Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Invertebrate Pathology 81 (2002) 175–184
Journal of INVERTEBRATE PATHOLOGY
www.academicpress.com

Hirsutella thompsonii and *Metarhizium anisopliae* as potential microbial control agents of *Varroa destructor*, a honey bee parasite

L.H.B. Kanga,[a,*] R.R. James,[b] and D.G. Boucias[c]

[a] *US Department of Agriculture, Agricultural Research Service, Beneficial Insects Research Unit, 2413 E. Highway 83, Weslaco, TX 78596, USA*
[b] *Department of Biology, US Department of Agriculture, Agricultural Research Service, Bee Biology and Systematics Laboratory, Utah State University, Logan, UT 84322-5310, USA*
[c] *Department of Entomology and Nematology, University of Florida, Gainesville, FL 32611, USA*

Received 23 July 2002; accepted 15 October 2002

Abstract

The potential of *Hirsutella thompsonii* Fisher and *Metarhizium anisopliae* (Metschinkoff) as biological control agents of the parasitic mite, *Varroa destructor* Anderson and Trueman was evaluated in the laboratory and in observation hives. In the laboratory, time required for 90% cumulative mortality of mites (LT_{90}) was 4.16 (3.98–4.42) days for *H. thompsonii* and 5.85 (5.48–7.43) days for *M. anisopliae* at 1.1×10^3 conidia mm^{-2}. At a temperature ($34 \pm 1\,^{\circ}C$) similar to that of the broodnest in a honey bee colony, *Apis mellifera* L., *H. thompsonii* [$LC_{90} = 9.90 \times 10^1$ (5.86–19.35) conidia mm^{-2} at Day 7] and *M. anisopliae* [$LC_{90} = 7.13 \times 10^3$ (2.80–23.45) conidia mm^{-2} at Day 7] both showed significant virulence against *V. destructor*. The applications of *H. thompsonii* to observation hives resulted in significant mortality of mites, and reduction of the number of mites per bee 21 and 42 days post-treatments. The treatments did not significantly affect the mite population in sealed brood. However, the fungus must have persisted because infected mites were still observed [$82.97 \pm (0.6)\%$] 42 days post-treatment. In addition, the fungus was found to sporulate on the host. A small percentage [$2.86 \pm (0.2)\%$] of dead mites found in the control hives also showed fungal infection, suggesting that adult bees drifted between hives and disseminated the fungus. *H. thompsonii* was harmless to the honey bees at the concentrations applied and did not have any deleterious effects on the fecundity of the queens. Microbial control with fungal pathogens provides promising new avenues for control of *V. destructor* and could be a useful component of an integrated pest management program for the honey bee industry.
Published by Elsevier Science (USA).

Keywords: *Varroa destructor*; *Apis mellifera*; *Hirsutella thompsonii*; *Metarhizium anisopliae*; Microbial control; Observation hive

1. Introduction

The honey bee, *Apis mellifera* L., is critical for crop pollination and honey production. The ecto-parasitic mite *Varroa destructor* Anderson and Trueman (formerly called *Varroa jacobsoni* Oudemans) is currently a worldwide and serious threat to beekeeping (De Jong et al., 1982). This parasitic mite causes weight loss, malformation, a shortened life span in honey bees (De Jong et al., 1982), and serves as a vector of virus diseases (Ball, 1994; Békési et al., 1999). Without adequate control of *Varroa* infestations, bee mortality approaches 100% in untreated colonies, which can perish within a few weeks (De Jong et al., 1982). The unique biology of this parasite has precluded many control strategies. Except for the mated female mite, all developmental stages live in the brood cells and feed on the hemolymph of developing honey bees. Adult female mites feed on both adult and immature honey bees, but reproduce only inside capped brood cells. It is because much of the life cycle of the mite occurs inside the capped brood cell that control is difficult.

Several ways to control *Varroa* have been developed, these include biotechnical, genetic, and chemical controls. Biotechnical control measures (e.g., natural products smoke and mite trapping devices) are labor-intensive

* Corresponding author. Fax: 1-956-969-4888.
E-mail address: lkanga@weslaco.ars.usda.gov (L.H.B. Kanga).

0022-2011/02/$ - see front matter. Published by Elsevier Science (USA).
PII: S0022-2011(02)00177-5

and provide various degrees of success (Fries and Hansen, 1993; Schmidt-Bailey et al., 1996). Chemical treatments are also necessary at present to ensure colony survival within the breeding programs for stocks resistant to *Varroa* in North America and Europe (Büchler, 1994; De Guzman and Delfinado-Baker, 1996; Rinderer et al., 1997). The major chemical control strategy is the use of plastic strips coated with the synthetic pyrethroid fluvalinate (Apistan®,[1] Wellmark International, Bensenville, IL), which are placed in the brood chamber either before or after honey flow. Although Apistan® strips have been very effective in controlling mites (Ferrer-Dufol et al., 1991), resistance has developed in several mite populations (Elzen et al., 1998; Milani, 1999). In addition, if improperly used, they can leave residues in wax and honey (Cabras et al., 1997; Wallner, 1999). More recently, plastic strips coated with the organophosphate coumaphos (CheckMite®, Bayer Shawnee Mission, KS) have been used under emergency registration in the United States (Sanford et al., 1999), but some resistance has already developed to this compound (Elzen and Westervelt, 2002). Chemical control agents, such as flumethrin, amitraz, cymiazole, and bromopropylate can leave toxic residues (Gamber, 1990; Lodesani et al., 1992; Wallner, 1999), and are difficult to register in North America. Organic acids (formic, lactic, and oxalic acids) have been investigated as a control strategy. Efficacy of these compounds depends on their ability to become volatile within the hive, and so most are temperature-dependent (Hoppe et al., 1989; Imdorf et al., 1999; Kraus and Berg, 1994; Mutinelli et al., 1997; Veen et al., 1998). Essential oils and essential oil components have also been investigated, but so far, they also lack consistency in their efficacy (Imdorf et al., 1999). Another promising approach has been the development of genetically resistant honey bee lines from queen bees originally collected in Russia (Rinderer et al., 1997, 2001).

The recent development of resistance to miticides in *V. destructor* populations has caused great concern to the honey bee industry (Elzen et al., 1998; Milani, 1999). As a result, there is an urgent need for alternative control strategies that are cost effective, environmentally friendly, and with no mammalian toxicity.

