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Synergism between *Metarhizium anisopliae* (Deuteromycota: Hyphomycetes) and Boric Acid against the German Cockroach (Dictyoptera: Blattellidae)

Ludek Zurek, D. Wes Watson, and Coby Schal[1]

Department of Entomology, Box 7613, North Carolina State University, Raleigh, North Carolina 27695-7613

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Mortality of German cockroaches, *Blattella germanica* (L.), caused by *Metarhizium anisopliae* (Metschnikoff) Sorokin strain AC-1 alone and in combination with different formulations of boric acid, was evaluated in laboratory bioassays. Topical application of *M. anisopliae* alone (8.96×10^9 conidia/m^2) required 28 days to cause >92% cockroach mortality ($LT_{50} = 10$ days). In contrast, in combination with boric acid (topically applied as a dust or in drinking water), *M. anisopliae* killed cockroaches significantly faster than without boric acid. *M. anisopliae* conidial dust (8.96×10^8 conidia/m^2) with either 12.5% (w/w) boric acid dust or 0.1% (w/v) boric acid in drinking water killed 100% of the cockroaches in only 8 days ($LT_{50} = 5$ days) and 10 days ($LT_{50} = 6$ days), respectively, without compromising the fungus emergence from cadavers. Replacement of *M. anisopliae* with flour dust or heat-killed *M. anisopliae* conidia eliminated this effect, demonstrating that it was not the consequence of greater boric acid ingestion due to more extensive cockroach grooming upon exposure to *M. anisopliae* conidia. Moreover, injections of a low dose of *M. anisopliae*, which caused only 30% mortality, together with sublethal concentrations of boric acid into the cockroach hemocoel resulted in a doubling of mortality. Statistical analysis demonstrated a synergistic interaction between these two insecticides. © 2002 Elsevier Science (USA)

***Key Words: Metarhizium anisopliae; Blattella germanica;* boric acid; dust; synergism.**

INTRODUCTION

The German cockroach, *Blattella germanica* (L.), is an important structural pest controlled primarily with synthetic organic insecticides (organophosphates, pyrethroids, and carbamates) (Schal and Hamilton, 1990; Rust *et al.*, 1993; Benson and Zungoli, 1997). Widespread resistance to these insecticides in populations of this cockroach (Cochran, 1989, 1995a; Scott *et al.*, 1990; Rust and Reierson, 1991; Rust *et al.*, 1993; Holbrook *et al.*, 1999; Valles and Yu, 1996), and concern about human safety and the environment have motivated investigations of alternative methods of cockroach control.

The entomopathogenic fungus, *Metarhizium anisopliae* (Metchnikoff) Sorokin (Deuteromycota: Hyphomycetes), has a wide insect host range that includes *B. germanica* (Gunner *et al.*, 1991). However, its slow action (usually ~30 days) and inconsistent efficacy have confounded effective use of this pathogen under field conditions, including the structural environment (Pachamutu *et al.*, 1999). For these reasons, sales of a commercial formulation of *M. anisopliae* for cockroaches, Bio-Path by EcoScience Corp. (Worcester, MA), were discontinued (Pachamutu *et al.*, 1999). Recently, several studies reported increased virulence of *M. anisopliae* against *B. germanica* in combination with the organic insecticides imidacloprid, chlorpyrifos, propetamphos, and cyfluthrin (Kaakeh *et al.*, 1997; Pachamutu *et al.*, 1999; Pachamutu and Kamble, 2000).

Various formulations of the inorganic insecticides boric acid (H_2BO_3) and borax (sodium tetraborate; $Na_2B_4O_7 \cdot 10H_2O$) have been used to control cockroaches since the middle of the 19th century (Lintner, 1882; cited in Ebeling, 1995). The mode of action of boric acid against insects is unknown, although destruction of the digestive tract wall and penetration of the exoskeleton have been reported (Ebeling, 1995). Cochran (1995b) reported that boric acid destroys the foregut epithelium and suggested that cockroaches might die from starvation. Boric acid has a very good safety record for mammals; it does not volatilize (in contrast to organic insecticides) and absorption of boric acid through unbroken skin is negligible (Pfeiffer, 1951; Valdes-Dupena and Arey, 1962; Ebeling, 1995). Yet, boric acid use has been limited, primarily because the organic insecticides provide much faster kill.

