VARIATION IN THE SUSCEPTIBILITY OF TURF-INFESTING WHITE GRUBS TO DIFFERENT CONTROL AGENTS AND OPPORTUNITIES FOR SYNERGISTIC COMBINATIONS OF BIOLOGICALS AND NEONICOTINOIDS

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ABSTRACT

White grubs (Coleoptera: Scarabaeidae) are the most widespread and damaging pests in turfgrass habitats of the Northeast U.S. and their management is highly dependent on chemical pesticides. Best IPM is constrained by widespread reliance on early season preventive applications of imidacloprid as well as by a lack of biologically-based control alternatives. Part of the solution would be curative alternatives that would permit sampling and better decision-making, and biobased alternatives that could supplant reliance on chemical insecticides. This project's focus on white grub control in turfgrass thereby represents the major pest complex in one of the most extensive and rapidly expanding components of our urban and rural landscape. As managed ecosystems, extensive and diverse turfgrass habitats require decision-making strategies to maintain them for their intended uses. Better managing this vast area will have huge positive impacts, especially considering that this habitat is intimately associated with human populations and that home owners can spray lawn chemicals with little regulation or training. Synergistic combinations of selected biological and chemical control products are one approach that might yield valuable alternatives for the management of soil insect pests.

Chapter 1 summarizes laboratory studies that were conducted on third instar white grubs challenged by individual control products. The objective was to measure variation in the susceptibility of four invasive species of white grubs (*Amphimallon majale*, *Anomala orientalis*, *Maladera castanea* and *Popillia japonica*) to 18 registered and experimental insecticides used as curative controls under controlled laboratory conditions. Across white grub species, the most efficacious biological and

chemical insecticide alternatives were the entomopathogenic nematode *Steinernema scarabaei* and chlorpyrifos, respectively. Biorationals were highly variable across target species. For biorational and chemical insecticides, *A. majale* was the least susceptible species. For biologicals, *P. japonica* was the least susceptible. Considering all control products, *A. orientalis* was the most susceptible.

Chapter 2 summarizes a series of laboratory, greenhouse and field trials that were conducted on third instar white grubs challenged by combinations of control products. The objective was to screen numerous combinations of biological and sublethal doses of neonicotinoid insecticides against third instars under controlled laboratory conditions, and then to characterize those interactions as synergistic, additive or antagonistic. The most promising combinations were advanced to greenhouse pot studies and then to microplot field trials. To reveal variation across white grub species, trials were conducted on *A. majale* and *P. japonica*. Among the combinations of biological and neonicotinoids tested here, results revealed that synergistic interactions are relatively uncommon, and involved only entomopathogenic nematodes and fungi. For *A. majale*, the most promising synergistic combinations were between *H. bacteriophora* and both neonicotinoids; across all laboratory, greenhouse and field trials. In contrast, for *P. japonica* the most promising synergistic combinations were between *B. bassiana* and *M. anisopliae* Met F52 with clothianidin and *M. anisopliae* NYSAES with imidacloprid. Like *A. majale*, this was discernible in each of the two laboratory trials, but did not persist through to the greenhouse and field. Finally, an antagonistic interaction between *Bt*-products and both neonicotinoids was common to both white grub species.

The magnitude of variation in susceptibility across white grub species supports the idea that a single product will not reliably suppress populations of all scarab taxa. This differential susceptibility could have broader consequences for grub management, if a numerically dominant target species is more completely suppressed than a co-occurring species. Synergistic combinations of biological control products with reduced rates of neonicotinoid insecticides could be a promising approach for the curative control of white grubs and as an IPM tool for the suppression of other soil insect pests.

BIOGRAPHICAL SKETCH

Anuar Morales Rodríguez was born in Bogotá, D.C., Colombia on January 8, 1966. He is the eldest of three siblings. After graduation in 1985 from the Instituto Nacional de Educación Media Dersificada, INEM Santiago Pérez El Tunal-Bogotá, he attended the Universidad Distrital Francisco José de Caldas in Bogotá, and received his B.S. in Biology with a major in Education in 1995. His undergraduate thesis was titled "Biological control of the coffee berry borer *Hypothenemus hampei* Ferrari (Coleoptera: Scolytidae) with different propagules of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuill." Before and after his graduation from the university, he worked with different agrochemical companies in Colombia until 1995. From 1995 to 2004 he worked at the International Center for Tropical Agriculture (CIAT), Colombia in the beans, forage and IPM entomology programs. From 2000 to 2001 he attended the Universidad del Valle in Cali, Colombia to obtain a "Specialization in Entomology." He moved to the United States in 2004 as a Visiting Scholar in the Department of Entomology at the New York State Agricultural Experimental Station (NYSAES) of Cornell University in Geneva, NY. In 2006 he was accepted into the graduate program (M.S.) in the Field of Entomology at Cornell University, and conducted his work at the NYSAES.

Este trabajo se lo dedico al motor de mi vida, mi familia.

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PREFACE

Turfgrass covers > 20 million ha in the United States. The maintenance and protection of that area is an economically important industry. Insect control programs are a major sector of that industry and almost all are based on the use of chemical insecticides. In turfgrass habitats of the Northeast U.S., root-feeding white grubs are a widespread and damaging pest complex of eight species. Four species are introduced exotics, including the Japanese beetle, *Popillia japonica* Newman; the oriental beetle, *Anomala orientalis* Waterhouse; the Asiatic garden beetle, *Maladera castanea* (Arrow); and the European chafer, *Amphimallon majale* (Razoumowsky). Four are native species, including the black turfgrass ataenius, *Ataenius spretulus* (Haldeman); the green June beetle, *Cotinis nitida* (L); the northern masked chafer, *Cyclocephala borealis* Arrow; and the May or June beetles, *Phyllophaga anxia* (LeConte). Their management in both preventive and curative windows is highly dependent on chemical pesticides. Some of the most commonly used insecticides in control programs include carbamates, diacylhydrazine, neonicotinoids and organophosphates. Biological control options are available but unreliable and infrequently used. Integrated pest management is constrained by widespread reliance on early season preventive applications of imidacloprid as well as by a lack of biologically-based control alternatives. In practical terms, this means that turfgrass managers have no quantitative way to decide when not to spray, other than making localized applications that target areas highly susceptible or traditionally affected by these pests. Pest management practitioners seeking non-chemical options are stymied by commercial formulations that are relatively difficult or expensive to apply, or yield such inconsistent results that they are impracticable. Searching for opportunities for pest management in this system would be enhanced by understanding how susceptibility to

control products varies across taxa. In addition, previous work has revealed promising possibilities for reduced-risk curative control by combining microbiological control agents and neonicotinoid insecticides. Synergistic combinations of select control products might yield valuable alternatives for the management of white grubs and other soil insect pests.

The first objective of this research was to measure under laboratory conditions the variation in susceptibility of four invasive white grub species to 18 registered and experimental insecticides used as curative controls. Across white grub species, the most efficacious biological and chemical insecticide alternatives were *Steinernema scarabaei* and chlorpyrifos, respectively. Biorationals were highly variable across target species. For biorational and chemical insecticides, *A. majale* was the least susceptible species. For biologicals, *P. japonica* was the least susceptible. Considering all control products, *A. orientalis* was the most susceptible. The magnitude of variation in susceptibility across white grub species supports the idea that a single product will not reliably suppress populations of all taxa, and highlights the need for pest management practitioners to diagnose and differentiate scarab species before intervention.

The second objective was to understand the breadth of potential synergies by screening numerous combinations of biological control agents with sublethal doses of neonicotinoid insecticides against third instar white grubs under controlled laboratory conditions. The most promising combinations were advanced to pot studies conducted in the greenhouse and then to field trials featuring microplots with artificially infested populations. To reveal variation across white grub species, trials were conducted on *Amphimallon majale* and *Popillia japonica*.

Among the combinations of biologicals and neonicotinoids tested, synergistic interactions were relatively uncommon, and involved only entomopathogenic

nematodes and fungi. Synergies were remarkably consistent across trials, were specific to white grub species, and diminished in strength from lab to greenhouse to field. For *A. majale*, the most promising synergistic combinations were between *H. bacteriophora* and both neonicotinoids; those results were discernible in all laboratory and greenhouse trials and into the field. In contrast, the most promising synergistic combinations for *P. japonica* were *B. bassiana* and *M. anisopliae* Met F52 with clothianidin and *M. anisopliae* NYSAES with imidacloprid. Like *A. majale*, this was discernible in each of the two laboratory trials, but did not persist through to the greenhouse and field. Finally, an antagonistic interaction between *Bt*-products and both neonicotinoids was common to both white grub species.

The differential susceptibility detected in this study could have broader consequences for grub management if a numerically dominant target species is more completely suppressed than a co-occurring species. On the other hand, synergistic combinations of biological control products with reduced rates of neonicotinoid insecticides could be a promising approach for the curative control of white grubs and as an IPM tool for the suppression of other soil insect pests.

CHAPTER ONE

Variation in the laboratory susceptibility of turf-infesting white grubs (Coleoptera: Scarabaeidae) to biological, biorational and chemical control products[1]

Abstract

BACKGROUND: White grubs are the most widespread and damaging pests in turfgrass habitats of the Northeast U.S. and their management is highly dependent on chemical pesticides. Because this complex includes eight species, opportunities for pest management in this system would be enhanced by understanding how susceptibility to control products varies across taxa. The objective of this laboratory study was to measure variation in the susceptibility of four invasive species of white grubs to 18 registered and experimental insecticides used as curative controls.
RESULTS: Across white grub species, the most efficacious biological and chemical insecticide alternatives were *Steinernema scarabaei* and chlorpyrifos, respectively. Biorationals were highly variable across target species. For biorational and chemical insecticides, European chafer, *Amphimallon majale*, was the least susceptible species. For biologicals, Japanese beetle, *Popillia japonica*, was the least susceptible. Considering all control products, Oriental beetle, *Anomala orientalis*, was the most susceptible.
CONCLUSION: The magnitude of variation in susceptibility across white grub species supports the idea that a single product will not reliably suppress populations of all taxa, and highlights the need for pest management practitioners to diagnose and differentiate scarab species before intervention. This differential susceptibility could

[1] This chapter was submitted to Pest Management Science.

have broader consequences for grub management, if a numerically dominant target species is more completely suppressed than a co-occurring species.

Keywords: *Amphimallon majale; Anomala orientalis*; *Maladera castanea*; *Popillia japonica*; turfgrass.

1 INTRODUCTION

For pest management practitioners who often contend with complexes of injurious species, a successful outcome depends on the ability to properly tailor intervention programs to the species present. It is unlikely that a single intervention tactic or control product will suffice. Therefore, it would help to have an understanding of how generalizable control method efficacy is across the members of the pest complex. Even among closely related species, details of variation in natural history may have repercussions for the outcome of a control program.

In the northeastern United States, a complex of eight white grub (Coleoptera: Scarabaeidae) species is highly damaging to turfgrass and nurseries. Four species are introduced exotics, including the Japanese beetle, *Popillia japonica* Newman, which was discovered in New Jersey in 1916; followed by the oriental beetle, *Anomala orientalis* Waterhouse, in Connecticut in 1920; the Asiatic garden beetle, *Maladera castanea* (Arrow), in New Jersey in 1921; and the European chafer, *Amphimallon majale* (Razoumowsky), in New York in 1940.[1,2,3,4] Today, all four species are widespread across the northeastern United States and are expanding into other regions of the country. Four native white grub species are also considered turfgrass pests in the Northeast. These include the black turfgrass ataenius, *Ataenius spretulus* (Haldeman); the green June beetle, *Cotinis nitida* (L); the northern masked chafer,

Cyclocephala borealis Arrow; and the May or June beetles, *Phyllophaga anxia* (LeConte).[1]

Turfgrass covers > 20 million ha in the United States.[5] The maintenance and protection of that area is an economically important industry. Insect control programs are a major sector of that industry and almost all are based on the use of chemical insecticides.[6] The pressure on turfgrass managers (golf course superintendents, lawn care providers and home owners) to maintain high aesthetic standards leaves little role for cultural or biological control. Unfortunately, there is no known specific host plant resistance for white grubs. This generates a high dependence on interventions through applications of control products, in particular chemical insecticides. Some of the most commonly used insecticides in control programs include carbamates, diacylhydrazine, neonicotinoids and organophosphates. Biological control options are available but unreliable and infrequently used. The most popular is *Paenibacillus* (=*Bacillus*) *popilliae* (Dutky), commonly referred to as "milky disease" or "milky spore disease".[7,8] Commercial formulations of nematodes from the genera *Heterorhabditis* and *Steinernema* are also available, as well as the fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin.[8] Most of the efficacy studies with biological control agents have been conducted as preventive controls of first or second instars where they have a better opportunity to suppress populations due to increased susceptibility. We found few reports where biological agents have been tested as curative controls of third instars.

"Biorational" or "biopesticide" is the generic name used for some insecticides derived from natural sources. These can include products from plants (neem and oils), insect pathogens (*Bt*-toxin), non insect pathogens (spinosyn), minerals or fossils (diatomaceous earth), and other ingredients such as soap.[9] Biorationals are desired by management practitioners because they are regarded as being innocuous, with low

environmental toxicity and minor impact to non-target organisms.[10] Among the *Bt*-insecticides, only some strains of *Bacillus thuringiensis* Berliner have been recommended for white grub control in turfgrass.

With two exceptions (*A. spretulus* and *P. anxia*), white grubs have a 1-yr life cycle with two principal application windows. In the preventive window, applications of control agents target small first and second instars, while in the curative window they target large third instars. Due to the relatively greater susceptibility of early life stages, there are more preventive control alternatives. However, since preventive interventions are made in a window prior to damage and feasible scouting, applications could be unnecessary or the area applied underestimated. Curative controls can be made after scouting to assess thresholds or in response to damage, but alternatives are few and there may be no opportunity to reapply if curative interventions fail.[11]

The decision on when to apply and what kind of control needs to be applied is also complicated by the common occurrence of two or more grub species at a site. Although some characteristics of the biology and behavior of the four invasive species are similar, others are subtly different. For instance, about 5-10% of the population of *A. majale* take 2 yr to complete their life cycle.[1] *Amphimallon majale* and *M. castanea* fly for a relatively short period (2-3 wk) in late spring while *P. japonica* and *A. orientalis* fly for longer periods (6-10 wk) during the summer and early fall. This means there are different times frames for the preventive control window as well as possible overlap in the curative and preventive windows for the different species in early fall. Another factor that complicates control decisions is the variation in action thresholds. Thresholds for third instars vary from 18-20 and 5-10 grubs per 0.1 m^2 in *M. castanea* and *A. orientalis*, respectively, to 4-5 grubs per 0.1 m^2 in *A. majale and P. japonica*.[1, 12]

Several chemical insecticides are recommended for the control of white grubs in general, and some variation in efficacy is documented across target species. For instance, laboratory tests showed halofenozide (N-[4-chlorobenzonyl]-N-benzoyl-terbutyl hydrazine) to be highly toxic to *P. japonica*, less toxic to *A. orientalis* and least toxic to *A. majale*.[13] A differential susceptibility was also found with bendiocarb, chlorpyrifos, diazinon, ethoprop and isofenphos against third instar *P. japonica*, *A. orientalis* and *A. majale*.[14] Variation in efficacy across white grub species also occurs with biological agents such as nematodes and fungi, as well as biorational controls such as azadirachtin and *Bt* serovar *japonensis* strain Buibui.[15, 16, 17, 18, 19] More studies to develop species-specific insecticide recommendations for the white grub complex are needed.[14]

The main objective of this study was to measure variation in the susceptibility of four invasive white grub species to 18 registered and experimental insecticides, including biological, biopesticide and chemical agents as curative controls. These studies were conducted under controlled laboratory conditions to reduce the possibility of interactions with other antagonists in the field. We sought information to help establish which groups of insecticides are the best options for the control of individual species, as well as the complex, and to ascertain whether the magnitude of variation in the efficacy of these insecticides has relevance for the design of grub control programs.

2 MATERIALS AND METHODS

2.1 White grubs

All studies were conducted on third instar white grubs collected from the field. We chose this life stage because it is the most difficult to control, it is the target for curative control in the field, and it can be maintained in the laboratory for several

months. This life stage was collected from infested turf in late fall before grubs descended in the soil profile for overwintering. *Maladera castanea* and *A. majale* were collected on 1 October 2004 in Lyons, NY (Wayne Hills Country Club, Wayne Co.). *Amphimallon majale* was also collected on 1-2 November 2004 in Saratoga Springs, NY (Saratoga Spa Golf, Saratoga Co.). *Anomala orientalis* was collected on 1-2 November 2004 in Saratoga Springs, NY (Saratoga Spa Golf, Saratoga Racing Cars and nearby residential lawn, Saratoga Co.). *Popillia japonica* was collected on 29 November 2005 in Fulton, NY (Battle Island Golf Course, Oswego Co.). The larvae were maintained in wooden boxes (30 cm wide x 50 cm long x 12 cm high) with soil and a piece of sod as food source from the same sites of collection. Boxes were held in a walk-in cooler at 10^{o}C until start of the trials.

