Chapter 15
Microbial Control for Invasive Arthropod Pests of Honey Bees

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Abstract Honey bees are critical to world agriculture because of their role in crop pollination. Unfortunately, the sustainability of this bee is threatened by an increasing number of invasive pests, particularly the tracheal mite, varroa mite, and small hive beetle. Integrated pest management has not been well utilized by beekeepers, partly due to a lack of biological control agents. Microbial control strategies have been investigated for varroa mites using fungal pathogens, but have produced variable results. Difficulties have arisen because bees maintain hives at temperatures that are detrimental to the fungi, and the immature stages of the mites can avoid the fungi. It is also difficult to mass produce highly virulent and persistent fungal spores, and products are not available for use. One option to investigate further is the search for pathogens of the pests in their native range, as has been done in the introduction of biological control agents to field crop pests. Also, pests that have part of their life cycle outside the hive, such as small hive beetles, may be more amenable to biological control.

15.1 How it All Started

I first met Vaclav (Bill) Ruzika in 1998 in Weslaco, Texas, which is located just north of the US–Mexican border in the Lower Rio Grande Valley. I was working there as a researcher for the USDA Agricultural Research Service. Ruzika is a character to remember, born and raised in Czechoslovakia, but when I knew him, he lived in Canada where he was, and still is, a retired engineer-turned-entrepreneur, running a ski resort and a string of honey bee hives. I think that at the time he was selling both honey and packaged bees. He came to Weslaco to meet with Bill Wilson and me about a fungus-based product called Mycar® (Abbott Laboratories, North Chicago, IL) and claimed that it had the ability to control varroa mites (*Varroa destructor*

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(Acari: Varroidae)) in honey bees. Mycar® was composed of dried hyphae of the fungus *Hirsutella thompsonii* and was originally marketed for control of the citrus rust mite (*Phyllocoptruta oleivora* (Acari: Eriophyidae)) (McCoy & Couch 1982), although by the time of our meeting, it was no longer on the market. Ruzika said that a honey bee researcher in Czechoslovakia, Oldrich Haragsim, had found that Mycar® reduced the reproduction of varroa mites in honey bee hives. So Ruzika had done his homework and obtained a live culture of the Mycar®-strain from Clayton McCoy in Florida (where Ruzika spent his winter holidays). McCoy had done the original research that lead to the development of Mycar® (McCoy & Couch 1982). Ruzika's idea brought Bill Wilson and me together because he was an experienced honey bee researcher, and I specialized in microbial control of insect pests, and we had previously discussed the idea of trying some kind of microbial control for honey bee pests. This visit was the impetus we needed to set the idea in motion. However, we were only one stop on Ruzika's way home to Alberta from Florida; he stopped at every honey bee research facility he could, including ones as out of the way as ours, and as a result, a few other people caught on to his idea and began looking into the possibility of microbial control for varroa mites. In addition, as it turned out, research on the same topic had already begun at the Rothamsted Experiment Station in the United Kingdom.

What was so appealing to me about this idea, besides my general interest in both microbial control and bees, was the potential to help fulfill the great need for biological control in honey production. At the time, no alternatives to chemical control were available for varroa mites, yet honey often appeals to people because it is seen as a pure and natural product. Furthermore, one of the biggest problems we have in developing microbials for field crops is poor persistence of the microbes after they are applied to crops and exposed to sunlight and drying conditions. The honey bee hive is dark and humid. Are these not the perfect conditions for a fungal microbial control agent? Initially, the problems I was concerned about were finding a microbe pathogenic to the mites, yet not to the honey bees, and making sure that honey bee housekeeping practices were not so thorough that they cleaned out all the spores applied to the hive. As it turned out, there were more complications than these three factors, but first, let me give you a little background in honey beekeeping and the invasive pests that have come into North America in the last few decades.

15.2 Honey Beekeeping

Honey bees are generally known for three things: they produce honey, they sting, and they pollinate flowers. Managed bees are indeed vital to world agriculture, providing pollination service for more than 90 crops, including legumes, rape, almonds, apples, berries, and numerous seed crops. However, honey bees have also played an important role in European and American culture as well, where their industriousness and social behaviors have been repeatedly used as metaphors for human political and social agendas (Horn 2005). The honey bees belong to the genus *Apis*,

and the most commonly managed bees are *A. mellifera* and *A. cerana*. In the US, farmers rent more than two million *A. mellifera* hives a year (Morse & Calderone 2000), but the sustainability of this bee is being threatened by an increasingly large complex of problems, making them less and less economical to manage.

For the region of North and Central America, somewhere between 2600 and 4900 species of bees have been described, depending on the taxonomic classification you choose (Krombein *et al*. 1979, Michener *et al*. 1994); however, none of the bees native to this region are honey bees. *Apis mellifera* was first brought into North America in the 17th century with the early European settlers, in part to help fulfill the ideal of the New World being the land of milk and honey (Horn 2005), and the bees then spread across the continent with the advancement of European settlements. According to Thomas Jefferson, Native Americans called honey bees the white man's fly, and "considered their approach as indicating the approach of the settlements of the whites" (Jefferson 1785). As agriculture has industrialized and increased in scale, the importance of the honey bee for pollination, rather than solely for honey production, has increased. For example, Burgett (2004) evaluated the economics of beekeeping in Oregon for the year 2003, and found that renting hives for pollination was essential for most beekeepers because honey sales were not profitable enough to off-set expenses. Of course, the profitability of honey varies from year to year depending on the current market price, but also, the costs of production have increased over the years due to increasing needs for pest management.

