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Impact of the Entomopathogenic Fungus *Beauveria bassiana* on the Honey Bees, *Apis mellifera* (Hymenoptera: Apidae)

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Abstract: The impact of entomopathogens on non-target organisms is critical information for the registration of microbial control agents. *Beauveria bassiana* is an excellent potential candidate for biological control of a variety of pests and efforts are being made for its registration in Canada. Four isolates of *B. bassiana* were evaluated to determine their pathogenicity to caged honey bees, *Apis mellifera carnica*. Results showed that three of the tested isolates: GHA, London BB001 and Arkansas ARSEF 3769 caused high mortalities to the caged bees when dusted with a dry formulation at high concentrations (10^8-10^9 CFU g^{-1}). However, exposure of honey bee hives to high inoculum densities of *B. bassiana* resulted in very low mortality that was not different from the control regardless of the isolate. Those results suggest that *B. bassiana* can be applied for pest control in fields where honey bees are used for pollination.

Key words: Beauveria · *Apis mellifera* · formulations · mortality

INTRODUCTION

There have been many reports of the decline of pollinators in agroecosystems all over the world in the last few years [1, 2]. The use of wide spectrum pesticides along with habitat destruction and fragmentation, diseases and parasites are factors contributing to this loss of pollinators [2]. A more sustainable approach for pest control relies on integrated pest management (IPM) which utilizes ecological factors such as parasites and predators as well as microbial control agents. Most microbial control agents are considered generally to be host specific and pose a lower risk than that associated with many chemical pesticides. However, microbial control agents can have a potential environmental risk depending on the nature of the pathogen in question and its pattern of use [3]. This is especially important with microbial control agents that have a wide host range. For example, *Beauveria bassiana* (Balsamo) has a host range of over 700 species including many beneficial insects such as the worker honey bee, *Apis mellifera* [4]. However, epizootics among beneficial insect populations appear to be uncommon with the exception of hibernating coccinelids [4]. Moreover, most isolates of *B. bassiana* tend to be more host specific [4].

When *B. bassiana* was evaluated against queenless caged adult honey bees, it reduced bee longevity at high concentrations and caused mycosis among treated bees at all the tested concentrations that ranged between approximately 10^6-10^8 spores/bee [5]. The effects of *B. bassiana* on the Africanized honey bees was evaluated using caged bees as well as whole bee hives [6]. The entomopathogen was administered to caged bees either orally with sucrose solution at a dose of $5X10^6$ conidia/bee or topically by spraying at a dose of $1X10^8$ conidia/ml. In both cases, caged bees showed higher mortalities than the controls. Bees that received the oral dose suffered higher mortalities (76-90%) than bees that received the topical treatment (50-84%) [6]. Although caged bees suffered high mortalities, whole bee hives exposure showed very little mortality [6]. The susceptibility of honey bees to the commercial strain of *B. bassiana* (GHA) was also evaluated using honey bee workers isolated from their hive. The GHA strain was virulent against the bees and the 7d LD_{50} was calculated as $2.2X10^5$ conidia per bee [Mycotech Corp., unpublished].

B. bassiana has been used on a commercial scale in the former USSR, China and Brazil [4]. More recently, this fungus was registered for commercial use on field crops, vegetables, fruit trees and greenhouse crops in the USA [3]. *B. bassiana* is not yet registered in Canada and its registration requires information about its effects on non-target organisms, especially pollinators such as

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Table 1: *B. bassiana* isolates that were tested against caged adult worker honey bees and the isolate's original hosts

Isolates	Original host
Arkansas (ARSEF 3769)	*Lygus lineolaris* (Hemiptera: Miridae)
New York (NY)	*Lygus lineolaris* (Hemiptera: Miridae)
London BB001 (LON)	*Leptinotarsa decemlineata* (Chrysomelidae: Coleoptera)
GHA	*Melanoplus sanguinipes* (Orthoptera: Acrididae)

honey bees. This study was carried out to evaluate the impact of *B. bassiana* on honey bees applied as a dry formulation.

