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Feasibility Study into In-Hive Fungal Bio-Control of Small Hive Beetle

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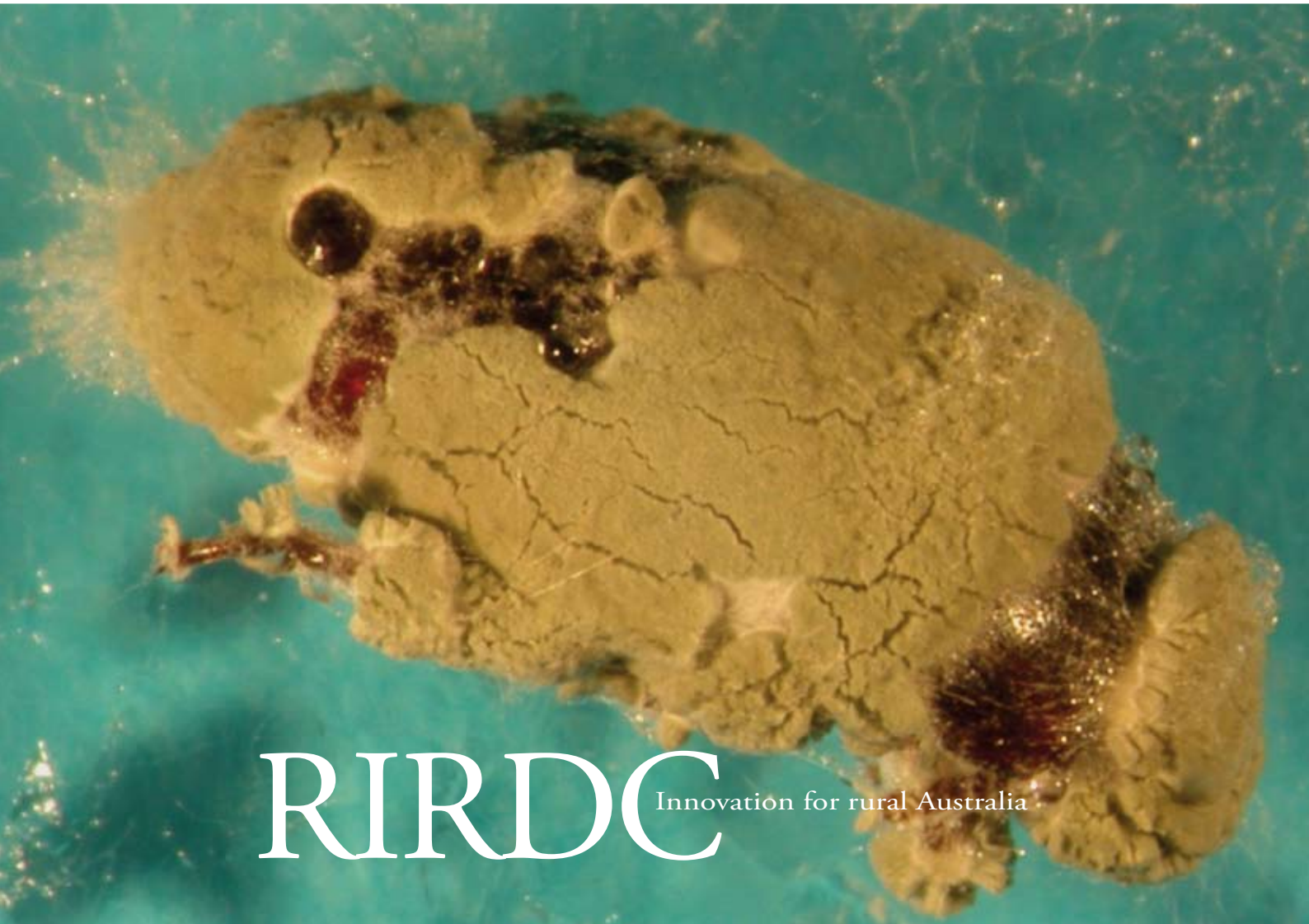

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Feasibility Study into In-Hive Fungal Bio-Control of Small Hive Beetle

by Diana Leemon and Jacinta McMahon

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Foreword

The Small Hive Beetle (SHB) is a foreign pest of bee hives that was first reported in Australia in 2002. Larvae of these beetles cause extensive damage to honey frames and combs, stored honey and pollen when they feed and leave wastes behind. The resulting fermented honey is rejected by honeybees and cannot be marketed by the beekeeper. Heavy infestations may also result in queens ceasing to lay eggs or bees abandoning their hives.

Since SHB was first reported it has spread at a rapid rate and is now regarded as a serious pest of hives in the eastern states of Australia. Chemical control of these pests in hives is limited by the possible toxicity of chemicals to the bees, unacceptable chemical residues in honey and the possible development of resistance to active ingredients. Therefore it is necessary to look for alternate and novel control strategies.

One strategy is to consider fungal biopesticides for the control of SHB. A fungal biopesticide for SHB control will have a dramatic impact on the honeybee industry by controlling a serious exotic pest without the use of chemicals. The magnitude of the benefits will go further through developing principles of fungal biocontrol of hive pests that can potentially be applied to the control of another extremely serious exotic pest of bee hives, the Varroa mite (*Varroa destructor*). Although this pest has not yet been recorded in Australia, it recently established in New Zealand; a strategy of preparedness for when Varroa does reach Australia should be regarded as critical. This project addresses both a current pest problem of the Australian honeybee industry, and develops the principles of fungal bio-control in bee hives for future pest problems.

