Pathogenicity Enhancement of *Metarhizium anisopliae* and *Beauveria bassiana* to First Instars of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) with Sublethal Doses of Imidacloprid

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ABSTRACT *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin combined with sublethal doses of imidacloprid as a contact or oral treatment increased synergistically mortality and mycosis of 1st instars of *Diaprepes abbreviatus* (L.). Time to death was also reduced significantly through the synergistic effect of the fungal/chemical treatment. Synergism only occurred at concentrations of imidacloprid of 100 ppm or greater. At fungal concentrations of 10^6 and 10^7 conidia per milliliters and imidacloprid at doses of 100 ppm or greater, larval mortality and mycosis reached 90–100%. Mycosis was reduced with imidacloprid at 1,000 ppm. There were no differences in larval mortality or mycosis by *M. anisopliae* when imidacloprid was administered by contact or per os. The active ingredient rather than the inert carrier was the main component responsible for the synergism between imidacloprid and entomopathogenic fungi.

KEY WORDS root weevil, *Diaprepes abbreviatus*, *Beauveria bassiana*, *Metarhizium anisopliae*, entomopathogenic fungi, chloronicotinyl

THE UBIQUITOUS FUNGI *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin are common disease agents associated with dead and moribund insects in nature (McCoy et al. 1988). These fungi have been scrutinized worldwide as microbial control agents of soil-inhabiting insects in particular (e.g., weevils, grubs, and ants [McCoy 1990]). In Florida, these entomopathogenic fungi infect both larval and adult stages of the root weevil *Diaprepes abbreviatus* (L.) (Beavers et al. 1972, 1983). Larvae of this species are of significant economic importance because of the severe root injury they cause to the citrus tree (Schroeder and Sutton 1977). Commercially produced conidia of *B. bassiana* and mycelia of *M. anisopliae* have suppressed root weevil larval populations in groves when applied at high inoculum rates (McCoy 1990, Schwarz 1995). Also, fungal persistence in the soil has usually been short (Storey et al. 1989; Studdert and Kaya 1990; Quintela et al. 1992, 1994).

The soil ecosystem beneath the citrus tree canopy is generally shaded thereby moderating soil temperatures, reducing solar radiation, and minimizing loss in soil moisture. Although these factors appear favorable for fungal survival and potential use of microbial control agents, field results suggest that some soil insect larvae are resistant to fungal diseases and the soil is strongly fungistatic (Wraight and Roberts 1987, Keller and Zimmermann 1989). In fact, healthy insect larvae actively moving within a soil substrate appear to void attached conidia from their cuticle (Quintela 1996).

Using chemicals to enhance efficacy of entomopathogenic fungi has been tested using organophosphates, carbamates, and organochlorines such as DDT (Fargues 1973; 1975; Anderson et al. 1989; Hassan and Charnley 1989). In these studies, no consistent interaction of entomopathogenic fungi was observed with insecticides.

In 1991, Bayer AG introduced new pesticide chemistry with the neurotoxic chloronicotinyl insecticide imidacloprid (Elbert et al. 1991). This nitromethylene heterocycle analogue is systemic in plants and is effective against a wide range of insects, including citrus root weevils (Abbink 1991, Elbert et al. 1991, McCoy et al. 1995). Sublethal concentrations of imidacloprid induced rapid kill of termites in soil containing *M. anisopliae* and *Conidiobolus coronatus* (Costantin) Batko (W. M. Zeck, personal communication). Strong synergism was detected when sublethal doses of imidacloprid were combined with conidia of *M. anisopliae*, *Paecilomyces farinosus* (Dickson), *C. coronatus*, *B. bassiana*, and *Actinomucor* sp. against the eastern subterranean termite, *Reticulitermes flavipes* (Kollar). Quintela and McCoy (1997) determined the effect of

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both sublethal and lethal doses of imidacloprid on survival, ecdysis, and mobility of 1st instars of *D. abbreviatus*, using contact and oral exposure bioassays with and without a soil substrate in the laboratory. Imidacloprid fed per os at ≥12.5 ppm reduced feeding, slowed larval development, and reduced ecdysis. Imidacloprid by contact exposure at doses >100 ppm also affected larval mobility and development, both in and outside the soil. However, larvae recovered mobility when they were placed in untreated soil.

In view of the physiological effects of imidacloprid on 1st instars of *D. abbreviatus*, laboratory studies were conducted to determine the effect of sublethal concentrations of imidacloprid and different concentrations of fungal conidia on larval mortality, mycosis, and ecdysis. This article addresses the effects of the fungi, *B. bassiana* and *M. anisopliae*, with and without imidacloprid, on 1st instars of *D. abbreviatus*.

Materials and Methods

General Procedures. First instars of *D. abbreviatus*, <48 h old, were obtained from eggs laid by field-collected adult females that were confined to screened cages in the greenhouse at 27 ± 2°C as described by McCoy et al. (1995). Before each experiment, vigorous 1st instars were selected from the whole test population as described in Quintela and McCoy (1997).

A commercial formulation of imidacloprid (Admire 2 F [flowable] 21.4% [AI], technical grade 1-[(6-chloro-3-pyridyl)methyl]-N-nitro-2-imidazolidin-imine) and inert carrier (Bayer, Kansas City, MO) was used in all experiments.

Metarhizium anisopliae was isolated from *D. abbreviatus* larvae collected from soil at Apopka, FL, and designated MADA strain. Strain GHA of *B. bassiana*, commercialized as Mycotrol (Mycotech, Butte, MT), was used in the experiments. Conidia of both fungi were harvested from fungal cultures that were produced on potato dextrose agar plus 0.4% yeast extract (PDAy) and incubated for 10–15 d at 25 ± 1°C. Conidial inoculum was taken from pure fungal cultures, with no more than 2 serial passages from a host insect. Conidial viability was determined by counting germ tubes produced on PDAy after 18 h using a phase contrast microscope at 400×. Conidial viability was 98–100%.

Cuticular and Oral Treatment of First Instars with Imidacloprid and Conidia of *M. anisopliae* and *B. bassiana*. In the 1st bioassay, ≈50 first instars were placed into 1.5-ml microcentrifuge tubes for group treatment with imidacloprid alone at 0 and 100 ppm (AI) or conidia of *M. anisopliae* alone at concentrations of 0, 10^4, 10^5, 10^6, and 10^7 conidia per milliliter and in combination. All conidial suspensions and chemical solutions were prepared in 0.05% aqueous Tween 80 (Fisher, Pittsburgh, PA). Tween 80 was included as a control. The tubes were shaken gently by hand for 30 s before larval removal. Larvae were then counted for each replicate and held at 28°C in the dark in 30-ml clear plastic cups containing a filter paper disc (1.5 cm diameter) moistened with sterile distilled water. A thin slice of raw carrot was supplied as a food source. Each treatment was replicated 6 times with 6 larvae per replicate. The filter paper was moistened every other day and the carrots replaced when they were desiccated or began to deteriorate. Larval mortality was recorded daily by microscopic examination at 10–16×, beginning 2 d after treatment. Dead larvae were removed daily and placed into 35-mm petri dishes containing moist filter paper to determine mycosis from *M. anisopliae*. In bioassay 2, *B. bassiana* was substituted for *M. anisopliae* as the fungal source and each treatment was replicated 5 times with 10 first instars per replication.

