.022) and 12 wk (F=4.41, d.f.=1, *P*=0.046) after treatment. Overall, *B. bassiana* ES caused higher mortality than control within 12 wk (F=10.8, d.f.=1, *P*=0.003), and grub mortality increased significantly over time (F=64.99, d.f.=5, *P*<0.0001) (two-way ANOVA) (Fig. 2). It suggests that longer time of exposure may be desirable for the fungus to take effect.

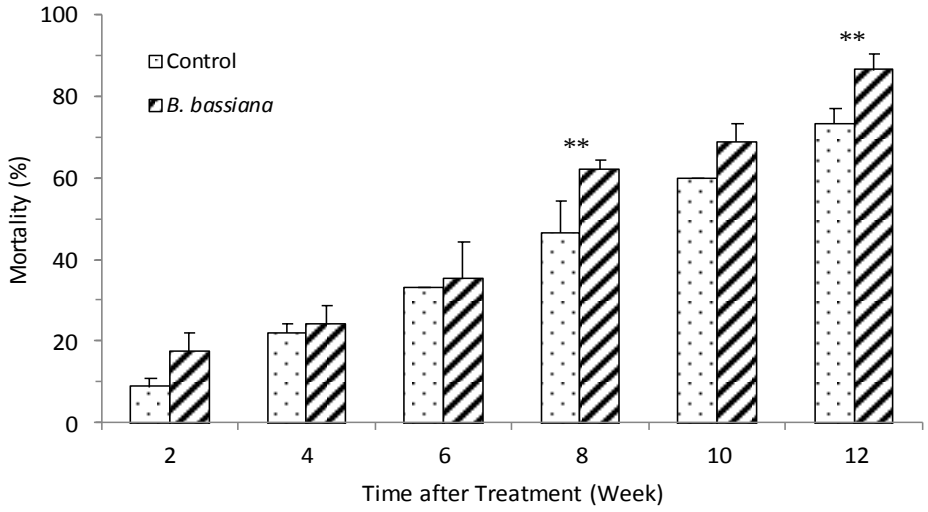

Fig. 2. Mortality of 3rd instar masked chafers in the untreated control and *Beauveria bassiana* ES over 12 wk after treatment. ** indicates significant difference between *B. bassiana* ES and control at each time after treatment (α=0.05).

1.2. Interaction of *M. anisopliae* / *B. bassiana* ES and *H. bacteriophora* on 3rd instar masked chafers in Fall 2011

Additive or synergistic interactions were observed between *M. anisopliae* / *B. bassiana* ES and *H. bacteriophora* when the nematode was added 0, 2, or 4 wk after the fungi (Table 3).

Table 3. Interaction of *Heterorhabditis bacteriophora* and *Metarhizium anisopliae* or *Beauveria bassiana* against pre-wintering 3rd instar masked chafers under 20 °C and LD 13:11.

EPF Species	Intervals[a]	Wk[b]	Observed mortality (%)[c]	Expected Mortality (%)[d]	X^2	Type of Interaction
M. anisopliae	0 wk	2	35.6	37.8	0.13	additive
M. anisopliae	0 wk	4	40.9	42.8	0.09	additive
M. anisopliae	2 wk	2	29.6	23.8	1.41	additive
M. anisopliae	2 wk	4	29.3	27.4	0.13	additive
M. anisopliae	4 wk	2	31.7	16.4	14.39	synergistic
M. anisopliae	4 wk	4	39.0	37.2	0.09	additive
B. bassiana ES	0 wk	2	42.2	34.8	1.58	additive
B. bassiana ES	0 wk	4	43.2	40.0	0.25	additive
B. bassiana ES	2 wk	2	20.5	20.0	0.01	additive
B. bassiana ES	2 wk	4	26.8	29.3	0.21	additive
B. bassiana ES	4 wk	2	22.0	18.6	0.59	additive
B. bassiana ES	4 wk	4	26.8	39.1	3.84	additive

a. Wk intervals between the application of *M. anisopliae* or *B. bassiana* and *H. bacteriophora*;

b. Wk after the application of *H. bacteriophora*;

c. Observed mortality was corrected for control mortality with Abbott's formula (Abbott 1925);

d. Expected mortality $M_E = M_N + M_F (1-M_N)$, where M_F and M_N are the observed proportional mortalities relatively caused by fungi and *H. bacteriophora* alone.

In pre-wintering 3rd instar masked chafers, the effect of EPF was not significantly different from the untreated control when EPF was applied alone, or in combination with *H. bacteriophora* added simultaneously with, or 2, 4 wk after the fungi, except for *B. bassiana* ES combined with the nematode in simultaneous applications (F=3.62, d.f.=4, *P*=0.045 for *B. bassiana* ES; F=2.26, d.f.=4, *P*=0.134 for *M. anisopliae*). Also, for both *B. bassiana* ES and *M. anisopliae*, the effect on grub mortality did not differ significantly with or without adding *H. bacteriophora*. No significant difference was detected between the two fungal species when they were applied alone or in combination with the nematode at various time intervals (*P*>0.05) (Fig. 3).

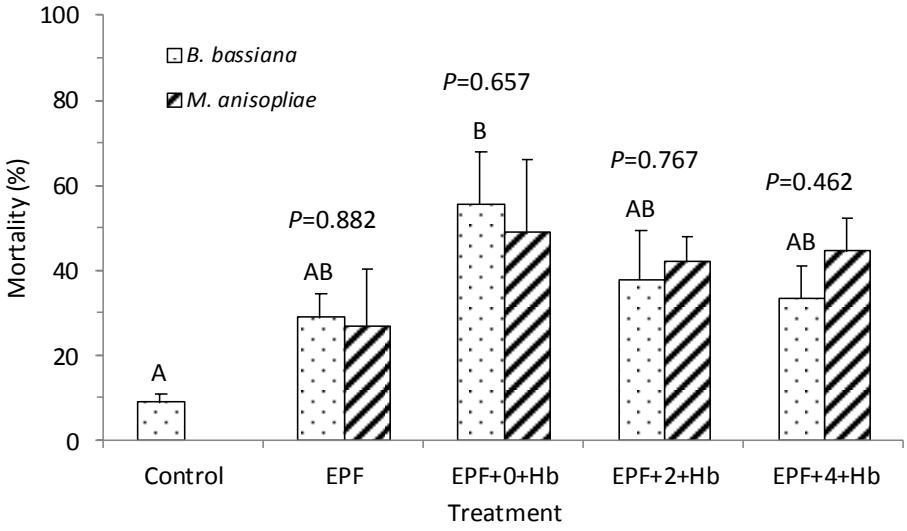

Fig. 3. Mortality of 3rd instar masked chafers 8 wk after treatment with entomopathogenic fungi (EPF) (*Beauveria bassiana* ES or *Metarhizium anisopliae* EC) alone, or in combination with *Heterorhabditis bacteriophora* at 0 (EPF+0+Hb), 2 (EPF+2+Hb), or 4 wk (EPF+4+Hb) interval (mean±SEM). Different letters indicate significant difference between treatment effects for *B. bassiana* ES (Tukey's HSD, α=0.05). No significant differences were found among treatments with *M. anisopliae*. *P* value indicates significance between *B. bassiana* ES and *M. anisopliae* under each treatment.

1.3. Effect of temperature on the efficacy of *M. anisopliae* and *H. bacteriophora* on 3rd instar masked chafers

Additive interactions were found between *M. anisopliae* and *H. bacteriophora* when they were applied simultaneously at 12, 20 °C, and in the 2 wk observation at 28 °C (Table 4). However, an antagonistic effect showed in the interaction of the two agents in the 4 wk observation at 28 °C. This was probably due to high mortality in the untreated control at 28 °C (Fig. 4), since data were corrected for control mortality under each temperature before analysis on the type of interaction.

Temperature had significant impact on the efficacy of the nematode and fungus. Within 4 wk after application, there were significant differences among various treatments (F=17.24, d.f.=3, *P*<0.0001) and among temperatures (F=56.5, d.f.=2, *P*<0.0001), but not in the interaction of treatments and temperatures (F=2.1, d.f.=6, *P*=0.091) (two-way ANOVA) (Fig. 4). It appeared that grub mortality in all treatments increased significantly as temperature increased. The treatment with *M. anisopliae* alone did not cause significant mortality relative to the untreated control. However, adding *H. bacteriophora* significantly improved the efficacy, although there was no difference in effect for the nematode applied alone or in combination with the fungus.

Table 4. Effect of temperature on the interaction between *Heterorhabditis bacteriophora* and *Metarhizium anisopliae* against 3rd instar masked chafers when the nematode and fungus were applied simultaneously.

Temperature (°C)	Wk[a]	Observed mortality (%)[b]	Expected Mortality (%)[c]	X^2	Type of Interaction
12	2	0	0	----	----
12	4	7.1	9.5	0.60	additive
20	2	35.6	37.8	0.13	additive
20	4	40.9	42.8	0.09	additive
28	2	75.6	79.1	0.15	additive
28	4	64.3	83.4	4.39	antagonistic

a. Wk after the application of *H. bacteriophora*;

b. Observed mortality was corrected for control mortality with Abbott's formula (Abbott 1925);

c. Expected mortality $M_E = M_N + M_F (1-M_N)$, where M_F and M_N are the observed proportional mortalities relatively caused by *M. anisopliae* and *H. bacteriophora* alone.

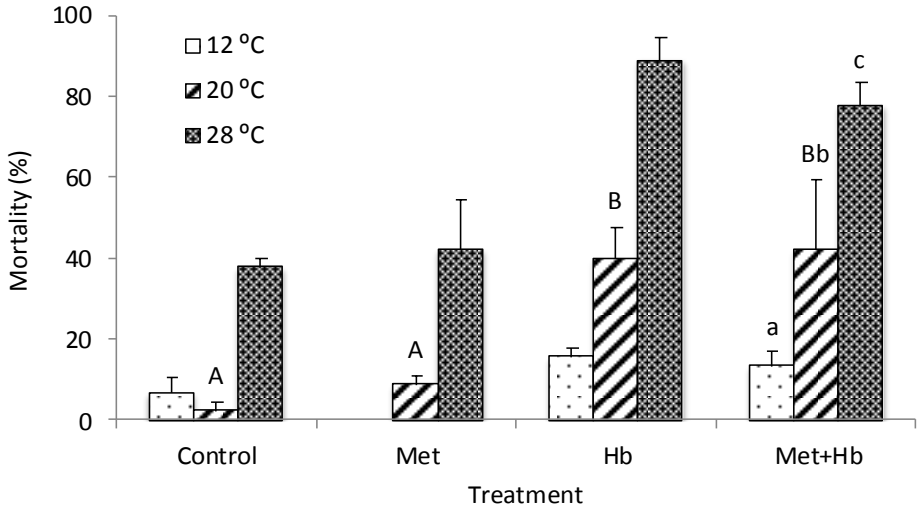

Fig. 4. Mortality of 3rd instar masked chafers within 4 wk after the treatment with *Metarhizium anisopliae* alone (Met), or *Heterorhabditis bacteriophora* alone (Hb), or both simultaneously (Met+Hb) (mean ± SEM). Capital and lower case letters indicate significant differences between treatment effects and between temperatures, respectively (Tukey's HSD, α=0.05).

2. Interaction of *M. anisopliae* / *B. bassiana* and *H. bacteriophora* on overwintered masked chafers

Additive interactions were found between *H. bacteriophora* and *M. anisopliae* when *H. bacteriophora* was added 0 or 4 wk after *M. anisopliae*. Similarly, additive or synergistic interactions were found

between *H. bacteriophora* and *B. bassiana* in both emulsifiable and wettable powder formulations when the nematodes were added 4 wk after the fungus (Table 5).

Table 5. Interaction of *Heterorhabditis bacteriophora* and *Metarhizium anisopliae* or *Beauveria bassiana* against overwintered masked chafer grubs under 20 °C and LD 13:11.

EPF Species	Intervals[a]	Wk[b]	Observed mortality (%)[c]	Expected Mortality (%)[d]	X^2	Type of Interaction
M. anisopliae	0	2	64.4	69.6	0.38	additive
M. anisopliae	0	4	76.2	83.3	0.61	additive
M. anisopliae	4	2	69.2	56.3	2.98	additive
M. anisopliae	4	4	73.5	69.6	0.23	additive
B. bassiana ES	4	2	79.5	52.5	13.84	synergistic
B. bassiana ES	4	4	85.3	52.9	19.77	synergistic
B. bassiana WP	4	2	46.2	52.5	0.77	additive
B. bassiana WP	4	4	41.2	54.3	3.18	additive

a. Wk intervals between the application of *M. anisopliae* or *B. bassiana* and *H. bacteriophora*;

b. Wk after the application of *H. bacteriophora*;

c. Observed mortality was corrected for control mortality with Abbott's formula (Abbott 1925);

d. Expected mortality $M_E = M_N + M_F (1-M_N)$, where M_F and M_N are the observed proportional mortalities relatively caused by fungi and *H. bacteriophora* alone.

When EPF was applied alone, efficacy varied significantly with fungal types (F=29.41, d.f.=2, *P*=0.001). *M. anisopliae* caused the highest grub mortality, whereas *B. bassiana* WP and ES did not have any significant effect compared with the control within 8 wk (Tukey's HSD, α=0.05) (Fig. 5). After adding *H. bacteriophora* to EPF 4 wk later, significant differences were found among fungal types in control effect (F=22.62, d.f.=2, *P*=0.002). However, after adding *H. bacteriophora*, mortalities caused by *M. anisopliae* and *B. bassiana* ES were not significantly different from each other, while *B. bassiana* WP caused the lowest mortality among the three fungal treatments within 8 wk period. Noticeably, compared with EPF applied alone, adding *H. bacteriophora* to EPF significantly increased grub mortality from 51.1% to 80% for *M. anisopliae* (F=39.0, d.f.=1, *P*<0.0001), from 22.2% to 88.9% for *B. bassiana* ES (F=207.68, d.f.=1, *P*<0.0001), and from 26.7% to 55.6% for *B. bassiana* WP (F=39.0, d.f.=1, *P*<0.0001) (Fig. 5).

No significant differences were detected between the treatments with *H. bacteriophora* applied alone, or in combination with *M. anisopliae* in IJs production (*t*-test: t=0.55, d.f.=51, *P*=0.588) (Table 6). Also, the nematode infection rate did not differ significantly between *H. bacteriophora* alone and *H.*

bacteriophora applied in combination with *M. anisopliae* simultaneously (paired *t*-test: t=0, d.f.=4, *P*=1.000). These results indicate that *M. anisopliae* had no significant impact on *H. bacteriophora* infection and production in overwintered masked chafers.

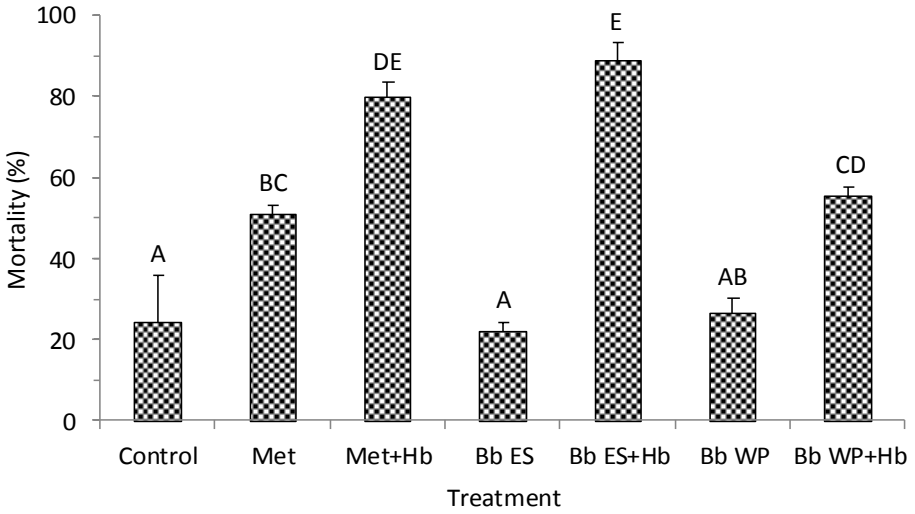

Fig. 5. Mortality of overwintered masked chafer grubs within 8 wk after fungal application, for *Metarhizium anisopliae* (Met), *Beauveria bassiana* ES (Bb ES) & WP (Bb WP) applied alone, or with *Heterorhabditis bacteriophora* (Hb) added 4 wk later (mean ± SEM) (F=25.43, d.f.=6, *P*<0.0001). Different letters indicate significant difference between treatment effects (Tukey's HSD, α=0.05).

Table 6. Rate of infection with *Heterorhabditis bacteriophora* in 3 wk after treatment, and IJs produced from overwintered masked chafer grubs treated with *H. bacteriophora* alone, or in combination with *Metarhizium anisopliae* applied simultaneously under 20 °C and LD 13:11.

Treatment	No. grubs	Mean IJs (±SEM)	Infection (±SEM)%
H. bacteriophora	27	62479 (±7722)	68.9 (±9.7)
H. bacteriophora +*M. anisopliae*	26	68713 (±8434)	68.9 (±5.9)

3. Interaction of *M. anisopliae* / *B. bassiana* and *H. bacteriophora* on 2nd instar masked chafers

When *B. bassiana* ES and *M. anisopliae* were applied alone, they caused an average mortality of 37.8% and 33.3%, respectively in 8 wk after treatment, which were not significantly different from the control mortality (22.2%) (F=0.83, d.f.=2, *P*=0.481) (Fig. 6). However, additive or synergistic interactions were found between *B. bassiana* ES and *H. bacteriophora* when the nematodes were added 0, 2, or 4 wk later; additive interactions were detected between *M. anisopliae* and *H. bacteriophora* when the nematode was added 0 or 2 wk later, but not after 4 wk (Table 7).

Table 7. Interaction of *Heterorhabditis bacteriophora* and *Metarhizium anisopliae* or *Beauveria bassiana* against 2nd instar masked chafers under 20 °C and LD 13:11.

EPF Species	Intervals[a]	Wk[b]	Observed mortality (%)[c]	Expected Mortality (%)[d]	X^2	Type of Interaction
M. anisopliae	0	2	59.1	51.1	1.24	additive
M. anisopliae	0	4	70.0	74.6	0.28	additive
M. anisopliae	2	2	62.5	56.1	0.74	additive
M. anisopliae	2	4	67.6	56.6	2.12	additive
M. anisopliae	4	2	8.1	30.1	16.07	antagonistic
M. anisopliae	4	4	2.9	31.4	25.99	antagonistic
B. bassiana ES	0	2	56.8	51.1	0.63	additive
B. bassiana ES	0	4	80.0	72.5	0.77	additive
B. bassiana ES	2	2	55.0	52.5	0.12	additive
B. bassiana ES	2	4	56.8	61.9	0.42	additive
B. bassiana ES	4	2	43.3	38.6	0.57	additive
B. bassiana ES	4	4	51.4	36.0	6.60	synergistic

a. Wk intervals between the application of *M. anisopliae* or *B. bassiana* and *H. bacteriophora*;

b. Wk after the application of *H. bacteriophora*;

c. Observed mortality was corrected for control mortality with Abbott's formula (Abbott 1925);

d. Expected mortality $M_E = M_N + M_F (1-M_N)$, where M_F and M_N are the observed proportional mortalities relatively caused by fungi and *H. bacteriophora* alone.

Despite that there was no effect for EPF applied alone compared with the untreated control, adding *H. bacteriophora* to *B. bassiana* ES significantly improved the fungal performance in causing grub mortality (F=12.18, d.f.=4, *P*=0.001), especially when the nematode and fungus were applied simultaneously. After adding the nematode 0 or 2 wk after the fungal application, the efficacy of *M. anisopliae* tended to be higher than applied alone although statistically not significant. There was an overall significant effect in causing grub mortality compared with the untreated control for *M. anisopliae* (F=7.52, d.f.=4, *P*=0.005). No significant difference was detected between *B. bassiana* ES and *M. anisopliae* in grub control when they were applied alone, or in combination with *H. bacteriophora* at 0 or 2 wk interval. However, when the nematode was added to EPF after 4 wk, *B. bassiana* ES caused significantly higher grub mortality than *M. anisopliae* did (F=9.66, d.f.=1, *P*=0.006) (Fig. 6).

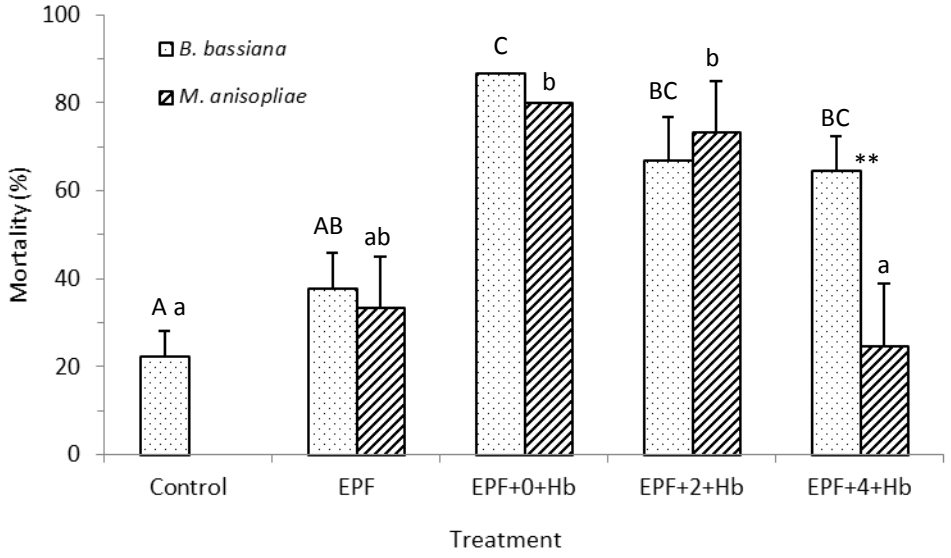

Fig. 6. Mortality of 2nd instar masked chafers 8 wk after the application of entomopathogenic fungi (EPF) (*Metarhizium anisopliae* or *Beauveria bassiana* ES), for the treatment with EPF applied alone, or in combination with *Heterorhabditis bacteriophora* at 0 (EPF+0+Hb), 2 (EPF+2+Hb) or 4 wk (EPF+4+Hb) interval (mean ± SEM). Different capital and lower case letters indicate significant difference between treatments for *B. bassiana* ES and *M. anisopliae*, respectively (Tukey's HSD, α=0.05); ** represents significant difference between fungal species under each treatment at α=0.05.

Mortality for the treatment with *H. bacteriophora* added 4 wk after *M. anisopliae* was significantly lower than that with the nematode added simultaneously or 2 wk later, whereas there was no difference in the effect of time intervals between the application of *B. bassiana* ES and *H. bacteriophora* (Tukey's HSD, α=0.05) (Fig. 6). A possible explanation for the effect of time intervals between the nematode and fungal applications in *M. anisopliae* is that grubs grew larger when the nematodes were added 4 wk after the fungus, and thus became less susceptible to the agents than the younger stages when the nematodes were added 0 or 2 wk after the fungus. However, this effect might be also due to the difference in exposure time to the nematode, as grubs in treatment EPF+4+Hb were only exposed to *H. bacteriophora* for 4 wk, compared with 6 wk in EPF+2+Hb and 8 wk in EPF+0+Hb, when all grubs received fungal treatment for 8 wk.

When EPF was applied in combination with the nematode simultaneously, there was no significant difference between *B. bassiana* ES and *M. anisopliae* in causing grub mortality for observations made 2, 4, 6, or 8 wk post application ($P>0.05$). However, significant differences were found for observations made at different time after treatment ($F=77.78$, d.f.=3, $P<0.0001$ for *B. bassiana* ES; $F=4.8$, d.f.=3, $P=0.034$ for *M. anisopliae*). For both *B. bassiana* ES and *M. anisopliae*, grub mortality increased within 4 wk after treatment, but did not change significantly after 4 wk (Tukey's HSD, α=0.05) (Fig. 7).

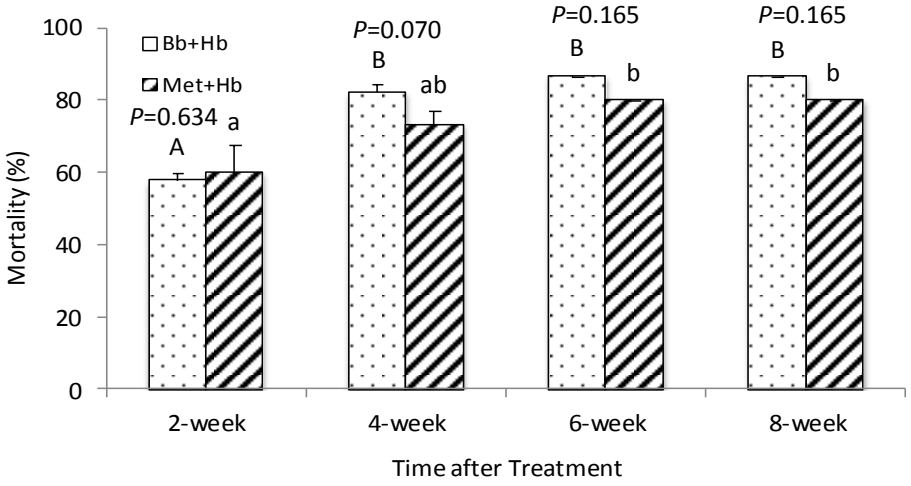

Fig. 7. Mortality of 2nd instar masked chafers within 2, 4, 6 or 8 wk after treatment, for *Metarhizium anisopliae* (Bb+Hb) or *Beauveria bassiana* ES (Met+Hb) applied in combination with *Heterorhabditis bacteriophora* simultaneously (mean ± SEM). Different capital and lower case letters indicate significant difference between time after treatment for Bb+Hb and Met+Hb, respectively (Tukey's HSD, α=0.05). *P* value indicates significance between Bb+Hb and Met+Hb for observations made at each time after treatment (α=0.05).

Discussion

When applied alone, *B. bassiana* ES & WP and *M. anisopliae* did not have any significant effects in reducing masked chafer numbers at various developmental stages (Fig. 2-6), except for the use of *M. anisopliae* against overwintered grubs (Fig. 5). In Experiment 1.3 & 2, adding EPN to EPF significantly improved the efficacy in grub control compared with EPF applied alone (Fig. 4 & 5). In Experiment 3, the treatment with *H. bacteriophora* and *B. bassiana* ES combined and applied simultaneously caused significantly higher mortality of 2nd instars than when *B. bassiana* ES was applied alone; most other treatments also showed a trend of increased mortality by adding the nematode, although statistically not significant (Fig. 6). However, in Experiment 1.2 against 3rd instars, no significant difference was found for EPF applied with or without adding EPN (Fig. 3). *H. bacteriophora* caused significantly higher grub mortality than *H. megidis* when they were applied alone, or in combination with *B. bassiana* ES at 0 or 2 wk interval, but not for the nematode added 4 wk after the fungus (Fig. 1). Compared with nematodes applied alone, adding *B. bassiana* ES or *M. anisopliae* to nematodes did not significantly improve the effect in grub control (Fig. 1 & 4).

Additive interactions were observed between EPN and EPF in most treatments against overwintered, 2nd and pre-wintering 3rd instar masked chafers, with an exception of a few treatments that showed synergistic or antagonistic effects. Synergism appeared in the 6 wk observation for the combined application of *H.*

bacteriophora and *B. bassiana* ES simultaneously against pre-wintering 3rd instars in 2010 (Table 1), in the 2 wk observation when *H. bacteriophora* was added 4 wk after *M. anisopliae* on 3rd instars in 2011 (Table 3), when *H. bacteriophora* was added 4 wk after *B. bassiana* ES against overwintered grubs (Table 5), and also in the 4 wk observation for *H. bacteriophora* added 4 wk after *B. bassiana* ES on 2nd instars (Table 7). Antagonistic effects were only detected when observations were made at 4 wk after nematode application for *H. megidis* added 2 or 4 wk after *B. bassiana* ES, when *H. megidis* was added 6 wk and *H. bacteriophora* was added 4 wk after the fungus against 3rd instars in fall 2010 (Table 1), and between *M. anisopliae* and *H. bacteriophora* when the nematodes were added 4 wk later against 2nd instars (Table 7). The antagonism was probably due to the high mortality in the untreated control in Table 1, and decreased grub susceptibility to the nematode and fungus as grubs grew larger in Table 7.