This research identified a number of local isolates of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* that show good efficacy against adult and larval small hive beetles. The *B. bassiana* isolates were more effective against the adult beetles, while the *M. anisopliae* isolates were more effective against the larval beetles. The susceptibility of beetles to fungal infection did not appear to increase with age. Although isolates of *M. anisopliae* did not kill high numbers of adult beetles there was a trend of lowered fecundity in the beetles surviving the treatment that is worthy of further investigation. Spores applied as a dry loose powder to hives were rapidly cleaned up by bees in strong hives. The spores appeared to have an effect on the bees in the treated hives, although it was short term. It was also noted that fungal spores contaminating honey lose their viability rapidly.

This project was funded by the RIRDC Honeybee R&D Program and co-funded by the State of Queensland acting through the Department of Primary Industries and Fisheries.

This report, an addition to RIRDC's diverse range of over 1800 research publications, forms part of our Honeybee R&D Program, which aims to improve the understanding and management of bee diseases and pests.

Most of RIRDC's publications are available for viewing, downloading or purchasing online at www.rirdc.gov.au. Purchases can also be made by phoning 1300 634 313.

Peter O'Brien
Managing Director
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Abbreviations

DPI&F – Department of Primary Industries and Fisheries

RIRDC – Rural Industries Research and Development Corporation

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Executive Summary

What the report is about

The small hive beetle (*Aethina tumida)*, a native to South Africa where is it only a minor pest, was discovered in Australia in 2002. Since this time small hive beetles have become an increasing problem in bee hives in the eastern states of Australia and appear to be spreading at a rapid rate. There is concern for even greater damage from this pest to the beekeeping industry in the warm moist regions of Australia where it can reproduce faster.

Who is the report targeted at?

This report is targeted at the beekeeping industry in Australia, particularly those in the warmer regions affected by small hive beetles.

Background

Larvae of *A. tumida* cause extensive damage to honey combs, stored honey, pollen and brood when they feed and leave wastes behind. Resulting fermented honey is rejected by honeybees and cannot be marketed by the beekeeper. Heavy infestations often result in hive death, queens ceasing to lay eggs or bees abandoning their hives. Eradication of *A. tumida* from Australia is not regarded as practical and thus research is focussing on management strategies to control beetle numbers and minimise damage to honey production and pollinating bees.

Chemical control of these pests in hives is limited by the possibilities of toxicity of chemicals to the bees, and accumulation of unacceptable chemical residues in honey, and the possible development of resistance to active ingredients. Therefore it is necessary to look for alternative and novel control strategies. One strategy is to consider fungal biopesticides for the control of *A. tumida*. The principles of fungal biopesticide control rely upon the application of large numbers of formulated spores to a target insect to ensure a rapid death. Many fungal bio-control products registered around the world for insect control are based on isolates of the cosmopolitan fungi *Metarhizium* and *Beauveria*. In Australia there are four *Metarhizium* products registered for use against agricultural insect pests.

Aims/objectives

The aim of this project was to carry out a feasibility study into in-hive fungal bio-control of small hive beetle using endemic isolates of the fungi *Metarhizium anisopliae* and *Beauveria bassiana.* This research addresses a current and increasing pest problem of the Australian honeybee industry.

Methods used

Laboratory investigations developed assay systems to screen local isolates of *M. anisopliae* and *B. bassiana* against both adult and larval *A. tumida*. Some of the *M. anisopliae* isolates originated as infections in dead *A. tumida* adults collected from bee hives. The toxicity of fungal spores to bees when applied as a dry powder was also assessed as was the viability of fungal spores contaminating honey.

Results/key findings

This research identified a number of local isolates of the entomopathogenic fungi *M. anisopliae* and *B.bassiana* that show good efficacy against adult and larval small hive beetles. The *B. bassiana* isolates were more effective against the adult beetles, while the *M. anisopliae* isolates were more effective against the larval beetles. The susceptibility of beetles to fungal infection did not appear to increase with age. Although isolates of *M. anisopliae* did not kill high numbers of adult beetles there was a trend of lowered fecundity in the beetles surviving the treatment that is worthy of further

investigation. Spores applied as a dry loose powder were rapidly cleaned up by bees in strong hives, and did not appear to have any long term effect on the bees. Fungal spores contaminating honey lose their viability rapidly.

Implications for relevant stakeholders for:

As the study reported here was an investigation into the feasibility of a fungal control option for small hive beetle there are no implications for stakeholders other than the recommendation below.

Recommendations

Based on the results of the current project, development of a fungal biopesticide for in-hive control of small hive beetles may be feasible. However, further research needs to be conducted with isolates of *Beauveria bassiana* to first develop a formulation and application strategy for adult small hive beetle control, then to test this in hives.

Introduction

The small hive beetle (*Aethina tumida)* is native to South Africa where is it only a minor pest restricted to infesting weak, stressed or diseased bee colonies (Ellis and Hepburn 2006; Hood 2004; Neumann and Elzen 2004). It was discovered in South Carolina in 1996, and since then has spread to over 30 states along the eastern coast of the United States (Neumann and Elzen, 2004), and has now reached major pest status in the United States. In October 2002 *A.tiumida* was confirmed in beehives in New South Wales and Queensland followed by Victoria and the Goulburn Valley by August 2005 (Knoxfield and Ararat, 2005; Fletcher and Cook, 2005; Hood, 2004). Since this time small hive beetle has become an increasing problem in bee hives in SE Queensland and appears to be spreading at a rapid rate. *A. tumida* is now present as far north as Tully in northern Queensland (Warhurst, *pers. Comm.*). There is concern that in the tropical conditions of northern Queensland the beetles will thrive and cause significant damage to bee colonies (Fletcher and Cook, 2005). Ellis *et al,* (2004) and Guzman and Frake (2007) noted that *A. tumida* reproduces faster in warm moist conditions such as those found in tropical regions.