In bioassays 3, 4, and 5, imidacloprid was tested at the following 3 concentrations: (1) 0, 25, 50, 100, 150, and 200 ppm (AI), with conidial suspensions of *B. bassiana* at 0, and 10^6 conidia per milliliter alone and in combination; (2) 0, 100, 250, 500, and 1,000 ppm (AI), with conidial concentrations of *M. anisopliae* at 0 and 10^7 conidia per milliliter alone and in combination; (3) 0, 100, 500, and 1,000 ppm (AI) with conidial suspensions of *B. bassiana* at 0, 10^5, 10^6, and 10^7 conidia per milliliter alone and in combination. For each bioassay, each treatment was replicated 5 times with 10 larvae per replication. Larval mortality was recorded daily by microscopic examination. In addition, larval ecdysis was recorded daily for bioassay 5.

To determine the effect of oral and cuticular exposure on larval mortality, mycosis, and ecdysis (bioassay 6), imidacloprid was administered 2 ways, at concentrations of 0 and 100 ppm (AI) and *M. anisopliae* as conidial suspensions at concentrations of 0, 10^5, 10^6, and 10^7 conidia per milliliter alone and in combination with imidacloprid. Larvae were treated orally by feeding them continuously on carrot previously soaked in the imidacloprid solutions for 30 min. Larvae were treated by contact by dipping as described for bioassay 1. Larvae were held in plastic cups containing a carrot slice (8 mm diameter). Each treatment was replicated 5 times with 10 larvae per replication. Diagnosis for larval mortality and mycosis were the same as described above.

The proportion mortality, mycosis, and ecdysis were transformed using arcsine $\sqrt{x}$. For the fungal treatments, a log-dose transformation was also performed. A factorial analysis (PROC GLM, SAS Institute 1985) was performed on bioassay data to determine the main effects of imidacloprid and fungal treatments and their interaction (nonadditivity). The model was considered additive if the factorial analysis for the interaction fungus/chemical was not significant (slopes were parallel) (e.g., mortality = effect of fungus + effect of chemical). The model was synergistic or antagonistic if the interaction was significant (slopes were not parallel) (e.g., mortality = effect of fungus + effect of chemical + effect of interaction). To furthe

Table 1. Summary of factorial analysis for ares with *B. bassiana* (Bb) or *M. anisopliae* (Ma), and i

Bioassay	Factor	*F*
1	Ma	23.0
	IMI	45.9
	Ma × IMI	2.6
2	Bb	14.6
	IMI	63.4
	Bb × IMI	4.2
3	Bb	43.8
	IMI	15.6
	Bb × IMI	7.1
4	Ma	90.5
	IMI	8.9
	Ma × IMI	1.3

synergistic or antagonistic effect, the d
percent larval mortality or mycosis b
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synergism was present the difference
larval mortality or mycosis increased as
tration of fungus increased. If antagon
ferences in percent larval mortality
would decrease with an increase in the
centration. Time–mortality regression
some tests was performed by probit a
ing the correlation in the data (SAS In
Failure of 95% CI overlap was used as
to determine significant differences
ment means.

Cuticular Exposure to Formulated and Each Component Separately with *anisopliae*. Formulated imidacloprid (AI), active ingredient (listed purity ppm, and inert carrier at 1,836 ppm (c equal to the amount found in formula prid at 500 ppm of the active ingredien with conidia of *M. anisopliae* at conce and 10^7 conidia per milliliter. Larvae w each suspension in the manner des ously. Aqueous Tween 80 at 0.05% wa trol. Treatments were replicated 5 t larvae per replication. Larvae were h slices as described above. Larval mor cosis were recorded daily as describ Proportion mortality and mycosis w using PROC GLM (SAS Institute 198 formation to arsine √proportion means were compared using the T significant difference (HSD) test at Institute 1985).

Results

Cuticular and Oral Treatment o with Imidacloprid and Conidia of *M. B. bassiana*: Comparison Between (bioassay 1) and *B. bassiana* (bioass *pliae*, *B. bassiana*, and imidacloprid h effect on larval mortality (Table 1; F

Table 1. Summary of factorial analysis for arcsine $\sqrt{}$ proportion of larval mortality and mycosis of *D. abbreviatus* after treatment with *B. bassiana* (Bb) or *M. anisopliae* (Ma), and imidacloprid (IMI) alone or in combination

Assay	Factor	Larval mortality			Larval mycosis		
		F	df	*P*	*F*	df	*P*
1	Ma	23.0	4, 50	0.0001	75.6	4, 50	0.0001
	IMI	45.9	1, 50	0.0001	92.4	1, 50	0.0001
	Ma × IMI	2.6	4, 50	0.0456	10.2	4, 50	0.0001
2	Bb	14.6	4, 40	0.0001	25.2	4, 40	0.0001
	IMI	63.4	1, 40	0.0001	63.4	1, 40	0.0001
	Bb × IMI	4.2	4, 40	0.0065	4.6	4, 40	0.0039
3	Bb	43.8	1, 48	0.0001	412.8	1, 48	0.0001
	IMI	15.6	5, 48	0.0001	18.7	5, 48	0.0001
	Bb × IMI	7.1	5, 48	0.0001	18.4	5, 48	0.0001
4	Ma	90.5	1, 40	0.0001	336.8	1, 40	0.0001
	IMI	8.9	4, 40	0.0001	5.0	4, 40	0.0021
	Ma × IMI	1.3	4, 40	0.2964	3.2	4, 40	0.0216

+ effect of interaction). To further elucidate a synergistic or antagonistic effect, the differences in percent larval mortality or mycosis between fungus/chemical and fungus alone were plotted. When synergism was present the differences in percent larval mortality or mycosis increased as the concentration of fungus increased. If antagonistic, the differences in percent larval mortality or mycosis would decrease with an increase in the fungal concentration. Time–mortality regression analysis for some tests was performed by probit analysis ignoring the correlation in the data (SAS Institute 1985). Failure of 95% CI overlap was used as the criterion to determine significant differences among treatment means.

Cuticular Exposure to Formulated Imidacloprid and Each Component Separately with Conidia of *M. anisopliae*. Formulated imidacloprid at 500 ppm (AI), active ingredient (listed purity 97.5%) at 500 ppm, and inert carrier at 1.836 ppm (concentration equal to the amount found in formulated imidacloprid at 500 ppm of the active ingredient) were mixed with conidia of *M. anisopliae* at concentrations of 0 and 10^7 conidia per milliliter. Larvae were dipped in each suspension in the manner described previously. Aqueous Tween 80 at 0.05% was used as control. Treatments were replicated 5 times with 10 larvae per replication. Larvae were held on carrot slices as described above. Larval mortality and mycosis were recorded daily as described previously. Proportion mortality and mycosis were analyzed using PROC GLM (SAS Institute 1985) after transformation to arsine $\sqrt{}$proportions. Treatment means were compared using the Tukey honestly significant difference (HSD) test at $P = 0.05$ (SAS Institute 1985).

