Biological control of *Varroa destructor* by fungi

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The mite *Varroa destructor* is a pest in bee colonies (*Apis mellifera*). The mites feed on haemolymph of the bees and their larvae and they can transmit viruses. Little work has been done on biological control of this mite. Only in the last few years the effect of insect pathogenic fungi on *Varroa* mite is investigated. Literature shows that several insect parasitic fungi are able to infect and kill *Varroa* without being a threat to honey bees.
In this paper three products based on the fungi *Metarhizium anisopliae* and *Lecanicillium lecanii*, which are currently sold in The Netherlands for the control of insect pests, were tested in bee hives for their effect on *Varroa*. Neither of them was effective against *Varroa*, at least not in the currant formulation. For the control of *Varroa* we should search for fungi that work under bee hive circumstances, high temperature and low humidity, and a good application method.

Keywords: Metarhizium anisopliae, *Lecanicillium lecanii*, bee hive, insect pathogenic fungi, Bio1020, Mycotal, Vertalec

The mite *Varroa destructor* is a pest in bee colonies (*Apis mellifera*). The mites feed on haemolymph of the bees and their larvae and they can transmit viruses like Deformed Wing Virus. Little work has been done on biological control of this mite (Chandler *et al.* 2001). Only in the last few years the effect of insect pathogenic fungi on *Varroa* mite is investigated. Shaw *et al.* (2002), Peng *et al.* (2002) and Davidson *et al.* (2003) showed in laboratory tests that several insect parasitic fungi are able to infect and kill *Varroa* without being a threat to honey bees. For example *Metarhizium* spp., *Hirsutella* spp. and *Lecanicillium lecanii* look very promising for control of *Varroa*. Kanga *et al.* (2002 en 2003) tested a product based on *Metarhizium anisopliae* in a field trial and found very good control of *Varroa*.

Products based on *M. anisopliae* and *L. lecanii* are currently sold in The Netherlands for the control of insect pests. Since the registration of products based on fungi is often expensive and very time consuming, it was decided first

to check the possibilities of the existing fungal-products in the control of *Varroa* in The Netherlands.

Mycotal and Vertalec, both containing *L. lecanii*, are used for control of insects in greenhouses. Bio1020 contains *M. anisopliae* and is used for control of soil insects. Shaw *et al.* (2002) tested the pathogenicity of Vertalec and Mycotal in laboratory tests. They found at 25°C 100% *Varroa* mortality for both fungi and at 30°C 40% for Mycotal and 100% for Vertalec. Davidson *et al.* (2003) tested growth of Vertalec, Mycotal and Bio1020 on agar plates at several temperatures. At 30°C Bio1020 showed best growth. At 35°C non of the three fungi showed growth any more.

Even though there is no fungal growth at 35°C, the temperature in the brood nest, mortality at 30°C looks promising enough to test these fungi in a field trial.

MATERIAL AND METHODS

Bee colonies

Out of 80 *A. mellifera* colonies in 'Mini Plus' hives (polystyrene hives 30x30x60cm, 3000 to 5000 bees) 48 colonies where selected with *Varroa* infestation. All colonies contained brood in all stages. Each treatment contained 7 colonies except for the control treatment C1, which contained only 6 colonies. Pretreatment mite fall per colony varied from 0.4 to 11.4 per day. The colonies where divided over the treatments in a way that mean mite fall per treatment group (7 colonies) varied between 2.3 and 3.4 per colony per day.

In the experiment of Kanga *et al.* (2003) fungal spores spread over all treatments, also the control treatment. To prevent this, three locations where used within a circle of 3 km diameter. One location contained all control treatments, one location contained all treatments with *Metarhizium anisopliae* and the last location contained all treatments with *Lecanicillium lecanii*.

Fungal treatments

Experiments were conducted between August 19 and October 7. Bio1020 was received from Bayer Cropscience B.V. This product consists of *Metarhizium* spores on rice. This product was used in two different ways. The first group of colonies (treatment M1) was treated with a suspension of *Metarhizium* spores in 0.01% Triton X-100. This suspension was made by washing 100 g product (rice grains) in 500 ml 0.01% Triton X-100 solution and removing the rice. Per colony 50 ml suspension was sprayed over the bees and combs. The concentration of spores in the suspension was determined directly before treatment, by plating dilutions on specific agar-medium (see below) and came to more then 10^7 spores/ml. In the second group (M2) 40 gram of the Bio1020 rice-grains was sprinkled over the top of the frames in each hive.