A few similar studies have been reported on the interaction between EPF and EPN in the management of white grubs. For example, the combined application of *H. bacteriophora* and *M. anisopliae* isolate MM increased larval mortality of the barley chafer grub, *C. curtipennis* in an additive and synergistic manner (Anbesse et al., 2008). Similar results also appeared in the combined application of *M. anisopliae* CLO 53 and *H. megidis* or *S. glaseri* against 3rd instar *H. philanthus* under laboratory and greenhouse conditions (Ansari et al., 2004), and occurred between *M. anisopliae* CLO 53 and *H. bacteriophora* in the field (Ansari et al., 2006). In addition, additive or synergistic effects from the combined application of EPN or EPF with other control agents in grub control has also been reported, for example, between EPN and bacterium *Bacillus thuringiensis* subspecies *japonensis* Buibui strain (Koppenhöfer et al., 1999; Koppenhöfer and Kaya, 1997), between EPN and insecticides (Koppenhöfer et al., 2002; Koppenhöfer et al., 2000b; Koppenhöfer and Kaya, 1998), and between EPF *M. anisopliae* and bacterium *Serratia entomophila* Grimont et al. (Glare, 1994).

It is suggested that stressed insects are generally more susceptible to pathogen infection (Steinhaus, 1958). A stressor like EPF or EPN may weaken the target insects and increase their susceptibility to other control agents to enhance the insect mortality or facilitate the speed of kill, which eventually leads to an additive or synergistic effect for the combined application of agents in insect control. For example, it was demonstrated that milky spore disease bacterium, *Bacillus popilliae* Dutky, acted as a stressor in the increased susceptibility of scarab larvae to nematode infection (Thurston et al., 1993; Thurston et al., 1994). Ansari et al. (2004) hypothesized that grubs suffering from fungal infection may not be able to feed or utilize food normally. In the current study, however, the application of *B. bassiana* and *M. anisopliae* did not have significant impact on the weight gain in the body mass in 2nd instar masked chafers (Wu et al. unpublished data). The underlying mechanism for the interaction between EPF and EPN against *Cyclocephala* spp. remains unknown.

Both Ansari et al. (2004) and Anbesse et al. (2008) stated that grubs had to be exposed to the fungus for at least 3 or 4 wk before the addition of nematodes to achieve stronger synergistic effects. In the current study, no advantage in enhancing efficacy was observed for the delayed application of nematodes. In Experiment 1.1 on pre-wintering 3rd instar masked chafers, there were no significant differences in grub mortality for EPN *H. bacteriophora* and *H. megidis* applied alone, or added 0, 2, or 4 wk after *B. bassiana* ES (Fig. 1). Similarly, in Experiment 1.2, the efficacy of both *B. bassiana* ES and *M. anisopliae* did not differ significantly with or without adding *H. bacteriophora* at different time intervals between applications (Fig. 3). Also, there was no effect of time intervals between the application of *B. bassiana* and *H. bacteriophora* on the mortality of 2nd instars, whereas *H. bacteriophora* added 4 wk after *M. anisopliae* caused significantly lower mortality than the treatments with the nematode added simultaneously or 2 wk later (Fig 6).

It was reported that temperature affected the relative competitive abilities of *H. bacteriophora* (*H. heliothidis*) and S. *carpocapsae* (*S. feltiae*) with *B. bassiana* (Barbercheck and Kaya, 1990). In the current study, temperature was found to have a significant impact on the efficacy of *H. bacteriophora* and *M. anisopliae*, and grub mortality in all treatments increased significantly as temperature increased, especially for the treatments with nematodes applied (Fig. 4). Additive interactions were found between *M. anisopliae* and *H. bacteriophora* when they were applied simultaneously at 12, 20 ^{o}C, and in the 2 wk observation at 28 ^{o}C, but not in the 4 wk observation at 28 ^{o}C (Table 4), which was probably due to high mortality in the untreated control at 28 ^{o}C. In addition, under all three temperatures, *H. bacteriophora* caused significantly higher grub mortality than *M. anisopliae* did when applied alone, and the combined application did not cause an effect different from *H. bacteriophora* alone. This indicates that *H. bacteriophora* outcompeted *M. anisopliae*, and increase in temperature from 12 to 28 ^{o}C did not enhance the competitive ability of the fungus, despite grub mortality increasing in the treatment with the fungus applied alone.

Barbercheck and Kaya (1990) stated that *H. bacteriophora* and *S. carpocapsae* were not compatible with *B. bassiana* in dually infected hosts, and usually only nematodes or fungus developed and produced progeny in *G. mellonella* exposed to both agents. Nematodes and their symbiotic bacteria prevented or inhibited the growth of *B. bassiana* if nematodes were applied within 24 h after fungal application, and the fungus was detrimental to the development of nematodes when applied more than 48 h ahead. Also, Ansari et al. (2005) reported that EPN symbiotic bacterium *Photorhabdus luminescens* was antagonistic to *M. anisopliae, B. bassiana, B. brongniartii* and *Paecilomyces fumosoroseus* by inhibiting their growth and conidial production, while another EPN symbiont *Xenorhabdus poinarii* had no inhibitory effect. In the current study, *B. bassiana* ES had no significant impact on the infection with *H. megidis* or IJs

production from grub carcasses, when *H. megidis* was applied at various time intervals after fungal application against 3rd instar *Cyclocephala* spp. (Table 2). Similarly, *M. anisopliae* did not significantly affect *H. bacteriophora* infection and production in overwintered grubs when applied simultaneously (Table 6). It is possible that the two types of pathogens acted independently with each other, and thus avoided the competition for resources within the same host. Or, the low fungal efficacy might have minimized the impact on nematode IJs infection and reproduction in the insect. For the combined application of EPN and EPF, dead grubs showed the symptoms of either nematode or fungal infection, and no grub showed both fungal sporulation and nematode development.

There was no obvious difference for the interaction of EPN and EPF on masked chafer white grubs at various developmental stages, and additive interactions were detected in most observations. The low efficacy of fungal application might account for the lack of a stronger interactive effect in *Cyclocephala* spp.

References

Abbott, W. S., 1925. A method for computing the effectiveness of an insecticide. J. Econ. Entomol. 18**,** 265-267.

Anbesse, S. A., Adge, B. J., Gebru, W. M., 2008. Laboratory screening for virulent entomopathogenic nematodes (*Heterorhabditis bacteriophora* and *Steinernema yirgalemense*) and fungi (*Metarhizium anisopliae* and *Beauveria bassiana*) and assessment of possible synergistic effects of combined use against grubs of the barley chafer *Coptognathus curtipennis*. Nematology. 10**,** 701-709.

Ansari, M. A., Shah, F. A., Tirry, L., Moens, M., 2006. Field trials against *Hoplia philanthus* (Coleoptera: Scarabaeidae) with a combination of an entomopathogenic nematode and the fungus *Metarhizium anisopliae* CLO 53. Biol. Control. 39**,** 453-459.

Ansari, M. A., Tirry, L., Moens, M., 2004. Interaction between *Metarhizium anisopliae* CLO 53 and entomopathogenic nematodes for the control of *Hoplia philanthus*. Biol. Control. 31**,** 172–180.

Ansari, M. A., Tirry, L., Moens, M., 2005. Antagonism between entomopathogenic fungi and bacterial symbionts of entomopathogenic nematodes. BioControl. 50**,** 465–475.

Barbercheck, M. E., Kaya, H. K., 1990. Interactions between *Beauveria bassiana* and the entomogenous nematodes, *Steinernema feltiae* and *Heterorhabditis heliothidis*. J. Invertebr. Pathol. 55**,** 225-234.

Choo, H. Y., Kaya, H. K., Huh, J., Lee, D. W., Kim, H. H., Lee, S. M., Choo, Y. M., 2002. Entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis bacteriophora*) and a fungus *Beauveria brongniartii* for biological control of the white grubs, *Ectinohoplia rufipes* and *Exomala orientalis*, in Korean golf courses. BioControl. 47**,** 177-192.

Dimock, W. J., Spatial factors affecting white grub presence and abundance in golf course turf. Virginia Polytechnic Institute and State University,, Blacksburg, VA, 2004.

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

Georgis, R., Gaugler, R., 1991. Predictability in biological control using entomopathogenic nematodes. J. Econ. Entomol. 84**,** 713-720.

Glare, T. R., 1992. Fungal pathogens of scarabs. In: T. A. Jackson, T. R. Glare, (Eds.), Use of Pathogens in Scarab Pest Management. Intercept, Andover, UK, pp. 63-77.

Glare, T. R., 1994. Stage-dependent synergism using *Metarhizium anisopliae* and *Serratia entomophila* against *Costelytra zealandica*. Biocontrol Sci. Technol. 4**,** 321–329.

Glazer, I., 2002. Survival biology. In: R. Gaugler, (Ed.), Entomopathogenic Nematology. CABI Publishing, Wallingford, UK, pp. 169–187.

Jaques, R. P., Morris, O. N., 1981. Compatibility of pathogens with other methods of pest control and with different crops. In: H. D. Burges, (Ed.), Microbial Control of Pests and Plant Disease1970–1980. Academic Press, London, pp. 695–715.

Kaya, H. K., 1990. Soil ecology. In: R. Gaugler, H. K. Kaya, (Eds.), Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, FL, pp. 93-115.

Kaya, H. K., 2002. Natural enemies and other antagonists. In: R. Gaugler, (Ed.), Entomopathogenic Nematology. CABI Publishing, Wallingford, UK, pp. 189–203.

Kaya, H. K., Koppenhöfer, A. M., 1996. Effects of microbial and other antagonistic organism and competition on entomopathogenic nematodes. Biocontrol Sci. Technol. 6**,** 357-371.

Kaya, H. K., Stock, S. P., 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, et al., (Eds.), Nematodes and the Biological Control of Insect Pests. CSIRO, East Melbourne, Australia, pp. 49–58.

Koppenhöfer, A. M., Choo, H. Y., Kaya, H. K., Lee, D. W., Gelernter, W. D., 1999. Increased field and greenhouse efficacy against scarab grubs with a combination of an entomopathogenic nematode and *Bacillus thuringiensis*. Biol. Control. 14**,** 37-44.

Koppenhöfer, A. M., Cowles, R. S., Cowles, E. A., Fuzy, E. M., Baumgartner, L., 2002. Comparison of neonicotinoid insecticides as synergists for entomopathogenic nematodes. Biol. Control. 24**,** 90-97.

Koppenhöfer, A. M., Fuzy, E. M., Crocker, R., Gelernter, W., Polavarapu, S., 2004. Pathogenicity of *Heterorhabditis bacteriophora*, *Steinernema glaseri*, and *S. scarabaei* (Rhabditida: Heterorhabditidae, Steinernematidae) against 12 white grub species (Coleoptera: Scarabaeidae). Biocontrol Sci. Technol. 14**,** 87-92.

Koppenhöfer, A. M., Grewal, P. S., Fuzy, E. M., 2006. Virulence of the entomopathogenic nematodes *Heterorhabditis bacteriophora*, *Heterorhabditis zealandica*, and *Steinernema scarabaei* against five white grub species (Coleoptera: Scarabaeidae) of economic importance in turfgrass in North America. Biol. Control. 38**,** 397-404.

Koppenhöfer, A. M., Grewal, P. S., Kaya, H. K., 2000. Synergism of imidacloprid and entomopathogenic nematodes against white grubs: the mechanism. Entomol. Exp. Appl. 94**,** 283-293.

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

Koppenhöfer, A. M., Kaya, H. K., 1998. Synergism of imidacloprid and an entomopathogenic nematode: A novel approach to white grub (Coleoptera: Scarabaeidae) control in turfgrass. J. Econ. Entomol. 91**,** 618-623.

Lacey, L. A., Brooks, W. M., 1997. Initial handling and diagnosis of diseased insects. In: L. A. Lacey, (Ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, CA pp. 5.

McVay, J. R., Gudauskas, R. T., Harper, J. D., 1977. Effect of *Bacillus thuringiensis* and chemical insecticides on *Spodoptera littoralis* (Lepidoptera: Noctuidae). J. Econ. Entomol. 77**,** 590-885.

Smits, P. H., 1996. Post-application persistence of entomopathogenic nematodes. Biocontrol Sci. Technol. 6**,** 379-387.

Steinhaus, E. A., 1958. Stress as a factor in insect disease. Proceedings of the Xth International Congress on Entomology. 4**,** 725–730.

Thurston, G. S., Kaya, H. K., Burlando, T. M., Harrison, R. E., 1993. Milky disease bacteria as a stressor to increase susceptibility of scarabaeid larvae to an entomopathogenic nematode. J. Invertebr. Pathol. 61**,** 167–172.

Thurston, G. S., Kaya, H. K., Gaugler, R., 1994. Characterization of enhanced susceptibility of milky disease infected scarabaeid grubs to entomopathogenic nematodes. Biol. Control. 4**,** 67–73.

Wraight, S. P., Inglis, G. D., Goettel, M. S., 2007. Fungi. In: L. A. Lacey, H. K. Kaya, (Eds.), Field Manual of Techniques in Invertebrate Pathology. Springer, Dordrecht, The Netherlands, pp. 223-248.

CHAPTER 4

Efficacy of Entomopathogenic Nematodes and Fungi against 3rd instar Masked Chafers, *Cyclocephala* spp. (Coleoptera: Scarabaeidae), under Greenhouse Conditions

Abstract

The efficacy of using entomopathogenic nematodes and entomopathogenic fungi and their interaction were evaluated against 3rd instar masked chafers, *Cyclocephala* spp., under greenhouse conditions for 3 yr. Two nematodes, *Heterorhabditis bacteriophora* or *H. megidis*, and two fungal species, *Beauveria bassiana* strain GHA or *Metarhizium anisopliae* strain F-52, were used. *B. bassiana* was used in emulsifiable (ES) and wettable powder (WP) formulations, and *M. anisopliae* as oil emulsifiable (EC) and granular (G) formulations. Additive or synergistic interactions were found between *H. bacteriophora* and most fungal types, except for *B. bassiana* ES, in both 2011 and 2012. The interactions between *B. bassiana* ES and the two nematode species were masked due to the high grub mortality in the untreated control in 2010. Significant improvement from the combination of a nematode and a fungus over the fungus alone was shown for *B. bassiana* ES and *H. bacteriophora* / *H. megidis* in 2010, and in *H. bacteriophora* and *M. anisopliae* EC / *B. bassiana* WP in 2011. However, such combined applications did not cause significantly higher grub mortality than the nematodes used alone. There appears to be a trend indicating that combined use of a fungus and *H. bacteriophora* achieved higher grub mortality than a single agent applied alone, except for *B. bassiana* ES plus *H. bacteriophora* in 2012. Overall, the use of the nematodes and/or fungi provided control efficacy comparable to the imidacloprid insecticide against 3rd instar *Cyclocephala* spp.

Keywords: white grub, *Cyclocephala* spp., entomopathogenic nematode, entomopathogenic fungi

Introduction

In Virginia, Japanese beetle (*Popillia japonica* Newman) and masked chafers (*Cyclocephala* spp.) are the most important white grub species in turfgrass (Dimock, 2004). The masked chafers comprise > 80% of the general white grub species in some areas (e.g. Blacksburg, Bristol) in the state in recent years (Wu et al. unpublished data). Successful control of *Cyclocephala* spp. may play an important role in turfgrass management. Currently, the predominant strategy for the management of these pests is to apply conventional insecticides, such as imidacloprid and clothianidin, on a preventative basis before damage occurs. For such a method to be successful, large areas need to be sprayed to achieve adequate coverage. As these chemicals are more effective against young grubs, damage can occur late in the season if early attempts fail to control them. Environmental and safety concerns rising from long-term use of the

insecticides in home lawns and commercial turf have led to the development of biological control of these pests.

We explored the potential for combined use of entomopathogenic nematodes (EPN) and entomopathogenic fungi (EPF) for white grub control. The role of EPN against white grubs has been studied by many researchers, as summarized by Grewal et al. (2005) and Klein (1990). Several EPN species [*Heterorhabditis bacteriophora* Poinar, *H. zealandica* Poinar, *Steinernema scarabaei* Stock & Koppenhöfer, *S. glaseri* (Steiner)] may have potential against southern (*C. lurida* Bland) and northern (*C. borealis* Arrow) masked chafers, although they are generally less susceptible than *P. japonica* (Koppenhöfer et al., 2004; Koppenhöfer et al., 2006). However, their field efficacies are often inconsistent and unsatisfactory (Georgis and Gaugler, 1991; Klein, 1993), due to various biotic (Kaya, 2002; Kaya and Koppenhöfer, 1996) and abiotic factors (Glazer, 2002; Kaya, 1990; Smits, 1996). Combining the application of EPN and EPF may achieve an improved level of control over white grub numbers. This was shown in the combination of *H. bacteriophora* with *Metarhizium anisopliae* (Metschn.) Sorokin isolate MM against the barley chafer grub, *Coptognathus curtipennis* Faimaire (Anbesse et al., 2008), *S. carpocapsae* (Weiser) with *B. brongniartii* (Saccardo) Petch against grub *Exomala orientalis* (Waterhouse) (Choo et al., 2002), *M. anisopliae* CLO 53 with *H. megidis* Poinar, Jackson & Klein or *S. glaseri* against 3rd instar *Hoplia philanthus* Füessly under laboratory and greenhouse conditions (Ansari et al., 2004), and with *H. bacteriophora* in the field (Ansari et al., 2006).

In the current study, the efficacy of EPN *H. bacteriophora* / *H. megidis* and EPF *M. anisopliae* / *Beauveria bassiana* (Balsamo) Vuillemin applied alone or in combination, and their possible interaction against 3rd instar *Cyclocephala* spp. were evaluated under greenhouse conditions. It was anticipated that the combined application of the nematodes and fungi would achieve enhanced efficacy by additive or synergistic interactions with the goal of better management of these pests.

Materials and Methods

1. Efficacy of *B. bassiana ES* and *H. bacteriophora* / *H. megidis* in 2010

Insects used were 3rd instar masked chafers collected from Virginia Tech Turfgrass Research Center. EPF *B. bassiana* strain GHA in emulsifiable formulation (*B. bassiana* ES containing 2.1×10^{10} viable spores/ml), and EPN *H. bacteriophora* and *H. megidis* (Becker Underwood) were used at the rate of 25.6 L/ha, and 2.1 billion infective juveniles (IJs)/ha (LC 50 rate of *H. bacteriophora* in 2 wk), respectively. *B. bassiana* ES is a commercially available product labeled as BotaniGard ES from Laverlam International Co. (Butte, MT). All EPNs applied in this and following experiments were cultured with full grown wax moth larvae, *Galleria mellonella* (L.), and collected with the White trap (Kaya and Stock,

1997) within 5 d. There were six treatments, including a water control, *B. bassiana*, *H. bacteriophora*, *H. megidis*, *B. bassiana* plus *H. bacteriophora*, and *B. bassiana* plus *H. megidis*. EPNs were added 6 wk after the application of *B. bassiana* ES. Four blocks were used, in randomized complete block design.

Treatments were applied in 2.5-liter pots with a soil surface area of 160.6 cm^2. Each pot was seeded with 3 g perennial ryegrass (*Lolium perenne* L.) seeds that were allowed to grow for 24 d before the introduction of white grubs. Scissors were used to trim the grass weekly to 4 cm. There were 24 pots in total, and each pot contained 15 grubs. The grubs not entering the soil within 24 h were replaced. 120 ml tap water was added to wash the spores off the grass blade and sheath. The grass pots were watered every other day with 80 ml water. Results were evaluated 4 wk after the nematode application. Soil used was a loamy sand texture, comprised of 79.3% sand, 13.7% silt, 7.0% clay, and 1% organic matter with a pH of 4.9. The average soil and air temperature, soil moisture and R.H. were 18.6 ^oC, 20 ^oC, 20.4% VWC (volumetric water content), and 27.5%, respectively.

2. Efficacy of *M. anisopliae EC* / *B. bassiana* ES & WP and *H. bacteriophora* in 2011

This experiment was comprised of nine treatments and four blocks in randomized complete block design, and was replicated twice. Treatments were: a water control, Merit 75 WP, *M. anisopliae*, *B. bassiana* ES, *B. bassiana* WP, *H. bacteriophora*, *H. bacteriophora* plus *M. anisopliae* EC, *H. bacteriophora* plus *B. bassiana* ES, and *H. bacteriophora* plus *B. bassiana* WP. *H. bacteriophora* in all treatments were added 4 wk after fungal application. *B. bassiana* ES and *H. bacteriophora* were used at the same rate as in experiment 1. *B. bassiana* strain GHA in wettable powder (WP) formulation (BotaniGard WP from Laverlam International Co., containing 4.4×10^{10} viable spores/g) was used at the rate of 12.3 kg/ha. *M. anisopliae* strain F-52 (5.5×10^9 conidia/g) in oil emulsifiable formulation was used at the rate of 6.4 L/ha. *M. anisopliae* used in this and following experiments was provided by Dr. Jarrod E. Leland from Novozymes Biologicals, Inc. (Salem, VA). Merit 75 WP, an insecticide (75% imidacloprid in wettable powder formulation) from Bayer Co. labeled for preventative control of white grubs, was applied at the rate of 451 g/ha.

There were 72 1-liter grass pots (surface area: 95 cm^2) used in total. Each pot was seeded with approximately 1.5 g perennial ryegrass seeds 3 wk before the introduction of grubs. Each pot contained 10 grubs, which were surface-sterilized with 0.5% sodium hydrochloride to remove external contaminants (Lacey and Brooks, 1997) before being placed into grass pots. Grubs that did not enter soil within 24 h were replaced. An auto-watering system with two nozzles was set up above the bench. Grass pots were watered twice a day for 2 min each time, with total amount of 2.8 mm per day during the experiment. Soil used was a sandy loam texture, and was composed of 73.8% sand, 18.1% silt, 8.1% clay and 1.2%

organic matter with a pH of 5.8. The average air temperature and R.H. during the experiment were 21.1 ^{o}C and 47%. Results were assessed 4 wk after the application of *H. bacteriophora*.

3. Efficacy of *M. anisopliae* EC & G / *B. bassiana* ES & WP and *H. bacteriophora* in 2012

M. anisopliae F52 granular formulation (Met 52 G, containing 9×10^{8} colony forming units (CFU)/g), and wetting agent Silwet L-77 (organosilicone surfactant) were added in this experiment, and were used at the rate of 98.2 kg/ha and 0.25% v/v, respectively. Other materials and rates used were the same as in Experiment 2, except *H. bacteriophora* was applied at 5 billion IJs/ha. There were 13 treatments, including a water control, Merit 75 WP, *B. bassiana* ES, *B. bassiana* WP, *M. anisopliae* EC, *M. anisopliae* EC + Silwet L-77, *M. anisopliae* G, *H. bacteriophora*, *B. bassiana* ES + *H. bacteriophora*, *B. bassiana* WP + *H. bacteriophora*, *M. anisopliae* EC + *H. bacteriophora*, *M. anisopliae* EC + Silwet L-77 + *H. bacteriophora*, and *M. anisopliae* G + *H. bacteriophora*. Four blocks and randomized complete block design were used. *M. anisopliae* G was dissolved in water and filtered with cloth before use.

Pots with a volume of 2.5-liter were filled with soil (surface area: 169 cm^2), and seeded with 3 g perennial ryegrass seeds each. Grass was allowed to grow for 8 wk before introducing grubs. Each pot contained 15 masked chafer grubs. Other experimental procedures were the same as in experiment 2. Different from Experiment 1 & 2, all agents were applied at the same time. Soil used was a loamy sand texture, comprised of 86.7% sand, 7.6% silt, 5.7% clay and 1.9% organic matter with a pH of 5.1. During the experiment, the average temperature, R.H. and soil moisture were 21.6 ^{o}C, 42.9% and 19.7% VWC, respectively. Live and dead grub counts were made at 34 DAT.

Data Analysis

A X^2 test was used to test the interaction of EPF and EPN. Before analysis, all mortality data were corrected for control mortality (Abbott, 1925). The method of determining the type of interaction (synergistic, additive, or antagonistic) was first described by Finney (1964), and then modified by McVay et al. (1977). The expected additive proportional mortality M_E for the EPN / EPF combinations was calculated by $M_E = M_N + M_F (1\text{-}M_N)$, where M_N and M_F are the observed proportional mortalities relatively caused by EPN and EPF alone. A X^2 test was then carried out using the formula $X^2 = (M_{NF} - M_E)^2 / M_E$, where M_{NF} represents the observed mortality for the EPN / EPF combination. The calculated value from the X^2 test was then compared with the X^2 table value for 1 degree of freedom. If calculated values are greater than the table value ($X^2_{1,\ 0.05} = 3.84$), non-additive effects, e.g. synergistic or antagonistic, could be suspected between the two agents (Finney, 1964). If the differences $M_{NF} - M_E = D$ had a positive value, the interaction was considered synergistic, and interaction was considered antagonistic if D was negative.

In addition, one-way ANOVA was used to test the significant difference among treatments with software JMP 10.0 (SAS, Cary, NC). Differences between means were considered significant when $P \leq 0.05$.

Results

1. Efficacy of B. bassiana ES and H. bacteriophora / H. megidis in 2010

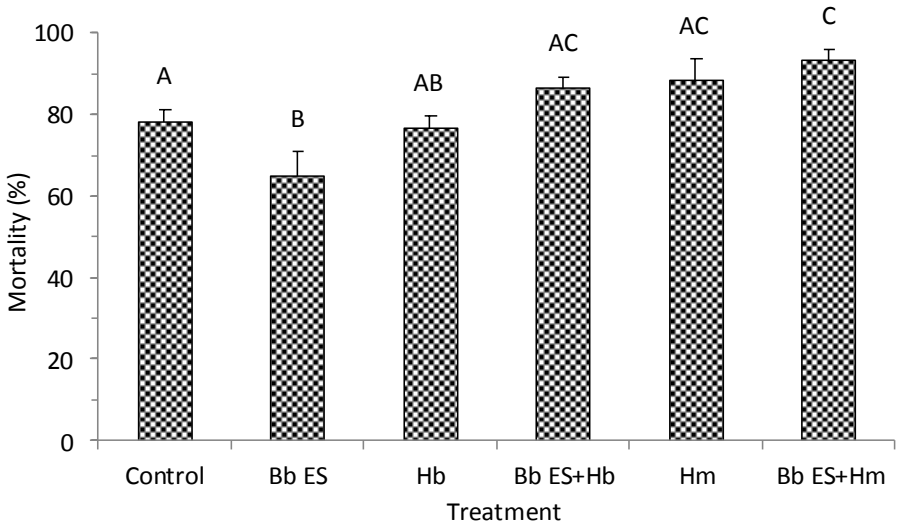

Fig. 1. Mortality of 3rd instar masked chafers 4 wk after nematode application under greenhouse conditions in 2010 (mean±SEM). Bb ES=*Beauveria bassiana* ES; Hb= *Heterorhabditis bacteriophora*; Hm=*H. megidis*. Hb & Hm were added 6 wk after fungal application. Different letters indicate significant difference between treatments (Fisher's LSD, α=0.05).

There were significant differences among treatments in efficacy against 3rd instar masked chafers 4 wk after the application of *H. bacteriophora* / *H. megidis* (F=5.75, d.f.=5, *P*=0.002), although high mortality in the untreated control might have masked the effect of some treatments. Among all treatments applied, only the combination of *B. bassiana* ES with *H. megidis* (Bb ES+Hm) showed a significant effect in grub control. Improved efficacy from the combined use of a nematode and *B. bassiana* ES over the fungus alone appeared for both nematode species (Bb ES versus Bb ES+Hb; Bb ES versus Bb ES+Hm). However, compared with *H. bacteriophora* or *H. megidis* applied alone, the combination with *B. bassiana* ES did not increase grub mortality (Hb versus Bb ES+Hb; Hm versus Bb ES+Hm) (Fisher's LSD, α=0.05) (Fig. 1). In addition, *H. megidis* appeared to be more effective than *H. bacteriophora* when applied alone or in combination with the fungus, although statistically not significant. The interaction between the nematodes and the fungus was masked due to high mortality in the untreated control.