Larvae of *A. tumida* cause extensive damage to honey combs, stored honey, pollen and brood when they feed and leave wastes behind. Resulting fermented honey is rejected by honeybees and cannot be marketed by the beekeeper. Heavy infestations may also result in hive death, queens ceasing to lay eggs or bees abandoning their hives (Hood, 2004; MAAREC, 2000). The USA loss attributed to destruction by *A. tumida* in 1998 was estimated at US$3 million (Sommerville, 2003; Ellis *et al.,* 2002). The degree of damage caused by *A. tumida* in Australia is not yet well documented. Small hive beetle invasion has also negatively affected the queen and package bee production business and there are concerns for other commodities such as fruits and possible threats to bumblebee and other non-*Apis* species (Hood, 2004).

Various methods for controlling all life stages of *A. tumida* have been trialled. Cultural control methods include maintaining strong, clean colonies (Waite and Brown, 2003), encouraging hygienic behaviour in bee colonies (Ellis *et al.,* 2003) and modifying hive entrances to impede beetle access (Ellis *et al.,* 2002). Mechanical control methods include in-hive traps (Hood and Miller, 2003) and light traps (Neumann and Elzen, 2004). Chemical methods of control include coumaphos and fluvalinate in-hive treatments and the treatment of soil surrounding hives using permethrin (Hood 2000). Somerville (2003) conducted a comprehensive study of small hive beetle and its control in the USA. This review noted ways Australia can learn from the USA experience and better manage the beetle problem.

Eradication of *A. tumida* from Australia is not regarded as possible and thus research is focussing on management strategies to minimise damage to honey production and pollinating bees. The use of chemicals such as CheckMite + Strips (coumaphos) and Apistan (fluvalinate) within the hive for small hive beetle control is limited by their toxicity to bees and mammals, and there are also issues relating to development of resistance and risk of contamination and residues in honey and wax (Sugden *et al.* 1995). Gardstar (permethrin), a soil drench targeting the soil-dwelling stage of the beetle, is also highly toxic to bees so extreme caution must be taken to avoid contact with any bees, hive equipment and other surfaces to which bees may come into contact (Hood, 2007; RIRDC 2005).

In addition, the use of traps containing beetle attractants has proved to be ineffective, most likely due to competing hive odours emanating from nearby honeybee colonies (Hood, 2004; Sugden *et al.,* 1995). Inconsistent beetle control has been reported with the use of an upper hive entrance opposed to a lower hive entrance. Decreased production of adult bees and brood, impaired thermoregulation, excessive floor debris and poor drainage have also been associated with use of an upper hive entrance (Hood, 2004). Traps containing oil such as the “West Beetle Trap” require hives to be completely level to prevent oil leakage and subsequent bee mortality, and supers to be removed in order to be an effective beetle control (Hood, 2004). It has also been reported that sticky traps are ineffective as beetles can walk straight across them (Sugden *et al.,* 1995).

The failure of chemical treatments and physical traps to effectively control small hive beetle infestations without the development of resistance, bee mortality or contamination of honey with undesirable residues necessitates alternative control strategies. One strategy could involve the use of a fungal biopesticide based on spores of *Metarhizium anisopilae* or *Beauveria bassiana*.

Metarhizium anisopilae and *Beauveria bassiana* are entomopathogenic fungi that occur worldwide in the soil and have evolved to infect and kill insects. The spores of these fungi adhere to an insect's surface, then germinate and penetrate the insect, killing it as the fungus proliferates throughout the body of the insect. The first use of entomopathogenic fungi for insect pest bio-control occurred in the late nineteenth century. The principles of biopesticide control rely upon the application of large numbers of formulated spores to a target insect to ensure a rapid death. In 2000 there were 19 fungal bio-control products based on *Metarhizium* and *Beauveria* registered around the world for insect control (Butt *et al.*, 2001). Since this time more products have been developed and registered. In Australia there are four *Metarhizium* products produced and marketed by Becker Underwood for the control of pecan borer, locusts and grasshoppers, sugar cane beetle larvae and southern cockchafer larvae.

Studies by Muerrle *et al*, (2006) indicated the potential for entomopathogenic fungi such as *M. anisopliae* and *B. bassiana* to be used to control *A. tumida.* They found an increased mortality in *A. tumida* treated with *B. bassiana* (74%) and *M. anisopliae* (28%) compared to control insects. It has also been reported recently that the fungi *Aspergillus flavus* and *A. niger* may cause mortality of small hive beetle larvae and pupae (Ellis et al., 2004; Richard *et al.*, 2005). However, these fungi are also known to kill larval and adult honeybees (Morse and Flottum 1997, Swart *et al.*, 2001) and cause serious disease in humans (Bennet and Kilch 2003). As a result these fungi would be unsuitable for *A. tumida* control. In contrast, *M. anisopliae* and *B. bassiana* are non-toxic to humans and mammals (Langewald *et al.*, 1997; USEPA 1999, 2003) and honeybees (Jones, 2004; Kanga *et al.* 2003; Kanga *et al*, 2002) and therefore could be implemented into a control strategy for *A. tumida*. The virulence of isolates of *Metarhizium* and *Beauveria* endemic to Queensland has not been tested against small hive beetle.

The aim of this project was to carry out a feasibility study into in-hive fungal bio-control of small hive beetle in Australia using endemic isolates of the fungi *Metarhizium anisopliae* and *Beauveria bassiana.*

Objectives

1. Refine laboratory assays to screen a range of fungal isolates against adult beetles and larvae to allow different methods of spore application for *in vivo* testing.