Entomopathogenic fungi offer promise for biological control of *Varroa* because they infect their arthropod hosts directly through the exoskeleton and do not have to be consumed (Chandler et al., 2000). In addition, certain fungus-based products can be inexpensive to produce, and the registration process for microbial pesticides is sometime less extensive than for chemical pesticides in the US and Canada. However, unlike chemical miticides, fungal pathogens often take between 3 and 10 days to kill their hosts, and most fungal pathogens will only germinate between 25 and 32 °C (Chandler et al., 2000), and so may not be pathogenic at brood nest temperatures (~35 °C) (Kraus et al., 1998).

Here, we present the results of several studies conducted under laboratory conditions and in observation hives to investigate: (a) the pathogenicity of *Hirsutella thompsonii* Fisher and *Metarhizium anisopliae* (Metschinkoff) against *V. destructor*, (b) the effectiveness of *H. thompsonii* in controlling *V. destructor* in observation hives, and (c) the effect of *H. thompsonii* on honey bee populations.

2. Materials and methods

2.1. Mite collection

To test the pathogenicity of selected isolates of entomopathogenic fungi against *V. destructor* under laboratory conditions, we collected female mites from infested frames of sealed brood taken from honey bee colonies maintained at the USDA/ARS Center in Weslaco, TX. We opened drone and worker brood cells and female mites were collected from larvae and pupae using a camel-hair brush. Additional mites were collected from nurse and adult bees on the frames. The mites were placed into glass scintillation vials (20 ml) containing two late instar honey bee larvae as food source. Twenty to thirty mites were held in each glass vial and used in the bioassays within an hour after they were collected.

2.2. Fungal cultures and production of conidia

The fungi selected for assays were *H. thompsonii* UF1 5858, a polyblastic strain isolated from the citrus rust mite, *Phyllocoptruta oleivora* L. (Prostigmata: Eriophyidae) and *M. anisopliae* (Bio-Blast®, EcoScience, New Brunswick, NJ). Stock cultures of *H. thompsonii* were stored at −78 °C in a Revco Ultima II freezer (Revco/Lindberg, Asheville, NC). The isolates were cultured on potato dextrose agar (PDA), conidia suspensions were held in 1.5-ml centrifuge tubes, and kept at −78 °C. Subcultures from the conidial suspensions were grown one time on PDA, incubated at 25 ± 1 °C, 85% RH, and 13:11 (L:D) h photoperiod, and used in each laboratory experiment. *H. thompsonii* conidia were harvested from 19 to 21-day-old cultures by flooding the plates with 5 ml of a sterile solution of 0.01% Triton X-100 in deionized water, and working the conidia loose from the culture with a sterile metal spreader. The conidia suspension was filtered through sterile Miracloth (Calbiochem, La Jolla, CA) and then through sterilized paper filters (4–6 μm pore size). Conidia

[1] Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

suspensions were washed once with 5 ml solution of 0.01% Triton X-100 and concentrated by centrifuging at 5000 rpm for 20 min. The conidia pellets were resuspended in sterile deionized water to produce the conidia concentrations desired for the bioassays. Conidia concentration was determined using a hemocytometer.

In all experiments conducted with *M. anisopliae*, we used the commercial product Bio-Blast® as supplied by the manufacturer. The Bio-Blast® used in the experiments was a formulated product containing about of 4×10^9 viable conidia per gram. The fungus was serially diluted in a solution of 0.02% Silwet L-77® (Loveland Industries, Greeley, CO) to provide the concentrations needed for the bioassays.

2.3. Laboratory bioassays

2.3.1. Treatments

Both isolates were tested at different concentrations; 10^5, 10^6, 10^7, 10^8, and 10^9 conidia ml^{-1} resulting in coverage of 0.53, 5.30, 5.30×10^1, 5.30×10^2, and 5.30×10^3 conidia mm^{-2}, respectively. This experiment was repeated on four different dates and mite mortalities recorded daily for 14 days. For each concentration, 20–30 mites were transferred to a glass petri plate containing a wet Whatman filter paper (90 mm diameter) (Whatman International, Maidstone, England) and sprayed with 1 ml of the conidial suspension using a Potter Precision Spray Tower (Burkhard Manufacturing, Rickmansworth, England) with 0.7 $kg\,cm^{-2}$ pressure and a 0.25 mm orifice diameter nozzle. Mites treated with deionized water containing 0.01% Triton X-100 served as controls. After being sprayed, groups of three mites were transferred to each cell of a 24-well tissue culture plate (Corning Glass Works, Corning, NY) containing one late instar honey bee larva as food. The mites were incubated in the dark at $34 \pm 1\,^{\circ}C$, 85% RH in a Percival Scientific incubators (autoregulated relative humidity and lighting) (Percival Manufacturing Company, Boone, Iowa). A Hobo® data logger (Onset Computer, Bourne, MA) was placed in each incubator to record the temperature and relative humidity.

We set up 6–10 groups of three treated mites per fungal concentration and control, giving a total of 108–180 mites per experiment. We repeated the experiments on four different dates, giving a total of 432–720 mites tested. Mite mortality was recorded daily for 14 days. In a separate bioassay, both isolates were also tested at single concentration of 2×10^8 conidia ml^{-1} (1.1×10^3 conidia mm^{-2}) to determine time–mortality responses. Mite mortality was recorded daily for 4 days, and the experiments were repeated on four different dates. Separate bioassays were set up to determine mortality after 7 and 14 days, and these were also repeated four different times.

2.3.2. Conidia viability

To determine conidia viability at the time of each experimental run, each concentration of fungal suspension was sprayed onto three petri dishes containing PDA. The conidia were incubated for 20 h at $34 \pm 1\,^{\circ}C$, 85% RH. After incubation, three droplets of lactophenol cotton blue stain (0.5% cotton blue) were added to each petri dish to fix and stain the conidia, preventing any further germination from occurring in the sample. The droplets were covered with a glass slide and evaluated using 400× phase-contrast magnification. The number of conidia that germinated in the first 100 conidia observed under the microscope was determined for each of the three droplets on each slide. Thus, 300 conidia were counted for each time period, concentration, and replicate. A conidium was considered to be viable if it germinated (the length of the germ tube was visible and greater than or equal to the width of the conidium). Viability estimates for all treatments were based on the proportion of conidia that had germinated after incubation (Goettle and Inglis, 1997).

2.3.3. Spray density

To determine spray density (the number of conidia per millimeter on the treated surface that a given conidia concentration produces in the Potter Spray Tower), five blocks of agar ($10 \times 10 \times 3$ mm each) were placed at equal distance to each other on a circular plastic disc of 140 mm diameter (four agar blocks on the side and one in the center). The blocks were sprayed under the Potter Precision Spray Tower with 1 ml of conidial suspensions. For each isolate, five concentrations (8.0×10^6, 2.1×10^7, 5.1×10^7, 8.5×10^7, and 2.0×10^8 conidia ml^{-1}) were tested. Each concentration was repeated three times. After spraying the blocks, three droplets of lactophenol cotton blue stain were added to each agar block to fix and stain the conidia. The droplets were covered with a glass slide and the density of conidia on the blocks was determined at 400× magnification using phase contrast as described by Wraight et al. (1998). Regression analysis (SAS Institute, 1996) was used to determine the relationship between the conidia concentration (conidia ml^{-1}) and the spray density (conidia mm^{-2}).