2.2 Trial protocols

One larva was housed per 30-ml plastic cup filled with approximately 30 g of soil and a pinch of grass seed (AgWay Shady-Green) included as a food source. Cup diameter was 4.0 cm with a surface area of 12.6 cm^2 at the top of the cup. Sandy soil (82.9% sand, 12.1% silt, 5.0% clay, 0.79% organic matter, pH 7.09) was screened and raised to 10.5% (w/w) moisture.

After the grub had successfully burrowed into the soil, both liquid and wettable powder formulations of control products were applied in 1 ml of water over the soil surface. Granular treatments were applied on the surface followed by an additional 1 ml of water. Cups were capped after treatments had been applied. In addition to an untreated check (water), 18 experimental treatments were applied representing biological, biopesticide and chemical insecticides (Table 1). The application rate for each insecticide was based on the highest label rate for white grubs or on direct recommendations from the manufacturer.

Table 1. Control products (18) evaluated in controlled laboratory bioassays against third instar of four species of white grubs.

Control agent	Active ingredient	Commercial name	Source	Rate[a]
Biologicals:				
Bacterial pathogen	*Paenibacillus popilliae*	Milky Spore	St. Gabriel Laboratories, Orange, VI	24.76 kg (AI) ha^{-1}
Entomopathogenic nematode	*Heterorhabditis bacteriophora*	Heteromask	BioLogic, Willow Hill, PA	2.04 X 10^9 IJ3 ha^{-1}
	Heterorhabditis sp.	---	NYSAES, Geneva, NY	2.04 X 10^9 IJ3 ha^{-1}
	Steinernema scarabaei	---	Rutgers University, New Brunswick, NJ	8.19 X 109 IJ3 ha^{-1}
Fungal entomopathogen	*Beauveria bassiana* GHA	Botanigard ES	Emerald BioAgriculture Okemos, MI	8.14 X10^{15} con ha^{-1}
	Metarhizium anisopliae F52	---	Novozymes Biologicals, Salem, VA	7.94 X 10^{15} con ha^{-1}
Biopesticides:				
Azadirachtin	Azadirachtin	Ornazin 3% EC	SePRO Carmel, IN	0.022 L (AI) ha^{-1}
Bacillus thuringiensis	*Bt* var. *galleriae*	---	(Proprietary)	0.65 kg (AI) ha^{-1}
	Bt var. *tenebrionis*	Novodor FC	Valent BioSciences, Libertyville, IL	0.09 kg (AI) ha^{-1}
Diatomaceous earth	Diatomaceous earth	Concern	Woodstream, Lititz, PA	7949.14 kg ha^{-1}
Spinosad	Spinosad	Conserve SC	Dow Agrosciences, Indianapolis, IN	0.44 L (AI) ha^{-1}
Chemicals:				
Neonicotinoid	Clothianidin	Arena 50 WDG	Valent USA Corporation, Walnut Creek, CA	0.45 kg (AI) ha^{-1}
	Dinotefuran	Safari 20 SG	Valent USA Corporation, Walnut Creek, CA	0.61 kg (AI) ha^{-1}
	Imidacloprid	Merit 0.2%	Bayer, Durham, NC	0.39 kg (AI) ha^{-1}
	Thiamethoxam	Flagship 25 WG	Syngenta, Wilmington, DE	0.30 kg (AI) ha^{-1}
Organophosphate	Chlorpyrifos	GrubGuard	Grotech, Melbourne, Australia	2.0 kg (AI) ha^{-1}
	Trichlorfon	Dylox 80	Bayer, Durham, NC	9.12 kg (AI) ha^{-1}
Pyrethroid	Bifenthrin	Talstar GC Flowable	FMC, Philadelphia, PA	0.24 kg (AI) ha^{-1}
Untreated check	Untreated check	---	---	1 ml water cup^{-1}

[a] AI = active ingredient, IJ3 = third instar infective juvenile nematodes, con = conidia

The registered and experimental biologicals were represented by *B. bassiana, H. bacteriophora*, *Heterorhabditis* sp., *M. anisopliae, P. popilliae* and *Steinernema scarabaei* Stock and Koppenhöfer. The *Heterorhabditis* sp. strain NYSAES was isolated from soil samples from Fulton, NY in 2005 (Battle Island Golf Course, Oswego Co.). It was cultured in the last instar of the greater wax moth, *Galleria mellonella* L. The emerging infective juveniles (IJ3) were harvested from white traps the same day of application.[20] The biopesticides were represented by azadirachtin, two *Bt*- products (*Bt.* var. *galleriae* in an experimental formulation and *Bt.* var. *tenebrionis* as a commercial product), diatomaceous earth and spinosad. Most commercial *Bt*-products contain the protein toxin and spores, but some are cultured in a manner that yields only the toxin component. Since the insecticidal activity of Bt-products is largely attributed to the toxin (in some cases the spore has no direct effect on mortality), for the purposes of this manuscript we categorized them as biopesticides. The neonicotinoid insecticides were represented by clothianidin, dinotefuran, imidacloprid and thiamethoxam. The organophosphates were represented by chlorpyrifos (bait formulation) and trichlorfon. The pyrethroids were represented by bifenthrin. All products were tested against all four species with the exception that azadirachtin, bifenthrin and dinotefuran were not tested on *A. orientalis* and *M. castanea.*

We conducted five repetitions, each with 20 grubs. Studies were initiated for *A. orientalis, M. castanea* and *A. majale* on 7 January 2005 and for *P. japonica* on 19 December 2005. After application the cups with insects were maintained in a walk-in environmental chamber under controlled climate conditions (complete darkness, 90-95% HR, 25^{o}C) at the NYSAES, Geneva, NY. Evaluations were made at 10, 20 and

30 days after treatment (DAT) to measure mortality rates. To do this, the contents of each cup were emptied onto a piece of paper, the status of the grub was assessed, and the contents were replaced in the cup with a pinch of additional grass seed.

2.3 Data analyses

We calculated mean mortality rates for each treatment based on the five repetitions. Treatment mortality data were corrected for mortality in the untreated check using Abbott's formula.[21] Percentage data was normalized using an arcsine square root transformation. Data were analyzed with Proc Mixed using SAS 9.1 as least-square means (LSMEANS statement), where repetition was treated as a random factor and treatment and species were treated as fixed factors within a repeated-measures design. Each experimental treatment was compared with the untreated check using Dunnett's test. For treatments with significant effects, mortality was defined as low when 0-29% of the insects died, moderate at 30-79% and high at 80-100%. All statistical analyses were performed using SAS.[22]

3 RESULTS

3.1 Biologicals

There were significant effects of treatment, white grub species, time after application, and all interactions on mortality (Table 2). Among the treatments, only *S. scarabaei* and *H. bacteriophora* caused a significant mortality in all four of the white grub species (Fig. 1). Among white grub species, *A. orientalis* was significantly more susceptible than *A. majale* and *M. castanea*, followed by *P. japonica,* which was significantly less susceptible ($P \leq 0.05$, LSD). With respect to interactions among

treatments, white grub species and time after application, mortality due to *H. bacteriophora* and *S. scarabaei* in *P. japonica,* for instance, was already expressed at 10 DAT and fully expressed by 20 DAT. In contrast, mortality was not expressed in *A. orientalis* until 30 DAT. In general, mortality due to nematode treatments was expressed faster than fungal or bacteria treatments. For instance, at 30 DAT in *A. majale,* both *S. scarabaei* and *M. anisopliae* caused significant mortality. At 10 DAT, however, mortality was already > 90% for *S. scarabaei*, but not significant for *M. anisopliae*.

Among the three nematode treatments, *S. scarabaei* caused high mortality in all four white grub species (Table 3). Significant mortality was already expressed at 10 DAT and by 30 DAT it was >97% for all species. There was no significant effect of white grub species ($F = 1.14$; df = 3, 48; $P = 0.341$) or time ($F = 2.12$; df = 2, 48; $P = 0.058$).

Table 2. PROC MIX of percent mortality (arcsine square root transformed) of white grubs (*Anomala orientalis*, *Amphimallon majale*, *Maladera castanea*, *Popillia japonica*) in response to biologicals under laboratory bioassay conditions.

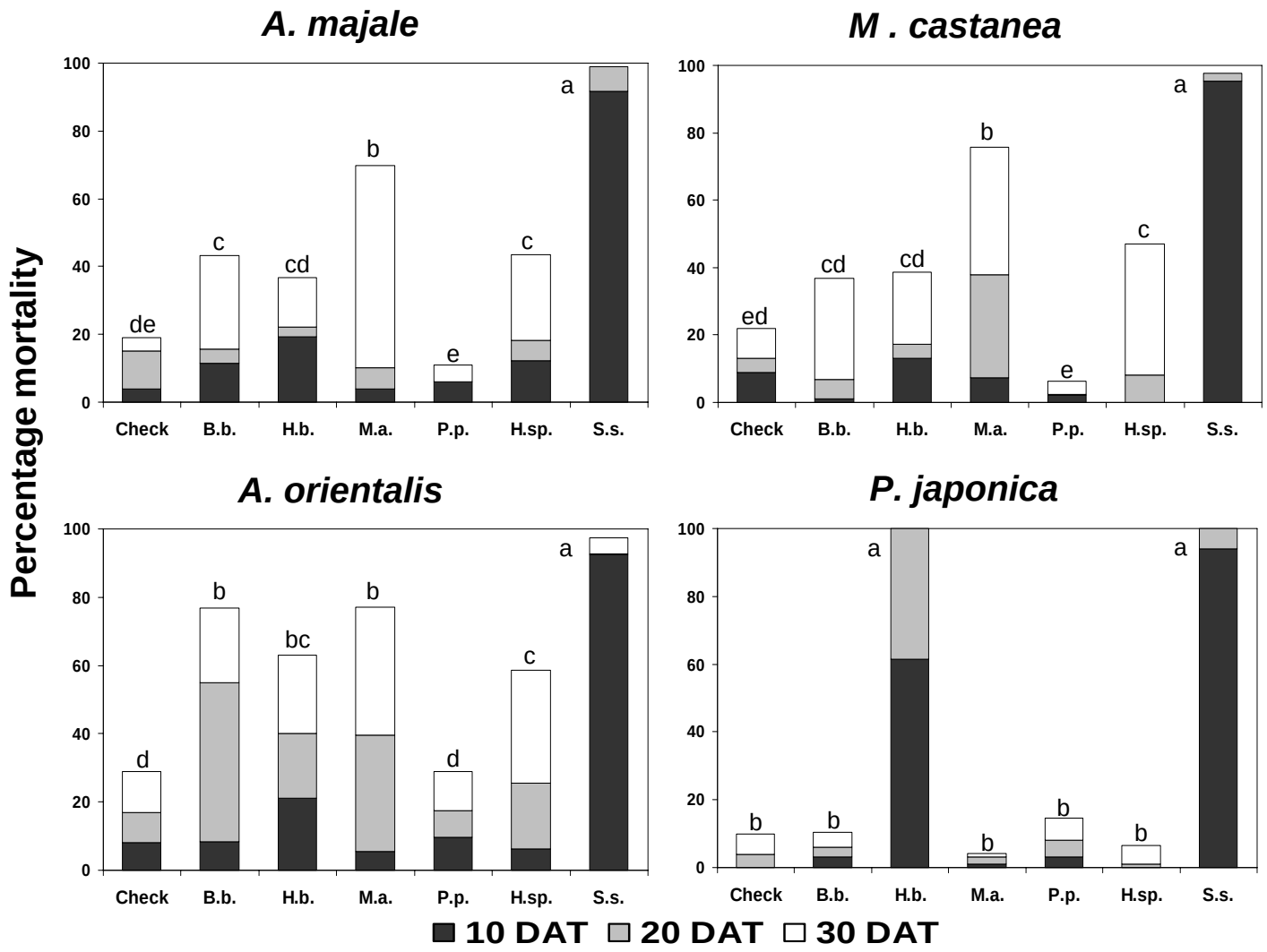
Source	Num df	Den df	*F* value	Pr > F
Treatment	6	332	167.67	<0.0001
Species	3	332	30.33	<0.0001
Time	2	332	129.43	<0.0001
Treatment*species	18	332	15.26	<0.0001
Species*time	6	332	6.05	<0.0001
Treatment*time	12	332	6.85	<0.0001
Treatment*species*time	36	332	1.48	0.0412

Table 3. Mean (± SE) percent mortality four species of white grubs at 10, 20 and 30 days after treatment (DAT) with biological insecticides.

Treatment	White grub species	10 DAT [a]	20 DAT [a]	30 DAT [a]
Nematodes:				
H. bacteriophora	*A. majale*	19.2 ± 9.2*	22.2 ± 12.3*	36.7 ± 14.7**
	A. orientalis	21.1 ± 6.5**	40.1 ± 8.2*	63.0 ± 8.2***
	M. castanea	13.0 ± 5.8*	17.3 ± 6.2 NS	38.7 ± 12.5***
	P. japonica	61.4 ± 4.3***	100.0 ± 0.0***	100.0 ± 0.0***
Heterorhabditis sp.	*A. majale*	12.1 ± 4.0 NS	18.1 ± 4.9 NS	43.5 ± 3.2**
	A. orientalis	6.3 ± 3.0 NS	25.6 ± 9.7*	58.6 ± 9.2***
	M. castanea	0.0 ± 0.0 NS	8.0 ± 4.4 NS	47.0 ± 5.1**
	P. japonica	0.0 ± 0.0 NS	1.1 ± 1.1 NS	6.7 ± 4.4 NS
S. scarabaei	*A. majale*	91.6 ± 5.2***	98.9 ± 1.1***	98.8 ± 1.3***
	A. orientalis	92.5 ± 2.8***	92.9 ± 2.7***	97.4 ± 1.61***
	M. castanea	95.4 ± 2.2***	97.6 ± 1.5***	97.3 ± 1.7***
	P. japonica	94.0 ± 4.0***	100.0 ± 0.0***	100.0 ± 0.0***
Bacteria:				
P. popilliae	*A. majale*	6.0 ± 4.0 NS	5.4 ± 3.5 NS	10.4 ± 4.3 NS
	A. orientalis	9.6 ± 4.8 NS	17.4 ± 6.6 NS	28.8 ± 4.4**
	M. castanea	2.0 ± 2.0 NS	2.2 ± 2.2 NS	5.1 ± 3.0 NS
	P. japonica	3.2 ± 2.0 NS	5.2 ± 2.3 NS	6.4 ± 2.6 NS
Fungi:				
B. bassiana GHA	*A. majale*	11.6 ± 5.3 NS	15.6 ± 2.6 NS	43.4 ± 8.4**
	A. orientalis	8.5 ± 3.8 NS	54.8 ± 5.7***	77.0 ± 4.0***
	M. castanea	1.1 ± 1.1 NS	6.9 ± 4.2 NS	36.8 ± 7.0 NS
	P. japonica	3.1 ± 2.0 NS	6.1 ± 3.7 NS	10.4 ± 5.5 NS
M. anisopliae	*A. majale*	4.0 ± 1.9 NS	10.1 ± 4.1 NS	69.8 ± 7.3***
	A. orientalis	5.5 ± 2.5 NS	39.6 ± 5.7***	77.2 ± 6.1***
	M. castanea	7.2 ± 4.5 NS	37.9 ± 8.7***	75.6 ± 5.8***
	P. japonica	1.0 ± 1.0 NS	3.2 ± 1.3 NS	4.2 ± 2.6 NS

[a] Means are significantly different from the untreated check at *0.05, **0.01 and ***0.001 (Dunnett's test), NS = Not significant.

Figure 1. Percent mortality of four species of white grubs at 10, 20 and 30 days after treatment (DAT) with biologicals. B.b. = *Beauveria bassiana*, H.b. = *Heterorhabditis bacteriophora*, M.a. = *Metarhizium anisopliae*, P.p. = *Paenibacillus popilliae*, H. sp. = *Heterorhabditis* sp., S.s. = *Steinernema scarabaei*. For each species, bars with the same letter are not significantly different for cumulative mortality by 30 DAT (Tukey's test, $P < 0.005$).


A. majale
M . castanea
A. orientalis
P. japonica
Percentage mortality
100
80
60
40
20
0
Check
B.b.
H.b.
M.a.
P.p.
H.sp.
S.s.
de
c
cd
b
e
c
a
ed
cd
cd
b
e
c
a
d
b
bc
b
d
c
a
b
b
a
b
b
b
a
10 DAT
20 DAT
30 DAT

For *H. bacteriophora,* there was a significant effect of white grub species ($F = 32.18$; $df = 3, 48$; $P < 0.0001$) and time ($F = 16.87$; $df = 2, 48$; $P < 0.0001$). Moderate mortality in *P. japonica* was already expressed at 10 DAT, increasing to 100% mortality by 20 DAT (Table 3). There was moderate mortality in *A. orientalis, A. majale* and *M. castanea* at 30 DAT.

In the third nematode treatment, *Heterorhabditis* sp., there was also a significant effect of white grub species ($F = 33.10$; $df = 3, 48$; $P < 0.0001$) and time ($F = 63.63$; $df = 2, 48$; $P < 0.0001$) (Table 3). There was significant moderate mortality in *A. orientalis, M. castanea* and *A. majale* but this was not expressed until 30 DAT. There was no significant effect on *P. japonica.*

For *P. popilliae,* there was a significant effect of white grub species ($F = 32.18$; $df = 3, 48$; $P < 0.0001$) and time ($F = 15.22$; $df = 3, 48$; $P = 0.0002$). There was significant low mortality in *A. orientalis* at 30 DAT. There was no significant effect on any of the other three grub species (Table 3).

