**Original Article**

# A Field Experiment to Assess the Rate of Infestation in Honey Bee Populations of Two *Metarhizium anisopliae* Isolates on *Varroa destructor* (Acari: Mesostigmata)

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## Abstract

**Background:** The protective effect of two isolates of an entomopathogenic fungus, *Metarhizium anisopliae* (DEMI 002 and Iran 437C) on the adult stage of *Varroa destructor* was evaluated in comparison with fluvalinate strips in the field.

**Methods:** A total of 12 honey bee colonies were provided from an apiculture farm. The selected hives were divided into 4 groups (3 hives per group). The first group was the control, treated with distilled water. The other two groups were exposed to different fungi (*M. anisopliae* isolates DEMI 002 and Iran 437C) and the last group was treated with one strip of fluvalinate per colony. The number of fallen mites was counted using sticky traps during a 6-day period, six days before and after treatments. A fungal suspension at a concentration of $5\times 10^6$ conidia/mL was sprayed onto the frames and the number of fallen mites was counted.

**Results:** *Metarhizium anisopliae* DEMI 002 and Iran 437C isolates were as effective (i.e., caused as much mite fall) as the fluvalinate strip in controlling bee colonies than no treatment.

**Conclusion:** Both *M. anisopliae* isolates are promising candidates as agents in the control of *Varroa* mites under field conditions. Isolate DEMI 002 can be considered as a possible non-chemical biocontrol agent for controlling bee infestation with *V. destructor* in the field. In order to substantiate this hypothesis, tests are currently being performed using larger colonies and larger doses than tested in the present study in our beekeeping.

**Keywords:** *Apis mellifera*, Biological control, Entomopathogenic fungi, Fluvalinate, *Metarhizium anisopliae*, *Varroa destructor*

## Introduction

The honey bee, *Apis mellifera*, is an important insect for honey production and crop pollination. The ectoparasitic mite of the genus *Varroa* is currently the most serious threat to beekeeping around the world (De Jong et al. 1982, Anderson and Trueman 2000). This parasitic mite causes weight loss, malformation, and shortens the life span of honey bees, it also serves as a disease vector (Chen and Siede 2007). Bee mortality, due to infestation with *Varroa* mite, reaches up to 100% in untreated colonies. Miticides, especially used in the area around Iran, are fluvalinate (fluvalinate, Vita (Europe) Ltd.) and coumaphos, which remain the most cost-effective and widely used chemicals of mite control for management of honey bee colonies (Ferrer-Dufol et al. 1991). However, these chemicals leave

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residues in wax and honey (Cabras et al. 1994, Wallner 1995, 1999). Other chemical control agents such as flumethrin, amitraz, cymiazole and bromopropylate are also associated with toxic residues (Gamber 1990, Wallner 1995). Considering these problems and the development of resistance in *Varroa* mite populations to fluvalinate and coumaphos (Elzen et al. 1998, Elzen and Westervelt 2002), it is critical to develop new and safer alternatives for better control measures.

There has been an increasing interest to search for alternative sustainable control methods of varroosis in recent years. Several biological control programs have been developed for *Varroa* control and some studies have used herbal medicine such as essential oils of aromatic plants and organic acids (Imdorf 1997, 1999). A relatively new alternative to varroosis control is based on biological control using entomopathogenic fungi, natural enemies of mites (Chandler et al. 2001). Entomopathogenic fungi are known to infect different tick species, among which *Beauveria bassiana* and *Metarhizium anisopliae* have received major attention (Kalsbeek et al. 1995). The mechanism by which fungi infect arthropods is not fully understood, but it involves the production of some important fungal enzymes such as chitinases, proteases, lipases and also fungal structures that penetrate the cuticle (St Leger et al. 1987, 1997, Campos et al. 2005). Fungi are widely used for the control of agricultural and forest pests and in recent years efforts have been made to evaluate the biological control potential of these fungi against important arthropod vectors of human and animal diseases (Pirali-Kheirabadi et al. 2007a, b). The successful biological control of *Varroa destructor* and *V. jacobsoni* using entomopathogenic fungi has previously been achieved under laboratory and field assay conditions (Shaw et al. 2002, Kanga et al. 2006, Garcia-Fernandez et al. 2008).