15.3 Exotic, Invasive Invertebrate Pests of Honey Bee Hives

Prior to 1984, the greatest pests in honey beekeeping were diseases, and American foulbrood was the disease that caused the greatest concern. In 1984, a mite parasitic on honey bees, the tracheal mite (*Acarapis woodi* (Acari: Tarsonemidae)), entered the US from Mexico (Shimanuki *et al*. 2006). This mite is called the tracheal mite because it infests and reproduces in the tracheae, or breathing tubes, of honey bees. The mites feed on the hemolymph of the bee by puncturing the tracheal wall. Female mites enter the tracheae of young adult bees, those less than 1 week old, and lay eggs. It takes 3–4 days for the eggs to hatch, and about 12 days for the young mites to mature and mate. Newly mature, mated female mites disperse by leaving the trachea and attaching to the hairs of the host, where they wait for a new host to come along, and the lifecycle is then repeated. Heavy mite infestations are most likely to cause high mortality of the adult bees during the early spring, before the bees begin their spring population growth.

In 1987, another mite parasitic to honey bees was detected in the US. This new invader, the varroa mite (*Varroa destructor*), was first found in Wisconsin on a package of bees from Florida. The varroa mite was originally a parasite of the Asian honey bee (*Apis cerana*) but was already well adapted to *A. mellifera* when it came into contact with this new host. It was probably introduced at least twice into the US, first from Korea, most likely by way of Europe (where varroa was already widespread)

into Florida, and then later from Japan or Thailand (de Guzman *et al*. 1997, 1999, Anderson & Trueman 2000). The mite is now widespread in *A. mellifera* colonies throughout North America. The adult females are much larger than tracheal mites, large enough to see with the naked eye, and they ride around on adult worker bees as a means of phoresy, and they also feed on the hemolymph of adults. I have found these mites to occur most commonly on the intersegmental membranes of adult honey bees, between the second and third sternites of the abdomen, but sometimes between the first and second sternites, and often embedding themselves partially under the sternal plate.

Honey bees raise their young in a wax comb and all stages of immature bees are referred to as "brood." The queen bee lays eggs individually in the cells of the comb, and the attending adult bees then feed the developing larvae until they become pre-pupae and begin to spin cocoons, at which time the attending adults produce a cap over each cell, sealing the brood inside. Worker brood (non-reproductive females) become pre-pupae 6 days after the eggs have hatched and drone brood (males) become pre-pupae 8 days after hatching. When a female adult varroa mite detects a cell containing 5 day old brood, it leaves the adult host and enters the brood cell. After the cell is capped, this mother mite feeds on the developing brood and lays an egg. This first egg will develop into a female mite. The second egg laid by the mother mite will become a male and subsequently the mother mite only lays female eggs, but no more than one per day. After the mite eggs hatch, the developing immatures feed on the host in the capped cell. The cells remain capped for approximately 10.5 days for workers and 12 days for drones. When the host completes development, the young adult bee emerges from the cell, and the mature female mites emerge with it. These females will have mated with their brother before emergence. The mother mite also emerges, being able to infest more than one cell during her lifetime. The male and any immature female mites probably die after the host emerges from the cell, although males can occasionally be found wandering around on the comb. This parasite has proven to be a much more devastating pest than the tracheal mite, to the extent that many beekeepers have somewhat forgotten about tracheal mites and concentrate their efforts on controlling varroa mites. It is possible that some of the chemical control measures for varroa mites also control the tracheal mite secondarily. I will discuss the varroa mite impact and control efforts in more detail later.

The newest invasive invertebrate pest is the small hive beetle (*Aethina tumida* (Coleoptera: Nitidulidae)). It was first found in the US in 1996 in a colony of *A. mellifera* in Florida (Shimanuki *et al*. 2006). The beetle now has been found throughout the US, but it is primarily a problem in the southeastern US. Adult beetles invade honey bee colonies in large numbers and feed on the pollen, honey and brood. They lay eggs inside the hive, hiding the eggs in areas where the bees either will not detect them or cannot get access to them. The larvae also develop in the pollen, honey and brood, and produce a large amount of a slimy substance with a putrefied smell. A yeast associated with this slime produces compounds that attract more beetles to the hive (Torto *et al*. 2007). The beetle can often be controlled by maintaining strong bee colonies and using hive entrance reducers so that the bees can defend themselves more easily. The beetle can also be a pest of stored combs.

15.4 Integrated Pest Management and Honey Beekeeping

After World War II and the invention of DDT, insect control in the US took a dramatic turn. This new chemical pesticide that had low plant and mammalian toxicity was a wonder drug, and its success stimulated the development of other synthetic insecticides. However, the glamour died away as the shortcomings of this approach began to present themselves. Initially, the chemicals that were favored by farmers were those with long environmental persistence and broad spectrum activity. However, they also killed the invertebrate natural enemies, and this trait led to outbreaks of secondary pests that had previously been controlled via natural biological control. The most persistent insecticides also bio-accumulated, that is, they increased in concentration through the food chain, leading to high concentrations in fish, predatory birds and mammals. Thus, even though the mammalian toxicity may have been low, some animals at the top of the food chain ended up getting exposed to concentrations that were detrimental to their reproduction. In addition, the extensive use of these highly effective chemicals sometimes induced pest populations to develop resistance to the pesticides, and with no natural enemies around, this again led to severe pest outbreaks.