[1] To whom correspondence should be addressed: Fax: (919) 515-7746. E-mail: coby_schal@ncsu.edu.

TABLE 1

Mortality of *Blattella germanica* Adults after Topical Application of Boric Acid (Ba), *Metarhizium anisopliae* (Ma), Flour (Fl), Ba + Ma, and Ba + Fl Dust Formulations

	Treatment										
	Ba				Ma		Ba + Ma			Ba + Fl	Fl
Concentration (g/m^2)	0.20	0.10	0.05	0.025	0.40	0.04	0.20 + 0.40	0.05 + 0.40	0.025 + 0.04	0.20 + 0.40	0.40
n(F:M = 1:1)	100	100	100	100	100	100	100	100	100	100	100
% Mortality ± SE	100	88.0 ± 8.5	38.5 ± 3.5	9.5 ± 0.7	92.5 ± 6.3	36.5 ± 4.9	100	100	83.0 ± 1.4	100	0
LT_{50} (days)	5	5	na	na	10	na	3	5	8	5	na
95% CI (days)	4–6	4–6	na	na	8–12	na	3–4	4–5	8–10	4–6	na
Fungal growth (%)			na		100	100	86	100	100	na	na

Note. n, Number of cockroaches; F, female; M, male; SE, standard error of mean; na, not applicable; CI, confidence interval for LT_{50}.

Our goal has been to develop and implement alternatives to the organic insecticides for cockroach control in residential settings and in the structural animal production industry. Toward this end, we sought to accelerate the activity of *M. anisopliae* and boric acid with combinations of these two insecticidal agents against adult *B. germanica.*

MATERIALS AND METHODS

Insects and Fungus

Adults (7–10 days old) were collected from a synchronously reared laboratory colony of insecticide-susceptible cockroaches. Cockroaches were supplied *ad libitum* with Purina No. 5012 Rat Chow (Purina Mills, St. Louis, MO), and water was provided in glass tubes with cotton stoppers. Each cage (19 × 14 cm) was provided with a paper egg carton shelter. All colonies were kept in an incubator at 27°C and a 12:12 h light:dark cycle.

M. anisopliae strain AC-1 was originally isolated in our laboratory from the cadaver of an American cockroach (*Periplaneta americana* L.) found at the NCSU Lake Wheeler Field Experimental Station. The fungus was cultured aerobically on potato dextrose agar (Difco, Detroit, MI) plates and stored at 4°C. For mass-production, conidia were aseptically harvested by loop and transferred to sterile 3.0 mM potassium phosphate buffer with 0.05% Triton X-100 (PPBT). A 0.5-L heat-sealed plastic pouch (Kapak Corp., Minneapolis, MN), containing ~200 g of autoclaved rice (Ibrahim and Low, 1993), was aseptically injected with fungus in 1 ml PPBT. The pouches were cultured at room temperature for 25 days and then left open at room temperature for 5 days to reduce the moisture content. Conidia were then harvested by sifting through a 300-μm mesh screen and stored at 4°C in sterile plastic containers.

Bioassays with Dust Formulations

For each treatment, 50 adult cockroaches (25 males, 25 females) were transferred to a 10-L plastic container (0.25 m^2 internal surface) with an egg carton shelter and allowed to acclimate for 24 h. Cockroaches were then topically treated with one of the following: boric acid (Ba) dust (Acros Organics, Pittsburgh, PA) (0.2, 0.1, 0.05, 0.025 g/m^2), *M. anisopliae* (Ma) conidial dust (0.40 g/m^2 [8.96 × 10^9 conidia], 0.04 g/m^2 [8.96 × 10^8 conidia]), or flour dust (Fl) (0.40 g/m^2) (bleached wheat flour; Kroger, Cincinnati, OH). Cockroaches were also treated with different combinations of boric acid and either *M. anisopliae* or flour dust at a high concentration (0.2 g Ba + 0.4 g Ma or 0.4 g Fl per m^2), medium concentration (0.05 g Ba + 0.4 g Ma per m^2), or low concentration (0.025 g Ba + 0.04 g Ma per m^2) (Table 1). An additional bioassay was conducted with heat-inactivated *M. anisopliae* conidial dust (conidia autoclaved for 20 min) instead of flour: boric acid dust alone (0.2 g/m^2 Ba), heat-inactivated conidial dust alone (0.4 g/m^2 Mah), and the combination of Ba and Mah. All treatments were applied with a hand duster (AgrEvo, Montvale, NJ). Cockroach mortality was monitored daily for 28 days and each bioassay was replicated twice over time.

