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## Efficacy of Two Fungus-based Biopesticide Against the Honeybee Ectoparasitic Mite, *Varroa destructor*

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**Abstract:** The varroa mite, *Varroa destructor* (Anderson and Trueman) (Acari: Varroidae), is known as the most serious ectoparasitic mite on honeybee, *Apis mellifera* (Hymenoptera: Apidae) in the world. Based on the spores of entomopathogenic fungi, two commercial preparations; Bioranza (*Metarhizium anisopliae*) and Biovar (*Beauveria bassiana*) were evaluated through application into the hives against varroa mite. Data showed significant differences between treatments with Bioranza and Biovar, the results were significant after 7 and 14 days post-treatment. Mean a daily fallen mite individual was significantly different between the hives before and after the applications of the two biopesticides and wheat flour. Also, mites' mortality was, significantly, different between the hives before and after treatments. There were significant differences between treatments with the two biopesticides in worker's body weight. Bioranza and Biovar did not infect the honeybee in larval, prepupal, pupal and adult stages. Scanning and transmission electron microscopy images showed spores and hyphae penetration through stigma and wounds on varroa. The results suggest that Bioranza and Biovar are potentially are effective biopesticides against *V. destructor* in honeybee colonies.

**Key words:** *Apis mellifera*, *Varroa destructor*, biopesticides, *M. anisopliae*, *B. bassiana*, electron microscopy

### INTRODUCTION

The parasitic mite *V. destructor* is currently a worldwide parasite on the honeybee (*Apis mellifera* L.). It feeds on the hemolymph of immature and adult bees (Harbo and Harris, 2001). This hemophagous mite species reproduces within sealed brood, showing strong preference for drone brood more than worker brood (Martin, 2001). The honeybee is considered of great economic importance, not only for honey production but also for crop pollination. A balanced host-parasite relationship is established in the sense that the host fitness loss due to parasitism is limited because mite reproduction occurs in drone brood only (Peng *et al.*, 1987; Tewarson *et al.*, 1992). This parasitic mite causes many biological effects like, weight loss, morphological abnormalities and reduces the lifespan of infested individuals (De Jong *et al.*, 1982).

Chemical control methods and alternative methods including biotechnical and genetic have been assayed against *V. destructor* (Fries and Hansen, 1993, De Guzman and Delfinado-Baker, 1996, Schmidt-Bailey *et al.* 1996; Rinderer *et al.* 1997; El-Ghamdi and Hoopingarner, 2004). Development of resistance in *V. destructor* mite populations to coumaphos was studied by Elzen *et al.*,

(1998) and Elzen and Westervelt (2002). Control of varroa mites with organic acids often requires multiple applications to achieve a high degree of control and pose a safety way to beekeepers (Veen *et al.* 1998). Essential oils and their components have also been tested (Calderone *et al.*, 1997). In previous studies, the dust or strip applications of *M. anisopliae* to honey bee hives provided satisfactory control of varroa mite under field conditions (Kanga *et al.*, 2003). The fungi *B. bassiana* has been used as mycopesticide to control of many pests belong to arthropod species (Goettel and Johnson, 1992). From honeybee colonies collected in France, Meikle *et al.* (2006) discovered several isolates of *B. bassiana* from varroa mites. Collecting fungal or bacteria isolates from the target pests themselves is intended to increase the probability of finding the best suitable isolates against these pests.

The present study aimed to determine the effect of two biopesticides, namely Bioranza (*M. anisopliae*) and Biovar (*B. bassiana*) against *V. destructor*, a honeybee ectoparasitic mite in Egypt. Compare between the two commercial formulations and inert powder on the fallen mites and mean weights of honeybee worker. Scanning and transmission electron microscopy images were, also, used to show penetration of the spores and hyphae.

## MATERIAL AND METHODS

### Efficacy of the two fungus-based biopesticide and inert materials

**Biopesticides:** Two commercial formulations: Bioranza (10 % WP) of dry conidia of fungus *M. anisopliae* and Biovar (10% WP) of dry conidia of fungus *B. bassiana*. A weight of 0.1 gm of each formulation was suspended in 50 mL of water to be used per each colony.