MATERIALS AND METHODS

Tests with caged honey bees: Four isolates of *B. bassiana* (Table 1) were evaluated for virulence against adult worker honey bees. The isolates were cultured on Sabouraud's dextrose agar (Difco, Detroit, MI.) for 2 weeks in the dark at 24-25°C. Conidia were then harvested with a spatula and stored at 4°C until used. To estimate the CFU per unit weight of the harvested conidia, six 0.1 g samples taken at random were each suspended in 100 ml sterile distilled water and 0.1% Tween 80 and agitated on a rotary shaker at 125 rpm for 2 h. Three 0.1 aliquots of 10 fold serial dilutions of each suspension were spread on oatmeal agar Petri plates amended with 550 μg ml^{-1} Dodine, 400 μg ml^{-1} penicillin G., 1000 μg ml^{-1} streptomycin sulfate and 5 g ml^{-1} crystal violet [7]. The plates were incubated at 24 -25°C in darkness for 4 to 5 d. after which colony forming units (CFU) were counted on each plate. To determine the viability of the cultured conidia, six 0.01 g of conidia were suspended in 100 ml distilled water and 0.1% Tween 80. Then 200 μl of the conidial suspension was added to 1 ml of Sabouraud's dextrose broth amended with 1% yeast extract in a sterile test tube. The suspension was incubated at 24 -25°C for 24 h. Four subsamples from each sample each consisting of 200 conidia were examined for germination using a hemacytometer under a compound microscope. Only batches that showed more than 90% germination were used in the experiment. Four concentrations ($1x10^6$, $1x10^7$, $1x10^8$, $1x10^9$ CFU g^{-1}) from each isolate were prepared by mixing dry conidia with corn flour.

Young honey bee workers were collected from the center of brood chamber of honey bee hives by brushing into ventilated Perspex cages (9X11X8 cm height) with approximately 100 bees in each cage. Bees were then immobilized using CO_2 gas and treated by the inocula mixture using a pollen applicator (Firman Pollen, Yakima, WA). Each cage received 0.5 g of the inoculum mixture. There were three cages for each isolate/concentration combination. To determine the density of *B. bassiana* on treated bees, three honey bees were collected from each cage and then agitated individually in flasks containing 100 ml sterilized distilled water and 0.1% Tween 80 on a rotary shaker for 2 h. the suspensions were treated as above to determine CFU per individual bees. Caged bees were kept at 24±1°C and a 16 h photo period. Sucrose syrup (50%) and irradiated honey bee collected pollen mixed with 50% (wt/wt) sucrose syrup were provided for feeding. Honey bee mortality was recorded daily for 14 d after treatment. Dead bees were removed and placed in Petri plates lined with moist filter paper to favor external growth and sporulation of the fungus. The experiment was repeated with fresh patches of fungal conidia and honey bees so each treatment combination of isolate and concentration was replicated six times.

Tests with honey bee hives: Three of the tested isolates in the caged bee experiment, ARSEF 3687, GHA and NY, were selected for the whole bee hive experiment. Conidia of the three isolates were produced as above. One single concentration of each isolate was prepared by mixing the conidia with corn flour to a concentration of $1X10^9$ CFU g^{-1}. Twenty honey bee colonies each one with 20,000-25,000 bees were used in the experiment (Townsend House Apiculture Field Laboratory, University of Guelph, Canada). A hygienic test as described by Hajek and Goettel [9] was applied to the hives to assess the hygienic behavior of each hive. Each hive was then given a score from one to four as follows: 1 for highly hygienic hives, 2 for hygienic, 3 for partially hygienic and 4 for non-hygienic. Based on the hive scoring, treatments were assigned to the hives so that the hygienic categories were almost equally distributed among the treatments. There were five treatments in the experiment; the three tested isolates (each one a separate treatment), corn flour only and a control with no treatment. Each treatment was applied to four hives using pollen applicators (Firman Pollen, Yakima, WA). All the frames from each treated hive were taken out of the brood chamber and the bees were dusted directly while on the frames. Each colony received 5 g of the corresponding treatment. To determine the density of *B. bassiana* on

treated bees, ten honey bees were collected from each colony (40 per treatment) and were treated as above. Two days before the application of the fungus, under-basket dead bee traps [10] were fastened to the entrance of each hive to collect dead bees. The traps were checked and dead bees were removed and counted every 3 d. Dead bees were removed and placed in Petri plates lined with moist filter paper to encourage external mycosis. Temperature and relative humidity were monitored inside the hives by temperature/humidity probes (Hycal Co., Elmonte, CA). Probes were placed in the middle of the brood chamber between the frames.

