Efficacy of *Beauveria bassiana* for Red Flour Beetle When Applied with Plant Essential Oils or in Mineral Oil and Organosilicone Carriers

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ABSTRACT The carriers mineral oil and Silwet L-77 and the botanical insecticides Neemix 4.5 and Hexacide were evaluated for their impacts on the efficacy of *Beauveria bassiana* (Balsamo) Vuillemin conidia against red flour beetle, *Tribolium castaneum* (Herbst), larvae. The dosages of liquid treatments were quantified by both conidia concentration in the spray volume and conidia deposition on the target surface. The latter approach allowed comparison with dry, unformulated conidia. The median lethal concentrations of *B. bassiana* in 0.05% Silwet L-77 solution or without a carrier were approximately double that for conidia in mineral oil. Carriers had highly significant effects on the efficacy of *B. bassiana*. The lower efficacy of conidia in aqueous Silwet L-77 may have been the result of conidia loss from the larval surface because of the siloxane's spreading properties. Neemix 4.5 (4.5% azadirachtin) delayed pupation and did not reduce the germination rate of *B. bassiana* conidia, but it significantly reduced *T. castaneum* mortality at two of four tested fungus doses. Hexacide (5% rosemary oil) caused significant mortality when applied without *B. bassiana*, but it did not affect pupation, the germination rate of conidia, or *T. castaneum* mortality when used in combination with the fungus.

KEY WORDS *Beauveria bassiana*, mineral oil, Silwet L-77, botanical insecticides, *Tribolium castaneum*

OILS AND WETTING AGENTS have been extensively investigated and adopted as means of enhancing the delivery, persistence, and efficacy of mycoinsecticides. Oil-based formulations of *Beauveria bassiana* (Balsamo) Vuillemin were introduced by Prior et al. (1988), who reported coconut oil to be a more efficient carrier of *B. bassiana* conidia than 0.01% aqueous Tween 80 for the weevil *Pantorhytes plutus* (Oberthur). They suggested that oil-based formulations have the advantage of allowing better adhesion of conidia to the hydrophobic cuticle of insects and reported better conidial survival in coconut oil than in water. More recently, oil-based formulations of mycopesticides have been tested against various insect pests with positive results (Bateman et al. 1993; Filho et al. 1995; Inglis et al. 1996a, b; Hidalgo et al. 1998; Legaspi et al. 2000; Malsam et al. 2002). Because of the lipophilic nature of phialoconidia that do not bear a mucus coating, they can be easily suspended in oils to achieve greater efficacies than when used in water (Bateman et al. 1993, David-Henriet et al. 1998). Cottonseed, soybean, and mineral oils reportedly do not adversely affect the viability of *B. bassiana* (Smart and Wright 1992, Grimm 2001). Bateman et al. (1993) speculated that, for *Metarhizium anisopliae* (Metschnikoff) Sorokin variety *acridium* Driver and Milner (formerly identified as *Metarhizium flavoviride* Gams & Rozsypal), the need for high relative humidity and dosage could be reduced if its conidia were formulated in oil. Like oils, organosilicone surfactants are widely used in formulating and tank-mixing pesticides (Stevens 1993). Silwet L-77 is an organosilicone that is commonly used to prepare spray suspensions of *B. bassiana*, for which a wetting agent is needed to suspend the hydrophobic conidia in water.

Naturally occurring botanical insecticides such as neem oil and other plant essential oils, defined as sources of characteristic plant odors, have long been used in Asia for protection against stored-grain insect pests. More recently, researchers in the Western Hemisphere have begun to assess their use as alternatives to fumigants and other chemical insecticides (Su 1991, Rice and Coats 1994, Ho et al. 1997, Isman 2000, Enan 2001, Wang et al. 2001). The main active component in neem seed extract is azadirachtin, which has deterrent, antifeedent, anti-ovipositional, growth regulating, and fecundity-reducing effects on various insects (Schmutterer 1990). Repellent, as well

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as growth-regulating, effects of neem on some stored-product insects, including the red flour beetle, *Tribolium castaneum* (Herbst), have been reported previously (Jilani and Su 1983, Jilani et al. 1988). Xie et al. (1995) found that concentrations of azadirachtin as low as 10 ppm repelled *T. castaneum;* rusty grain beetle, *Cryptolestes ferrugineus* (Stephens); and rice weevil, *Sitophilus oryzae* (L.), with *T. castaneum* being the most sensitive.