Both fungal treatments caused moderate to high mortality in *A. orientalis*, and *A. majale*, but not in *P. japonica* or *M. castanea.* For *M. anisopliae,* there was a significant effect of white grub species ($F = 56.17$; $df = 3, 48$; $P < 0.0001$) and time ($F = 107.78$; $df = 2, 48$; $P < 0.0001$). Significant mortality in *M. castanea* and *A. orientalis* was already expressed at 20 DAT and at 30 DAT there was significant mortality in *A. majale, M. castanea* and *A. orientalis.* For *B. bassiana*, there was a significant effect of white grub species ($F = 26.41$; $df = 3, 48$; $P < 0.0001$) and time ($F = 48.81$; $df = 2, 48$; $P < 0.0001$). Significant mortality in *A. orientalis* was expressed at 20 DAT and this increased at 30 DAT. Significant moderate mortality in *A. majale*

was expressed at 30 DAT. There was no significant effect of *B. bassiana* on *M. castanea* or *P. japonica*.

Table 4. PROC MIX of percent mortality (arcsine square root transformed) of white grubs (*Anomala orientalis*, *Amphimallon majale*, *Maladera castanea*, *Popillia japonica*) treated with biopesticide insecticides under laboratory bioassay conditions.

Source	Num df	Den df	*F* value	Pr > F
Treatment	5	260	14.27	<0.0001
Species	3	260	4.15	0.0067
Time	2	260	131.63	<0.0001
Treatment*species	13	260	15.69	<0.0001
Species*time	6	260	7.08	<0.0001
Treatment*time	10	260	2.29	0.0138
Treatment*species*time	26	260	1.26	0.1836

3.2 Biorationals

There were significant effects of treatments, white grub species, time after application and all 2-way interactions on mortality (Table 4). Among the treatments, only diatomaceous earth caused a significant mortality in all four of the white grub species (Fig. 2). Among white grub species, *A. orientalis* was significantly more susceptible than *P. japonica* and *M. castanea*, followed by *A. majale,* which was significantly less susceptible ($P \leq 0.05$, LSD). With respect to the interaction between white grub species and time after application, mortality due to spinosad in *M. castanea* was not significant at 20 DAT but increased significantly by 30 DAT (Table 5). With respect to the interaction between treatment and time after application, mortality due to diatomaceous earth, for instance, caused significant mortality in *A. majale* and *P.*

japonica by 10 DAT, but this was not expressed in *A. orientalis* and *M. castanea* until 30 DAT (Table 5).

For azadirachtin there was a significant effect of white grub species ($F = 86.79$; $df = 1, 24$; $P < 0.0001$) and time ($F = 6.52$; $df = 2, 24$; $P = 0.0055$). Moderate mortality was expressed in *P. japonica* by 10 DAT and this increased to high mortality by 30 DAT. In contrast, azadirachtin had no effect on *A. majale*. For the mechanical insecticide treatment, diatomaceous earth, there was a significant effect of time ($F = 14.65$; $df = 2, 48$; $P < 0.0001$) but not white grub species ($F = 1.10$; $df = 3, 48$; $P = 0.359$). Moderate mortality was expressed in *P. japonica* and *A. majale* at 10, 20 and 30 DAT. There was also a significant effect on *M. castanea* and *A. orientalis* at 30 DAT but no significant effect was detected at 10 or 20 DAT. Among the microbial-derived biopesticides, spinosad showed a significant effect of white grub species ($F = 5.99$; $df = 2, 48$; $P = 0.0015$) and time ($F = 39.50$; $df = 2, 48$; $P < 0.0001$). Mortality was not expressed until 30 DAT for *M. castanea*, *A. orientalis* and *A. majale.* There was no effect on *P. japonica.*

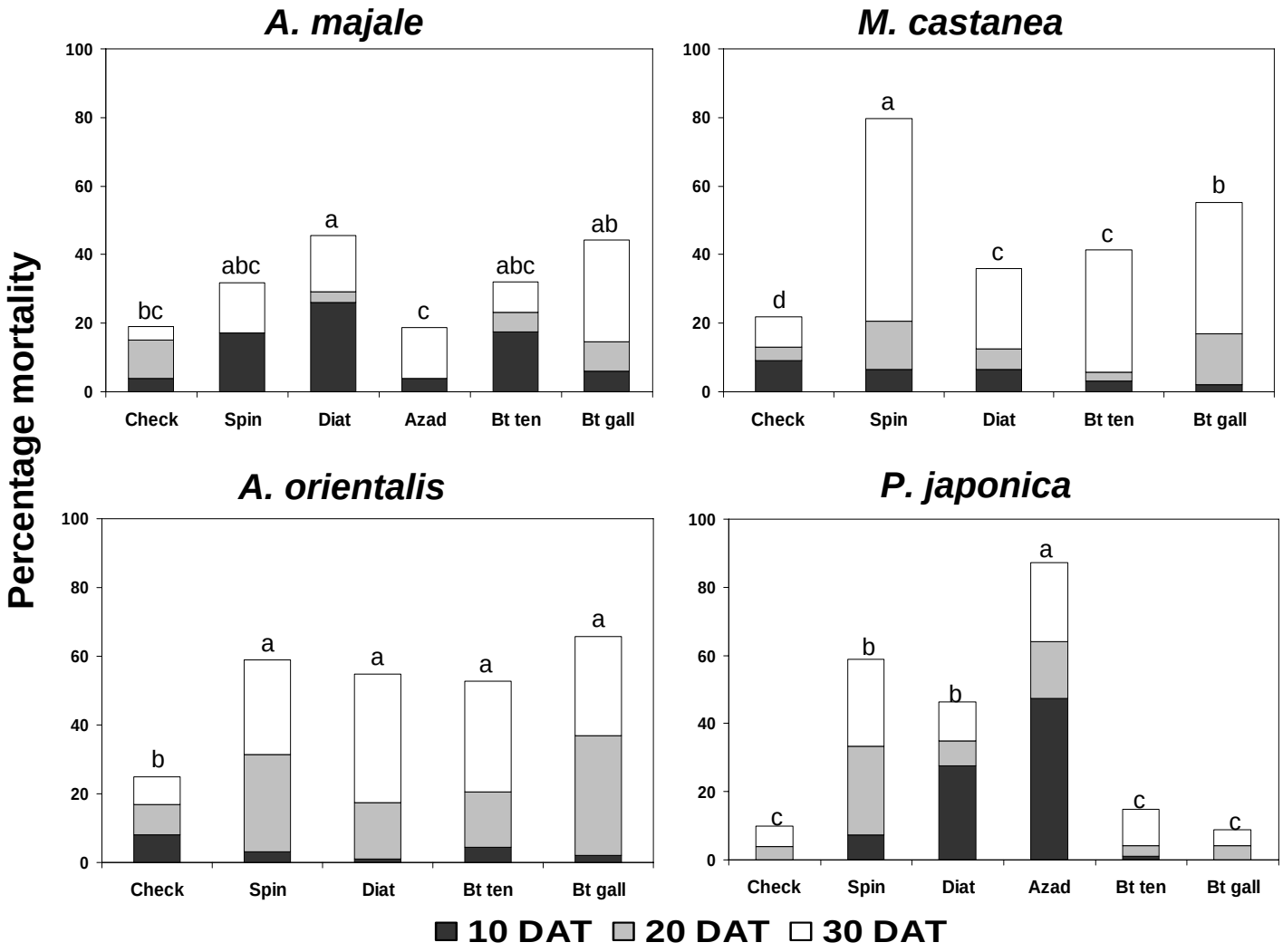
Both *Bt* treatments caused low to moderate mortality in some species but this was not expressed until 30 DAT. For *Bt* var. *galleriae* there was a significant effect of white grub species ($F = 39.43$; $df = 2, 48$; $P < 0.0001$) and time ($F = 104.22$; $df = 2,48$; $P < 0.0001$). For *Bt* var. *tenebrionis* there was also significant effect of white grub species ($F = 7.27$; $df = 2, 48$; $P = 0.0004$) and time ($F = 22.68$; $df = 2, 48$; $P < 0.0001$). Both strains caused significantly moderate mortality to *A. majale, A. orientalis* and *M. castanea* at 30 DAT and had no effect on *P. japonica.*

Table 5. Mean (± SE) percent mortality of four species of white grubs at 10, 20 and 30 days after treatment (DAT) with biopesticide insecticides.

Treatment	White grub species	10 DAT [a]	20 DAT [a]	30 DAT [a]
Botanical:				
Azadirachtin	*A. majale*	4.0 ± 2.5 NS	1.1 ± 1.1 NS	15.8 ± 8.3 NS
	A. orientalis	NA	NA	NA
	M. castanea	NA	NA	NA
	P. japonica	47.3 ± 7.0***	64.1 ± 9.2***	87.2 ± 5.7***
Mechanical:				
Diatomaceous earth	*A. majale*	26.0 ± 5.6***	29.1 ± 10.1*	45.5 ± 13.8**
	A. orientalis	1.0 ± 1.0 NS	17.4 ± 4.8 NS	54.8 ± 2.5***
	M. castanea	6.5 ± 3.0 NS	12.6 ± 4.4 NS	35.9 ± 6.5***
	P. japonica	27.7 ± 4.8***	34.8 ± 9.5**	46.3 ± 11.9***
Microbial-derived:				
Bt var. *galleriae*	*A. majale*	6.1 ± 1.9 NS	14.7 ± 4.6 NS	44.2 ± 3.9**
	A. orientalis	2.1 ± 1.3 NS	37.1 ± 2.8 NS	65.7 ± 5.8***
	M. castanea	2.0 ± 2.0 NS	17.0 ± 3.8 NS	55.2 ± 2.9***
	P. japonica	0.0 ± 0.0 NS	4.2 ± 3.1 NS	8.9 ± 5.4 NS
Bt var. *tenebrionis*	*A. majale*	17.4 ± 5.9 NS	23.1 ± 7.9 NS	32.1 ± 9.5**
	A. orientalis	4.3 ± 2.7 NS	20.4 ± 8.3 NS	52.8 ± 9.5***
	M. castanea	3.1 ± 2.0 NS	5.7 ± 2.6 NS	41.3 ± 3.8 ***
	P. japonica	1.0 ± 1.0 NS	4.2 ± 3.1 NS	14.9 ± 7.2 NS
Spinosad	*A. majale*	17.1 ± 8.9 NS	11.8 ± 9.5 NS	26.5 ± 10.5 NS
	A. orientalis	3.2 ± 1.3 NS	31.4 ± 8.2**	59.0 ± 14.9***
	M. castanea	6.6 ± 2.2 NS	20.6 ± 6.4 NS	79.6 ± 2.1***
	P. japonica	7.3 ± 2.3 NS	33.5 ± 4.8**	58.9 ± 8.5***

[a] Means are significantly different from the untreated check at *0.05, **0.01 and ***0.001 (Dunnett's test), NS = Not Significant.

Figure 2. Percent mortality of four species of white grubs at 10, 20 and 30 days after treatment (DAT) with biopesticides. Spin. = Spinosad, Diat. = Diatomaceous earth, Azad = Azadirachtin, *Bt* ten = *Bacillus thuringiensis* var. *tenebrionis*, *Bt* gall = *Bacillus thuringiensis* var. *galleriae*. For each species, bars with the same letter are not significantly different for cumulative mortality by 30 DAT (Tukey's test, $P < 0.005$).


A. majale
M. castanea
A. orientalis
P. japonica
Percentage mortality
100
80
60
40
20
0
bc
abc
a
c
abc
ab
d
a
c
c
b
b
a
a
a
a
c
b
b
a
c
c
Check
Spin
Diat
Azad
Bt ten
Bt gall
10 DAT
20 DAT
30 DAT

3.3 Chemicals

There were significant effects of treatment, white grub species, time after application and all interactions on mortality (Table 6). Among the treatments, chlorpyrifos and trichlorfon caused significant mortality in all four of the white grub species (Fig. 3). Among white grub species, *A. orientalis* was significantly more susceptible than *P. japonica* and *M. castanea*, followed by *A. majale,* which was significantly less susceptible ($P \leq 0.05$, LSD). With respect to interactions among treatment, white grub species and time after application, mortality due to clothianidin in *A. orientalis*, for instance, was already expressed at 10 DAT. In contrast, mortality was not expressed in *A. majale* until 30 DAT (Table 7). For *A. majale* at 30 DAT, both chlorpyrifos and clothianidin caused significant mortality. At 10 DAT, however, mortality was already > 40% for chlorpyrifos, but was not significant for clothianidin.

Table 6. PROC MIX of percent mortality (arcsine square root transformed) of white grubs (*Anomala orientalis*, *Amphimallon majale*, *Maladera castanea*, *Popillia japonica)* treated with chemical insecticides under laboratory bioassay conditions.

Source	Num df	Den df	*F* value	Pr > F
Treatment	7	332	280.11	<0.0001
Species	3	332	189.41	<0.0001
Time	2	332	94.53	<0.0001
Treatment*species	17	332	37.30	<0.0001
Species*time	6	332	1.16	0.3294
Treatment*time	14	332	5.58	<0.0001
Treatment*species*time	34	332	1.12	0.2963

Among the four neonicotinoid treatments, thiamethoxam caused the highest overall mortality to all species, except *A. majale* (Table 7). There was a significant effect of white grub species ($F = 32.95$; df = 3, 54; $P < 0.0001$) and time ($F = 50.31$; df = 2, 54; $P < 0.0001$). Significant mortality was already expressed at 10 DAT in *P.*

japonica and *A. orientalis*, at 20 DAT in *M. castanea* and only until 30 DAT in *A. majale*.

For clothianidin there was a significant effect of white grub species ($F = 98.55$; $df = 3, 54$; $P < 0.0001$) and time ($F = 53.93$; $df = 2, 54$; $P < 0.0001$). At 10 DAT significant mortality was already expressed in *A. orientalis* and *M castanea*. At 30 DAT significant mortality was expressed for all four species but mortality was higher for *M. castanea* and *A. orientalis* than *A. majale* and *P. japonica*.

For imidacloprid there was also a significant effect of white grub species ($F = 2.81$; $df = 3, 54$; $P = 0.048$) and time ($F = 24.23$; $df = 2, 54$; $P < 0.0001$) (Table 7). Imidacloprid had a significant effect on *P. japonica* and *A. orientalis* at 20 DAT and on *A. majale* at 30 DAT. Imidacloprid had no effect on *M. castanea* (Table 7).

Finally, for dinotefuran there was a significant effect of white grub species ($F = 131.75$; $df = 2, 26$; $P < 0.0001$) and time ($F = 5.54$; $df = 2, 26$; $P = 0.0072$). It caused significant mortality on *P. japonica*, which was already expressed at 10 DAT and increased at 30 DAT. There was no effect on *A. majale.*

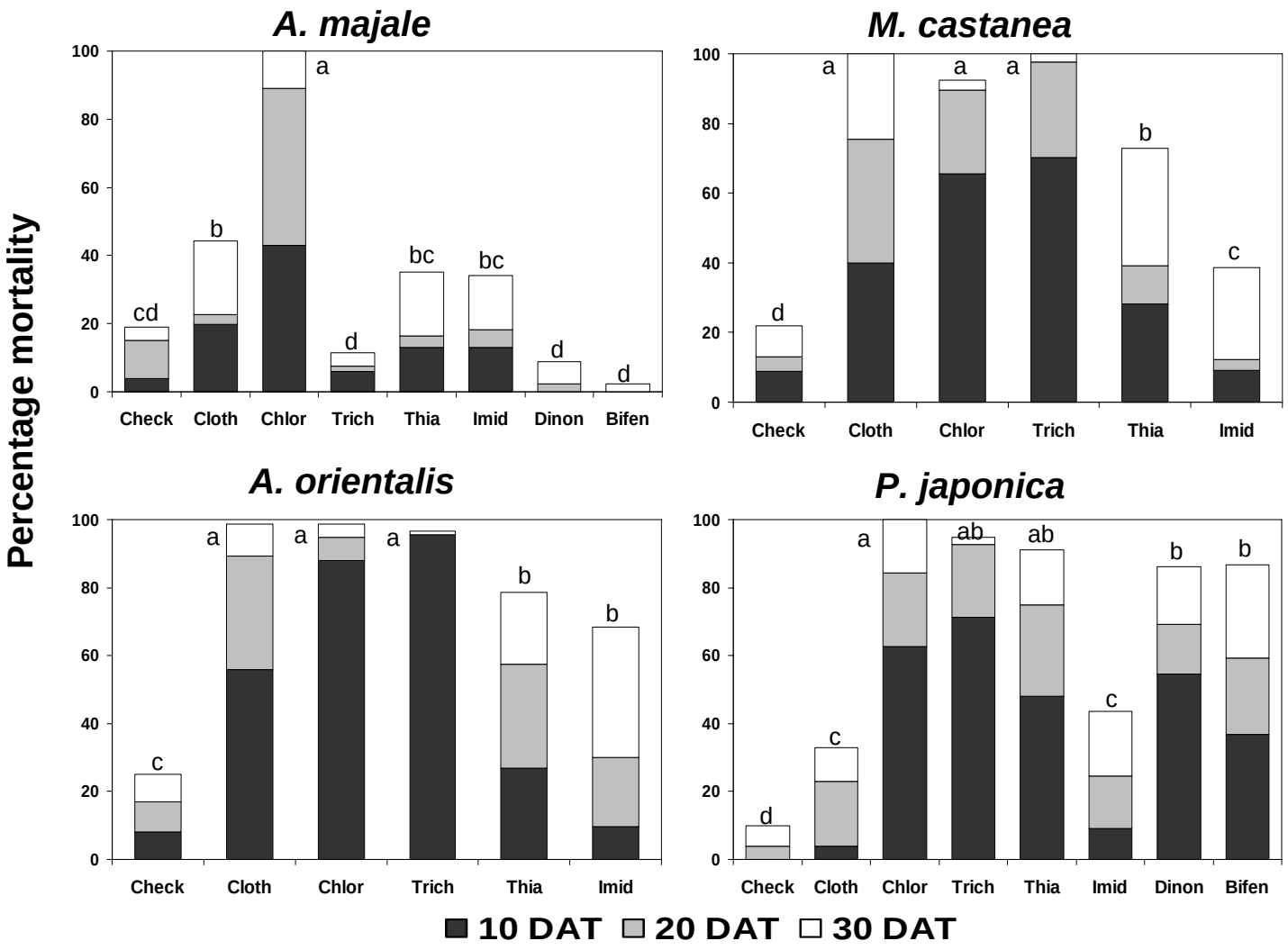
For both organophosphate insecticides, there was a significant effect of species ($F = 7.91$; $df = 3, 48$; $P = 0.0002$ for chlorpyrifos and $F = 114.22$; $df = 3, 48$; $P < 0.0001$ for trichlorfon) and time ($F = 146.96$; $df = 2, 48$; $P < 0.0001$ for chlorpyrifos and $F = 10.54$; $df = 2, 48$; $P = 0.0002$ for trichlorfon). For chlorpyrifos there was significant mortality on all four species at 10, 20 and 30 DAT. The highest mortality at 10 DAT was expressed in *A. orientalis* followed by *M. castanea*, *P. japonica* and *A. majale* (Table 7). Mortality was high at 30 DAT for all four species, but only for *A. majale* and *P. japonica* was 100%.