Bioassays with Injections

To determine whether the combined effects of *M. anisopliae* and boric acid were due to a heavier dust load that would elicit more grooming and thus dust ingestion, we bypassed ingestion by injecting the fungus and boric acid into the hemocoel. *M. anisopliae* was transferred to sterile PPBT and diluted to 5.1 × 10^4, 5.1 × 10^3, and 5.1 × 10^2 conidia/ml. Boric acid was diluted in sterile PPBT to 5.0, 2.0, 1.0, and 0.1% (w/v). For each treatment, 10 or 20 adult females were injected with 1 or 2 μl (two injections, 1 μl each) of either PPBT alone (control), 1 or 2 μl (two injections, 1 μl each) of PPBT with boric acid (PPBT-Ba), 1 μl PPBT with *M. anisopliae* (PPBT-Ma), and combinations of PPBT-Ba and PPBT-Ma (two injections, 1 μl each) (Table 2). Cockroaches were briefly immobilized with CO_2, and a 32-gauge needle attached to a 10-μl sterile syringe (Hamilton, Reno, NV) was inserted between

TABLE 2

Mortality of *Blattella germanica* Adult Females Challenged by Injections of Boric Acid and/or *Metarhizium anisopliae* Conidia into the Hemocoel

	Treatment											
	PPBT-Ba					PPBT		PPBT-Ma[a]			PPBT-Ba-Ma[a]	
Dose	0.1%	1.0%	2.0%	5.0%	5.0%	na		5.1×10^4	5.1×10^3	5.1×10^2	$5.0\% + 5.1 \times 10^3$	$5.0\% + 5.1 \times 10^2$
Total volume (μl)	1	1	1	1	2	1	2	1	1	1	1 + 1	1 + 1
n	10	10	10	20	20	10	10	20	20	20	20	20
Mortality (%)	0	0	0	0	100	0	0	100	60	30	100	65
LT_{50} (days)	na	na	na	na	7	na	na	2	9	na	5	8
95% CI (days)	na	na	na	na	6–7	na	na	2–3	8–9	na	5–6	8–9
Fungal growth (%)			na					100	100	100	100	100

Note. PPBT, potassium phosphate buffer with 0.1% Tween 80 (v/v); PPBT-Ba, PPBT with boric acid; PPBT-Ma, PPBT with *M. anisopliae;* PPBT-Ba-Ma, PPBT with boric acid and *M. anisopliae; n,* number of cockroaches injected; na, not applicable; CI, confidence interval for LT_{50}.

[a] Concentration of *M. anisopliae* = number of conidia/ml of PPBT.

abdominal sternites 6 and 7. Cockroach mortality was monitored daily for 28 days after injection.

Bioassays with Boric Acid in Water

To further dissociate the interactive effects of dusts, boric acid was delivered in drinking water. The treatment cages were set up as with dust applications, but after the 24-h acclimation period, the drinking water was replaced with 0.1% boric acid solution (w/v) in glass tubes with cotton stoppers. *M. anisopliae* (0.40 g/container = 8.96×10^9 conidia/m^2) or flour dust (0.40 g/container) were dusted throughout the container with the hand duster. Cockroach mortality was monitored daily for 28 days and each bioassay was replicated twice over time.

Fungus Emergence and Virulence

For all bioassays, cadavers were immediately removed and placed into sterile petri dishes. All dead cockroaches from bioassays with *M. anisopliae* were surface sterilized with 70% ethanol and 0.05% sodium hypochlorite as previously described (Zurek and Keddie, 2000) and placed on sterile wet filter paper in sterile petri dishes that were then sealed with parafilm and kept at room temperature. The emergence of hyphae was monitored for 10 days. To evaluate their virulence, conidia were sampled by loop from cadavers from the high boric acid and *M. anisopliae* dust treatment and aseptically transferred to sterile PPBT. This suspension was then used to inoculate the rice substrate. Harvested conidia were used for the virulence bioassay: 50 adult cockroaches (25 males, 25 females) were dusted with *M. anisopliae* (0.40 g/m^2, as above) and mortality was monitored daily for 28 days.