2. Efficacy of M. anisopliae EC / B. bassiana ES & WP and H. bacteriophora in 2011

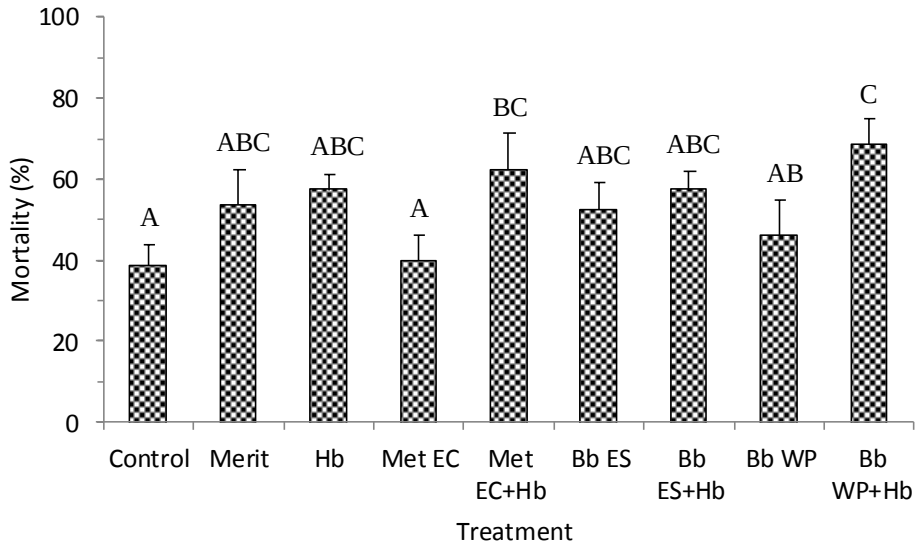

Fig. 2. Mortality of 3rd instar masked chafer in various treatments 4 wk after the application of *Heterorhabditis bacteriophora* (Hb) under greenhouse conditions in 2011 (mean±SEM). Merit=insecticide Merit 75 WP; Met EC=*Metarhizium anisopliae* EC; Bb ES=*Beauveria bassiana* ES; Bb WP=*B. bassiana* WP. Hb was added 4 wk after fungal application. Different letters indicate significant difference between treatments (Fisher's LSD, α=0.05).

Table 1. Interaction of *Heterorhabditis bacteriophora* and *Beauveria bassiana* or *Metarhizium anisopliae* against 3rd instar masked chafers under greenhouse conditions in 2011.

Treatment [a]	Raw mortality (%)	Corrected mortality (%)[b]	Expected Mortality (%)[c]	X^2	Type of Interaction
Bb ES+Hb	57.5	30.6	46.2	5.25	antagonistic
Bb WP+Hb	68.8	49.0	39.1	2.49	additive
Met EC+Hb	62.5	38.8	32.0	1.42	additive

a. Hb=*H. bacteriophora*, Bb ES=*B. bassiana* ES, Bb WP=*B. bassiana* WP, Met EC=*M. anisopliae* EC; Hb was added 4 wk after the fungi;

b. Mortality was corrected for control mortality with Abbott's formula (Abbott 1925);

c. Expected mortality $M_E = M_N + M_F (1-M_N)$, where M_F and M_N are the observed proportional mortalities relatively caused by fungi and *H. bacteriophora* alone.

A significant difference was detected among various treatments in causing mortality of 3rd instar masked chafers 4 wk after the application of *H. bacteriophora* (F=2.1, d.f.=8, *P*=0.049). The treatment with *H. bacteriophora* applied in combination with *M. anisopliae* EC (Met EC+Hb), or combined with *B. bassiana* WP (Bb WP+Hb) caused significantly higher grub mortality than the untreated control, whereas

other treatments including the insecticide Merit 75 WP and all fungi alone were not significantly different (Fisher's LSD, α=0.05) (Fig. 2).

For both *M. anisopliae* EC and *B. bassiana* WP, efficacy improved significantly after adding *H. bacteriophora* to the fungus 4 wk later, compared with the fungus applied alone. However, no significant improvement in grub mortality was shown in the combined application of *H. bacteriophora* and fungi than the nematode alone. Additive interaction was also detected between *H. bacteriophora* and *M. anisopliae* EC or *B. bassiana* WP, but antagonism was found between *H. bacteriophora* and *B. bassiana* ES (Table 1).

3. Efficacy of *M. anisopliae* EC & G / *B. bassiana* ES & WP and *H. bacteriophora* in 2012

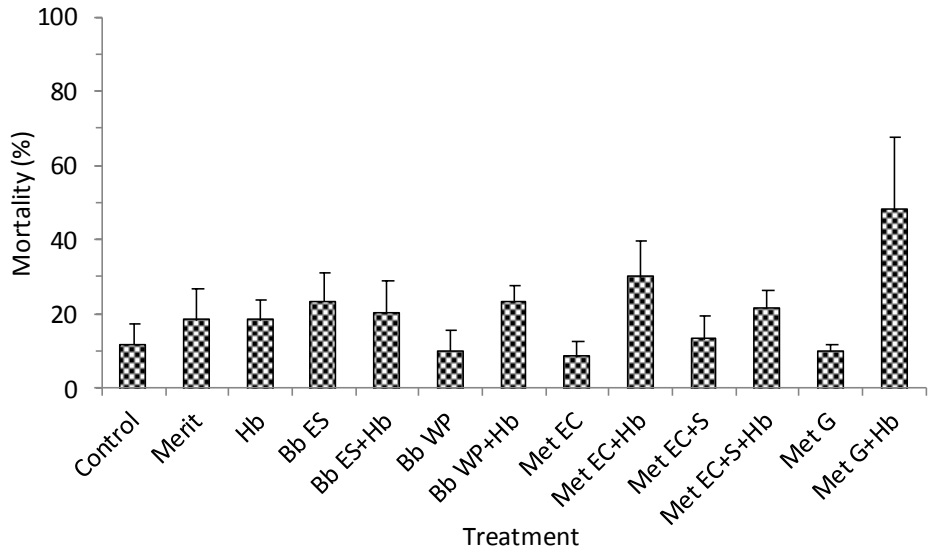

Fig. 3. Efficacy of the nematode and fungi applied simultaneously against 3rd instar masked chafers 34 DAT under greenhouse conditions in 2012 (mean±SEM). Hb=*Heterorhabditis bacteriophora*; Bb ES=*Beauveria bassiana* ES; Bb WP=*B. bassiana* WP; Met EC=*Metarhizium anisopliae* EC; Met G=*M. anisopliae* G; SW=Silwet L-77. Data were transform with the formula y=$\sqrt{x+0.5}$ before analysis. Untransformed data are shown in the Figure.

Overall, the effect of various treatments were not significantly different from each other (F=1.42, d.f.=12, *P*=0.198), although the combined application of *M. anisopliae* G and *H. bacteriophora* incurred higher grub mortality than other treatments. It is worthwhile to mention that on average 36.7% (range from 0% to 80%) grubs were infected with *H. bacteriophora*, and 1 grub was infected with *M. anisopliae* in this treatment. Except for the treatment with fungi *B. bassiana* WP alone, *M. anisopliae* EC alone or added with Silwet L-77, and *M. anisopliae* G alone, all other treatments caused an effect comparable to or higher than Merit, but were not statistically significant (Fig. 3). There appears to be a trend in the combined use of EPF and *H. bacteriophora* achieving higher grub mortality than a single agent applied

alone, except for *B. bassiana* ES plus *H. bacteriophora* (Bb ES+Hb), but this was not significantly different. Synergism was detected between the interaction of *H. bacteriophora* and *B. bassiana* WP, *M. anisopliae* EC or *M. anisopliae* G, and additive effect was found between the nematode and *M. anisopliae* EC plus Silwet L-77 (Met EC+S), whereas *H. bacteriophora* and *B. bassiana* ES showed antagonistic interaction, when the nematode and fungi were applied simultaneously. Among them, *H. bacteriophora* and *M. anisopliae* G achieved the strongest interaction for the combined application (Table 2).

Table 2. Interaction of *Heterorhabditis bacteriophora* and *Beauveria bassiana* or *Metarhizium anisopliae* against 3rd instar masked chafers under greenhouse conditions in 2012.

Treatment [a]	Raw mortality (%)	Corrected mortality (%) [b]	Expected Mortality (%) [c]	X^2	Type of Interaction
Bb ES+Hb	20.0	9.4	19.8	5.40	antagonistic
Bb WP+Hb	23.3	13.2	7.6	4.24	synergistic
Met EC+Hb	30.0	20.8	7.6	23.08	synergistic
Met EC+S+Hb	21.7	11.3	9.3	0.44	additive
Met G+Hb	48.3	41.5	7.6	152.75	synergistic

a. Hb=*H. bacteriophora*, Bb ES=*B. bassiana* ES, Bb WP=*B. bassiana* WP, Met EC=*M. anisopliae* EC, Met G=*M. anisopliae* G, S=Silwet L-77; Hb was added simultaneously to the fungal application;

b. Mortality was corrected for control mortality with Abbott's formula (Abbott 1925);

c. Expected mortality $M_E = M_N + M_F (1-M_N)$, where M_F and M_N are the observed proportional mortalities relatively caused by fungi and *H. bacteriophora* alone.

Discussion

When two control agents are applied together against a single pest, they may act independently and cause an additive effect, or interact with each other in a synergistic or antagonistic way (Jaques and Morris, 1981). In previous laboratory experiments, additive effects were found in the interaction of EPN *H. bacteriophora* / *H. megidis* and EPF *B. bassiana* / *M. anisopliae* against 3rd instar masked chafers (Wu et al. unpublished data). Similarly, in the current study, additive or synergistic interactions were detected between the nematode and fungal species, except for *H. bacteriophora* combined with *B. bassiana* ES simultaneously (2012, Table 2) or added 4 wk after the fungal application (2011, Table 1). Similar studies on the interaction of EPN and EPF against white grubs have been reported, e.g. additive or synergistic interactions in the combined application of *H. bacteriophora* and *M. anisopliae* isolate MM against the barley chafer, *C. curtipennis* (Anbesse et al., 2008), between *M. anisopliae* CLO 53 and *H. megidis* or *S. glaseri* against 3rd instar of *H. philanthus* under laboratory and greenhouse conditions

(Ansari et al., 2004), and between *M. anisopliae* CLO 53 and *H. bacteriophora* in the field (Ansari et al., 2006).

In this study, increased mortality from the combination of a nematode and a fungus over the fungus alone was shown for *B. bassiana* ES and *H. bacteriophora* / *H. megidis* in 2010 (Fig. 1), *H. bacteriophora* and *M. anisopliae* EC / *B. bassiana* WP in 2011 (Fig. 2). However, such combined applications did not cause significantly higher grub mortality than nematodes alone (Fig. 1 & 2). Also, although statistically not significant, a trend was shown in 2012 that the combined use of a fungus and *H. bacteriophora* achieved higher grub mortality than a single agent applied alone, except for *B. bassiana* ES plus *H. bacteriophora* (Fig. 3). Such a phenomenon is consistent with the laboratory and field results (Wu et al. unpublished data). Under laboratory conditions, adding EPN to EPF significantly improved the efficacy in grub control compared with EPF applied alone; compared with nematodes used alone, adding EPF did not significantly increase mortality. Similarly, the combined use of EPF and EPN improved the efficacy over either pathogen applied alone in part of the field treatments. Some combinations significantly reduced grub numbers over fungi alone, despite not having an effect higher than nematodes applied alone.

These results indicate that EPF played a less important role than EPN for their combined application in grub control, or the nematodes out-competed the fungi when they targeted a single host, although low efficacy of the fungi applied may also provide an explanation. EPNs generally kill the host within 48 h by releasing the symbiotic bacteria, i.e. *Photorhabdus* spp. associated with *H. bacteriophora* / *H. megidis*. In contrast, time until death is much longer for fungus-infected grubs, and the nematodes may have killed the host early in this period. A much higher percentage of EPN-infected carcasses were found than EPF-infected grubs. This provides further evidence of nematode surpassing fungi infectivity. Overall, the use of the nematodes and/or fungi achieved efficacy comparable to the imidacloprid insecticide in the control of 3rd instar *Cyclocephala* spp. More virulent fungal strains or species may be required to achieve a stronger interactive effect with the nematodes.

References

Abbott, W. S., 1925. A method for computing the effectiveness of an insecticide. J. Econ. Entomol. 18**,** 265-267.

Anbesse, S. A., Adge, B. J., Gebru, W. M., 2008. Laboratory screening for virulent entomopathogenic nematodes (*Heterorhabditis bacteriophora* and *Steinernema yirgalemense*) and fungi (*Metarhizium anisopliae* and *Beauveria bassiana*) and assessment of possible synergistic effects of combined use against grubs of the barley chafer *Coptognathus curtipennis*. Nematology. 10**,** 701-709.

Ansari, M. A., Shah, F. A., Tirry, L., Moens, M., 2006. Field trials against *Hoplia philanthus* (Coleoptera: Scarabaeidae) with a combination of an entomopathogenic nematode and the fungus *Metarhizium anisopliae* CLO 53. Biol. Control. 39**,** 453-459.

Ansari, M. A., Tirry, L., Moens, M., 2004. Interaction between *Metarhizium anisopliae* CLO 53 and entomopathogenic nematodes for the control of *Hoplia philanthus*. Biol. Control. 31**,** 172–180.

Choo, H. Y., Kaya, H. K., Huh, J., Lee, D. W., Kim, H. H., Lee, S. M., Choo, Y. M., 2002. Entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis bacteriophora*) and a fungus *Beauveria brongniartii* for biological control of the white grubs, *Ectinohoplia rufipes* and *Exomala orientalis*, in Korean golf courses. BioControl. 47**,** 177-192.

Dimock, W. J., Spatial factors affecting white grub presence and abundance in golf course turf. Virginia Polytechnic Institute and State University,, Blacksburg, VA, 2004.

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

Georgis, R., Gaugler, R., 1991. Predictability in biological control using entomopathogenic nematodes. J. Econ. Entomol. 84**,** 713-720.

Glazer, I., 2002. Survival biology. In: R. Gaugler, (Ed.), Entomopathogenic Nematology. CABI Publishing, Wallingford, UK, pp. 169–187.

Grewal, P. S., Koppenhöfer, A. M., Choo, H. Y., 2005. Lawn, turfgrass and pasture applications. In: P. S. Grewal, et al., (Eds.), Nematodes as Biocontrol Agents. CABI Publishing, Wallingford, UK, pp. 115-146.

Jaques, R. P., Morris, O. N., 1981. Compatibility of pathogens with other methods of pest control and with different crops. In: H. D. Burges, (Ed.), Microbial Control of Pests and Plant Disease1970–1980. Academic Press, London, pp. 695–715.

Kaya, H. K., 1990. Soil ecology. In: R. Gaugler, H. K. Kaya, (Eds.), Entomopathogenic Nematodes in Biological Control. CRC Press, Boca Raton, FL, pp. 93-115.

Kaya, H. K., 2002. Natural enemies and other antagonists. In: R. Gaugler, (Ed.), Entomopathogenic Nematology. CABI Publishing, Wallingford, UK, pp. 189–203.

Kaya, H. K., Koppenhöfer, A. M., 1996. Effects of microbial and other antagonistic organism and competition on entomopathogenic nematodes. Biocontrol Sci. Technol. 6**,** 357-371.

Kaya, H. K., Stock, S. P., 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., 1990. Efficacy against soil-inhabiting insect pests. In: R. Gaugler, H. K. Kaya, (Eds.), Entomopathogenic Nematodes in Biological Control. CRC press, Boca Raton, FL, pp. 195-214.

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

Koppenhöfer, A. M., Fuzy, E. M., Crocker, R., Gelernter, W., Polavarapu, S., 2004. Pathogenicity of *Heterorhabditis bacteriophora*, *Steinernema glaseri*, and *S. scarabaei* (Rhabditida: Heterorhabditidae, Steinernematidae) against 12 white grub species (Coleoptera: Scarabaeidae). Biocontrol Sci. Technol. 14**,** 87-92.

Koppenhöfer, A. M., Grewal, P. S., Fuzy, E. M., 2006. Virulence of the entomopathogenic nematodes *Heterorhabditis bacteriophora*, *Heterorhabditis zealandica*, and *Steinernema scarabaei* against five white grub species (Coleoptera: Scarabaeidae) of economic importance in turfgrass in North America. Biol. Control. 38**,** 397-404.

Lacey, L. A., Brooks, W. M., 1997. Initial handling and diagnosis of diseased insects. In: L. A. Lacey, (Ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, CA pp. 5.

McVay, J. R., Gudauskas, R. T., Harper, J. D., 1977. Effect of *Bacillus thuringiensis* and chemical insecticides on *Spodoptera littoralis* (Lepidoptera: Noctuidae). J. Econ. Entomol. 77**,** 590-885.

Smits, P. H., 1996. Post-application persistence of entomopathogenic nematodes. Biocontrol Sci. Technol. 6**,** 379-387.

CHAPTER 5

Field Efficacy of Entomopathogenic Nematodes and Entomopathogenic Fungi against White Grub (Coleoptera: Scarabaeidae) Complex in Turfgrass

Abstract

Entomopathogenic nematodes (EPN) and entomopathogenic fungi (EPF) were evaluated for their efficacy against the white grub complex, including Japanese beetle (*Popillia japonica*) and masked chafers (*Cyclocephala* spp.), at different developmental stages under field conditions for up to 4 consecutive yr. Two EPN species, *Heterorhabditis bacteriophora* and *H. megidis*, and two EPF species, *Beauveria bassiana* and *Metarhizium anisopliae*, in different formulations were used. No significant difference was detected between *H. megidis* and *H. bacteriophora* alone or between nematode applications made on Jul-28 and Aug-13, 2010. The combined use of EPF and EPN improved the efficacy compared with nematodes or fungi applied alone in some of the treatments. EPN and EPF applied alone or in combination were not more effective than the imidacloprid insecticide in grub control in >50% field trials or treatments, but some EPN + EPF treatments were more effective than the insecticide in reducing grub numbers. The efficacy of EPF and EPN varied dramatically with field sites and conditions, and those that provided good grub control in some trials were not equally effective in others. Environmental conditions, especially temperature, might explain the inconsistent efficacy of nematodes and fungi in white grub management. EPN and EPF showed better potential for providing extended control of white grubs in the next generation than insecticides.

Keywords: white grub, turfgrass, entomopathogenic nematode, entomopathogenic fungus

Introduction

Japanese beetle (*Popillia japonica* Newman) and masked chafers (*Cyclocephala* spp.) are the most important white grub species in Virginia (Dimock, 2004). In some areas, e.g. Blacksburg and Bristol (VA), masked chafer numbers have largely surpassed Japanese beetle in recent years, and become the predominant white grub species in turfgrass (Wu et al. unpublished data). Heavily infested turfgrass turns brown and becomes spongy making it easy to be pulled up, and the grass eventually dies from dehydration. More often, the most significant damage is caused by small vertebrates, like birds and skunks, tearing the turf into pieces when they hunt for grubs as food.

Currently, the most prevalent method in white grub management is to apply insecticides, e.g. imidacloprid or clothianidin, on a preventative basis before damage occurs, and large spraying areas are generally required to provide good coverage. However, these chemicals are only effective against white grubs in the earliest life stages as efficacy declines on larger grubs. Thus, if early attempts fail, vigorously

feeding 3rd instars can cause severe damage in the fall. Difficulties in achieving long-term and consistent efficacy against white grubs with chemical approaches, and the increasing public concern about the impact of insecticides on the environment, have given more importance to the development of biological control strategies.

Entomopathogenic nematodes (EPN) and entomopathogenic fungi (EPF) are environmentally safe, and appear to be good alternatives to chemical insecticides in white grub control (Jackson and Glare, 1992). Klein (1990) summarized the field efficacy of using EPN against various white grub species, including *P. japonica* and *Cyclocephala* spp. In addition, several studies have updated the efficacy of EPN in white grub control in the field (Cappaert and Koppenhöfer, 2003; Grewal et al., 2004; Koppenhöfer et al., 2000a; Koppenhöfer et al., 1999; Koppenhöfer et al., 2002; Koppenhöfer and Fuzy, 2003a; Koppenhöfer and Fuzy, 2003b; Koppenhöfer et al., 2000c). According to these studies, EPN may have potential in white grub management, but their efficacies vary significantly under field conditions. EPF species like the green muscardine fungus *Metarhizium anisopliae* (Metschn.) Sorokin and white muscardine fungus *Beauveria bassiana* (Balsamo) Vuillemin are pathogenic to white grubs (Glare, 1992), but their field performances are also constrained by environmental conditions (Wraight et al., 2007).

The combined application of EPN and EPF may achieve a higher level of control against white grubs than either alone. For example, additive or synergistic interactions have been reported from the combination of EPN *Heterorhabditis bacteriophora* Poinar and EPF *M. anisopliae* isolate MM against the barley chafer grub, *Coptognathus curtipennis* Faimaire (Anbesse et al., 2008). Similar effects were also seen in the interaction between *M. anisopliae* CLO 53 and nematode *H. megidis* Poinar, Jackson & Klein or *Steinernema glaseri* (Steiner) against 3rd instar *Hoplia philanthus* Füessly under laboratory and greenhouse conditions (Ansari et al., 2004), and between *M. anisopliae* CLO 53 and *H. bacteriophora* in the field (Ansari et al., 2006). In addition, Choo et al. (2002) also reported that the combination of *S. carpocapsae* (Weiser) with *B. brongniartii* (Saccardo) Petch resulted in a significant increase in mortality of grub *Exomala orientalis* (Waterhouse) over the application of the fungus alone.

In previous laboratory experiments, two EPN species *H. bacteriophora* and *H. megidis*, and two EPF *M. anisopliae* and *B. bassiana* were selected for the efficacy against masked chafer grubs, and additive interaction had been detected from the combined use of a nematode and a fungus (Wu et al. unpublished data). The objective of the current study was to test the field efficacy of the selected EPN and EPF species against the white grub complex, especially masked chafers, at different developmental stages, and to explore the potential for improving the management effect by combining the two types of agents in field applications.

Materials and Methods

1. Efficacy of *M. anisopliae* F-52 EC (Met 52 EC) in 2009

The experiment was conducted in late July at Tazewell Country Club (Pounding Mill, VA) and Virginian Country Club (Bristol, VA) in 2009 to evaluate the efficacy of various rates of Met 52 EC against white grubs on golf course turf. Met 52 EC is *M. anisopliae* strain F-52 formulated in oil emulsifiable concentration containing 5.5×10^9 conidia/g, provided by Dr. Jarrod E. Leland from Novozymes Biologicals, Inc. (Salem, VA). The turfgrass sward was comprised of 80% tall fescue (*Festuca arundinacea* Schreb.) and 20% Kentucky bluegrass (*Poa pratensis* L.) at both sites. For each site, five rates plus one untreated control (control, 0.4, 0.8, 1.6, 3.2 and 6.4 L/ha) were applied in four blocks arranged in a randomized complete block design. Treatments in this and following experiments were applied as foliar sprays using a CO_2 backpack sprayer equipped with 4, 8008VS stainless steel spray tips and calibrated to deliver 748 L water per hectare at the pressure of 2.76×10^5 Pa. Each plot size was 1.2 m by 1.5 m. At both sites, approximately 12.7 mm of overhead irrigation water was applied immediately after treatments were applied. No fungicides were applied at either site. White grub counts were taken about 2 months after application at the depth of 3.8 cm with a sod cutter. Both thatch and soil in the sampled area were thoroughly checked. The mean temperature was 20 ^oC (7.8 - 28.9 ^oC) at the Tazewell Country Club site, and 21.1 ^oC (5.6 - 30.6 ^oC) at the Virginian Country Club site. The average precipitation was 1.3 mm per day at the Virginian Country Club; precipitation data were not available for the Tazewell site. Soil type was unknown at either site.

2. Efficacy of *B. bassiana* ES and two EPN species against 1st and 2nd instars in 2010

The field experiment was conducted at the Virginia Tech Turfgrass Research Center (VT TRC) (Blacksburg, VA) in late summer to fall 2010 to target the 1st and 2nd instar white grubs. The site chosen had a variety of grass types (Kentucky bluegrass *Poa pratensis* L. 56%, perennial ryegrass *Lolium perenne* L. 33%, creeping bentgrass *Agrostis stolonifera* L. 9%, tall fescue *Festuca arundinacea* Schreb. 2%), with a history of white grub infestation, and was not sprayed with any insecticide for over 3 yr. EPF *B. bassiana* strain GHA in emulsifiable formulation (*B. bassiana* ES), EPNs *H. bacteriophora* and *H. megidis*, and a standard insecticide Merit 75 WP were used in this experiment, at the rates listed in Table 1. Merit 75 WP was an imidacloprid insecticide from Bayer Co. labeled for preventative control of white grubs. *B. bassiana* ES was a commercially available product labeled as BotaniGard ES from Laverlam International Co. (Butte, MT). The nematodes used in this and following experiments were provided by Becker Underwood Co. (Ames, IA). There were 9 treatments, including untreated check, Merit 75 WP, *B. bassiana* ES, *B. bassiana* ES + *H. bacteriophora*, *B. bassiana* ES + *H. megidis*, *H. bacteriophora* alone on July 28 or August 13, *H. megidis* alone on July 28 or August 13. Merit 75 WP and *B. bassiana* ES

were applied on July 28, while treatments with both *B. bassiana* ES and EPNs (*H. bacteriophora* or *H. megidis*) had *B. bassiana* ES applied on July 28 with added EPNs on August 13.

Table 1. Materials and rates used in field trials.

Material	Active Ingredient	Rate
Merit 75 WP	Imidacloprid 75% wettable powder	451 g/ha
BotaniGard ES (*B. bassiana* ES)	*Beauveria bassiana* GHA emulsifiable solution (2.1×10^{10} viable spores/ml)	25.6 L/ha
BotaniGard WP (*B. bassiana* WP)	*B. bassiana* GHA wettable powder (4.4×10^{10} viable spores/g)	12.3 kg/ha
Met 52 EC (*M. anisopliae* EC)	*Metarhizium anisopliae* F-52 oil emulsifiable concentration (5.5×10^{9} colony forming units (CFU)/g)	6.4 L/ha
Met 52 G (*M. anisopliae* G)	*M. anisopliae* F-52 granular formulation (9×10^{8} colony forming units (CFU)/g)	98.2 kg/ha
Silwet L-77	Organosilicone surfactant	0.25% v/v
Nemasys H	*Heterorhabditis megidis* infective juveniles (IJs)	5 billion IJs/ha
Nemasys G	*H. bacteriophora* IJs	5 billion IJs/ha

Four blocks and a randomized complete block design were used. Each plot size was 1.2 m by 1.5 m. In addition, there was a 0.9 m-wide buffer area between adjacent treatments. Mesh filters on the spray tips were removed to allow the nematodes to pass. The microbials were applied at sunset to avoid sun exposure. The treated area was overhead irrigated with a sprinkler before and immediately after the application for 12.7 mm, and then on a daily basis for 10 d. The results were assessed on October 1, 2010 and September 2, 2011 (1 yr after application). The mean air temperature and mean precipitation were 21 ^{o}C (range of 7 to 33 ^{o}C) and 4 mm per day for the period from July 28 to October 1, and were 12.8 ^{o}C (range of -14.4 to 33.9 ^{o}C) and 3.6 mm per day from July 28, 2010 to September 2, 2011. The soil was a loam texture, comprised of 44.8% sand, 45.8% silt, 9.4% clay and 4% organic matter with a pH of 5.8.

3. Efficacy of EPF and *H. bacteriophora* on white grubs at various developmental stages in 2011

3.1. Effect of EPF and *H. bacteriophora* on 1st to 2nd instars

Microbial agents *B. bassiana* GHA in emulsifiable (*B. bassiana* ES) and wettable powder (*B. bassiana* WP) formulations, *M. anisopliae* EC, *H. bacteriophora,* and Merit 75 WP, were applied at the rates listed in Table 1. This experiment was comprised of nine treatments, including a water control, Merit 75 WP,

M. anisopliae EC; *B. bassiana* ES; *B. bassiana* WP; *H. bacteriophora*; *H. bacteriophora* + *M. anisopliae* EC; *H. bacteriophora* + *B. bassiana* ES; *H. bacteriophora* + *B. bassiana* WP. Merit and fungi were applied at the same time in early July, while all IJs were added 4 wk later. Four blocks and randomized complete block design were used. The experiment was replicated at three sites; two (Site 1 and 2) were located at Virginia Tech Golf Course (Blacksburg, VA) fairway (70% Zoysia grass *Zoysia japonica* L., 20% Kentucky bluegrass, and 10% tall fescue), and one (Site 3) at Virginian Country Club rough area (100% Kentucky bluegrass).