2.3.4. Infection determination

Dead mites were collected daily from the fungal treatments, and tested in the following way to see if mortality was due to infection. Mites were surface-sterilized by dipping them for 1 min in a sterilant-disinfectant (Exspor®, Alcide, Redmond, WA), and rinsing them with 95% ethanol (in preliminary tests, conidia of both isolates were killed after 10-s exposure to either Exspor® or 95% ethanol). The mites were then transferred with a camel-hair brush to petri dishes containing water–agar and incubated at 25 °C for 4–10 days. Mites

that died in the controls were also surface-sterilized and incubated as described above. The petri dishes were sealed with parafilm prior to incubation and the dead mites were observed daily for the presence of external fungal hyphae. The number of dead mites with external hyphae was counted, and to reduce the possibility of cross contamination, these mites were removed from the petri dishes. Only mites that showed fungal growth were considered to have died of infection.

2.3.5. Data analysis

Mite mortality in laboratory bioassays was recorded daily, and cumulative mortality over 4, 7, and 14 days was subjected to probit analysis to generate dose–mortality regression lines using the POLO program (Russell et al., 1977). Dose–mortality responses were corrected for control mortality by the POLO program. Virulence of the fungal isolates were considered significantly different if the 95% confidence limit (CL) of the LC_{50} ratios did not include 1.0, as described by Robertson and Preisler (1992). Dose–mortality regression lines were generated for Days 4, 7, and 14 separately, and the slopes and LC_{50}s were compared as described by Robertson and Preisler (1992). A likelihood ratio test of equality was conducted to determine whether the regression lines of the two fungi were equal (the slopes and intercepts of the two lines are the same). Similarly, a likelihood ratio test of parallelism was run to determine whether the regression lines were parallel (the slopes of the two lines are the same) (Robertson and Preisler, 1992). Percent mortality for the single dose bioassay was corrected for control mortality using Abbott's (1925) formula, and data from each time group were subjected to probit analysis (logistic transformation) to generate lethal time response (LT) (SAS Institute, 1996). The slopes of the time-mortality regression lines and LT_{50}s were compared as described above.

2.4. Observation hives

2.4.1. Establishing the observation hives

The observation hives used in these experiments were established by dividing larger honey bee colonies that were severely infested with *V. destructor* into 12 nucleus colonies, in Weslaco, TX. To ensure uniform bee and mite populations, 15 hives were de-queened, and all the bees from them were shaken into a large cage and placed in a refrigeration unit (4 °C, 85% RH) for 24 h to allow the bees to mix with each other. These bees were then divided into 0.92-kg packages. Each package was placed in a Langstroth hive body containing two frames of brood, two frames of honey, and one frame of foundation. A new marked queen was introduced into each of these nucleus colonies, and in order to reduce genetic variability between colonies, we used queens that were progeny of the same mother. The nucleus colonies were placed about 15 m apart from each other. Twelve days after they were established, eight were sampled using ether rolls as described by Delaplane and Hood (1997) to estimate initial mite levels, and then relocated to just outside the laboratory where the observation hive exits were located. After 48 h, two frames of bees were moved from each of the eight nucleus colonies to the observation hives. The eight observation hives were kept in a room maintained at 34 ± 1 °C and 65% RH. These hives had access to the out-of-doors. Outside, the bee exits and flyways were separated by 2 m along the wall. Four-meter high potted *ficus* trees (*Ficus* sp., Moraceae) were set in rows perpendicular to the wall (5 m in length) between the bee exits and along the length of the flyway to minimize drifting of the bees between colonies.

2.4.2. Observation hive treatments

Two experimental runs were done, one was conducted from April through May, 2001; and the second from August to October, 2001. A new set of observation hives was established for each experimental run. The eight colonies were randomly divided into two treatment groups (four treated with *H. thompsonii* and four control hives) for each experimental run. The fungal treatments were 2×10^8 conidia ml^{-1} in 0.01% Triton X-100, and the control treatment was deionized water containing 0.01% Triton X-100. To treat the colonies, each observation hive was taken outside in either the early morning or the late afternoon (each time a treatment was made, all the hives were treated at the same time), opened, and each frame (both sides) was sprayed with 15 ml of either the fungal suspension or the deionized water, using a Paasche® (Harwood Heights, IL) airbrush with $0.7\ kg\ cm^{-2}$ pressure and a 0.25-mm nozzle orifice diameter. After being treated, the observation hives were placed back in the room maintained at 34 ± 1 °C and 65% RH.

For the first experimental run, two applications were made, on May 5 and May 8, 2001 (Fig. 1). Data on mite mortality were recorded daily for 21 days after the first treatment. For the second experimental run, the fungus was applied four times: first on August 30, and then on September 6, 10, and 14, 2001 (Fig. 2). For this experiment, data on mite mortality were recorded every day for a period of 42 days after the first treatment. The fungal applications for both experiments were conducted as described above.

2.4.3. Data collection

Before and after the fungal applications, the mite infestation levels in the colonies were estimated using sticky-boards and ether rolls (Calderone and Turcotte, 1998; De Jong, 1990; Delaplane and Hood, 1997; Delaplane, 1998). The sticky-boards were 13.8×49.3 cm paper cards with a printed grid area of 10.0×44 cm.