Results

Cuticular and Oral Treatment of First Instars with Imidacloprid and Conidia of *M. anisopliae* and *B. bassiana*: Comparison Between *M. anisopliae* (bioassay 1) and *B. bassiana* (bioassay 2). *M. anisopliae*, *B. bassiana*, and imidacloprid had a significant effect on larval mortality (Table 1; Fig. 1 A and B). Larval mortality caused by cuticular exposure to imidacloprid alone at 100 ppm ranged from 18 to 30% compared with 10 to 18% in the untreated controls in bioassays 1 and 2, respectively (Fig. 1 A and B). In bioassay 2, slightly higher mortality was probably the result of accidental fungal contamination (Fig. 1F). Larval mortality and larval mycosis increased gradually with an increase in conidial concentration of either *M. anisopliae* and *B. bassiana* (Fig. 1 A, B, E, F). No larval mycosis from *M. aniso-*

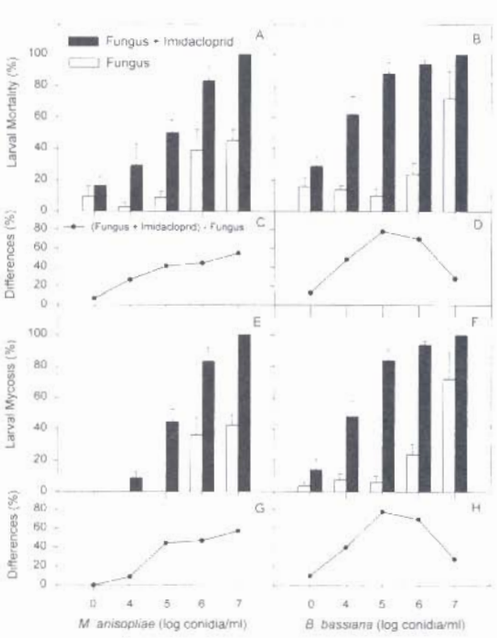

Fig. 1. Effect of different conidial concentrations of *M. anisopliae* or *B. bassiana* applied to the cuticle alone (white bars) or in combination with a sublethal dose of imidacloprid at 100 ppm (AI) (black bars) on larval mortality (A, B) and mycosis (E, F) of 1st instars of *D. abbreviatus* after 6 d. Differences between the chemical with fungus and *M. anisopliae* or *B. bassiana* alone (solid line, black dots) for larval mortality (C, D) and mycosis (G, H).

Table 2. Probit analysis of bioassay data where different conidial concentrations of *M. anisopliae* and *B. bassiana* with and without a sublethal dose of imidacloprid were tested against 1st instars of *D. abbreviatus*

Imidacloprid, ppm	Fungus (conidia/ml)	*M. anisopliae*		*B. bassiana*	
		Slope ± SE[a]	LT_{50}[b] (95% CI), d	Slope ± SE[a]	LT_{50}[b] (95% CI), d
0	0	1.1 ± 0.6	16.0 (∞)	1.9 ± 0.7	10.5 (7.7–34.7)
	10^4	1.5 ± 1.2	16.6 (∞)	3.3 ± 1.1	9.0 (7.2–16.5)
	10^5	1.5 ± 0.9	12.9 (∞)	4.2 ± 1.8	9.1 (7.3–21.5)
	10^6	3.2 ± 0.7	5.9 (4.6–18.1)	3.2 ± 0.8	7.6 (6.5–10.5)
	10^7	3.8 ± 0.7	5.5 (4.5–9.0)	3.8 ± 0.5	3.9 (2.3–5.4)
100	0	2.1 ± 0.7	9.3 (7.1–19.2)	3.8 ± 0.8	7.4 (6.2–11.1)
	10^4	1.6 ± 0.5	8.0 (6.2–15.7)	6.3 ± 0.9	5.1 (4.2–7.2)
	10^5	4.4 ± 0.8	5.6 (5.1–6.4)	5.9 ± 0.7	3.7 (2.4–4.7)
	10^6	6.5 ± 1.2	3.9 (2.7–5.0)	4.3 ± 0.6	2.1 (1.8–2.5)
	10^7	12.3 ± 1.8	2.5 (2.3–2.7)	7.3 ± 1.1	2.0 (1.7–2.3)

Bioassay data are means of 5 or 6 replicates for *B. bassiana* (n = 50) and *M. anisopliae* (n = 36), respectively.
[a] Slope from regression of cumulative percent mortality (probit) on log time.
[b] Time until death of 50% of inoculated larvae.

pliae occurred at concentrations of 10^4 and 10^5 conidia per milliliter (Fig. 1E).

When a sublethal dose (100 ppm) of imidacloprid was included with different concentrations of either *M. anisopliae* or *B. bassiana* and applied directly to the larval cuticle, there was a significant interaction between imidacloprid and fungal concentrations for both larval mortality and larval mycosis (Table 1, bioassays 1 and 2). Synergism between the fungi and chemical was indicated by the significant interaction, and the increased differences in larval mortality or mycosis between fungus/imidacloprid and fungus alone (Fig. 1 C, D, G, H). Mortality to 1st instars of *D. abbreviatus* by *M. anisopliae* and *B. bassiana* with imidacloprid at 100 ppm ranged from 1.7- to 10.5-fold and 1.4- to 8.6-fold higher than for the fungi alone, respectively. Larval mortality and larval mycosis increased with an increase in conidial concentration of either *M. anisopliae* or *B. bassiana* in combination with 100 ppm imidacloprid. At lower conidial concentrations (10^4–10^5 conidia per milliliter), mycosis by either fungus in combination with imidacloprid was evident and exceeded 80% at concentrations of 10^6 and 10^7 conidia per milliliter (Fig. 1 E and F).

The time required to kill 50% of the larvae (LT_{50}) decreased significantly with the inclusion of imidacloprid in the conidial inoculum of both *M. anisopliae* and *B. bassiana* (Table 2). For example, *M. anisopliae* had an LT_{50} of 5.5 d alone at 10^7 conidia per milliliter and 2.5 d at 10^7 conidia per milliliter with 100 ppm imidacloprid (Table 2).

Effect of Lower Sublethal Doses of Imidacloprid (bioassay 3). Only small differences in larval mortality were detected among different concentrations of imidacloprid by contact exposure alone (Fig. 2A). For the fungus/chemical combination, however, both larval mortality and larval mycosis generally increased with an increase in imidacloprid concentration (Fig. 2 A and C).