**Inert materials:** Both of wheat and maize flour were used to make the treatments more economical for beekeepers. Five grams of powder of maize or wheat flour were used per each colony.

**Experimental honeybee colonies:** The present investigation was carried out in special apiary during spring season of 2011. Fifteen colonies of hybrid Carniolan honeybees, *A. mellifera* naturally heavily infested by varroa mites were used.

**Mite collection:** To test the efficacy of selected commercially formulated of entomopathogenic fungi against *V. destructor*, female mites were collected from infested frames of sealed brood taken from honeybee colonies and 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 instars honeybee 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.

**Treatments:** Four groups of colonies and control, all treated by spraying with fine atomizer. The 1st treated with Bioranza, the 2nd with Biovar, the 3rd with wheat flour, the 4th was treated with maize flour and 5th is the control. Mite mortality was recorded daily for 7 days and the experiments were repeated three times. Also, all treatments were repeated with using wheat flour. Dead mites were collected daily from the two biopesticides treatments and tested in the following way to see if mortality was due to infection.

**Establishing the hives:** The hives used in these experiments were established by dividing larger honeybee colonies that were severely infested with varroa into 15 nucleus colonies to ensure uniform bee and mite populations. Used queens were progeny of the same mother. The nucleus colonies were placed about 1.5 m apart from each other. Twelve days after they were established, hives were ready for treatment. Honeybee workers were collected from each treatment and weighed.

**Hive treatments:** The experimental started from April to October (2011). A new set of hives was established for

each experimental run. To treat the colonies, each hive was opened and each frame (both sides) was sprayed. Data on mite mortality were recorded daily for 14 days.

**Data collection:** Before and after the fungal applications, the mite infestation levels in the colonies were estimated using sticky-boards (De Jong, 1990; Delaplane and Hood, 1997; Delaplane, 1998; Calderone and Turcotte, 1998). These were coated on the upper surface with a clear adhesive material 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 1-7 days before the treatment in order to determine pretreatment mite fall. The mites that fell onto the boards over a period of 24 h were counted and removed. The boards remained sticky for up to 7 days and then they were replaced by a new set of sticky boards. The collected mites were daily assessed for fungal infection by surface-sterilizing and incubating them as described above for the laboratory assays. The number of mites was then recorded.

**Electron microscopy examination:** Mites were surface sterilized by dipping them for 1 min in a sterilant-disinfectant and rinsing them with 95% ethanol. The mites were then transferred with a camel-hair brush to Petri-dishes containing water-agar and incubated at 25°C for 4-7 days. Also, dead mites in the controls were also surface-sterilized and incubated as described above. Mites were observed daily for the presence of external fungal hyphae. The numbers of mites with external hyphae were counted; these mites were removed from the Petri dishes. Only mites that showed fungal growth were considered to have died by infection and photographed with electron microscopy.

**Statistical analysis:** The data was analyzed using analysis of variance (ANOVA) followed by the Student-Newman Keuls test to determine significance between different groups. These tests were performed using a computer software CoStat system for Windows, version 6.311, CoStat Program, 2006, Berkeley, CA, USA.

## RESULTS

**Efficacy of the two fungus-based biopesticide:** Data in Table 1 showed significant differences between treatments with Bioranza and Biovar. Results were significant after 7 and 14 days of application in the treatment with Bioranza which was more effective than Biovar which differed in its effect from 7 and 14 days. Daily mite mortality was significantly different between

Table 1: Mean number of fallen *V. destructor* mites on sticky boards by using formulations of fungi and inert materials in hives during 2011

Treatment	Mean No. of fallen varroa/day		
	0 day	1-7 days	8-14 days
Biovar	8.2 <sup>a</sup>	12.2 <sup>c</sup>	11.1 <sup>d</sup>
Bioranza	8.5 <sup>a</sup>	12.6 <sup>c</sup>	8.2 <sup>a</sup>
Wheat flour	8.1 <sup>a</sup>	14.4 <sup>b</sup>	13.6 <sup>c</sup>
Maize flour	8.2 <sup>a</sup>	14.2 <sup>b</sup>	15.9 <sup>b</sup>
Control	8.2 <sup>a</sup>	16.4 <sup>a</sup>	22.4 <sup>a</sup>
L.S.D.	0.463	0.588	0.613