Two products based on *Lecanicillium lecanii* where used: Mycotal and Vertalec, both provided by Koppert BV. From both products a suspension was

made according to the description on the box. The concentration of spores in the suspension was determined directly before treatment, by plating dilutions on specific agar-medium (see below) and came to 1.5 10^6 spores/ml for Mycotal and 1.7 10^4/ml for Vertalec. Per colony 50 ml suspension was sprayed over bees and combs.

Control treatments

The experiment contained three control groups. The colonies of group C1 were not treated. The colonies of treatment C2 were treated with a 0.01% Triton X-100 solution. Per colony 50 ml of the solution was sprayed over the bees and combs.

To compare the fungal treatments with a currently used method of control, a treatment with Thymovar was conducted. Per colony half a strip of Thymovar was put on top of the frames. After 3 weeks the strips were replaced by new strips, after another 3 weeks the strips were removed.

Persistence of the fungi

To investigate the persistence of the fungi in the bee colony, 30 bees per colony were randomly collected from each of the six treatment groups at 1, 4 and 7 days after treatment. The same method was used one day before treatment to detect natural occurrence of the fungus.

The tubes (Greiner 50 ml) with bees were kept cool during field sampling and processed within the hour in the lab. Per tube 20 ml of sterile 0.01% Triton X-100 solution was added and vortexed for 30 seconds. The solution was poured into a sterile tube and dilutions (10x, 100x, 1000x) were made with 0.01% Triton X-100. An aliquot 0.1 ml of the dilutions and the undiluted suspension (not on day 1) was spread onto a selective agar medium (9 cm Petri dish).

Selective media

The selective medium for *Metarhizium* consist of: 72.5 g/l Bacto oatmeal agar (Difco), 10mg/ml crystal violet, 250 mg/l chloramphenicol, 8 mg/l banlate (50% benomyl). For *Lecanicillium* the selective medium consist of: SDAY (10 g/l mycological peptone, 40 g/l dextrose (D-glucose), 2 g/l yeast extract, 15 g/l agar) containing 100 mg/l chloramphenicol. The agar plates were incubated in the dark at 25°C. After 3, 7 and 10 days fungus growth was checked.

Mite mortality

The mite fall was registered by collecting and counting mites on a sticky board (Brinkman) on the bottom board of the bee hive. The mite fall was counted from three days before treatment of the colonies till 56 days after treatment, every 3 or 4 days.

To investigate whether the mites were infected with fungi, 30 mites per treatment were removed from the sticky boards with tweezers and externally sterilized for 1 min in a Petri dish with 70% ethanol. Subsequently the mites were

washed twice with sterile tab-water. The mites were put onto water agar plates (12.5 g/l agar, 9 cm Petri dish), 30 mites per plate, one plate per treatment. The agar plates were incubated in the dark at 25°C. After 3, 7 and 10 days fungus growth was checked.

Bee mortality

To investigate the impact of fungal application on honey bees, a dead-bee-trap was placed at the entrance of each bee hive. Since there was no dead-bee-trap available for the Mini Plus hives, it was designed. Once or twice a week the dead bees were counted, till 56 days after treatment.

To investigate whether the bees were infected with fungi 21 dead bees were put in a 50 ml Greiner-tube and 30 ml 70% ethanol was added to externally sterilize the bees. Subsequently the bees were washed with 30 ml sterile 0.01% Triton X-100 and 30 ml sterile tab-water. The bees were put onto water agar plates (12.5 g/l agar, 9 cm Petri dish), 7 bees per plate, 3 plates per treatment. The agar plates were incubated in the dark at 25°C. After 3, 7 and 10 days fungus growth was checked.

Mathematics

Since treatment C1 only contained 6 hives instead of 7 in all other treatments, calculations were made to compare for this: #(dead bees or mites)/6x7=result.