Each plot size was 1.5 m by 1.5 m, with a 1.5 m wide buffer (Site 1 and 2) or 0.9 m wide (Site 3) area between adjacent treatments. Grub counts were made 4 wk after the nematode application. Soil at all sites was a silt loam texture. Site 1 was comprised of 21.4% sand, 65.8% silt, 12.8% clay, 3.9% organic matter with a pH of 5.78; Site 2 was 35.2% sand, 54.1% silt, 10.7% clay, 8.1% organic matter with a pH of 6.1; and Site 3 was 40.9% sand, 49.6% silt, 9.5% clay and 10% organic matter with a pH of 5.6. The mean temperature and average precipitation were 22.8 ^{o}C (8.9 - 33.9 ^{o}C) and 3.8 mm per day for site 1 & 2, 22.8 ^{o}C (11.7 - 32.8 ^{o}C) and 2.5 mm per day for site 3.

3.2. Effect of EPF and *H. bacteriophora* on 2nd to 3rd instars

The target insects in this experiment were 2nd to 3rd instars. Experimental design and procedures were the same as in Experiment 3.1, except that the application time was 40 d later for both the nematode and fungi. Sites chosen were adjacent to those in 3.1. The mean temperature and average precipitation were 17.8 ^{o}C (3.3 - 32.2 ^{o}C) and 3.3 mm per day at Virginia Tech Golf Course site, 18.3 ^{o}C (2.8 - 32.8 ^{o}C) and 2.3 mm per day at Virginian Country Club site.

4. Efficacy of EPF and *H. bacteriophora* against white grubs in 2012

4.1. Spring trials

The field trials testing the efficacy of *H. bacteriophora* and *M. anisopliae* or *B. bassiana* were carried out in early May to target the overwintered white grubs. The experiment was repeated at two sites, one at Tazewell Country Club and one at VT TRC. Grass type in both sites was 100% Kentucky bluegrass. Materials and the rate used are listed in Table 1. There were 13 treatments, including an untreated control, Merit 75 WP, *B. bassiana* ES, *B. bassiana* WP, *M. anisopliae* EC, *M. anisopliae* EC + silwet L-77, *M. anisopliae* G, *H. bacteriophora*, *B. bassiana* ES + *H. bacteriophora*, *B. bassiana* WP + *H. bacteriophora*, *M. anisopliae* EC + *H. bacteriophora*, *M. anisopliae* EC + Silwet L-77 + *H. bacteriophora*, *M. anisopliae* G + *H. bacteriophora*. All agents were delivered to the field at the same time. Four blocks and randomized complete block design were used. *M. anisopliae* G was dissolved in water and filtered with cloth before spray.

Plot size was 1.5 m × 1.5 m, with a 0.9 m-wide buffer between adjacent treatments. Grub counts were made 3 wk after the application, before the insects reached the adult stage. The residual effect of agents was evaluated in September, 2012. The mean temperature and average precipitation were 17.8 ^{o}C (2.2 - 29.4 ^{o}C) and 3.8 mm per day for 21 days after treatment (DAT), 20.6 ^{o}C (2.2 - 35 ^{o}C) and 3.3 mm per day for 133 DAT at VT TRC site; 18.3 ^{o}C (5 - 30.6 ^{o}C) 22 DAT, and 20.6 ^{o}C (5 - 36.7 ^{o}C) 136 DAT at the Tazewell site. Precipitation data were not available for the Tazewell site. Soil was a loam soil texture at both VT TRC site (41.7% sand, 45.3 silt, 13% clay; 5.6% organic matter; pH=7.2) and Tazewell site (36.3% sand, 47.5% silt, 16.2% clay; 5.3% organic matter; pH=6.4).

4.2. Fall trials

The field trials were carried out in late August, 2012 to target 3rd instars. The experiment was replicated at one site at Tazewell Country Club (Kentucky bluegrass 75%, tall fescue 12.5%, perennial ryegrass 12.5%) and two sites at VT TRC (VT TRC 1: Kentucky bluegrass 65%, fine fescue (*Festuca rubra* L.) 25%, tall fescue 10%; VT TRC 2: Kentucky bluegrass 100%). Treatments and experimental design were the same as the spring trials. Live grub counts were made 4 wk after application. The mean temperature was 18.9 ^{o}C (2.8 - 31.7 ^{o}C) at Tazewell site, and 17.8 ^{o}C (1.7 - 31.1 ^{o}C) at VT TRC sites. The average precipitation was 3.6 mm per day at VT TRC sites; precipitation data were not available for the Tazewell site. Soil was a silt loam texture at the Tazewell site (15.3% sand, 65.3% silt, 19.4% clay; 6% organic matter; pH=5.7), and was a loam texture at both VT TRC 1 (47.3% sand, 39.8% silt, 12.9% clay; 2.8% organic matter; pH=5.6) and VT TRC 2 (42.5% sand, 45.2% silt, 12.3% clay; 3% organic matter, pH=5.7).

Data Analysis

Analysis of Variance (ANOVA) with software JMP 10.0 (SAS, Cary, NC) was used to detect significant differences among treatments at α=0.05. Grub counts were transformed with the formula $y=\sqrt{x+0.5}$ before analysis. Untransformed data were presented in Tables as mean ± SEM.

Results

1. Efficacy of *M. anisopliae* F-52 EC (Met 52 EC) in 2009

At Site 1 (Tazewell Country Club), 69 white grubs representing four genera were collected and identified (Table 2). At Site 2 (Virginian Country Club), 100% of the 27 total white grubs collected were identified as masked chafers.

The treatment source of variation for white grubs was not significant at either Site 1 (F=0.14, d.f.=5, *P*=0.980) or Site 2 (F=0.36, d.f.=5, *P*=0.867). At Site 1, percentage of white grub control ranged from 0% in the 0.4 L/ha treatment to 57% in the 3.2 L/ha treatment. At Site 2, control efficacy ranged from 0% in

the 0.8 L/ha treatment to 100% in the 6.4 L/ha treatment (Table 3). Although high rates of Met 52 EC were not statistically different from the lower rates in live grub counts, the high rates, 3.2 and 6.4 L/ha, did show certain efficacy in reducing grub population.

Table 2. White grub species identified in 2009 field trial site at Tazewell Country Club.

Common name	Species	% of total (n)
Masked chafers	*Cyclocephala* spp.	84.1 (n = 58)
Japanese beetle	*Popillia japonica*	11.6 (n = 8)
Black turfgrass ataenius	*Ataenius spretulus*	2.9 (n = 2)
Green June beetle	*Cotinis nitida*	1.4 (n = 1)

Table 3. Field efficacy of *Metarhizium anisopliae* F-52 EC (Met 52 EC) at various rates at Site 1-Tazewell Country Club and Site 2-Virginian Country Club in 2009 (mean white grubs/m^2 ±SEM) (% control in parentheses).

Treatment (Rate)	Site 1	Site 2
Control	14.3 ± 9.8	20.3 ± 20.3
Met 52 EC (0.4 L/ha)	15.3 ± 8.6 (0)	2.8 ± 2.8 (86)
Met 52 EC (0.8 L/ha)	9.0 ± 5.4 (37)	21.5 ± 21.5 (0)
Met 52 EC (1.6 L/ha)	11.7 ± 6.8 (18)	5.5 ± 5.5 (73)
Met 52 EC (3.2 L/ha)	6.2 ± 2.7 (57)	5.5 ± 5.5 (73)
Met 52 EC (6.4 L/ha)	8.1 ± 3.1 (43)	0.0 ± 0.0 (100)

2. Efficacy of *B. bassiana* ES and two EPN species against 1st and 2nd instars in 2010

At VT TRC site, 70.5% and 67.4% white grubs collected were identified as masked chafers in 2010 and 2011, respectively (Table 4), and were used for data analysis.

Merit 75 WP was most effective in reducing both the white grub complex and masked chafer grub numbers 65 DAT, followed by the combined use of *B. bassiana* ES and *H. bacteriophora*, which tended to perform better than a single agent. *H. bacteriophora* applied alone on Jul. 28 was least effective and

barely had any effect on grub control. However, there were no significant differences in live grub counts among various treatments either for the white grub complex (*P*=0.090) or masked chafers (*P*=0.139).

Table 4. White grub species at VT TRC site in 2010 and 2011.

White grub species	Scientific name	2010 (%)	2011 (%)
Masked chafers	*Cyclocephala* spp.	70.5	67.4
Asiatic garden beetle	*Maladera castanea*	21.9	29.1
Black turfgrass ataenius	*Ataenius spretulus*	5.5	--------
Green June beetle	*Cotinis nitida*	1.4	3.5
Japanese beetle	*Popillia japonica*	0.7	--------
Total grubs (N)	--------	146	141

There was no significant difference between *H. megidis* and *H. bacteriophora* alone 49-65 DAT (F=0.00, d.f.=1, *P*=0.979 for the grub complex; F=0.24, d.f.=1, *P*=0.635 for masked chafer), or between the two application times (F=0.19, d.f.=1, *P*=0.668 for the grub complex; F=0.01, d.f.=1, *P*=0.917 for masked chafer), and no significant interaction was found between nematode species and the time applied (F=2.75, d.f.=1, *P*=0.123 for the grub complex; F=0.98, d.f.=1, *P*=0.343 for masked chafer) (two-way ANOVA). Live grubs collected from the field trial were placed in individual solo cups and cultured in an incubator of LD 13:11, 20 ^{o}C for further observation. No grub showed white fungus or nematode infection after 4 wk.

One year post application, significant difference was observed among various treatments against the grub complex (*P*=0.031). *H. bacteriophora* applied alone on Aug. 13 had the highest efficacy, and provided 85% control of the grub complex in the next generation. *B. bassiana* ES + *H. bacteriophora* (Bb ES+Hb), and *H. megidis* applied alone on Aug. 13 also reduced the grub complex / masked chafer densities, but grub counts were not significantly different from the control. The insecticide Merit 75 WP was not effective 401 DAT in grub control. Masked chafer grub counts were not significantly different among treatments (*P*=0.367), although *H. bacteriophora* applied alone on Aug. 13 controlled 88% of the population (Table 5). Noticeably, treatments that provided residual efficacy against grubs in the new generation were mostly consistent with those giving considerable control of grub numbers in 2010, except Merit 75 WP and *H. megidis* applied on Jul. 28.

Table 5. Live white grubs counts from various treatments at VT TRC on Oct. 01, 2010 (49-65 DAT), and Sep. 02, 2011 (1 yr after treatment) (mean white grubs/m^2±SEM) (% control in parentheses).

Treatment [a]	Oct. 01, 2010		Sep. 02, 2011	
	Grub Complex	Masked chafer	Grub Complex	Masked chafer
Control	29.6 ± 12.1	26.9 ± 13.4	35.0 ± 7.8 a	22.9 ± 10.8
Merit 75 WP	1.3 ± 1.3 (96)	0.0 ± 0.0 (100)	51.1 ± 13.5 a (0)	24.2 ± 14.2 (0)
Bb ES	29.6 ± 11.1 (0)	17.5 ± 7.1 (35)	51.1 ± 26.9 a (0)	45.7 ± 28.6 (0)
Hb (Jul-28)	32.3 ± 9.1 (0)	24.2 ± 10.9 (10)	64.6 ± 24.5 a (0)	51.1 ± 26.1 (0)
Hm (Jul-28)	17.5 ± 6.0 (41)	9.4 ± 2.6 (65)	37.7 ± 12.8 a (0)	29.6 ± 13.5 (0)
Bb ES + Hb	12.1 ± 5.1 (59)	6.7 ± 1.3 (75)	21.5 ± 6.2 ab (39)	13.5 ± 6.8 (41)
Bb ES + Hm	29.6 ± 14.8 (0)	14.8 ± 9.7 (45)	53.8 ± 13.9 a (0)	21.5 ± 11.6 (6)
Hb (Aug-13)	17.5 ± 9.2 (41)	17.5 ± 9.2 (35)	5.4 ± 3.1 b (85)	2.7 ± 2.7 (88)
Hm (Aug-13)	26.9 ± 5.8 (9)	21.5 ± 7.9 (20)	24.2 ± 5.2 ab (31)	21.5 ± 4.4 (6)
Statistics *F*	*1.97*	*1.72*	*2.58*	*1.14*
Statistics *d.f.*	*8*	*8*	*8*	*8*
Statistics *P*	*0.090*	*0.139*	*0.031**	*0.367*

[a] Treatment Bb=*Beauveria bassiana* ES; Hb=*Heterorhabditis bacteriophora*; Hm=*H. megidis.* Bb+Hb (or Hm) = *B. bassiana* ES applied on Jul-28 with *H. bacteriophora* (or *H. megidis*) added on Aug-13.

* Indicates significant difference among treatments at α=0.05. Different letters indicate significance from multiple comparisons (Fisher's LSD, α=0.05).

3. Efficacy of EPF and *H. bacteriophora* on white grubs at various developmental stages in 2011

3.1. Effect of EPF and *H. bacteriophora* on 1st to 2nd instars

At Site 1 and 3, due to the low grub activities, data were not available for analysis on efficacy of agents applied. At Site 2, white grubs were comprised of 47.4% masked chafer (n=18) and 52.6% black turfgrass ataenius (n=20). Merit 75 WP tended to be most effective, followed by *B. bassiana* WP + *H. bacteriophora* and *M. anisopliae* EC. Also, a trend showed that the combination of *B. bassiana* WP and *H. bacteriophora* outperformed the treatment with either *B. bassiana* WP alone or *H. bacteriophora* alone. However, no significant difference was observed among various treatments for the white grub

complex (F=1.05, d.f.=8, *P*=0.425), or masked chafer grubs (F=0.12, d.f.=8, *P*=0.381) (Table 6). The low grub densities might have masked some treatment effects.

Table 6. Live white grub counts from various treatments against 1st-2nd instars 4 wk after nematode application at Site 2 (Virginia Tech Golf Course) (mean white grubs/m^2 ±SEM) (% control in parentheses).

Treatment [a]	White grub complex	Masked chafer
Control	13.5 ± 7.8	12.1 ± 7.1
Merit 75 WP	0.0 ± 0.0 (100)	0.0 ± 0.0 (100)
Beauveria bassiana ES	8.1 ± 8.1 (40)	8.1 ± 8.1 (33)
B. bassiana WP	26.9 ± 13.5 (0)	16.1 ± 16.1 (0)
Metarhizium anisopliae EC	5.4 ± 5.4 (60)	0.0 ± 0.0 (100)
Heterorhabditis bacteriophora	10.8 ± 0.0 (20)	0.0 ± 0.0 (100)
B. bassiana ES+*H. bacteriophora*	8.1 ± 8.1 (40)	0.0 ± 0.0 (100)
B. bassiana WP+*H. bacteriophora*	2.7 ± 2.7 (80)	0.0 ± 0.0 (100)
M. anisopliae EC+*H. bacteriophora*	13.5 ± 13.5 (0)	0.0 ± 0.0 (100)

[a] *Heterorhabditis bacteriophora* IJs were applied 4 wk after fungal application.

3.2. Effect of EPF and *H. bacteriophora* on 2nd to 3rd instars

Similar to Site 1 and 3 in Experiment 3.1 on 1st to 2nd instar grubs, few to no grubs were recovered in the untreated control in all 3 sites, and the remaining plots were not sampled. Lack of grub abundance in the field sites affected data analysis and the role of each agent under the field conditions.

4. Efficacy of EPF and *H. bacteriophora* against white grubs in 2012

The percentage of each white grub species collected from 2012 spring and fall field sites were listed in Table 7. For spring sites, scarabs collected 3 wk after treatment included a proportion of pupae and were not identified to species; data were analyzed in white grub/pupa complex. For data collected in fall sites and 133-136 DAT in spring sites, both the grub complex and the major species were used for data analysis.

Table 7. White grub species in spring and fall field sites in 2012.

White Grub Species	Scientific Name	Spring Sites		Fall Sites		
		Tazewell	VT TRC	Tazewell	VT TRC 1	VT TRC 2
Japanese beetle	*Popillia japonica*	65.6%*	1.9%	50.5%*	0.4%	3.4%
Masked chafer	*Cyclocephala* spp.	4.5%	80.8%*	33.6%*	94.2%*	89.4%*
May/June beetle	*Phyllophaga* spp.	15.6%	--------	14.3%	2.7%	3.4%
Asiatic garden beetle	*Maladera castanea*	14.3%	14.7%	1.6%	2.7%	3.4%
Green June beetle	*Cotinis nitida*	--------	2.6%	--------	--------	0.4%
Total grubs (N)	--------	578	266	307	259	265

*means the major grub species used for data analysis at each site.

4.1. Spring trials

At Tazewell site, no significant difference was found among treatments 22 DAT (P=0.212), which was probably due to the low scarab densities. *H. bacteriophora* alone and *B. bassiana* ES alone tended to be more effective than Merit 75 WP, but statistically not significant (Table 8). In addition, 12 grubs and pupae were found infected with EPF 22 DAT.

For data collected 136 DAT, significant differences were found among various treatments for the white grub complex (P=0.003) and Japanese beetle (P=0.003). Merit 75 WP significantly reduced both the white grub complex and Japanese beetle numbers. Among treatments with EPN and/or EPF, *B. bassiana* WP + *H. bacteriophora* (Bb WP+Hb) and *M. anisopliae* G + *H. bacteriophora* (Met G+Hb) reduced grub numbers, but grub counts were not significantly different from the untreated control (Table 8). In addition, an EPN-infected grub and an EPF-infected grub were found in the treatment with *B. bassiana* ES + *H. bacteriophora* (Bb ES+Hb), and Bb WP+Hb, respectively.

The combination of *B. bassiana* WP with *H. bacteriophora* showed improved efficacy over that of *B. bassiana* WP alone against the grub complex / Japanese beetle. In addition, *M. anisopliae* granular formulation (Met G) appeared to be more effective than the oil emulsifiable formulation (Met EC) in reducing grub numbers when applied alone or in combination with *H. bacteriophora*, but they were not significantly different. Adding wetting agent Silwet L-77 to *M. anisopliae* EC showed no significant reduction in grub counts (Table 8).

Table 8. Field efficacy of various treatments applied in spring 2012 against overwintered grub/pupa complex (22 DAT) and the residual effect on the grub complex and Japanese beetle (136 DAT) in the next generation at Tazewell Country Club (mean white grub/m^2±SEM) (% control in parentheses).

Treatment [a]	22 DAT	136 DAT	
	Grub/pupa complex	White grub complex	Japanese beetle
Control	29.6 ± 4.7	103.6 ± 15.9 abcd	75.3 ± 7.3 abc
Merit 75 WP	18.8 ± 5.2 (36)	16.1 ± 5.4 e (84)	2.7 ± 2.7 d (96)
Bb ES	8.1 ± 5.2 (73)	94.2 ± 33.9 bcd (9)	64.6 ± 25.2 abc (14)
Bb WP	16.1 ± 5.4 (46)	183.0 ± 37.5 a (0)	123.8 ± 36.6 a (0)
Met EC	29.6 ± 10.2 (0)	169.5 ± 26.5 ab (0)	91.5 ± 32.4 abc (0)
Met EC+S	26.9 ± 10.3 (9)	126.5 ± 5.2 abcd (0)	86.1 ± 15.2 abc (0)
Met G	26.9 ± 9.3 (9)	107.6 ± 27.8 abcd (0)	91.5 ± 28.6 abc (0)
Hb	5.4 ± 3.1 (82)	107.6 ± 9.8 abcd (0)	43.1 ± 13.9 bc (43)
Bb ES+Hb	35.0 ± 6.8 (0)	115.7 ± 50.8 abcd (0)	86.1 ± 31.7 abc (0)
Bb WP+Hb	26.9 ± 9.3 (9)	61.9 ± 24.2 de (40)	32.3 ± 7.6 c (57)
Met EC+Hb	16.1 ± 5.4 (46)	150.7 ± 36.5 abc (0)	105.0 ± 22.1 ab (0)
Met EC+S+Hb	16.1 ± 3.1 (46)	134.6 ± 29.3 abcd (0)	80.7 ± 19.9 abc (0)
Met G+Hb	16.1 ± 6.9 (46)	80.7 ± 26.9 cd (22)	51.1 ± 26.1 bc (32)
Statistics *F*	*1.39*	*3.16*	*3.26*
Statistics *d.f.*	*12*	*12*	*12*
Statistics *P*	*0.212*	*0.003**	*0.003**

[a] Treatment Bb ES=*Beauveria bassiana* ES; Bb WP=*B. bassiana* WP; Met EC=*Metarhizium anisopliae* EC; Met G=*M. anisopliae* G; Hb=*Heterorhabditis bacteriophora*; S=Silwet L-77.

* Indicates significant difference among treatments at α=0.05. Different letters indicate significance from multiple comparisons (Fisher's LSD, α=0.05).

Table 9. Field efficacy of various treatments applied in spring 2012 against overwintered grub/pupa complex (21 DAT) and the residual effect on the grub complex and masked chafers (133 DAT) in the next generation at VT TRC (mean white grub/m^2±SEM) (% control in parentheses).

Treatment [a]	21 DAT	133 DAT	
	Grub/pupa complex	White grub complex	Masked chafer
Control	10.8 ± 2.2	48.4 ± 16.0	28.3 ± 9.2
Merit 75 WP	5.4 ± 5.4 (50)	40.4 ± 17.8 (17)	35.0 ± 17.8 (0)
Bb ES	10.8 ± 4.4 (0)	40.4 ± 25.8 (17)	37.7 ± 23.5 (0)
Bb WP	18.8 ± 5.2 (0)	21.5 ± 14.6 (56)	18.8 ± 15.5 (34)
Met EC	18.8 ± 6.8 (0)	51.1 ± 28.3 (0)	45.7 ± 28.6 (0)
Met EC+S	18.8 ± 8.1 (0)	102.3 ± 25.8 (0)	99.6 ± 24.2 (0)
Met G	2.7 ± 2.7 (75)	32.3 ± 13.2 (33)	26.9 ± 11.2 (5)
Hb	18.8 ± 5.2 (0)	51.1 ± 26.9 (0)	26.9 ± 16.1 (5)
Bb ES+Hb	2.7 ± 2.7 (75)	61.9 ± 18.3 (0)	48.4 ± 16.1 (0)
Bb WP+Hb	13.5 ± 5.2 (0)	70.0 ± 29.3 (0)	61.9 ± 26.5 (0)
Met EC+Hb	13.5 ± 6.8 (0)	53.8 ± 20.6 (0)	40.4 ± 22.1 (0)
Met EC+S+Hb	13.5 ± 6.8 (0)	21.5 ± 15.2 (56)	13.5 ± 8.1 (52)
Met G+Hb	5.4 ± 3.1 (50)	72.7 ± 52.4 (0)	67.3 ± 50.2 (0)
Statistics *F*	*1.71*	*0.80*	*0.88*
Statistics *d.f.*	*12*	*12*	*12*
Statistics *P*	*0.103*	*0.651*	*0.575*

[a] Treatment Bb ES=*Beauveria bassiana* ES; Bb WP=*B. bassiana* WP; Met EC=*Metarhizium anisopliae* EC; Met G=*M. anisopliae* G; Hb=*Heterorhabditis bacteriophora*; S=Silwet L-77.

At VT TRC, no significant differences were found among treatments for the white grub complex 21 DAT (*P*=0.103) or 133 DAT (*P*=0.651), or for masked chafer grubs 133 DAT (*P*=0.575) (Table 9). One nematode-infected pupa and 5 EPF-infected grubs were observed 21 DAT; compared with the untreated control, lower grub numbers were apparent in *M. anisopliae* G alone, Met G+Hb, and Bb ES+Hb, but

these were not significantly different. For data collected 133 DAT, one nematode-infected grub was found in treatment with Bb ES+Hb. Decline in grub density occurred in *B. bassiana* WP alone, *M. anisopliae* G alone, and in the combined use of *M. anisopliae* EC + Silwet L-75 and *H. bacteriophora* (Met EC+S+Hb), but grub counts were not significantly different from the control. Different from the Tazewell site, Merit 75 WP did not provide satisfactory control of the white grub complex or masked chafer numbers 133 DAT at VT TRC. Compared with *H. bacteriophora* or fungi alone, improved but not significantly different efficacy from the combined application was found in Bb ES+Hb 21 DAT against the grub complex and Met EC+S+Hb against the grub complex/masked chafer 133 DAT.

4.2. Fall trials

Data analyses were carried out for the white grub complex, masked chafer, and Japanese beetle separately at the Tazewell field site (Table 10). Significant differences were found among treatments for Japanese beetle grubs collected (P=0.048), although grub counts in the various treatments were not significantly different from the control (Table 10). Masked chafer grub numbers in various treatments were not significantly different from each other (P=0.605). Marginal significance was detected in the white grub complex (P=0.063), with a trend similar to that in Japanese beetle grubs. Two grubs were found infected with EPF.

Merit 75 WP tended to be more effective than treatments with EPN and/or EPF. *H. bacteriophora* alone, Bb ES+Hb, and the combined use of *H. bacteriophora* and *M. anisopliae* in different formulations reduced grub numbers, but were not significantly different from the untreated control. For both *B. bassiana* ES and *M. anisopliae* EC, the treatment with the combination of the nematode and a fungus had significantly lower Japanese beetle grub numbers than the fungus applied alone. Compared with *H. bacteriophora* alone, adding a fungus to the nematode did not improve the efficacy in grub control.

Table 10. Field efficacy of various treatments applied in fall 2012 against the white grub complex (WG), 3rd instar masked chafer (MC) or Japanese beetle (JB) at three sites (mean white grub/m^2 ±SEM) (% control in parentheses).

Treatment [a]	Tazewell			VT TRC 1		VT TRC 2	
	WG	MC	JB	WG	MC	WG	MC
Control	56.5±19.0	13.5±3.5	32.3±12.6 abcd	76.7±31.3	76.7±31.3	64.6±16.3	56.5±13.6
Merit 75 WP	13.5±6.8 (76)	2.7±2.7 (80)	2.7± 2.7 d (92)	0.0±0.0 (100)	0.0± 0.0 (100)	48.4±9.3 (25)	40.4±11.9 (28)

Bb ES	75.4±20.6 (0)	16.1±12.8 (0)	48.4±6.9 ab (0)	32.3±28.8 (58)	32.3±28.8 (58)	48.4±28.0 (25)	43.1±26.0 (24)
Bb WP	75.4±30.8 (0)	21.5±8.8 (0)	51.1±26.9 abc (0)	10.8±7.6 (86)	10.8±7.6 (86)	35.0±14.8 (46)	29.6±13.5 (48)
Met EC	113±25.4 (0)	35.0±31.5 (0)	67.3±16.1 a (0)	24.2±24.2 (68)	24.2±24.2 (68)	75.4±26.0 (0)	67.3±18.3 (0)
Met EC+S	64.6±4.4 (0)	29.6±9.2 (0)	32.3±7.6 abc (0)	91.5±33.3 (0)	78.0±26.9 (0)	67.3±22.1 (0)	64.6±20.1 (0)
Met G	99.6±11.9 (0)	40.4±15.5 (0)	37.7±10.3 abc (0)	45.7±45.7 (40)	45.7±45.7 (40)	51.1±29.6 (21)	40.4±22.1 (28)
Hb	40.4±20.3 (28)	18.8±9.2 (0)	10.8±7.6 cd (67)	78.0±28.3 (0)	70.0±20.4 (9)	24.2±9.2 (63)	21.5±9.8 (62)
Bb ES+Hb	45.7±18.3 (19)	26.9±6.9 (0)	13.5±10.2 cd (58)	40.4±20.8 (47)	37.7±20.8 (51)	48.4±16.7 (25)	45.7±14.2 (19)
Bb WP +Hb	72.7±30.9 (0)	16.1±10.3 (0)	40.4±26.9 abcd (0)	88.8±30.6 (0)	88.8±30.6 (0)	45.7±5.2 (29)	45.7±5.2 (19)
Met EC +Hb	37.7±19.9 (33)	26.9±16.1 (0)	10.8±4.4 cd (67)	53.8±33.8 (30)	51.1±31.5 (33)	48.4±11.2 (25)	43.1±9.8 (24)
Met EC+S +Hb	43.1±15.2 (24)	10.8±10.8 (20)	24.2±15.5 bcd (25)	51.1±19.3 (33)	40.4±18.8 (47)	29.6±2.7 (54)	24.2±2.7 (57)
Met G +Hb	32.3±8.8 (43)	5.4±3.1 (60)	13.5±5.2 bcd (58)	26.9±14.2 (65)	24.2±11.9 (68)	61.9±15.5 (4)	59.2±15.5 (0)
Statistics *F*	*1.92*	*0.85*	*2.03*	*1.28*	*1.23*	*0.72*	*0.94*
Statistics *d.f.*	*12*	*12*	*12*	*12*	*12*	*12*	*12*
Statistics *P*	*0.063*	*0.605*	*0.048**	*0.269*	*0.296*	*0.722*	*0.518*

[a] Treatment Bb ES=*Beauveria bassiana* ES; Bb WP=*B. bassiana* WP; Met EC=*Metarhizium anisopliae* EC; Met G=*M. anisopliae* G; Hb=*Heterorhabditis bacteriophora*; S=Silwet L-77.