Some plant essential oils block the octopamine neuroreceptors that regulate the movement, heart rate, behavior, metabolism, and pupation of insects (Hirashima et al. 1998, Enan 2001). Hexacide is an organically certified insecticide that is labeled for use in agronomic and horticultural crops against a broad range of insect pests (EcoSmart Technologies Inc. 2002). Its active ingredient is essential oil of rosemary, an octopamine antagonist. *T. castaneum* has shown susceptibility to various essential oils (Sighamony et al. 1984, Su 1991, Rice and Coats 1994, Huang et al. 1997, Liu and Ho 1999, Tripathi et al. 2000), but the effectiveness of essential oil of rosemary against *T. castaneum* is as yet unknown.

Entomopathogenic fungi such as *B. bassiana* adhere to, germinate on, and penetrate through their host integument to achieve infection. Attached conidia may be shed during ecdysis without completing penetration. We hypothesized that the prolongation of the intermolt and prepupal periods caused by the reported growth-regulating action of botanicals such as neem and essential oil of rosemary may extend the intermolt period during which the fungus could establish itself on the host cuticle and complete penetration. It is also possible that the botanicals can alter the insect's behavior in a manner that reduces loss of conidia, as has been suggested for some insects when treated with imidacloprid and fungi (Boucias et al. 1996; Quintela and McCoy 1998a, b).

We chose *T. castaneum* as the target insect for these studies because it is one of the most important and difficult to control insects among the stored-grain pests. Our previous testing of unformulated conidia of *B. bassiana* against *T. castaneum* adults and larvae showed them to have insufficient efficacy for commercial use (Akbar et al. 2004). Therefore, we proposed that greater efficacy might be achieved via carrier effects or combination with other agents. In most previous studies, the dose effects of entomopathogenic fungi in liquid carriers have been reported with dosage quantification in terms of conidia or colony-forming units per milliliter of suspension, but, to our knowledge, the relationship between the concentration of conidia in the applied suspension and the number that actually impinge on the target have not been reported. In the present article, we use two approaches to the dose quantification: spray concentrations and number of conidia per unit area. The latter is not only a more direct measure but also allows for efficacy comparisons with conidia in their unaltered, dry condition. Our objectives were 1) to determine the effects of two widely used carriers on the efficacy of *B. bassiana;* 2) to compare methods of dose quantification in spray bioassays; and 3) to assess the interaction of *B. bassiana* with the minimally toxic botanical insecticides, neem, and rosemary oil.

Materials and Methods

Insects and Fungus. The experiments were conducted at the USDA Grain Marketing and Production Research Center, Manhattan, KS, with a colony of *T. castaneum* that has been maintained in the laboratory since 1958 and has eastern Kansas origin. Commercially produced, unformulated conidia of *B. bassiana* strain GHA, provided by Emerald BioAgriculture, Butte, MT, were used in all experiments. The *B. bassiana* technical powder contained 9.4×10^{10} conidia/g. To check for viability, the conidia were spread on Sabouraud dextrose agar (SDA) with a cotton swab, and the presence or absence of germ tubes on 200 conidia was recorded after 18 h of incubation at 26°C. Germination rates were at least 90% throughout the study.