RESULTS

Survival of the fungi

On day 1, 4 and 7 after treatment the presence of viable conidia on bees was determined. None of the plates with solution washed from live bees contained viable conidia of either *Metarhizium* or *Lecanicillium*. This means that the bee samples (30 bees) contained less then 2000 conidia on day 1 and less then 200

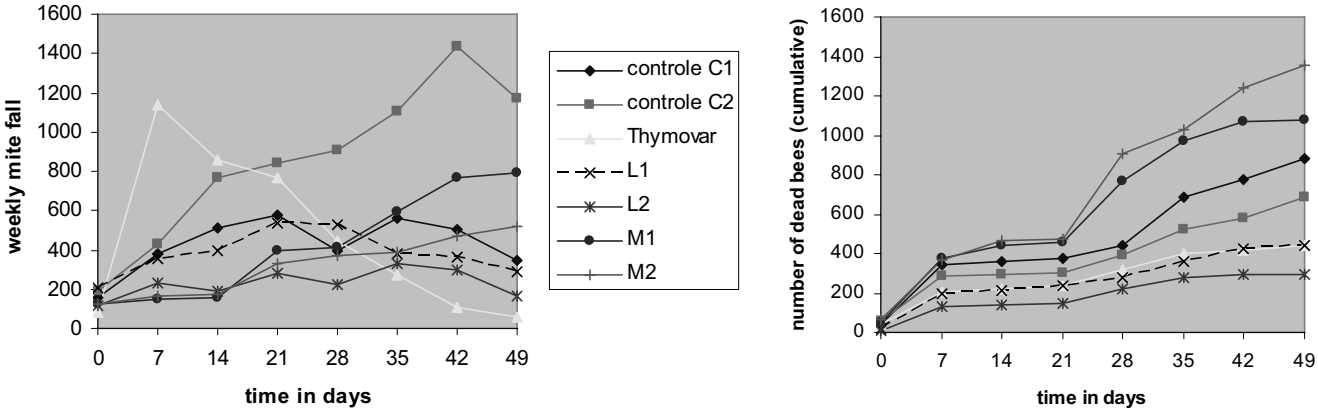

Figure 1. The effect of different treatments on mite fall (*Varroa destructor*) and bee mortality (*Apis mellifera*). C1 is not treated, C2 is sprayed with 0.01% Triton X-100 solution. L1 and L2 are treated with *Lecanicillium lecanii*, respectively with Mycotal and Vertalec. M1 and M2 are treated with *Metarhizium anisopliae* Bio1020, respectively in suspension and on rice grain. Weekly mite fall is the total of 7 hives.

conidia on day 4 and 7. Before the treatment *Metarhizium* was found in one of the samples from the location on which the *Metarhizium* treatment was going to take place. Since this was only one colony it could be a chance hit.

Mite mortality

Figure 1 (left) shows the mite fall per treatment. Dead mites were checked for fungus infection on day 4, 11 and 26 after treatment. On day 4 and 26 no mites infected with *Metarhizium* or *Lecanicillium* were found. On day 11 both treatment M1 and M2 contained one mite infected with *Metarhizium*. A lot of mites were infected with bacteria or other fungi.

Bee mortality

Figure 1 (right) shows the bee mortality. The colonies treated with *Metarhizium* were located in a moist area under trees. Because of this the containers of the dead-bee-traps, in which the dead bees were collected, were often wet, even though there were drain holes in the container. Living bees, who fell into the container by accident, sometimes became so wet that they couldn't crawl out any more. When the containers with 'dead' bees were brought into the lab for counting, part of the bees started moving again in this warm and dry surroundings. Unfortunately some of the containers were checked in the field and the bees were thrown out. Therefore part of the living bees in treatment M1 and M2 were counted as dead.

Dead bees were checked for fungus infection on day 4, 7, 14, 21, 28 and 35 after treatment. Only on day 7 we found one bee infected with *Metarhizium*. Most other bees were infected with bacteria or other fungi. Part of the dead bees were in the bee trap for a few days before they were put on agar plates. The trap was emptied every 3 or 4 days. Therefore all kind of secondary infections could have taken place.

DISCUSSION

The effect of different treatments on *Varroa* is determined by the mite fall. There is a clear effect of Thymovar on *Varroa*. Directly after the treatment many dead mite fell down, after that the mite fall is less. Thus, the mite population decreases continuously in these colonies. In the control colonies without treatment (C1) the mite fall increases a bit and than stays at a constant level. In this case the mite population increases a bit and than stays at the same level. In the treatment with Triton X-100 (C2) there is a constant increase of the mite fall. At first this looks like a treatment with Triton X-100 improves mite population growth. However, in this treatment two of the seven hives had very high mite fall. Probably the starting population of these two hives was higher than the starting population of the other hives, resulting in an exponential growth of the population and a high mite fall.