Integrated pest management (IPM) is a pest control strategy that emerged as a result of the crises generated by pesticides (Perkins 1982). From one perspective, the base for IPM is biological control, and IPM replaced control strategies where pesticides were at the core. In IPM programs, pesticides are used, but they are used in a strategic manner to avoid disrupting benefits that can be gleaned from biological control. For example, pesticides were developed that were less broad in their target range and were applied more selectively, such as only during the time when they would be most effective, and only when the benefits outweighed the costs based on the population levels of the pests (these critical population levels are termed "thresholds"). Biological control became an important area of research for entomologists, and researchers began "foreign explorations" for the natural enemies of exotic pests for the purpose of releasing them in the US. In addition, other strategies began to be employed, such as the development of insect pheromones to trap pests, monitor their populations, or disrupt their mating. Thus the name "integrated" pest management, because the objective was to integrate the use of several strategies to control a complex of pests in each crop.

The impetus for IPM for field crops and forestry began in the 1970s (Perkins 1982), but the idea of using IPM strategies for honey bee hives is much newer. I remember the idea being discussed at a national honey bee meeting in 2000. Many beekeepers had not heard of IPM at that time, and if they had, they did not really know what it was. Still today, no real IPM strategies are being used in beekeeping. A few research papers exist that attempt to develop some of the basic data needed for an IPM program. For example, an economic threshold for varroa mite control has been described (Delaplane & Hood 1999), the most effective time to apply miticides has been evaluated (Strange & Sheppard 2001, Gatien & Currie 2003), and strategies for coping with miticide resistance have been evaluated, but a general IPM strategy for hive pests has not been widely implemented.

Part of the problem has been a lack of any natural biological control in the beekeeping ecosystem. This problem may not be unique for honey bee colonies, but rather, typical of both highly artificial agricultural systems and invasive pests. Pest control options for beekeepers are limited mainly by two factors. First, the bees are arthropods and easily killed by many pesticides, and second, it is critical not to contaminate the honey and other hive products. Beekeepers try to maintain colonies vigorous enough that the bees can manage their own pest problems, which perhaps is one form of biological control. For example, some genetic lines of honey bees can detect and remove brood that is infested with varroa (Spivak & Reuter 2001), or corral and disable small hive beetles (Neumann *et al*. 2001). However, the general lack of natural biological control means that no natural enemies exist to either be released or protected in the hive. Furthermore, even if we are able to find more natural enemies, the hive environment poses special problems that are very different from those encountered in developing biological control for a forest or an agricultural field. In particular, the hive is a somewhat contained system with a carefully controlled environment that is not always accessible (or amenable) to human manipulations. Thus, some of the IPM approaches taken in the past will not work for bees.

Microbial control may be more adaptable to bee systems than the introduction of parasitoids or predators because honey bees are less likely to remove a microbe from the hive than they would an insect or mite that was introduced as a biological control agent. Parasites and predators of honey bees must be able to deceive the host into letting them into the hive environment, often using chemically-mediated behaviors, or by being too small to be detected and removed. For this reason, microbial control of invasive hive pests probably holds the most promise for future biological control, and is a reason for the considerable interest that has been generated in developing a microbial control for varroa mite, a pest that easily ranks number one in the honey bee world.

15.5 Microbial Control of Varroa Mite

15.5.1 The Identification of Potential Biological Control Agents

Several chemical and cultural controls have been developed for varroa mites, but none of them are entirely effective, and the mites have developed resistance to those that were most effective. Thus, varroa still remains a significant pest of honey bees, and is central to any pest management program. New strategies for its control are constantly being sought. This brings me back to the visit from Bill Ruzika in Texas. The fungus he brought to us was *H. thompsonii*, and we tested it in the laboratory and found it to be a very poor pathogen of varroa mites. However, we also tested several other strains of *H. thompsonii* that originally came from the citrus rust mite, and some of these strains had very high virulence. Our best strain (ARSEF[1] 5858)

[1] ARSEF is the USDA Agricultural Research Service's Entomopathogenic Fungus Collection in Ithaca, NY.

had an LC_{90} of 6.3×10^2 conidia/mm^2, 4 days after a spray application (Kanga & James 2002). Compare this to our best strain of *M. anisopliae* (isolated from a product called Bioblast®), that had an LC_{90} of 1.6×10^4 conidia/mm^2 (Kanga & James 2002).