2. Characterisation of new fungal isolates with respect to optimal growth temperature, vigour and sporulation characteristics.

3. Produce test quantities of spores from selected fungal isolates.

4. Check for *Metarhizium* spore survival in honey and toxicity towards bees in hives.

5. Screen fungal isolates against adult and larval beetles to find most virulent isolates.

6. Determine the minimum fungal spore dose required for adult and larval control with most virulent fungal strains.

7. Develop a formulation and application method for applying spores of selected fungal isolates to hives to control larvae and beetles.

Methodology

Fungal isolates

All *Metarhizium* and *Beauveria* isolates used in these studies were from the Queensland DPI&F entomopathogenic fungal culture collection housed at the Animal Research Institute. These isolates were obtained from either soil samples or dead insects, including small hive beetle adults and larvae, collected in Queensland. Cultures are stored at 4°C and -22°C on agar slants of Malt Extract agar (*Beauveria* isolates) and Sabouraud's Dextrose agar (*Metarhizium* isolates).

The thermal growth characteristics of isolates were determined by measuring radial growth on Sabouraud's Dextrose agar over 14 days at a range of temperatures from 20°C to 35°C, then calculating average daily growth rates.

Spores were produced via a biphasic process. A liquid culture was first grown to inoculate solid media. The liquid culture consisted of 150 ml of sterile yeast peptone broth in 250 ml Erlenmeyer flasks inoculated with spores scraped from Oatmeal agar (Difco™) plates. Cultures were grown for 5 days at 28°C on an orbital shaker. Mushroom spawn culture bags containing 500 g steam sterilised rice were chemically sterilised with 60 ml 1.5% sodium metabisulphite for 24 hours, then neutralised with 12 ml saturated sodium bicarbonate. Each bag was inoculated with 75 ml of the liquid culture. Extra sterile water was added to the bags to bring the total moisture to 40%. Inoculated bags were incubated for seven days at 28°C on wire racks; the solid cultures were then broken up and left for further 10 days of growth. Bags were opened and left to air dry for 3-4 days at 19°C in a de-humidified room. Spores were harvested from the dried grain through a series of sieves (1 mm, 300 μm and 150 μm) on an Endicott sieve shaker. Spore powder was stored at 4°C.

Small Hive Beetle Colony

A laboratory colony of small hive beetle was established with larvae from infested bee hives. Beetles were kept in plastic boxes (29 cm x 21.5 cm x 7.5 cm) lined with damp sponge cloth (Wettex™) to maintain high humidity; pieces of crumpled paper towel were provided as harbourages. Rearing boxes were ventilated by cutting a hole in the lid (16 cm x 10 cm) and sandwiching a piece of cloth gauze between the lid and container base. All rearing boxes were kept in a dark cupboard at 27°C and 60% RH. Beetles were maintained on a diet of granulated sucrose supplied in a 9 cm Petri dish. When either larvae or eggs were required a Feedbee™ pollen patty was added to each container in a 9 cm Petri dish. Beetles were allowed to oviposit in the dish of protein for 2-3 days, before the dish was transferred to another plastic container (17 cm x 12 cm x 20.5 cm) filled with moist sand to a depth of 15 cm (10% moisture by weight). After hatching, larvae fed on the protein for 2 weeks before wandering from the food source to pupate in the sand. Adult beetles emerged after 4-6 weeks and were transferred from the sand to new rearing containers to continue the cycle.

Bee Hives

Two groups of double storey hives were maintained at the Animal Research Institute at Yeerongpilly, Brisbane. Hives were initially placed in two locations separated by approximately 200 m and several building structures. Later the control hives were moved 22 km away to a property south-west of Brisbane in preparation for any in-hive testing of isolates against beetles.

Larval SHB Studies

Fungal screening

To screen isolates of *M. anisopliae* and *B. bassiana* against *A. tumida* larvae, the fungal spores were mixed through a pupation media to which wandering larvae were added. A range of doses was tested initially to determine the most effective dose for discriminating between isolates. A spore suspension of each isolate was prepared by mixing 0.1 g of spores in 100 ml of sterile 0.1% Tween 80 giving approximate concentrations of 4×10^7 spores/ml for *M. anisopliae* and 8×10^7 spores/ml for *B. bassiana*. Plastic containers (500 ml) were prepared with 200 g sand moistened with 30 ml of a

spore suspension. Containers had gauze inserts in the lids for air exchange. Twenty larvae were added to each container, and all treatments were replicated three times. Replicated controls were treated with 30 ml of 0.1% Tween 80. Containers were incubated in the dark at 27ºC and 65% relative humidity. The numbers of live and dead larvae were counted at 7 and 14 days. Cadavers removed from the treatments were surface sterilised with 70% ethanol, plated on water agar amended with 0.01% chloramphenicol and incubated at 28°C to confirm fungal infection. Twenty isolates of *M. anisopliae* and 2 isolates of *B. bassiana* were screened. Each isolate was screened at least twice.

Adult SHB Studies

Fungal screening

To determine the optimum way of screening fungal isolates against *A. tuminda* adults, spores of three isolates (M10, M16, M63) were presented to beetles as five different 'doses'. These included a spore solution in which beetles were dipped, spore powder mixed with either dried pollen or sugar presented to beetles for feeding, spore powder in a Corflute refuge or dry spore powder in which beetles were dipped. The tests were then standardised with beetles dipped in 0.05 g of spore powder from *M. anisopliae* and *B. bassiana* isolates. Twenty dipped beetles per treatment were incubated in the dark at 27°C and 65 % RH in a 1L rectangular plastic container with a crumpled paper harbourage, moistened sponge (Wettex™) and carbohydrate supply (granulated sucrose). Containers had gauze inserts in the lid for air exchange and a second layer of gauze under the lid to prevent beetle escape. Each treatment was replicated three times. The numbers of live and dead beetles were recorded at 7 and 14 days. Dead beetles were removed and isolations performed to confirm fungal infection. Seven isolates of *M. anisopliae* and 7 isolates of *B. bassiana* were screened. Each isolate was screened at least twice.