* indicates significant difference among treatments at α=0.05. Different letters indicate significance from multiple comparisons (Fisher's LSD, α=0.05).

At VT TRC sites, data for the white grub complex and masked chafer grubs were used for analyses. There were no significant differences among treatments in the white grub complex or masked chafer grub

counts in either site (P>0.05) (Table 10), despite one EPF-infected grub being found in the treatment with *M. anisopliae* EC + Silwet L-77 (Met EC+S) at VT TRC 2.

At VT TRC 1, Merit appeared to be most effective; fungi applied alone achieved a certain level of grub control, except Met EC+S; *H. bacteriophora* alone and Bb WP+Hb were not effective. Treatments with the combination of a fungus and *H. bacteriophora* tended to have better efficacy than the nematode alone, except for Bb WP+Hb. Adding the nematode to Met EC+S and *M. anisopliae* G tended to improve efficacy in grub control, but the combination of the nematode and other fungal types did not perform better than fungi alone (Table 10).

At VT TRC 2, among all agents applied, the treatment with *H. bacteriophora* alone appeared to be most effective, followed by Met EC+S+Hb and *B. bassiana* WP, but they were not statistically different from Merit 75 WP. Among fungal treatments, only *M. anisopliae* EC and Met EC+S showed enhanced efficacy after adding the nematodes. The combined application of the nematodes and fungi did not have an improved effect in grub management than nematodes alone (Table 10). Further studies are required to verify the significance of treatment effects that showed certain trends that were not statistically different.

Discussion

The results showed that masked chafers were the predominant white grub species at the field sites at VT TRC (Blacksburg, VA) and Virginian Country Club (Bristol, VA), whereas both masked chafers and Japanese beetle were the most important species at Tazewell Country Club (Pounding Mill, VA) (Table 2, 4 & 7). With *M. anisopliae* EC applied alone, although treatments with higher rates tended to have better efficacy in reducing grub densities, no significant difference in live grub counts was detected among the rates applied (Table 3). Also, no significant difference was found either between *H. megidis* and *H. bacteriophora* alone or between nematode applications at Jul-28 and Aug-13 for 49-65 DAT in 2010 (Table 5). In laboratory experiments, *H. bacteriophora* was more effective than *H. megidis*, when it was applied alone, or in combination with *B. bassiana* ES (Wu et al. unpublished data).

Steinhaus (1958) stated that stressed insects are generally more susceptible to pathogen infection. A stressor like EPF or EPN may weaken the target insects and increase their susceptibility to other control agents and enhance insect mortality or facilitate the speed of kill that eventually leads to an additive or synergistic effect for the combined application of various agents in insect control. For example, it was demonstrated that milky spore disease bacterium, *Bacillus popilliae* Dutky, acted as a stressor in the increased susceptibility of scarab larvae to nematode infection (Thurston et al., 1993; Thurston et al., 1994). Additive or synergistic effect from the combined application of EPN or EPF with other control agents in grub control has been reported, e.g. EPN and bacterium *Bacillus thuringiensis* subspecies

japonensis Buibui strain (Koppenhöfer et al., 1999; Koppenhöfer and Kaya, 1997), EPN and insecticides (Koppenhöfer et al., 2002; Koppenhöfer et al., 2000b; Koppenhöfer and Kaya, 1998), EPF *M. anisopliae* and bacterium *Serratia entomophila* Grimont et al. (Glare, 1994), EPF and EPN (Anbesse et al., 2008; Ansari et al., 2006; Ansari et al., 2004). In previous laboratory experiments, additive interaction had been detected from the combined use of EPN *H. bacteriophora* or *H. megidis*, and EPF *M. anisopliae* or *B. bassiana* against masked chafer grubs (Wu et al. unpublished data).

The combined application of EPF and EPN might perform better than EPF alone. For example, improved efficacy was shown by the combination of *B. bassiana* WP with *H. bacteriophora* over that of *B. bassiana* WP alone against the grub complex / Japanese beetle (Table 8). Also, at the Tazewell site in fall 2012, the combination of *H. bacteriophora* and *B. bassiana* ES or *M. anisopliae* EC significantly improved efficacy against the Japanese beetle than with fungi applied alone. In the same trial, a trend of lower grub numbers appeared in the combined application of *H. bacteriophora* with other fungal agents than fungi alone, although these were not significantly different (Table 10). In addition, a similar but not statistically significant trend was also seen in Bb ES+Hb 49-65 DAT in Table 5; Bb WP+Hb in Table 6; Met EC/G +Hb and Met EC+S+Hb 22 DAT, and Met G+Hb 136 DAT in Table 8; Bb ES+Hb 21 DAT, and Met EC+S+Hb 133 DAT in Table 9; Met EC+S+Hb and Met G+Hb at VT TRC 1, and Met EC+Hb or Met EC+S+Hb at VT TRC 2 in Table 10. These findings were consistent with the laboratory studies that adding EPN to EPF significantly enhanced the effect in grub control compared with EPF alone (Wu et al. unpublished data). Similar to the current study, the combination of *S. carpocapsae* with *B. brongniartii* led to a significant increase in mortality of grub *E. orientalis* over the application of the fungus alone (Choo et al., 2002).

When comparing the field efficacy of EPN and EPF with Merit 75 WP, the insecticide achieved a significantly higher level of grub control for the data collected 136 DAT at the Tazewell site treated in spring 2012 (Table 8). A similar but not significantly different trend showed in 65 DAT in fall 2010 (Table 5), fall 2011 site 2 (Table 6), and sites at Tazewell & VT TRC 1 in fall 2012 (Table 10). However, some treatments with EPN and/or EPF achieved an effect higher than or comparable to the insecticide, e.g. 1 yr after treatment in fall 2010 (Table 5), 22 DAT at Tazewell site in spring 2012 (Table 8), VT TRC site treated in spring 2012 (Table 9) and VT TRC 2 site in fall 2012 (Table 10). Although nematode and fungal treatments tended to be less effective than Merit 75 WP in > 50% field trials, reduction in grub population was shown in some treatments with nematode and fungi alone or in combination in each trial. A series of efficacy trials revealed that the performance of EPF and EPN was not consistent. It varied dramatically with field sites and conditions, and the agents that provided good grub control in some trials were not equally effective in others, as seen in Table 8 versus Table 9, and comparing three sites in Table

10. Georgis and Gaugler (1991) summarized 82 nematode field trials against Japanese beetle larvae and showed that successful control occurred with *H. bacteriophora* when the following conditions were met: applications made in the fall, soil temperatures were > 20 ^{o}C, soil type was silt clay, irrigation frequency was at 1-4 d intervals, and thatch depth was < 10 mm. In the current study, temperature fluctuated vastly, with the lowest below 5 ^{o}C and highest above 30 ^{o}C in most field trials. This might account for the lack of an adequate and consistent effect in white grub management in the field.

Despite this, the use of EPN and EPF in white grub control has potential of providing extended control of grub populations in the next generation when insecticides fail to do so. EPN and EPF had been reported to recycle or persist in the field for a relatively long period of time when hosts are present (Forschler and Gardner, 1991b; Kaya et al., 1993; Rath et al., 1995b; Yokoyama et al., 1998). For example, Kaya et al. (1993) found that *S. feltiae* (Filipjev) could be recovered for 550 d and *H. bacteriophora* could be recovered for 260 d after application. Also, Rath et al. (1995b) demonstrated excellent soil persistence of *M. anisopliae* DAT F-001, and the level of the fungus in the soil increased dramatically by recycling on its scarab host. The potential of residual effects from the use of EPN and EPF was promising in the current study. *H. bacteriophora* applied alone on Aug. 13, 2010 significantly reduced grub numbers 1 yr after treatment; Bb ES + Hb and *H. megidis* applied alone on Aug. 13 also appeared to reduce grub densities, although the differences were not significant. In contrast, the insecticide showed no residual effect in grub control (Table 5). Similar but not statistically different trends in grub reduction also appeared in Bb WP+Hb and Met G+Hb for 136 DAT at the Tazewell site (Table 8); *B. bassiana* WP alone, *M. anisopliae* G alone, and Met EC+S+Hb 133 DAT at the VT TRC site sprayed in spring 2012 (Table 9). These indicate that the nematode and fungi might have recycled in the grub or other alternative hosts in the field, and provided additional control of the next generation. In support of this, an EPN-infected grub and an EPF-infected grub were found 136 DAT at the Tazewell site, and one grub was infected with EPN 133 DAT at VT TRC. Similarly, Klein and Georgis (1992a) reported that *H. bacteriophora* gave ≥ 90% control of the next generation of *P. japonica* 386 DAT.

Georgis and Gaugler (1991) summarized that the nematode application needed to be made in the fall to achieve successful control of Japanese beetle grubs. Also, according to Koppenhöfer and Fuzy (2004), nematodes might be more effective against white grubs in the early instars. In the current study, because of the inconsistent and inadequate performance of EPN and EPF, there was no dramatic difference among grub developmental stages in the field efficacy of the agents applied. Agents, especially EPN, applied in the spring would require alternative hosts to survive the drought in summer when annual species like Japanese beetle and masked chafers are not present as grub stages in the field, as persistence of EPN is poor in the absence of hosts (Smits, 1996). EPN and EPF applied in the fall may have better potential in

magnifying the level of IJs / conidia by recycling on the scarab hosts, especially when grubs are abundant, although their persistence and survival may depend on various biotic and abiotic factors (Koppenhöfer, 2007b; Wraight et al., 2007). Young larvae might be more easily controlled, but they hold less potential than older grubs in providing resources for the proliferation of nematodes and fungi, to infect the new population. This was seen in Table 5 that EPN species applied on August 13 alone or in combination with *B. bassiana* ES provided extended control of grubs in the next generation 1 yr after treatment, whereas the nematodes applied on July 28 failed to do so.

Field studies with different fungal types revealed that formulation might affect the efficacy of fungal agents in white grub control. *M. anisopliae* granular formulation (Met G) tended to be more effective than the oil emulsifiable formulation (Met EC) in reducing grub population when applied alone or in combination with *H. bacteriophora*, and adding wetting agent Silwet L-77 to *M. anisopliae* EC (Met EC+S) may improve the efficacy (Table 8-10). Further studies are required to confirm the significance of the different effects. In field applications, grass thatch may retain the active materials to prevent the conidia flowing into soil, and the delivered fungal agents thus fail to take effect for lack of adequate contact with the target insect, especially for materials poorly dissolvable in the carrier (water). Because of the oil formulation, Met EC dissolved more poorly in water than Met G, but becomes more dissolvable after adding wetting agent Silwet L-77. This might explain the less effectiveness of Met EC than Met G, and the improved efficacy after adding Silwet L-77 in grub control. As for the emulsifiable formulation and wettable powder of *B. bassiana*, it was not clear which formulation performed better than the other, as the trial results were inconsistent. Similarly, *M. anisopliae* tended to be more effective than *B. bassiana* in some treatments or trials, but less in others.

Six field trials were conducted in late summer to fall in 2011 to target white grubs at early or late stages. Unfortunately, very few grubs were recovered in these sites, and analysis on the field efficacy of the agents applied was either not available, or masked by the low grub densities (Table 6). The low grub activities were probably due to the drought during oviposition or egg hatch period in summer. Meanwhile, grass type might have also affected white grub activities, as the sites in Virginia Tech Golf Course were mainly covered with Zoysia grass, which is a warm season grass with strong and hardened root system developed across the field. In contrast to the low grub activities in these sites, a high masked chafer grub density of 50-100/m^2 was seen within 50 m where cool season grasses (tall fescue and Kentucky bluegrass) grew.

The nematodes were added 2 wk after fungal application in the 2010 trial on 1st to 2nd instars, 4 wk later in 2011 trials on all grub stages, but applied simultaneously with fungi in both spring and fall trials in 2012. Initially this design was to test the possible effect of time intervals between nematode and fungal

applications on the efficacy in grub control. Both Ansari et al. (2004) and Anbesse et al. (2008) stated that grubs had to be exposed to the fungus for at least 3 or 4 wk before the addition of nematodes to achieve stronger synergistic effects. In the current study, due to the low grub activities in field sites in 2011, the best time interval between the applications was not clear. However, in laboratory experiments, no advantage in enhancing efficacy was gained from the delayed delivery of nematodes 2 or 4 wk after fungal application (Wu et al. unpublished data).

In summary, EPN and EPF failed to show significant effects comparable to the imidacloprid insecticide in > 50% field trials or treatments, but reduction in grub numbers appeared in some nematode and/or fungal treatments in each trial. EPN and EPF have potential for recycling in the field to provide a residual effect in grub control, where these agents out-compete the insecticide. Environmental conditions, especially temperature, might explain the inconsistent and inadequate efficacy in white grub management with nematode and fungal agents in the field.

References

Anbesse, S. A., Adge, B. J., Gebru, W. M., 2008. Laboratory screening for virulent entomopathogenic nematodes (*Heterorhabditis bacteriophora* and *Steinernema yirgalemense*) and fungi (*Metarhizium anisopliae* and *Beauveria bassiana*) and assessment of possible synergistic effects of combined use against grubs of the barley chafer *Coptognathus curtipennis*. Nematology. 10**,** 701-709.

Ansari, M. A., Shah, F. A., Tirry, L., Moens, M., 2006. Field trials against *Hoplia philanthus* (Coleoptera: Scarabaeidae) with a combination of an entomopathogenic nematode and the fungus *Metarhizium anisopliae* CLO 53. Biol. Control. 39**,** 453-459.

Ansari, M. A., Tirry, L., Moens, M., 2004. Interaction between *Metarhizium anisopliae* CLO 53 and entomopathogenic nematodes for the control of *Hoplia philanthus*. Biol. Control. 31**,** 172–180.

Cappaert, D. L., Koppenhöfer, A. M., 2003. *Steinernema scarabaei*, an entomopathogenic nematode for control of the European chafer. Biol. Control. 28**,** 379-386.

Choo, H. Y., Kaya, H. K., Huh, J., Lee, D. W., Kim, H. H., Lee, S. M., Choo, Y. M., 2002. Entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis bacteriophora*) and a fungus *Beauveria brongniartii* for biological control of the white grubs, *Ectinohoplia rufipes* and *Exomala orientalis*, in Korean golf courses. BioControl. 47**,** 177-192.

Dimock, W. J., Spatial factors affecting white grub presence and abundance in golf course turf. Virginia Polytechnic Institute and State University,, Blacksburg, VA, 2004.

Forschler, B. T., Gardner, W. A., 1991. Field efficacy and persistence of entomogenous nematodes in the management of white grubs (Coleoptera: Scarabaeidae). J. Econ. Entomol. 84**,** 1454–1459.

Georgis, R., Gaugler, R., 1991. Predictability in biological control using entomopathogenic nematodes. J. Econ. Entomol. 84**,** 713-720.

Glare, T. R., 1992. Fungal pathogens of scarabs. In: T. A. Jackson, T. R. Glare, (Eds.), Use of Pathogens in Scarab Pest Management. Intercept, Andover, UK, pp. 63-77.

Glare, T. R., 1994. Stage-dependent synergism using *Metarhizium anisopliae* and *Serratia entomophila* against *Costelytra zealandica*. Biocontrol Sci. Technol. 4**,** 321–329.

Grewal, P. S., Power, K. T., Grewal, S. K., Suggars, A., Haupricht, S., 2004. Enhanced consistency in biological control of white grubs (Coleoptera: Scarabaeidae) with new strains of entomopathogenic nematodes. Biol. Control. 30**,** 73–82.

Jackson, T. A., Glare, T. R., 1992. Use of Pathogens in Scarab Pest Management. Intercept, Andover, UK.

Kaya, H. K., Klein, M. G., Burlando, T. M., 1993. Impact of *Bacillus popilliae*, *Rickettsiella popilliae* and entomopathogenic nematodes on a population of the scarabaeid, *Cyclocephala hirta*. Biocontrol Sci. Technol. 3**,** 443–453.

Klein, M. G., 1990. Efficacy against soil-inhabiting insect pests. In: R. Gaugler, H. K. Kaya, (Eds.), Entomopathogenic Nematodes in Biological Control. CRC press, Boca Raton, FL, pp. 195-214.

Klein, M. G., Georgis, R., 1992. Persistence of control of Japanese beetle (Coleoptera: Scarabaeidae) larvae with steinernematid and heterorhabditid nematodes. J. Econ. Entomol. 85**,** 727-730.

Koppenhöfer, A. M., 2007. Nematodes. In: L. A. Lacey, H. K. Kaya, (Eds.), Field Manual of Techniques in Invertebrate Pathology. Springer, Dordrecht, The Netherlands, pp. 249–264.

Koppenhöfer, A. M., Brown, I. M., Gaugler, R., Grewal, P. S., Kaya, H. K., Klein, M. G., 2000a. Synergism of entomopathogenic nematodes and imidacloprid against white grubs: greenhouse and field evaluation. Biol. Control. 19**,** 245-251.

Koppenhöfer, A. M., Choo, H. Y., Kaya, H. K., Lee, D. W., Gelernter, W. D., 1999. Increased field and greenhouse efficacy against scarab grubs with a combination of an entomopathogenic nematode and *Bacillus thuringiensis*. Biol. Control. 14**,** 37-44.

Koppenhöfer, A. M., Cowles, R. S., Cowles, E. A., Fuzy, E. M., Baumgartner, L., 2002. Comparison of neonicotinoid insecticides as synergists for entomopathogenic nematodes. Biol. Control. 24**,** 90-97.

Koppenhöfer, A. M., Fuzy, E. M., 2003a. Biological and chemical control of the Asiatic garden beetle, *Maladera castanea* (Coleoptera: Scarabaeidae). J. Econ. Entomol. 96**,** 1076–1082.

Koppenhöfer, A. M., Fuzy, E. M., 2003b. *Steinernema scarabaei* for the control of white grubs. Biol. Control. 28**,** 47-59.

Koppenhöfer, A. M., Fuzy, E. M., 2004. Effect of white grub developmental stage on susceptibility to entomopathogenic nematodes. J. Econ. Entomol. 97**,** 1842-1849.

Koppenhöfer, A. M., Grewal, P. S., Kaya, H. K., 2000b. Synergism of imidacloprid and entomopathogenic nematodes against white grubs: the mechanism. Entomol. Exp. Appl. 94**,** 283-293.

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

Koppenhöfer, A. M., Kaya, H. K., 1998. Synergism of imidacloprid and an entomopathogenic nematode: A novel approach to white grub (Coleoptera: Scarabaeidae) control in turfgrass. J. Econ. Entomol. 91**,** 618-623.

Koppenhöfer, A. M., Wilson, M., Brown, I., Kaya, H. K., Gaugler, R., 2000c. Biological control agents for white grubs (Coleoptera: Scarabaeidae) in anticipation of the establishment of the Japanese beetle in California. J. Econ. Entomol. 93**,** 71-80.

Rath, A. C., Worledge, D., Koen, T. B., Rowe, B. A., 1995. Long-term field efficacy of the entomogenous fungus *Metarhizium anisopliae* against subterranean scarab, *Adoryphorus couloni*. Biocontrol Sci. Technol. 5**,** 439–451.

Smits, P. H., 1996. Post-application persistence of entomopathogenic nematodes. Biocontrol Sci. Technol. 6**,** 379-387.

Steinhaus, E. A., 1958. Stress as a factor in insect disease. Proceedings of the Xth International Congress on Entomology. 4**,** 725–730.

Thurston, G. S., Kaya, H. K., Burlando, T. M., Harrison, R. E., 1993. Milky disease bacteria as a stressor to increase susceptibility of scarabaeid larvae to an entomopathogenic nematode. J. Invertebr. Pathol. 61**,** 167–172.

Thurston, G. S., Kaya, H. K., Gaugler, R., 1994. Characterization of enhanced susceptibility of milky disease infected scarabaeid grubs to entomopathogenic nematodes. Biol. Control. 4**,** 67–73.

Wraight, S. P., Inglis, G. D., Goettel, M. S., 2007. Fungi. In: L. A. Lacey, H. K. Kaya, (Eds.), Field Manual of Techniques in Invertebrate Pathology. Springer, Dordrecht, The Netherlands, pp. 223-248.

Yokoyama, T., Hasegawa, M., Fujiie, A., Sawada, M., Noguchi, K., 1998. Microbial control of scarab beetle larvae by a formulation of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) in a sweet potato field. Appl. Entomol. Zool. 33**,** 215–218.

CHAPTER 6

Sub-lethal Effects of Entomopathogenic Fungi on Southern Masked Chafer, *Cyclocephala lurida* (Coleoptera: Scarabaeidae)

Abstract

The sub-lethal effects of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, on southern masked chafer, *Cyclocephala lurida*, were investigated. Neither *M. anisopliae* nor *B. bassiana* had a significant impact on grub fitness in terms of weight gain. Adult longevity of this insect was not significantly affected by treatments, and male and female longevity were not significantly different. Oviposition was strongly correlated with the time duration of male presence and female longevity, but neither fungal species had a significant impact. Pupation rate for the treatment with *M. anisopliae* was significantly lower than that with *B. bassiana*, but there were no significant differences from the control. Neither the treatment of *M. anisopliae* or *B. bassiana* had any significant impact on adult eclosion. Neither fungus had a sub-lethal effect on masked chafer grub weight gain, adult longevity, oviposition, pupation and eclosion.

Keywords: white grub, *Cyclocephala lurida*, entomopathogenic fungi, sub-lethal effect

Introduction

Cyclocephala lurida Bland is among the most abundant and damaging white grub species in turfgrass of Virginia. Heavily infested turfgrass turns brown and becomes spongy, making it easily dislodged, and the grass dies from dehydration. More often, the most damage is caused by small vertebrates, like birds and skunks, tearing the turf into pieces when they hunt for grubs as food. Currently, the control of this pest mainly relies on the application of chemical insecticides on a preventative basis before damage occurs. Large spraying areas are generally required to achieve adequate coverage before the grub problem emerges. Also, these insecticides are generally more effective against grubs at the earlier stages. If early attempts fail, grubs are more difficult to control as late instars, and they cause severe damage. Difficulties in achieving consistent long-term efficacy against white grubs with chemical approaches, and increasing public concern about the use of insecticides, have given more importance for development of biological control strategies.

For these reasons, the potential of using entomopathogenic fungi (EPF) like *Metarhizium anisopliae* (Metschn.) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin have been explored for the control of this pest. Results from laboratory and field studies indicate that efficacies of these fungi in grub control are generally low in terms of causing direct mortality (Wu et al. unpublished data). Despite this, as EPF can persist in soil for a relatively long time if environmental conditions are suitable (Latch and Falloon,

1976; Milner and Lutton, 1976; Müller-Kögler and Stein, 1976; Rath, 1992; Samuels and Pinnock, 1988), there is a potential for these fungi in lowering grub abundance by causing both direct mortality and through sub-lethal effects. Although significant mortality is the desired effect for most studies on the use of EPF in pest control, sub-lethal effects from fungal application should not be underestimated. They may provide control or suppression of pest numbers by lowering pest fitness of individuals or populations, which may become more obvious when the direct mortality caused by fungal infection is low.

Pre- or sub-lethal effects of *B. bassiana* and *M. anisopliae* have been substantially investigated on many insects. Such effects include reduced feeding (Arthurs and Thomas, 2000; Blanford et al., 2011; Darbro et al., 2012; Ekesi and Maniania, 2000; Maehara et al., 2007; Scholte et al., 2006; Tefera and Pringle, 2003), a reduction in fecundity or reproductive potential (Darbro et al., 2012; Dembilio et al., 2010; Hajek et al., 2008; Kaur et al., 2011; Liu and Bauer, 2008; Quesada-Moraga et al., 2004; Scholte et al., 2006), and decreased longevity (Dubois et al., 2004; Gindin et al., 2006; Hajek et al., 2008; Liu and Bauer, 2008; Pereira et al., 2011). In addition, EPF infection may also interfere with insect developmental process, e.g. deformation (Kaur et al., 2011), molting process (Torrado-León et al., 2006), and developmental rate (Kaur et al., 2011; Liu and Bauer, 2008). Other sub-lethal effects of EPF on target pests include reduction in host-finding behavior (George et al., 2011), decreased flight capacity (Blanford et al., 2011; Seyoum et al., 2002; Seyoum et al., 1994), and increased susceptibility to predation (Arthurs and Thomas, 2001; Thomas et al., 1998), etc.

Studies on the sub-lethal effects of EPF on white grubs are more limited. Villani et al. (1994) reported that the application of *M. anisopliae* affected the behavior of Japanese beetle *Popillia japonica* Newman at both larval and adult stages. The grubs avoided soil contaminated with high concentrations of *M. anisopliae*, whereas the incorporation of mycelial particles increased oviposition. Besides, Lacey et al. (1995) investigated the flight activity of *P. japonica* after the treatment with *M. anisopliae*, and found significantly fewer treated beetles recaptured than the untreated control. Other than those mentioned above, there are very few reports on the sub-lethal effects of EPF on white grubs. Our experiments were carried out based on the hypothesis that, beside causing direct mortality of southern masked chafer, *M. anisopliae* and *B. bassiana* may have sub-lethal effects by reducing grub fitness, oviposition, adult longevity, or interference with the pupation or eclosion process, and thus provide additional suppression of this pest.

Materials and Methods

1. Effect of EPF on grub fitness in terms of weight gain in body mass

Grubs used in this experiment were 2nd instar southern masked chafers collected from VT golf course (Blacksburg, VA) around August 01, 2011 using a sod cutter. Grubs were surface sterilized with 0.5% sodium hydrochloride (Lacey and Brooks, 1997), and were then stored individually in egg cells filled with solarized soil for at least 3 d before being used in the experiment. Treatments included: water control; *M. anisopliae*; *B. bassiana*. *B. bassiana* GHA strain in emulsifiable formulation (BotaniGard ES, containing 2.1×10^{10} viable spores/ml), and *M. anisopliae* F-52 in oil emulsifiable formulation (Met 52 EC, containing 5.5×10^{9} colony forming units (CFU)/g) used at the rate of 25.6 L/ha, 6.4 L/ha, respectively. There were 3 replicates per treatment, with 15 grubs per replicate. Grubs were weighed individually before treatment, and only live grubs were weighed at 4 and 8 wk after treatment. Grubs were surface-cleaned to be weighed. Body size and weight prior to treatment were used to group grubs by stages of development. Based on body size, grubs whose weight ranged from 50-80 mg, 81-100 mg and 101-135 mg were considered to be at the stage of early-, mid- and late-2nd instar, respectively.

Grubs were treated individually in 30 ml cups filled with 25g soil (soil surface area: 12.15 cm^2). Perennial ryegrass (*Lolium perenne* L.) seeds were added to the soil surface and allowed to germinate to provide food for them. Soil in individual cups was moistened with 2.5 ml water before introduction of grubs. Grubs that did not enter the soil within 4 h were replaced. The final moisture content was adjusted to 18% (v/w). The cups were placed in trays, and covered with lids to maintain the moisture content. There were 15 holes punctured on each lid with a thumb tack to allow for air exchange. One ml of distilled water was added to each cup every other wk to compensate for water loss. The experiment was conducted in an incubator at the photoperiod of LD 13:11 (light 13h: dark 11h), 20 ^{o}C and R.H. of 80%. Grubs were weighed individually after being cleaned with distilled water at the start of the experiment, and then every 4 wk for up to 8 wk. Soil used was a sandy loam texture comprised of 74.1% sand, 19.6% silt, 6.3% clay and 3.2% organic matter with pH of 5.2. Before application, the soil was covered with a clear plastic cloth for solarizing in the greenhouse for at least one month in summer.