Means followed by the same letter (s) within each vertical column are not significantly different

Table 2: Mean weights of honeybee workers before and after using formulations of fungi and inert materials in hives during 2011

Treatment	Mean weights mg/bee worker	
	Before	After
Biovar	227.1 <sup>a</sup>	247.8 <sup>a</sup>
Bioranza	226.8 <sup>a</sup>	246.9 <sup>a</sup>
Wheat flour	225.7 <sup>a</sup>	234.5 <sup>b</sup>
Maize flour	227.5 <sup>a</sup>	235.4 <sup>b</sup>
Control	226.2 <sup>a</sup>	219.7 <sup>c</sup>
L.S.D.	1.392	1.998

Means followed by the same letter(s) within each vertical column are not significantly different

the hives before and after the applications of the fungus. Treating the hives with Bioranza resulted in a significant increase in mites' mortality. After treatments were initiated, mite populations were found to be significantly smaller in treated hives than in the control hives. Mites' mortality varied significantly over time within observation hives.

Table 2 showed significant differences between treatments with Bioranza and Biovar in workers body weights. Results were significant after treatments than before; the fungus Bioranza was the highly effective fungus, followed by Biovar. Mean workers weight was not significantly different between the hives before and after the applications of the fungus. Overall, *V. destructori* was found to be a suitable host for the entomopathogenic fungi, Bioranza was more virulent to varroa because it killed the host more quickly. Fungal applications did seem to affect mite infestation levels in hives. It is possible that the mites could become infected after they emerged from the brood cells if sufficient quantities of conidia are present. Mite infestation level varied, significantly, between treatments 7 and 14 days post-treatments.

### ELECTRON MICROSCOPY EXAMINATION

**Scanning electron microscopy (sem):** The present scanning electron microscopy (SEM) study describes the external development of *B. bassiana* and *M. anisopliae* on the surface (cuticle) of the mite *V. destructor*. In Fig. 1 to 5 illustrates the scanning electron micrographs of the

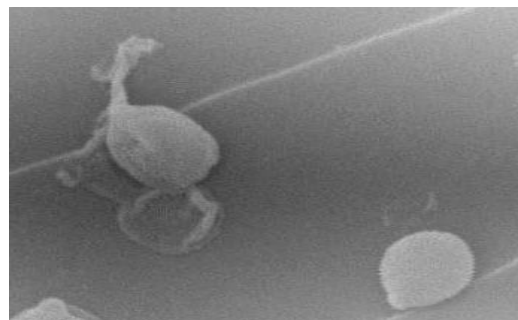


Fig. 1: Germ tubes emerged from a single conidia and extended over the cuticle, Selected examples of scanning electron micrographs showing the external development of *B. bassiana* and *M. anisopliae* on the cuticle of the mite *V. destructor*

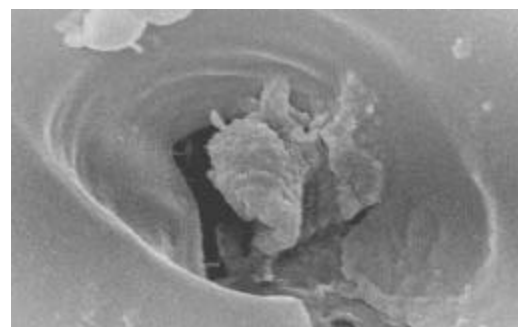


Fig. 2: Penetration the cuticle to find the more suitable exo-penetration sites, Selected examples of scanning electron micrographs showing the external development of *B. bassiana* and *M. anisopliae* on the cuticle of the mite *V. destructor*.