Application of Conidia with or without Carriers. We used light, white mineral oil (Aldrich Chemical Co., Milwaukee, WI) and a solution of Silwet L-77 (Loveland Industries, Greeley, CO) as carriers for *B. bassiana* conidia in spray applications to *T. castaneum* larvae. Conidia were suspended in oil or 0.05% Silwet L-77 at five concentrations: 2.5, 5, 10, 15, and 20 mg of conidia/ml. Twenty larvae, 15 d postoviposition, were placed in 90-mm polystyrene petri dishes and were sprayed with 40 μl of each concentration by using a Badger model 100 Airbrush spray apparatus (Air-Brush Spray Co., Franklin Park, IL) at a pressure of 62 kPa and a distance of 23 cm. In our preliminary tests, 40 μl of mineral oil or 0.05% Silwet L-77 caused <10% mortality. A vacuum desiccator was used to apply dry, unformulated conidia. Twenty larvae were placed in a petri dish that was placed inside a desiccator with conidia on a small weighing paper. Vacuum was drawn in the desiccator for 2 min and then rapidly released to disperse the conidia. After applications, the larvae were transferred to clean petri dishes and provided with 30 mg of flour. The larval mortality was recorded after 8 days of incubation at 75 ± 1% RH (over saturated NaCl) and 26 ± 1°C. Carrier controls and untreated controls were handled in the same manner. With each application, four glass coverslips (18 by 18 mm) also were placed in each petri dish to quantify the surface distribution of conidia. Each coverslip was washed in a small beaker with 2 ml of 0.2% Silwet L-77 solution and a stir bar rotated at high speed for 1 min. The conidia were counted with a hemacytometer with phase contrast illumination. Two counts were made for each wash. Treatment effects were evaluated by spray concentration and by deposition of conidia. There were six replications of each treatment in one experiment, and the experiments were carried out three times.

Tests for Interactions between *B. bassiana* and Botanical Insecticides. Two insecticidal botanical oils, Hexacide (active ingredient 5.0% rosemary oil) (EcoSmart Technologies Inc., Franklin, TN) and Nee-

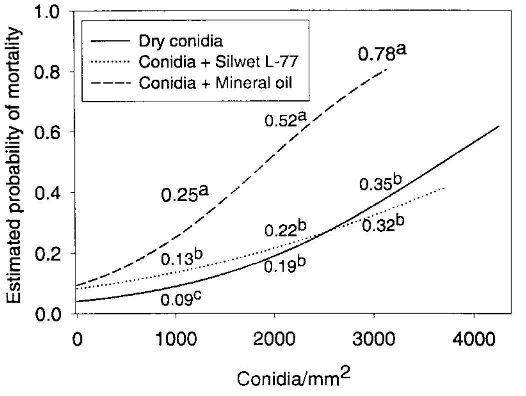

Fig. 1. Dose-mortality estimate curves of *B. bassiana* against *T. castaneum* larvae, with and without a carrier, on the basis of the number of conidia delivered per square millimeter. The estimated probabilities of mortality at each concentration followed by same letter do not differ significantly from each other at $P > 0.05$.

mix 4.5 (active ingredient 4.5% azadirachtin) (Certis USA, Columbia, MD) were tested to determine whether they enhanced *B. bassiana* efficacy. Conidia were mixed thoroughly into crimped hard red winter wheat kernels (moisture content 11.6%) at rates of 0, 125, 250, 500, and 1000 mg/kg. Ten-grams lots of the grain were transferred to plastic weigh boats and were sprayed with 40 μl of botanical oil with an airbrush spray apparatus as described previously or not treated with oil. The treated grain was poured into 30-ml plastic cups, and 16 beetle larvae were placed in each cup. To allow growth regulator effects on pupation during the assay, the larvae were in the fourth instar and were cultured at 30°C for 20 d from hatch. There were three cups per treatment and the experiment was replicated four times, with different generations of insects. Incubation was at 75 ± 1% RH and 26 ± 1°C for 8 d before pupation rates and mortality of all stages were scored. Pupation rates were analyzed for survivors of fungus-free treatments only, providing a conservative estimate of the oils' growth regulator effects.

The effect of the oils on germination of *B. bassiana* was assessed with conidia in nine cups per treatment. The conidia were washed from the grain with 0.1% Silwet L-77, and 0.1 ml of each wash was spread on SDA. After 18 h at 26°C, 100 conidia per replicate in clump-free zones of the agar were scored for presence or absence of a germ tube.