2. Effect of EPF on adult longevity, oviposition, pupation and eclosion rate in *C. lurida*

Overwintered masked chafer grubs were collected from the VT golf course in early May, 2012. Treatments and rates applied were the same as Experiment 1. All treatments were replicated four times, with 15 grubs per replicate. Before adult eclosion, the experimental procedures were similar to Experiment 1. Soil used was a sandy loam texture consisting of 73.8% sand, 18.1% silt, 8.1% clay and 1.2% organic matter with pH of 5.8.

Dates of grub pupation and adult eclosion were recorded individually. Upon eclosion, pairs of southern masked chafer adults were transferred to 120 ml (4 oz.) cups, a quarter filled with moist soil, to mate and lay eggs. Filter cloth was used to cover the cup with a rubber band. Soil was kept moist around 18% (v/w) during the whole experimental process. Egg counts were made weekly after introduction of adults. Date of death for each adult was recorded. If the male died before the female, it was removed and replaced with another male. Observation ceased at the death of female.

Data Analysis

Software JMP 10.0 (SAS, Cary, NC) was used for data analysis. Analysis of Variance was used to analyze data on grub fitness, adult longevity, pupation and eclosion rate, and Generalized Linear Regression was used to test the correlation between oviposition and other factors studied.

Results

1. Effect of EPF on 2nd instar grub fitness in terms of weight gain in body mass

Masked chafers treated with the water control, *B. bassiana*, or *M. anisopliae* gained an average weight of 35-145 mg, 55-110 mg, 45-165 mg in body mass in 4 wk; and 174-244 mg, 195-243 mg, 159-312 mg in 8 wk, respectively. Grub weight gain differed significantly among treatments ($F=4.1$, d.f.=2, $P=0.019$), and among developmental stages ($F=25.44$, d.f.=2, $P<0.0001$) 4 wk after treatment; but no significant interaction was found between treatments and stages ($F=1.84$, d.f.=4, $P=0.128$) (two-way ANOVA, $\alpha=0.05$). Differences among treatments showed that grubs treated with *B. bassiana* gained significantly less weight than those treated with *M. anisopliae*; however, neither fungus had any significant effect on grub growth when compared with the control (Tukey's HSD, $\alpha=0.05$) (Fig. 1). Within 8 wk after treatment, significant differences were detected among developmental stages ($F=8.2$, d.f.=2, $P=0.001$), but not among treatments ($F=1.09$, d.f.=2, $P=0.342$); no significant interaction was found ($F=1.12$, d.f.=4, $P=0.352$) (two-way ANOVA, $\alpha=0.05$). A trend showed that larger grubs gained more weight than smaller ones despite treatments. The results suggest that neither *M. anisopliae* nor *B. bassiana* had any significant impact on masked chafer grub fitness in terms of weight gain.

2. Effect of EPF on adult longevity, oviposition, pupation and eclosion rate in *C. lurida*

2.1. Adult longevity

Neither *B. bassiana* nor *M. anisopliae* had a significant effect on the adult longevity of *C. lurida* ($F=0.53$, d.f.=2, $P=0.593$). The longevity of male adults was not significantly different from females ($F=0.26$, d.f.=1, $P=0.614$), and there was no significant interaction between treatments and the sex of adults ($F=0.52$, d.f.=2, $P=0.594$) (Fig. 2).

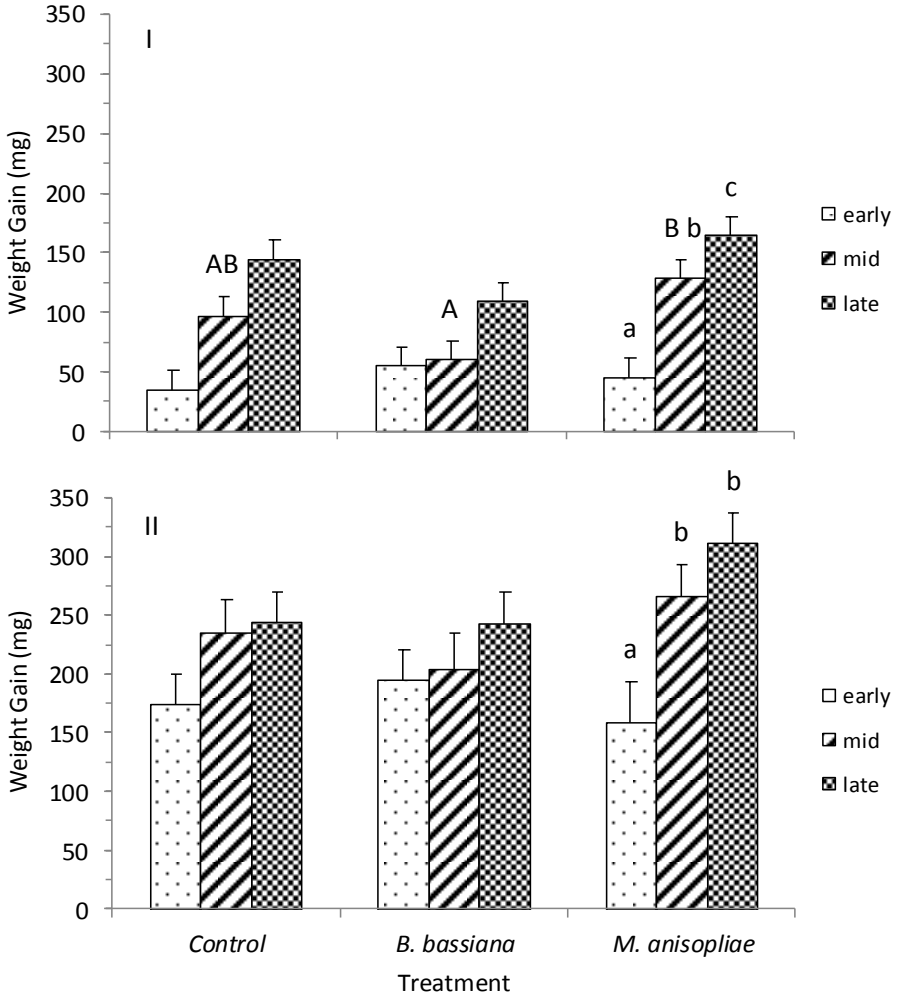

Fig. 1. Weight gain in 2nd instar *Cyclocephala lurida* within 4 (I) or 8 wk (II) after treatment with *Beauveria bassiana* or *Metarhizium anisopliae* at various stages (early, mid and late 2nd instar) (n=11-13 for each bar in I; n=6-11 for each bar in II). Different capital and lower case letters indicate significant differences between treatments and between developmental stages, respectively (Tukey's HSD, α=0.05).

2.2. Oviposition

The oviposition of southern masked chafer was positively correlated to the duration of male presence (r=0.72) and female longevity (r=0.56). However, treatments had no significant effect on oviposition (X^2=0.69, d.f.=2, *P*=0.708) (Fig. 3).

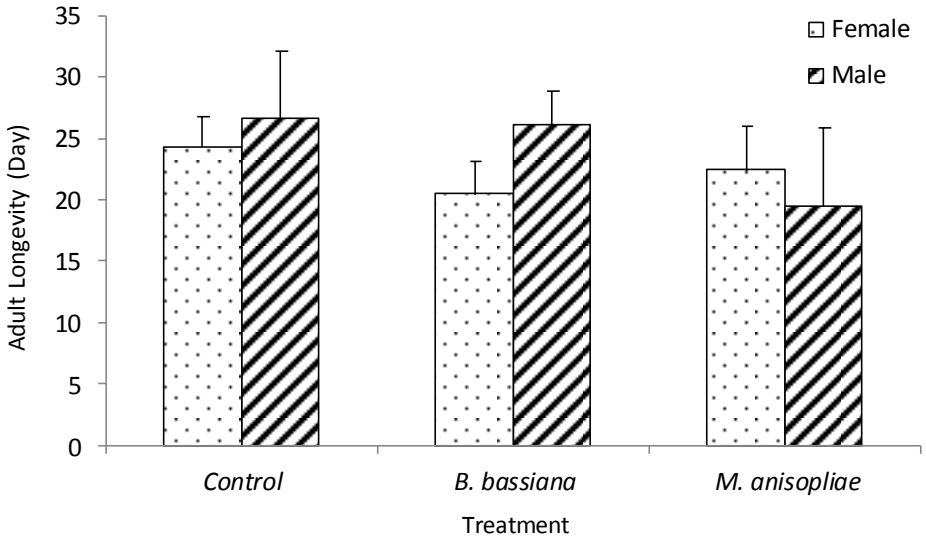

Fig. 2. Effect of *Beauveria bassiana* and *Metarhizium anisopliae* on the longevity of male and female *Cyclocephala lurida* adults (N=17-21 female; 4-14 male for each treatment).

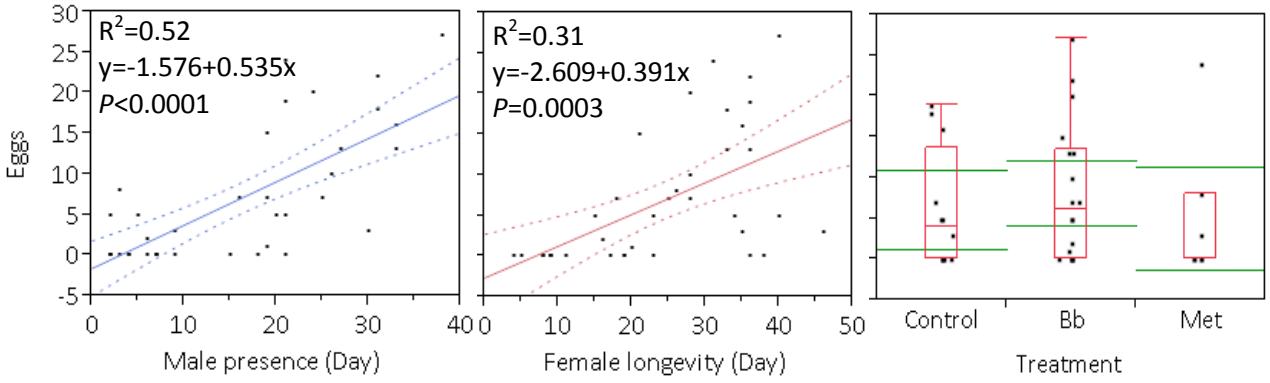

Fig. 3. Correlation of duration of male presence, female longevity and treatment (control, *Beauveria bassiana*-Bb, *Metarhizium anisopliae*-Met), with oviposition in *Cyclocephala lurida*. In left and middle diagrams, $P<0.05$ indicates that linear regression model fits the data. In right diagram, 90% CI lines of mean were added to the box-plot (No. pairs=7-18 for each treatment).

2.3. Pupation and eclosion rate

There were significant differences among treatments on the successful pupation rate of *C. lurida* ($F=8.74$, d.f.=2, $P=0.008$). *M. anisopliae* had a significantly lower pupation rate than *B. bassiana*, but neither fungus had any significant impact on the rate when compared with the control (Tukey's HSD, $\alpha=0.05$) (Fig. 4).

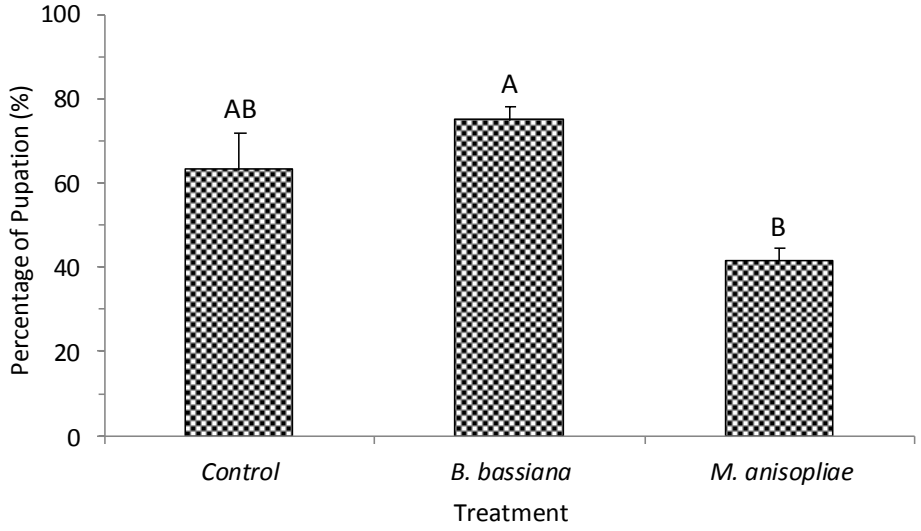

Fig. 4. Effect of *Beauveria bassiana* and *Metarhizium anisopliae* on the pupation rate in *Cyclocephala lurida*. Different letters indicate significance between treatments (Tukey's HSD, α=0.05).

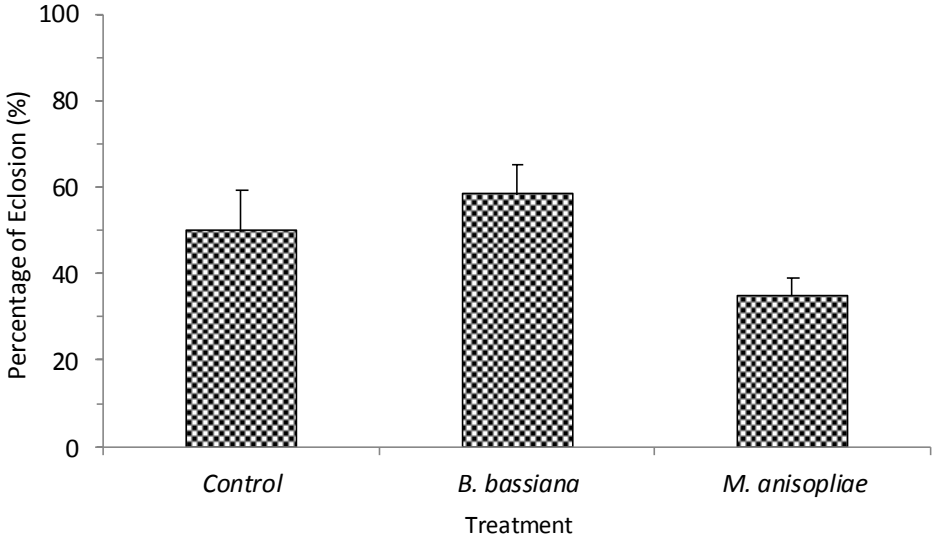

Fig. 5. Effect of *Beauveria bassiana* and *Metarhizium anisopliae* on the adult eclosion rate in *Cyclocephala lurida*.

Time of pupation was recorded individually for each grub. Most of the differences in pupation rate occurred after June 05, as no significant difference was observed before June 05 (F=1.39, d.f.=2, *P*=0.298), when the average percentage of pupation was 43.3%, 51.7%, 35% for the control, *B. bassiana*, *M. anisopliae*, respectively. After June 27, an average of 8.3% individuals in the control, 5% in *B. bassiana* and 6.7% in *M. anisopliae* treatments remained in grub stage, and did not pupate before death; no significant differences were detected among treatments (F=0.41, d.f.=2, *P*=0.676).

Neither *M. anisopliae* nor *B. bassiana* had a significant impact on the adult eclosion of *C. lurida* (F=2.67, d.f.=2, *P*=0.123) (Fig. 5). This indicates that the time of eclosion for this insect was not affected by the treatment of these fungi.

Discussion

Hornbostel et al. (2004) found that *M. anisopliae* reduced the body mass of the tick *Ixodes scapularis* Say in all active stages. In the current study, neither *M. anisopliae* nor *B. bassiana* had a significant impact on 2nd instar masked chafer fitness in terms of weight gain in body mass 4 wk or 8 wk after treatment (Fig. 1), although in each treatment there were a few individuals that barely gained any weight in the 4 wk period and died probably from starvation or dehydration. This indicates that the fungi did not have an obvious direct effect in interfering with the normal growth and food consumption of the 2nd instar of this insect, although it was possible that those affected individuals died and thus their body weight gain was not counted. There have been several reports on reduced feeding of the target pests from fungal infection, e.g. EPF-infected mosquitoes (Blanford et al., 2011; Darbro et al., 2012; Scholte et al., 2006), thrips *Megalurothrips sjostedti* Trybom (Ekesi and Maniania, 2000), pine sawyer *Monochamus alternatus* Hope (Maehara et al., 2007), *Chilo partellus* (Swinhoe) (Tefera and Pringle, 2003), and the brown locust *Locustana pardalina* (Walker) (Arthurs and Thomas, 2000). However, in the current study, reduction in feeding behavior was not observed in masked chafers that survived fungal treatment.

Decreased longevity from fungal treatment has also been reported in several insects, e.g. the red palm weevil *Rhynchophorus ferrugineus* (Olivier) treated with *M. anisopliae* and *B. bassiana* (Gindin et al., 2006), the Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky) infected with *Beauveria* spp. or *M. anisopliae* (Dubois et al., 2004; Hajek et al., 2008), emerald ash borer, *Agrilus planipennis* Fairmaire from *B. bassiana* infection (Liu and Bauer, 2008), and the mealybug *Pseudococcus viburni* (Signoret) from *M. anisopliae infection* (Pereira et al., 2011). In addition, Dubios et al. (2004) noticed significant difference in longevity between insect sexes in *A. glabripennis*, and longevity of the untreated male was shorter than untreated female. Although longevity of both sexes was significantly higher in the non-treated than the fungus-treated individuals, females showed higher longevity in treatment with *B. bassiana* GHA than *B. brongniartii* (Saccardo) Petch NBL 851, whereas no significant difference was observed for males treated with these two fungal species. In the current experiment, neither *B. bassiana* nor *M. anisopliae* had a significant effect on the adult longevity of *C. lurida*, and no significant difference was observed between male and female longevity due to fungal treatment (Fig. 2).

Reduction in fecundity or reproductive potential of target insects from fungal infection has been studied in several insect species, e.g. *M. anisopliae*-infected mosquito *Anopheles gambiae* Giles (Scholte et al., 2006), *A. glabripennis* (Hajek et al., 2008), German cockroach *Blatella germanica* (L.) (Quesada-Moraga

et al., 2004); *B. bassiana*-infected tobacco caterpillar *Spodoptera litura* (Fabricius) (Kaur et al., 2011), *A. planipennis* (Liu and Bauer, 2008), *Aedes aegypti* (L.) (Darbro et al., 2012), and *R. ferrugineus* (Dembilio et al., 2010). In this experiment, neither *M. anisopliae* nor *B. bassiana* was found to have significant impact on oviposition in *C. lurida* (Fig. 3, right). However, this could be due to the low number of females being tested, especially for the treatment with *M. anisopliae*, which only included seven female adults paired with males because of high mortality before adult eclosion in this treatment. Among these seven females tested in *M. anisopliae* treatment, only three laid eggs, although the control and *B. bassiana* treatment also had five out of 12 and 18 females that did not lay eggs, respectively. Hornbostel et al. (2004) also reported that due to *M. anisopliae* application, only 33% of treated females of *I. scapularis* oviposited. However, in the current experiment, no or low oviposition in *C. lurida* may also be explained by the short duration of male presence or female longevity, as data analyses reveal that ovipostion was strongly correlated with the two factors (Fig. 3, left & middle).

Noticeably, in the control and *M. anisopliae* treatment, there was one female that laid two and three eggs, respectively, without mating. Thus, egg hatch rate might be another valuable criterion for evaluation of the fungal effect. Unfortunately, these data were unavailable due to mold infection of eggs in this experiment. Also, in a previous experiment testing the effect of EPF on oviposition in *C. lurida*, the untreated control, *B. bassiana* and *M. anisopliae* treatments had an average of 8.2, 5.6, 5.6 eggs laid, respectively. These data were obtained from the cage experiment with 15 females plus 21 males in the control, 11 females plus 15 males in *B. bassiana*, and 16 females plus 20 males in the *M. anisopliae* treatments. A trend was observed showing that the control had higher oviposition than fungal treatment, although analysis of variance was not available.

EPF has also been reported to have an impact on insect developmental rate. For example, Liu and Bauer (2008) found that treatment of *B. bassiana* strain GHA prolonged the larval development of *A. planipennis*. Conversely, in *S. litura*, larval period decreased significantly as compared with the control, due to infection with *B. bassiana* (PDBC-Bb-5a) (Kaur et al., 2011). However, in the current study, neither *M. anisopliae* nor *B. bassiana* had a significant effect on the larval development rate of *C. lurida*. Grubs from different treatments experienced a similar period of time to reach the 3rd instar stage, although differences were found between groups by developmental stages (i.e. individuals treated at the late 2nd instar required less time than those at the early 2nd instar to reach the 3rd instar). Fungal treatment also did not have an impact on the time for pupation when overwintered 3rd instar grubs were treated approximately 3 wk prior to pupation. Although *M. anisopliae* reduced the total pupation rate (Fig. 4), this was probably due to the relatively high mortality incurred by this fungus, as most grubs that did not pupate died from fungal infection.

EPF infection may also cause deformation in insects during their developmental process. Kaur et al. (2011) reported that *B. bassiana* induced pupal and adult deformities in *S. litura*. Also, in *Bemisia tabaci* (Gennadius) due to infection with *B. bassiana*, about 30% imagos from treated nymphs were unable to detach completely from the exuviae (Torrado-León et al., 2006). In this experiment, a few individuals died in pre-pupa stage or during pupating process, and some pupae died before adult eclosion, which were probably due to fungal infection, as some of them had fungal growth eventually while others did not. Meanwhile, only one pupa from *B. bassiana* treatment died during eclosion; one pupa from *M. anisopliae* treatment died immediately upon eclosion and showed sign of deformity. However, the majority of grubs survived the fungal treatment, pupated and eclosed into adults normally. Statistically, neither *M. anisopliae* nor *B. bassiana* had a significant impact on the adult eclosion in *C. lurida* (Fig. 5).

References

Arthurs, S., Thomas, M. B., 2000. Effects of a mycoinsecticide on feeding and fecundity of the brown locust *Locustana pardalina*. Biocontrol Sci. Technol. 10**,** 321-329.

Arthurs, S., Thomas, M. B., 2001. Behavioural changes in *Schistocerca gregaria* following infection with a fungal pathogen: implications for susceptibility to predation. Ecol. Entomol. 26**,** 227–234.

Blanford, S., Shi, W., Christian, R., Marden, J. H., Koekemoer, L. L., Brooke, B. D., Coetzee, M., Read, A. F., Thomas, M. B., 2011. Lethal and pre-Lethal effects of a fungal biopesticide contribute to substantial and rapid control of malaria vectors. PLoS ONE. 6**,** e23591.

Darbro, J. M., Johnson, P. H., Thomas, M. B., Ritchie, S. A., Kay, B. H., Ryan, P. A., 2012. Effects of *Beauveria bassiana* on survival, blood-feeding success, and fecundity of *Aedes aegypti* in laboratory and semi-field conditions. Am. J. Trop. Med. Hyg. 86**,** 656–664.

Dembilio, O., Quesada-Moraga, E., Santiago-Alvarez, C., Jacas, J. A., 2010. Potential of an indigenous strain of the entomopathogenic fungus *Beauveria bassiana* as a biological control agent against the red palm weevil, *Rhynchophorus ferrugineus*. J. Invertebr. Pathol. 104**,** 214-221.

Dubois, T., Hajek, A. E., Jiafu, H., Li, Z., 2004. Evaluating the efficiency of entomopathogenic fungi against the Asian longhorned beetle, *Anoplophora glabripennis* (Coleoptera: Cerambycidae), by using cages in the field. Environ. Entomol. 33**,** 62-74.

Ekesi, S., Maniania, N. K., 2000. Susceptibility of *Megalurothrips sjostedti* developmental stages to *Metarhizium anisopliae* and the effects of infection on feeding, adult fecundity, egg fertility, and longevity. Entomol. Exp. Appl. 94**,** 229–236.

George, J., Blanford, S., Domingue, M. J., Thomas, M. B., Read, A. F., Baker, T. C., 2011. Reduction in host-finding behaviour in fungus-infected mosquitoes is correlated with reduction in olfactory receptor neuron responsiveness. Malaria Journal. 10**,** 219.

Gindin, G., Levski, S., Glazer, I., Soroker, V., 2006. Evaluation of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* against the red palm weevil *Rhynchophorus ferrugineus*. Phytoparasitica. 34**,** 370-379.

Hajek, A. E., Lund, J., Smith, M. T., 2008. Reduction in fitness of female Asian longhorned beetle (*Anoplophora glabripennis*) infected with *Metarhizium anisopliae*. J. Invertebr. Pathol. 98**,** 198–205.

Hornbostel, V. L., Ostfeld, R. S., Zhioua, E., Benjamin, M. A., 2004. Sublethal effects of *Metarhizium anisopliae* (Deuteromycetes) on engorged larval, nymphal, and adult *Ixodes scapularis* (Acari: Ixodidae). J. Med. Entomol. 41**,** 922-929.

Kaur, S., Kaur, H. P., Kaur, K., Kaur, A., 2011. Effect of different concentrations of *Beauveria bassiana* on development and reproductive potential of *Spodoptera litura* (Fabricius). Journal of Biopesticides. 4**,** 161-168.

Lacey, L. A., Amaral, J. J., Coupland, J., Klein, M. G., Simoes, A. M., 1995. Flight activity of *Popillia japonica* (Coleoptera: Scarabaeidae) after treatment with *Metarhizium anisopliae*. Biol. Control. 5**,** 167-172.

Lacey, L. A., Brooks, W. M., 1997. Initial handling and diagnosis of diseased insects. In: L. A. Lacey, (Ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, CA pp. 5.

Latch, C. C. M., Falloon, R. E., 1976. Studies on the use of *Metarhizium anisopliae* to control *Oryctes rhinoceros*. Entomophaga. 21**,** 39-48.

Liu, H., Bauer, L. S., 2008. Microbial control of emerald ash borer, *Agrilus planipennis* (Coleoptera: Buprestidae) with *Beauveria bassiana* strain GHA: Greenhouse and field trials. Biol. Control. 45**,** 124–132.

Maehara, N., He, X., Shimazu, M., 2007. Maturation feeding and transmission of *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae) by *Monochamus alternatus* (Coleoptera: Cerambycidae) inoculated with *Beauveria bassiana* (Deuteromycotina: Hyphomycetes). J. Econ. Entomol. 100**,** 49-53.

Milner, R. J., Lutton, G. G., *Metarhizium anisopliae*: survival of conidia in soil. Proceedings of the 1st International Colloquium of Invertebrate Pathology, Kingston, Canada, 1976, pp. 428-429.

Müller-Kögler, E., Stein, W., 1976. Gewachshausversuche mit *Metarhizium anisopliae* (Metsch.) Sorok. zur Infektion von *Sitona lineatus* L. im Boden. Pflanzenschutzberichte. 83**,** 96-108.

Pereira, A., Casals, P., Salazar, A. M., Gerding, M., 2011. Virulence and pre-lethal reproductive effects of *Metarhizium anisopliae* var. *anisopliae* on *Pseudococcus viburni* (Hemiptera: Pseudococcidae). Chilean Journal of Agricultural Research. 71**,** 554-559.

Quesada-Moraga, E., Santos-Quirós, R., Valverde-García, P., Santiago-Álvarez, C., 2004. Virulence, horizontal transmission, and sublethal reproductive effects of *Metarhizium anisopliae* (Anamorphic fungi) on the German cockroach (Blattodea: Blattellidae). J. Invertebr. Pathol. 87**,** 51–58.

Rath, A. C., 1992. *Metarhizium anisopliae* for control of the Tasmanian pasture scarab *Adoryphorus couloni*. In: T. A. Jackson, T. R. Glare, (Eds.), Use of Pathogens in Scarab Pest Management. Intercept, Andover, pp. 217-227.

Samuels, K. D. Z., Pinnock, D. E., Control of Australian soldier fly and scarabaeid larvae with *Metarhizium anisopliae*. Abstracts of the 11th Annual Meeting of the Society for Invertebrate Pathology, University of California, San Diego, 1988, pp. 32.

Scholte, E.-J., Knols, B. G. J., Takken, W., 2006. Infection of the malaria mosquito *Anopheles gambiae* with the entomopathogenic fungus *Metarhizium anisopliae* reduces blood feeding and fecundity. J. Invertebr. Pathol. 91**,** 43–49.

Seyoum, E., Bateman, R. P., Charnley, A. K., 2002. The effect of *Metarhizium anisopliae* var *acridum* on haemolymph energy reserves and flight capability in the desert locust, *Schistocerca gregaria*. J. Appl. Entomol. 126**,** 119-124.