Statistical Analysis

In all experiments, estimates of survival probabilities and LT_{50} were determined by the Kaplan–Meier procedure (Kalbfleisch and Prentice, 1980) using PROC LIFETEST in SAS version 6.12 for the personal computer (SAS Institute Inc., 1996). Log-rank test (SAS Institute Inc., 1996) was used to compare survival curves for different treatments (Kalbfleisch and Prentice, 1980). Binomial test and comparison of the expected (P_E) and observed (P_0) percentage mortality was used for analysis of additive, synergistic, and antagonistic interactions (Nishimatsu and Jackson, 1998).

RESULTS

A concentration of 0.2 g boric acid dust/m^2 caused 100% mortality in 15 days (LT_{50} = 5 days), while lower concentrations failed to kill all cockroaches in the 28-day bioassay (Table 1, Fig. 1A). The lowest concentration tested, 0.025 g boric acid dust/m^2, killed only 9.5% of the cockroach population (Table 1).

The highest concentration of *M. anisopliae* conidial dust (0.4 g conidia/m^2, equivalent to 8.96×10^9 conidia) killed 92.5% of the cockroaches, while a 10-fold lower concentration of the fungus resulted in only 36.5% mortality within 28 days of exposure (Table 1); *M. anisopliae* grew from all cadavers in the fungal treatments (Table 1).

Combined applications of 0.4 g fungus/m^2 and 0.2 g (high concentration) or 0.05 g (medium concentration) boric acid/m^2 resulted in much faster mortality, suggesting a high compatibility of these two insecticides. Both concentrations of the combined dust application killed 100% of the cockroaches within 8 days (LT_{50} = 3 days for the high concentration, LT_{50} = 5 days for the medium concentration) (Figs. 1A and 1B, Table 1), significantly faster than either the fungus alone or the high (Fig. 1A) and medium (Fig. 1B) concentrations of boric acid alone. Similarly, the combined dusting with a low concentration of boric acid (0.025 g/m^2) and a low concentration of *M. anisopliae* (0.04 g/m^2), which alone killed only 9.5 and 36.5% of the cockroaches, respec-