Beetle Age

The effect of age on adult beetle susceptibility to fungal infection was tested using a 2×2 factorial with beetle age (young, old) by treatment (control, spores). The young beetles were freshly emerged; old beetles were more than five months old. Beetles were dipped in 0.05 g of spore powder of isolate M81. Twenty beetles were added to a plastic container as described above with damp sponge, harbourage and granulated sucrose. Each treatment was replicated three times in each experiment. The experiment was replicated twice. The numbers of live and dead beetles were recorded after 7 and 14 days.

Fecundity

The effect of *M. anisopliae* infection on beetle fecundity was investigated. Twenty adult beetles were exposed to 0.05 g spores of isolate FI 1268 mixed with 3 g sugar for 9 days. The number of live and dead beetles was recorded. A pollen patty was added to each group of surviving beetles for four days to stimulate breeding, after which the patty was removed to a pupation box. Additional pollen patty was added as necessary until larvae had burrowed into the pupation medium. Larvae were allowed four weeks to pupate; the numbers of live beetles emerging were then recorded three times per week for two weeks.

In-hive Testing

Metarhizium toxicity to bees

M. anisopliae spores were dispersed through beehives to determine if they were pathogenic to bees. Twenty grams of spores of isolate M16 were added to each of three hives. Approximately 10 g of spores was distributed across the tops of the frames in the brood box; the other 10 g was distributed across the tops of frames in the super. Three untreated hives approximately 200 m away were used as control hives. Exit traps attached to the front of the hives forced bees to drop detritus being removed from hives. Dead bees (adults and undeveloped adults) were collected from these traps and counted five times per week, beginning seven days before treatment and continuing for another 23 days post treatment. A visual inspection was made inside hives at 7 days and 21 days post treatment. Isolations were carried out on dead adult bees collected from treated hives 4-7 days post treatment.

Larval treatment

Pupation boxes were set up under hives to measure the effect of *M. anisopliae* on *A. tumida* larvae under field conditions. Each box (60 cm *w* × 110 cm *l* × 15 cm *d*) was filled with washed river sand. Dried rice covered in *M. anisopliae* isolate FI1268 (500 g) was added as a thin layer to the surface of treatment boxes. The treatment was replicated three times as were the control pupation boxes that did not have added spores. Samples of sand (~ 250 g) were taken weekly for six weeks to assess the number of live and dead *A. tumida* larvae and pupae.

Spore Survival in Honey

The survival of fungal spores in honey was investigated by adding spores to fresh honey collected from the hives at the Animal Research Institute and commercially purchased Manuka UMF 20^{+} honey. *M. anisopliae* spores, isolate M16 (0.1g) and *B. bassiana* spores, isolate B27 (0.1 g) were added to 50 ml samples of each type of honey. These mixtures were incubated at 34°C then sampled 1 and 6 days later. The samples were diluted × 2000 and aliquots (20 µl and 100 µl) were streaked across plates of CAD selective agar (Chloramphenicol-Actidione-Dodine); the number of colony forming units (CFUs) was recorded after 14 days.

Statistical Analyses

All analyses were conducted in Genstat (2008). Mortality data were subjected to binary analyses via a generalised linear regression with binomial distribution and logit link (McCullagh and Nelder, 1989), or analyses of corrected mortalities (Abbott, 1925) using the angular transformation. The full model was assessed first; non-significant terms were progressively removed from the model to arrive at the final model.

Count data were skewed, and $\log_{10}(x+1)$ transformed prior to analysis. Where appropriate, repeated-measures analysis of variance (Rowell and Walters, 1976) was used.

Results

Fungal Isolates

The isolates used in the investigations with both larval and adult SHB are identified in Table 1 with their ARI isolate number, species and source, and temperature characterisation. The temperature characterisation is given as the average daily growth rate at 30°C and 35°C. These temperatures are relevant to the interior of a bee hive. The *Metarhizium* isolates had a higher thermal tolerance than the *Beauveria* isolates. Small quantities of spore powder were produced from these isolates and stored for use in laboratory assays against larvae and adult beetles as well as the honey investigations. Larger quantities of isolate M16 were produced for the in-hive investigations.

Table 1. Characterisation and source of fungal isolates used in Small Hive Beetle investigations