Table 7. Mean (± SE) percent mortality of four species of white grubs at 10, 20 and 30 days after treatment (DAT) with chemical insecticides.

Treatment	White grub species	10 DAT [a]	20 DAT [a]	30 DAT [a]
Neonicotinoid:				
Clothianidin	*A. majale*	19.8 ± 4.1 NS	22.6 ± 4.4 NS	44.3 ± 3.6**
	A. orientalis	56.0 ± 5.7***	89.3 ± 0.9***	98.6 ± 1.43***
	M. castanea	39.8 ± 9.9*	75.7 ± 5.2***	100.0 ± 0.0***
	P. japonica	4.0 ± 2.5 NS	22.9 ± 2.5**	32.8 ± 4.8***
Dinotefuran	*A. majale*	0.0 ± 0.0 NS	2.2 ± 2.2 NS	8.9 ± 5.4 NS
	A. orientalis	NA	NA	NA
	M. castanea	NA	NA	NA
	P. japonica	54.4 ± 8.2***	69.3 ± 7.7***	86.2 ± 6.5***
Imidacloprid	*A. majale*	13.0 ± 6.4 NS	18.3 ± 10.7 NS	34.2 ± 9.9**
	A. orientalis	9.7 ± 2.7 NS	30.1 ± 8.4***	68.3 ± 8.4***
	M. castanea	9.2 ± 5.6 NS	12.2 ± 5.1 NS	38.8 ± 10.9 NS
	P. japonica	9.1 ± 3.3 NS	22.6 ± 3.9*	41.7 ± 5.6***
Thiamethoxam	*A. majale*	13.1 ± 8.1 NS	16.4 ± 9.7 NS	35.1 ± 9.5**
	A. orientalis	27.0 ± 1.8***	57.5 ± 4.5***	78.7 ± 5.0***
	M. castanea	28.2 ± 5.1 NS	39.1 ± 9.1***	72.8 ± 5.4***
	P. japonica	48.1 ± 3.1***	74.9 ± 5.0***	91.0 ± 3.9***
Organophosphate:				
Chlorpyrifos	*A. majale*	43.0 ± 3.0***	89.0 ± 2.9***	100.0 ± 0.0***
	A. orientalis	87.9 ± 2.3***	94.8 ± 3.2***	98.6 ± 1.4***
	M. castanea	65.5 ± 5.7***	89.6 ± 4.3***	92.4 ± 3.6***
	P. japonica	62.7 ± 5.7***	84.4 ± 2.9***	100.0 ± 0.0***
Trichlorfon	*A. majale*	6.0 ± 2.5 NS	2.6 ± 7.4 NS	7.4 ± 8.7 NS
	A. orientalis	95.6 ± 2.1***	95.1 ± 2.2***	96.0 ± 1.7***
	M. castanea	70.3 ± 16.6***	97.7 ± 1.4***	100.0 ± 0.0***
	P. japonica	71.3 ± 1.6***	92.6 ± 2.8***	94.8 ± 2.7***
Pyrethroid:				
Bifenthrin	*A. majale*	0.0 ± 0.0 NS	0.0 ± 0.0 NS	0.0 ± 0.0 NS
	A. orientalis	NA	NA	NA
	M. castanea	NA	NA	NA
	P. japonica	36.9 ± 3.3***	59.3± 3.7***	86.7 ± 5.7***

[a] Means are significantly different from the untreated check at *0.05, **0.01 and ***0.001 (Dunnett's test), NS=Not Significant. NA = Not Applied in this species.

Figure 3. Percent mortality of four species of white grubs at 10, 20 and 30 days after treatment (DAT) chemical. Cloth = clothianidin, Chlor = chlorpyrifos, Trich = trichlorfon, Thia = thiamethoxam, Imid = imidacloprid, Dino = dinotefuran, Bifen = bifenthrin. For each species, bars with the same letter are not significantly different for cumulative mortality by 30 DAT (Tukey's test, $P < 0.005$).


A. majale
M. castanea
A. orientalis
P. japonica
Percentage mortality
Check
Cloth
Chlor
Trich
Thia
Imid
Dinon
Bifen
10 DAT
20 DAT
30 DAT

For both organophosphate insecticides, there was a significant effect of species ($F = 7.91$; $df = 3, 48$; $P = 0.0002$ for chlorpyrifos and $F = 114.22$; $df = 3, 48$; $P < 0.0001$ for trichlorfon) and time ($F = 146.96$; $df = 2, 48$; $P < 0.0001$ for chlorpyrifos and $F = 10.54$; $df = 2, 48$; $P = 0.0002$ for trichlorfon). For chlorpyrifos there was significant mortality on all four species at 10, 20 and 30 DAT. The highest mortality at 10 DAT was expressed in *A. orientalis* followed by *M. castanea*, *P. japonica* and *A. majale* (Table 7). Mortality was high at 30 DAT for all four species, but only for *A. majale* and *P. japonica* was 100%.

Trichlorfon caused significant and high mortality in *A. orientalis* and moderate mortality in *P. japonica* and *M. castanea* at 10 DAT. Mortality was 100% at 30 DAT for *M. castanea* and significantly high for *A. orientalis* and *P. japonica*. In contrast, there was no effect on *A. majale* at any time after application (Table 7).

For the last chemical treatment, bifenthrin, there was a significant effect of white grub species ($F = 154.96$; $df = 3, 141$; $P < 0.0001$) and time ($F = 7.92$; $df = 2, 141$; $P = 0.0021$). Significant mortality was expressed in *P. japonica* at 10 DAT, which increased significantly at 20 and 30 DAT. There was no effect on *A. majale.*

4 DISCUSSION

There is broad variation in the effectiveness of the 18 insecticides evaluated across white grub species. Only two, for instance, showed a high efficacy to all four species: chlorpyrifos in a bait formulation and the entomopathogenic nematode *S. scarabaei.* Four other products showed a significant effect on all four species: *H. bacteriophora*, diatomaceous earth, clothianidin and thiamethoxam. Only one showed low effect to all four species: a commercial formulation of *P. popilliae*, which only caused

significant but low mortality (28.8%) on *A. orientalis* at 30 DAT. Efficacy varied across white grub species for the other 12 insecticides.

There was also broad variation in the susceptibility of grub species to the insecticides. Overall, *A. majale* and *P. japonica* were the least susceptible to all treatments; 6 of 18 treatments had no effect. In contrast, 3 of 15 treatments for *M. castanea* had no effect and all 15 treatments had effect on *A. orientalis.*

4.1 Biologicals

In general, entomopathogenic nematodes were more virulent than entomopathogenic fungi except for *A. orientalis* where there was no significant difference between the two pathogen groups. However, different results are reported from golf courses in Korea for *A. orientalis* where *Beauveria brongniartii* (Sacc.) caused lower mortality and more variability than *Steinernema carpocapsae* (Weiser), *Steinernema glaseri* Steiner or *H. bacteriophora*.[17]

Among nematodes, *S. scarabaei* was more effective than *H. bacteriophora* because it led to 100% mortality in all four species by 10 DAT (Fig 1). This confirms results obtained by Koppenhöfer and Fuzy (2004), where the same rate of 100 IJs larva^{-1} caused > 95% mortality in third instars of the same four white grub species. In a subsequent study, Koppenhöfer et al. (2002) reported no difference in mortality of *A. majale, A. orientalis* and *P. japonica* with 50 and 400 IJs larvae^{-1} at 7 and 14 DAT.[15, 23] Efficacy against *M. castanea*, however, was significantly less at the low rate. *Heterorhabditis bacteriophora* was more effective against *P. japonica* than the other three white grub species. At 20 DAT it caused 100% mortality in *P. japonica* but did not cause significant mortality in *A. majale* or *M. castanea.* Significant mortality

was observed against *A. orientalis* at 30 DAT. These results are similar to those reported by Koppenhöfer et al. (2002), where *H. bacteriophora* caused ≈ 90% mortality in *P. japonica* but < 30% in the other three species.[15]

Among fungi, there was no effect of either species against *P. japonica*. Among the other three white grub species, *M. anisopliae* was more pathogenic than *B. bassiana* (Fig 1). There are some reports of successful control of white grubs using fungi. In Belgium *M. anisopliae* suppressed *Hoplia philanthus* Füessly in a sports field, and in Europe and Korea, *B. brongniartii* controlled *Melolontha* spp. and *A. orientalis*, respectively.[16, 17, 24] In North America, however, only a few formulations of *B. bassiana* and only one of *M. anisopliae* are registered and marketed for the control of white grubs; there are no specific reports of their effectiveness. In addition, while *M. anisopliae* and *B. bassiana* occur under natural conditions, there are no reports of outbreaks on natural populations. Low efficacy of entomopathogenic fungi has been reported in diverse studies against the four pest species that we tested in this study.[25, 26, 27]

For *P. popilliae*, several studies showed ambiguous efficacy against white grubs, and specifically *P. japonica*.[28] In 1946, the incidence of this bacterial disease was 41.5% in a field survey in Kentucky. By 1995 only 0.2% of *P. japonica* larvae collected from golf courses in Kentucky showed evidence of the disease.[28] In this study, we found no significant effect of the commercial formulation of milky spore against *P. japonica* and this result was similar for the other three white grub species. Lack of efficacy is not surprising given that first instars are more susceptible to milky spore than later instars. In addition, third instars collected in the late summer or early spring are more susceptible than those collected in the late fall.[29] Production *in-vivo*

and *in-vitro* as well as the formulation of milky spore present problems resulting in an unreliable product.[30] There is also the possibility of an increase in the degree of resistance by white grubs.[28]

4.2 Biorationals

For azadirachtin, our results showed no effect on *A. majale* but high efficacy (>87%) versus *P. japonica.* George and Potter (2008), however, showed poor results in greenhouse and field trials where the label rate had no effect on third instar *P. japonica* and five times the label rate was needed to kill second instars.[31] Azadirachtin has an antifeedant effect in many species of insects, causing death by starvation.[32] However, late third instar of *A. majale* can survive long periods without eating (Morales A, pers. obs.), which means that an insecticide with antifeedant properties could have minimal effect.

Among the biorational products, *Bt*–based insecticides are most effective against lepidopteran larvae. However, there are some *Bt* subspecies and strains with specific toxicity to white grubs.[33] *Bt* var *japonensis* strain Buibui, isolated from soil samples in Japan, exhibits a strong effect against different scarab grubs.[18] Our results showed a low to moderate mortality in *A. orientalis* and *M. castanea* with both *Bt*-products tested, moderate mortality in *A. majale* with *Bt* var t*enebrionis* and no effect on *P. japonica* for both formulations. High mortality (88 - 99%) in *P. japonica* and *A. orientalis* has been reported in other studies using *Bt.* var *japanensis* strain Buibui.[18, 34] However, due to formulation and commercial development challenges there is no commercial product currently available.

The other biorational product tested spinosad, is produced from a soil bacterium through a fermentation process and is labeled for the control of caterpillars and fire ants in turf. Efficacy versus white grubs is not documented. While our results revealed no effect in *A. majale,* spinosad had a significant effect on *P. japonica*, *A. orientalis* and especially in *M. castanea*. Greenhouse and field studies are necessary to establish the potential of spinosad as a control alternative.

4.3 Chemicals

Our results showed that the susceptibility of white grub species varies for most insecticides applied (Fig 3). Among the chemical insecticides, only chlorpyrifos showed consistently high mortality (>90%) across all four species. Baker reported a LD_{50} 8.5 times higher for *A. orientalis* than *P. japonica*.[35] The use of technical quality materials (chlorpyrifos 95%) and topical application versus a new experimental bait formulation in this study was the main difference between the two studies. The bait formulation has been shown to be effective for other species of white grubs in sugar cane crops.[36] In contrast, trichlorfon, the other organophosphate widely used for curative control, showed no effect on *A. majale* but high mortality (90%) on the other three species. In this study bifenthrin, the pyrethroid insecticide, showed high control of *P. japonica* but poor control of *A. majale.* Pyrethroids are generally not used for white grub control because they penetrate poorly into the soil zone where the grubs are active.[11]

Among the neonicotinoid insecticides, imidacloprid had the lowest mortality on all four species. *Amphimallon majale* and *M. castanea* were the least susceptible and *A. orientalis* the most susceptible. In a laboratory experiment testing

imidacloprid, Koppenhöfer et al. (2004) reported < 20% mortality against third instar *A. orientalis*, *A. majale* and *M. castanea* with a rate of 360 g AI ha^{-1}, and 5-20% mortality of *P. japonica* with 50, 100 and 200 g AI ha^{-1}.[38] Clothianidin and thiamethoxam in this study showed high (≥ 72.8%) control of *P. japonica*, *A. orientalis* and *M. castanea* and low (≤ 35.1%) control against *A. majale.* Few or no studies have reported the efficacy of both insecticides against the four species, however thiamethoxam caused low mortality (< 25%) on *P. japonica* and *A. orientalis* at 14 DAT using different doses (50, 100 and 200 g AI ha^{-1}).[23] Grewal found a significant population reduction of *P. japonica* at 31 DAT with imidacloprid but not with thiamethoxam or halofenozoide.[37]

4.4 Implications

The results of this study show that with the exception of *S. scarabaei* and chlorpyrifos, none of the control agents evaluated can be reliably used for the curative control of the exotic white grub complex in the Northeast United States. Variation in efficacy among pest species means that decisions on product selection have to be made based on the pest species' identity. Trichlorfon, widely relied on as a late season curative or rescue treatment, may not be reliable against *A. majale,* so turf managers should focus on preventive control in areas dominated by this species. Moreover, some insecticides with high efficacy are not commercially available due to production and marketing limitations. For instance, mass production of *S. scarabaei* has not yet proven to be feasible.[38] Another difficulty is the restricted use designation in turfgrass of insecticides with high efficacy, such as chlorpyrifos. This chemistry is no longer

allowable in turfgrass settings due to a review by the Food Quality Protection Act (FQPA).[38]

Variation in efficacy measured in this study could be attributed to several factors including insect morphology, behavior and physiology, insect immune response and insecticide mode of action. However, the variability generated by many of these factors were likely minimized under the experimental and laboratory conditions. In the field, due to grub-soil and control agent-soil interactions, higher variation in the efficacy of the different control agents is expected, even among closely related taxa with similar resource use and habitat requirements. This differential susceptibility could have another ecological consequence for grub management. In areas where two or more pest species are present, insecticide applications could favor populations of non-target grub species, thereby increasing their potential to emerge as consequential pests.

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CHAPTER TWO

Synergies between biological and neonicotinoid insecticides for the curative control of the white grubs *Amphimallon majale* and *Popillia japonica*.

