JOURNAL OF INVERTEBRATE PATHOLOGY **72,** 220–230 (1998)
ARTICLE NO. IN984791

Conidial Attachment of *Metarhizium anisopliae* and *Beauveria bassiana* to the Larval Cuticle of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) Treated with Imidacloprid

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Received August 1, 1997; accepted May 12, 1998

A series of experiments was conducted to determine the effect of imidacloprid on the number of *Metarhizium anisopliae* and *Beauveria bassiana* conidia found on the cuticle of first instar *Diaprepes abbreviatus* following different treatments. Larvae treated with *M. anisopliae* conidia and imidacloprid by dipping removed significantly fewer conidia from their cuticle when in contact with soil or a food source compared with fungal-treated larvae alone. In addition, more *M. anisopliae* and *B. bassiana* conidia were found on the cuticle of larvae treated with imidacloprid while exposed to soil at 7 and 14% moisture resulting in higher larval mortality and mycosis. Conidial attachment to cuticles of untreated larvae was higher at <1% compared with 7 and 14% soil moistures. *M. anisopliae* conidia were distributed uniformly over the pleural membrane of the larval cuticle of both untreated and imidacloprid-treated larvae. However, fewer conidia were attached to specific sites such as setae and setal sockets of treated larvae. At 12 h after treatment, imidacloprid-treated larvae had fewer conidia removed from exposed cuticle, setae, and spiracles than did untreated larvae. Cuticular exposure to imidacloprid at doses >0.01% (AI) affected conidial attachment of *M. anisopliae* negatively. Conidial number decreased sevenfold at 0.1% (AI). Comparative data on the effect of imidacloprid formulation on conidial attachment showed that components of the inert ingredient were responsible for lower conidial attachment on larval cuticle at higher insecticidal doses.

Key Words: **entomopathogenic fungi; root weevil; conidial attachment; insect cuticle; imidacloprid; insecticide.**

INTRODUCTION

For many years, the use of chemicals or other biological agents as "stressors" to enhance the efficacy of mycopathogens has been proposed (Steinhaus, 1958; Benz, 1971; Ferron, 1978; Anderson *et al.*, 1989; Hassan and Charnley, 1989; Hassan *et al.*, 1989). In 1982, Dr. Walter M. Zeck, a member of the Bayer Research Group, Vero Beach, Florida, discovered that sublethal doses of a number of insecticidal nitroguanidine compounds, including imidacloprid, increased the susceptibility of subterranean termites to various opportunistic fungi including *Conidiobolus coronatus, Paecilomyces farinosus, Beauveria bassiana, Metarhizium anisopliae,* and *Actinomucor* sp. (W. M. Zeck, personal communication). These findings led to a number of recent reports on the synergistic effect of imidacloprid on the entomopathogenic fungi *B. bassiana* and *M. anisopliae.* Boucias *et al.* (1996) showed that conidia of *B. bassiana* infected imidacloprid-treated termites more readily then non-treated termites. They observed that imidacloprid disrupted the grooming behavior and other social activities common to termites. Removal of fungal conidia via grooming is an innate defense mechanism of termites, and body paralysis caused by imidacloprid inhibited grooming thereby increasing the susceptibility of the termites to fungal infection. Quintela and McCoy (1997a,b, 1998) demonstrated the synergistic effects of imidacloprid on both *M. Anisopliae*- and *B. bassiana*-treated larvae of a soil inhabiting root weevil, *Diaprepes abbreviatus.* Larval mobility, both in and out of the soil, was inhibited due to temporary muscular paralysis caused by imidacloprid. We speculated that the loss of larval mobility interfered with normal conidial voidance behavior accomplished by larvae when moving on or within a substrate. Kaakeh *et al.* (1997) found that the German cockroach, *Blatella germanica,* when fed imidacloprid-impregnated bait following topical treatment with conidia of *M. anisopliae,* died more quickly than roaches not exposed to the chemical. Steinkraus and Tugwell (1997) showed higher tarnished plant bug, *Lygus lineolaris,* mortality

0022-2011/98 $25.00

with the combination of Mycotrol WP (*B. bassiana* conidia) and imidacloprid.

To further understand the relationship between imidacloprid and mycopathogen infection to the host insect, SEM and fluorescence microscopy were conducted to determine conidial number and distribution at different cuticular sites of imidacloprid-treated larvae of *D. abbreviatus* compared with larvae exposed only to the fungus. In addition, studies were conducted to determine the effect of imidacloprid on the attachment and loss of *M. anisopliae* conidia on the larval cuticle following exposure to different soil moistures. Various doses of formulated imidacloprid, as well as active and inert ingredients, were compared to determine their effects on conidial attachment to the larval cuticle.

MATERIALS AND METHODS

General Procedures

First instars of *D. abbreviatus*, <48 h old, were obtained from eggs laid by field-collected adult females confined to screened cages in the greenhouse at 27 ± 2°C as described by McCoy *et al.* (1995). For each experiment, vigorous first instars were selected in the manner described by Quintela and McCoy (1997b).

M. anisopliae (Strain MADA) was isolated from *D. abbreviatus* larvae collected from citrus grove soil at Apopka, Florida. Conidia of *B. bassiana* (Strain GHA) were isolated from formulated Mycotrol produced by Mycotech Corporation (Butte, MT). Conidia of both fungi were produced in quantity in the manner described in Quintela and McCoy (1997a).

A formulation of imidacloprid (Admire 2F 21.4% AI) or technical grade 1-[(6—chloro—3-pyridyl)methyl)]-*N*-nitro-2-imidazolidin-imine and the inert ingredients in a formulation blank were supplied by Bayer Corporation (Kansas City, MO).

The soil used in the tests was classified as a Candler fine sand (Typic Quartzipsamments, hyperthermic, uncoated) with a particle size distribution of 96.7% sand, 0.8% silt, and 2.5% clay. The organic matter content was 0.80%, bulk density 1.47 g/cm^3, and the pH was 5.7. Only unsterilized air-dried soil was used in the experiments.

Statistical Analysis

All experimental data were analyzed using Proc GLM and treatment means were compared using the Tukey's honestly significant difference test at $P = 0.05$ unless stated otherwise (SAS Institute, 1985).

Microscopic Procedures

Fluorescence microscope. Conidia of *M. anisopliae* and *B. bassiana* were suspended in 10 ml of a 0.05 M carbonate–bicarbonate buffer, pH 9.2, in 0.01% Tween 80. The suspensions were vortexed at high speed and then filtered through a Nytex screen (Fisher Scientific) of 125-μm mesh size. The suspension was then centrifuged at 4500 rpm for 5 min. Most of the supernatant was then decanted. The resuspended conidial pellet was transferred to a 1.5-ml centrifuge tube and centrifuged, and the supernatant was removed. The conidial suspension was stained with 1 mg fluorescein isothiocyanate (FITC, Sigma Chemical Co., St. Louis, MO) in 1 ml of carbonate-bicarbonate buffer for 1 h at room temperature according to Hung and Boucias (1992). Stained conidia were washed five times by centrifugation for 5 min at 4500 rpm with sterile distilled water to remove excess stain.

Scanning electron microscopy. First instars were submerged for 30 s in a suspension of *M. anisopliae* at 10^7 conidial/ml alone or in combination with imidacloprid at 0.02%. Larvae were prepared for SEM as described by Grodowitz *et al.* (1982). The larvae were coated with gold:palladium (80:20) and examined with a Hitachi S-530 scanning electron microscope operating at 20 kV. Photographs were taken with Polaroid 55 film.