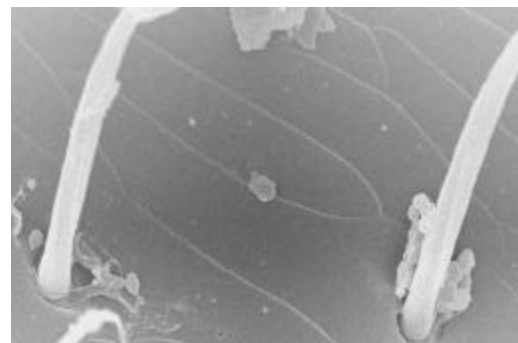


Fig. 3: Mycelium development on the host, Selected examples of scanning electron micrographs showing the external development of *B. bassiana* and *M. anisopliae* on the cuticle of the mite *V. destructor*.

cuticle of *V. destructor* infected with *M. anisopliae* and *B. bassiana*. Conidia of *M. anisopliae* were observed adhering to all parts of the body of the mite and the fungus rapidly colonized the surface of the host's cuticle. Germ tubes grew from conidia and extended over the cuticle (Fig. 1). The fungus was found to form germ-tubes and started to penetrate the structures of the host within 24- 48 hrs after inoculation. Penetration started in the epicuticle, then to the other regions of the cuticle and thereby inside the mite's body.

The epicuticle was penetrated without formation of an appressorium-like structure. Later on, an appressorium was formed and full development of the germination tube which started to find possible site of penetration. The present observations demonstrate the following sequence of events in the infection of the mite *V. destructor* as a host by *M. anisopliae*: (1) Attachment to the host (2) Conidium germination and formation of germ tube

(3) Mycelium development on the host (Figs. 3 and 4) (4) Penetration and growth of the pathogen on and in the host and (5) Sporulation on the surface of the host's body (Fig. 5).

**Transmission electron microscopy (tem):** Transmission Electron Microscopy (TEM) studies were carried out to describe the internal development of *M. anisopliae* and *B. bassiana* inside the body-cavity of the mite *V. destructor*. TEM sections of the mite individuals infected with *M. anisopliae* are shown in Fig. 6 to 9. It is well known that the infection mainly takes place through the cuticle due to body contamination with the conidia of the entomopathogenic fungus (Fig. 6). Therefore, the first step of infection is to adhere to the cuticle to find the more suitable exopenetration sites. After the exopenetration, cylindrical conidia spread

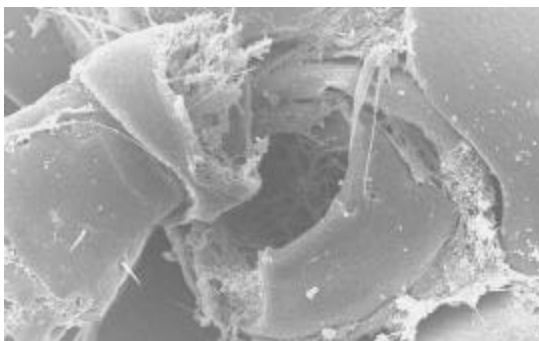


Fig. 4: Growth of the pathogen on and in the host, Selected examples of scanning electron micrographs showing the external development of *B. bassiana* and *M. anisopliae* on the cuticle of the mite *V. destructor*.

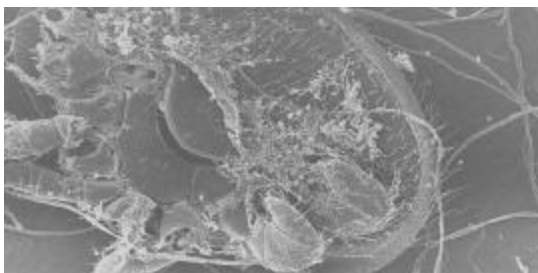


Fig. 5: Sporulation on the surface of the hos's body, Selected examples of scanning electron micrographs showing the external development of *B. bassiana* and *M. anisopliae* on the cuticle of the mite *V. destructor*