Table 1. Growth and pathogenicity of different fungal strains at different temperatures: comparison of literature and this research.

Fungal strain	fungal growth on agar (mm/day)[1]		*Varroa*-mortality at 30°C in lab bioassay (%)[2]	Control of *Varroa* in bee eehivehives (*ca.* 35°C)
	at 30°C	at 35°C		
Mycotal	0.20	0.00	47	not demonstrated[4]
Vertalec	0.18	0.00	100	not demonstrated[4]
Bio1020	0.99	0.00	nt[3]	not demonstrated[4]
Bio-Blast	1.72	0.23	100	very well[5]

[1] from Davidson *et al.* 2003; [2] from Shaw *et al. 2*002; [3] nt= not tested; [4] this paper; [5] from Kanga *et al.* 2003

The big difference between the two control treatments shows that *Varroa* population density should be taken into account when looking at the effect of the treatments. Normally an after-treatment, with a chemical that kills all mites, is used at the end of the experiment, to count the whole mite population per hive, after which a percentage effect of the treatment can be determined. However, because of resistance to Apistan there was no chemical available for this after-treatment. In this experiment it was tried to divide the hives over the treatments in a way that all treatments had similar starting populations. Because of this and because all fungi treatments can be compared to two negative treatments (C1 and C2) and one positive treatment (Thymovar) it is still possible to draw reasonable conclusions from these experiments according to the mite fall.

If the fungal treatments would have been effective it was expected that in the beginning there would be a lot of mite fall and the mite fall would decrease towards the end. The first mite fall would not be so soon and so extreme as in the Thymovar treatment because it takes some time for the fungi to kill the mites. This pattern is not seen in any of the fungal treatments. In the *Metarhizium* treatments the mite fall slowly increases. In the *Lecanicillium* treatments the mite fall stays almost at a constant level, just like control treatment C1.

The fungal products used are on the market for the control of insect pests in horti- and agriculture. *Metarhizium* Bio1020 is used against soil insects and the soil temperature is mostly between 15 and 20°C. Both *Lecanicillium* products are used in greenhouses at a temperature between 15 and 25°C. In bee hives the temperature is 35°C (a bit lower outside the brood nest). Davidson *et al.* (2003) showed that the three fungal products used here do not grow on agar at 35°C. The fungi did grow at 20 and 30°C. This is probably the reason why there is no effect of the fungal treatments: they are not active at 35°C. At 25°C both *Lecanicillium* strains worked very well against *Varroa* in a lab test; after 7 days 98% and 100% mortality (Shaw *et al.* 2002). Vertalec showed even at 30°C 100% mite mortality (Shaw *et al.* 2002). It is possible that Vertalec would have shown

better results in our test at a higher spore concentration in the spray-solution. 10^4 spores per ml is rather low, Shaw *et al.* (2002) used 10^8 spores per ml. Normally a Vertalec spray-solution is supposed to have a higher concentration.

Already after one day the fungal spores could not be detected on the bees. It is not clear if the bees removed the fungal spores (washing, eating?) or if the spores were not viable any more after the extreme circumstances in the hive. Another possibility is that the detection level of our test method was too low.

Kanga *et al.* (2002) used Bio-Blast (Ecoscience, New Brunswick, NJ, USA), which contains another strain of *Metarhizium*. This strain grows well at 35°C (Davidson *et al.* 2003) and was very successful in a field test (Kanga *et al.* 2002; Table 1). This product was a powder and was dusted in the hives. Possibly this application method is more effective than our spaying method. They could still detect the fungi on the bees after 42 days.

The fungi have little effect on the bees. This was to be expected since test on honey bees are required for registration. The amount of dead bees in the Vertalec and Mycotal treatments was lower then in the control treatments. In the *Metarhizium* treatments there were more dead bees than in the control treatments. However, it is more likely that this is caused by the location than by the treatment. Because of the humidity on this location many healthy bees could not leave the bee trap because they were too wet. On the other locations it was less humid (more sun, wind) so it is difficult to compare the bee fall in these treatments.

In conclusion, literature shows that insect parasitic fungi are suited for the control of *Varroa*. However, the fungal strains on the Dutch market can not be used for the control of *Varroa*, at least not in the currant formulation. For the control of *Varroa* we should search for fungi that work under bee hive circumstances, high temperature and low humidity, and a good application method.

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