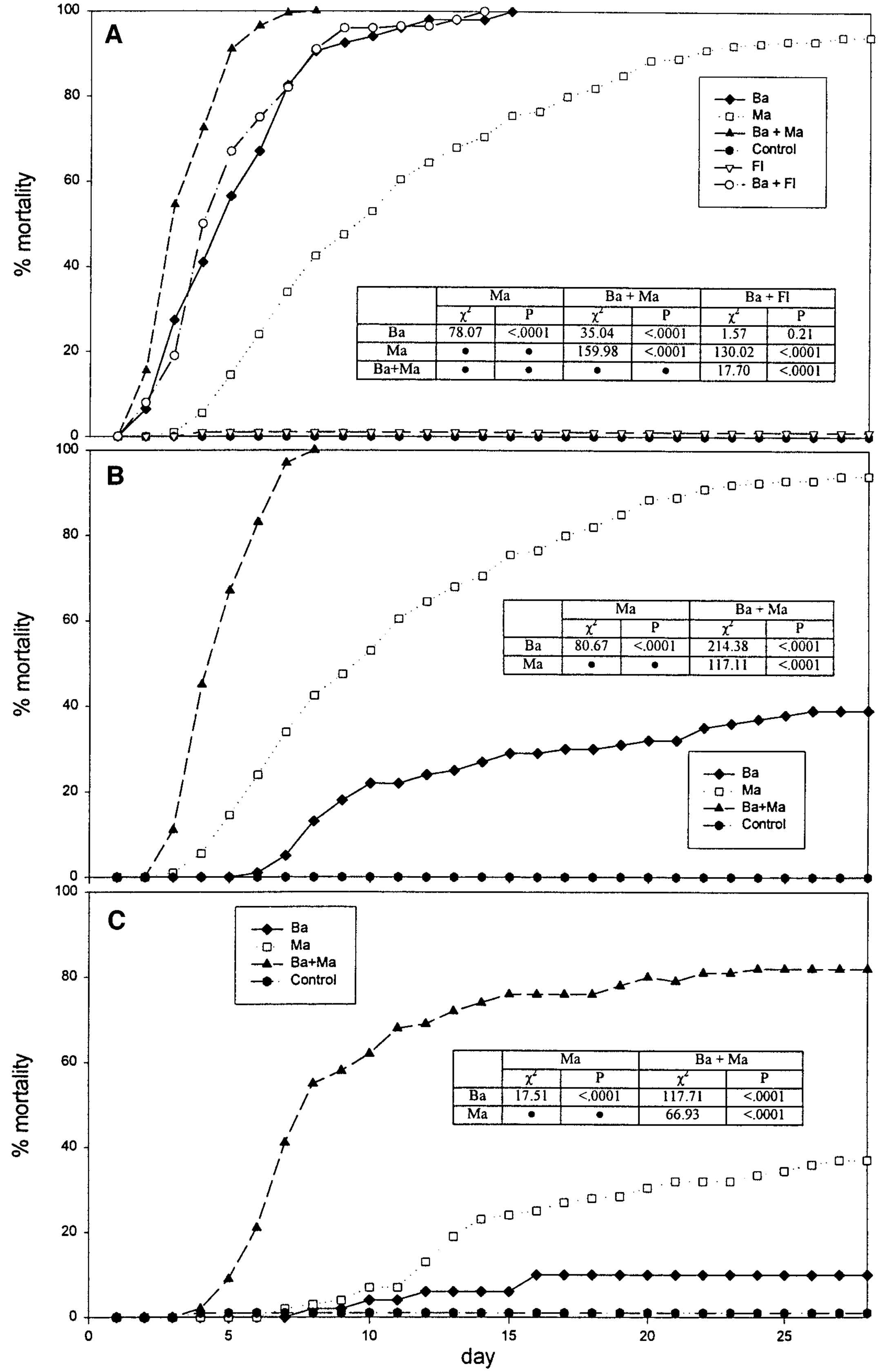
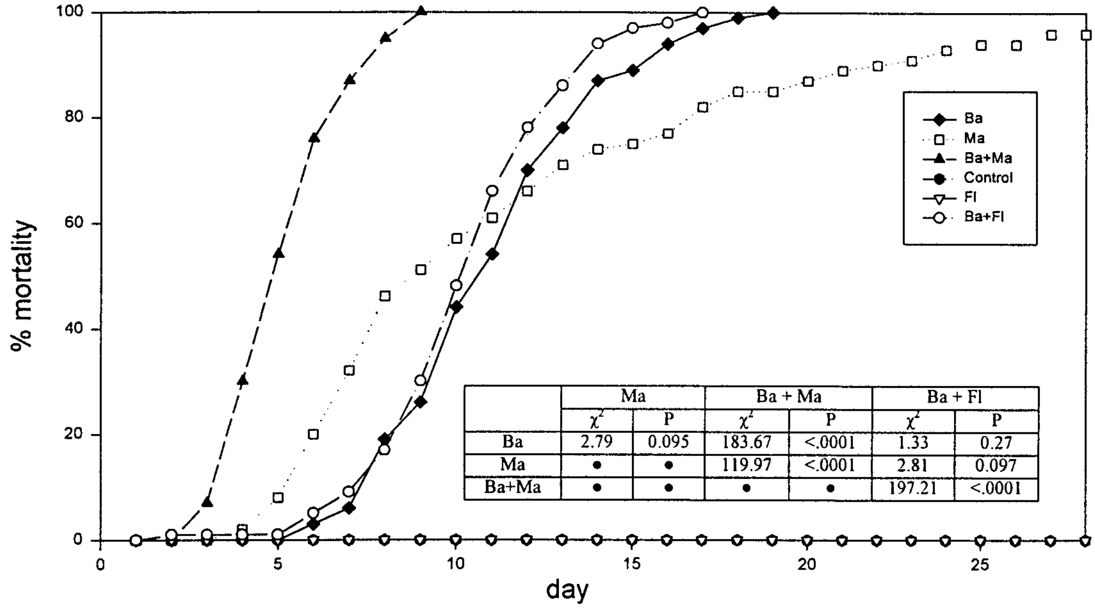


FIG. 1. Mean cumulative mortality of *Blattella germanica* exposed to dust treatments. Fifty adult cockroaches (25 males, 25 females) were dusted with boric acid (Ba), *Metarhizium anisopliae* (Ma), or flour (Fl) in various combinations. Each bioassay was replicated twice. Log-rank test (SAS Institute Inc., 1996) was used to compare survival curves for different treatments. (A) High concentration; (B) medium concentration; (C) low concentration.