ABSTRACT

Synergistic combinations of select biological and chemical control products might yield promising alternatives for the management of soil insect pests. Root-feeding white grubs are a widespread and damaging pest complex in turfgrass habitats of the Northeast U.S. Their management is highly dependent on chemical pesticides, but previous work has demonstrated feasibility for reduced-risk curative control via synergies between entomopathogenic nematodes and neonicotinoid insecticides. To understand the breadth of potential synergies, we screened numerous combinations of biological control agents with sublethal doses of neonicotinoid insecticides against third instar white grubs under controlled laboratory conditions. The most promising combinations were advanced to greenhouse pot studies and then to field trials featuring microplots with artificially infested populations. To reveal variation across white grub species, trials were conducted on *Amphimallon majale* and *Popillia japonica*. For *A. majale*, synergistic combinations of *Heterorhabditis bacteriophora* with imidacloprid and clothianidin were detected in the laboratory, greenhouse and in the field. Under field conditions, some synergistic interaction was detected among overwintered insects (174 days after treatment) but not late fall insects (30 DAT). For *A. majale*, interactions with fungal entomopathogens were largely additive and *Bt*-products largely antagonistic. For *P. japonica,* synergistic combinations of *B. bassiana* and *M. anisopliae* with both neonicotinoids were detected in the laboratory and greenhouse, but effects did not persist in the field. For *P. japonica*, interactions with entomopathogenic nematodes were largely additive and *Bt*-products largely

antagonistic. Synergistic combinations of biological control products with reduced rates of neonicotinoid insecticides could be a promising approach for the curative control of white grubs and as an IPM tool for the suppression of other soil insect pests.

Keywords: *Beauveria bassiana*; clothianidin; European chafer; *Heterorhabditis bacteriophora*; imidacloprid; Japanese beetle; *Metarhizium anisopliae*; *Paenibacillus popilliae*; soil insect pests; synergism; turfgrass.

1. INTRODUCTION

The simultaneous application of biological and chemical insecticides to achieve a greater total effect than the sum of their individual effects may be a promising approach for insect pest management in different agricultural systems (Anderson et al., 1989; Furlong and Groden, 2001). For instance, the combination of the fungal entomopathogen *Beauveria bassiana* (Balsamo) Vuill. with a low rate of the insecticide imidacloprid against Colorado potato beetle (*Leptinotarsa decemlineata* Say) can exert a significant synergistic effect on mortality and mycosis (Boucias et al., 1996; Quintela and McCoy, 1997; Furlong and Groden, 2001). For the suppression of soil insect pests, in particular, enhanced effects of bacteria, fungi and nematodes in combination with chemical insecticides have been reported (Boucias et al., 1996; Koppenhöfer and Kaya, 1998; Quintela and McCoy, 1998; Koppenhöfer et al., 2003; Jaramillo et al., 2005). For instance, Jaramillo et al. (2005) reported low doses of imidacloprid as a synergist for the fungal entomopathogen *Metarhizium anisopliae* (Metchnikoff) Sorokin against the burrower bug *Cyrtomenus bergi* Froeschner under laboratory and greenhouse conditions. Quintela and McCoy (1998) observed a significant increase in the mortality of the root weevil *Diaprepes abbreviatus* (L.) when *B. bassiana* was applied in combination with imidacloprid.

Synergistic combinations of biological controls with reduced rates of chemical insecticide synergists may therefore represent a valuable approach for the suppression of soil insect pests.

Root-feeding white grubs (Coleoptera: Scarabaeidae) are a major global pest of diverse agricultural crops (Jackson and Klein, 2006). They are the most damaging group of soil insects in turfgrass, nurseries and ornamentals in extensive areas of the United States (Fleming, 1972; Potter, 1998; Wright et al., 1988; Alm et al., 1999; Vittum et al., 1999). In the Northeast United States, a complex of eight scarab species is problematic in turf. The most damaging among these are four exotic species introduced in the early 1900's. These include the Japanese beetle, *Popillia japonica* Newman; the oriental beetle, *Anomala orientalis* (Waterhouse), the Asiatic garden beetle, *Maladera castanea* (Arrow); and the European chafer, *Amphimallon majale* (Razoumowsky) (Vittum et al., 1999).

All four exotic species have a 1-yr life cycle in the Northeast U.S. Adults emerge late in the spring (*A. majale* and *M. castanea*) or during the summer (*A. orientalis* and *P. japonica*) to lay eggs in the soil (Potter, 1998; Vittum et al., 1999). First instars develop over 4-6 wk, when they can be effectively targeted for suppression with a variety of preventive insecticides. Larvae of all four species are third instar by the end of the summer; at this point suppression shifts to faster acting curative insecticides. Third instars feed voraciously on roots which can lead to extensive loss of turf. Additional indirect damage can be experienced due to the activity of mammalian predators digging for grubs, even in areas where direct damage is not visible. After overwintering, third instars emerge in early spring to feed for another couple weeks before pupation (Potter, 1998; Vittum et al., 1999).

While there are non-chemical control alternatives for the control of white grubs, all have severe limitations (Koppenhöfer and Kaya, 1998; Potter, 1988; Vittum et al.,

1999). Among the biological insecticides, a commercial formulation of the bacteria that causes "milky disease", *Paenibacillus popilliae* Dutky, is mainly effective against first instar *P. japonica* and even then it has only a slow effect (Koppenhöfer and Kaya, 1998). Entomopathogenic nematodes of the genera *Heterorhabditis* and *Sterneinema* are commercially available but are not reliable enough to provide consistent control, especially due to problems related with the formulation and quality of the final product (Koppenhöfer and Kaya, 1998; Grewal and Peters, 2005). Most recently, in the U.S the first commercial formulation of *M. anisopliae* (strain F52) was approved and this product includes turf-infesting white grubs on the label (USEPA, 2003).

Several studies have shown that third instar white grubs are more susceptible to nematodes when challenged by other antagonists such as reduced rates of neonicotinoids or endophytic host plants (Grewal et al., 1995; Koppenhöfer and Kaya, 1998; Koppenhöfer et al., 2000a, 2002, 2003). The first evidence was obtained by Koppenhöfer and Kaya (1998) who reported a synergistic interaction between *H. bacteriophora* and imidacloprid against *Cyclocephala hirta* (LeConte) and *C. pasadenae* Casey. Later work revealed that imidacloprid and two other neonicotinoids (acetamiprid and thiamethoxam) were synergists for *H. bacteriophora* against *A. orientalis* but not against *A. majale* or *M. castanea* (Koppenhöfer et al., 2002).

While the mechanisms involved in the interaction between neonicotinoids and biological insecticides are not established, among white grubs it is likely that disruption of the insect's grooming behavior facilitates host attachment of infective juvenile nematodes (Koppenhöfer et al., 2000b). Another possibility is blocking the evasive response of the insect to avoid places with natural enemies. That evasive behavior was reported for larvae of *P. japonica* where white grubs move away from sites applied with *M. anisopliae* (Villani et al., 1994). The same evasive behavior has also been observed in *A. majale* to *M. anisopliae*, and additionally to sublethal doses

of imidacloprid (Morales, A. unpublished data). *Diaprepes abbreviatus* increased mobility with a single application of *B. bassiana* or in combination with imidacloprid, but with a single application of imidacloprid the mobility of larvae decreased (Quintela and McCoy, 1998).

In order to broaden our understanding of which non-chemical control products could be synergized by neonicotinoid insecticides, our goal was to screen numerous combinations against third instars under controlled laboratory conditions. This life stage is the most damaging, the most difficult to control, the easiest to manipulate, and is targeted by late season curative applications. The interactions were characterized as synergistic, additive or antagonistic. The most promising combinations (based on synergy and efficacy) were advanced to greenhouse pot studies, and then to field studies featuring microplots with artificially infested populations. To understand how synergistic combinations might vary with white grub species, trials were conducted on two of the dominant species in the Northeast U.S., *P. japonica* and *A. majale*.

2. METHODOLOGY

2.1. *Source of insects*

Third instar white grubs collected from the field were used to conduct all laboratory, greenhouse and field experiments. *Popillia japonica* was collected on 29 November 2005 in Fulton, NY (Battle Island Golf Course, Oswego Co.), on 7-8 November 2006 in Geneva, NY (Seneca Lake Country Club, Ontario Co.) and on 6-7 November 2007 in Victor, NY (Parkview Fairways Golf Course, Ontario Co.). *Amphimallon majale* was collected on 15-16 November 2005 and on 31 October 2006 in Lake George, NY (Queensbury Country Club, Warren Co.) and on 6-7 November 2007 in Victor, NY (Parkview Fairways Golf Course, Ontario Co.). The larvae were maintained in wooden boxes with soil and a piece of sod as a food source from the

same sites of collection. Boxes were held in a walk-in cooler at 10°C until the start of trials.

2.2. *Treatments*

We applied registered and experimental biological insecticides at recommended rates in combination with low doses of two chemical insecticides. The chemical insecticides were clothianidin (Arena 50 WDG; Valent, Walnut Creek, CA) and imidacloprid (Merit 75 WP; Bayer, Durham, NC) applied at ½ and ¼ the label rate of 0.45 kg AI/ha. The biologicals included products based on entomopathogenic fungi, nematodes and bacteria.

The fungal entomopathogens were represented by *Beauveria bassiana* (Botanigard ES; Emerald BioAgriculture, Okemos, MI) (8.14×10^{15} conidia/ha) and *Metarhizium anisopliae* (two strains: Met F52 Novozymes Biologicals, Salem, VA and Met NYSAES) (7.94×10^{15} con/ha). The NYSAES strain (ARSEF pending) was isolated from *P. japonica* collected from Fulton, NY (Battle Island Golf Course, Oswego Co.) and was propagated on sterile Sabouraud Dextrose Agar (10 g peptone, 40 g dextrose, 15 g agar, 1 liter distilled water) plus 1% yeast (10 g yeast extract). After inoculation with conidia, Petri dishes were maintained for 18-20 d in a growth chamber at 27ºC and photoperiod of 12:12 h light:dark. For the application, conidia were scraped from the plate into a 1-liter flask containing 500 ml of sterilized distilled water with 0.05% Tween 80 (Fisher Scientific, Pittsburgh, PA). The conidial concentration was determined with a hematocytometer (Bright-Line®; American Optical, Buffalo, NY) and adjusted to the required concentration.

The entomopathogenic nematodes were represented by *Heterorhabditis bacteriophora* (Heteromask; BioLogic, Willow Hill, PA) (2.04×10^{9} IJ3/ha) and *Heterorhabditis* sp. (two NYSAES strains) (2.04×10^{9} IJ3/ha). The NYSAES Nema

1 strain was isolated from soil samples from Fulton, NY and the NYSAES Nema 2 strain was isolated from a third instar *A. majale* collected from Saratoga Springs, NY (Saratoga Spa Golf, Saratoga Co.). Both strains were cultured in the last instar of the greater wax moth, *Galleria mellonella* L. The emerging infective juveniles (IJ3) were harvested from white traps the same day of application (Kaya and Stock, 1997).

The bacterial products were represented by *Paenibacillus popilliae* (Milky Spore; St. Gabriel Laboratories, Orange, VA) (2.5 kg AI/ha) and *Bacillus thuringiensis*. The *Bt*- products were *Bt* var. *galleriae* in an experimental formulation (650 g AI/ha) and *Bt* var. *tenebrionis* (Novodor FC; Valent BioSciences, Libertyville, IL) (935 ml AI/ha).

2.3. *Laboratory experiments*

For the laboratory experiments, insects were maintained in a walk-in environmental chamber under controlled climate conditions (complete darkness, 90-95% RH, 25^{o}C) at the NYSAES, Geneva, NY. Assay units consisted of 30-ml plastic cups filled with 30 g of screened soil raised to 10.0% (w/w) moisture. The soil was sandy loam (83.0% sand, 12.0% silt, 5.0% clay) with 0.79% organic matter and pH 7.09. A pinch of grass seed (Shady-Green; Agway, Richmond, VA) was added as a food source. After being held at room temperature for 24 h, individual larvae were released into each cup. Larvae that did not burrow into the soil within 3 h were replaced. The treatment applications consisted of the full rate of the biological alone, ½ or ¼ rate of the neonicotinoid alone and the combination of biological and neonicotinoid. Treatments were applied in 2 ml of water total: 1 ml for the biological and 1 ml for the chemical insecticide. When only one control agent was applied, 1 ml of water was added. Untreated checks received 2 ml of water.

Each assay had six repetitions of 10 cups and 10 grubs, and was conducted once each year. In 2005 the studies were initiated on 20 December (*P. japonica*) and 22 December (*A. majale*). In 2006 the studies were initiated on 15 December (*P. japonica*) and 18 December (*A. majale*). In 2006, neither *Heterhabditis* sp. Nema 1 nor *Heterhabditis* sp. Nema 2 could be applied against *P. japonica* or *A. majale*. Evaluations were made at 10, 20 and 30 d after treatment (DAT) to measure mortality rates. To do this, the contents of each cup were emptied onto a piece of paper, the status of the grub was assessed, and the contents were replaced in the cup with a pinch of additional grass seed. These protocols were modified from Morales et al. (submitted).

2.4. *Greenhouse experiments*

Greenhouse experiments were conducted at the NYSAES, Geneva, NY. The average greenhouse temperature was 14.0°C (8.5-27.5°C) and the photoperiod was 14:10 hr light:dark. One-liter pots filled with soil were seeded with perennial ryegrass (Tri-Rye; Agway, Richmond, VA). The potting soil was sandy loam (84.0% sand, 11.0% silt, 5.0% clay) with 8.43% organic matter and pH 6.85. Grass was maintained for 10-12 wk, watered every 2 d, and cut to a height of 5.0 cm and fertilized (20-20-20, Scotts Miracle-Gro Products, Marysville, OH) every week.

Five larvae were released into each pot. Larvae that did not burrow into the soil within 24 h were replaced. Treatments were applied in 100 ml of water: 50 ml for the biological and 50 ml for the chemical insecticide. When only one control agent was applied, 50 ml of water was added. Untreated checks received 100 ml of water. Each assay had 10 pots (repetitions) and was conducted once each year. In 2006 the studies were initiated on 20 December (*P. japonica*) and 22 December (*A. majale*). In

2007 the studies were initiated on 15 January (*P. japonica*) and 29 January (*A. majale*).

Treatments for each grub species were selected based on those that exhibited a synergistic interaction in laboratory assays. The *P. japonica* treatments were *B. bassiana* and clothianidin at ½ and ¼ label rate alone and in combination; *M. anisopliae* Met 52 and clothianidin at ½ label rate alone and in combination; and *M. anisopliae* strain NYSAES and imidacloprid at ½ label rate alone and in combination. The *A. majale* treatments were *H. bacteriophora* and clothianidin and imidacloprid at ½ and ¼ label rate alone and in combination. After applications, all pots were arranged in a completely randomized design. Destructive evaluations were made at 30 DAT to measure mortality rates.

2.5. *Field experiments*

Field experiments were conducted in microplots with artificially infested populations. Studies with *P. japonica* were conducted on irrigated turf at the Turf and Landscape Research Center, Cornell University, Ithaca, NY. Mowing height was 6.5 cm, thatch depth was 0.2-0.5 cm and turf composition was ryegrass (*Lolium* spp. L.) (35%), annual bluegrass (*Poa annua* L.) (19%), fescue (*Festuca* spp. L.) (2%) and broad-leaf weeds (44%). Soil was sandy loam (63.0% sand, 26.0% silt, 9.0% clay) with 2.6% organic matter and pH 5.8. Previous to the start of the experiment, natural populations of white grubs were detected at a density of 30-40 grubs/m^2, with a species composition of 90-95% *P. japonica* and 5-10% *A. majale*.

Experiments with *A. majale* were conducted in an experimental turf area at the NYSAES, Geneva, NY. Mowing height was 8.5 cm, thatch depth was 0.4-0.6 cm and turf composition was ryegrass (18.8%), crabgrass (*Digitaria* spp.) (75.0%)

and broad-leaf weeds (6.2%). Soil was silty clay loam (12.5% sand, 55.0% silt, 32.5.0% clay) with 5.7% organic matter and pH 6.5. Previous to the start of the experiment, a low natural population of *P. japonica* was detected at a density of ≤ 5 grubs/m^2. Neither of the two locations had been treated with insecticides within 5 yr.

The microplots were PVC rings (30.5 cm diameter, 7.6 cm height) that were pushed completely into the soil 1 wk before infestation. Fifteen *P. japonica* or 10 *A. majale* larvae (third instar) were released into each ring 24 h before treatment application. Larvae that did not burrow into the soil within 3 h were replaced. There were ten replicated microplots per treatment and these were arranged in a randomized block design. The *P. japonica* treatments were applied on 29 October 2007 (air temperature 5.5°C, soil temperature 10.7°C at 2.5 cm depth and 10.9°C at 7.6 cm depth; sunny). The *A. majale* treatments were applied on 26 October 2007 (air temperature 13.0°C, soil temperature 11.9°C at 2.5 cm depth and 12.2°C at 7.6 cm depth; partly cloudy). The treatments applied in the field were the same treatments applied in the greenhouse for each species. Applications were made in 500 ml of water and the untreated check received only water. All applications were made using a watering can followed by 0.95 cm of irrigation. Destructive evaluations were made in five microplots at 30 DAT (late fall) and five at 174 DAT (early spring) to measure mortality rates.