Conidial Recovery from Different Substrates

Quintela (1996) conducted a preliminary test to determine the most efficient method for recovering conidia of *M. anisopliae* from the carrot used to feed the larvae treated with fungus. Maceration of the carrots was seven times more efficient (455.6 ± 32.0 conidia recovered) than a vortex method (66.2 ± 16.8 conidia recovered). In the maceration method, the carrot was macerated with a mortar and pestle with 400 μl of 0.05% Tween 80. Two hundred microliters of each suspension was then transferred to 90-mm-diameter petri dishes containing selective dodine oatmeal agar medium (0.46 g AI/liter) (Beilharz *et al.*, 1982) and maintained at 28°C. After 7 days, the number of colony-forming units was counted in 40 petri dishes.

The plastic wells that held larvae were also tested for the presence of *M. anisopliae* conidia. This was done by adding 200 μl of 0.05% Tween 80 to each well and brushing the conidia from the container walls. The 200 μl suspension was plated onto 90-mm petri dishes containing the above selective media and maintained at 28°C. After 7 days, the number of colony-forming units were counted in 37 dishes.

Larval Treatment with Fungal Conidia and Imidacloprid for Microscopic Studies of the Larval Cuticle

In test 1, we examined conidial number and distribution at different cuticular sites on imidacloprid-treated

larvae compared to fungus-treated larvae alone. Approximately 50 first instar larvae were placed in 1.5-ml micro-centrifuge tubes containing FITC-labeled conidia of *M. anisopliae* at a concentration of 10^7 conidia/ml or in a tube containing the same dose of conidia and imidacloprid at 0.01% (AI). The tubes were shaken gently by hand for 30 s before larval removal. Larvae were not rinsed in water after the 30-s exposure to remove nonattached conidia. Larvae were held individually in tissue culture plates (2.0-mm-diameter wells) at 28°C in the dark. Each well contained an 8-mm-diameter slice of raw carrot as a food source. At 0, 12, and 24 h post-treatment, larvae were frozen at −50°C for 30 min. Dead larvae were mounted in 1,4-diazabicyclo[2.2.2]octane (DABCO). Conidial number and distribution were determined on different abdominal segments of the cuticle pleural membrane by examination at 600× magnification using an epifluorescent Leica microscope with an exciter filter BP 450–490 nm and a barrier filter of 0–530 nm. Twenty-five microscopic fields were examined at 0 h and 50 fields were examined at 12 and 24 h for each treatment. Photographs were taken using a Kodak TMAX 400 ASA 35-mm film. The plastic wells and the carrots that were used to feed the larvae were examined for the presence of conidia as described previously.

In test 2, the loss of conidia from the larval cuticle after movement in soil was determined at different moistures. First instars were submerged in a suspension of FITC-labeled conidia at 10^7 conidia/ml or in an imidaclopride–conidial mixture at 0.05% (AI) for 30 s. Twenty inoculated larvae were placed on the surface of untreated soil (1 cm depth) in bioassay columns (15 cm high, 2.0 cm diameter) constructed from polystyrene tubes similar to those described by Hamlen and Beavers (1975). Columns were attached to a containment cell to catch the invasive larvae. Units were closed tightly and held at 28°C. The soil water content was adjusted to <1, 7, and 14% (v/w). For <1% moisture, treated soil was air-dried in a dark incubator at 28°C by continuous exposure to air flow for 24 h before experimentation. Each treatment was replicated four times. After 24 h, larvae recovered from containment cells and the soil in each tube were mounted in DABCO. Conidia attached to the pleural membrane of the fifth and sixth abdominal segment were counted with a fluorescence microscope at 630× magnification. Spores were counted in 15 microscope fields for each treatment.

Soil Treatment with Fungal Conidia and Imidacloprid for Microscopic Studies

A 1.5-ml suspension containing FITC-labeled conidia of *M. anisopliae* at 0, 10^7, and 10^8 conidia/ml or *B. bassiana* at 0 and 10^8 conidia/ml were added alone or in combination with imidacloprid at a concentration of 0 and 0.05% (AI) to 30 g of Candler soil. These concentrations are equivalent to *M. anisopliae* at 0, 5×10^5, and 5×10^6 conidia/g soil; *B. bassiana* at 0 and 5×10^6 conidia/g soil; and imidacloprid at 0 and 25 μg (AI)/g soil, respectively. The soil moisture was adjusted to <1, 7, and 14%. For the *B. bassiana* test, the soil was held at a constant 7% water content. Treated soils were added to a bioassay columns (15 cm high, 2.0 cm diameter). Twenty 48-h-old first instar larvae were placed on the soil surface of each container. Units were closed tightly and held at 28°C. Each treatment was replicated three times. For *M. anisopliae* at 5×10^5 conidia/g soil, 10 first instar larvae were tested in five replications. After 22 h, larvae recovered from containment cells and those remaining in the soil in each tube were mounted in DABCO. Conidial attachment to the larval cuticle on the fifth- to sixth abdominal segment on the pleural membrane was examined at 630× magnification. One field was examined on each of 10 larvae from each treatment. For *M. anisopliae* at 5×10^5 conidia/g soil, one field was examined on each of 15 larvae. After 7 days, the number of live and dead larvae recovered from the containment cells as well as those remaining in the soil was recorded by microscopic examination. Dead larvae were held in 35-mm petri dishes with moistened filter paper to confirm mycosis by *B. bassiana* or *M. anisopliae*. All bioassay data were transformed to arcsine $\sqrt{\times}$ before performing factorial analyses using the analysis of variance procedure (SAS Institute, 1985). The model was considered additive if the factorial analyses for the interaction fungus/chemical was not significant (slopes were parallel) (e.g., mortality = effect of fungus + effect of chemical). The model was synergistic if the interaction was significant (slopes were not parallel) (e.g., mortality = effect of fungus + effect of chemical + effect of interaction).

Larval Treatment with Fungal Conidia and Components of Formulated Imidacloprid

In test 1, approximately 50 first instar larvae were placed in 1.5-ml microcentrifuge tubes containing 10^7 FITC-labeled *M. anisopliae* conidia/ml alone or in combination with imidacloprid at 0.01, 0.05, and 0.1% (AI). The tubes were agitated gently for 30 s. After treatment, larvae were killed by freezing at −50°C for 30 min. Dead larvae were mounted in DABCO. Conidial attachment to the cuticle on the fifth- to sixth abdominal segments on the pleural membrane was determined. One field on each of 10 larvae was examined for each treatment. Regression analysis was performed on the data using the general linear models procedure (SAS Institute, 1985).

In test 2, formulated imidacloprid at 0.05%, technical product (AI) at 0.05%, and the blank formulation at 0.184% (concentration equal to the amount found in

formulated imidacloprid at 0.05% of the active ingredient) were mixed with FITC labeled *M. anisopliae* conidia at concentrations of 0 and 10^7 conidia/ml. Larvae were dipped in each suspension in the manner described previously. Conidial attachment on the cuticle on the fifth- to sixth abdominal segment on the pleural membrane was determined. One field on each of 10 larvae was examined for each treatment.

RESULTS

Conidial Attachment of M. anisopliae to Different Regions of the Larval Cuticle Treated with Imidacloprid

Regardless of treatment, FITC-labeled *M. anisopliae* conidia applied directly to *D. abbreviatus* larvae were uniformly attached to the 10 abdominal segments on the pleural membranes at 0 and 12 h after exposure ($P > 0.78$, 0.95, respectively) (Fig. 1). At 0 h, no significant difference was found in the number of conidia on treated and untreated larval cuticle ($P > 0.11$). At 12 h, however, more conidia had been removed from the cuticles of untreated larvae than from those of larvae treated with imidacloprid at 0.01% (AI) ($P < 0.001$).