Based on their findings and on our previous data on the pathogenicity of these fungal isolates on *Rhipicephalus (Boophilus) annulatus* (Acari Ixodidae) (Pirali-Kheirabadi et al. 2007a, b), we decided to evaluate the efficacy of two Iranian isolates, DEMI 002 and Iran 437C, of the entomopathogenic fungus *Metarhizium anisopliae.* These isolates were used for the first time in this field trial to determine their ability to be used as promising biological control agents for *Varroa* mite control in honey bee colonies. The aim of this study was to ultimately determine the pathogenicity of both isolates against adult stages of *V. destructor*, with special reference to mortality rate.

## Materials and Methods

### Fungal isolates

*Metarhizium anisopliae* isolates DEMI 002 and Iran 437C were obtained from the fungal culture collection of the Department of Mycology of the Iranian Research Institute of Plant Protection, Tehran, Iran (Table 1). Fluvalinate was prepared as recommended by the manufacturer (Sichuan Wang's Animal Health Co., Ltd.) and used as a treatment comparison against the two fungal isolates (Table 1).

### Preparation of conidial suspension

The fungi were cultured on potato dextrose agar (PDA, E. Merck) plates for 2 weeks at 25 °C and 70% relative humidity (RH) in the dark. To avoid bacterial contamination, an antibiotic, ampicillin (200 µg/mL, Biotika) was mixed with 10 mL of ethanol and added to the medium after filter sterilizing. Conidia were harvested by washing the plates with an aqueous solution of sterilized distilled water with 0.005% Tween 80 under a laminar flow hood. The conidial suspension was filtered through four layers of sterilized (muslin) gauze to remove fungal mycelia and other debris. Conidial numbers


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were determined using a Neubauer camera under a light microscope (Olympus CX41) at 400X magnification and the concentration of conidia was adjusted to $5\times 10^6$ conidia/ mL, which was sprayed evenly onto the frames in hives as described in more detail below. For this purpose frames were drawn out of the hives and the suspension was sprayed directly onto the frames using a microporous sprayer.

**Treatment of colonies with conidial suspension and chemical acaricide**

The treatments were conducted early in the morning. Both *M. anisopliae* isolates were used at a concentration of $5\times 10^6$ conidia/ mL (every hive received 10 mL of conidial suspension). These fungi isolates have no adverse effects on bees, animals, humans and the environment (Pirali-Kheirabadi et al. 2007a, b). Treatment with the fluvalinate strip was carried out following the methods of Kanga et al. (2006) and the number of fallen mites was counted using sticky traps during a 6-day period, even though the experiment spanned for 6 days prior to the sampling period and 6 days after.

**Experimental design**

Twelve bee hives of *A. mellifera* infested with *Varroa* mites were identified in an apiary located in the north of Iran (Lavizan, Tehran) (35° 38' 34" N, 51° 21' 27" E). The type of beehive was Langstroth and the colonies had at least 4 frames within each hive. Established groups were separated from the rest by about 3–4 m. The experiment was carried out in autumn, in September-October, when the temperature was between 15 and 25 °C and the RH was 50%. The mass, status and other parameters (age of queen, honey and pollen reserves and number of brood frames) were homogeneous. The selected hives were divided into 4 groups (3 hives per group). The first group was the control, which was treated with distilled water. The other two groups were exposed to different fungi (*M. anisopliae* isolates DEMI 002 and Iran 437C) and the last group was treated with one strip of fluvalinate (Apistan, 10% tau-Apistan, 800 mg per strip, Vita Europe Ltd. Co.) per colony. The fallen mites were collected daily from each hive during the first 6 days (this experiment spanned for 12 days).

**Statistical analysis**

Pre- and post-treatment data were used in the analysis to determine the efficacy of the fungal treatments. The experiment was organized in a complete randomized block design (CRBD). There were three replicates per block and data was collected daily. The total number of collected mites for each hive throughout the experiment was recorded. Daily percentage of collected mites was calculated for each hive. The effect of the main factors, i.e. day and type of treatment (control, isolate type (Iran 437C and DEMI 002) and fluvalinate) on the percentage of collected mites was analyzed using the General Linear Model (GLM) procedure of SAS. Least square means (LS Means) were used with the SAS pdiff test (SAS statistics, 9.2). The overall effects of treatments on the mean percentage of collected mites from hives before and after treatments were analyzed using the GLM procedure of SAS. Data was expressed as LSMeans ± SEM.

