Journal of Invertebrate Pathology 111 (2012) 237–243

Contents lists available at SciVerse ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

# Entomopathogenic fungi as potential biocontrol agents of the ecto-parasitic mite, *Varroa destructor*, and their effect on the immune response of honey bees (*Apis mellifera* L.)

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ARTICLE INFO

*Article history:*
Received 20 April 2012
Accepted 7 September 2012
Available online 19 September 2012

*Keywords:*
*Varroa destructor*
Immune-related gene expression
Induced resistance
*Hymenoptaecin*
*Metarhizium anisopliae*
*Beauveria bassiana*

ABSTRACT

Three isolates of each of the entomopathogenic fungi, *Metarhizium anisopliae*, *Beauveria bassiana* and *Clonostachys rosea*, were assessed for their pathogenicity to the honey bee parasitic mite, *Varroa destructor*. The fungi were applied to varroa mites by immersing them in a spore solution, and then the inoculated mites were placed on honey bee brood inside capped cells. At 7 days post inoculation (dpi), the three fungi caused significant varroa mortality compared to non-inoculated mites. In brood treated only with varroa mites, expression of the honey bee genes, *hymenoptaecin* and poly U binding factor 68 Kd (*pUf68*), decreased over time, while expression of blue cheese (*BlCh*) and single minded (*SiMd*) was not affected. In brood inoculated directly only with *M. anisopliae* or *B. bassiana*, the emerged adults showed reduced weight indicating infection by the fungi, which was confirmed by observation of hyphae in the brood. Fungal infection of the brood resulted in increased expression of *hymenoptaecin*, *pUf68* and *BlCh*, but not *SiMd*. In brood treated with varroa mites that had been inoculated with the fungi, expression of *hymenoptaecin*, *pUf68* and *BlCh*, but not *SiMd*, was even more up-regulated. While varroa mites can suppress gene expression in honey bee brood, varroa mites infected with entomopathogenic fungi induced their expression. This may be due to a low level of fungal infection of the bee, which negated the immunosuppression by the mites. Therefore, entomopathogenic fungi could reduce varroa mite damage to honey bee brood by both infecting the parasite and preventing varroa-associated suppression of honey bee immunity.



## 1. Introduction

Beekeepers, crop growers, scientists and the general public are concerned with the mysterious die-offs of honey bee (*Apis mellifera*) colonies that have occurred during the last 5 years in many countries around the world. The phenomenon has been termed colony collapse disorder (CCD) in the USA. While many suspects have been suggested as causes of these losses, no clear explanation has yet been found (vanEngelsdorp et al., 2008). However, many scientists believe that it is due to a combination of factors (Stankus, 2008).

One important factor associated with bee mortality is the parasitic mite, *Varroa destructor*. Without treatment, honey bee colonies typically die within 2 years after initial varroa infestation (De Jong et al., 1982). Thus, several synthetic miticides are used by beekeepers for their control. Although initially effective, the continuous use of these pesticides has led to the development of miticide resistance within a few years (Milani, 1999). Miticide resistance is now widespread in Europe, USA and Canada (Elzen et al., 1998, 1999; Milani, 1999; Sprefacio et al., 2001; Elzen and Westervelt, 2002; Thompson et al., 2002; Skinner et al., 2003). A recent study showed that most (>85%) colony fatality cases during winter in Canada were significantly associated with varroa mite infestation despite colonies being treated with synthetic miticides (Guzman-Novoa et al., 2010). This suggests that mite populations are becoming more difficult to control in recent years. In addition, the use of synthetic miticides in bee hives raises the risk of contamination of honey and other hive products (Ruijter, 1995; Wallner, 1999). These disadvantages are a considerable incentive to develop new strategies for mite control that minimize miticide resistance and miticide accumulation in bee products.

The ideal varroa control treatment should be environmentally friendly (i.e., limited non-target effects), varroa-selective (i.e., kills varroa at doses that are relatively harmless to bees) and should leave little to no residues in honey and wax. An alternative strategy for mite control in hives, therefore could be the use of entomopathogenic fungi as biological control agents of *V. destructor*. They can infect varroa, are non-toxic to humans, can be mass-cultured and occur naturally in the environment.

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0022-2011/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jip.2012.09.001

The entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, have been used as biocontrol agents against several insects in the genera *Eurygaster* and *Aelia* (Aquino de Muro et al., 2005), as well as cattle ticks (Frazzon et al., 2000). Application of *M. anisopliae* and *B. bassiana* to honey bee colonies has provided some degree of control against *V. destructor* under field conditions (Kanga et al., 2003, 2006; Meikle et al., 2007, 2008), although not in all cases (James et al., 2006). However, the relative pathogenicity of these fungi to bees and brood is largely unknown. Additionally, it would be desirable to test other fungi that have shown potential for the control of insects, such as *Clonostachys rosea* (Toledo et al., 2006; Vega, 2008).

There are no reports thus far about whether exposure to entomopathogenic fungi can cause induction or suppression of gene expression in the honey bee. However, varroa mite parasitism can affect the expression of a number of honey bee genes (Yang and Cox-Foster, 2005; Navajas et al., 2008). For example, *hymenoptaecin*, which encodes an antimicrobial peptide as part of the immune system (Casteels-Josson et al., 1994), was down regulated by *V. destructor* parasitism (Yang and Cox-Foster, 2005). Similarly, both the genes for poly U binding factor 68Kd, *pUf68*, involved in pre-mRNA splicing (Foley and O'Farrell, 2004), and the basic helix-loop-helix-PAS transcription factor, *SiMd*, involved in locomotory behavior (Pielage et al., 2002), were down regulated by varroa mites (Navajas et al., 2008). In contrast, expression of the gene for the autophagy-linked FYVE protein, *BlCh*, involved in autophagasome trafficking to lysosomes and prevention of neural degeneration during aging (Finley et al., 2003), was up-regulated by varroa mite parasitism (Navajas et al., 2008).