Conidial distribution on various regions of larval cuticle is presented in Fig. 2. Immediately after treatment no difference in conidial number was observed on the cuticle of untreated larvae compared to treated larvae. *M. anisopliae* conidia tended to attach to epicuticular folds, around sensilla organelles or in depressions of the larval cuticle (Figs. 3-A and 3B). Conidia were also attached to setae and around setal sockets (Fig. 3-C). At 0 h, conidial number on setae and around setal sockets was significantly higher for larvae treated with the fungus only compared to fungus + imidacloprid-treated larvae (Fig. 2). Conidial attachment to the spiracular region was very low for both fungus-treated only and fungus + imidacloprid-treated larvae and no difference was observed between treatments. At 12 h after treatment, conidial number was significantly lower on the cuticle, setae, and spiracles of larvae treated with fungus alone compared with the fungus + chemical (Fig. 2). There was no difference in attachment of conidia to the setal socket between fungus-treated and imidacloprid/fungus-treated larvae.

Within 12 h post-treatment, a marked reduction of FITC-labeled conidia from the cuticle of both fungus-treated and imidacloprid + fungus-treated larvae was observed (Fig. 4). These results suggest that the initial loss of conidia was due to the removal of nonattached conidia since larvae were not rinsed in water following treatment. However, fungus-treated larvae removed

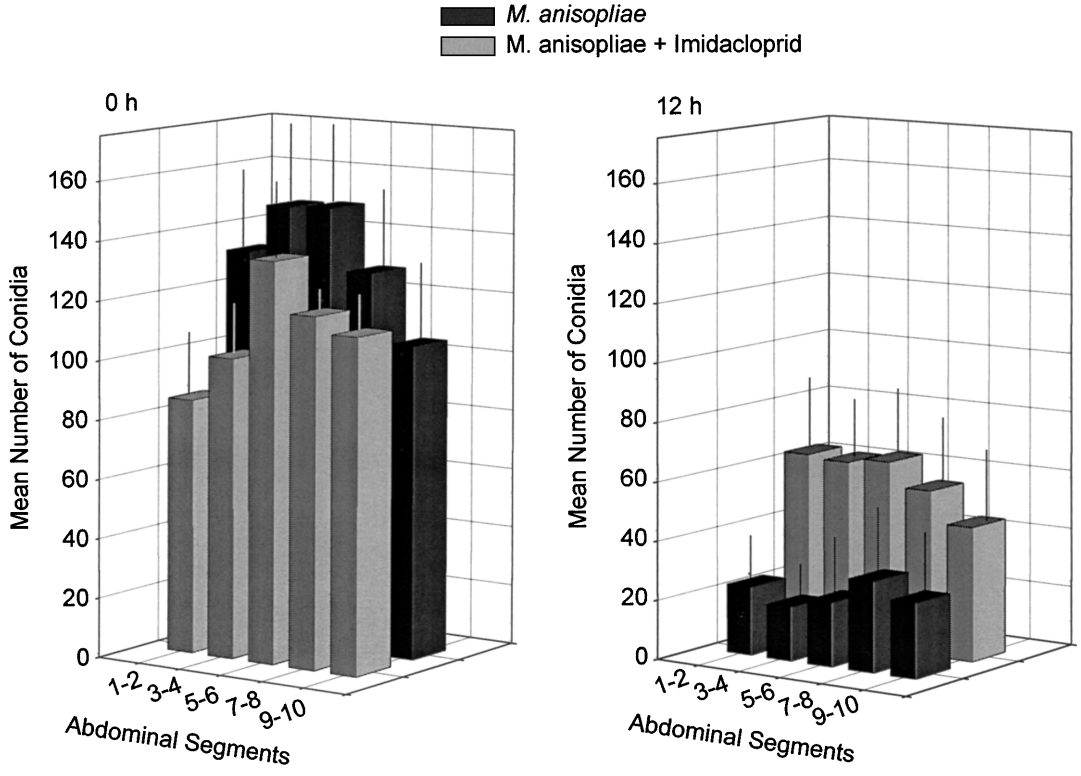

FIG. 1. Mean number of conidia of *Metarhizium anisopliae*, attached to pleural abdominal segments on the cuticle of first instars of *Diaprepes abbreviatus* following contact application with and without imidacloprid at 0.01% (AI). Mean values based on 25 and 50 microscope fields (0.013 mm^2 area at 600×) at 0 and 12 h after treatment, respectively.

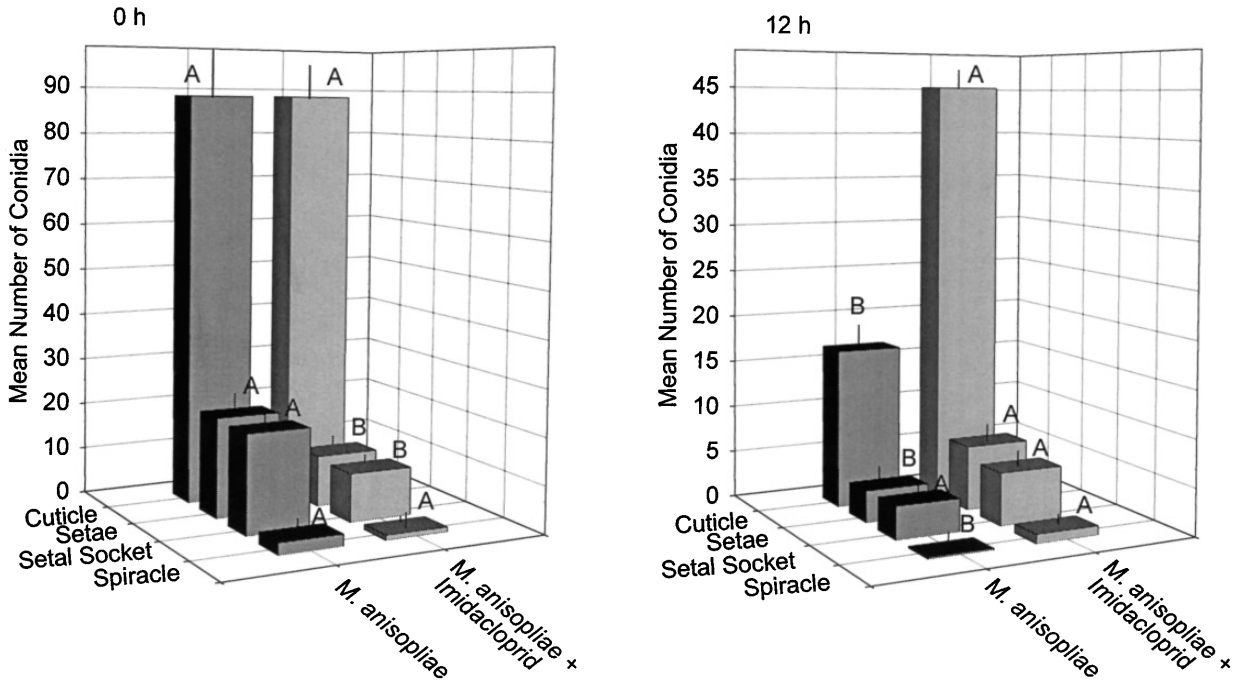

FIG. 2. Mean number of conidia of *Metarhizium anisopliae*, attached to different sites on the cuticular pleural membrane of first instars of *Diaprepes abbreviatus* following inoculation with fungus alone or fungus + imidacloprid at 0.01% (AI). Mean values based on 25 and 50 microscope fields (0.013 mm^2 area at 600×) at 0 and 12 h after treatment, respectively. Bars with the same letter within a region are not significantly different as determined by Tukey's test ($P < 0.05$).

significantly more conidia (82%) compared with fungus + imidacloprid-treated larvae (58.2%) ($P < 0.0001$). By 24 h, only 2.2 ± 0.26 conidia per microscope field were observed on larval cuticle treated with fungus only and many larvae examined had no conidia. Larvae exposed to imidacloprid did not show this marked reduction in conidial numbers (Fig. 4). By 24 h, 53.5% of conidia were still present on imidacloprid-treated larvae; a number significantly different from the control ($P < 0.0001$). Conidia were readily removed from larvae treated with fungus only as they crawled over the food source (carrot) and the walls of the plastic container. Conidial recovery was significantly higher from the carrots (154.1 ± 26.8 conidia) ($P < 0.0001$) and the inner wall of plastic cups (10.8 ± 2.0 conidia) ($P < 0.0001$) containing control larvae compared to imidacloprid-treated larvae from carrots (69.5 ± 15.8 conidia) and plastic cups (0.7 ± 0.2 conidia).