2.6. *Statistics*

In the laboratory and greenhouse experiments, percent mortality was corrected for mortality in the untreated check (Abbott, 1925). In the field experiments, mortality was corrected for the average number of larvae recovered from the untreated check. Percent mortality data were normalized using an arcsine square root transformation

and subjected to analysis of variance (ANOVA). Synergistic, additive and antagonistic interactions between agents in the combination treatments were determined using a X^2 test (Benz, 1971; MacVay et al., 1977; Koppenhöfer and Kaya, 1998; Koppenhöfer and Fuzy, 2003). The expected interaction mortality value, M_E, for combined agents was calculated using the formula $M_E = M_B + M_N (1- M_B/100)$, where M_B and M_N are the observed percent mortalities caused by the biological and neonicotinoid products alone, respectively. Results from a X^2 test were compared to the X^2 table value for 1 df, using the formula $X^2 = (M_{BN}- M_E)^2 / M_E$, where M_{BN} is the observed mortality for the biological - neonicotinoid combinations. A non-additive effect between the two agents was suspected when the X^2 value exceeded the table value (Koppenhöfer and Fuzy, 2003). If the difference $M_{BN} – M_E$ had a positive or negative value, a significant interaction was then considered synergistic or antagonistic, respectively. Data from the field experiment were also assessed for an effect of overwintering. To do that each treatment mean was tested individually in a contrast between 30 and 174 DAT evaluations. Differences among means were considered significant at $P < 0.05$. All statistical analyses were performed using SAS (SAS Institute, 2002).

3. RESULTS

3.1. *Laboratory experiments*

Mortality in the untreated check ranged from 5 - 15% for both species. Because there was an effect of year ($F = 10.15$; df = 1, 714; $P = 0.0015$) on mortality, each year was analyzed separately. In 2005, there was a significant effect of treatment ($F = 27.95$; df = 49, 500; $P < 0.0001$), white grub species ($F = 1440.51$; df = 1, 500; $P < 0.0001$) and their interaction ($F = 6.73$; df = 50, 500; $P < 0.0001$) on mortality at 30 DAT. This result was consistent in 2006 for treatment ($F = 13.14$; df = 49, 500; $P < 0.0001$), white grub species ($F = 649.69$; df = 49, 500; $P < 0.0001$) and their

interaction ($F = 6.52$; $df = 101, 714$; $P < 0.0001$). Overall, *P. japonica* was significantly more susceptible to fungi-neonicotinoid combinations than *A. majale*. Of the 24 combinations evaluated over the 2005 and 2006 studies, 11 were synergistic for *P. japonica* but none for *A. majale* (Tables 1-4). In contrast, *A. majale* was significantly more susceptible to *H. bacteriophora* combinations than *P. japonica.* Of the 16 combinations, 7 were synergistic for *A. majale* and only 1 for *P. japonica* (Tables 1-4). For the bacteria-neonicotinoid combinations, only additive or antagonistic effects were detected. Of the 24 combinations, 8 were antagonistic for *A. majale* and 11 for *P. japonica*.

For *P. japonica* and *B. bassiana*, a synergistic interaction was detected for clothianidin-½ in both years (Tables 1 and 2). There was an additional synergy for imidacloprid-½ and clothianidin-¼ in 2005 and imidacloprid-¼ in 2006. For Met F52, a synergistic interaction was detected for clothianidin-½ in both years. There was an additional synergy for imidacloprid-½ in 2005. For Met NYSAES, a synergistic interaction was detected for imidacloprid-½ in both years. There was an additional synergy for clothianidin-½ in 2005. An antagonistic interaction was detected for clothianidin-¼ in 2006. All other interactions between fungal entomopathogens and neonicotinoids were additive.

For *A. majale a*nd *B. bassiana*, an antagonistic interaction was detected for imidacloprid-¼ and clothianidin-¼ in 2006 (Tables 3 and 4). For Met F52, an antagonistic interaction was detected with clothianidin-¼ and ½ and imidacloprid-½ in 2006. For Met NYSAES, an antagonistic interaction was detected for clothianidin-½ in 2006. All other interactions between fungal entomopathogens and neonicotinoids were additive (Tables 3 and 4).

Table 1. Laboratory mortality (mean ± SE) of third instar *Popillia japonica* and the interaction among different combinations of biological and neonicotinoid insecticides at 30 DAT in 2005.

Treatment	Measurement[a]	Rate 1/2[b]			Rate 1/4[c]		
		Mortality	χ^2	Effect	Mortality	χ^2	Effect
B. bassiana GHA							
Imidacloprid	Observed	75.0 ± 5.0	7.76	Synergistic	53.3 ± 5.6	0.27	Additive
	Expected	58.3 ± 6.3			56.7 ± 8.7		
Clothianidin	Observed	83.3 ± 4.9	70.48	Synergistic	80.0 ± 8.6	23.80	Synergistic
	Expected	36.7 ± 8.6			50.0 ± 8.3		
M. anisopliae Met F52							
Imidacloprid	Observed	78.3 ± 4.7	46.17	Synergistic	61.7 ± 7.5	0.81	Additive
	Expected	40.0 ± 5.1			53.3 ± 5.6		
Clothianidin	Observed	78.3 ± 4.8	46.17	Synergistic	61.7 ± 7.0	3.27	Additive
	Expected	61.7 ± 9.3			60.0 ± 7.2		
M. anisopliae NYSAES							
Imidacloprid	Observed	83.3 ± 4.9	9.33	Synergistic	45.0 ± 9.9	3.05	Additive
	Expected	68.3 ± 7.7			66.7 ± 6.7		
Clothianidin	Observed	66.7 ± 9.2	41.67	Synergistic	56.7 ± 9.5	0.29	Additive
	Expected	46.7 ± 5.4			60.0 ± 7.1		
H. bacteriophora							
Imidacloprid	Observed	100.0 ± 0.0	0.0	Additive	100.0 ± 0.0	0.0	Additive
	Expected	100.0 ± 0.0			100.0 ± 0.0		
Clothianidin	Observed	100.0 ± 0.0	0.0	Additive	100.0 ± 0.0	0.0	Additive
	Expected	100.0 ± 0.0			100.0 ± 0.0		
Heterorhabditis sp. Nema 1							
Imidacloprid	Observed	58.3 ± 5.4	0.06	Additive	53.3 ± 4.9	0.05	Additive
	Expected	61.7 ± 6.2			60.0 ± 5.8		
Clothianidin	Observed	63.3 ± 7.1	18.74	Synergistic	53.3 ± 6.1	0.38	Additive
	Expected	40.0 ± 5.6			53.3 ± 5.1		

Table 1. (Continued).

Heterorhabditis sp. Nema 2							
Imidacloprid	Observed	55.5 ± 5.7	1.13	Additive	53.3 ± 4.2	0.19	Additive
	Expected	50.0 ± 4.9			63.3 ± 6.1		
Clothianidin	Observed	48.3 ± 10.1	2.06	Additive	60.0 ± 5.7	1.63	Additive
	Expected	41.3 ± 8.7			56.7 ± 4.9		
P. popilliae							
Imidacloprid	Observed	26.7 ± 7.1	24.26	Antagonistic	50.0 ± 7.3	3.80	Additive
	Expected	83.3 ± 5.6			81.7 ± 9.2		
Clothianidin	Observed	38.3 ± 8.7	3.67	Additive	53.3 ± 4.2	1.03	Additive
	Expected	61.6 ± 7.3			75.0 ± 6.8		
Bt var. *galleriae*							
Imidacloprid	Observed	26.7 ± 6.2	16.38	Antagonistic	38.3 ± 5.4	5.49	Antagonistic
	Expected	53.3 ± 4.7			51.7 ± 8.1		
Clothianidin	Observed	46.7 ± 6.1	1.89	Additive	48.3 ± 6.0	0.05	Additive
	Expected	41.7 ± 5.1			45.0 ± 5.2		
Bt var. *tenebrionis*							
Imidacloprid	Observed	33.3 ± 8.4	13.35	Antagonistic	59.4 ± 9.5	3.94	Antagonistic
	Expected	73.3 ± 9.4			100.0 ± 0.0		
Clothianidin	Observed	48.3 ± 13.2	0.22	Additive	48.3 ± 4.8	0.95	Additive
	Expected	51.7 ± 9.3			65.0 ± 5.7		

[a] Observed = efficacy of both control agents applied at the same time. Expected = sum of efficacy of each control agent applied separately.

[b] ½ = half of the high label rate recommendation for white grub control.

[c] ¼ = quarter of the high label rate recommendation for white grub control

Table 2. Laboratory mortality (mean ± SE) of third instar *Popillia japonica* and the interaction among different combinations of biological and neonicotinoid insecticides at 30 DAT in 2006.

Treatment	Measurement[a]	Rate 1/2[b]			Rate 1/4[c]		
		Mortality	χ^2	Effect	Mortality	χ^2	Effect
B. bassiana GHA							
Imidacloprid	Observed	38.3 ± 9.5	1.39	Additive	43.3 ± 2.1	3.92	Synergistic
	Expected	41.7 ± 5.4			35.0 ± 8.5		
Clothianidin	Observed	63.3 ± 2.1	15.78	Synergistic	45.0 ± 4.3	2.23	Additive
	Expected	43.3 ± 7.6			40.0 ± 7.7		
M. anisopliae Met F52							
Imidacloprid	Observed	26.7 ± 3.3	1.65	Additive	23.3 ± 3.3	1.80	Additive
	Expected	21.7 ± 3.1			18.3 ± 3.1		
Clothianidin	Observed	58.3 ± 4.8	41.68	Synergistic	28.3 ± 4.8	1.54	Additive
	Expected	26.7 ± 4.2			23.3 ± 2.1		
M. anisopliae NYSAES							
Imidacloprid	Observed	55.0 ± 2.2	8.16	Synergistic	41.7 ± 6.0	1.27	Additive
	Expected	41.7 ± 5.4			38.3 ± 4.0		
Clothianidin	Observed	41.7 ± 10.8	0.01	Additive	25.0 ± 6.2	4.87	Antagonistic
	Expected	46.7 ± 9.3			43.3 ± 4.9		
H. bacteriophora							
Imidacloprid	Observed	33.3 ± 4.2	0.59	Additive	23.3 ± 5.6	0.35	Additive
	Expected	31.7 ± 5.4			28.3 ± 3.1		
Clothianidin	Observed	28.3 ± 4.0	0.78	Additive	30.0 ± 3.7	0.01	Additive
	Expected	36.7 ± 4.2			33.3 ± 4.9		
P. popilliae							
Imidacloprid	Observed	16.7 ± 3.3	1.38	Additive	18.3 ± 3.1	1.38	Additive
	Expected	23.3 ± 3.3			20.0 ± 4.5		
Clothianidin	Observed	13.3 ± 2. 1	6.88	Antagonistic	15.0 ± 2.2	3.26	Additive
	Expected	28.3 ± 4.0			25.0 ± 2.2		

Table 2. (Continued).

Bt var. galleriae							
Imidacloprid	Observed	20.0 ± 2.6	2.88	Additive	23.3 ± 6.1	0.35	Additive
	Expected	31.7 ± 4.0			28.3 ± 1.7		
Clothianidin	Observed	10.0 ± 2.6	16.41	Antagonistic	23.3 ± 4.9	1.73	Additive
	Expected	36.7 ± 4.2			33.3 ± 3.3		
Bt var. *tenebrionis*							
Imidacloprid	Observed	20.0 ± 5.2	11.27	Antagonistic	26.7 ± 3.3	4.06	Antagonistic
	Expected	46.7 ± 6.1			43.3 ± 3.3		
Clothianidin	Observed	23.3 ± 4.9	10.59	Antagonistic	26.7 ± 4.2	6.08	Antagonistic
	Expected	51.7 ± 6.0			48.3 ± 4.8		

[a] Observed = efficacy of both control agents applied at the same time. Expected = sum of efficacy of each control agent applied separately.

[b] ½ = half of the high label rate recommendation for white grub control.

[c] ¼ = quarter of the high label rate recommendation for white grub control.

For *P. japonica* and *Heterorhabditis* sp. Nema 1, a synergistic interaction was detected for clothianidin-½ in 2005 (Table 1). For *P. japonica*, all other interactions between entomopathogenic nematodes and neonicotinoids were additive.

For *A. majale* and *H. bacteriophora,* a synergistic interaction was detected for imidacloprid-½ and ¼ and clothianidin-½ and ¼ in both years, with the exception of clothianidin-¼ in 2006 which was only additive (Tables 3 and 4). For *A. majale*, all other interactions with *Heterhabditis* sp. Nema 1 and *Heterhabditis* sp. Nema 2 were additive.

For *P. japonica* and *P. popilliae*, an antagonistic interaction was detected for imidacloprid-½ and ¼ in 2005 and clothianidin-½ in 2006 (Table 1 and 2). For *Bt* var *galleriae,* an antagonistic interaction was detected for imidacloprid-½ and ¼ in 2005 and clothianidin-½ in 2006. For *Bt* var *tenebrionis,* an antagonistic interaction was detected for imidacloprid-½ and ¼ in 2005 and imidacloprid-½ and ¼ and clothianidin-½ and ¼ in 2006. For *P. japonica*, all other interactions between bacterial products and neonicotinoids were additive.

For *P. popilliae* and *A. majale,* an antagonistic interaction was detected for imidacloprid-½ and ¼ in 2005 and clothianidin-½ rate in 2006 (Tables 3 and 4). For *Bt* var *galleriae,* an antagonistic interaction was detected for imidacloprid-½ in 2005 and clothianidin-½ in 2006. For *Bt* var *tenebrionis,* an antagonistic interaction was detected for imidacloprid-¼ in both years. There was an antagonism for clothianidin-¼ in 2006. For *A. majale*, all other interactions between bacterial products and neonicotinoids were additive.

Table 3. Laboratory mortality (mean ± SE) of third instar *Amphimallon majale* and the interaction among different combinations of biological and neonicotinoid insecticides at 30 DAT in 2005.

		Rate 1/2 [b]			Rate 1/4[c]		
Treatment	Measurement[a]	Mortality	χ^2	Effect	Mortality	χ^2	Effect
B. bassiana GHA							
Imidacloprid	Expected	10.0 ± 4.5	0.0	Additive	6.7 ± 3.3	1.66	Additive
	Observed	10.0 ± 4.4			10.0 ± 2.6		
Clothianidin	Expected	6.7 ± 4.2	1.66	Additive	16.7 ± 8.2	1.21	Additive
	Observed	10.0 ± 3.7			6.7 ± 2.1		
M. anisopliae Met F52							
Imidacloprid	Expected	10.0 ± 4.5	1.11	Additive	6.7 ± 3.3	3.75	Additive
	Observed	6.7 ± 3.3			11.7 ± 1.6		
Clothianidin	Expected	6.7 ± 4.2	0.41	Additive	6.6 ± 4.3	2.72	Additive
	Observed	5.0 ± 2.2			1.7 ± 1.7		
M. anisopliae NYSAES							
Imidacloprid	Expected	15.0 ± 5.0	3.38	Additive	11.7 ± 5.4	3.62	Additive
	Observed	23.3 ± 6.1			20.0 ± 5.2		
Clothianidin	Expected	13.3 ± 5.5	0.15	Additive	20.0 ± 5.8	2.88	Additive
	Observed	10.0 ± 3.7			11.7 ± 4.0		
H. bacteriophora							
Imidacloprid	Expected	33.3 ± 9.5	62.45	Synergistic	30.0 ± 7.7	93.44	Synergistic
	Observed	75.0 ± 6.7			80.0 ± 3.7		
Clothianidin	Expected	28.3 ± 9.1	31.41	Synergistic	23.3 ± 6.6	62.45	Synergistic
	Observed	58.3 ± 6.0			41.7 ± 7.5		
Heterorhabditis sp. Nema 1							
Imidacloprid	Expected	11.7 ± 4.8	0.29	Additive	8.3 ± 4.0	3.17	Additive
	Observed	13.3 ± 4.2			13.3 ± 7.1		
Clothianidin	Expected	10.0 ± 3.7	0.0	Additive	16.7 ± 3.3	3.37	Additive
	Observed	8.3 ± 1.7			6.7 ± 3.3		

Table 3. (Continued).

Heterorhabditis sp. Nema 2							
Imidacloprid	Expected	11.7 ± 4.8	0.12	Additive	8.3 ± 4.0	0.0	Additive
	Observed	13.3 ± 4.2			13.3 ± 7.1		
Clothianidin	Expected	10.0 ± 3.7	3.00	Additive	16.7 ± 3.3	3.67	Additive
	Observed	8.3 ± 1.7			6.7 ± 3.3		
P. popilliae							
Imidacloprid	Expected	16.7 ± 5.6	10.02	Antagonistic	13.3 ± 3.3	9.77	Antagonistic
	Observed	3.3 ± 2.1			1.7 ± 1.7		
Clothianidin	Expected	8.3 ± 4.0	0.64	Additive	16.7 ± 5.6	0.41	Additive
	Observed	10.0 ± 3.7			8.3 ± 3.1		
Bt var. *galleriae*							
Imidacloprid	Expected	10.0 ± 4.4	6.94	Antagonistic	6.7 ± 3.3	1.67	Additive
	Observed	1.7 ± 2.1			3.3 ± 1.7		
Clothianidin	Expected	6.7 ± 4.2	0.41	Additive	16.7 ± 7.6	3.79	Additive
	Observed	8.3 ± 4.0			8.3 ± 5.4		
Bt var. *tenebrionis*							
Imidacloprid	Expected	11.7 ± 4.0	5.79	Antagonistic	8.3 ± 3.0	2.91	Additive
	Observed	3.3 ± 2.1			3.3 ± 3.3		
Clothianidin	Expected	8.3 ± 4.0	3.17	Additive	16.7 ± 5.8	3.22	Additive
	Observed	13.3 ± 4.9			5.0 ± 2.2		

[a] Observed = efficacy of both control agents applied at the same time. Expected = sum of efficacy of each control agent applied separately.