Much of the initial search for an appropriate fungal species and strain focused on finding a fungal pathogen of any arthropod that might show high virulence towards varroa. Many pathogens are known to infect mites (Poinar & Poinar 1998, Chandler *et al*. 2000, Van der Geest *et al*. 2000). The most frequently encountered mite pathogens are fungi, and among the fungi, the most common are the Entomophthorales (Poinar & Poinar 1998). Other common fungal pathogens are in the genus *Hirsutella*, and *M. anisopliae* has been tested for microbial control of ticks (Zhioua *et al*. 1997, 1999, Guedes Frazzon 2000). Rickettsiae, protozoa, microsporidia, and viruses have also been shown to infect mites, but in our initial approach to biological control we needed to focus our efforts because even laboratory-based bioassays were very labor intensive and time consuming. Varroa mites had to be collected directly from honey bee hives just prior to each bioassay. This meant maintaining several varroa-infested honey bee colonies. Keeping the hives infested, yet still viable, required constant monitoring of infestation levels and moving frames of brood from strong colonies to weaker ones. Also, once the mites were collected for a bioassay, they had to be fed live bee pupae several times a week. A few anamorphs of hypocealean fungi emerged as the preferred biocontrol candidates for the following reasons:

(1) The spores of these fungi infect their hosts when they come into contact with the cuticle. Varroa are parasites with sucking mouthparts, and so it would be difficult to apply any pathogen or toxin that needs to go through the gut, such as most bacteria and viruses, because the mites are not likely consume a sufficient number of infective propagules to initiate infection.
(2) These fungi are relatively easy to mass culture. Production methods for infective propagules of Entomophthorales on artificial media do not exist.
(3) Many of the insect-pathogenic Hypoceales are very heat sensitive, but some *Hirsutella* and *Metarhizium* species are more heat tolerant and would be able to survive and infect in the honey bee hive environment. Honey bees regulate the temperature of the hive at approximately 34 °C, which is too high for many entomopathogenic fungi.
(4) Drion Boucias and Clayton McCoy (University of Florida) had a large collection of *H. thompsonii* strains isolated from the citrus rust mite, and many species of *M. anisopliae* have been isolated and stored in general collections, providing a large stock of potential strains to screen.

Although it is generally true that entomopathogenic Hypoceales are easy to mass produce, not all are, and *Hirsutella* is one of the more difficult genera, although the strain of *H. thompsonii* that Ruzika provided us had once been produced on a commercial scale, so it is possible. Stephan Jaronski, a former Abbott Laboratory production scientist (now with the USDA, Agricultural Research Service), said that he had difficulty finding an adequate formulation for *H. thompsonii* spores, and as a result, Mycar® was primarily a hyphal product, and spores were produced by

the fungus after the hyphae were applied in the field. This was to prove to be an insurmountable problem later. Like Abbott Laboratories, I was never able to produce a stable spore product that could be applied in the field, even though some of the *H. thompsonii* strains were highly virulent. One of the problems may have been that *H. thompsonii* spores do not readily adhere to varroa cuticle, other than on their feet as they walk around contaminated surfaces (Peng *et al*. 2002). In addition, we could not obtain a reasonable shelf life for the spores once they were harvested from the culture medium, and if spores did not survive well under controlled storage, survival was not likely to improve after being released into the hive environment.

Our studies, and those of others at the time, focused on fungal pathogens known from other species of mites, or from insects. None of the pathogens initially tested had been found to naturally occur on varroa. Our reasoning was that many of these fungi have broad host ranges and could potentially be more pathogenic to varroa than to their natural host, in much the same way that varroa has a much greater impact on the survival of *A. mellifera* than it has on its native host, *A. cerana*. Sometimes when a pathogen switches to a new host, it can be more virulent than it is on its native host. This approach proved fruitful, initially. In general, varroa mites were found to be more susceptible than other mite species to fungal pathogens, and Shaw *et al*. (2002) speculate that this may be due to the mites evolving in a relatively pathogen-free environment in the bee hive.

Meikle *et al.* (2006, 2007) looked for pathogens of varroa mites in bee hives and obtained six isolates of *Beauveria bassiana* (Meikle *et al.* 2006), and then tested two of these naturally occurring pathogens in the field to see if they could get them established in the hive (Meikle *et al*. 2007). I had not previously considered using *B. bassiana* as a biocontrol agent for varroa because this species has been shown several times to be very susceptible to high temperatures, with a significant inhibition of germination and growth above 32 °C (Walstad *et al*. 1970, Hywel-Jones & Gillespie 1990, James *et al*. 1998). However, contrary to expectations, it was found to occur in the hive environment (Meikle 2006) and to be pathogenic to varroa (Shaw *et al*. 2002, Meikle 2006).

15.5.2 Field Trials

Once potential biocontrol agents had been identified, the next logical step was to begin field trials. Several things are necessary to successfully accomplish a field trial, including:

(1) Propagating a sufficient amount of the control agent, or finding a partner in commercial fungal fermentation who can supply spore material;
(2) Developing an application strategy and formulation, or several strategies;
(3) Establishing the amount of control agent material that will be needed, based on the laboratory bioassays;
(4) Developing, at least a method for evaluating varroa populations inside a bee colony, but in addition, methods to evaluate infection rates, honey bee colony strength, and spore survival rates, would be ideal.