Data Analysis. For each carrier treatment of *B. bassiana* conidia, the larval responses were analyzed by using a logistic regression model. Goodness-of-fit was checked using deviance/DF statistics and was always close to 1. The estimates for regression coefficients were obtained with maximum-likelihood estimation procedures in PROC GENMOD (SAS Institute 2002). Separate curves were fitted for each treatment in which significant treatment by *B. bassiana* interactions were observed. Pairwise comparisons among treatments were done at selected conidia per square millimeter counts and at tested concentrations of suspensions. The LC_{25} values with 95% confidence interval (CI) values of *B. bassiana* conidia, alone and in combination with a carrier, were calculated from PROC GENMOD output (SAS Institute 2002). The data for *B. bassiana* with botanical insecticides were assessed with PROC GLM with Fisher's least significant difference. The mortality and pupation data were transformed to arcsines; germination data were not.

Voucher Specimens. Specimens of *T. castaneum* have been placed in The Kansas State University Museum of Entomological and Prairie Arthropod Research, Department of Entomology, Kansas State University, under voucher number 148.

Results

For each of the three selected conidial densities, the probability of *T. castaneum* larval mortality was significantly greater when the conidia were in mineral oil than when they were mixed in Silwet L-77 or applied without a carrier, both in number of conidia per unit area (Fig. 1) and in concentration of conidia per unit volume of the carrier (Table 1). At the lowest deposition rate (1000 conidia/mm^2), the efficacies of the three preparations differed significantly from each other ($P < 0.05$ (Fig. 1). At the two higher deposition rates, 2,000 and 3,000 conidia/mm^2, the efficacies of dry conidia and conidia in Silwet L-77 did not differ significantly from each other ($P > 0.05$), but both had significantly less efficacy than the conidia in mineral oil. The estimated LC_{25} values were 2,292 conidia/mm^2 (95% CI = 2,092–2,505 conidia/mm^2) for the dry conidia, 2,830 conidia/mm^2 (95% CI = 2,144–3,572 conidia/mm^2) for conidia in Silwet L-77, and 1,053 conidia/mm^2 (95% CI = 780–1,270 conidia/mm^2) in mineral oil.

The pairwise efficacy comparison of conidia in mineral oil and Silwet L-77 on the basis of suspension

Table 1. Pairwise comparison of probabilities of mortalities of *T. castaneum* larvae at tested concentrations of conidia in Silwet L-77 and mineral oil

Carrier	Concn (mg of conidia/ml of carrier)					
	0[a]	2.5	5	10	15	20
	Probability of mortality (95% CI)[b]					
Mineral oil	0.12 (0.09–0.15)a	0.14 (0.12–0.16)a	0.18 (0.16–0.20)a	0.28 (0.26–0.30)a	0.42 (0.39–0.45)a	0.57 (0.53–0.61)a
Silwet L-77	0.08 (0.07–0.10)a	0.10 (0.08–0.12)b	0.12 (0.10–0.13)b	0.17 (0.15–0.18)b	0.23 (0.21–0.25)b	0.31 (0.27–0.35)b

[a] 40 μl of carrier alone.
[b] Probabilities within columns followed by same letter do not differ significantly from each other at $P > 0.05$.

Table 2. Percentage mortality ± SE of *T. castaneum* larvae in *B. bassiana*-treated wheat with and without Neemix 4.5 or Hexacide

Oil	*B. bassiana* (mg/kg)				
	0	125	250	500	1000
	% mortality[a]				
Control	0.5 ± 0.5b	50.0 ± 1.5a	49.0 ± 5.9a	62.5 ± 5.4a	67.5 ± 5.7a
Hexacide	5.2 ± 1.0a	36.5 ± 9.4ab	53.7 ± 4.8a	53.1 ± 3.2ab	61.5 ± 6.7a
Neemix 4.5	1.6 ± 0.5b	24.0 ± 5.4b	37.0 ± 1.7a	41.1 ± 3.7b	48.4 ± 3.4a

[a] Mortalities within columns followed by same letter do not differ significantly from each other at $P > 0.05$.

concentrations showed significant differences in the probabilities of mortality at each of the selected concentrations ($P < 0.05$) (Table 1). A decrease in the median lethal concentrations of *B. bassiana* in the presence of mineral oil also was noted. The LC_{50} in the presence of mineral oil was 17.6 mg/ml (95% CI = 16.5–18.8 mg/ml), and the LC_{50} was 29.9 mg/ml (95% CI = 26.5–34.9 mg/ml) when Silwet L-77 was the carrier.