[b] ½ = half of the high label rate recommendation for white grub control.

[c] ¼ = quarter of the high label rate recommendation for white grub control.

Table 4. Laboratory mortality (mean ± SE) of third instar *Amphimallon majale* and the interaction among different combinations of biological and neonicotinoid insecticides at 30 DAT in 2006.

		Rate 1/2 [b]			Rate 1/4[c]		
Treatment	Measurement[a]	Mortality	χ^2	Effect	Mortality	χ^2	Effect
B. bassiana GHA							
Imidacloprid	Observed	20.0 ± 2.6	2.88	Additive	28.3 ± 4.7	0.14	Additive
	Expected	31.7 ± 7.0			28.3 ± 6.0		
Clothianidin	Observed	21.7 ± 5.4	7.18	Antagonistic	21.7 ± 5.4	4.08	Antagonist
	Expected	40.0 ± 6.3			36.7 ± 6.1		ic
M. anisopliae Met F52							
Imidacloprid	Observed	6.7 ± 3.3	4.63	Antagonistic	11.7 ± 1.6	0.0	Additive
	Expected	15.0 ± 3.4			11.6 ± 4.0		
Clothianidin	Observed	5.0 ± 2.2	14.40	Antagonistic	1.7 ± 1.7	16.81	Antagonist
	Expected	23.3 ± 3.3			20.0 ± 3.7		ic
M. anisopliae NYSAES							
Imidacloprid	Observed	31.7 ± 7.0	1.40	Additive	48.3 ± 7.9	3.69	Additive
	Expected	43.3 ± 4.2			40.0 ± 3.7		
Clothianidin	Observed	41.7 ± 10.7	0.25	Additive	25.0 ± 6.2	7.32	Antagonist
	Expected	51.7 ± 4.0			48.3 ± 4.8		ic
H. bacteriophora							
Imidacloprid	Observed	58.3 ± 3.1	13.45	Synergistic	48.3 ± 3.1	6.30	Synergisti
	Expected	40.0 ± 4.5			36.7 ± 4.9		c
Clothianidin	Observed	58.3 ± 3.0	5.90	Synergistic	51.7 ± 4.0	3.40	
	Expected	48.3 ± 4.0			45.0 ± 5.0		Additive

Table 4. (Continued).

P. popilliae							
Imidacloprid	Observed	16.7 ± 3.3		Additive	30.0 ± 8.5	1.83	Additive
	Expected	28.3 ± 3.1			25.0 ± 5.6		
Clothianidin	Observed	18.3 ± 3.1		Antagonistic	23.3 ± 2.1	1.75	Additive
	Expected	36.7 ± 4.2			33.3 ± 4.2		
Bt var. *galleriae*							
Imidacloprid	Observed	25.0 ± 4.3	0.10	Additive	26.7 ± 6.1	1.86	Additive
	Expected	25.0 ± 4.3			21.7 ± 3.1		
Clothianidin	Observed	13.3 ± 3.3	10.07	Antagonistic	28.3 ± 6.5	0.01	Additive
	Expected	33.3 ± 3.3			30.0 ± 3.7		
Bt var. *tenebrionis*							
Imidacloprid	Observed	20.0 ± 5.2	?.16	Additive	26.7 ± 5.6	7.22	Antagonist
	Expected	30.0 ± 5.2			38.3 ± 4.8		ic
Clothianidin	Observed	23.3 ± 4.9	3.79	Additive	35.0 ± 5.6	1.33	
	Expected	38.3 ± 6.5			26.7 ± 4.2		Antagonist ic

[a] Observed = efficacy of both control agents applied at the same time. Expected = sum of efficacy of each control agent applied separately.

[b] ½ = half of the high label rate recommendation for white grub control.

[c] ¼ = quarter of the high label rate recommendation for white grub control.

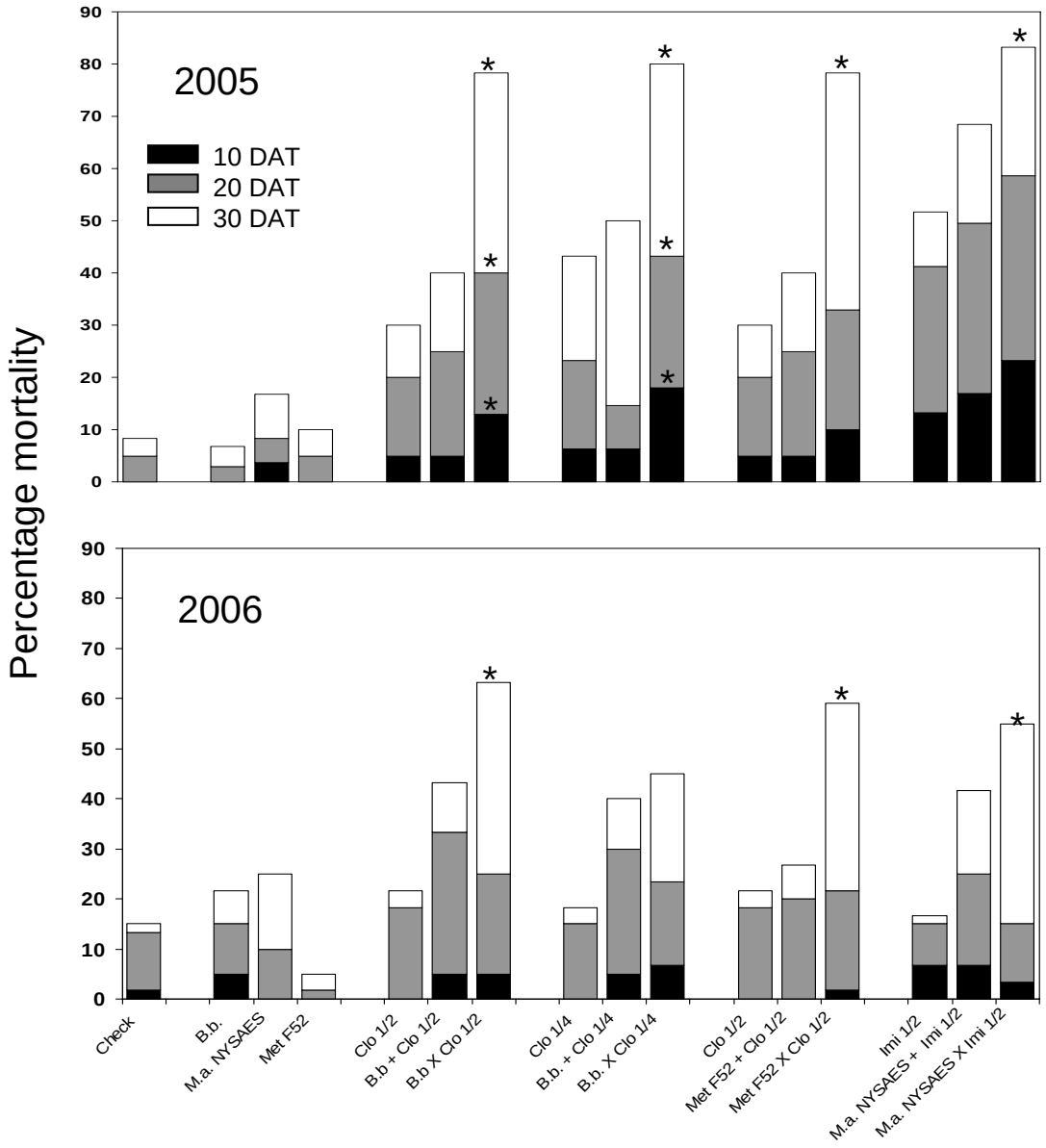

Figure 1. Percent mortality of *Popillia japonica* at 10, 20 and 30 days after treatment (DAT) with different insecticide combinations under laboratory conditions. B.b. = *Beauveria bassiana*, M.a. = *Metarhizium anisopliae*, Clo-1/4 = clothianidin quarter label rate, Clo-1/2 = clothianidin half label rate, Imi-1/2 = imidacloprid half label rate, + = expected mortality base on mortalities of both insecticides applied separately, x = mortality observed when both insecticides are applied at the same time,* = significant synergistic interaction between biological and chemical insecticide, X^2 test value for 1 df.

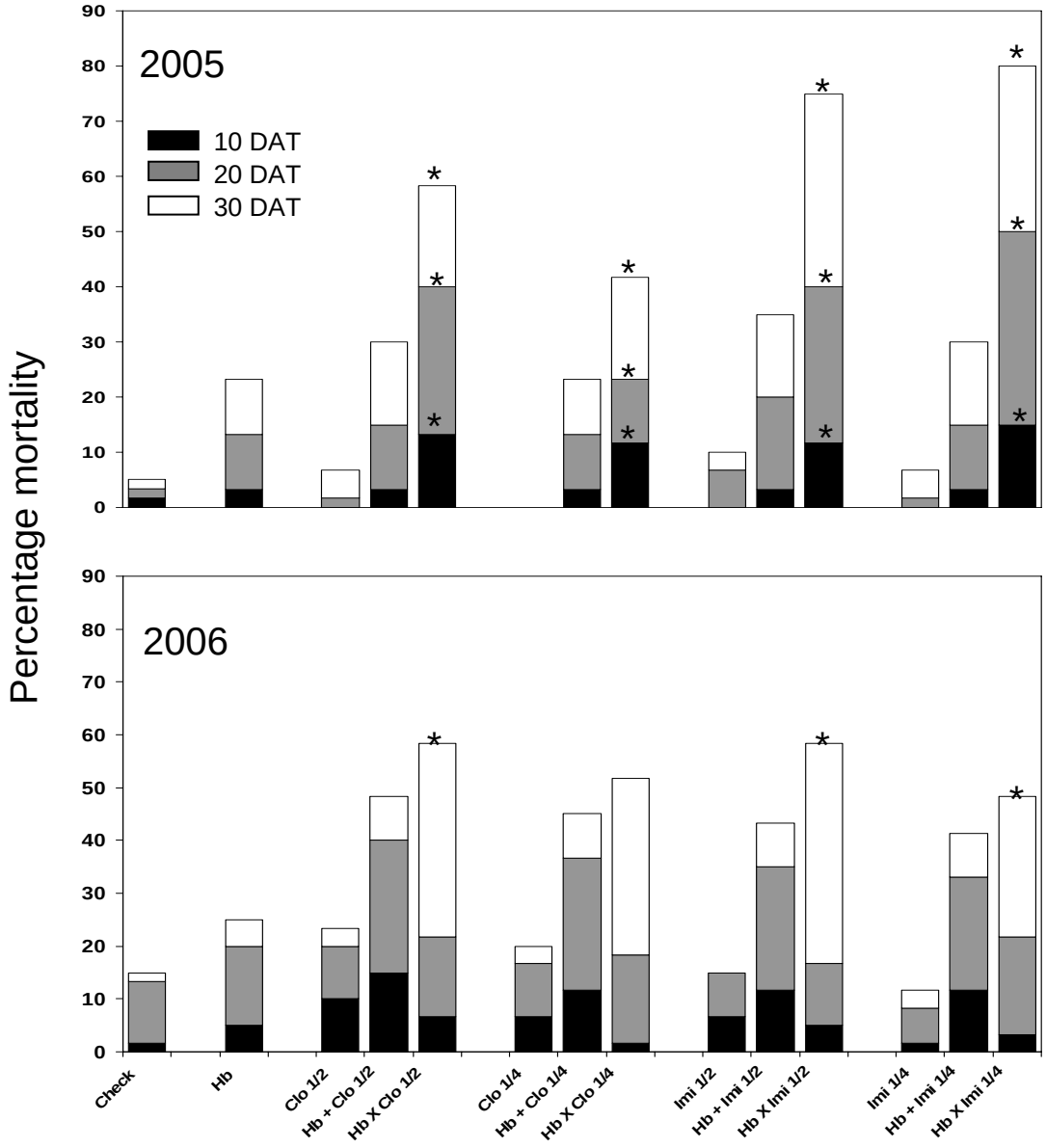

Figure 2. Percent mortality of *Amphimallon majale* at 10, 20 and 30 days after treatment (DAT) with different insecticide combinations under laboratory conditions. H.b. = *Heterorhabditis bacteriophora*, Clo-1/4 = clothianidin quarter label rate, Clo-1/2 = clothianidin half label rate, Imi-1/4 = imidacloprid quater label rate, Imi-1/2 = imidacloprid half label rate, + = expected mortality base on mortalities of both insecticides applied separately, x = mortality observed when both insecticides are applied at the same time, * = significant synergistic interaction between biological and chemical insecticide, X^2 test value for 1 df.

Among all the treatments that showed synergistic interactions against *P. japonica* at 30 DAT in 2005, only *B. bassiana* in combination with clothianidin-½ and ¼ expressed the effect at 10 and 20 DAT (Fig. 1). Neither *M. anisopliae* NYSAES in combination with imidacloprid-½ nor Met F52 in combination with clothianidin-½ expressed the effect earlier than 30 DAT. In 2006, none of the synergies was expressed before 30 DAT. For *A. majale*, in 2005 all treatments that showed synergistic interaction effects were expressed as early as 10 and 20 DAT (Fig. 2). In 2006, none of the synergies was expressed before 30 DAT.

3.2. *Greenhouse experiments*

Mortality in the untreated check ranged from 4 - 18% for both species. In general, treatment mortalities declined in the greenhouse with respect to the laboratory. For *P. japonica*, mortality due the combination of *B. bassiana* and clothianidin-½ was 83.3% in 2005 and 63.3% in 2006 for the laboratory, but was 29.5% in 2006 and 53.0% in 2007 in the greenhouse. For *M. anisopliae* NYSAES in combination with clothianidin-½, laboratory mortalities of 83.3 and 41.7% fell to 18.0 and 34.0% in the greenhouse, respectively (Tables 3, 4 and 5). For *B. bassiana*, a synergy was maintained for clothianidin-½ in both years, but was only additive for clothianidin-¼ (Table 5). For *M. anisopliae* Met F52, a synergy was also maintained for clothianidin-½. All other interactions were additive.

For *A. majale* laboratory mortalities due to the combination of *H. bacteriophora* and clothianidin-½ and ¼ were 58.3 and 41.7% in 2005 and 58.3 and 51.7% in 2006 for each clothianidin rate, respectively (Table 5). Greenhouse mortalities fell to 32 and 28% for 2006 and 36 and 18% in 2007 for each clothianidin

Table 5. Greenhouse mortality (mean ± SE) of third instar *Popillia japonica* and *Amphimallon majale* and the interaction among different combinations of biological and neonicotinoid insecticides at 30 DAT.

		2006			2007		
Treatment[b]	Measurement[a]	Mortality	χ^2	Effect	Mortality	χ^2	Effect
P. japonica:							
B. bassiana							
Clothianidin-½	Observed	29.5 ± 5.6	9.36	Synergistic	53.0 ± 4.1	13.78	Synergistic
	Expected	17.5 ± 3.0			44.0 ± 4.5		
Clothianidin-¼	Observed	21.5 ± 3.1	0.00	Additive	40.0 ± 4.9	0.96	Additive
	Expected	15.0 ± 3.3			37.7 ± 6.7		
M. anisopliae Met F52							
Clothianidin-½	Observed	28.5 ± 3.7	19.50	Synergistic	13.0 ± 4.4	0.03	Additive
	Expected	17.0 ± 3.6			30.0 ± 3.6		
M. anisopliae NYSAES							
Imidacloprid-½	Observed	18.0 ± 4.7	0.98	Additive	34.0 ± 4.0	0.44	Additive
	Expected	14.5 ± 4.4			33.0 ± 4.7		
A. majale:							
H. bacteriophora							
Clothianidin-½	Observed	32.0 ± 3.3	12.54	Synergistic	36.0 ± 5.8	14.80	Synergistic
	Expected	18.0 ± 3.6			20.0 ± 5.2		
Clothianidin-¼	Observed	22.0 ± 3.6	1.19	Additive	18.0 ± 3.8 22.0	0.39	Additive
	Expected	18.0 ± 2.0			± 4.4		
Imidacloprid-½	Observed	40.0 ± 7.5	37.50	Synergistic	42.0 ± 7.6	34.58	Synergistic
	Expected	16.0 ± 2.7			18.0 ± 5.5		
Imidacloprid-¼	Observed	51.0 ± 6.0	97.79	Synergistic	58.0 ± 4.7	94.32	Synergistic
	Expected	14.0 ± 3.1			19.0 ± 4.7		

[a] Observed = efficacy of both control agents applied at the same time. Expected = sum of efficacy of each control agent applied separately.