	Ma		Ba + Ma		Ba + Fl	
	χ^2	P	χ^2	P	χ^2	P
Ba	2.79	0.095	183.67	<.0001	1.33	0.27
Ma	•	•	119.97	<.0001	2.81	0.097
Ba+Ma	•	•	•	•	197.21	<.0001

FIG. 2. Mean cumulative mortality of *Blattella germanica* exposed to combinations of dusts and boric acid (Ba) in drinking water. Fifty adult cockroaches (25 males, 25 females) were exposed to Ba in drinking water and dusted with *Metarhizium anisopliae* (Ma) or flour (Fl). Each bioassay was replicated twice. Log-rank test (SAS Institute Inc., 1996) was used to compare survival curves for different treatments.

tively, significantly elevated cockroach mortality to 83% (Table 1, Fig. 1C). Statistical analysis for additive, synergistic, and antagonistic interaction clearly demonstrated a synergistic interaction (P_0 = 83.0%, P_E = 43.1%, χ^2 = 65.3, $P < 0.01$). As before, fungus emerged from 86–100% of the cadavers in these treatments, indicating that boric acid did not compromise the virulence of the fungus (Table 1). Moreover, virulence bioassays with conidia produced from the emerged fungus killed 92.0% of the cockroach population within 28 days after exposure.

Dusting cockroaches with 0.4 g flour/m^2 alone did not cause any mortality (Table 1), and in combination with 0.2 g boric acid/m^2, flour did not enhance the insecticidal effects of boric acid (Table 1, Fig. 1A). The same results were observed with the application of the heat-inactivated conidial dust instead of the flour dust (mortality from Ba, Mah, Ba + Mah, and Control was 100% within 13 days, 0% within 28 days, 100% within 12 days, and 0% within 28 days, respectively). These results suggest that the synergistic interaction of boric acid with *M. anisopliae* is unlikely due to elevated ingestion of dust particles (flour or inactivated conidia).

Injected boric acid was relatively nonlethal to cockroaches, except at high concentration. A dose of 100 μg boric acid (2 μl of a 5% boric acid solution) killed all adult females within 8 days but doses of $\leq$50 μg failed to kill any cockroaches within 28 days (Table 2). The sublethal dose of 50 μg was therefore selected for combined injections with *M. anisopliae.*

Injection of a high dose of *M. anisopliae* conidia (5.1 $\times$ 10^4) killed all cockroaches within 4 days (LT_{50} = 2 days) while 10- and 100-fold lower doses caused only 60 and 30% mortality, respectively (Table 2). As before, cotreatment with the fungus and boric acid resulted in faster mortality than either insecticide alone. Injection of a sublethal dose of boric acid (50 μg) together with 5.1 $\times$ 10^3 *M. anisopliae* conidia resulted in 100% mortality within 11 days (LT_{50} = 5 days), compared with no mortality with boric acid alone and 60% mortality with fungus alone (Table 2). Injection of 50 μg boric acid with only 5.1 $\times$ 10^2 conidia killed 65% of the cockroaches within 28 days (LT_{50} = 8 days), compared with 30% when the fungus was injected alone (Table 2). Fungus grew from all the cadavers (Table 2).

Finally, the efficacy of the interaction between boric acid and the fungus was evaluated with boric acid delivered in drinking water. Aqueous 0.1% boric acid killed all cockroaches in 20 days (LT_{50} = 11 days, 95% Confidence Interval (CI) = 10–12 days), whereas dusting with a high concentration of *M. anisopliae* (0.4 g/m^2 = 8.96 $\times$ 10^9 conidia) killed 96.2% of the cockroaches in 28 days (LT_{50} = 10 days, 95% CI = 9–12 days) (Fig. 2). In contrast, the combination of boric acid and *M. anisopliae* killed all cockroaches in only 10 days (LT_{50} = 6 days, 95% CI = 5–6 days), a significant acceleration of mortality (Fig. 2) without compromising fungus emergence from cadavers (not shown). Flour dust by itself did not kill any of the cockroaches, and when dusted in combination with 0.1% boric acid in drinking water, it had no significant effect on cockroach mortality (Fig. 2).

DISCUSSION

Our results demonstrate a synergistic interaction between boric acid and *M. anisopliae.* Sublethal concentrations of boric acid dust, combined with low concentrations of *M. anisopliae,* resulted in 40–45% greater mortality in German cockroaches. Although the principle of the synergy is not known, it is clear that German cockroaches challenged with a sublethal dose of boric acid are more susceptible to *M. anisopliae* infection.