Neither Hexacide nor Neemix 4.5 significantly increased *B. bassiana*'s efficacy (Table 2) for red flour beetle larvae at any of the *B. bassiana* concentrations tested ($P > 0.05$). There was significantly greater mortality of larvae that were exposed to Hexacide without the fungus than of the controls ($F = 11.17$; df = 2, 9; $P = 0.004$), but Hexacide did not significantly change the mortality for any rate of *B. bassiana.* In contrast, although there was no significant difference between control mortality and mortality with fungus-free Neemix 4.5, there was significantly less mortality with Neemix 4.5 than without it at *B. bassiana* rates of 125 mg/kg ($F = 4.28$; df = 2, 9; $P = 0.049$) and 500 mg/kg ($F = 5.97$; df = 2, 9; $P = 0.02$). At *B. bassiana* rates of 250 mg/kg ($F = 3.32$; df = 2, 9; $P = 0.09$) and 1000 mg/kg ($F = 3.18$; df = 2, 9; $P = 0.09$), the reductions in mortality with Neemix 4.5 followed the same trend but were not statistically significant.

Mean pupation of surviving beetles was 94.6% in controls, which was significantly greater than the 66.1% for those treated with Neem without fungus, but not significantly greater than the mean of 82.5% for those treated with Hexacide without fungus ($F = 4.34$; df = 2, 9; $P = 0.048$). The mean germination rates of conidia after incubation, with or without the oils, remained in excess of 90% and did not differ significantly among the treatments ($F = 2.95$; df = 2, 24; $P = 0.07$).

Discussion

Improvements in the efficacy of entomopathogenic fungi by using oil formulations have been reported for a wide range of insect pests, including locusts (Lomer et al. 1993, Milner et al. 1994), thrips (Van der Pas et al. 1998), and beetles (Ibrahim et al. 1999, Wraight and Ramos 2002), but comparisons among different carriers were lacking. Our results indicate that mineral oil is a more efficient carrier of *B. bassiana* for *T. castaneum* than a Silwet L-77 solution, when quantified either in spray suspension concentrations or in number of conidia that impinge on the target. For the weevil *P. plutus,* Prior et al. (1988) reported a 36-fold decrease in the LD_{50} and a 111-fold decrease in the LD_{95} of *B. bassiana* when formulated in coconut oil versus water. Our findings showed that the median lethal concentration of *B. bassiana* in mineral oil was less than one-half the value for conidia in Silwet L-77 solution. Bateman et al. (1993) reported a similar decrease in the median lethal concentration of *M. anisopliae* variety *acridum* conidia in cottonseed oil compared with suspensions in water, when tested against the locust *Schistocerca gregaria* (Forskal). The authors speculated that the suspension in oil might help the process of infection by compromising the waxy layer of the insect's cuticle. In other studies, better performance of *B. bassiana* conidia in oil has been speculatively attributed to enhanced attachment to the hydrophobic cuticle (Prior et al. 1988) or to a better spread of conidia and better germination rates in oil (Ibrahim et al. 1999). Improved spread may carry conidia into intersegmental membranes where the microclimate and thin cuticle are favorable for infection (Burges 1998). Inglis et al. (1996a, b) reported enhancement of *B. bassiana* efficacy for *Melanoplus sanguinipes* (F.) by oil formulation and demonstrated that fluorescent dye incorporated into oil formulations spread over the insect's surface but dye in water did not. According to Locke (1984), the entomopathogenic fungi may gain entry into the host insect by replacing epicuticular lipids with an aqueous phase. He stated that this would happen in the presence of oil because the lipids in the insect cuticle may rush out, followed by an aqueous cuticular fluid, covering the surface with watery droplets. Under these conditions, conidial germination would be expected to increase.