Statistical analysis: For data on caged adult honey bee tests, probit analysis was used to estimate LC_{50}, LD_{50} and LT_{50} values for the tested isolates. When Pearson's goodness of fit test (Chi-square was significant at the 0.05 level) indicated significant departure from the probit model, all variances and co-variances were multiplied by the heterogeneity factor H [10]. The tested isolates were compared by using 95% Confidence intervals (95% CL) for the LC_{50}, LD_{50} and LT_{50} values. Overlapping 95% CL were considered not significant at 0.05 level.

Percentage mortalities of honey bees in the whole hive tests were analyzed using logistic regression [10]. Mean cumulative mortalities were then compared by polynomial contrast comparisons. The type I error rate (%) was set at 0.05 level for all tests. Mortality data were back transformed to the original scale before presentation in tables.

RESULTS

Tests with caged honey bees: Caged honey bees suffered mortality that ranged between 10-94, 5-93, 8-93 and 9-27% for ARK, GHA, LON and NY isolates, respectively. Mortality in the bees treated with corn flour only and the control ranged between 5-12 and 9-15%, respectively.

The concentration-mortality response (LC_{50}) and dose-mortality response (LD_{50}) of caged adults honey bees treated with different isolates of *B. bassiana* are presented in Table 2. There were no significant differences in the virulence of ARK, GHA and LON isolates as indicated by no significant differences between the LC_{50} and LD_{50} values of the three strains. However, the NY isolate was less virulent to caged honey bees as it had significantly lower LC_{50} and LD_{50} than the other isolates (Table 2).

Mortality of bees treated with the NY isolate was less than 28% at all tested concentrations. Consequently, the LT_{50} value and 95% CL for NY isolate was not calculated. No significant differences were found between the LT_{50} values of the isolates ARK, LON and GHA at concentrations of 10^8 and 10^9. However, the LT_{50} values were significantly lower for the isolates ARK and GHA at a concentration of $1X10^9$ CFU g^{-1} compared to the concentration $1X10^8$ CFU g^{-1} but not for the LON isolate (Table 3).

Tests with honey bee hives: Very few bee cadavers were collected on day one after the treatment. Most cadavers

Table 2: Concentration-mortality response (LC_{50}) and 95% CL and dose-mortality response (LD_{50}) and 95% CL of caged adults honey bees treated with four isolates of *B. bassiana* applied at four concentrations ranging from $1X10^6$ to $1X10^9$ CFU g^{-1} corn flou

Isolates	LC_{50}	95% CL	LD_{50}	95% CL
ARK	3.1X108a	1.4X108-5.2X108	5.4X105a	3.1X105-8.2X105
GHA	3.2X108a	1.2X108-5.6X108	5.4X105a	2.7X105-8.6X105
LON	3.3X108a	1.4X108-5.9X108	5.2X105a	2.7X105-8.5X105
NY	3.5X109b	2.3X109-8.5X109	5.9X106b	3.9X106-1.4X107

LC_{50} and LD_{50} within columns with different letters are significantly different at 0.05 as indicated by non-overlapping 95% CL. Overlapping 95% CL's are not significantly different

Table 3: Time-mortality response (LT_{50}) values and 95% CL for caged adults honey bees treated with different isolates of *B. bassiana* at concentrations of $1X10^8$ and $1X10^9$ CFU g^{-1} corn flour

	Concentration CFU g^{-1} corn flour			
	$1.0X10^8$		$1.0X10^9$	
Isolates	LT_{50}	95% CL	LT_{50}	95% CL
ARK	11.2a	10.4-12.0	9.0a*	8.4-9.6
GHA	11.5a	10.7-12.4	9.5a*	8.9-10.2
LON	10.9a	10.1-11.8	9.7a	8.8-10.5