Conidial Removal from Larvae Treated with Imidacloprid after Vertical Movement in Soil at Different Moistures

Following application of *M. anisopliae* conidia to the larval cuticle, larvae removed 68.4–90.8% of the conidia during movement through untreated Candler soil at all soil moistures (Fig. 5). The number of conidia remaining on the cuticle of larvae exposed to untreated soil was highest at <1% compared with 7 and 14% moisture. When larvae were treated with imidacloprid at 0.05%, impairing larval mobility, significantly fewer conidia were removed (0–2.8%) during exposure to soil.

Conidial Attachment on Insect Cuticle in Soil Treated with Imidacloprid at Different Moistures

M. anisopliae conidia attached to the larval cuticle regardless of the presence or absence of imidacloprid (Figs. 6A and 7A). The mean number of conidia attached to larval cuticle following soil exposure to *M. anisopliae* at 5×10^5 conidia/g was similar among soil moistures (Fig. 6A, Table 1). Although conidia attached to the larval cuticle at 7% soil moisture, no mycosis was caused by *M. anisopliae* alone (Fig. 6C). These results suggest that larvae removed fungal conidia prior to germination and penetration during exposure to soil. With the exception of soil at <1% moisture, conidial attachment was higher in soil treated with imidacloprid (Fig. 6A, Table 1). In addition, larval mortality and mycosis were significantly higher in soil treated with imidacloprid + fungus at 7 and 14% moisture compared with soil treated with fungus alone (Figs. 6-B and 6C, Table 2). At 5×10^6, conidial attachment on larvae held in soil treated with fungus only was significantly higher at <1% compared with 7 and 14% soil moistures (Fig. 7A, Table 1). At <1% soil moisture, conidial attachment was similar for fungus only and imidacloprid/fungus treatments. However,

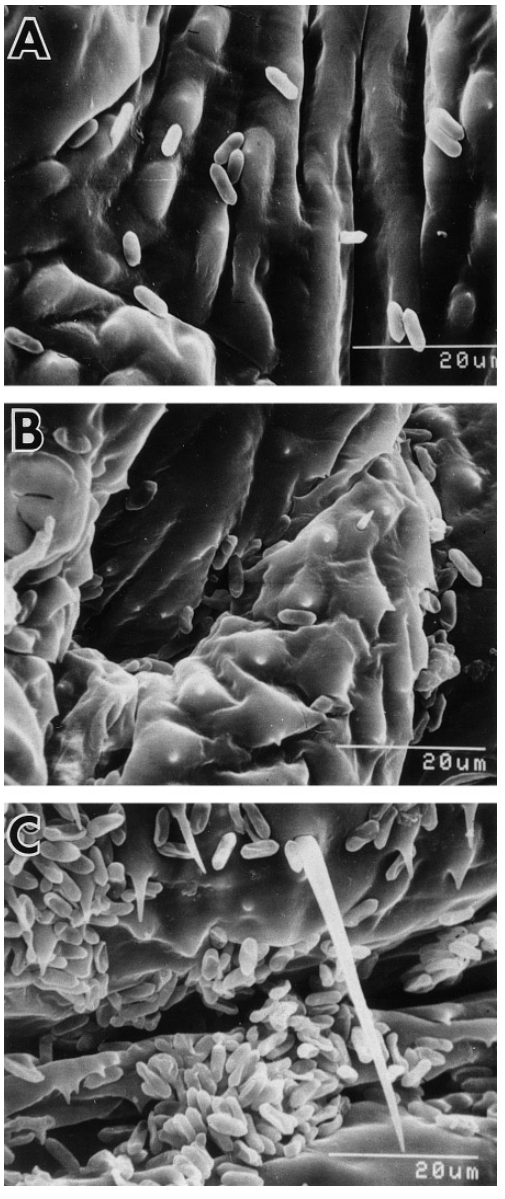

FIG. 3. Scanning electron micrographs of *Metarhizium anisopliae* conidia attached to first instars of *Diaprepes abbreviatus*. (A) Conidia attached to larval cuticle; (B) conidia in depressions of the larval cuticle; (C) conidia attached to setae and around setal socket.

larval mortality and mycosis at <1% were significantly higher in soil treated with the imidacloprid + fungus combination compared with fungus alone (Figs. 7B and 7C, Table 2). According to factorial analyses, the effect of imidacloprid on conidial attachment, larval mortality, and mycosis was dependent on the concentration of the fungus and synergism was expressed at 7 and 14% soil moisture (Figs. 7-A–7C, Tables 1 and 2). Conidial attachment and larval mycosis in soil at 7% moisture with *B. bassiana* at 5×10^6 conidia/g soil was significantly higher in imidacloprid-treated soil (Table 3).

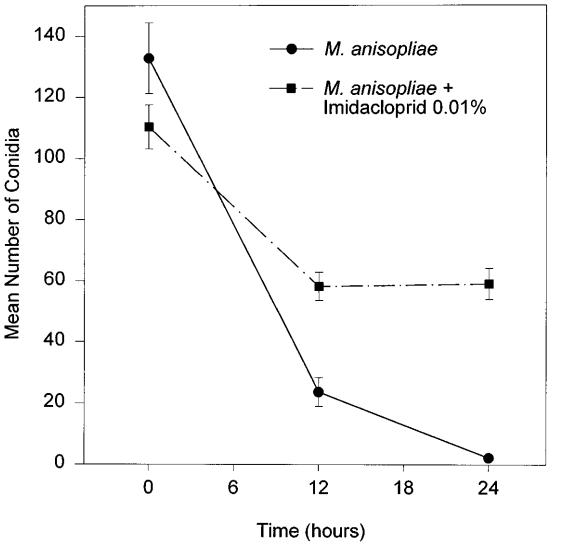

FIG. 4. Mean number of *Metarhizium anisopliae* conidia attached to the cuticle of first instars of *Diaprepes abbreviatus* at 0, 12, and 24 h post-treatment following inoculation with fungus alone or fungus + imidacloprid at 0.01% (AI). Mean values based on 25 fields at 0 h and 50 microscope fields at 12 and 24 h (0.013 mm^2 area at 600×).