Our first experiments on honey bee colonies were done using small observation hives (two-frame hives behind glass) (Kanga & James 2002). These experiments tested the effects of *H. thompsonii* spores in an aqueous suspension sprayed on the bees in the observation hives, and then we monitored varroa mortality (based on the number of dead mites that fell to the bottom of the hive each day). Queen egg-laying and bee death rates were also monitored. In these experiments, approximately 50% of the dead mites were found to be infected with the biocontrol agent after application, but the increases in mortality rates were small, and not statistically greater than the controls (Kanga & James 2002). I repeated these small hive tests in the field, but could not induce infection or any measurable effects on the mite population levels, probably due to poor activity of the spores in the beehives. In these later experiments, we used a dry spore and mycelia powder, and this formulation may not have been as effective as spraying fresh spores. Unlike our spray applications to observation hives, we were not able to recover any viable spores in the hive after application.

While we were experimenting with *H. thompsonii*, two companies (Sylvan and Earth Bioscience) expressed interests in producing *M. anisopliae* spores for biocontrol in the US. Sylvan wanted to produce a strain of the fungus that had previously been used in a product for termite control (called Bioblast) that had been produced by EcoScience. Earth Biosciences was interested in producing a fungus that had been developed by Bayer (called Bio 1020 by Bayer) for biocontrol of several insects (including ticks). Earth Bioscience referred to this as Strain F52. Several field experiments have been conducted with these strains, but with mixed results, as described below.

15.5.2.1 Experiments with the Bioblast Strain of *M. anisopliae*

In the spring of 2002, we conducted a small scale field trial using "nuclear colonies" of honey bees. Nuclear colonies are essentially mini-colonies created by shaking adult bees from full-sized colonies and placing them in a small hive that consists of 5-frames of comb foundation (a standard brood box in the US contains 10 frames of comb) and a new queen. For our experiments, we used 0.9 kg of adult bees and Italian queens. The nuclear colonies were treated with a spore preparation containing 1.07×10^9 spores/g, which was produced at a commercial facility using rice as the substrate. The spores were applied at a rate of 46.8 g/hive (5×10^{10} spores/hive) as either a dry spore powder, or by adhering the spores to plastic strips with vegetable oil. These strips were then inserted between the comb frames. These application methods reduced varroa mite levels to below that of control hives, and to a level that was similar to hives treated with the miticide fluvalinate (Kanga *et al*. 2003). This field trial was conducted in southern Texas, which has a subtropical climate. Kanga *et al*. (2006) repeated these experiments using 3.0×10^{11} spores per hive and a similar number of bees. These spores were produced in-house on Sabouraud dextrose agar (SDA). With this higher dosage, Kanga *et al*. (2006) again obtained significant control of the mites, especially if the colonies were broodless. When colonies are broodless, all the mites in the hive are on adult bees and thus exposed to the fungus.

Furthermore, with no brood present, the mites are not able to reproduce. A broodless condition, or nearly so, may occur during late fall and winter in northern climates, and sometimes during mid-summer in sub-tropical climates. In these experiments, however, they did not use a control that was broodless, so mite levels were compared to those in a reproducing colony, which may be somewhat misleading.

In October 2002, I applied a dry spore powder to colonies of bees obtained from a commercial bee keeper in Logan, Utah. These colonies consisted of two 10-frame hive boxes (called "supers") with approximately 3.5–4.5 kg of bees. Logan has a dry, temperate climate with winter high temperatures usually below freezing between December and March. Spores were applied using talc as an inert ingredient. *Metarhizium anisopliae* was again produced on SDA. The night before treatments were to be applied, the spores were scraped off the culture plates and air dried in a laminar-flow hood over night. The next morning, 30 g of talc powder was mixed with 57 g of spores, yielding a mix that was 1.75×10^{10} spore/g. This mix was then measured into three different treatment rates: H (5×10^{10} spores, 2.86 g), M (2.5×10^{10} spores, 1.43 g), and L (1.0×10^{10} spores, 0.6 g). Controls were not treated, and extra talc was not added to the M and L rates to balance the bulk of material added to the hives, a technique we used later (see below). The spore treatments were applied to the hives by sprinkling half the spores between the frames in each of the supers. Spore viability at the time of treatment was 98.6%, as determined by plating the spores on SDA and then counting the ratio of germinated to ungerminated spores under a microscope (for 500 spores) after the spores had been incubated 18 hours at 25 °C. The hives were then left for the winter. The resulting number of mites per bee did not differ significantly between treatments the next spring, however, the proportion of colonies that were still alive at the end of the winter increased as the application rate increased (Table 15.1).

In September 2003, we repeated the overwintering experiments that we had conducted in Logan, but relocated the hives from the mouth of a canyon (where they were located in 2002) to the floor of Cache Valley, with the hopes that we could improve overwintering survival since the valley was not as cold and windy as the previous location. Also, this time we cultured the fungus on sterile rice, and used

Table 15.1 Effect of *Metarhizium anisopliae* treatments on the overwintering survival of honey bee colonies in 2002