Seyoum, E., Moore, D., Charnley, A. K., 1994. Reduction in flight activity and food consumption by the desert locust, *Schistocerca gregaria* Forskål (Orth., Cyrtacanthacrinae), after infection with *Metarhizium flavoviride*. J. Appl. Entomol. 118.

Tefera, T., Pringle, K. L., 2003. Food consumption by *Chilo partellus* (Lepidoptera: Pyralidae) larvae infected with *Beauveria bassiana* and *Metarhizium anisopliae* and effects of feeding natural versus artificial diets on mortality and mycosis. J. Invertebr. Pathol. 84**,** 220-225.

Thomas, M. B., Blanford, S., Gbongbui, C., Lomer, C. J., 1998. Experimental studies to evaluate spray applications of a mycoinsecticide against the rice grasshopper, *Hieroglyphus daganensis*, in northern Benin. Entomol. Exp. Appl. 87**,** 93-102.

Torrado-León, E., Montoya-Lerma, J., Valencia-Pizo, E., 2006. Sublethal effects of *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina: Hyphomycetes) on the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) under laboratory conditions. Mycopathologia 162**,** 411–419.

Villani, M. G., Krueger, S. R., Schroeder, P. C., Consolie, F., Consolie, N. H., Preston-Wilsey, L. M., Roberts, D. W., 1994. Soil application effects of *Metarhizium anisopliae* on Japanese beetle (Coleoptera: Scarabaeidae) behavior and survival in turfgrass microcosms. Environ. Entomol. 23**,** 502-513.

CHAPTER 7

Interaction between *Heterorhabditis bacteriophora* and *Metarhizium anisopliae*: Role of Infective Juvenile Nematodes in the Vertical Distribution of Fungal Conidia in Soil

Abstract

Interaction between an entomopathogenic nematode, *Heterorhabditis bacteriophora*, and an entomopathogenic fungus, *Metarhizium anisopliae* strain F52, was investigated for the nematode potential in improving fungal distribution in soil. In sandy loam soil without grass thatch, a significantly higher level of *M. anisopliae* conidia was recovered in the combined use with *H. bacteriophora* than the fungus alone. However, in sandy soil with grass thatch, conidia recovered from *M. anisopliae* alone or combined with *H. bacteriophora* were not significantly different. Also, there were no significant differences in nematode infective juveniles (IJs) recovered from *H. bacteriophora* alone or combined with *M. anisopliae*. In both soil types, soil depths had significant effects on nematode and fungal distributions, which were mostly in 5 and 10 cm in sandy loam soil without grass thatch, and in 10 and 15 cm in sandy soil with grass thatch, respectively. In water profile, *M. anisopliae* conidia germinated hyphae tubes that attached to the outer cuticle (sheath) of *H. bacteriophora* IJs. The IJs molt within 8-11 d to detach from the fungus. IJs mortality and virulence were not negatively affected by the presence of *M. anisopliae*. When wetting agent Triton X-100 was added, the combination of *H. bacteriophora* and *M. anisopliae* appeared to have lower IJs mortality and significantly higher virulence than the nematode alone. The results indicate positive interaction between *H. bacteriophora* and *M. anisopliae*, and potential for improved efficacy in pest control from the combined use of the nematode and fungus, although grass thatch might mitigate the impact.

Keywords: *Heterorhabditis bacteriophora*, *Metarhizium anisopliae*, interaction, infective juvenile, molt

Introduction

The entomopathogenic nematode, *Heterorhabditis bacteriophora* Poinar (Order: Rhabditida), is lethal to a range of insects, including soil-borne pests like some weevil larvae and scarab grubs (Klein, 1990), by releasing the symbiotic bacterium *Photorhabdus luminescens* (Thomas and Poinar) (Family: Enterobacteriaceae). The infective juvenile, also called 'dauer juvenile', is the 3rd stage juvenile nematode that retains the 2nd stage cuticle as a sheath. Infective juvenile is the only free-living stage when nematodes actively search for host insects, and they normally do not feed or molt before entering a potential host. The nematode offers an environmentally safe and integrated pest management compatible alternative to chemical insecticides (Georgis et al., 1991), but does not always provide adequate pest control in practical field applications (Georgis and Gaugler, 1991; Georgis et al., 2006; Klein, 1990; Klein,

1993). The combined use of the nematode with other control agents like entomopathogenic fungi is thus considered (Anbesse et al., 2008; Ansari et al., 2006; Barbercheck and Kaya, 1991; Choo et al., 2002).

For about 130 years, the entomopathogenic fungus, *Metarhizium anisopliae* (Metschn.) Sorokin (Hyphomycetes), has been used for biological control of pest insects, and the first attempt to use the fungus against insects was against the scarab, wheat chafer *Anisoplia austriaca*, in Russia (Glare, 1992). *M. anisopliae* conidia may persist in soil for a relatively long period of time and provide an environmentally friendly and sustainable alternative control for soil-borne insect pests (Rath et al., 1995b; Yokoyama et al., 1998). However, in practical use in lawn and turf, a large proportion of fungal conidia may retain in the grass thatch and lack adequate contact with the target hosts, affecting overall efficacy of the fungus. This problem may be more important for oil emulsifiable formulated fungal products, which are poorly dissolvable in the carrier (water) in field applications.

The objective of the current study was to explore the interaction between *H. bacteriophora* and *M. anisopliae*, and the potential for enhancing the vertical spread of fungal conidia in soil by adding the nematode. It was anticipated that the nematode infective juveniles (IJs) would assist the spread of the fungus in soil, and thus achieve improved efficacy in pest control.

Materials and Methods

1. Interaction between *H. bacteriophora* and *M. anisopliae* in soil without grass thatch

Four-segmented PVC tubes with an inner diameter of 5 cm and a height of 5 cm per segment were used to evaluate the vertical distribution of *M. anisopliae* conidia in different soil depths (0-5 cm; 5-10 cm; 10-15 cm; 15-20 cm). *M. anisopliae* strain F-52 used in this and following experiments was an oil emulsifiable formulation containing 5.5×10^9 conidia/g, provided by Dr. Jarrod E. Leland from Novozymes Biologicals, Inc. (Salem, VA). *H. bacteriophora* (Becker Underwood) IJs were cultured with full grown larvae of the greater wax moth, *Galleria mellonella* (L.), and collected with the White trap (Kaya and Stock, 1997) within 5 d.

PVC segments were attached with a duct tape, and a petri dish (dia. 10 cm) was attached at the bottom to prevent the soil from falling out. Approximately 520 g soil were used to fill up the 4-segmented PVCs. Two treatments (*M. anisopliae* only; *M. anisopliae* mixed with *H. bacteriophora* IJs) were used in three replicates. *M. anisopliae* and *H. bacteriophora* were applied at 1×10^6 conidia/g soil and 5 billion IJs/ha, respectively, by drenching the soil surface. For the combined treatment with *M. anisopliae* and *H. bacteriophora*, the fungus and nematodes were mixed together and shaken well before application. The final soil moisture was adjusted at 18.4%. Soil used was a sandy loam texture, comprising of 55.4% sand, 30.9% silt, 13.7% clay and 1.8% organic matter with a pH of 6.0.

After treatment, soil was allowed to sit at room temperature for 24-hour to allow nematodes to move into the profile. Then, soil from each layer was mixed well, and an amount of approximately 5 g soil was arbitrarily taken and transferred to 99 ml sterile phosphate buffer added with 0.5 ml 10% tween 20. The bottle was shaken well for 30 s to make the 1^{st} solution. An amount of 11 ml from 1^{st} solution was transferred to 99 ml phosphate buffer to make the 2^{nd} solution. Finally, an amount of 0.1 ml was taken from each solution and spread evenly with a spreader on Veen's medium plates (dia. 10 cm). Plates were placed under 20 ^{0}C for 4 to 5 d to allow the conidia to germinate and grow to a countable size. The remaining soil from the treatment involving *H. bacteriophora* was then transferred to Baermann funnel, and sat for at least 12 h to allow the IJs to move to the bottom of the pipe. Nematodes collected were identified and counted.

In addition, an untreated water control and a spike treatment were used with the same procedures in 65 g soil, in three replicates. The spike treatment was *M. anisopliae* applied alone at the same rate as above, but it differed from *M. anisopliae* treatment in that fungal conidia in spike were mixed thoroughly with the soil instead of surface-drenched. Conidia concentration per gram of soil was determined, and was compared with conidia recovered in different soil depths in each treatment. Fungal conidia were not considered uniformly distributed, if conidia levels in various soil depths were significantly different from the spike.

2. Interaction between *H. bacteriophora* and *M. anisopliae* in soil with grass thatch

Materials used and experimental procedures were similar to Experiment 1, except PDA with Rose Bengal medium plates was used for fungal growth, and two plates were used for each solution. An area of bentgrass (*Agrostis stolonifera* L.) green growing in uniform sandy soil in the Virginia Tech Turf Research Center was chosen to drill soil cores with PVC tubes. The thatch depth was 2.5 cm, and the grass height was 0.4 cm. The edge of the bottom segment of PVC was sharpened to drill into the turf thatch. Four treatments (water control; *M. anisopliae* only; *H. bacteriophora* only; *M. anisopliae* mixed with *H. bacteriophora* IJs) and three replicates were used. *M. anisopliae* was applied at 1×10^6 conidia/g soil with a germination rate of 10.7%; *H. bacteriophora* at 25 billion IJs/ha, by drenching at the thatch surface. One drop (0.02 ml) of wetting agent Triton X-100 was added to *M. anisopliae* when applied alone or in combination with *H. bacteriophora*. For the combined treatment, the fungus and nematodes were mixed together and shaken well before application. In addition, a spike treatment with *M. anisopliae* alone in three replicates was used similar to Experiment 1. The final soil moisture was adjusted to 19.5%. The soil in all PVC segments was uniform sandy texture, comprising of 98.0% sand, 1.2% silt, 0.8% clay, and 0.6% organic matter with a pH of 6.5.

3. Interaction between *H. bacteriophora* and *M. anisopliae* in water

A water profile was used to test the interaction of *H. bacteriophora* IJs and *M. anisopliae* conidia, and to evaluate the impact of oil-formulated *M. anisopliae* on nematode mobility, mortality and virulence. This experiment was conducted in an incubator at 20 ^{o}C and LD 13:11, and was repeated twice (Trial 1 & 2).

Trial 1 included two treatments: *H. bacteriophora*; *H. bacteriophora* mixed with *M. anisopliae*. Petri dishes (dia. 25 cm) containing 20 ml solution were used. The rates of *H. bacteriophora* and *M. anisopliae* used were 400 IJs/ml and 2.8×10^{7} conidia/ml, respectively. Nematode activities and mortalities were recorded 48, 96, 192 h after treatment. After 192 h, 0.1ml solution was transferred to Veen's media to confirm the viability and growth potential of *M. anisopliae* conidia; 1 ml solution was taken to inoculate 10 full grown wax moth larvae in a petri dish (dia. 10 cm) lined with filter paper to test IJs virulence with or without *M. anisopliae*, in three replicates. Wax moth mortality was assessed after 1 wk.

Trial 2 included five treatments: a water control; *H. bacteriophora*; *M. anisopliae* plus 1 drop Triton X-100; *H. bacteriophora* mixed with *M. anisopliae*; and *H. bacteriophora* mixed with *M. anisopliae* plus 1 drop Triton X-100. *H. bacteriophora* and *M. anisopliae* were applied at the same rates as in Trial 1. Wax moth larvae were used to test the virulence of IJs with or without *M. anisopliae*, with 15 larvae / group, replicated six times. Larval mortality was assessed at 6, 12 or 22 days after treatment (DAT). The treatment *H. bacteriophora* mixed with *M. anisopliae* only (without adding Triton X-100) was not tested for virulence, as live nematodes were taken out to make slides for observations.

Data Analysis

Software JMP 10.0 (SAS, Cary, NC) was used for data analysis. Analysis of Variance (ANOVA) was used to detect the significant difference in *M. anisopliae* conidia and *H. bacteriophora* IJs distribution among treatments and among different depths of soil at α=0.05. Data for *M. anisopliae* conidia and *H. bacteriophora* IJs were transformed with $\log_{10}$ (formula $y=\log_{10}(x+1)$) and log, respectively, before analysis. Untransformed data were presented in Tables or Figures.

Results

1. Interaction between *H. bacteriophora* and *M. anisopliae* in soil without grass thatch

For both *M. anisopliae* alone and combined with *H. bacteriophora*, soil in 0-5 and 5-10 cm depth had more conidia recovered than the spike, although statistically not significant; soil in the depth of 10-15 and 15-20 cm had a significantly lower level of conidia than the spike (Table 1). This indicates that soil is

among the major factors affecting conidial distribution; otherwise, conidia in various soil depths would not be different from the spike, if they were uniformly distributed in soil.

Table 1. Vertical distribution of *Metarhizium anisopliae* conidia and *Heterorhabditis bacteriophora* IJs in different treatments and/or depths of soil without grass thatch.

Treatment	Soil depth (cm)	No. conidia/g soil (±SEM) [a]		No. IJs/100 g soil (±SEM) [b]	
M. anisopliae	0-5	186812 (±17192)	A	----------	
	5-10	156435 (±17795)	A	----------	
	10-15	0 (±0)	D	----------	
	15-20	0 (±0)	D	----------	
M. anisopliae	0-5	347617 (±137580)	A	384 (±107)	a
+*H. bacteriophora*	5-10	340611 (±98653)	A	35 (±3)	b
	10-15	1381 (±650)	C	39 (±8)	b
	15-20	0 (±0)	D	5 (±4)	c
Spike	-------	56955 (±5501)	AB	----------	
Control	-------	2336 (±1134)	BC	----------	

[a] Data were transformed with formula $y=\log_{10}(x+1)$ before analysis with one-way ANOVA ($F=43.76$, d.f.=9, $P<0.0001$). Different capital letters indicate significant difference between treatments (Tukey's HSD, $\alpha=0.05$).

[b] Data were log transformed before analysis with one-way ANOVA ($F=18.96$, d.f.=3, $P=0.001$). Different lower case letters indicate significant difference for IJs recovered in various soil depths (Tukey's HSD, $\alpha=0.05$).

Two-way ANOVA was used to test the effect of treatments with *M. anisopliae* alone or combined with *H. bacteriophora*, effect of soil depths on conidial distribution, and the interaction between treatments and soil depths. Significantly higher level of *M. anisopliae* conidia was recovered in the combination with *H. bacteriophora* than the fungus alone ($F=5.83$, d.f.=1, $P=0.028$). Also, significant differences were found in conidial distribution in different soil depths ($F=99.34$, d.f.=3, $P<0.0001$). Most fungal conidia were recovered within 10 cm, and there was no significant difference between 0-5 and 5-10 cm. Very few conidia were found within 10-20 cm, and were significantly lower than those in 0-10 cm; no significant difference was detected between 10-15 and 15-20 cm (Tukey's HSD). There was a significant interaction between treatments and soil depths ($F=3.32$, d.f.=3, $P=0.047$), probably because a level of 1381 conidia/g soil on average was found in the depth of 10-15 cm for the combination application, whereas no conidia were recovered in the same soil depth for *M. anisopliae* alone. This suggests that treatment effects exist, and the nematode might have assisted the vertical distribution of the fungus.

In addition, significant differences were found in IJs recovery among different soil depths (P=0.001). The depth of 0-5 cm had the highest amount of IJs recovered, and 15-20 cm had the lowest; IJs levels in 5-10 cm and 10-15 cm were not significantly different from each other (Table 1).

2. Interaction between *H. bacteriophora* and *M. anisopliae* in soil with grass thatch

Table 2. Vertical distribution of *Metarhizium anisopliae* conidia and *Heterorhabditis bacteriophora* IJs in different treatments and/or depths of soil with grass thatch.

Treatment	Soil depth (cm)	No. conidia/g soil (±SEM) [a]	No. IJs/100 g soil (±SEM) [b]
Spike	-------	72016 (±5149) ABC	----------
Control	0-5	6888 (±2857) CD	----------
	5-10	92 (±60) E	----------
	10-15	69 (±69) E	----------
	15-20	139 (±44) E	----------
M. anisopliae	0-5	438133 (±55045) AB	----------
	5-10	161088 (±31261) ABC	----------
	10-15	27523 (±2685) ABC	----------
	15-20	2613 (±1588) DE	----------
M. anisopliae	0-5	592395 (±41882) A	391 (±29) ab
+*H. bacteriophora*	5-10	172837 (±18461) ABC	127 (±5) bc
	10-15	10070 (±610) BC	71 (±15) c
	15-20	480 (±295) E	61 (±26) c
H. bacteriophora	0-5	----------	500 (±96) a
	5-10	----------	276 (±43) ab
	10-15	----------	57 (±16) c
	15-20	----------	39 (±3) c

[a] Data were transformed with formula $y=\log_{10}(x+1)$ before analysis with one-way ANOVA (F=28.47, d.f.=12, P<0.0001). Different capital letters indicate significant difference between treatments (Tukey's HSD, α=0.05).

[b] Data were log transformed before analysis with one-way ANOVA (F=15.83, d.f.=7, P<0.0001). Different lower case letters indicate significant difference for IJs recovered in various soil depths (Tukey's HSD, α=0.05).

Significant differences were found among various treatments and soil depths for conidia recovered (P<0.0001) (Table 2). Despite a trend showing fewer conidia in deeper soil, conidia levels in 0-5, 5-10 and 10-15 cm soil depths were not significantly different from the spike; those in 15-20 cm were significantly lower than the spike for *M. anisopliae* alone or in combination with *H. bacteriophora*. This suggests that a significant amount of conidia moved down to the depth of 15 cm in sandy soil.

Two-way ANOVA was used to examine effects of fungal treatments with or without *H. bacteriophora*, effects of soil depths, and their interactions on conidial distribution. Conidia recovered in *M. anisopliae* alone or combined with *H. bacteriophora* were not significantly different from each other (F=0.73, d.f.=1, *P*=0.399). Soil depths had significant effects on conidia distribution (F=51.26, d.f.=3, *P*<0.0001). Soil in depth of 0-5 cm and 5-10 cm had significantly more conidia recovered than in 10-15 and 15-20 cm. Although 0-5 cm appeared to have more conidia recovered than 5-10 cm, the difference was not statistically significant. Soil at 15-20 cm had significantly fewer conidia than 10-15 cm (Tukey's HSD, α=0.05). No significant interaction was found between treatments and soil depths (F=0.59, d.f.=3, *P*=0.625).

There were significant differences for IJs recovered in different treatments combined with different soil depths (*P*<0.0001) (Table 2). Two-way ANOVA was used to test effects of treatments, soil depths and their interactions. There were no significant treatment effects for IJs recovered from *H. bacteriophora* alone or combined with *M. anisopliae* (F=0.36, d.f.=1, *P*=0.555). Only live and mobile nematodes were collected by the funnel, indicating that *M. anisopliae* had no obvious negative impact on *H. bacteriophora* IJs mobility and survival. IJs distributions varied significantly in different soil depths (F=34.95, d.f.=3, *P*<0.0001). Soil at 0-5 cm had the highest amount of IJs recovered, followed by 5-10 cm; soil at 10-15 cm and 15-20 cm had significantly less IJs, and were not significantly different from each other. No significant interaction was found between treatments and soil depths (F=1.9, d.f.=3, *P*=0.174). A similar trend in IJs distribution is also seen in Table 1. It suggests that soil and/or thatch might be the major factor affecting the nematode movement.

3. Interaction between *H. bacteriophora* and *M. anisopliae* in water

In water profile in Trial 1, nematode IJs were observed to be trapped by *M. anisopliae* conidia, which germinated hypha tubes that attached to the outer cuticle of IJs; no hyphae were found penetrating the inner cuticle (Fig. 1 A & B). After 192 h (8 d), the IJs shed the outer cuticle (sheath) to detach from the trap of *M. anisopliae* (Fig. 1 C & D I-III).

Mortality of *H. bacteriophora* IJs was less than 10% within 192 h after treatment. Although IJs mortality increased over time (F=13.89, d.f.=2, *P*=0.001), the treatment with the combination of *H. bacteriophora* plus *M. anisopliae* did not have significantly higher IJs mortality than *H. bacteriophora* alone (F=2.92, d.f.=1, *P*=0.113). No significant interactions were found between treatments and time of exposure (F=3.03, d.f.=2, *P*=0.086) (two-way ANOVA, α=0.05) (Fig. 2).

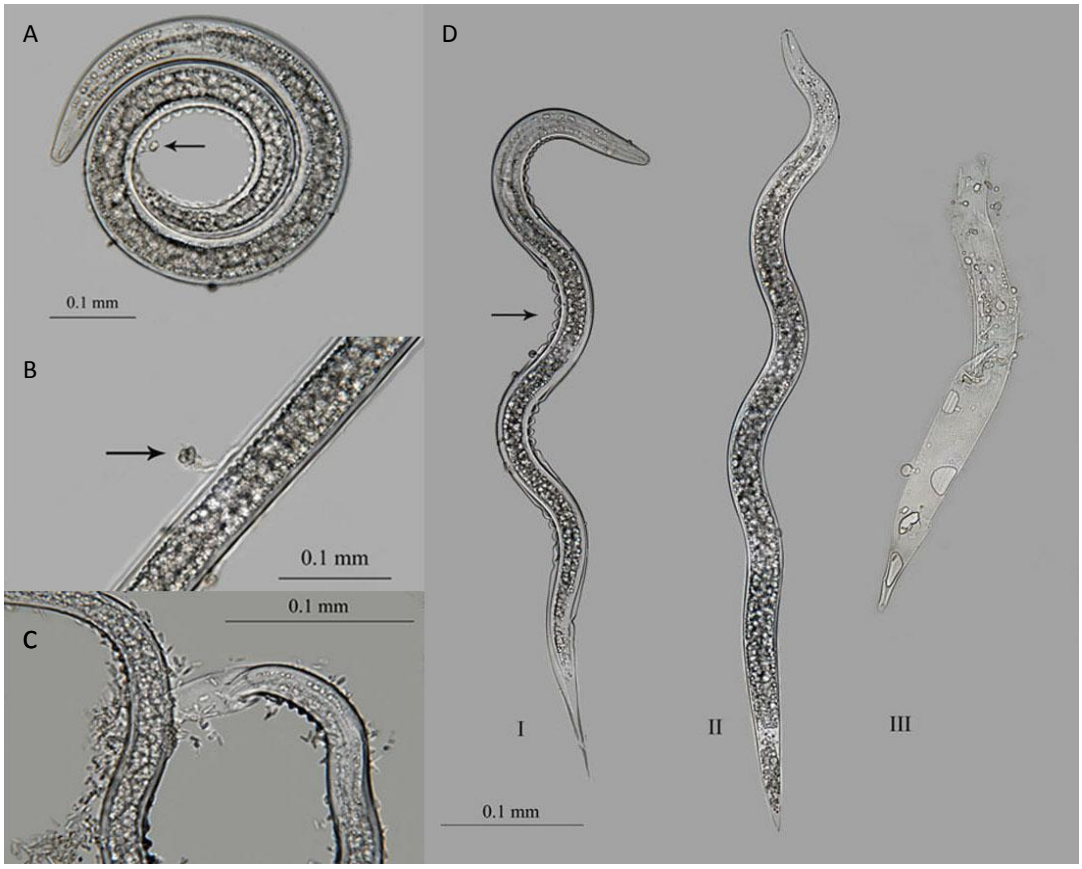

Fig. 1. *Metarhizium anisopliae* conidia germinated and attached to the sheath of IJs 96 h after mixture with *Heterorhabditis bacteriophora* IJs (arrows in A & B); IJs molted to detach from *M. anisopliae* within 192 h (C), showing IJs before molting (I: sheath wrinkles pointed by an arrow), after molting (II), and the shed cuticle (III).

Full grown wax moth larvae were used to test nematode virulence after molting. Within 1 wk, *H. bacteriophora* alone or combined with *M. anisopliae* caused mortality of 86.7 (±3.3)%, and 93.3 (±6.7)%, respectively; no significant difference was found between them (pooled *t*-test: t=0.89, d.f.=4, *P*=0.422). After 192 h, the mixture of *H. bacteriophora* and *M. anisopliae*, *M. anisopliae* was still capable of growing colonies in Veen's medium.

In Trial 2, a similar phenomenon of *H. bacteriophora* IJs being trapped by *M. anisopliae* conidia and hyphae was observed. *H. bacteriophora* IJs in the combination with *M. anisopliae* molted within 11 DAT, which was 3 d delayed compared with that in Trial 1. Within 13 DAT, similar to Trial 1, *M. anisopliae* had no significant impact on IJs mortality (F=4.66, d.f.=2, *P*=0.060). The combination of *H. bacteriophora* with *M. anisopliae* plus Triton X-100 (Hb+Met+T) had a slightly lower IJs mortality than *H. bacteriophora* alone, or combined with *M. anisopliae*, but they were not significantly different (Fig. 3).

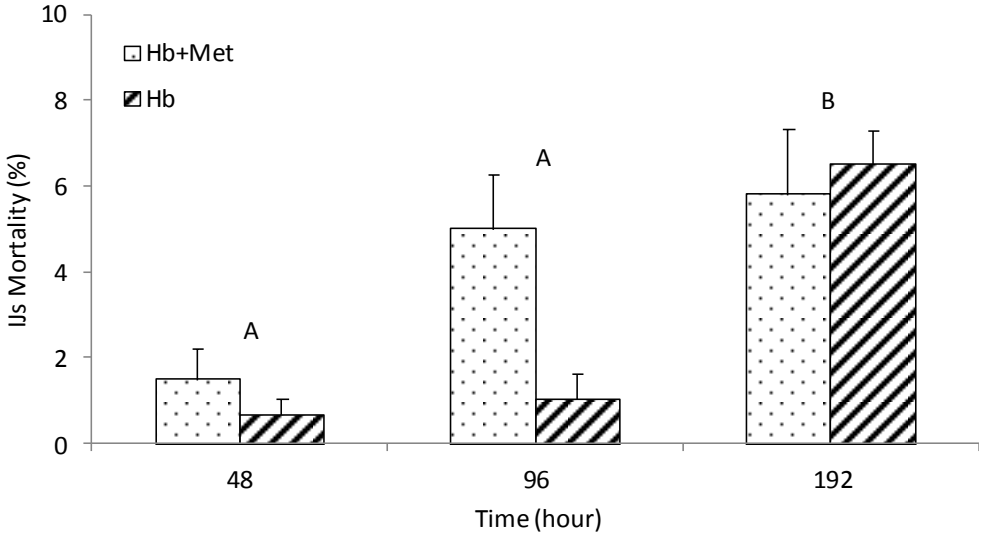

Fig. 2. Mortality of *Heterorhabditis bacteriophora* IJs 48, 96 and 192 h after treatment with *H. bacteriophora* alone (Hb), or mixed with *Metarhizium anisopliae* (Hb+Met) in Trial 1 (mean±SEM). Different letters indicate significant differences between times after treatment (Tukey's HSD, α=0.05).

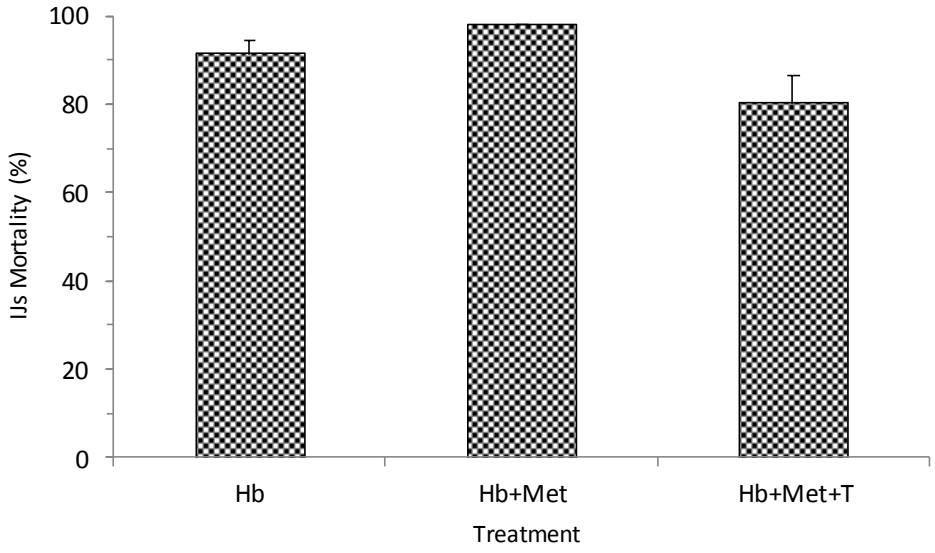

Fig. 3. Mortality of *Heterorhabditis bacteriophora* IJs in 13 DAT with *H. bacteriophora* alone (Hb), combined with *Metarhizium anisopliae* (Hb+Met), or with *M. anisopliae* plus Triton X-100 (Hb+Met+T) in Trial 2.