The objectives of this study were to evaluate the efficacy of selected entomopathogenic fungi as potential biocontrol agents of varroa mites on honey bee brood, and to investigate whether such entomopathogenic fungi could also detrimentally affect brood, as well as up or down regulate the expression of honey bee genes, such as those previously shown to be suppressed or induced by *V. destructor* parasitism.

## 2. Materials and methods

### 2.1. Collection of V. destructor

Experiments were conducted at the Honey Bee Research Centre, University of Guelph, Guelph, Ontario, Canada. Adult *V. destructor* from heavily infested honey bee colonies that had not been treated with miticides for at least 6 months were harvested from brood cells using a fine paint brush. The harvested mites were held in Petri dishes lined with moist filter paper, and two white-eyed bee pupae collected from a non-infested colony served as the food source for the parasites. Varroa mites were used within 2 h from the time of collection.

### 2.2. Entomopathogenic fungal cultures

From the University of Alberta Microfungi Collection, fungal isolates were obtained for *M. anisopliae* UAMH 4450, UAMH 9197, UAMH 9198, *B. bassiana* UAMH 1069, UAMH 9744 and *C. rosea* UAMH 7494, UAMH 9161. From the University of Guelph, *C. rosea* isolate Endofine® was provided by Dr. John Sutton, and *B. bassiana* isolate GHA (Botanigard 22WP®) was obtained from Laverlam International Corporation, Butte, MT, USA. All isolates were grown on Potato Dextrose Agar (PDA) containing 100 mg/L streptomycin sulfate at 26 °C and 80% RH. Conidia were harvested from 21-day-old cultures by flooding the dishes with sterile 0.03% Tween 80 and gently scraping the surface of the culture with a soft-tipped sterile spatula. After vortexing, the conidial suspensions were filtered through sterilized double cheese cloth. The conidial concentration was then adjusted to $1 \times 10^8$ conidia/mL in dd $H_2O$ (Shaw et al., 2002; Kanga et al., 2003; Rodriguez et al., 2009).

### 2.3. Honey bee brood inoculation with fungi-treated varroa mites

Groups of 30 *V. destructor* were inoculated with the nine fungal isolates by individually immersing them in 5 mL of the conidial suspension for 10 s. The varroa mites were then dried by placing them onto filter paper in a Petri dish, which was sealed with micro-perforated Parafilm. To infest honey bee brood with varroa mites, groups of 10 newly-capped brood cells each from three brood frames obtained from a healthy colony, were colored on their outer rims with water based, non-toxic, paint markers (L551P2, Hunt Int., Mississauga, Ont., Canada). Colored capped cells containing brood were opened by cutting a thin slit approximately 2 mm long using a sterile blade, and then three fungal-inoculated *V. destructor* were transferred into each cell using a fine paintbrush. The slit was resealed by lightly brushing it with liquid beeswax. As the control, 30 varroa mites were used per replicate that had been immersed in dd $H_2O$ before introducing them into brood cells. The three frames were placed inside screened cages in an incubator at 33–35 °C and 80% RH for 10 days. Varroa mite mortality in each inoculated cell was recorded every 48 h by cutting open the cell cap as described above, removing the brood with a fine forceps and examining for dead mites. A mite was considered dead if it did not move after being probed with a pin. If a mite was alive, it was then transferred to another cell containing live brood in the same frame, and the cell was sealed and marked. Dead varroa mites retrieved from the treated cells were surface sterilized in 90% ethanol, cultured on PDA with streptomycin, and incubated for 36 h at 26 °C and 80% RH. The presence of mycelia growing from a varroa mite cadaver indicated fungal-caused mortality. The experiment was replicated three times for a total of 300 mites. Test frames were examined daily for emerged worker bees, which were swept off the frames to prevent them from removing brood in infested cells.

### 2.4. Honey bee brood inoculation with selected fungal isolates

Inoculation of honey bee brood with *M. anisopliae* UAMH 9198 or *B. bassiana* GHA, selected for their high pathogenicity to varroa mites from the previously described experiment, was done by collecting three brood frames containing newly capped cells and incubating them overnight at 33–35 °C and 60% RH. Rows of brood cells on each side of each frame were transected and color-coded as described above, with each color corresponding to a fungal isolate or control treatment. Each transect was randomly assigned to be inoculated with 5 μL of the conidial suspension of *B. bassiana*, *M. anisopliae* or water. Each fungal treatment was applied to 30 brood in newly capped cells that were opened and resealed as previously mentioned. The brood cells were covered with a wire mesh screened cage that was manually embedded on the comb to capture the bees emerging from the treated cells. The frames were returned to the incubator and observed for bee emergence. Hatched bees were removed and held in feeding cages ($12.7 \times 8.5 \times 14.5$ cm) provided with water and sucrose syrup in gravity feeding bottles. Total number of bees that emerged and brood mortality (i.e., brood that did not hatch) were recorded. Body weights of emerged adult bees were determined for bees anesthetized using carbon dioxide.

To determine the timing and extent of fungal colonization of the honey bee brood, cells were uncapped, and inoculated brood were removed with a fine forceps at 1 and 7 days post-inoculation (dpi). They were then washed with 90% ethanol and transferred to microscope slides where they were crushed and stained with lac-