Imidacloprid, *B. bassiana*, and the interaction had a significant effect on larval mortality (Table 1, bioassay 3). Synergism was observed when concentrations of imidacloprid were 100 ppm or greater

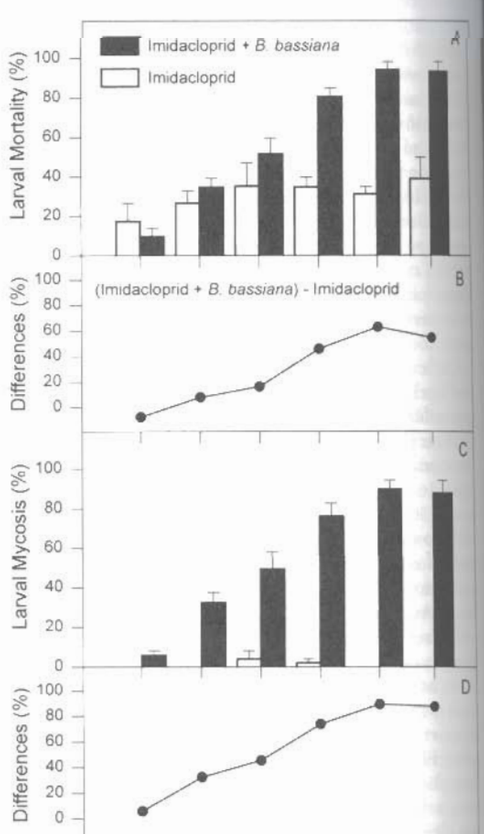

Fig. 2. Effect of different doses of imidacloprid applied to the cuticle alone (white bars) or in combination with *B. bassiana* at 10^6 conidia per milliliter (black bars) on mortality (A) and mycosis (C) of 1st instars of *D. abbreviatus* after 6 d. Differences between chemical with *B. bassiana* and imidacloprid alone (solid line, black dots) for larval mortality (B) and mycosis (D).

Table 3. Probit analysis of mortality data where centrations of imidacloprid with and without conidia were tested against 1st instars of *D. abbreviatus*

B. bassiana (conidia/ml)	Imidacloprid, ppm	Slope ± SE[a]	LT_5
0	0	0.6 ± 0.4	16
	25	0.9 ± 0.4	10
	50	2.4 ± 0.5	6
	100	2.0 ± 0.4	7
	150	2.1 ± 0.5	7
	200	2.0 ± 0.4	6
10^6	0	1.0 ± 0.5	14
	25	3.1 ± 0.6	6
	50	3.7 ± 0.9	5
	100	6.1 ± 1.3	
	150	4.9 ± 1.1	
	200	6.4 ± 0.6	

Mortality data are means of 5 replicates of 10 l
[a] Slope from regression of cumulative percent mo
on log time.
[b] Time until death of 50% of inoculated larvae.

(Fig. 2A). At concentrations <100 ppm in larval mortality were <20% between alone and the combination (Fig. 2B).

Larval mycosis was significant for bo and fungus alone, and the interactio bioassay 3). Some fungal contaminat tected at 50 and 100 ppm in the chemic (Fig. 2C). The synergistic effect of chemical combination was confirmed creases in the difference in mycosis be acloprid alone and the combinat chemical (Fig. 2 D).

The LT_{50}s for imidacloprid at 100, ppm combined with the fungus at 10 milliliter were significantly lower co chemical alone (Table 3). Below 100 CI for LT_{50} values overlapped for alone.

Effect of Higher Doses of Imidaclo 4). Only 20–40% larval mortality among the different concentrations of alone (Fig. 3A). Slight increases in la and mycosis occurred as the concentra ical and fungus combinations increa and C).

Larval mortality was significantly both chemical and fungus (Table 1 However, the fungal/chemical intera significant, suggesting an additive ef ference in larval mortality between alone and the fungal/chemical intera increase with an increase in the che tration, confirming the additive effe

Larval mycosis was significant for *M. anisopliae*, and the fungus/chemi (Table 1, bioassay 4). No larval m pected from imidacloprid alone, yet was detected at 100, 500, and 1,000 p from accidental contamination (Fig. ergistic effect of the fungus/chemic was confirmed by the increase in the

Table 3. Probit analysis of mortality data where different concentrations of imidacloprid with and without conidia of *B. bassiana* were tested against 1st instars of *D. abbreviatus*

bassiana conidia/ml)	Imidacloprid, ppm	Slope ± SE[a]	LT_{50}[b] (95% CI), d
0	0	0.6 ± 0.4	16.5 (∞)
	25	0.9 ± 0.4	10.7 (7.3–59.7)
	50	2.4 ± 0.5	6.7 (5.8– 8.3)
	100	2.0 ± 0.4	7.0 (6.0– 9.3)
	150	2.1 ± 0.5	7.1 (6.1– 9.7)
	200	2.0 ± 0.4	6.6 (5.7– 8.5)
10^6	0	1.0 ± 0.5	14.8 (∞)
	25	3.1 ± 0.6	6.4 (4.9–20.4)
	50	3.7 ± 0.9	5.1 (3.9–11.1)
	100	6.1 ± 1.3	3.9 (2.6– 5.3)
	150	4.9 ± 1.1	3.3 (2.7– 3.8)
	200	6.4 ± 0.6	3.7 (3.1– 4.3)

Mortality data are means of 5 replicates of 10 larvae ($n = 50$).
[a] Slope from regression of cumulative percent mortality (probit) on log time.
[b] Time until death of 50% of inoculated larvae.

Fig. 2A). At concentrations <100 ppm, differences in larval mortality were <20% between the chemical alone and the combination (Fig. 2B).

Larval mycosis was significant for both chemical and fungus alone, and the interaction (Table 1, bioassay 3). Some fungal contamination was detected at 50 and 100 ppm in the chemical treatment (Fig. 2C). The synergistic effect of the fungus/chemical combination was confirmed by the increases in the difference in mycosis between imidacloprid alone and the combination fungus/chemical (Fig. 2 D).

The LT_{50}s for imidacloprid at 100, 150, and 200 ppm combined with the fungus at 10^6 conidia per milliliter were significantly lower compared with chemical alone (Table 3). Below 100 ppm, the 95% CI for LT_{50} values overlapped for imidacloprid alone.

Effect of Higher Doses of Imidacloprid (bioassay 4). Only 20–40% larval mortality was detected among the different concentrations of imidacloprid alone (Fig. 3A). Slight increases in larval mortality and mycosis occurred as the concentration of chemical and fungus combinations increased (Fig. 3 A and C).

Larval mortality was significantly influenced by both chemical and fungus (Table 1, bioassay 4). However, the fungal/chemical interaction was not significant, suggesting an additive effect. The difference in larval mortality between imidacloprid alone and the fungal/chemical interaction, did not increase with an increase in the chemical concentration, confirming the additive effect (Fig. 3B).

Larval mycosis was significant for imidacloprid, *M. anisopliae*, and the fungus/chemical interaction (Table 1, bioassay 4). No larval mycosis was expected from imidacloprid alone, yet some mycosis was detected at 100, 500, and 1,000 ppm most likely from accidental contamination (Fig. 3C). The synergistic effect of the fungus/chemical combination was confirmed by the increase in the differences in mycosis between imidacloprid alone and the combination (Fig. 3D).