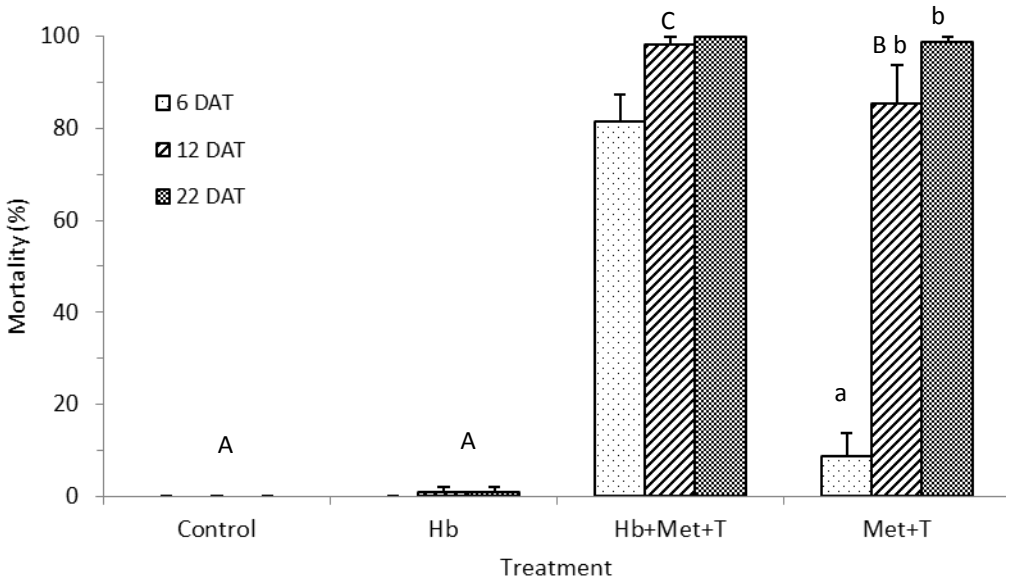

Fig. 4. Mortality of greater wax moth larvae treated with *Heterorhabditis bacteriophora* alone (Hb), or combined with *Metarhizium anisopliae* plus Triton X-100 (Hb+Met+T), or *M. anisopliae* plus Triton X-100 (Met+T) only, in 6, 12 or 22 DAT. Different capital and lower case letters indicate significant difference between treatments, and between times after exposure, respectively (Tukey's HSD, α=0.05).

Wax moth larval mortality was significantly different among treatments (F=543.92, d.f.=3, *P*<0.0001), among different times after inoculation (F=76.89, d.f.=2, *P*<0.0001), and in the interaction between treatments and time after treatment (F=51.57, d.f.=6, *P*<0.0001). Within 6 DAT, the combination of *H. bacteriophora* with *M. anisopliae* plus Triton X-100 (Hb+Met+T) caused an average mortality of 81.7%, whereas *H. bacteriophora* alone (Hb) and *M. anisopliae* plus Triton X-100 (Met+T) killed 0% and 8.9% larvae, respectively. Within 22 DAT, larval mortality increased to 100% in Hb+Met+T, and was improved significantly from 8.9% to 98.9% in Met+T, but only increased to 1.1% in Hb; there were no significant changes from 12 to 22 DAT (Fig. 4). It suggests that *H. bacteriophora* alone was much less virulent than the combination of the nematode and the fungus plus Triton X-100. This was probably due to the relatively lower IJs mortality in Hb+Met+T as shown in Fig. 3.

In the treatment with Hb+Met+T, Hb infected 78.3 (±6.9) % larvae in 6 DAT, and the remaining insects showed fungal infection from 6 to 22 DAT, with 6.7% in green and 15% remained in white color for > 48 d; nematode-infected larvae excluded fungal growth. In Met+T, infection rate with *M. anisopliae* increased significantly from 8.9% in 6 DAT to 98.9% in 22 DAT (F=20.65, d.f.=2, *P*<0.0001) (Fig. 5); all fungal infected larvae turned green and sporulated successfully in 22 DAT.

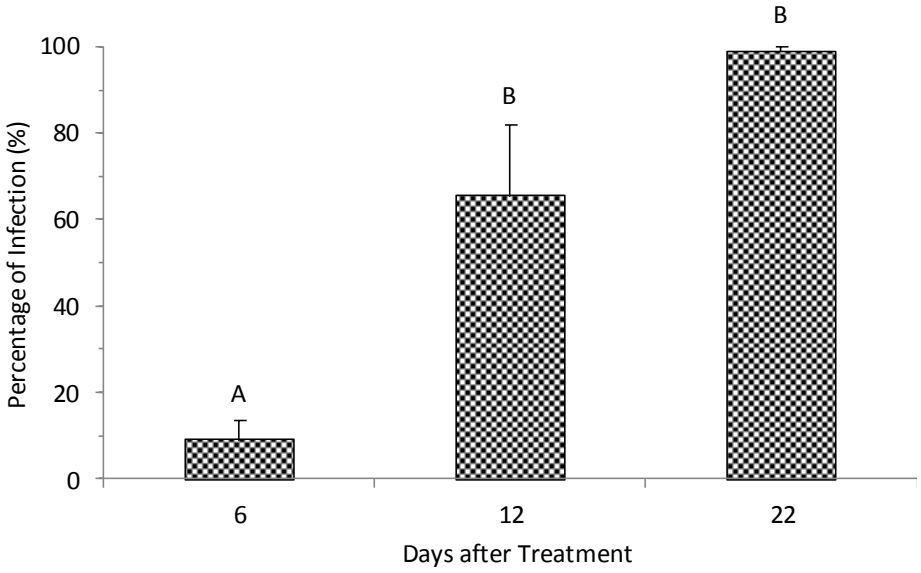

Fig. 5. Percentage of infection with *Metarhizium anisopliae* in greater wax moth larvae treated with *M. anisopliae* plus Triton X-100 in 6, 12 or 22 DAT. Different letters indicate significant difference between times after treatment (Tukey's HSD, α=0.05).

Discussion

In sandy loam soil without grass thatch (Experiment 1), a significantly higher level of *M. anisopliae* conidia were recovered in the combination with *H. bacteriophora* than the fungus alone. The difference showed that the combined treatment had 1381 conidia/g soil on average in soil depth of 10-15 cm, whereas *M. anisopliae* alone had no conidia recovered (Table 1). This suggests that the nematode might have had a positive interaction with the fungus, and played a role in the vertical distribution of the fungus. However, this was not repeated in the test using sandy soil with creeping bentgrass thatch (Experiment 2), in which conidia recovered in *M. anisopliae* alone or combined with *H. bacteriophora* were not significantly different from each other (Table 2). A possible explanation for such a difference is that soil and/or grass thatch restricted nematode movement, hence affecting the ability of nematode IJs in assisting the vertical distribution of fungal conidia. This was verified by nematode movement and distribution pattern in soil in both Experiments 1 and 2 (Table 1 & 2).

Soil depth and texture were among the major factors affecting distribution of *M. anisopliae*. Soil depths had significant effects on conidial distribution in both Experiment 1 and 2, with a trend showing fewer conidia in deeper soil. Soil texture might have also played a role in the vertical distribution of fungal conidia. In sandy loam soil tested in Experiment 1, most conidia were restricted within the depth of 10 cm, with only 0-1381 conidia/g soil found in 15 cm (Table 1); in sandy soil in Experiment 2, significant amounts of *M. anisopliae* conidia (10,070-27,523/g soil) were recovered at 15 cm (Table 2). This

indicates that fungal conidia were more uniformly distributed in sandy soil than in sandy loam soil, due to difference in size of pore spaces. In addition, soil texture also had an impact on nematode movement. Most nematode IJs were recovered within the depth of 5 cm in sandy loam soil (Table 1), compared with 10 cm in sandy soil (Table 2).

A significant interaction was observed between *M. anisopliae* and *H. bacteriophora* in water profile. *M. anisopliae* conidia germinated hyphae tubes that attached to the outer cuticle of IJs (Fig. 1 A & B), affecting the nematode mobility. It is possible that the nematode acted as a stimulus triggering fungal germination and growth. In Experiment 2, nematode IJs had a similar distribution pattern in treatments with or without adding *M. anisopliae* (Table 2), suggesting that the fungus had no negative impact on *H. bacteriophora* IJs mobility. This was probably due to lack of adequate and strong contact of IJs with fungal conidia, or insufficient time to interact with each other during application in sandy soil. The interaction between *M. anisopliae* and *H. bacteriophora* has potential for nematodes assisting the distribution of the fungus in the soil, enhancing fungal efficacy in pest control.

In Experiment 3, despite IJs being trapped by the fungus, no fungal hyphae were seen penetrating the inner cuticle and causing death of IJs. As in earlier experiments, *M. anisopliae* did not have any negative impact on IJs mortality in both Trials 1 and 2 (Fig. 2 & 3). This also indicates that nematodes were able to exchange air normally in solution with oil-based fungus. It is worthwhile to mention that the combination of *M. anisopliae* and *H. bacteriophora* plus Triton X-100 (Hb+Met+T) had a slightly lower IJs mortality than the nematode alone (Hb), or combined with *M. anisopliae* (Hb+Met), although statistically not significant (Fig. 3). A possible explanation is that shaking Triton X-100-added solution brought in air bubbles for nematodes to respire, in addition to improving dissolvability of the oil-formulated fungus in water.

An interesting phenomenon showed that affected IJs shed the outer cuticle (sheath) to detach from the trap of *M. anisopliae* (Fig. 1 C & D I-III). After molting, IJs virulence and physiological adaptations might be affected, as the sheath may afford protection against mechanical damage and environmental extremes during host searching. Wharton and Surry (1994) reported that the sheath of *H. zealandica* Poinar IJs prevented inoculative freezing, allowing the larva to supercool in the presence of external ice and to be freeze avoiding; the exsheathed IJs did not survive below -6 ^{o}C. Also, the sheath may protect the IJs from desiccation, as reported by Menti et al. (1997) that it slowed down the rate of drying of the enclosed juvenile of *H. megidis* Poinar, Jackson & Klein, enabling it to survive better than the exsheathed IJs. However, Timper et al. (1991) pointed out that the presence of a sheath may protect IJs against antagonistic organisms, such as pathogenic fungi, and may not necessarily indicate a role in desiccation survival. In the current experiment, we did not study the physiological tolerance of *H. bacteriophora* IJs

after molting, but the virulence of exsheathed IJs was not negatively affected under 20 ^{o}C. In addition, the combination of the nematode and fungus plus Triton X-100 was more virulent than the nematode alone against full grown wax moth larvae (Fig. 4). This was consistent with the higher survival rate of IJs in the combined treatment (Fig. 3).

M. anisopliae was less competitive than *H. bacteriophora* in virulence against *G. mellonella*. Within 6 DAT with Hb+Met+T, 78.3 (±6.9) % larvae showed nematode infection, and the remaining insects gradually showed symptoms of fungal infection with 6.7% sporulated and 15% not sporulated for > 48 DAT. It was interesting to find that sporulation in the combined treatment was much slower than in *M. anisopliae* alone. In Met+T, infection rate with *M. anisopliae* increased from 8.9% in 6 DAT to 98.9% in 22 DAT (Fig. 5); all fungal infected larvae turned green and sporulated successfully by 22 DAT. This indicates that sporulation might have been affected by the presence of *H. bacteriophora*, while the nematode excluded fungal growth on insects it killed, due to the symbiotic bacterium, *P. luminescens*. This is consistent with the study of Ansari et al. (2005) that *P. luminescens* was antagonistic to *M. anisopliae* by inhibiting growth and conidial production.

In Experiment 3, no insect showed symptoms of both nematode and fungal growth, indicating incompatibility in the same hosts. Barbercheck and Kaya (1990) stated that *H. bacteriophora* and *S. carpocapsae* (Weiser) were not compatible with *B. bassiana* in dually infected hosts, and usually only nematodes or fungus developed and produced progeny in *G. mellonella* exposed to both agents. Also, nematodes and their symbiotic bacteria prevented or inhibited the growth of *B. bassiana* if nematodes were applied within 24 h after fungal application, and the fungus was detrimental to the development of nematodes when applied more than 48 h ahead. In the current study, despite incompatibility in the same hosts, the combined application of *H. bacteriophora* and *M. anisopliae* plus Triton X-100 achieved higher mortality of *G. mellonella* than the nematode or fungus used alone (Fig. 4).

Overall, positive interaction was indicated between *H. bacteriophora* and *M. anisopliae*. *H. bacteriophora* IJs has potential in improving conidial distribution of *M. anisopliae* in soil, although the effect might be mitigated by grass thatch and soil texture. Higher efficacy is thus anticipated from the combined application of the nematode and fungus in pest control.

References

Anbesse, S. A., Adge, B. J., Gebru, W. M., 2008. Laboratory screening for virulent entomopathogenic nematodes (*Heterorhabditis bacteriophora* and *Steinernema yirgalemense*) and fungi (*Metarhizium anisopliae* and *Beauveria bassiana*) and assessment of possible synergistic effects of combined use against grubs of the barley chafer *Coptognathus curtipennis*. Nematology. 10**,** 701-709.

Ansari, M. A., Shah, F. A., Tirry, L., Moens, M., 2006. Field trials against *Hoplia philanthus* (Coleoptera: Scarabaeidae) with a combination of an entomopathogenic nematode and the fungus *Metarhizium anisopliae* CLO 53. Biol. Control. 39**,** 453-459.

Ansari, M. A., Tirry, L., Moens, M., 2005. Antagonism between entomopathogenic fungi and bacterial symbionts of entomopathogenic nematodes. BioControl. 50**,** 465–475.

Barbercheck, M. E., Kaya, H. K., 1990. Interactions between *Beauveria bassiana* and the entomogenous nematodes, *Steinernema feltiae* and *Heterorhabditis heliothidis*. J. Invertebr. Pathol. 55**,** 225-234.

Barbercheck, M. E., Kaya, H. K., 1991. Competitive interactions between entomopathogenic nematodes and *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) in soilborne larvae of *Spodoptera exigua* (Lepidoptera: Noctuidae). Environ. Entomol. 20**,** 707-712.

Choo, H. Y., Kaya, H. K., Huh, J., Lee, D. W., Kim, H. H., Lee, S. M., Choo, Y. M., 2002. Entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis bacteriophora*) and a fungus *Beauveria brongniartii* for biological control of the white grubs, *Ectinohoplia rufipes* and *Exomala orientalis*, in Korean golf courses. BioControl. 47**,** 177-192.

Georgis, R., Gaugler, R., 1991. Predictability in biological control using entomopathogenic nematodes. J. Econ. Entomol. 84**,** 713-720.

Georgis, R., Kaya, H. K., Gaugler, R., 1991. Effect of steinernematid and heterorhabditid nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) on nontarget arthropods. Environ. Entomol. 20**,** 815-822.

Georgis, R., Koppenhöfer, A. M., Lacey, L. A., Bélair, G., Duncan, L. W., Grewal, P. S., Samish, M., Tan, L., Torr, P., van Tol, R. W. H. M., 2006. Successes and failures in the use of parasitic nematodes for pest control. Biol. Control. 38**,** 103-123.

Glare, T. R., 1992. Fungal pathogens of scarabs. In: T. A. Jackson, T. R. Glare, (Eds.), Use of Pathogens in Scarab Pest Management. Intercept, Andover, UK, pp. 63-77.

Kaya, H. K., Stock, S. P., 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., 1990. Efficacy against soil-inhabiting insect pests. In: R. Gaugler, H. K. Kaya, (Eds.), Entomopathogenic Nematodes in Biological Control. CRC press, Boca Raton, FL, pp. 195-214.

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

Menti, H., Wright, D. J., Perry, R. N., 1997. Desiccation survival of populations of the entomopathogenic nematodes *Steinernema feltiae* and *Heterorhabditis megidis* from Greece and the UK. J. Helminthol. 71**,** 41–46.

Rath, A. C., Worledge, D., Koen, T. B., Rowe, B. A., 1995. Long-term field efficacy of the entomogenous fungus *Metarhizium anisopliae* against subterranean scarab, *Adoryphorus couloni*. Biocontrol Sci. Technol. 5**,** 439–451.

Timper, P., Kaya, H. K., Jaffee, B. A., 1991. Survival of entomogenous nematodes in soil infested with the nematode-parasitic fungus *Hirsutella rhossiliensis* (Deuteromycotina: Hyphomycetes). Biol. Control. 1**,** 42–50.

Wharton, D. A., Surry, M. R., 1994. Cold tolerance mechanisms of the infective larvae of the insect parasitic nematode *Heterorhabditis zealandica* Poinar. CryoLetters. 15**,** 353-360.

Yokoyama, T., Hasegawa, M., Fujiie, A., Sawada, M., Noguchi, K., 1998. Microbial control of scarab beetle larvae by a formulation of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) in a sweet potato field. Appl. Entomol. Zool. 33**,** 215–218.

SUMMARY

Maked chafers, *Cyclocephala* spp., are among the most widespread and damaging turfgrass pests in Virginia. Currently, the predominant strategy for the management of these pests is to apply conventional insecticides, such as imidacloprid and clothianidin, on a preventative basis before damage occurs. For such a method to be successful, large areas need to be sprayed. As these chemicals are more effective against young grubs, damage can occur late in the season if early attempts fail to control them. In addition, the impact from long-term use of insecticides on the environment, human health, natural enemies, and insecticide resistance has increased public awareness for a more bio-rational approach in managing these turfgrass pests. The desirability of developing environmentally safe alternatives to achieve persistent and sustainable control of the pests has led to the development of biological control agents including entomopathogenic nematodes (EPN) and entomopathogenic fungi (EPF). EPN and EPF may have potential in providing sustainable control of these pests. However, their field efficacies are often inconsistent and unsatisfactory, due to various environmental factors, and combining the application of EPN and EPF may achieve an improved level of control over white grub numbers. The goal for this research was to explore the potential for improved efficacy from the combined use of EPN and EPF against *Cyclocephala* spp. It was hypothesized that additive or synergistic interactions would be achieved from the combined use of the two types of pathogens and thus improve the overall efficacy in the management of these pests.

To verify the hypothesis, this research tested the interaction between two selected EPN species (*H. bacteriophora* and *H. megidis*), and two EPF species (*M. anisopliae* and *B. bassiana* in different formulations) under laboratory and greenhouse conditions, as well as their efficacy against white grubs at various stages in the field. Additive interactions were found between EPN and EPF in most combinations against *Cyclocephala* spp. under both laboratory and greenhouse conditions, with the exception of a few observations that showed antagonism or synergism. The low efficacy of fungal application might account for the lack of a stronger interactive effect in *Cyclocephala* spp. Under field conditions, the combined use of EPF and EPN improved control efficacy compared with nematodes or fungi applied alone in some trials, but efficacy of EPF and EPN varied dramatically between field sites. In addition, the sub-lethal effects of the two EPF species on southern masked chafer, *C. lurida*, were investigated. Neither *M. anisopliae* nor *B. bassiana* had a sub-lethal effect on masked chafer grub weight gain, adult longevity, oviposition, pupation and eclosion. Finally, interaction between *H. bacteriophora* and *M. anisopliae* was examined to determine the potential of the nematode in improving fungal distribution in soil. Fungal conidia attached to the sheath of nematode IJs without affecting the nematode virulence and viability, although IJs finally molted to detach from the fungus. Overall, these results indicate positive interactions

between EPN and EPF, and potential for improved efficacy from the combined use of nematodes and fungi against *Cyclocephala* spp. More virulent fungal strains or species may be required to achieve a stronger interaction with nematodes in the management of these pests.

Chapter 2

Virulence was tested on four EPN species (two *Heterorhabditis* spp. and two *Steinernema* spp.) against 3rd instar *Cyclocephala* spp. at two rates. *Heterorhabditis bacteriophora* was most virulent, followed by *H. megidis*; the two *Steinernema* spp. were less virulent, especially *S. riobrave* did not cause any infection in the insects. The rates of 2.5 and 5 billion IJs/ha were equally effective. In addition, bioassays were conducted on overwintered and pre-diapausing 3rd instar *Cyclocephala* spp. with *H. bacteriophora* and *H. megidis*, and two EPF species, *M. anisopliae* strain F52 and *B. bassiana* strain GHA. The LC_{25} and LC_{50} were 0.58 and 2.1 billion IJs/ha for *H. bacteriophora*; 0.48 and 2.2 billion IJs/ha for *H. megidis*, respectively, against pre-diapausing 3rd instars 2 wk after treatment. No LC values were obtained for either EPN species against overwintered grubs. Bioassay tests on *M. anisopliae* and *B. bassiana* did not show rate-dependent responses, except for *M. anisopliae* against pre-diapausing 3rd instars.

Chapter 3

Interactions between EPN (*H. bacteriophora* or *H. megidis*) and EPF (*B. bassiana* or *M. anisopliae*) were evaluated for efficacy against *Cyclocephala* spp. at the 2nd instar, pre-wintering 3rd instar and overwintered stages. When applied alone, *B. bassiana* ES & WP and *M. anisopliae* did not have a significant effect in reducing grub numbers at various stages, except for the use of *M. anisopliae* against overwintered grubs. Adding EPN to EPF significantly improved the efficacy in grub control compared with EPF applied alone. However, compared with nematodes applied alone, adding EPF did not significantly improve the effect of control. Additive interactions were found between EPN and EPF in most treatments against grubs at various stages, with the exception of a few observations that showed synergism or antagonism. The combined effect did not differ significantly for nematode and fungal applications made simultaneously or at different times. Temperature had a significant impact on efficacy of *H. bacteriophora* and *M. anisopliae*, and grub mortality was enhanced significantly as temperature increased. EPF had no significant impact on the EPN infection and IJs production in grub carcasses. No significant difference showed among developmental stages for the interaction of EPN and EPF on the grub.

Chapter 4

The efficacy of EPN and EPF and their interaction were evaluated against 3rd instar *Cyclocephala* spp. under greenhouse conditions for 3 yr. Additive or synergistic interactions were found between *H.*

bacteriophora and most fungal types, except for *B. bassiana* ES, in both 2011 and 2012. The interactions between *B. bassiana* ES and the two nematode species were masked due to the high grub mortality in the untreated control in 2010. Similar to laboratory findings, significant improvement occurred from the combination of a nematode and a fungus over the fungus alone, but not over the nematode alone. There appears to be a trend indicating that combined use of a fungus and *H. bacteriophora* achieved higher grub mortality than a single agent applied alone. Overall, the use of the nematodes and/or fungi provided a level of effect comparable to the insecticide Merit 75 WP in the control of 3rd instar *Cyclocephala* spp.

Chapter 5

Efficacies of EPN and EPF were evaluated against the white grub complex, including Japanese beetle (*Popillia japonica*) and masked chafers (*Cyclocephala* spp.), at different developmental stages under field conditions for up to 4 consecutive yr. No significant difference was detected between *H. megidis* and *H. bacteriophora* alone or between nematode applications made on Jul-28 and Aug-13, 2010. The combined use of EPF and EPN improved the efficacy compared with nematodes or fungi applied alone in some trials. EPN and EPF applied alone or in combination were not more effective than Merit 75 WP in grub control in >50% field trials or treatments, but some EPN + EPF treatments were more effective than the insecticide in reducing grub numbers. The efficacy of EPF and EPN varied dramatically with field sites and conditions, and those that provided good grub control in some trials were not equally effective in others. Environmental conditions, especially temperature, might explain the inconsistent efficacy of nematodes and fungi in white grub management. EPN and EPF showed better potential for providing extended control of white grubs in the next generation than insecticides.

Chapter 6

The sub-lethal effects of EPF were tested on southern masked chafer, *C. lurida*. Neither *M. anisopliae* nor *B. bassiana* had a significant impact on grub fitness in terms of weight gain. Adult longevity of this insect was not significantly affected by treatments, and male and female longevity were not significantly different. Oviposition was strongly correlated with the time duration of male presence and female longevity, but neither fungal species had a significant impact. Pupation rate for the treatment with *M. anisopliae* was significantly lower than that with *B. bassiana*, but there were no significant difference from the control. Neither the treatment of *M. anisopliae* or *B. bassiana* had any significant impact on adult eclosion. Both fungi had no sub-lethal effect on masked chafer grub weight gain, adult longevity, oviposition, pupation and eclosion.

Chapter 7

Interaction between EPN *H. bacteriophora* and EPF *M. anisopliae* was investigated for the nematode potential in improving fungal distribution in soil. In water profile, *M. anisopliae* conidia germinated hyphae tubes that attached to sheath of *H. bacteriophora* IJs. The IJs molt within 8-11 days to detach from the fungus. IJs mortality and virulence were not negatively affected by the presence of *M. anisopliae*. When wetting agent Triton X-100 was added, the combination of *H. bacteriophora* and *M. anisopliae* appeared to have lower IJs mortality and significantly higher virulence than the nematode alone. *M. anisopliae* was less competitive than *H. bacteriophora* in virulence against *G. mellonella*. In addition, significantly higher level of *M. anisopliae* conidia occurred in sandy loam soil without grass thatch, but not in sandy soil with thatch, when the fungus was combined with *H. bacteriophora* compared with the fungus alone. In both soil types, soil depths had significant effects on nematode and fungal distributions, which were mostly in 5 and 10 cm in sandy loam soil without grass thatch, and in 10 and 15 cm in sandy soil with grass thatch, respectively. The results indicate positive interaction between *H. bacteriophora* and *M. anisopliae*, and potential for improved efficacy in pest control from the combined use of the nematode and fungus, although soil texture and grass thatch might mitigate the impact.

Recommendation for turfgrass managers

For using these bio-control agents in turf, based on these studies, a recommendation for turfgrass managers or golf course superitendants is to apply the nematodes at the end-July or early to mid-August on an inundative basis to target masked chafer grubs in early instars to achieve highest efficacy. However, for nematode recycling in the field to provide sustainable control, it is preferable to apply them at the end of August when grubs are larger as more nematode IJs can be produced. The fungus *M. anisopliae* or *B. bassiana* can be added simultaneously or early in separate applications if the budget allows, but fungi alone may not achieve satisfactory control. The nematodes and fungi need to be applied no later than at the end of September as cold temperatures may affect their pathogenicity, especially nematode mobility and viability. Alternatively, they may be applied in late April or early May if overwintered grubs are a problem, but they may not provide extended control of the new generation in the fall as drought and high temperatures in summer threaten their survival. Annual applications may be required, as the potential for nematode surviving harsh winters remains unknown. However, in warmer climates they have potential for providing persistent and sustainable control of white grubs in soil with good moisture.

Selecting the proper nematode and fungal species/strains are critical for suscessful control. Because of this, grubs need to be sampled to determine the species and developmental stages for the best match. The nematodes and fungi can be applied with the same equipments used for chemical insecticides. Despite their compatibility with many pesticides, it is preferred to check the product labels to find out the time

intervals between applications of chemicals and nematode and/or fungi. In addition, they need to be applied at dawn or dusk, and preferably on a cloudy day or in rain, to avoid sun exposure. Over-head watering is required before and immediately after application, and also in 7-10 d to maintain the soil moisture.

Future research

For future research, more virulent pathogens are desirable to achieve higher efficacy in grub control. To be successful, grubs naturally infected with pathogens should be collected to filter pathogens virulent to the grubs. The pathogens may include but not limited to entomopathogenic fungi, nematode and bacteria. Another aspect for future research is to explore the potential for improving application strategies to increase nematode and fungal survival in the field, or their ability of penetrating thatch/soil for better contact with the target insects. This may involve mechanical devices for applications after aeration, or delivering insect carcasses infected with nematode or fungi to the field instead of direct application of materials in infective stages. In addition, site selection is critical for field studies to be productive. A site with a history of grub infestation is preferred. However, if it is not available, grub populations may be built up with a black light trap from the end of June to early-July along with proper irrigation in noncommercial turf, preferably with cool-season grasses, e.g. Kentucky bluegrass.