Conidial Attachment of M. anisopliae to Larval Cuticle Treated with Formulated Product and Active and Inert Ingredients

In test 1, the effect of different doses of formulated imidacloprid on conidial attachment on larval cuticle

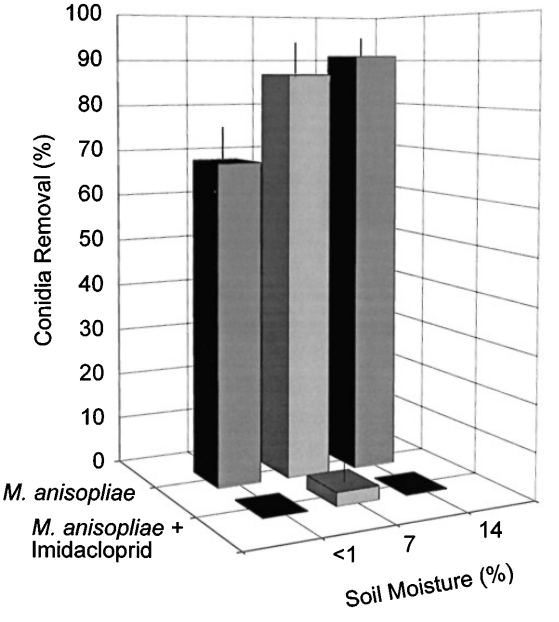

FIG. 5. Mean percent *Metarhizium anisopliae* conidia removed from the cuticle of first instars of *Diaprepes abbreviatus* following cuticular exposure with conidia alone or conidia + imidacloprid at 0.05% (AI), following inoculation on the surface of 1 cm untreated soil.

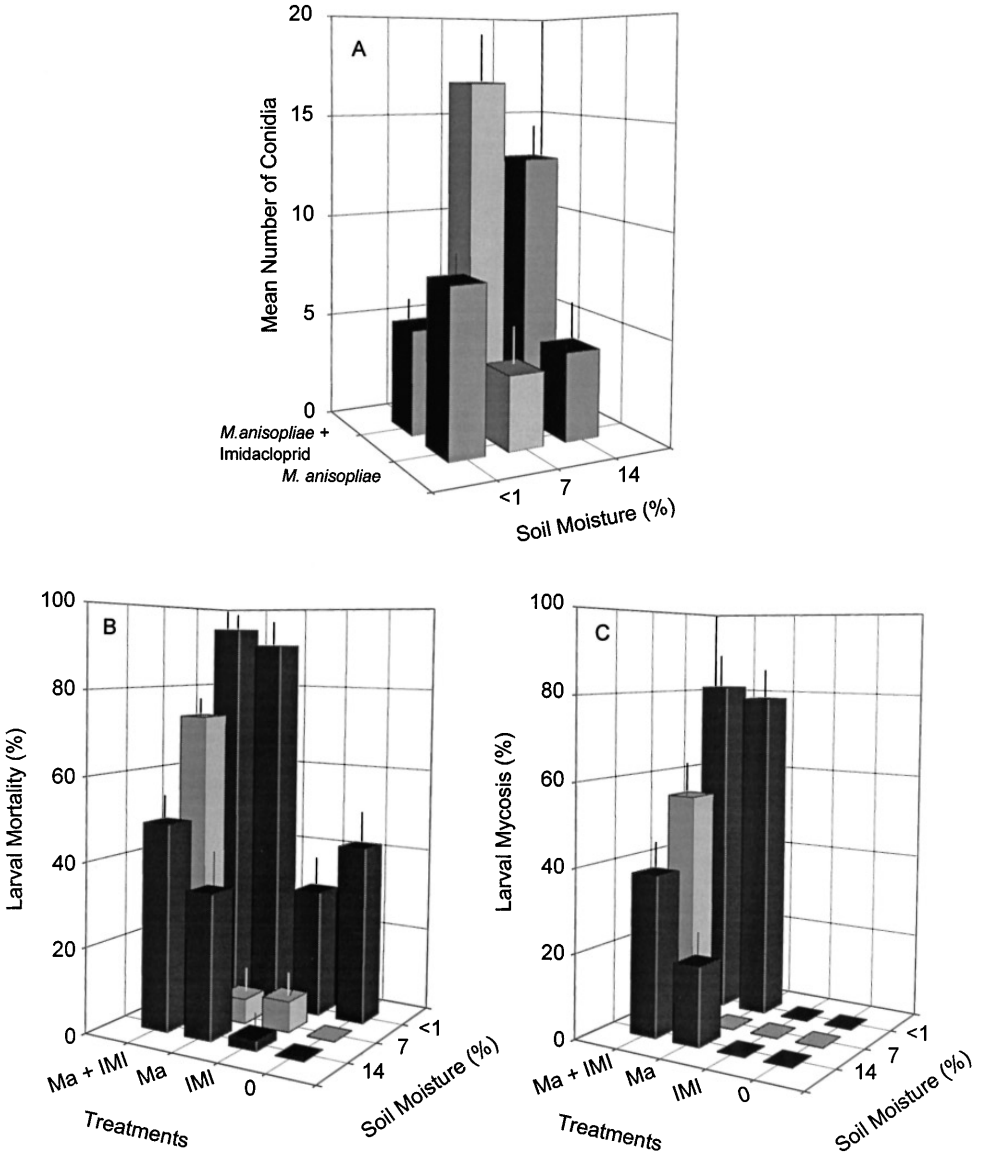

FIG. 6. Mean number of conidia of *Metarhizium anisopliae* found on cuticle (A), larval mortality (B), and mycosis (C) of first instars of *Diaprepes abbreviatus* after exposure to soil at 5×10^5 conidia/g soil at different soil moistures. Conidial attachment on larval cuticle was determined 24 h after exposure to treated soil. Larval mortality and mycosis were evaluated 7 days after larval inoculation in treated soil.

was determined. Conidial attachment to the larval cuticle decreased with an increase in dose of imidacloprid (Fig. 8) and best fit a second degree curve ($r^2 = 0.61$; $df = 2, 37$; $P < 0.0371$). Only 87.9 ± 9.3 conidia per microscope field were counted on the cuticle of larvae after cuticular exposure to imidacloprid at 0.1% compared to 604.2 ± 87.6 conidia for untreated larvae. Conidial attachment decreased one, three, and sevenfold at doses of 0.01, 0.05, and 0.1% of imidacloprid, respectively.

In test 2, the effect of each component of the imidacloprid formulation on conidial attachment to larval cuticle was evaluated using 0.1% concentration. The active ingredient had no effect on conidial attachment compared with the fungus control (Fig. 9). However, both the inert ingredient and the formulated product reduced conidial attachment significantly compared with the control. Only 71.2 ± 9.0 and 126.9 ± 4.9 conidia were found attached to larval cuticle for formulated product and inert ingredients, respectively. Clearly, the inert ingredient is responsible for lower conidial attachment to the larval cuticle.