Isolate Identification	Species	Source within Queensland	Average growth rate (mm/day) at 30°C	Average growth rate (mm/day) at 35°C
M2	*Metarhizium anisopliae*	Soil, Currumbin Valley	4.1	0.2
M4	*Metarhizium anisopliae*	Soil, Cunungra	4.6	0.2
M6	*Metarhizium anisopliae*	Soil, Nunimbah Valley	4.1	0.3
M9	*Metarhizium anisopliae*	Soil, Eacham	3.3	2.0
M10	*Metarhizium anisopliae*	Soil , South Johnstone	4.9	0.4
M13	*Metarhizium anisopliae*	Soil , South Johnstone	4.9	0.7
M16	*Metarhizium anisopliae*	Soil, Aratula	4.8	0.7
M32	*Metarhizium anisopliae*	Soil, South Johnstone	2.2	0.9
M45	*Metarhizium anisopliae*	*Musca domestica*	4.6	0.3
M63	*Metarhizium anisopliae*	Laboratory mutant	4.9	0.3
M71	*Metarhizium anisopliae*	*Aethina tumida* larva, lab colony	3.8	2.0
M72	*Metarhizium anisopliae*	*Aethina tumida* adult, lab colony	3.8	2.0
M80	*Metarhizium anisopliae*	*Aethina tumida* adult, lab colony	4.1	1.0
M81	*Metarhizium anisopliae*	*Aethina tumida* adult, lab colony	4.0	1.0
M82	*Metarhizium anisopliae*	*Aethina tumida* adult, lab colony	4.5	0.7
M85	*Metarhizium anisopliae*	*Aethina tumida* adult, beehive	4.8	1.1
M86	*Metarhizium anisopliae*	*Aethina tumida* adult, beehive	4.7	1.0
M87	*Metarhizium anisopliae*	*Aethina tumida* adult, beehive	4.6	0.9
M88	*Metarhizium anisopliae*	*Aethina tumida* adult, beehive	4.7	1.0
FI 1268	*Metarhizium anisopliae*	Ex CSIRO, originally Locust	4.2	0.2
B4	*Beauveria bassiana*	*Lucilia cuprina*	2.6	0.2
B14	*Beauveria bassiana*	*Musca domestica*	2.0	0
B23	*Beauveria bassiana*	*Haemotobia irritans exigua*	2.1	0.3
B25	*Beauveria bassiana*	*Bovicola ovis*	1.1	0
B26	*Beauveria bassiana*	*Bovicola ovis*	1.3	0
B27	*Beauveria bassiana*	*Bovicola ovis*	0.9	0
B28	*Beauveria bassiana*	*Bovicola ovis*	0.8	0

Larval Studies

There was variation in the virulence of different isolates to *A. tumida* larvae as measured by the average percent mortality at days 7 and 14. There were no significant interactions, and the effects of dose and assay were both significant ($P<0.01$). As expected, higher doses were more effective. The trend in results at days 7 and 14 was similar, except at day 14 half of the isolates had caused 100 % mortality. Hence the day 7 results were more discriminating.

Figure 1 shows the percent mortality at day 7 of the *Metarhizium* isolates. The two *Beauveria* isolates did not perform as well as the *Metarhizium* isolates. The asterisks indicate the best isolate (FI1268) along with the three next most virulent isolates (M81; M82; M80) whose activities were not significantly ($P<0.05$) different from it. Isolations confirmed the presence of fungal infection (Figure 2) in dead larvae from the spore treatments.

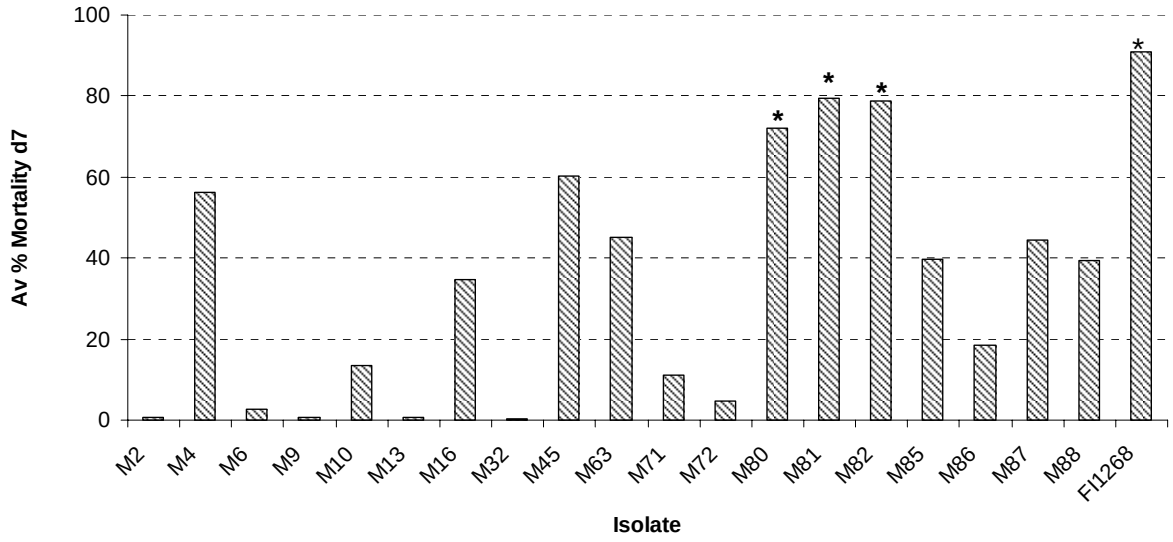

Figure 1. Average per cent mortality in larval *A. tumida* after 7 days exposure to different *Metarhizium* isolates. The four most virulent isolates ($P<0.05$) are marked with asterisks.

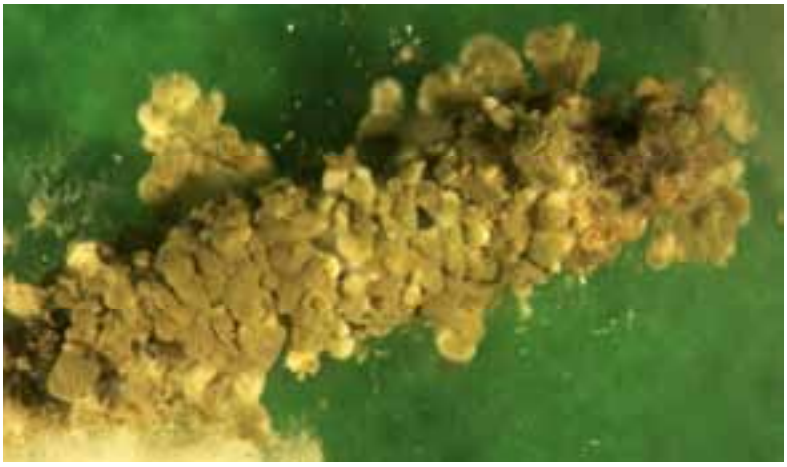

Figure 2. *Metarhizium* growing from *A. tumida* larva exposed to spores in pupation media

Adult Studies

Screening

Generally *Metarhizium* isolates were less effective against adult *A. tumida* than against larval *A. tumida*. Most *Beauveria* isolates were more virulent towards adult *A. tumida* than the *Metarhizium* isolates.