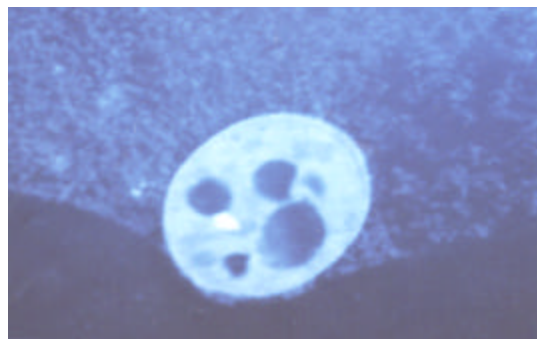


Fig. 6: The infection mainly takes place through the cuticle, Selected examples of transmission electron micrographs showing the internal development of *M. anisopliae* inside the body-cavity of the mite *V. destructor*

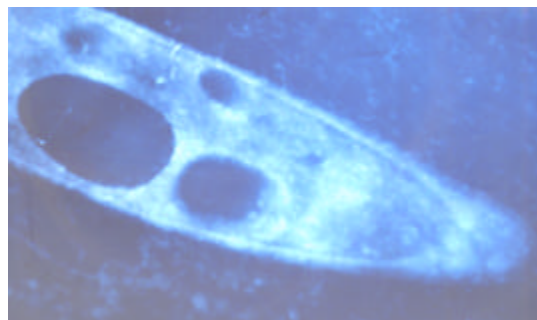


Fig. 7: The nucleus of a mother conidium starts to divide into two nuclei, Selected examples of transmission electron micrographs showing the internal development of *M. anisopliae* inside the body-cavity of the mite *V. destructor*

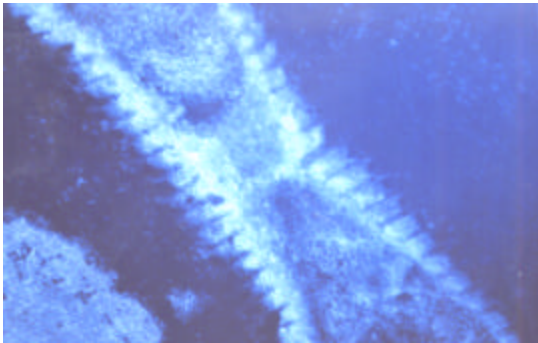


Fig. 8: The nucleus of a mother conidium divide, forming two sister conidia, Selected examples of transmission electron micrographs showing the internal development of *M. anisopliae* inside the body-cavity of the mite *V. destructor*

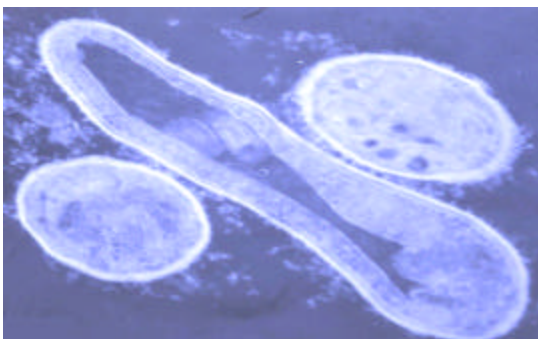


Fig. 9: Death of the host might be due to the effect on hemolymph cells and hemolymph characters, Selected examples of transmission electron micrographs showing the internal development of *M. anisopliae* inside the body-cavity of the mite *V. destructor*

through the body fluid and started to produce a toxin (s) that weakness the host's immune system and supply the fungus conidia with the favorable conditions to start the division process Fig. 7. An electron micrograph of a full structure of *M. anisopliae* conidium showed its main components (cell wall, mitochondria, vacuole and nucleus). The nucleus of a mother conidium starts to divide into two nuclei, forming two sister conidia Fig. 8. The division of the conidia of the fungus *M. anisopliae* inside the body-cavity of the mite is a mitosis one with the haploid nuclei. Conidium division is apparently required. The resulting increase in number of conidia makes it possible to excrete more toxin(s) to suppress the host's immunity system and complete the life-cycle within the host which eventually lead to host's mortality. Death of

the host might be due to the effect on hemolymph cells and hemolymph characters (Fig. 9). Also, conidiagerminate in the hemolymph and penetrate muscles, nervous system, malpighian tubules etc.