DISCUSSION

Microscopic data presented here support previous research (Quintela and McCoy, 1997a,b, 1998) that suggests fungal synergism by imidacloprid is caused, in

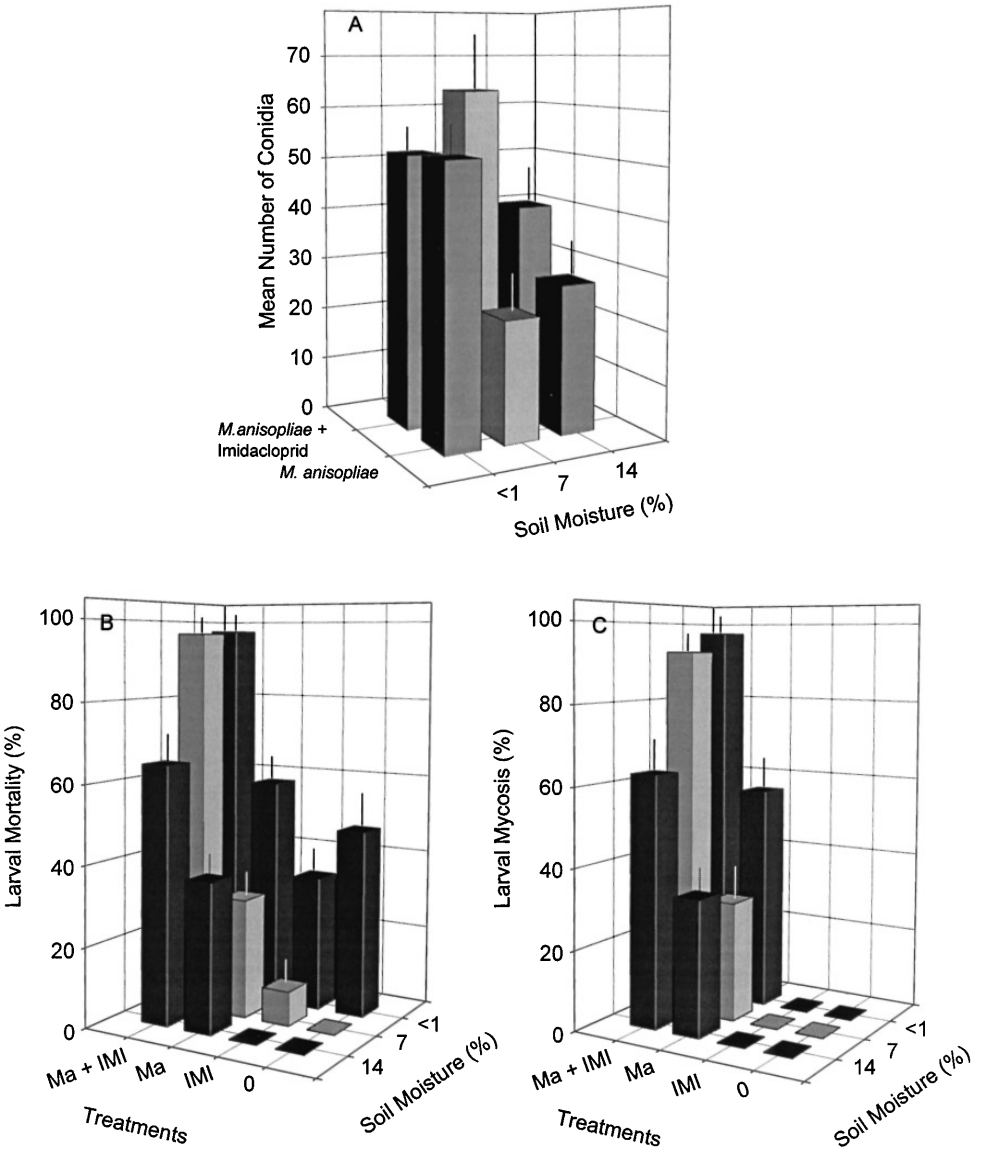

FIG. 7. Mean number of conidia of *Metarhizium anisopliae* found on cuticle (A), larval mortality (B), and mycosis (C) of first instars of *Diaprepes abbreviatus* after 24-h exposure to soil at 5×10^6 conidia/g soil at different soil moistures. Conidial attachment on larval cuticle was determined 24 h after exposure to treated soil. Larval mortality and mycosis were evaluated 7 days after larval inoculation in treated soil.

part, by larval behavioral modification involving reduced or temporary loss of mobility for <48 h caused by neurotoxicity. Apparently, healthy larvae can void their cuticles of conidia prior to germination and penetration when contacting soil or other substrates. Failure to do so results in higher fungal infection. This hypothesis is supported by the work of Boucias *et al.* (1996), who found that termites void themselves of fungal conidia via normal grooming behavior. Termite intoxication by imidacloprid prevented grooming, resulting in increased fungal infection.

Furthermore, our studies showed that conidial number on the larval cuticle of untreated larvae was higher in dry soil (<1% soil moisture) than in soil with higher moistures. Quintela and McCoy (1997b) found that neonate *D. abbreviatus* mobility is severely impaired in dry soil. These findings support our hypothesis and suggest that regulation of soil moisture can indirectly affect the performance of fungi as microbial control agents. For example, Quintela and McCoy (1997c) demonstrated that larval mortality and mycosis of *D. abbreviatus* by *M. anisopliae* decreased as moisture increased from <1 to 14%. Krueger *et al.* (1991) and Studdert and Kaya (1990) also found that more chinch bugs, *Blissus leucopterus leucopterus* Say, and soybean caterpillars, *Spodoptera exigua* Hübner, became infected by *B. bassiana* when exposed to drier soils compared with wetter soils.

TABLE 1

Summary of Factorial Analyses for Number of Conidia of *Metarhizium anisopliae* Found on Cuticle of First Instar *Diaprepes abbreviatus* Subsequent to Soil Treatment with *M. anisopliae* and Imidacloprid Alone and in Combination under Different Soil Moisture[a]

M. anisopliae (conidia/g soil)	Factor	*F*	*df*	*P*
5×10^5	Imidacloprid (IMI)	17.14	1, 84	0.0001
	Moisture (moist)	0.18	2, 84	0.8390
	IMI × moist	10.99	2, 84	0.0001
5×10^6	Imidacloprid (IMI)	7.94	1, 54	0.0067
	Moisture (moist)	3.62	2, 54	0.0335
	IMI × moist	3.92	2, 54	0.0258

[a] Data transformed to $\sqrt{x} + 1$.

TABLE 3

Mean Number of Conidia of *Beauveria bassiana* Found on Cuticle and Subsequent Larval Mortality and Mycosis of First Instar *Diaprepes abbreviatus* after Exposure to Soil Treated with Fungus at 5×10^6 Conidia/g Soil Alone or in Combination with Imidacloprid at 25 μg/g Soil at 7% Soil Moisture

Treatment	Mean no. conidia on larval cuticle[a]	Larval mortality[b] (%)	Larval mycosis[b] (%)
B. bassiana	14.8 ± 6.2 a	19.6 ± 7.8 a	19.6 ± 7.8 a
B. bassiana + imidacloprid	32.3 ± 4.8 b	60.6 ± 7.8 b	60.6 ± 7.8 b

[a] Mean number of conidia found on larval cuticle after exposure to treated soil for 22 h. Ten larvae examined/treatment.

[b] Larval mortality and mycosis were evaluated 7 days after larval inoculation in treated soil. Twenty larvae/treatment ($n = 60$) in three replications. Larval mortality in the untreated control and imidacloprid at 25 μg/g soil was 0.0 and 7.1 ± 6.6%, respectively.

Zacharuk (1970) demonstrated that conidia of *M. anisopliae* attached nonspecifically over the entire cuticular surface of three species of larval elateridae and can be removed from smooth sclerites more readily than from epicuticular folds. Sosa-Gomez *et al.* (1997) showed that both topography and chemistry of the stink bug cuticle affect conidial adhesion of *M. anisopliae.* We observed uniform nonspecific distribution of conidia of *M. anisopliae* on the cuticle of both imidacloprid-treated and untreated *D. abbreviatus* larvae. However, in less exposed regions of the larval cuticle, such as the setal socket, conidial removal by healthy larvae was less than on smooth areas. Imidacloprid-treated larvae had higher conidial numbers over the entire cuticle after 12 h.