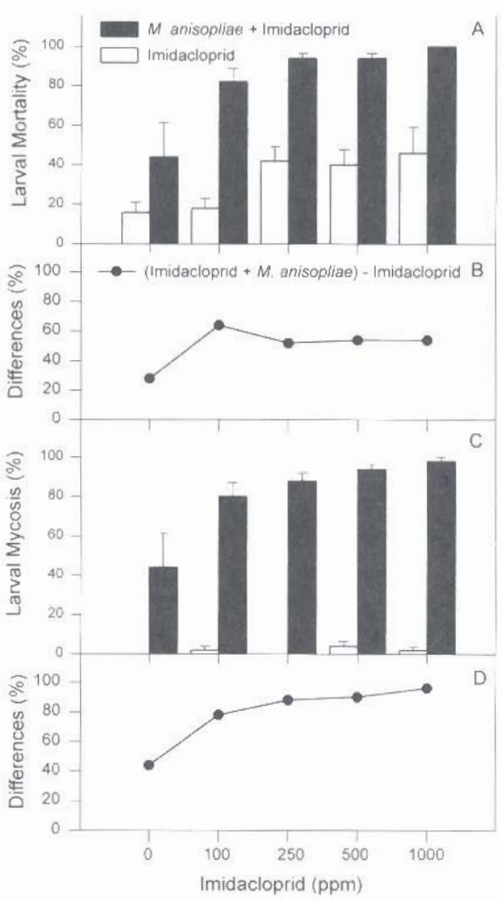

Fig. 3. Effect of different doses of imidacloprid applied to the cuticle alone (white bars) or in combination with *M. anisopliae* at 10^7 conidia per milliliter (black bars) on mortality (A) and mycosis (C) of 1st instars of *D. abbreviatus* after 4 d. Differences in larval mortality (B) and mycosis (D) between the combination with *M. anisopliae* and imidacloprid alone (solid line, black dots)

Larval mortality was low for all doses of the fungus applied alone to the insect cuticle in bioassay 5 (Fig. 4A). Even the highest dose of the fungus (10^7 conidia per milliliter) killed only 18.6% of larvae after 8 d. As the concentration of imidacloprid alone increased from 0 to 1,000 ppm, mortality increased from 16.9 to 57.3%. However, when these same concentrations of imidacloprid were combined with the fungus at 10^5 conidia per milliliter, larval mortality increased significantly. For example, larval mortality was 12.7% at 0 ppm, 31.4% at 100 ppm, 58.4% at 500 ppm, and 80.9% at 1,000 ppm of chemical. When imidacloprid at 500 and 1,000 ppm was combined with the fungus at 10^6 and 10^7 conidia per milliliter, however, larval mortality did not increase. At 10^6

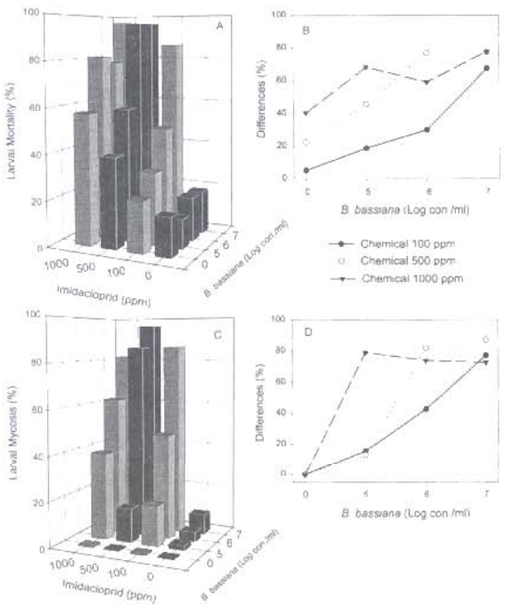

Fig. 4. Effect of different doses of imidacloprid applied to the cuticle alone or in combination with different conidial concentrations of *B. bassiana* on mortality (A) and mycosis (C) of 1st instars of *D. abbreviatus* after 8 d. Differences between the fungus/chemical combination and *B. bassiana* alone for larval mortality (B) and mycosis (D).

conidia per milliliter, larval mortality was higher when combined with 500 ppm (96%) than 1,000 ppm (78%). In addition, there was no difference in larval mortality when imidacloprid at 500 or 1,000 ppm was combined with the fungus at 10^7 conidia per milliliter.

Both larval mortality and mycosis were significantly influenced by *B. bassiana* and imidacloprid alone (Table 4). The *B. bassiana*–imidacloprid interaction also was significant. The synergistic effect of the fungus/chemical combination was confirmed as the difference in mortality or mycosis between fungus alone and the combination, generally increased with an increase in fungal conidial concentration (Fig. 4 B and D).

Larval mycosis was <10% for all fungus doses alone (Fig. 4C). However, when *B. bassiana* at 10^6 and 10^7 conidia per milliliter was combined with imidacloprid at 500 ppm, the percent infection was 86 and 96%, respectively. When the same conidial concentrations of the fungus were combined with imidacloprid at 1,000 ppm, mycosis were reduced to 62 and 81%, respectively.

Table 5. Probit analysis of mortality data where different doses of imidacloprid with and without different conidial concentrations of *B. bassiana* were tested against 1st instars of *D. abbreviatus*

Imidacloprid, ppm	*B. bassiana* (conidia/ml)	Slope ± SE[a]	LT_{50}[b] (95% CI)
0	0	1.8 ± 0.9	14.2 (10.0-[illegible])
	10^5	5.1 ± 2.7	11.1 (9.1- [illegible])
	10^6	4.0 ± 1.5	10.6 (8.9- [illegible])
	10^7	2.6 ± 1.1	12.2 (9.5- [illegible])
100	0	1.2 ± 0.8	15.7 (∞)
	10^5	3.0 ± 0.9	7.8 (6.8- [illegible])
	10^6	3.1 ± 0.7	7.8 (6.8- [illegible])
	10^7	2.8 ± 0.6	3.6 (1.8- [illegible])
500	0	2.1 ± 0.7	9.5 (7.9- [illegible])
	10^5	3.4 ± 0.7	7.2 (6.4- [illegible])
	10^6	7.0 ± 0.8	4.9 (4.5- [illegible])
	10^7	5.4 ± 0.7	4.1 (3.4- [illegible])
1000	0	2.2 ± 0.6	7.2 (6.1- [illegible])
	10^5	4.8 ± 1.8	6.4 (5.8- [illegible])
	10^6	3.9 ± 0.6	5.2 (4.5- [illegible])
	10^7	5.2 ± 0.7	3.5 (2.7- [illegible])

Mortality data are means of 5 replicates of 10 larvae ($n = 50$).
[a] Slope from regression of cumulative percent mortality (probit) on log time.
[b] Time until death of 50% of inoculated larvae.