## Results

The total (mean/hives) number of collected mites from the control, fluvalinate, *Metarhizium* DEMI 002 and Iran 437C isolates was 40 (1.1±0.02), 155 (4.3±0.6), 170 (4.7±1.3) and 74 (2.05±0.05), respectively. Fig. 1 shows the percentage of collected fallen mites before and after treatment within each group. The mean percentage of total collected fallen mites before treatments was not significantly different between groups (Table 2). There was no statistical difference


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between collected mites before and after treatment in the control group (Table 2). The mean percentage of total collected mites was higher after treatment than before within the three groups tested (Table 2). However, there was no difference in the mean percentage of total mites collected after treatments among the three treatment groups (Table 2). Fig. 2 shows the daily changes in cumulative percentages of the collected fallen mites during the treatment for the four established groups.

**Table 1.** General characteristics of *Metarhizium anisopliae* fungal isolates used in this study

| Origin | Host | Strain |
|---|---|---|
| **Rasht, Iran** | *Chilo suppressalis* | IRAN 437 C |
| **Noor, Iran** | *Rhychophorus ferrugine* | DEMI 002 |

**Table 2.** Total collected mites and the percentages (LS Mean ± SEM) of collected mites (per day) before (7 days) and after (5 days) treatment within experimental groups

| Groups | Total collected mites | Mean daily percentages of collected mites during experiment | |
|---|---|---|---|
| | | Before treatment | After treatment |
| **Control** | 40 | 9.24±5.8Aa | 7.05±5.8Aa |
| **Apistan® Strip** | 155 | 3.5±5.8Aa | 15.5±5.8Bb |
| **Ma DEMI002** | 170 | 5.7±5.8Aa | 12.05±5.8Bb |
| **M.a. Iran 437 C** | 74 | 4.9±5.8Aa | 13.1±5.8Bb |

[AB] Values with different upper case letters within rows differ significantly (P< 0.05).
[ab]Values with different lower case letters within columns differ significantly (P< 0.05).

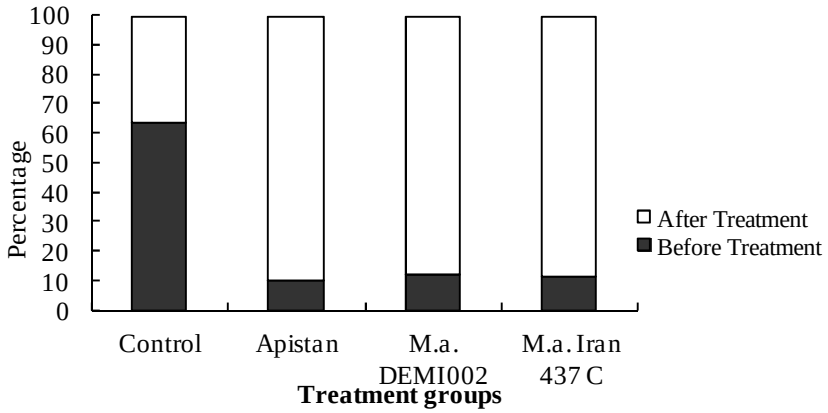


**Fig. 1.** The percentages of collected fallen mites before and after treatment within each group (Control: n= 40, Apistan: n= 155, *Metrhizium anisopliae* DEMI002: n= 170 and *M. anisopliae* Iran 437C: n= 74)


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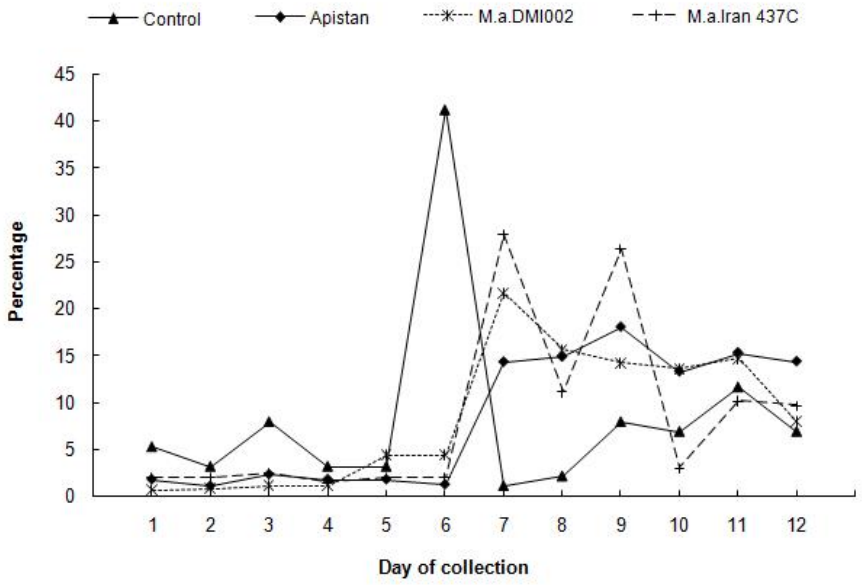