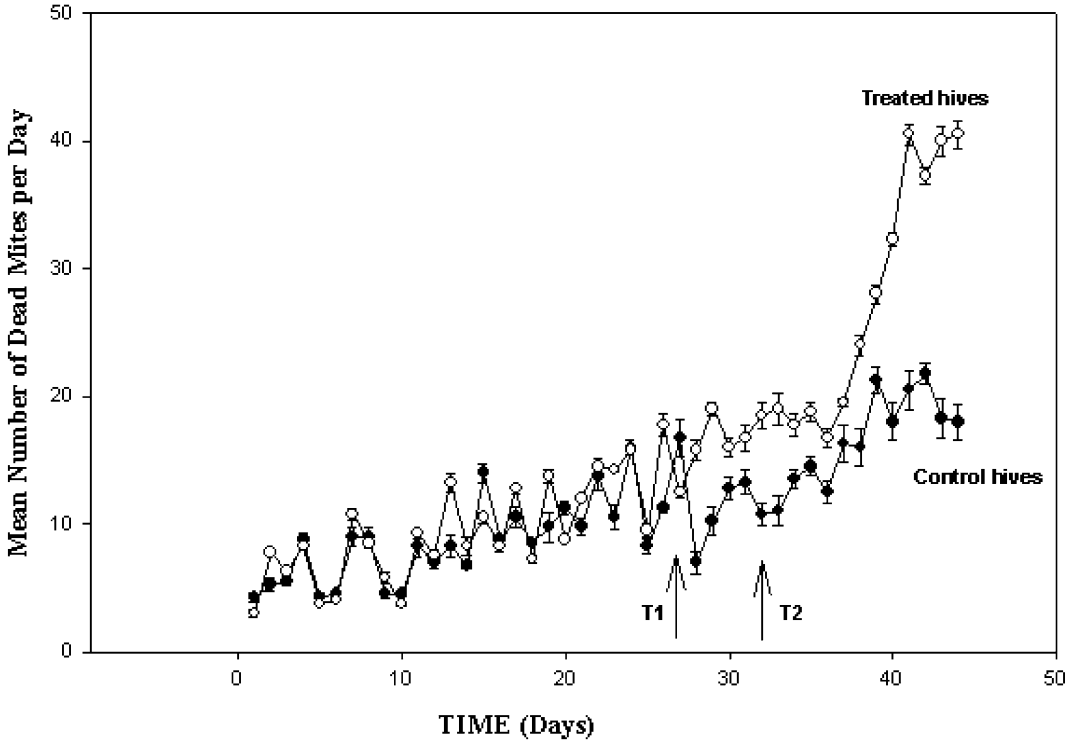

Fig. 1. Mortality of *V. destructor* in control hives and hives treated twice with *H. thompsonii* conidia (*T*1 and *T*2). Values are means (with SE bars) of daily mite mortality.

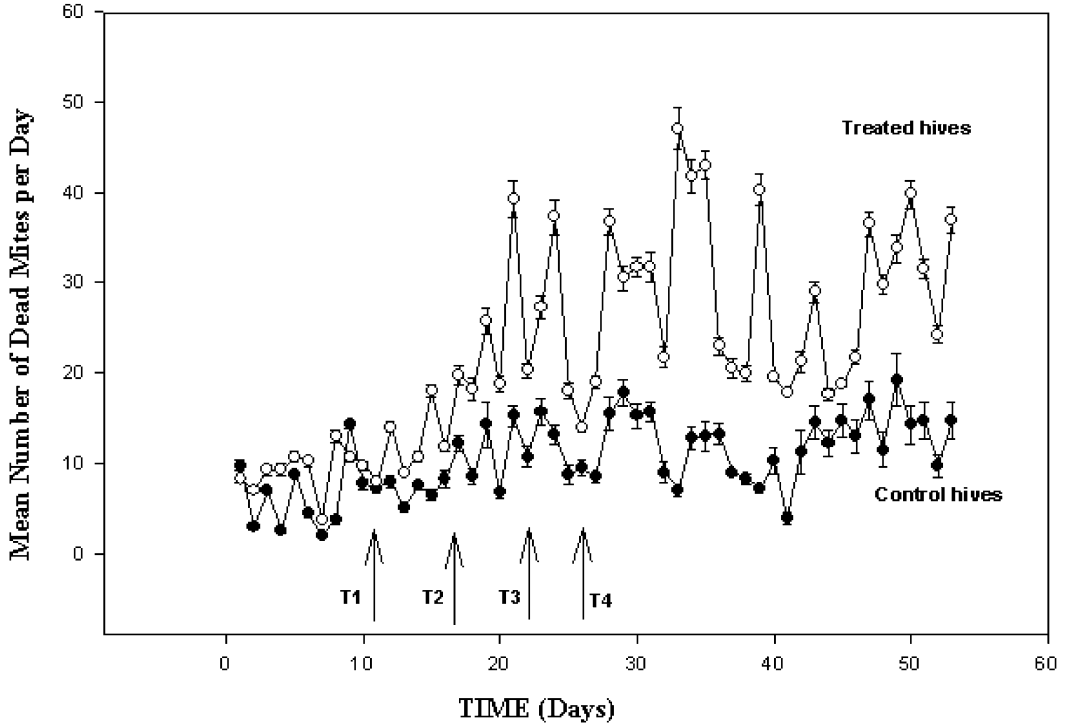

Fig. 2. Mortality of *V. destructor* in control hives and hives treated *with H. thompsonii* conidia four different times (*T*1–*T*4). Values are means (with SE bars) of daily mite mortality.

These were coated on the upper surface with a clear adhesive material (Tanglefoot®; Bio-Quip, Gardena, CA). The bottom board of the observation hive was removed and replaced with an 8-in mesh screen. The screen was used to prevent the bees from removing the mites and from becoming stuck to the cards. Mites that fell to the bottom of the hive passed through the screen and were trapped on the sticky board. The sticky boards were placed under each observation hive 15–20 days before the fungus was applied in order to determine pre-treatment mite fall. The mites that fell onto the boards over a period of 24 h were counted, and removed. Pollen and other debris that fell onto the sticky boards were also discarded every day and the boards were reused. The boards remained sticky for up to 7 days and then they were replaced by a new set of sticky boards. The mites collected daily were assessed for fungal infection by surface-sterilizing and incubating them as described above for the laboratory assays.

Ether roll counts were conducted in the evening (18 ± 2 h) of the day before each fungal application and at the end of each experimental run. The ether roll method involved brushing about 300 bees from the edge of the brood chamber into a 1-liter glass jar (Delaplane and Hood, 1997). The bees were sprayed with automotive starting fluid (diethyl ether) for 3 s and then the jar was capped and shaken for 30 s. The ether dislodged the mites from bees, and the mites stuck to the sides of the jar. The number of mites and bees was then recorded.

Pre- and post-fungal application, the number of mites within brood cells was determined by making a horizontal transect (in a crossway manner) across capped cells of a randomly chosen frame of broods. The cells within the transects were opened, the brood and mites were removed, and the adult mites were counted. A total of 200 cells per frame were uncapped in all control and treated hives at two different dates.

To assess the impact of the fungus on queen honey bee fecundity, the number of eggs laid per day were counted for five consecutive days between 7 and 14 days before the fungal treatments were applied. The impacts of fungal applications on honey bee colonies (larvae, pupae, and adults) were measured by placing a dead bee trap at the entrance of each observation hive. The dead bee trap used was a single-chamber Langstroth hive body covered with a mesh wire screen which caused the worker bees to drop into the trap any dead bees, larvae, and pupae they tried to remove from the hive. Dead larvae, pupae and adult bees were collected from the trap and counted every day. A sub-sample of dead pupae and adults were surface-sterilized and incubated on water–agar, as described previously for the mites, to determine if the bees were infected with the fungus. Regression analysis (SAS Institute, 1996) was used to determine the relationship between the number of mites collected daily on sticky traps from control and treated observation hives and the number of mites that died of fungal infection.