Virulence of *M. anisopliae* was greatly enhanced and cockroach mortality significantly accelerated by 20 days with the addition of 12.5% boric acid (w/w). It is important to emphasize that fungus emergence from the cadavers was generally not compromised. In the high-boric acid treatment, however, the fungus emerged from only 86% of the cadavers, because cockroaches that died within the first 2 days did not produce any hyphae. These cockroaches probably died from boric acid exposure before the fungus penetrated the cuticle and/or infected the hemocoel. In addition, results of the virulence bioassay show that conidia produced from emerged fungus on cadavers retained the same virulence as that of the original inoculum.

We hypothesized that the fungal conidia might accelerate ingestion of boric acid dust by eliciting grooming in cockroaches. However, replacement of the fungus with an inert flour dust or heat-killed conidia eliminated the synergistic effect, showing that the effect is specific to viable *M. anisopliae.* We also reduced or eliminated the dust load by formulating boric acid in drinking water or injecting both insecticidal agents into the hemocoel. In both cases, mortality of cockroaches was significantly accelerated, confirming that the interaction of *M. anisopliae* and boric acid is independent of the formulation. These data further suggest that although boric acid dust might abrade the cuticle (Ebeling, 1995), this effect probably does not make cockroaches more susceptible to *M. anisopliae* conidial dust.

Although the toxic mode of action of boric acid against insects remains unknown, destruction of the gut wall of *B. germanica* after boric acid ingestion has been suggested (Ebeling, 1995). Based on a histological study of the digestive tract of *B. germanica* after ingestion of boric acid, Cochran (1995b) concluded that destruction of the foregut epithelium might result in death from starvation. This is unlikely, however, because cockroaches exposed to boric acid die much faster than starved cockroaches (C. Schal, personal observation). Moreover, boric acid kills cockroaches when injected directly into the hemolymph (Table 2), showing that mechanical destruction of the digestive tract is not requisite for its insecticidal activity. Penetration of the cuticle of *B. germanica* by boric acid dust was also reported (Ebeling, 1995).

M. anisopliae enters the insect hemocoel after penetration of the exoskeleton, which is then followed by the production of hyphae and toxins that kill the host. We have shown that conidia injected directly into the cockroach hemolymph can germinate and kill the host, although relatively high doses are necessary to achieve 100% mortality.

The nature of the interaction between boric acid and *M. anisopliae* may be physicochemical or immunologically based. The various experimental treatments suggest that low concentrations of boric acid enhance the pathogenic activity of the fungus and not vice versa. Boric acid might physically damage the gut cuticle, thus facilitating penetration of *M. anisopliae.* The acid might also reduce hemolymph pH and alter its osmolarity in favor of fungal infection. It is also possible that boric acid, the fungus, or both, compromise the immune response of the cockroach, making it more susceptible to infection.

Several studies have focused on the potential use of entomopathogenic fungi primarily, *Beauveria bassiana* (Balsamo) Vuillemin and *M. anisopliae,* in combination with sublethal doses of organic insecticides against various insect pests (Anderson *et al.*, 1989; Moorhouse *et al.*, 1992; Li and Holdom, 1994; Quintela and McCoy, 1997, 1998; Furlong and Groden, 2001). Most recently, the compatibility (increased mortality and lower LT_{50}) of *M. anisopliae* was demonstrated with imidacloprid (Kaakeh *et al.*, 1997), chlorpyrifos, prometamphos, and cyfluthrin (Pachamutu *et al.*, 1999; Pachamutu and Kamble, 2000) against the German cockroach. Boric acid also enhanced the virulence of *Bacillus thuringiensis* (Berliner) subsp. *kurstaki* against *Mamestra configurata* (Walker) (Morris *et al.*, 1995) and nucleopolyhedrovirus against *Lymantria dispar* (L.) (Shapiro and Bell, 1982). Although the principle of the interaction is not known, the combined applications offer additional and safer methods of pest control while retaining a fast mode of action.

In conclusion, this study demonstrates a synergistic interaction between biological (*M. anisopliae*) and inorganic (boric acid) insecticides, each of which has a favorable human safety record. The main advantage of using *M. anisopliae*–boric acid combinations is a fast mode of action without compromising the fungus growth from cadavers that is crucial for inducing epizootics in cockroach populations.

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