	Spore application rate (per hive)			
	Control	Low 1.0×10^{10}	Medium 2.5×10^{10}	High 5.0×10^{10}
Hives treated in the fall	4	5	7	6
Hives alive in the spring	1	2	5	5
% Survival	25	40	71	83
Mites per 100 bees pre-treatment (mean ± SE)	9.2 ± 2.3	23 ± 5.1	7.6 ± 1.5	8.2 ± 2.3
Mites per 100 bees in the spring (mean ± SE)	–	3.4 ± 1.6	7.0 ± 2.9	5.0 ± 1.0

rice flour as the inert ingredient. To harvest the spores from the rice, the cultured rice was dried overnight in a laminar-flow hood, and the spores were removed by shaking the rice in a sieve. The yield was 5.50×10^9 spores/g. Due to the poor yield of spores, the treatments were altered and included: H (2.48×10^{10} spores, 4.5 g), M (1.24×10^{10} spores, 2.5 g spores +3.25 g rice flour), and the control (6.5 g rice flour), but these treatments were applied twice, once on September 29 and then again on October 8. On September 29, spore viability was 97% and percent moisture of the spores harvested was 67.8%. For the second treatment, a new batch of spores was produced in the same manner. Spore viability of the second batch was 96.5% with 30% moisture. To test how well spores prepared in this manner stored, unused portions of the second batch were stored at 10 °C. After 17 days viability was still 95%. Thus, the high moisture content did not adversely affect spore survival. In fact, we found that for this strain, dry spores did not survive as well in storage at 4 and 35 °C as non-dried spores (Fig. 15.1). Eight hives were treated for the control and M treatments, and seven hives for the H treatment.

Unfortunately, the treatments failed to increase winter survival in this second trial. Mite infestation levels at the beginning of the experiment (September 29, 2003) were lower this second year, with the mean number of mites per 100 bees being 9.69 (SE = 2.34), 7.93 (SE = 1.04) and 10.82 (SE = 2.30) for the control, M, and H treatments, respectively. The numbers of hives still alive on March 23, 2004, were 4 in the control, 1 in treatment M, and 3 in treatment H, yielding the following winter mortality rates: 50% in the control, 87.5% in the M treatment, and 57.1% in the H treatment. Due to the weak condition of the remaining colonies, we did not sample bees in the remaining hives to estimate mite infestation levels.

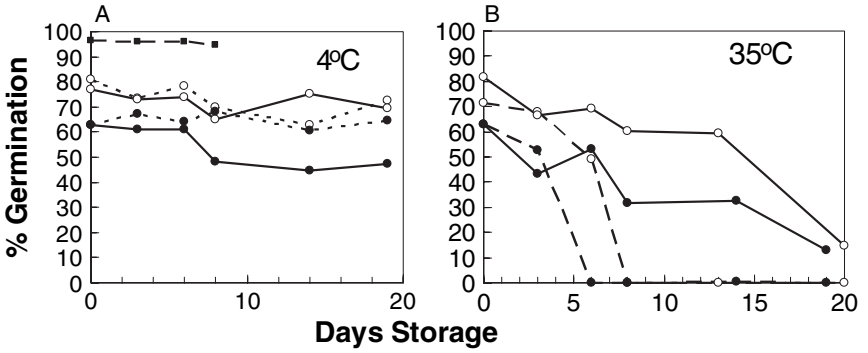

Fig. 15.1 Survival of *Metarhizium anisopliae* spores when stored at different temperatures. To determine viability, spores were tested for an ability to germination on nutrient agar after 24–48 hours incubation at 25 °C. Open circles are spores that were air-dried for 24 hours before being harvested from rice, and solid circles are spores that were air-dried for 48 hours. Dotted lines are spores that were stored as a powder (no formulation), and solid lines are spores that were attached to plastic strips using vegetable oil (cooking spray). Squares are spores that were not dried. Spores were stored at ambient RH (~ 15%) (800–1200 spores were counted for a given point)

15.5.2.2 Experiments with the Earth Bioscience Strain of *M. anisopliae*

In 2004, we began testing strain F52 in beehives. This experiment was conducted near Gainesville, Florida, which has a subtropical climate. The experiments were conducted in June when the bees experience a period of low bloom availability, and thus brood production is reduced. The fungal spores were produced commercially on rice and the spore concentration was 1.1×10^{10} spores/g. Spore viability at the time of treatments was 86% after 48 hours incubation on SDA. We treated commercial honey bee hives (of a similar size to those treated in Logan) with four different treatments: H (2.87×10^{10} spores/hive, 1.4 g spores +5.1 g rice flour), M (1.44×10^{10} spores/hive, 0.7 g spores +5.8 g rice flour), a rice flour control (6.5 g rice four), and an untreated control. We used ten hives for each treatment. Approximately 200 adult bees were sampled from one frame in each hive just before the treatments were applied, and then 3, 6, and 13 days after the treatments were applied. A repeated measures ANOVA was used to determine whether treatment had a significant effect on the mite infestation levels, after the data had been transformed using the arcsine-square root of the mean number of mites per bee. Again, treatments had no significant effect on mite infestation levels. This time, none of the hives died from mites, probably because the experiments were conducted over a shorter period than in the overwintering experiments, and the bees did not have to endure winter conditions.