2.4.4. Observation hive data analysis

Daily mite mortality collected from the sticky boards during the experiments were analyzed using a repeated measures analysis of a complete randomized model (PROC Mixed; SAS Institute, 1996). Treatments were modeled as fixed effects; date, and date-by-treatment interactions were modeled as random effects. A Dunnett test (SAS Institute, 1996) was used to compare means of treated and untreated hives before and after the fungal applications. Because both sticky board and ether roll data were best described by a negative binomial distribution, the data were transformed to $\log(x+1)$ to satisfy the assumptions of normality before analysis. Data on the effects of the fungal treatments on bee mortality, on queen fecundity, and ether roll data were $\log(x+1)$ transformed and subjected to a repeated measures analysis model (PROC Mixed; SAS Institute, 1996) with treatments modeled as fixed effects. Date and date-by-treatment interactions were modeled as random effects. Means from treated and control hives before and after the fungal applications, were compared using a Dunnett test (SAS Institute, 1996). The percent of sealed brood cells that were infested with *Varroa* were arcsine transformed and subjected to an analysis of covariance using pre-treatment data as the covariate (PROC GLM; SAS Institute, 1996). The means in percent of sealed brood infested with *Varroa* in the controls and treated hives, before and after treatments were initiated, were separated using a Dunnett test (SAS Institute, 1996). Each experimental run was analyzed separately.

3. Results

3.1. Laboratory bioassays

Mean (±SE) conidial germination across all bioassays was 95.7 (±0.7)% for *H. thompsonii* and 96.6 (±0.9) % for *M. anisopliae*. The relative virulence at LC_{90} of *H. thompsonii* 5858 as compared to *M. anisopliae* were 24.96- and 72.02-fold greater at 4 and 7 days post-treatments, respectively, and these differences were significant based on failure of the 95% CL of the ratios at LC_{90} to bracket 1.0 (Robertson and Preisler, 1992) (Table 1). The virulence of the two fungal isolates were similar at 14 days. The dose–mortality regression line for *H. thompsonii* was significantly different from that for *M. anisopliae* on Days 4 ($\chi^2 = 31.99$; df = 2; $P > 0.05$), and 7 ($\chi^2 = 54.17$; df = 2; $P > 0.05$) after treatments. Differences in the dose–mortality regression lines were not significant 14 days ($\chi^2 = 2.61$; df = 2; $P < 0.05$) post-treatments. Lethal time for 90% cumulative mortality of mites (LT_{90}) was 4.16 (3.98–4.42)

Table 1
Virulence of an isolate of *M. anisopliae* and an isolate of *H. thompsonii* against *V. destructor*

Fungal isolate	n[a]	Slope ± SE	LC_{50}[b] (95% CL)	LC_{90}[b] (95% CL)	Lethal dose ratio[c] (95% CL)	
					LC_{50}	LC_{90}
After 4 days						
M. anisopliae	540	0.53 ± 0.04	61.52 (33.69–114.39)	1.57×10^4 (0.61–5.21)	—	—
H. thompsonii	504	0.95 ± 0.07	27.99 (19.93–39.63)	6.29×10^2 (3.70–12.36)	2.20 (1.08–4.47)	24.96 (7.25–85.03)
After 7 days						
M. anisopliae	540	0.54 ± 0.04	29.39 (16.15–54.27)	7.13×10^3 (2.80–23.45)	—	—
H. thompsonii	612	0.96 ± 0.07	4.51 (3.22–6.37)	9.90×10^1 (5.86–19.35)	6.52 (3.23–13.14)	72.02 (21.17–243.24)
After 14 days						
M. anisopliae	500	0.70 ± 0.05	2.77 (1.74–4.44)	2.01×10^2 (1.09–4.11)	—	—
H. thompsonii	620	0.68 ± 0.05	1.63 (1.02–2.63)	1.19×10^2 (0.64–2.44)	1.70 (0.86–3.30)	1.69 (0.44–4.57)

[a] Total number of mites tested.
[b] Concentrations are expressed in conidia mm^{-2}. Fungal suspension of 1×10^7 conidia ml^{-1} produced 53.00 ± 3.38 conidia mm^{-2}.
[c] Lethal dose ratios (relative virulence) calculated by dividing the LC_{50} or LC_{90} for *M. anisopliae* by the LC_{50} or LC_{90} for *H. thompsonii*.

days for *H. thompsonii* 5858 and 5.85 (5.48–7.43) days for *M. anisopliae* at 1.1×10^3 conidia mm^{-2}. The time–mortality regression line for *M. anisopliae* was significantly different from that of *H. thompsonii* 5858 at Day 4 ($\chi^2 = 28.26$; df = 1; $P > 0.05$) and Day 7 ($\chi^2 = 28.18$; df = 1; $P > 0.05$), but not at Day 14 ($\chi^2 = 0.147$; df = 1; $P < 0.05$).

3.2. Observation hives

3.2.1. Experiment 1

Daily mite mortality was not significantly different between the observation hives before the applications of the fungus, *H. thompsonii* ($F = 0.40$; df = 1, 6; $P = 0.532$). Treating the hives with *H. thompsonii* resulted in a significant increase in mite mortality ($F = 4.41$; df = 1, 6; $P = 0.035$). The date effect (a measure of within-hive variability over time) was statistically significant ($F = 2.35$; df = 20, 120; $P < 0.001$), but the date-by-treatment interactions were not ($P = 0.162$) (Table 2, Fig. 1). Mite infestation levels as determined by ether roll counts ($n = 300$ bees) were not significantly different between observation hives before the fungal applications ($F = 0.58$; df = 1, 6; $P = 0.474$). After treatments were initiated, mite populations were found to be significantly smaller in treated hives than in the control hives ($F = 8.63$; df = 1, 6; $P = 0.004$). The proportion of sealed brood infested with mites did not differ significantly between treated and control hives, before and after the fungal treatments ($F = 3.78$; df = 1, 2; $P = 0.191$). However, treated hives had lower average values of sealed brood infested with mites than untreated hives (Table 2).