The LT_{50} for the *Beauveria* treatments alone compared to *Beauveria*/imidacloprid interaction at 100 ppm was significantly different at 10^7 conidia per milliliter concentration (Table 5). Nevertheless, at 500 and 1,000 ppm, all LT_{50} values for the treatment combinations were significantly different from the single treatments. The 95% CI for the LT_{50}s overlapped for the fungus/chemical combination at 500 and at 1,000 ppm.

As concentrations of imidacloprid increased, larval ecdysis decreased in all treatments (Fig. 5). Virtually no larval ecdysis was observed at 500 and 1,000 ppm, and imidacloprid had a significant effect on ecdysis (Table 4). However, the fungus alone had minimal effect on larval ecdysis. Only an additive effect for larval ecdysis was observed because the interaction was not significant (Table 4).

Comparison Between Oral and Cuticular Exposure of Imidacloprid. In bioassay 6, mortality and mycosis among larvae treated with *M. anisopliae* alone for both exposure methods were <20% (Fig. 6 A, B, E, F). In fact, no larval mycosis was observed for *M. anisopliae* at 10^5 and 10^6 conidia per milliliter for both contact and oral exposure, respectively.

Table 4. Summary of factorial analysis for arcsine $\sqrt{}$ proportion of larval mortality, mycosis, and ecdysis of *D. abbreviatus* after treatment with *B. bassiana* and imidacloprid alone and in combination

Factor	Larval mortality			Larval mycosis			Larval ecdysis		
	F	df	*P*	*F*	df	*P*	*F*	df	*P*
B. bassiana (Bb)	15.7	3, 64	0.0001	90.4	3, 64	0.0001	4.6	3, 64	0.0057
Imidacloprid (IMI)	36.2	3, 64	0.0001	38.7	3, 64	0.0001	131.5	3, 64	0.0000
Bb × IMI	3.1	9, 64	0.0037	8.7	9, 64	0.0001	1.8	3, 64	0.0923

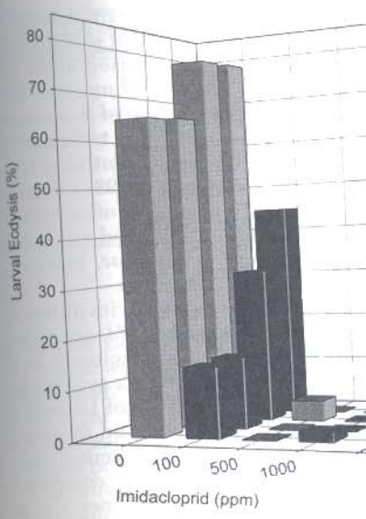

Fig. 5. Effect of different doses of imi directly to the cuticle of first instars of *D.* or in combination with different conidi of *B. bassiana* on larval ecdysis.

However, when *M. anisopliae* con bined with imidacloprid, a gradual i mortality and mycosis occurred as centration increased for both cont posure (Fig. 6 A, B, E, F).

Larval mortality and mycosis w influenced by both imidacloprid a for both contact and oral exposur fungus/chemical interaction was suggesting synergism (Table 6). T fect of the fungus/chemical combi or oral exposure was confirmed by ferences in percent larval mortali tween the combination and fungu ferences increased with an in conidial concentration (Fig. 6 C,

Ecdysis of contact-treated larva imidacloprid and *M. anisopliae* b was not significant, suggesting (Table 6). Larval ecdysis was 25.6 at 100 ppm, but when combined 10^5 and 10^7 conidia per milliliter 4 and 0%, respectively (Fig. 7A) larvae with imidacloprid had a larval development (Fig. 7B; T ecdysis was observed for chemic combination with fungus. Ho alone had no effect on larval ecd imidacloprid combination was

The time required to kill 50 creased with an increase in co of *M. anisopliae* alone or in comb ical for oral exposure (Table 7) methods, the LT_{50} values for t

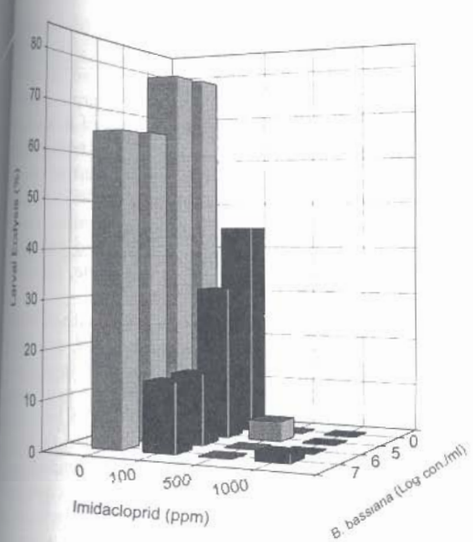

Fig. 5. Effect of different doses of imidacloprid applied directly to the cuticle of first instars of *D. abbreviatus* alone or in combination with different conidial concentrations of *B. bassiana* on larval ecdysis.

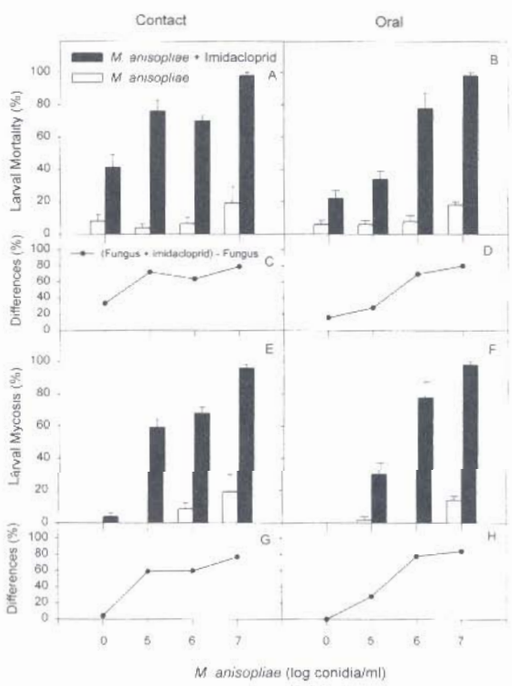

Fig. 6. Effect of different conidial concentration of *M. anisopliae* applied directly to the cuticle alone (white bars) or in combination with imidacloprid applied as contact (A–G) or oral (B–H) treatment at 100 ppm (AI) (black bars) on mortality (A, B) and mycosis (E, F) of 1st instars of *D. abbreviatus* after 6 d. Differences in larval mortality (C, D) and mycosis (G, H) between the fungus–imidacloprid combination and *M. anisopliae* alone for contact and oral exposure (solid line, black dots).

However, when *M. anisopliae* conidia were combined with imidacloprid, a gradual increase in larval mortality and mycosis occurred as the fungal concentration increased for both contact and oral exposure (Fig. 6 A, B, E, F).

Larval mortality and mycosis were significantly influenced by both imidacloprid and *M. anisopliae* for both contact and oral exposure (Table 6). The fungus/chemical interaction was also significant, suggesting synergism (Table 6). The synergistic effect of the fungus/chemical combination on contact or oral exposure was confirmed by plotting the differences in percent larval mortality or mycosis between the combination and fungus alone. The differences increased with an increase in fungal conidial concentration (Fig. 6 C, D, G, H).