Previous studies suggest that once hydrophobic conidial attachment by hyphomyceteous fungi occurs, they adhere strongly to the host cuticle (Boucias *et al.*, 1988). In their studies, cuticles were obtained from larvae that were boiled for 2 h in a suspension containing SDS at 2%. During this process, waxes, lipids, and other chemical compounds present on the epicuticle were altered, no doubt preventing strong attachment of conidia to the cuticle. The epicuticle in healthy insects is made up of lipids and waxes secreted by the epidermal cells through lipids channels. This hydrophobic layer provides a barrier to water loss (Hadley, 1981) and plays an important role in the attachment and germination of fungal propagules on the host cuticle (Boucias and Pendland 1991). In our studies where conidia were readily removed by larvae during normal movement in the soil or when contacting the surface of a given substrate, conidia were weakly attached on the host cuticle.

In summary, conidial attachment appears to be a

TABLE 2

Summary of Factorial Analyses for Larval Mortality and Mycosis of *D. abbreviatus* Subsequent to Soil Treatment with *M. anisopliae* and Imidacloprid Alone and in Combination under Different Soil Moisture

M. anisopliae (conidia/g soil)	Factor	Larval mortality[a]			Larval mycosis[a]		
		F	*df*	*P*	*F*	*df*	*P*
5×10^5	Imidacloprid (IMI)	15.40	1, 48	0.0003	11.16	1, 48	0.0016
	M. anisopliae (Ma)	125.73	1, 48	0.0001	183.57	1, 48	0.0001
	Moisture (moist)	65.08	2, 48	0.0001	22.17	2, 48	0.0001
	IMI × Ma	10.39	1, 48	0.0023	11.16	1, 48	0.0016
	IMI × moist	11.00	2, 48	0.0001	5.19	2, 48	0.0091
	Ma × moist	1.77	2, 48	0.1816	22.17	2, 48	0.0001
	IMI × Ma × moist	2.98	2, 48	0.0604	5.19	2, 48	0.0091
5×10^6	Imidacloprid (IMI)	13.59	1, 22	0.0013	67.69	1, 22	0.0001
	M. anisopliae (Ma)	74.57	1, 22	0.0001	800.68	1, 22	0.0001
	Moisture (moist)	15.63	2, 22	0.0001	11.30	2, 22	0.0004
	IMI × Ma	12.88	1, 22	0.0016	67.69	1, 22	0.0001
	IMI × moist	2.80	2, 22	0.0824	3.81	2, 22	0.038
	Ma × moist	2.77	2, 22	0.0843	11.30	2, 22	0.0004
	IMI × Ma × moist	0.94	2, 22	0.4057	3.81	2, 22	0.038

[a] Data transformed to arcsine $\sqrt{}$ proportion.

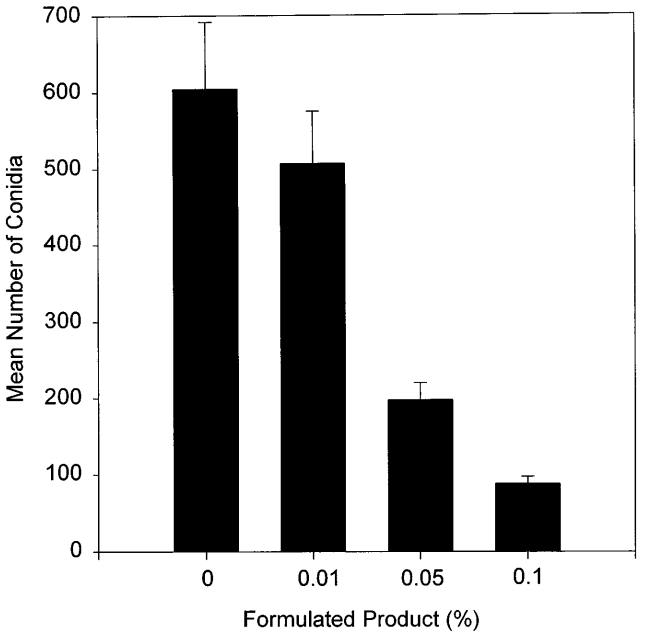

FIG. 8. Mean number of *Metarhizium anisopliae* conidia found on cuticle after cuticular exposure of first instars of *Diaprepes abbreviatus* to different doses of imidacloprid.

dynamic process involving a multiplicity of factors such as pathogen, host behavior, and its environment. Our data suggest that in nature, a host may come in contact with an infective conidial dose; however, through subsequent contact with normal soil or another substrate it may reduce the probability for infection through conidia voidance. One can assume, depending on host age, the conidial load will exceed or fall below a threshold for infection frequently during the host lifetime. In addition, environmental stress will also affect the susceptibility of a host population, by lowering its defenses enough to trigger fungal infection.

Conidial attachment on the larval cuticle of *D. abbreviatus* was significantly higher in soil treated with imidacloprid. However, cuticular exposure of *D. abbreviatus* larvae to doses of formulated imidacloprid greater than 0.01% affected conidial attachment of *M. anisopliae* negatively, but larval mycosis was not affected. Quintela and McCoy (1997), in a series of experiments, showed that larval mycosis of *D. abbreviatus* was not decreased when *M. anisopliae* or *B. bassiana* was combined with imidacloprid at doses >0.01%. Comparative experimentation showed that the factor(s) responsible for a sevenfold decrease in attachment was associated with the inert carrier. Further testing suggests that wetting agents found in the formulation are responsible (Quintela and McCoy, unpublished data). Boucias *et al.* (1991) showed that certain detergents, solvents, and high molecular weight proteins neutralized hydrophobic interactions and reduced conidial adhesion when mixed as a suspension. Our data support these findings.

Our overall results presented herein and with other published information (Quintela and McCoy, 1998) suggest that doses of formulated imidacloprid in soil ($^3\epsilon \geq 5$ µg (AI)/g) will prevent larvae from removing fungal conidia from the cuticle because of the temporary larval paralysis caused by the chemical. How does this translate to the field? Obviously, the field residual dose of chemical and fungus vary greatly in time due to environmental effects. Recent field studies (McCoy *et al.*, unpublished data) suggest, however, that an initial dose of formulated imidacloprid of 1.0 µg (AI)/g soil with *B. bassiana* conidia at 2.5×10^5 CFU/g soil is synergistic for 8–10 days. At this dose, imidacloprid reaching plant roots is systemic too (McCoy *et al.*, 1995). Most importantly, synergistic mycosis expressed both in the field and in the laboratory suggest that host behavioral modification stimulated by chemical alterations can improve the efficacy of some entomopathogenic fungi and should be tested against other invertebrate hosts.

ACKNOWLEDGMENTS

We are grateful to Carmi Louzada for technical assistance. We express our sincere appreciation to Dr. Daniel S. Gomez and Dr.

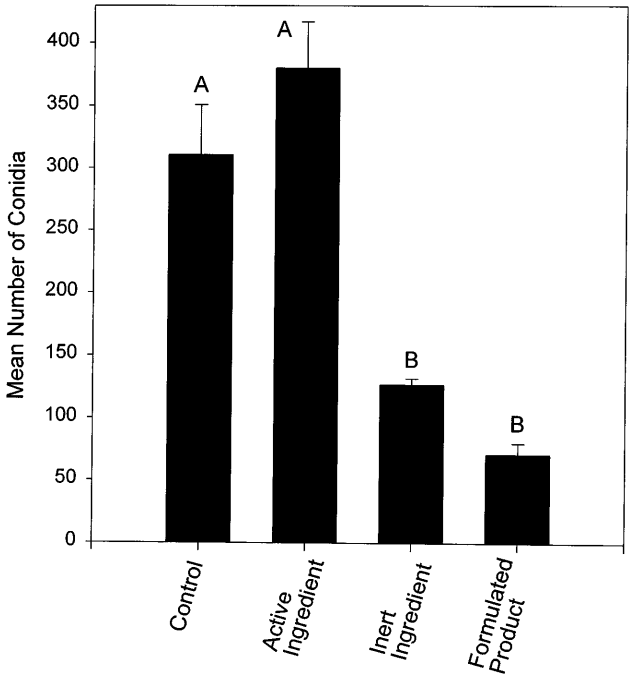

FIG. 9. Mean number of *Metarhizium anisopliae* conidia found on the cuticle of the first instars of *Diaprepes abbreviatus* after contact exposure to active ingredient and formulated imidacloprid at 0.05% (AI) and inert ingredient at 0.184% (concentration equal to the amount found in formulated imidacloprid at 0.05% of the active ingredient). Bars with the same letter are not significantly different by Tukey's test ($P < 0.05$).