3.2.2. Experiment 2

No significant differences in mite mortality occurred between the observation hives before the fungal applications ($F = 2.40$; df = 1, 6; $P = 0.172$). After the colonies were treated with the fungus, they had significantly smaller mite populations than the control colonies ($F = 44.74$; df = 1, 6; $P < 0.001$). As in the first experiment, mite mortality varied significantly over time within observation hives ($F = 3.11$; df = 41, 252; $P < 0.0010$), but the date-by-treatment interactions were not significant ($P = 0.536$) (Table 2, Fig. 2). Mite counts from the ether rolls did not differ significantly between observation hives before the fungal applications ($F = 0.05$; df = 1, 6; $P = 0.837$), but they did 42 days after the treatments began. The number of mites per bee was significantly lower in treated hives than in the control hives ($F = 5.18$; df = 1, 6; $P = 0.037$). Mite

Table 2
Effects of *H. thompsonii* on *V. destructor* and *A. mellifera* in observation hives

Parameters	Experiment	Before application (Means ± SE)		After application (Means ± SE)	
		Control	Treated	Control	Treated
Mite mortality per day	1	6.37 ± 1.15a	6.56 ± 1.05a	10.22 ± 1.49a	18.90 ± 1.46b
(# mites on sticky traps)	2	4.46 ± 1.20a	5.62 ± 1.4a	8.89 ± 2.53a	21.95 ± 2.81b
Mite infestations on adult bee	1	2.73 ± 0.02a	3.09 ± 0.02a	9.74 ± 0.03b	4.81 ± 0.02a
(from ether roll counts)	2	3.50 ± 0.02a	3.70 ± 0.02a	10.26 ± 0.03b	6.44 ± 0.02a
% Brood cells infested with *Varroa*	1	10.27 ± 2.49a	17.52 ± 4.17a	13.10 ± 3.57a	12.62 ± 2.50a
	2	6.74 ± 2.42a	7.44 ± 2.23a	25.38 ± 4.32b	23.99 ± 3.85b
Adult bee mortality per day	2	6.30 ± 1.05a	6.5 ± 1.01a	7.7 ± 1.22a	6.1 ± 1.09a
Queen fecundity (eggs laid/day)	2	264.51 ± 29.20a	213.43 ± 26.50a	210.84 ± 25.96a	234.92 ± 28.32a

Note. Means in row are not significantly different if followed by the same letter ($P > 0.05$, Dunnett test).

infestation of sealed brood cells was similar between control and treated hives before and after the fungal applications ($F = 0.06$; df $= 1, 3$; $P = 0.821$). In both treated and control hives the proportion of sealed brood cells that were infested with mites increased during the 42-day period of the experiment (Table 2).

No significant difference was found between the treated and the control hives in daily mortality of the honey bee populations before ($F = 0.20$; df $= 1, 6$; $P = 0.667$) or after the fungal applications ($F = 0.32$; df $= 1, 6$; $P = 0.593$) (Table 2). Similarly, queen fecundity (number of eggs per day) did not differ between treated and control hives before ($F = 1.61$; df $= 1, 6$; $P = 0.251$) or after treatments were initiated ($F = 0.22$; df $= 1, 6$; $P = 0.661$) (Table 2).

3.3. Fungal infections of dead mites

Mite mortality was highly correlated ($r^2 = 0.944$, $P < 0.0001$) with the occurrence of mycosis in dead mites collected in the fungal treated samples of Experiment 2 (Fig. 3). In contrast, there was no such correlation in control samples ($r^2 = 0.349$, $P > 0.05$). However, 2.86 (± 0.2)% of the dead mites were infected with the fungus in the control samples, and 82.97 (± 0.6)% of the mites caught in the sticky traps were infected with the fungus in the treated hives 42 days after the start of the fungal applications.

4. Discussion

Overall, *V. destructor* was found to be a suitable host for the entomopathogenic Hyphomycetes, *H. thompsonii* and *M. anisopliae*. *H. thompsonii* was more virulent to *Varroa* because it killed the host more quickly. Temperatures within bee hives do not appear to be a limiting factor for these fungi, as growth, and infection occurred at 34 ± 1 °C.

These fungi took 3–5 days to kill *Varroa* mites in the laboratory. McCoy (1981) reported that conidia of *H. thompsonii* killed *P. oleivora* L. within 3 days, and Smith et al. (2000) found the LT_{50} of *M. anisopliae* to be 2.7 days for the ectoparasitic mite, *Psoroptes ovis* (Hering). The reason that these fungi took longer to kill *Varroa* may be due to the fact that *Varroa* is considerably larger than these other mites, and the cuticle appears to be much more heavily scleritized. It is also possible that the temperature of the hive (~35 °C) (Hess, 1926) slowed growth of the fungi (Hall, 1981).

The treatments of observation hives with *H. thompsonii* have the potential to control *V. destructor* in honey bee colonies. Fungal applications did not seem to affect mite infestation levels in sealed brood. Mites are probably protected from exposure to the fungus when they are sealed inside brood cells. It is possible that the mites could become infected after they emerged from the brood cells if sufficient quantities of conidia are present

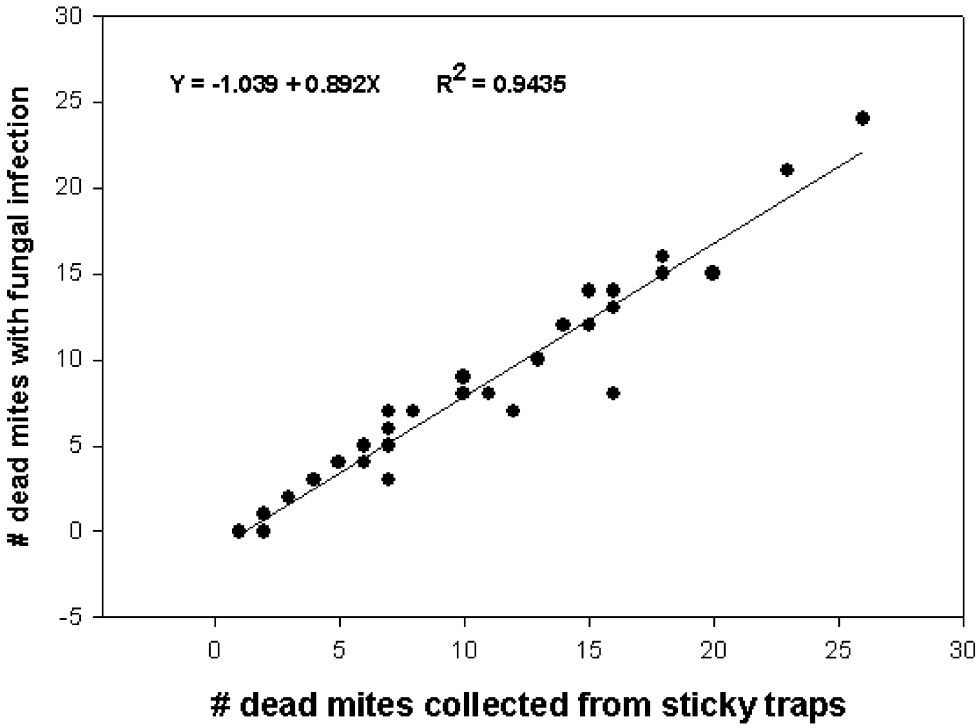

Fig. 3. Relationship between *V. destructor* collected daily from sticky traps and infection by *H. thompsonii* in Experiment 2. Dead mites collected daily from treated hives were surface-sterilized, plated onto petri dishes containing water–agar, and incubated at 25 °C for 4–10 days. Mites were collected after 24 h for 42 days, but only dead mites that were processed the day of the collection were used in the analysis.