In May 2005, we went back to our Logan, Utah, location to determine if we could improve varroa control using different application strategies (James *et al*. 2006). We ran these experiments for 62 days to see if there were any long term effects of the fungus. Using one dose, we tested four different application/packaging strategies and an untreated control group. The five treatments were (a) a removable hive frame that contained live, sporulating fungus cultured on a non-woven material, a preparation sometimes referred to as "fungal bands" (Xu *et al*. 2003, Dubois *et al*. 2004) (Fig. 15.2 A), (b) paper packets of dry spores laid across the tops of the frames (Fig. 15.2 B, the bees were expected to tear apart the packets in an attempt to remove them from the hive, and in this way, release the spores over a period of days), (c) dry spores fixed to plastic strips with vegetable oil, and inserted between two hive frames (Fig. 15.2 C,D), (d) dry spore powder dusted onto the bees between the frames; and (e) untreated controls. Treatments were applied to hives obtained from a commercial bee keeper (of approximately the same size as the first Logan experiments), using five hives per treatment. The dry spores were always applied using 1.4 g per hive, and were obtained from Earth Biosciences (produced on rice substrate). The preparation had 3.0×10^{10} spores/g, with a viability of 84% based on 24 hour germination counts, thus we applied 3.5×10^{10} viable spores per hive. The "fungal bands" were produced in-house and contained 2.1×10^{7} spores/cm^2, for a total of 3.69×10^{10} spores per band (James *et al*. 2006). Spore viability was 95% at the time of application.

We measured the number of mites per adult bee, and per brood cell, and estimated the total number of bees in the hive (adults and brood separately) so that we could

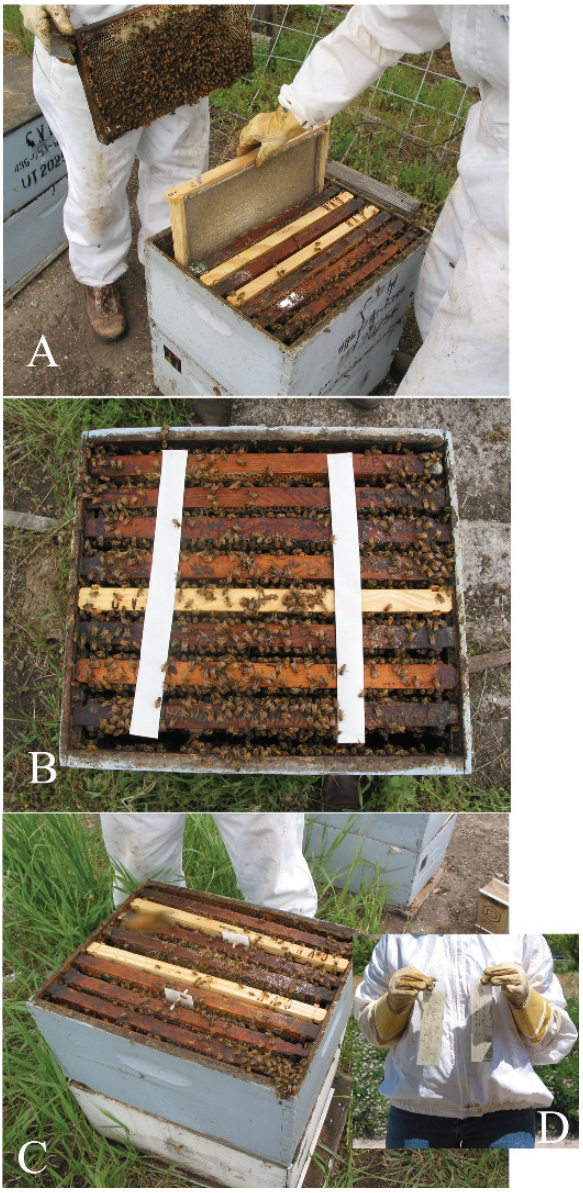

Fig. 15.2 A few *Metarhizium anisopliae* application methods, including (**A**) "fungal bands," (**B**) paper packets containing spores, (**C**) plastic strips coated with oil and spores, then placed in the hives, and (**D**) strips before being placed in the hives

then estimate the total number of mites in the hives. These estimates were made two days before treatments, and again 15, 32, and 62 days post treatment. A repeated measures ANOVA was used to determine whether treatment had a significant effect on the total number of mites and bees in the hive.

Mite numbers increased over the course of the experiment in the packets treatment, and in the plastic-strips treatment ($P = 0.07$ for packets and $P = 0.023$ for strips), but did not change significantly in the other treatments, including the controls. The only treatment that showed a decrease in mite numbers was the "fungal bands," but this effect was small and not statistically significant. The populations of adult bees and brood did not change significantly over the course of the experiment, or between treatments (James *et al*. 2006).

To determine viability of the spores in the hives, samples were taken from the "fungal bands" and from the paper packets on the day of application, and at regular intervals during the course of the experiment. Spore viability in the packets dropped from 84% to 40% in the first day, and then spores slowly lost all viability over the next 20 days. Spores in the "fungal bands" lost viability more rapidly, dropping from 95 to 20% in the first day, and losing all viability by day 14 (James *et al*. 2006).