In the initial screenings comparing a range of doses, dose was not found to be significant at either day 7 (P=0.088) or day 14 (P=0.147). However there was a trend that indicated that spores presented in a refuge were slightly more effective.

The effect of isolate was significant (P<0.01) at both 7 and 14 days. Adult beetles appeared to take longer to die than larvae so the percent mortalities at day 14 are presented in Figure 3. The asterisks indicate the best isolate B26 along with the two next most virulent isolates (B28; B25;) whose activities were not significantly (P<0.05) different from it. Isolations confirmed the presence of fungal infection (Figure 4) in dead beetles from the spore treatments.

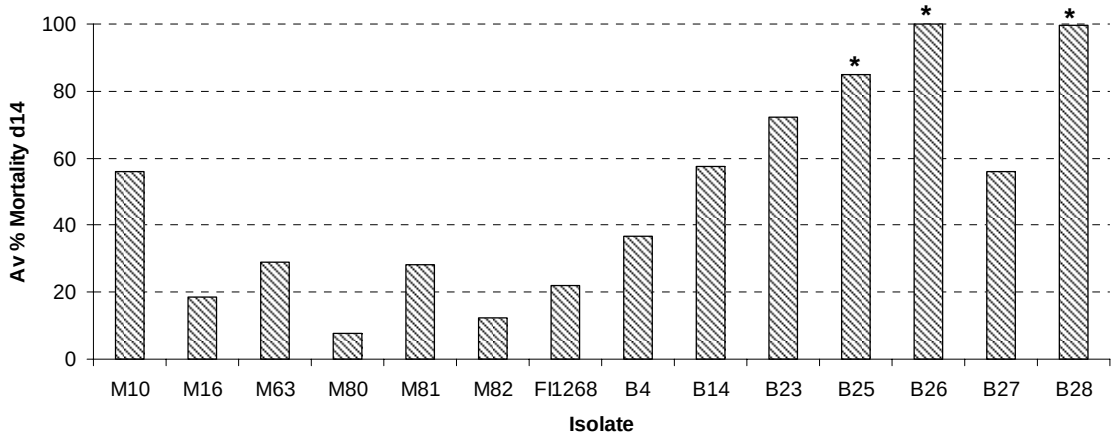

Figure 3. Average per cent mortality in adult *A. tumida* 14 days after exposure to different *Metarhizium* and *Beauveria* isolates. The three most virulent isolates (P<0.05) are marked with asterisks.

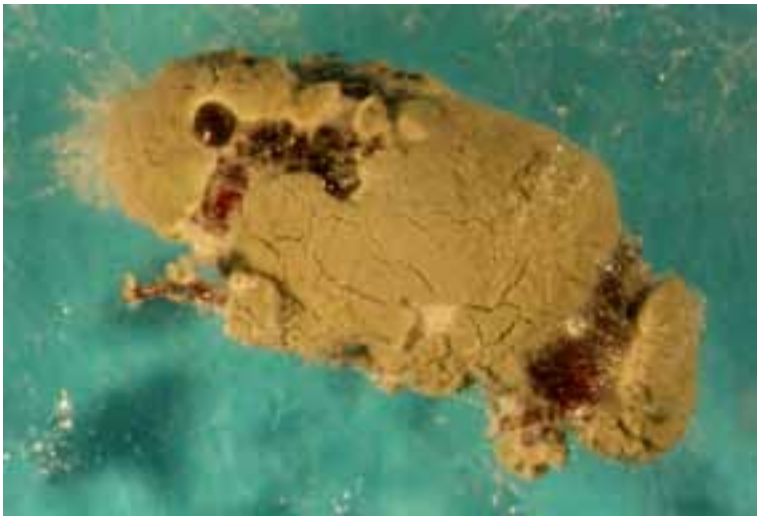

Figure 4. *Metarhizium* growing from *A. tumida* adult exposed to spores

Effect of Age on susceptibility to *Metarhizium*

There was no effect of age on beetle mortality while both assay and treatment did affect survival. The interaction between age and treatment was not significant at 7 days (P=0.343) or 14 days (P=0.910), neither was age significant at 7 days (P=0.959) or 14 days (0.910). Both assay and treatment were highly significant at 7 and 14 days (P<0.001). The mortalities in treated beetles are shown in Table 2, old and young beetles are combined because there was no significant difference between them.

Table 2. Per cent mortality (±SE) in old and young beetles treated with spores of fungal isolate M81 mixed with sugar

Treatment	7 days	14 days
Control	2 (±1)	3 (±1)
Spores	16 (±2)	43 (±3)

Fecundity

The results of the fecundity trial are shown below in Table 3. Exposure to spores caused 33 (±4)% mortality in adult beetles after 9 days, while the mortality in control beetles was unusually high at 21(±3)%; this difference between the treatments is significant (P<0.01). The total offspring produced by the surviving beetles was higher for the controls (186) than for Metarhizium-exposed beetles (65). There was no significant effect of treatment on fecundity (P=0.24) due to a very high variability in the control treatment. The average number of offspring per surviving beetle was 1.50 (±0.63) for the control group, compared to 0.67 (±0.17) for the treated group.