## DISCUSSION

**Efficacy of the two fungus-based biopesticide:** The biopesticide Bioranza which is dependent upon the entomopathogenic fungi, *M. anisopliae* was the most efficacious fungal formulation tested because it caused the highest reduction in mite counts in the hives. Mites are susceptible to entomopathogenic fungi (Chandler *et al.*, 2000). Among the pathogens of varroa, only entomopathogenic fungi have the desired characteristics of a control agent (Chandler *et al.*, 2001). Observations in this investigation indicated that it was critical to have fungal spores with good germination, pathogenicity and virulence. In the fungal treatments, mite infestations in the hives were significantly lower than those recorded in the control at day 14 after treatment. Fungi of the genus *Beauveria* can be considered as natural enemies of the mite since they have been found naturally-occurring on varroa (Steenberg *et al.*, 2010). Despite the fact that they show specificity towards the mite, results of field tests have been mixed, with some research groups reporting a measure of success and other groups reporting no effect as biopesticides (Meikle *et al.*, 2012). This could simplify future registration procedures. The overall differences between all treatments and the controls were statistically significant at the end of the experiments. All treatments caused significant change in mite infestations on adult bees over time and that the relationships between the treated and control hives varied over time. It was determined that fungal formulated spores provided successful control of mite populations in established honeybee colonies at 10 g of conidia per hive applied two times (day 0 and day 7). Microbial control of varroa mite with *M. anisopliae* is feasible and could be a useful component of an integrated pest management program for the honeybee industry. In addition, organic beekeepers, homeowner, hobby beekeepers should benefit from a novel, user-friendly and chemical-free strategy to manage the destructive pest of honeybees. Overall, this user-friendly delivery method for the fungus, *M. anisopliae* could provide beekeepers with effective, environmentally sound and sustainable control option for varroa mite populations in honeybee colonies. *B. bassiana* appeared to be harmless to honeybee workers (Martin, 2001; James and Hayes 2006; James *et al.*, 2006; Meikle *et al.*, 2006). The high correlation between mite mortality and fungal infection is an indication that the

fungus was the major mortality factor of the varroa mite population in the observation hive experiments. However, the fungus proved of good persistence as the infecting mites were recorded 14 days after the application. Also, the low infection rates found in the control hives may be an indication that bees carrying fungus-treated mites drifted the fungus between hives. The current and future target of the present research is to develop 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*.

**Electron microscopy examination:** The aforementioned explanations agree with Moino *et al.* (2002) who concluded that the infection processes varies in different entomopathogenic fungi. He reported that the initiation of *B. bassiana* conidial germination happened between 12 to 48 hrs after the inoculation of the subterranean termite *Heterotermis tenuis* (Order: Isoptera). Altre *et al.* (1999) reported that a higher germination percentage might help increase the probability of infection before spores are removed from the cuticle surface. An early event in germination of hydrophobic conidia is very probably favored by increasing fungal affinity to insect-like cuticle components. *B. bassiana* produce lipases, proteases and chitinases which can degrade insect cuticle (Charnley, 2003). These observations coincide with the commonly described sequence of events characterizing other entomopathogenic fungal infections (Askary and Yarmand, 2007). Therefore, the use of entomopathogenic fungi such as *B. bassiana*, alone or associated to safe chemicals is an efficient and environmentally favorable method for controlling the mite *V. destructor* attacking honeybee. In this respect, Fargues *et al.* (1994) stated that penetration, colonization and sporulation occurs faster in the fungus *M. anisopliae* than in *B. bassiana* resulting in the earlier death of hosts infected with the former fungus.

## CONCLUSION

In conclusion, results obtained from this study demonstrate that Bioranza which is dependent upon the entomopathogenic fungi, *M. anisopliae* was the most efficacious fungal formulation tested against *V. destructor* in honeybee colonies. Electron microscopy examination describes the external and the internal development of spores of the two fungus-based biopesticide on and inside the mite *V. destructor*.

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