Drion Boucias for technical assistance on the fluorescence microscopy technique. This research was conducted as partial fulfillment of the Doctor of Philosophy degree by the senior author at the Graduate school of the University of Florida. This work was supported in part by Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA-Brasília/Brazil), Conselho Nacional de Desenvolvimento Científico e Technológico (CNPq-Brasília/Brazil, Process No. 202780/91-0), the Florida Citrus Research Advisory Group, USDA-IR-4, and Bayer AG. Florida Agricultural Experiment Stations Journal Series No. R-60671.

REFERENCES

Anderson, T. E., Hajek, A. E., Roberts, D. W., Preisler, H. A., and Robertson, J. L. 1989. Colorado potato beetle (Coleoptera: Chrysomelidae): Effects of combinations of *Beauveria bassiana* with insecticides. *J. Econ. Entomol.* **82,** 83–89.

Beilharz, V. C., Parberry, D. G., and Swart, H. J. 1982. Dodine: A selective agent for certain soil fungi. *Trans. Br. Mycol. Soc.* **79,** 507–511.

Benz, G. 1971. Synergism of micro-organisms and chemical insecticides. *In* "Microbial Control of Insects and Mites" (H. D. Burges and N. W. Hussey, Eds.), pp. 327–355. Academic Press, New York.

Boucias, D. G., Pendland, J. C., and Latge, J. P. 1988. Nonspecific factors involved in attachment of entomopathogenic deuteromycetes to host insect cuticle. *Appl. Environ. Microbiol.* **54,** 1795–1805.

Boucias, D. G., and Pendland, J. C. 1991. The fungal cell wall and its involvement in the pathogenic process in insect hosts. *In* "Fungal Cell Wall and Immune Response" (J. P. Latgé and D. Boucias, Eds.), pp. 303–314. Springer-Verlag, Berlin-Heidelberg.

Boucias, D. G., Stokes, C., Storey, G., and Pendland, J. C. 1996. The effects of imidacloprid on the termite *Reticulitermes flavipes* and its interaction with the mycopathogen *Beauveria bassiana*. *Pflanzenshutz-Nachrichten Bayer* **49,** 105–151.

Ferron, P. 1978. Biological control of insect pests by entomogenous fungi. *Annu. Rev. Entomol.* **23,** 409–421.

Grodowitz, M. J., Krchma, J., and Broce, A. B. 1982. A method for preparing soft bodied larval Diptera for scanning electron microscopy. *J. Kansas Entomol. Soc.* **55,** 751–753.

Hadley, N. H. 1981. Cuticular lipids of terrestrial plants and arthropods, a comparison of their structure, composition and waterproofing function. *Biol. Rev.* **56,** 23–47.

Hamlen, R. A., and Beavers, J. B. 1975. Toxicity and persistence of surface applied and soil incorporated insecticides against *Diaprepes abbreviatus* larvae. *J. Econ. Entomol.* **72,** 131–134.

Hassan, A. E. M., and Charnley, A. K. 1989. Ultrastructural study of the penetration by *Metarhizium anisopliae* through dimilin affected cuticle of *Manduca sexta*. *J. Invertebr. Pathol.* **54,** 117–124.

Hassan, A. E. M., Dillon, R. J., and Charnely, A. K. 1989. Influence of accelerated germination of conidia on the pathogenicity of *Metarhizium anisopliae* for *Manduca sexta*. *J. Invertebr. Pathol.* **54,** 277–279.

Hung, S. Y., and Boucias, D. G. 1992. Influence of *Beauveria bassiana* on the cellular defense response of the beet armyworm, *Spodoptera exigua*. *J. Invertebr. Pathol.* **60,** 152–158.

Kaakeh, W., Reid, B. L., Bohnert, T. J., and Bennett, G. W. 1997. Toxicity of imidacloprid in the german cockroach (Dictyoptera: Blattellidae), and the synergism between imidacloprid and *Metarhizium anisopliae* (Imperfect Fungi: Hyphomycetes). *J. Econ. Entomol.* **90,** 473–482.

Krueger, S. R., Nechols, J. R., and Ramoska, W. A. 1991. Infection of chinch bug, *Blissus leucopterus leucopterus* (Hemiptera: Lygaeidae), adults from *Beauveria bassiana* (Deuteromycotina: Hyphomycetes) conidia in soil under controlled temperature and moisture conditions. *J. Invertebr. Pathol.* **58,** 19–26.

McCoy, C. W., Quintela, E. D., Simpson, S. E., and Fojtik, J. 1995. Effect of surface-applied and soil-incorporated insecticides for the control of *Diaprepes abbreviatus* larvae in container-grown citrus. *Proc. Fl. State Hort. Soc.* **108,** 130–136.

Quintela, E. D. 1996. "Synergistic Effect of Imidacloprid on Conidial Germination and the Pathogenicity of Two Entomopathogenic Fungi to Larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae)," Ph.D. dissertation, University of Florida, Gainesville, FL.

Quintela, E. D., and McCoy, C. W. 1997a. Enhancing the pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* to larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) with sublethal doses of imidacloprid. *Environ. Entomol.* **26,** 1173–1182.

Quintela, E. D., and McCoy, C. W. 1997b. Effects of imidacloprid on development, mobility, and survival of first instars of *D. abbreviatus* (Coleoptera: Curculionidae). *J. Econ. Entomol.* **90,** 988–995.

Quintela, E. D., and McCoy, C. W. 1998. Synergistic effect of imidacloprid and two entomopathogenic fungi on the behavior and survival of larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in Soil. *J. Econ. Entomol.* **91,** 110–122.

SAS Institute. 1985. "SAS User's Guide: Statistics," 5th ed. SAS Institute, Cary, NC.

Sosa-Gomez, D. R., Boucias, D. G., and Nation, J. L. 1997. Attachment of *Metarhizium anisopliae* to the southern green stink bug *Nezara viridula* cuticle and fungistatic effect of cuticular lipids and aldehydes. *J. Invertebr. Pathol.* **69,** 31–39.

Steinhaus, E. A. 1958. Stress as a factor in insect disease. *Proc. 10th Int. Congr. Entomol.* **4,** 725–30.

Steinkraus, D. C., and Tugwell, N. P. 1997. *Beauveria bassiana* (Deuteromycotina: Moniliales) effects on *Lygus lineolaris* (Hemiptera: Myridae). *J. Entomol. Sci.*, **32,** 79–90.

Studdert, J. P., and Kaya, H. K. 1990. Water potential, temperature, and clay-coating of *Beauveria bassiana* conidia: Effect on *Spodoptera exigua* pupal mortality in two soil types. *J. Invertebr. Pathol.* **56,** 327–336.

Zacharuk, R. Y. 1970. Fine structure of the fungus *Metarhizium anisopliae* infecting three species of larval Elateridae (Coleoptera). *J. Invertebr. Pathol.* **15,** 63–80.