in the hive. Overall, it may be that timing of the applications is critical for achieving optimum mite control. Fewer fungal applications may provide satisfactory control during times when no brood is being produced, or when brood production is very low (as would be the case in the early spring or late fall). When brood is not present, the mites do not reproduce, and thus most of the mites reside on adult bees and are more accessible to treatments (Calderone et al., 1997; Koeniger and Fuchs, 1989; Le Conte et al., 1998).

Hirsutella thompsonii appeared to be harmless to honey bee workers and brood, and did not have any deleterious effects on queen fecundity. We did not conduct hive trials with the Bio-Blast® strain of *M. anisopliae*, therefore, its effects on the honey bees have yet to be tested. Shaw et al. (2002) reported that four isolates of *M. anisopliae* caused significant mortality to caged honey bees sprayed with conidial suspensions (1×10^8 ml^{-1}) 14-day post-treatments. But, not all mortality could be attributed to fungal infection. Further, Butt et al. (1998) reported that honey bees are effective agents for the dissemination of *M. anisopliae* (an isolate highly pathogenic to adult honey bees in a bioassay) to control pollen beetles on oilseed rape, without any adverse effect on honey bee colonies.

The high correlation between mite mortality and fungal infection is an indication that the fungus was the major mortality factor in the *Varroa* mite population in the observation hive experiments. In the bioassays, both *H. thompsonii* and *M. anisopliae* were able to produce conidia on *V. destructor*. The honey bee hive is a somewhat closed system, and when conditions are favorable, the fungus could potentially proliferate on dead, infected mites, producing a new source of inoculum for uninfected mites. *H. thompsonii* caused significant mortality of *V. destructor* in treated hives, but infection levels did not increase over time without repeated applications of the fungus. However, the fungus must have good persistence because infected mites were recorded 30 days after the last fungal application. Also, the low infection rates found in the control hives may be an indication that bees carrying fungus-treated mites drifted between hives, such adrift of *Varroa* between treatments was also seen by Greatti et al. (1992).

No study has previously investigated fungal pathogens as control agents of *Varroa* mites in observation hives. Additional tests on the stability and persistence of these fungi in a hive, nontarget effects, formulations more appropriate for hive treatments, and treatment methodology are needed. The current and future direction of our research aims at developing a more efficient application technology to reduce the time required per application and to make the treatments more economical for beekeepers. Biological control could provide a key component in the development of a sustainable integrated pest management strategy for *V. destructor*.

Acknowledgments

We thank Sonia Gallegos, Carlos Gracia, Frank Eischen, and Henry Graham (USDA, ARS, Weslaco, TX) for their technical assistance with this study. We also thank Mamoudou Sétamou (Texas Agricultural Experiment Station, Weslaco, TX), Patrick Moran (USDA-ARS, Weslaco, TX), and Tong-Xian Liu (Texas Agricultural Experiment Station, Weslaco, TX) for providing useful discussions and reviews on earlier drafts of the manuscript.

References

Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18, 265–267.

Büchler, R., 1994. *Varroa* tolerance in honey bees occurrence, characters and breeding. Bee World 75, 54–70.

Ball, B.V., 1994. The development of control strategies for *Varroa jacobsoni* in colonies of *Apis mellifera*. Proceedings of the Brighton Crop Protection Conference—Pest and Disease 2, 569–576.

Békési, L., Brenda, V.B., Dobos-Kovács, M., Bakonyi, K., Rusvai, M., 1999. Occurrence of acute paralysis virus of the honey bee (*Apis mellifera*) in a Hungarian apiary infested with the parasitic mite *Varroa jacobsoni*. Acta Vet. Hung. 47, 319–319.

Butt, T.M., Carreck, N.L., Ibrahim, L., Williams, I.H., 1998. Honeybee-mediated infection of pollen beetle (*Meligethes aeneus* Fab.) by the insect-pathogenic fungus, *Metarhizium anisopliae*. Biocontrol Sci. Technol. 8, 533–538.

Cabras, P., Floris, I., Garau, V.L., Melis, M., Prota, R., 1997. Fluvalinate content of Apistan strips during treatment and efficacy in colonies containing sealed worker broods. Apidologie 28, 91–96.

Calderone, N.W., Wilson, W.T., Spivak, M., 1997. Plant extracts used for control of the parasitic mites, *Varroa jacobsoni* (Acari: Varroidae) and *Acarapis woodi* (Acari: Tarsonemidae) in colonies of *Apis mellifera* (Hymenoptera: Apidae). J. Econ. Entomol. 90, 1080–1086.

Calderone, N.W., Turcotte, R.M., 1998. Development of sampling methods for estimating levels of *Varroa jacobsoni* (Acari: Varroidae) infestation in colonies of *Apis mellifera* (Hymenoptera: Apidae). J. Econ. Entomol. 91, 851–863.

Chandler, D., Davidson, G., Pell, J.K., Ball, B.V., Shaw, K., Sunderland, K.D., 2000. Fungal biocontrol of acari. Biocontrol Sci. Technol. 10, 357–384.

De Guzman, L.I., Delfinado-Baker, M., 1996. A new species of *Varroa* (Acari: Varroidae) associated with *Apis koschevnikovi* (Hymenoptera: Apidae) in Borneo. Int. J. Acarol. 22, 23–27.

De Jong, D., De Jong, H.P., Gonçales, L.S., 1982. Weight loss and other damage to developing worker honey bees from infestation with *Varroa jacobsoni*. J. Apic Res. 21, 165–167.

De Jong, D., 1990. Mites: *Varroa* and other parasites of broods. In: Morse, R.A., Nowogrodzki, R. (Eds.), Honey Bee Pests, Predators and Diseases, second ed. Cornell University Press, Ithaca, NY, pp. 200–218.