Ecdysis of contact-treated larvae was affected by imidacloprid and *M. anisopliae* but the interaction was not significant, suggesting an additive effect (Table 6). Larval ecdysis was 25.6% for imidacloprid at 100 ppm, but when combined with the fungus at 10^5 and 10^7 conidia per milliliter ecdysis dropped to 4 and 0%, respectively (Fig. 7A). Oral treatment of larvae with imidacloprid had a greater effect on larval development (Fig. 7B; Table 6). No larval ecdysis was observed for chemical treatments or in combination with fungus. However, the fungus alone had no effect on larval ecdysis and the fungus/imidacloprid combination was not significant.

The time required to kill 50% of the larvae decreased with an increase in conidial concentration of *M. anisopliae* alone or in combination with chemical for oral exposure (Table 7). For both exposure methods, the LT_{50} values for the fungus/chemical treatment were significantly lower than that for fungus alone. The LT_{50} for imidacloprid at 100 ppm with fungus at 10^5 conidia per milliliter on contact exposure was significantly lower than for the oral treatment at this same dose of fungus/chemical combination. For the other treatments, the 95% CI for the LT_{50}s overlapped for oral and topical applications.

Cuticular Exposure to Formulated Imidacloprid and Each Component Separately with Conidia of *M. Anisopliae*. The active ingredient and the formulated product in combination with fungal conidia had a significantly greater effect on larval mortality and mycosis than the single effects of each component (Table 8). The LT_{50} values for these treatments were also reduced. The inert carrier alone had no effect on larval mortality compared with the control. When combined with *M. anisopliae* conidia, the inert carrier killed 10% more larvae than the fungus alone, but the difference was not significant. These results suggest that the active ingredient is the key component of the formulation responsible for the synergistic effect between imidacloprid and the entomopathogenic fungi.

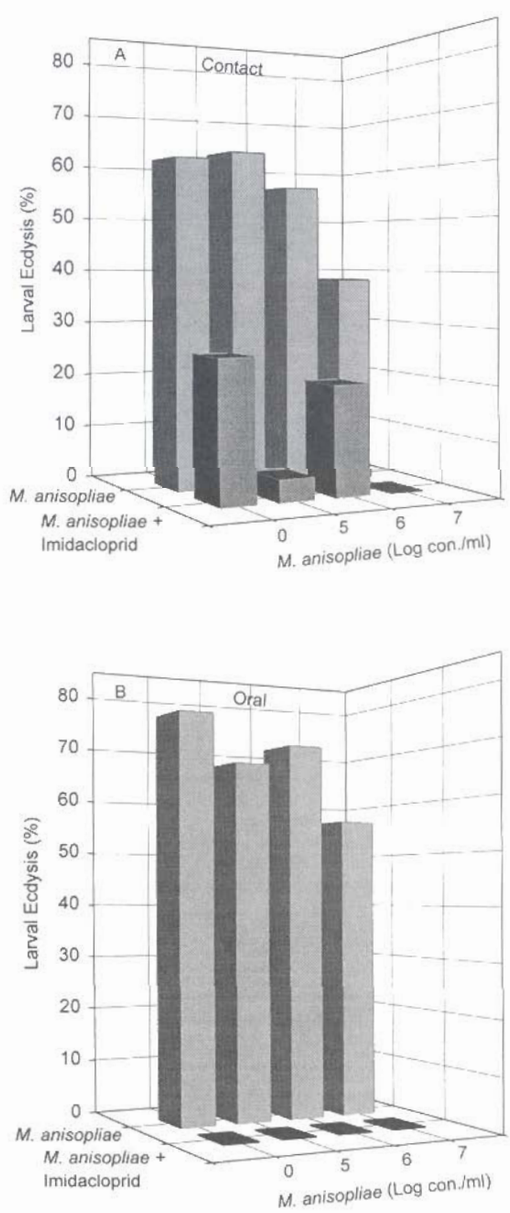

Fig. 7. Effect of different conidial concentrations of *M. anisopliae* applied alone to the cuticle of 1st instars of *D. abbreviatus* or with imidacloprid applied by contact (A) and oral (B) exposure on ecdysis.

Discussion

Early studies attempted to test synergism between entomopathogenic fungi and insecticides for insect control with no success. DDT did not predispose larvae of the Colorado potato beetle, *Leptinotarsa decemlineata*, to mycosis (Fargues 1973). In field cage studies, combinations of azinphos-ethyl and carbaryl with *B. bassiana* were not significantly more effective than the chemicals used alone for control of Colorado potato beetle (Fargues 1975). In addition, no significant interaction of *B. bassiana* with carbaryl, fenvaralate, abamectin, triflumuron, and thuringiensin was observed for control of Colorado potato beetle (Anderson et al. 1989). Hassan and Charnley (1989) observed that dimilin-treated cuticle of *Manduca sexta* (L.) provided enhanced penetration by hyphae of *M. anisopliae*, however, larval mortality enhancement was low.

In our studies, bioassays performed with different concentrations of either *B. bassiana* or *M. anisopliae* and different concentrations of imidacloprid as a contact or oral treatment resulted in a synergistic increase in both mortality and mycosis of 1st instars of *D. abbreviatus*. Benz (1971) suggested that synergism is temporal, that is, the fungal/chemical combinations reduce the time to mortality compared with 1 treatment alone. We observed synergism at concentrations of imidacloprid ≥100 ppm when applied as a contact treatment. Below 100 ppm, larval mortality and LT_{50} values for the combination were similar to imidacloprid alone. Because larvae recovered from chemical-induced paralysis in <24 h, conidial removal most likely occurred before germination and penetration. Quintela and McCoy (1997) showed conclusively that larval development and mobility are retarded at concentrations of imidacloprid of 100 ppm or greater. The loss of mobility prevented larval removal of conidia upon contact with the surface of a substrate (Quintela 1996). Similar results were observed by Boucias et al. (1996) working with the subterranean termite *R. flavipes*. Termites treated with imidacloprid lost their ability to remove conidia from each others' cuticle during social grooming. Although loss of larval mobility appears to be the most important factor responsible for synergism, it is true that other physiological factors could be enhancing fungal pathogenicity. For example, studies showed that certain components of formulated imidacloprid stimulated the rate and amount of conidial germination of *B. bassiana* and *M. anisopliae* on larval cuticle (Quintela 1996).

According to the literature, fungal conidia attached to the cuticle are normally removed during ecdysis (Boucias and Pendland 1991, Hassan et al. 1989). Although imidacloprid reduced larval ecdysis, failure of larvae to molt had no apparent effect on fungal/chemical synergism. This can be explained by the above. Most likely, conidia were removed by the larvae when contacting a substrate within a 3- to 4-d period before ecdysis began. This inference was further substantiated by observations that showed no conidia or mycelial growth on exuviae of fungus-treated larvae held at high humidity.