With the failure of our creativity in trying to develop a more effective application strategy, we decided to try one high dose of spores mixed with water and sprayed into the hives (Fig. 15.3). This method might not be practical for a beekeeper, but we could at least test the fungus under the best conditions that we could create for it – a high dose with moisture added. This experiment was conducted near Gainesville, Florida, during the winter (November). Twenty hives were randomly assigned to two treatments: treated and control (ten hives per treatment). Fungal spores were produced on rice at the USDA-ARS lab in Stoneville, MS, and spore viability was 96% at the time of treatments. The spore preparation contained 2.9×10^{10} spores/g. For the treated hives, the spores were mixed in a pressurized sprayer with 0.01% Tween 20 (to help disperse and wet the spores) in water at a rate of 17.65 g/L, and applied into the hives at a rate of 12 g/hive (3.34×10^{11} viable spores/hive). The control hives were treated with an equal volume (680 ml) of 0.01% Tween 20. Unfortunately, results from this experiment were no more encouraging. The number of mites per 100 adult bees increased over the course of the experiment, and was unaffected by the treatment (James *et al*. 2006).

Fig. 15.3 A wand sprayer being used to spray *Metarhizium anisopliae* spore suspension into a honey bee hive

15.5.2.3 Field Trials with *B. bassiana* Strains Isolated from Honey Bee Hives

Meikle *et al*. (2007) applied *B. bassiana* spores to hives. These spores were produced on SDA with yeast extract, then mixed with a silica powder and an electrostatic powder. They used 3.0×10^9 and 8.0×10^9 spores per hive, respectively, in two different experiments, and were able to increase "mite drop" (the number of mites that fall to the bottom board of the hive) after treatments. Significantly more of these trapped mites were infected with *B. bassiana* than in the controls. Unfortunately, the treatments did not have any measurable effect on varroa population levels in the hives. However, it is worth noting that the dosages Meikle *et al*. (2007) tested were several times lower than in all the experiments described above, yet he still saw infections in the hive.

15.5.2.4 General Conclusions Regarding Microbial Control of Varroa

The most frustrating aspect of all these experiments has been that the results are so variable; good control has occasionally been achieved, but not consistently, even under seemingly similar conditions. The substrate on which the spores are produced (rice versus nutrient agar) may have had some effect on the results; spores produced on the nutrient agar were associated with the best results above. This may have to do with how the spores were handled as much as the substrate itself, particularly with how the spores are dried (Hong *et al*. 2000).

I suspect that three main problems plagued this application of microbial control. First, it was difficult to direct applications to the target pest. Many of the mites are enclosed in sealed brood cells and avoid hive applications. Even when the mites are on the bees, we are not certain if they came into contact with the spores. I have found that spores disperse readily in the hive on the adult bees, but I do not know how many spores actually come into contact with the mites. The second problem is that the conditions in the hives are very warm and humid, to the extent that they reduce spore survival. As described above, spore survival in the hive after application is very poor for unformulated spores. Oil has been shown to increase the survival rate of spores under warm conditions (Hong *et al*. 1999), and that may explain the slightly greater success of the spores applied to hives on plastic strips treated with oil. The third problem is dose. The best results have occurred with the highest dosages. However, it has been difficult to obtain such large numbers of spores. To put this into perspective, the highest rates tested for varroa were 3×10^{11} spores per hive (Kanga *et al*. 2006), as compared to, say, the recommended rates of *B. bassiana* for whitefly control in greenhouses where 3×10^{13} spores are used for an entire hectare, although they must be applied every 5–7 days (Faria & Wraight 2001). The dose applied by Kanga *et al*. (2006) is so high that it cannot feasibly be applied all at once; as for whitefly control, it would have to be applied repeatedly. Furthermore, the colonies in those experiments were what beekeepers call "nuclear hives." That is, they only contained the small number of bees used to initiate a new colony, similar to what are sold as packages of bees. They are not the size of a normal, established colony. Thus, much higher doses are probably required for more typical colonies.

15.6 General Conclusions About Biological Control in Honey Bee Hives

Beekeeping seems to be vulnerable to invasion by a myriad of pests, as described earlier in this chapter. What makes the honey bee so susceptible? Is it the lack of any natural biological control agents? Even within the native range of honey bees (Africa, Asia, and Europe), not many natural enemies for honey bee pests have been reported. This is partly due to the social behavior of this bee. Honey bees protect their nests very aggressively, and are on careful watch for invaders, even of the same species. Any beneficial organism would have to be able to avoid this detection system, much as the pests themselves do. For this reason, microbial control seems the most promising biological control strategy, but very few studies have been conducted to identify microbes pathogenic to hive pests. However, the same problems encountered during our attempts to develop microbial control of varroa will be encountered again: high hive temperatures, an inability to easily access the target pest, and the resulting need for very high dosages.

The small hive beetle also spends time outside the bee colony, pupating in the soil and attacking stored comb. It may be easier to target this pest outside of the active bee colony. For example, pupae of the small hive beetle are occasionally killed by fungi in the soil (Ellis *et al*. 2004).

A troublesome area for microbial control of honey bees is availability of products. The number of honey bee hives in any country is relatively small and the value of these hives is also not especially great. As a result, beekeeping does not provide a very large market to support all of the costs associated with developing and registering new microbial control products. New products are most likely to be borrowed from other industries, such as the *M. anisopliae* strains we tested that had been developed for the control of termites and lawn pests. Alternatively, perhaps a more classical biological control approach could be achieved if the beekeeping industry or government agencies paid for the exploration and dispersal of agents with the intention of getting them established in hives, similar to the way the government supports biological control of weeds in rangelands and pastures.

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