Delaplane, K.S., Hood, W.M., 1997. Effects of delayed acaricide treatment in honey bee colonies parasitized by *Varroa jacobsoni* and a late-season treatment threshold of the south-eastern USA. J. Apic Res. 36, 101–108.

Delaplane, K.S., 1998. *Varroa* control: timing is everything. Am. Bee J. 138, 575–576.

Elzen, P.J., Eischen, F.A., Baxter, J.B., Pettis, J., Elzen, G.W., Wilson, W.T., 1998. Tau-fluvalinate resistance in *Varroa jacobsoni* from several geographic locations. Am. Bee J. 138, 674–676.

Elzen, P.J., Westervelt, D., 2002. Detection of coumaphos resistance in *Varroa destructor* in Florida. Am. Bee J. 142, 291–292.

Ferrer-Dufol, M., Martinez-Vinuales, A.I., Sanchez-Adedo, C., 1991. Comparative tests of fluvalinate and flumethrin to control *Varroa jacobsoni* Oudemans. J. Apic Res. 30, 103–106.

Fries, I., Hansen, H., 1993. Biotechnical control of *Varroa* mites in cold climates. Am. Bee J. 133, 435–438.

Gamber, W.R., 1990. Fluvalinate scare should serve as warning. Am. Bee J. 130, 629.

Goettle, M.S., Inglis, G.D., 1997. Fungi: Hyphomycetes. In: Lacey, L.A. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, pp. 213–249.

Greatti, M., Milani, N., Nazzi, F., 1992. Reinfestation of an acaricide-treated apiary by *Varroajacobsoni* Oud. Exp. Appl. Acarol. 16, 279–286.

Hall, R.A., 1981. The fungus *Verticilium lecanii* as a microbial insecticide against aphids and scales. In: Burges, H.D. (Ed.), Microbial of Pests and Plant Diseases, 1970–1980. Academic Press, London, pp. 483–498.

Hess, W.R., 1926. Die temperaturregulierung im bienenvolk. Zeitschrift fur Vergleichende. Physiologie 4, 465–487.

Hoppe, H., Ritter, W., Stephen, E.W.C., 1989. The control of parasitic mites: *Varroa jacobsoni*, *Acarapis woodi*, and *Tropilaelaps clareae* with formic acid. Am. Bee J. 129, 739–742.

Imdorf, A., Bogdanov, S., Ochoa, R.I., Calderone, N., 1999. Use of essential oils for the control of *Varroa jacobsoni* Oud. in honey bee colonies. Apidologie 30, 209–228.

Koeniger, N., Fuchs, S., 1989. Eleven years with *Varroa* experiences, retrospects and prospects. Bee World 70, 148–159.

Kraus, B., Berg, S., 1994. Effect of lactic acid treatment during winter in temperate climate upon *Varroa jacobsoni* Oud. and the bee (*Apis mellifera* L.) colony. Exp. Appl. Acarol. 18, 459–468.

Kraus, B., Velthuis, H.H.W., Tingek, S., 1998. Temperature profiles of the brood nests of *Apis cerana* and *Apis mellifera* colonies and their relation to varroasis. J. Apic Res. 37, 175–181.

Le Conte, Y., Colin, M.E., Paris, A., Crauser, D., 1998. Oil spraying as a potential control of *Varroa jacobsoni*. J. Apic Res. 37, 291–293.

Lodesani, M., Pellacani, A., Bergomi, S., Carpana, E., Rabitti, E., Lasagni, P., 1992. Residue determination for some products used against *Varroa* infestation in bees. Apidologie 23, 257–272.

McCoy, C.W., 1981. Pest control by the fungus *Hirsutella thompsonii*. In: Burges, H.D. (Ed.), Microbial Control of Pest and Plant Diseases, 1970–1980. Academic Press, London, pp. 499–512.

Milani, N., 1999. The resistance of *Varroa jacobsoni* Oud. to acaricides. Apidologie 30, 229–234.

Mutinelli, F., Baggio, A., Capolongo, F., Piro, R., Biasion, L., 1997. Oxalic acid in the control of varroosis. Ape Nostra Arnica 19, 4–7.

Rinderer, T.E., Kuznetsov, V.N., Danka, R.G., Delatte, G.T., 1997. An importation of potentially *Varroa*-resistant honey bees from far-eastern Russia. Am. Bee J. 137, 787–789.

Rinderer, T.E., De Guzman, L.I., Delatte, G.T., Stelzer, J.A., Lancaster, V.A., Kuznetsov, V., Beaman, L.D., Watts, R., Harris, J.W., 2001. Resistance to the parasitic mite *Varroa destructor* in honey bees from far-eastern Russia. Apidologie 32, 381–394.

Robertson, J.L., Preisler, H.K., 1992. Pesticide Bioassays with Arthropods. CRC, Boca Raton, FL.

Russell, R.M., Robertson, J.L., Savin, N.E., 1977. POLO: a new computer program for probit analysis. Bull. Entomol. Soc. Am. 23, 209–213.

Sanford, T., Flottum, K., Arthur, B., 1999. Focus on Bayer bee strips. Bee Culture 127, 32–35.

SAS Institute, 1996. SAS/STAT Software. Changes and Enhancements. SAS Institute, Gary, NC.

Schmidt-Bailey, J., Fuchs, S., Büchler, R., 1996. Effectiveness of drone brood trapping combs in broodless honey bee colonies. Apidologie 27, 293–295.

Shaw, K.E., Davidson, G., Clark, S.J., Ball, B.V., Pell, J.K., Chandler, D., Sunderland, D., 2002. Laboratory bioassays to assess the pathogenicity of mitosporic fungi to *Varroa destructor* (Acari: Mesostigmata), an ectoparasitic mite of the honey bee, *Apis mellifera*. Biol. Control 24, 266–276.

Smith, K.E., Wall, R., French, N.P., 2000. The use of entomopathogenic fungi for the control of theparasitic mites, *Psoroptes* spp. Vet. Parasitol. 92, 97–105.

van Veen, J., Calderon Fallas, R.A., Cubero Murillo, A., Arce Arce, H.G., 1998. *Varroa jacobsoni* in Costa Rica: detection, spread and treatment with formic acid. Bee World 79, 5–10.

Wallner, K., 1999. Varroacides and their residues in bee products. Apidologie 30, 235–248.

Wraight, S.P., Carruthers, R.I., Bradley, C.A., Jaronski, S.T., Lacey, L.A., Wood, P., Galaini-Wright, S., 1998. Pathogenicity of the entomopathogenic fungi *Paecilomyces* spp. and *Beauveria bassiana* against the silver leaf whitefly, *Bemisia argentifolii*. J. Invertebr. Pathol. 71, 217–226.