Metarhizium anisopliae or *B. bassiana* in combination with concentrations of imidacloprid >100 ppm did not result in higher larval mycosis. Mycosis was actually reduced when imidacloprid was used at

Table 6. Summary of factorial analysis for ar treatment with conidia of *M. anisopliae* (Ma) alon

Exposure	Factor	Larval mo	
		F	df
Contact	Ma	11.3	3, 32
	IMI	223.6	1, 32
	MA × IMI	5.9	3, 32
Oral	Ma	37.3	3, 32
	IMI	210.3	1, 32
	Ma × IMI	22.1	3, 32

1,000 ppm. Constant or lower larval my >100 ppm was probably related to red attachment. Quintela (1996) showed nent(s) of the inert carrier affecte conidial adhesion on the larval cutic mulated product was tested at doses

Our study showed a synergistic i tween sublethal doses of imidaclopr mopathogenic fungi for control of larvae outside the soil. Further resear conducted to determine whether th acloprid combination performs sim environment.

Table 7. Probit analysis of bioassay data v of imidacloprid were applied by contact and c

Imidacloprid, ppm	*M. anisopliae* (conidia/ml)	S
0	0	
	10^5	
	10^6	
	10^7	
100	0	
	10^5	
	10^6	
	10^7	

Bioassay data are means of 5 replicates (n
[a] Slope from regression of cumulative per
[b] Time until death of 50% of inoculated la

Table 8. Probit analysis of bioassay data of *M. anisopliae* were tested against 1st inst

M. anisopliae (conidia/ml)	Chemical ingredient[a]
0	0
	Inert carrier
	Active ingredient
	Formulated
10^7	0
	Inert carrier
	Active ingredient
	Formulated

Data are means of 5 replicates of 10 larv
test ($P = 0.05$).
[a] Active ingredient at 500 ppm and mea
[b] Slope from regression of cumulative p
[c] Time unitil death of 50% of inoculated

Table 6. Summary of factorial analysis for arcsine $\sqrt{}$ proportion of larval mortality, mycosis, and ecdysis of *D. abbreviatus* after treatment with conidia of *M. anisopliae* (Ma) alone or in combination with imidacloprid (IMI) applied as a contact or oral treatment

Exposure	Factor	Larval mortality			Larval mycosis			Larval ecdysis		
		F	df	*P*	*F*	df	*P*	*F*	df	*P*
Contact	Ma	11.3	3, 32	0.0001	34.6	3, 32	0.0001	3.5	3, 32	0.0274
	IMI	223.6	1, 32	0.0001	128.5	1, 32	0.0001	56.8	1, 32	0.0001
	Ma × IMI	5.9	3, 32	0.0024	10.3	3, 32	0.0001	1.0	3, 32	0.4162
Oral	Ma	37.3	3, 32	0.0001	95.6	3, 32	0.0001	0.8	3, 32	0.4760
	IMI	210.3	1, 32	0.0001	286.3	1, 32	0.0001	229.0	1, 32	0.0001
	Ma × IMI	22.1	3, 32	0.0001	46.0	3, 32	0.0001	0.8	3, 32	0.4760

1,000 ppm. Constant or lower larval mycosis at doses >100 ppm was probably related to reduced conidial attachment. Quintela (1996) showed that component(s) of the inert carrier affected negatively conidial adhesion on the larval cuticle when formulated product was tested at doses >100 ppm.

Our study showed a synergistic interaction between sublethal doses of imidacloprid with entomopathogenic fungi for control of *D. abbreviatus* larvae outside the soil. Further research needs to be conducted to determine whether the fungal/imidacloprid combination performs similarly in a soil environment.

Acknowledgments

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Table 7. Probit analysis of bioassay data where different conidial concentrations of *M. anisopliae* with and without a sublethal dose of imidacloprid were applied by contact and oral exposure against 1st instars of *D. abbreviatus*

Imidacloprid, ppm	*M. anisopliae* (conidia/ml)	Contact		Oral	
		Slope ± SE[a]	LT_{50}[b] (95% CI), d	Slope ± SE[a]	LT_{50}[b] (95% CI), d
0	0	1.1 ± 0.5	17.9 (11.1–2756.5)	1.6 ± 0.7	14.9 (10.3–59.2)
	10^5	9.8 ± 4.4	8.8 (7.7– 18.3)	4.0 ± 1.4	10.2 (8.4–16.8)
	10^6	2.3 ± 0.8	11.1 (8.9– 19.3)	4.6 ± 1.5	9.6 (8.2–14.5)
	10^7	1.9 ± 0.4	9.2 (7.7– 12.9)	2.5 ± 0.6	9.5 (8.0–13.1)
100	0	2.2 ± 0.4	6.9 (6.1– 8.3)	1.5 ± 0.4	9.7 (7.9–14.2)
	10^5	3.5 ± 0.4	4.4 (3.5– 5.3)	2.3 ± 0.4	7.8 (6.8– 9.7)
	10^6	3.0 ± 0.6	4.0 (1.5– 6.4)	3.0 ± 0.3	4.0 (3.6– 4.3)
	10^7	5.9 ± 0.6	2.0 (1.8– 2.2)	5.1 ± 0.5	2.4 (1.2– 3.1)

Bioassay data are means of 5 replicates (n = 50).
[a] Slope from regression of cumulative percent mortality (probit) on log time.
[b] Time until death of 50% of inoculated larvae.

Table 8. Probit analysis of bioassay data where inert carrier, active ingredient, and formulated imidacloprid with and without conidia of *M. anisopliae* were tested against 1st instars of *D. abbreviatus*

M. anisopliae (conidia/ml)	Chemical ingredient[a]	Slope ± SE[b]	LT_{50}[c] (95% CI), d	% mortality ± SE	% mycosis ± SE
0	0	4.2 ± 2.5	11.3 (8.6–8,562.4)	5.6 ± 3.7c	0c
	Inert carrier	0.9 ± 0.8	22.2 (∞)	6.2 ± 4.1bc	0c
	Active ingredient	2.7 ± 0.6	8.5 (7.3– 11.3)	30.4 ± 6.7b	6.2 ± 4.1bc
	Formulated	1.2 ± 0.5	11.0 (8.1– 33.2)	29.4 ± 8.4bc	0c
10^7	0	2.0 ± 0.7	11.5 (8.8– 25.4)	16.5 ± 2.3bc	8.7 ± 2.2bc
	Inert carrier	2.8 ± 0.7	8.7 (7.5– 11.8)	26.7 ± 5.3bc	14.4 ± 4.2b
	Active ingredient	5.1 ± 0.5	4.3 (2.8– 5.4)	87.7 ± 4.0a	70.8 ± 5.6a
	Formulated	4.3 ± 0.5	3.8 (2.2– 4.9)	91.8 ± 2.1a	83.5 ± 2.7a

Data are means of 5 replicates of 10 larvae (n = 50). Means followed by the same letter are not significantly different by the Tukey test (P = 0.05)
[a] Active ingredient at 500 ppm and inert carrier at 1,836 ppm.
[b] Slope from regression of cumulative percent mortality (probit) on log time.
[c] Time unitil death of 50% of inoculated larvae.

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