Treatments in assays with entomopathogenic fungi are frequently quantified in terms of inoculum per unit of spray volume. Here, we assessed dose both by volume and by deposition per unit of area. The latter approach has the considerable advantage of allowing more accurate interpretation of assays and better comparisons among assays with diverse targets. However, it requires considerably more effort, and compared doses must be obtained from regression rather than actual applied rates. It should be noted that, although the deposition rates of conidia in oil and in Silwet L-77 were directly proportional to their concentrations in the carrier, there was large variation among individual sprays for each concentration, demonstrating the need for careful and repetitive applications to obtain reliable data.

Solutions of Silwet L-77 and other siloxane wetting agents have low surface tensions and spread rapidly over the target's surface (Stevens 1993). They also have known insecticidal and miticidal properties (Purcell and Schroeder 1996, Cowles et al. 2000) due to surface wetting and resultant suffocation or the disruption of other physiological processes. In spite of a phytotoxicity risk (Liu and Stansly 2000), siloxane wetting agents are generally considered to be effective carriers of mycoinsecticides. In our studies, *B. bassiana* applications in a Silwet L-77 solution did not increase *T. castaneum* larval mortality, in spite of better spreading than mineral oil suspensions on the glass coverslips that were used for dosage quantification. Paradoxically, the lesser efficacy of conidia in Silwet L-77 solution, compared with oil, may be caused by the improved spreading resulting in a reduced number of conidia per unit area because of runoff. Silwet L-77, although excellent in preparations of spray suspensions, may have some negative consequences for delivery and efficacy of *B. bassiana* in direct applications.

The botanical oils used in this study did not enhance *B. bassiana*'s efficacy for *T. castaneum* larvae. Hexacide caused significant mortality without *B. bassiana,* but when combined with the fungus, the mortality was not significantly different from the mortality with fungus as the sole treatment. Neemix 4.5 significantly reduced the larval mortality at two of the tested conidial concentrations. This was surprising, considering that Neemix 4.5 delayed pupation, as anticipated. Inhibition of *B. bassiana* development by a neem-extract formulation has been reported (Ignatowicz et al. 1998). The formulation that was used in that study consisted primarily of ethyl and methyl alcohols, and the neem extract's role in the inhibition is dubious. The putative antifungal activity was not implicated in reduction of the *B. bassiana* efficacy that we detected, because the mean germination rate was not reduced by incubation in the presence of Neemix 4.5. Neem extracts repel *T. castaneum* (Jilani et al. 1988, Xie et al. 1995), and repellency may have played a role in the reduction of fungal efficacy. The botanical oils were in a heterogeneous mixture with the grain. Perhaps larval movement and consequently contact with conidia was reduced in the presence of Neem oil deposits. The mechanism of the antagonism observed here between Neemix 4.5 and *B. bassiana* is not apparent. It should be noted that our botanical oil dosages approach maximum challenge. Direct application to the larvae with these doses killed many within a few minutes (i.e., by physical means). We conclude that Neemix 4.5 and Hexacide are poor candidates for integration with *B. bassiana* for controlling *T. castaneum.*

Research on the development of suitable formulations of entomopathogenic fungi is necessary to maximize their efficacy and ease of use. Furthermore, widespread acceptance of mycopesticides will depend on the improvement of shelf life and field persistence. Use of oil formulations may allow *B. bassiana* and other fungal pathogens to fulfill these requirements. The reduced efficacy of *B. bassiana* with Silwet L-77 also indicates the potential for runoff of a mycopesticide with a highly efficient surfactant. The U.S. Food and Drug Administration has approved mineral oils for dust control in stored wheat and maize (Rulis 2000). Therefore, we suggest a *B. bassiana*–mineral oil combination as a potentially useful management tool for *T. castaneum* that may be applied in cracks and crevices or directly onto grain.

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