LT_{50} values within columns with different letters are significantly different at 0.05 level and within rows with asterisks are significantly different at 0.05 level as indicated by Overlapping 95% CL's

Table 4: Mean (S.E.) cumulative mortality and dose (S.E.) per bee of honey bees exposed to three isolates of *B. bassiana* applied to standard-sized honey bee hives at a concentration of $1X10^9$ CFU g^{-1} corn flour

Treatments	# of hives	CFU/bee (S.E.)	% mortality (S.E.)	% confirmed mycosis (S.E.)
ARK	4	$4.0X10^5$ ($4.5X10^4$)	3.1 (1.0)a	15.4 (3.0)
GHA	4	$4.9X10^5$ ($4.0X10^4$)	3.0 (1.4)a	12.1 (3.4)
NY	4	$4.4X10^5$ ($3.8X10^4$)	2.3 (0.4)a	3.7 (1.5)
Corn flour	4	NA	2.5 (0.9)a	0.1 (0.1)
Control	4	NA	2.1 (0.3)a	0.0

Means within columns with different letters are significantly different at 0.05 level using polynomial contrasts

were collected between 6-18 d after treatment. Total percentage mortality 36 days after treatment was very low and ranged between 2-3% for both *Beauveria* treated hives and the control hives (Table 4). No significant differences were found between the different isolates and the controls using contrast comparisons. Furthermore, no significant differences were found between the corn flour treatment and the control. Percent of cadavers with external mycosis was higher for the isolates ARK and LON compared to NY (Table 4). Only 2 cadavers with external mycosis were detected in the corn flour treatment. No cadavers with external mycosis were detected in all treatments after 21 days of the application of the fungus.

DISCUSSION

Caged adult honey bees suffered high mortalities when exposed to ARK, GHA and LON isolates of *B. bassiana*, but not to the NY isolate. It has been shown that the pathogenicity of entomopathogenic fungi, including *B. bassiana*, varies within species depending on the isolate [4]. The high mortality observed with caged bees was dependent on the dose; the higher doses of 10^8 and 10^9 resulted in higher mortality than did the lower doses of 10^6 and 10^7. Similar findings were reported by Alves *et al.* [6].

Although honey bees suffered high mortalities when exposed to *B. bassiana* in cages, exposure of whole honey bee hives under field conditions resulted in low mortality that was not different from the controls regardless of the isolate tested. Those findings coincide with previous reports that showed the virulence of *B. bassiana* against queenless caged honey bees but its safety to nuclear honey bee colonies [Mycotech Corp., unpublished] and whole hive Africanized honey bees [6]. The latter concluded that confining honey bees causes a stressful situation that rendered the bees more vulnerable to the fungus especially at sub-optimum temperature and humidity for the bees. Another factor that might explain the harmlessness of *B. bassiana* when applied to whole hives compared to caged bees is the temperature of the brood chamber in hives. Records of daily temperature and relative humidity during the course of our whole hive experiment showed that temperature and relative humidity in the brood chamber ranged between 32.1-36.3°C and 33.6-71.2 %RH, respectively. High temperatures above 30EC have been shown to adversely affect germination and development of *B. bassiana* [11]. Temperatures of the brood chambers were similar in the treated and control hives further confirming the safety of *B. bassiana* to honey bees as abnormal temperatures in the brood chamber are considered a sign of a stressed or a diseased hive [8].

The percentage of mycosed cadavers in the whole honey bee test was low for all isolates (Table 4). This further confirms the safety of *B. bassiana* to honey bees. No mycosed cadavers were detected 21 days post application which indicates that the disease was not established in the hives and no repeated cycles were initiated in the treated hives.

The current study results indicate that *B. bassiana* is safe when applied to honey bees under field conditions. The doses applied to honey bee hives in this experiment were high and honey bees are unlikely to be exposed to such doses under normal circumstances. Those findings indicate that this microbial control agent can be implemented in Integrated Pest Management (IPM) programs with harmless impact on honey bee.

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