**Fig. 2.** Mean percentage of daily collected fallen mites from four experimental groups. (Day 6= 3 days before treatment and Day 7= day of treatment commencement)

## Discussion

In this experiment the basal data depended on an equal number of fallen mites, considered at the beginning of the experiment. Thus, among the hive colonies selected for the experiment, the primary infestation (according to the fallen mites) was statistically negligible. Therefore it could be concluded that both *M. anisopliae* isolates reduced the number of mites per hive at the end of the experimental period, and the mean total number of fallen mites per hive was significantly higher in Iran 437C, DEMI 002 and the fluvalinate strip compared to the control (Table 2).

At the end of the experimental period, fungal treatments had significantly reduced mites, about 1.85- and 4.25-fold more than the control group for Iran 437C and DEMI 002 isolates, respectively. The number of fallen mites was 3.87-fold higher than the control using the fluvalinate strip (Table 2), which showed comparable effects on mites to *M. anisopliae* DEMI 002. Experiments considering more replicates in a different are with variable humidity and temperature are needed to assess the proper dosage and to improve the effect of these potential biocontrol agents.

Entomopathogenic fungi have been used widely for the control of agricultural and forest pests (Kaaya et al. 1996), and much attention has been paid to evaluate the biological control potential of entomopathogenic fungi against the important bee parasite *V. destructor* (syn. *Acari mesostigmata*) under laboratory and field conditions since 2000. Therefore, the biological control of *V. destructor* requires natural enemies from other hosts. At present, the management of *Varroa* is based on the use of chemical pesticides throughout the world, but resistance to the miticide fluvalinate in *Varroa* mite populations has become widespread since 1998 (Elzen et al. 1998) and resistance to coumaphos was documented since 2002 (Elzen and Westervelt 2002). There is a need, therefore, for alternative and sustainable forms of *Varroa* management. The present study


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shows clearly that these fungal isolates are promising candidates for the control of *V. destructor* in field conditions (Kanga et al. 2003, 2006).

Lower concentrations of fungal spores were tested in our experiment compared to those in other studies in which some isolates of *M. anisopliae* (Shaw et al. 2002, Kanga et al. 2006) and *Beauveria bassiana* were tested against *V. destructor* (Meikle 2006, 2008). A possible explanation for fairly different results relative to other studies may lie in the fact that in our assay we directly sprayed conidial suspensions onto the frames. In contrast, in most other studies, a strip coated with conidia was used (eg Kanga et al. 2006). The other possible explanation is that isolate Iran 437C truly has a powerful miticidal effect on mites at higher doses compared with the fluvalinate strip although different areas with variable humidity and temperature are needed to assess the proper dosage; these are currently being performed using more recipients and larger doses than those tested in our field experiment. The *M. anisopliae* fungal isolate DEMI 002 can control *V. destructor* effectively, more than isolate Iran 437C. The fluvalinate strip could inhibit mites during days 8 and 9 (2–3 days) post treatment but in the case of *M. anisopliae* isolates DEMI002 and Iran 437C caused most mortality during the first 72 h post treatment (3 days) (Garcia-Fernandez et al. 2008) (Fig. 2). Differences between treated groups were significant considering the sum of squares before and after treatment ($P< 0.05$).

Future research will aim to determine the most suitable method and concentration to deliver the fungus into the hives and also to determine the effect of these fungal isolates on non-target organisms. Unlike miticides, it is less likely that *Varroa* mite populations could develop resistance to fungal infections because of the multiple target receptors involved in fungal infections as compared to a miticide's mode of action (Chandler et al. 2001).

## Acknowledgements

The authors declare that there is no conflict of interest.

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