[b] ½ = half of the high label rate recommendation for white grub control and ¼ = quarter of the high label rate recommendation for white grub control

rate, respectively. For *H. bacteriophora* in combination with imidacloprid-½ and ¼, laboratory mortalities were 75.0 and 80.0% in 2006 and 58.3 and 48.3% in 2006 for each imidacloprid rate, respectively. Greenhouse mortalities fell to 40.0 and 51.0% in 2006 and 42 and 58% in 2007 for each imidacloprid rate, respectively. For *H. bacteriophora* a synergy was maintained for clothianidin-½ and imidacloprid-½ and ¼ in both years. All other interactions were additive (Table 5).

3.3. *Field experiments*

The average number of *P. japonica* recovered in the untreated check was 5.2 or 34.7% of the initial infestation at 30 DAT and 10.2 or 67.8% of the initial infestation at 174 DAT. Average recovery for *A. majale* was 9.1 or 90.1% of the initial infestation at 30 DAT and 6.9 or 69.3% of the initial infestation at 174 DAT.

For *P. japonica*, no mortality was detected at 30 DAT for any of the treatments (Table 6). At 174 DAT an antagonistic interaction was detected for *B. bassiana* with clothianidin-½. All other interactions were additive. Overwintering had a significant effect on mortality because there was a significant difference between 30 and 174 DAT for all treatments. Mortality was significantly higher at 174 DAT for *B. bassiana* with clothianidin-½ ($F = 17.61$; $df = 1, 32$; $P = 0.0002$) and clothianidin-¼ ($F = 23.01$; $df = 1, 32$; $P = 0.0001$), *M. anisopliae* Met F52 with imidacloprid-½ ($F = 17.61$; $df = 1, 32$; $P = 0.0002$) and *M. anisopliae* NYSAES with clothianidin-½ ($F = 29.12$; $df = 1, 32$; $P = 0.0001$).

For *A. majale*, all combinations were additive at 30 DAT (Table 6). At 174 DAT there was a synergy for *H. bacteriophora* in combination with clothianidin-½

Table 6. Field mortality (mean ± SE) of third instar *Popillia japonica* and *Amphimallon majale* and the interaction among different combinations of biological and neonicotinoid insecticides at 30 DAT.

		30 DAT			174 DAT		
Treatment[b]	Measurement[a]	Mortality	χ^2	Effect	Mortality	χ^2	Effect
P. japonica:							
B. bassiana							
Clothianidin-½	Observed	0.0 ± 0.0	0.00	Additive	20.6 ± 4.9	8.25	Antagonist
	Expected	0.0 ± 0.0			42.6 ± 5.5		ic
Clothianidin-¼	Observed	0.0 ± 0.0	0.30	Additive	23.5 ± 4.0	3.28	Additive
	Expected	0.3 ± 0.2			36.8 ± 5.0		
M. anisopliae Met F52							
Clothianidin-½	Observed	0.0 ± 0.0	0.00	Additive	20.6 ± 6.8	2.83	Additive
	Expected	0.0 ± 0.0			32.4 ± 5.2		
M. anisopliae NYSAES							
Imidacloprid-½	Observed	0.0 ± 0.0	0.00	Additive	26.5 ± 2.9	0.14	Additive
	Expected	0.0 ± 0.0			26.5 ± 4.3		
A. majale:							
H. bacteriophora							
Clothianidin-½	Observed	15.9 ± 9.5	0.29	Additive	42.3 ± 11.0	7.98	Synergistic
	Expected	14.4 ± 5.6			30.0 ± 3.6		
Clothianidin-¼	Observed	7.6 ± 4.7	4.20	Additive	36.5 ± 10.8	0.76	Additive
	Expected	16.5 ± 4.0			34.6 ± 5.6		
Imidacloprid-½	Observed	18.2 ± 6.7	1.47	Additive	45.4 ± 16.7	16.10	Synergistic
	Expected	26.9 ± 4.3			26.9 ± 4.3		
Imidacloprid-¼	Observed	22.0 ± 6.8	0.00	Additive	48.1 ± 11.6	20.70	Synergistic
	Expected	23.2 ± 7.2			26.9 ± 8.6		

[a] Observed = efficacy of both control agents applied at the same time. Expected = sum of efficacy of each control agent applied separately.

[b] ½ = half of the high label rate recommendation for white grub control and ¼ = quarter of the high label rate recommendation for white grub control.

and imidacloprid-½ and ¼. *H. bacteriophora* in combination with clothianidin-¼ was additive. Overwintering had a significant effect on mortality for only one combination. Mortality was significantly higher at 174 DAT for *H. bacteriophora* in combination with imidacloprid-¼ ($F = 3.15$; $df = 1, 32$; $P = 0.005$). No overwintering effect was detected for *H. bacteriophora* in combination with imidacloprid-½ ($F = 3.43$; $df = 1, 32$; $P = 0.072$), clothianidin-¼ ($F = 3.89$; $df = 1, 32$; $P = 0.057$) or clothianidin-½ ($F = 3.25$; $df = 1, 32$; $P = 0.081$).

4. DISCUSSION

Among the combinations of biological and neonicotinoids tested here, results reveal that synergistic interactions are relatively uncommon, and involved only entomopathogenic nematodes and fungi. Of the 80 experimental combinations evaluated, interactions were synergistic/additive/antagonistic 17/49/14 times for *A. majale* and 15/52/13 times for *P. japonica*. Moreover, synergies were remarkably consistent across trials, were specific to white grub species, and diminished in strength from lab to greenhouse to field. For *A. majale*, the most promising synergistic combinations were between *H. bacteriophora* and both neonicotinoids; those results were discernible in all laboratory and greenhouse trials and into the field. In contrast, the most promising synergistic combinations for *P. japonica* were *B. bassiana* and *M. anisopliae* Met F52 with clothianidin and *M. anisopliae* NYSAES with imidacloprid. Like *A. majale*, this was discernible in each of the two laboratory trials, but did not persist through to the greenhouse and field. Finally, an antagonistic interaction between *Bt*-products and both neonicotinoids was common to both white grub species. Further study of these non-additive interactions might shed light on how biological and chemical products could be combined to offer enhanced control of soil insect pests.

4.1 *Synergies are specific to grub species*

We showed that the same combination of insecticides have different effects in different species of white grubs. In this case, specific combinations of neonicotinoids and biological insecticides have a synergistic response in specific white grub species. Interactions between *H. bacteriophora* and both neonicotinoids were synergistic in *A. majale* but only additive in *P. japonica*. In contrast, interactions between *B. bassiana* and *M. anisopliae* and both neonicotinoids were synergistic in *P. japonica* but only additive in *A. majale*.

Synergies between entomopathogenic nematodes and different neonicotinoids against white grub species have previously been shown (Koppenhöfer and Kaya, 1998; Koppenhöfer et al., 2000a; 2000b; 2003 and 2006). Those reports are highly variable, however. The same group of researchers, for instance, reported synergies in *P. japonica* for *H. bacteriophora* and different doses of imidacloprid and thiamethoxam in one study (Koppenhöfer et al., 2002a), but only an additive effect in a later study (Koppenhöfer et al., 2003). In another study (Koppenhöfer et al., 2002a), *H. bacteriophora* combined with imidacloprid and acetamiprid had only an additive effect on *A. majale* and *M. castanea*, but an antagonistic effect with thiamethoxam; in *A. orientalis* there was a synergistic effect for all combinations. In contrast, the synergies that we showed between *H. bacteriophora* and both rates of imidacloprid (½, ¼) in *A. majale* were consistent across experiments and trials.

For *P. japonica,* a synergistic interaction was detected for some combinations of entomopathogenic fungi with both neonicotinoids under laboratory conditions, a few in the greenhouse and none in the field (Table 6). No other reports of synergistic interactions were found in the literature using fungi, bacteria and neonicotinoids either on *P. japonica* or *A. majale*. But synergisms have been reported for other soil insects using low doses of imidacloprid and entomopathogenic fungi. For instance, Jaramillo

et al. (2005) reported a synergistic effect of low doses of imidacloprid with *M. anisopliae* CIAT 224 on *C. bergi* nymphs under laboratory and greenhouse conditions. Moreover, Quintela and McCoy (1998) observed decreased larval movement and increased larval mortality of *D. abbreviatus* with *B. bassiana* and *M. anisopliae* in combination with low doses of imidacloprid.

The type of interaction and the strength of synergistic effects may depend on factors such as target species, doses (biological and neonicotinoid) insecticides and even strain of bacteria, fungi or nematode used (Jaramillo et al., 2005; Koppenhöfer et al., 2000a; 2000b; 2002 and 2003; Polavarapu et al., 2007). When Koppenhöfer et al. (2002) targeted *P. japonica,* for instance, *H. bacteriophora* and imidacloprid were synergistic under laboratory, greenhouse and field conditions. No synergy was detected when *A. majale* was the target species. With respect to dose, Polavarapu et al. (2007) reported a synergism in *A. orientalis* when *H. bacteriophora* was combined with a low dose of imidacloprid (84 mg AI/ha) but not with a high dose (168 mg AI/ha). The dose of the biological insecticide may also affect the type of interaction. For instance, Koppenhöfer et al. (2002) showed that at low dose (1.25×10^9 IJ/ha) of *H. bacteriophora* in combination with thiamethoxam was additive, but at a high dose (2.5×10^9 IJ/ha) it was antagonistic for *P. japonica* and *E. orientalis*. With imidacloprid, however, the low dose of *H. bacteriophora* was additive, and the high dose synergistic. The differences could be related to different nematode strains used for each study. Our study used a commercial strain (Heteromask) while Koppenhöfer et al. (2002) used non commercial strains (TF and NC1).

4.2 *Mechanisms and neonicotinoids effects*

Understanding how each insecticide affects the target insect when applied individually may shed light on the mechanism behind synergies. For instance,

neonicotinoid insecticides could affect normal behavior and make insects more susceptible to natural enemies. An immediate effect of neonicotinoids is antifeedant. While this is probably less important in the late fall when white grubs are preparing to overwinter, it could be very important in early spring when they reemerge to feed for a couple weeks more before pupation (Grewal et al., 2001). If the insect does not move to forage, it may be an easy target for fungi, nematodes as well as parasitoids. That defensive behavior may be efficient for systemic insecticides (such as neonicotinoids) and enterobacteria (such as *Bt* and *P. popilliae*) if the insect does not ingest enough product. Second, neonicotinoids also interfere with the insect nervous system, producing uncoordinated movements, tremor and paralysis, rendering white grubs less able to descend in the soil to avoid freezing temperatures during the winter or exposure to pathogens, parasitoids and predators (Ehler et al., 1998; Grewal et al., 2001). Third, Koppenhöfer et al. (2000b) hypothesized a blockade of the defensive behavior of white grubs due to the disruption of normal nerve function as a direct effect of the neonicotinoid in the cholinergic receptors in the postsynaptic membrane. This could produce a change in grooming behavior, such as a reduction in frequency of brushing, chewing and rubbing. In response to the presence of *H. bacteriophora* the frequency of these activities has been shown to increase (Koppenhöfer et al., 2000a). This behavior is probably linked to defense from nematodes, rather than fungi or bacteria where a cellular and humoral immune response is the main defense (Narayanan, 2004).

4.3. *Bt-products and neonicotinoids are antagonistic*

Beyond the synergistic interactions detected in this study, specific antagonistic interactions were also detected. In particular, under laboratory conditions for both white grub species, almost all combinations of bacteria and neonicotinoids were

antagonistic. Chemical insecticides may have inhibitory effects on bacteria and their prevalence in the field. For *P. popilliae*, 14 chemical pesticides (herbicides, fungicides and insecticides) were shown to reduce levels of spore viability, spore germination and/or vegetative cell growth (Dingman, 1994).

Other than that, the only other antagonistic interactions detected were for *A. majale* with some combinations of entomopathogenic fungi and both neonicotinoids. The efficacy of entomopathogenic fungi in combination with some chemical insecticides may be affected due to lower germination rate, decreased production of enzymes necessary for penetration of the insect's cuticle, and poor mycelium growth ratio. For instance, mycelium growth ratios of *B. brogniartti* and *B. bassiana* are inhibited by carbosulfan, but carbofuran stimulated the growth of *B. brogniartti* (Bednarek et al., 2004). Antagonistic interactions have been reported by Koppenhöfer et al. (2002) for *A. majale, A. orientalis, M. castanea* and *P. japonica* with combined application of thiamethoxam and *H. bacteriophora* under field conditions. An antagonistic effect was also reported for *A. majale* treated with a combination of *S. scarabaei* and imidacloprid, but that effect could be attributed to the high mortality produced by the nematode alone, which did not give room for the expression of any improvement in mortality, rather than inhibition of *S. scarabaei*, or no synergistic effect of imidacloprid (Cappaert and Koppenhöfer, 2003).

4.4. *Strength of synergies diminishes from laboratory to field*

For *P. japonica*, mortality in treatments with synergistic interactions was 2.2 times higher in the laboratory than the greenhouse, and 6.0 times higher than the field. For *A. majale*, mortality in the laboratory was 1.6 times higher than the greenhouse and 1.8 times higher than the field. The decline in mortality from laboratory to greenhouse and field experiments may be due to size of the experimental units and

temperature fluctuation. Laboratory bioassay cups were 33 times smaller than greenhouse pots and 185 times smaller than field arenas. The large arenas provided more possibilities to escape from the control agents even if the insecticide doses were applied at the same rate proportional to the area. Previous studies have shown evasive behavior in *P. japonica* to areas treated with *M. anisopliae* in soil microcosms (Villani et al., 1994; Fry et al., 1997). The same behavior has also been observed in *P. japonica* with imidacloprid and in *A. majale* with both *M. anisopliae* and imidacloprid (Morales, A. unpublished data).

Environmental conditions could induce changes in the behavior of white grubs that diminish the strength of synergistic interactions. After applications in the laboratory, the experimental units were maintained in walk-in environmental chambers under controlled climate conditions with little variation in temperature and humidity. In the greenhouse, even with an environmental control system, high variation was observed in the temperature (mean 13.5°C, range 9.0 - 25.0°C) and humidity (average 55%, range 35 - 95%). While more variation was expected in the field, it may be that the low temperatures of late fall had an inhibitory affect on both biological and chemical insecticides. The average temperature 7 DAT was 6.9°C (3.3 – 10.5°C) for *P. japonica* and 9.6°C (5.5 – 13.8°C) for *A. majale*. Low temperatures can inactivate or otherwise affect performance of biological insecticides. The optimal range of temperature is 20-30°C for *B. bassiana* and *M. anisopliae* and 15-30°C for *H. bacteriophora* (Bruck et al., 2008; Pandey, 2008).

In the fall evaluation, unusually low air temperatures for almost a week dropped soil temperatures by about 5-7ºC below average; *P. japonica* may have responded to the low temperatures by moving down in the soil profile (Vittum et al., 1999). We suspect that this is the reason why few larvae were collected in the field evaluation at 30 DAT in late fall. At 174 DAT with higher temperatures, twice as

many larvae were collected from the untreated checks. *Amphimallon majale* tolerates low temperatures better than *P. japonica* and moves down in the soil profile later in the fall (Morales, personal observation). For *P. japonica*, we could not compare the effect of the treatments at 30 and 174 DAT to establish any additional effect of treatments in the overwintering larvae. For *A. majale*, mortality was higher at 174 DAT than 30 DAT for *H. bacteriophora* and the high rate of clothianidin (½) and both rates of imidacloprid (½, ¼). This suggests that both neonicotinoids have an effect on overwintering larvae as Grewal et al. (2001) reported.

4.5. *Implications for soil insect pest management*

To validate and adopt synergistic combinations of biological and chemical insecticides as a new approach for turfgrass IPM programs, some hurdles remain. For instance, only two entomopathogenic fungi products are registered in the United States for control of white grubs in turfgrass. More species-specific nematodes are needed commercially. High standards of quality control are needed to avoid variability in biological control agents available in the market. Due to differential effects among white grub species (Morales et al., submitted), pest management practitioners need to diagnose and differentiate scarab species before any intervention.

As an alternative for curative control, synergistic interaction products could be used with less cost to turf managers. Low application rates (half of the recommended field rate) could be made in response to signs of damage and could be limited to only the affected area, in contrast to preventive applications where large areas are treated even when the degree of infestation is unknown. For white grub management, a synergistic interaction approach might be useful for curative control and thereby broaden opportunities for the use of biological insecticides beyond preventive control. Indeed, Koppenhöfer and Fuzy (2008) suggest an early curative control; nematode and

neonicotinoid combinations could be more effective against second and early third instars than late third instars.

Turfgrass managers prioritize the control of diseases and insect pests on fairways, tees and greens, and avoid pesticide application in low-value turf areas that can tolerate some damage, such as roughs. But each year untreated areas may become a source of new infestation for the whole golf course, thereby increasing reliance on insecticide applications. Combined applications of a biological with low doses of insecticide could help to control the problem at low cost while conserving natural enemies for the avoidance of future outbreaks.

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