Table 3. Effect on adult beetles of exposure to *Metarhizium* spores showing per cent mortality (±SE) and reduced production of offspring by beetles exposed to spores for 9 days

Treatment	% Mortality after exposure to spores	Total Offspring from surviving beetles	Ratio adults: progeny
Control	21 (±3)	186	1.50
Spore Exposure	33 (±4)	65	0.67

In-hive Testing

Metarhizium toxicity to bees

Repeated measures ANOVA on pre-treatment counts (log transformed) showed no treatment effect or treatment by time interaction, indicating a balanced allocation of the hives to treatment. There was no significant (P>0.05) relationship between pre and post treatment counts, so no covariate was used. Total numbers of bees showed a significant (P=0.047) interaction between treatment and days. This indicates a different pattern in the post treatment numbers as shown in Figure 5. This significant interaction is due to the increased number of dead bees collected from treated hives 4 to 7 days post treatment. After this time the numbers of dead bees collected from treated hives was similar to that collected from the control hives.

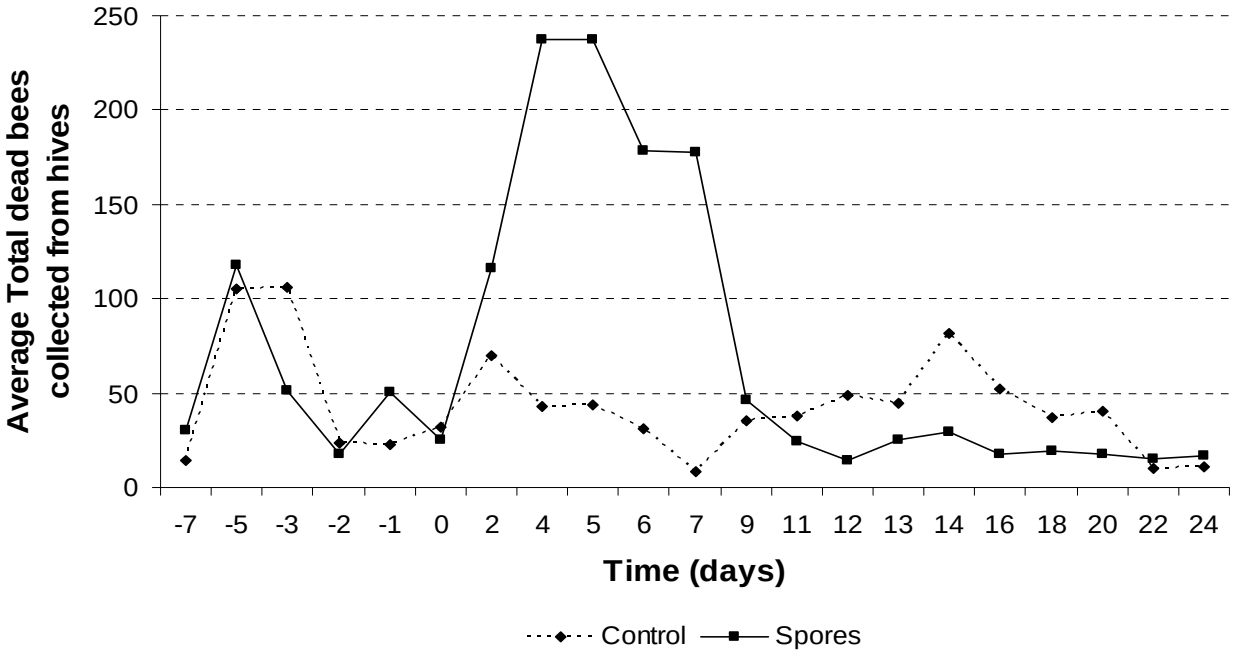

Figure 5. Average total numbers of dead adult and undeveloped bees collected before and after treatment of hives with fungal spores. Spores were added on day 0 after the dead bees were collected

Observation in the treated hives showed that in two hives the bees had cleaned up the spores after 7 days leaving little visual evidence of the spore treatment. In the third treated hive few spores appeared to have been cleaned up even after 21 days post treatment. It was noted that this hive was a much weaker hive than the other two treated hives in which the spores had been cleaned up. After 21 days it also became apparent that this hive was queen-less and the frames of brood and honey in the brood box had been destroyed by small hive beetle larvae.

Microscopic examinations of samples of dead bees collected from the treated hives 4 to 7 days post treatment found that some of cadavers were packed with fungal mycelium. Isolations confirmed the presence of *M. anisopliae* in these bees (Figure 6). No *Metarhizium* was isolated from dead bees collected from control hives.

Figure 6. *Metarhizium* growing out of dead bee recovered from a hive treated with *Metarhizium* spores

Larval treatment

No SHB larvae or pupae were collected from either the treated or control sand boxes under the hives.

Spore Survival in Honey

Spores stored in honey lost viability rapidly. After one day of storage at 34 °C there were fewer colonies growing from the honey samples than from the water controls (Table 4). After 6 days of storage no colonies grew from the honey samples while the number of colonies growing from the water control were too numerous to count.

Table 4. Number of colonies growing from aliquots of diluted samples of honey contaminated with spores of either M16 or B27 stored for 1 and 6 days at 34°C

		ARI Honey		**Manuka Honey**		**Water Control**	
		20 µl	100 µl	20µl	100 µl	20 µl	100 µl
	T1	4	4	8	13	TNTC	TNTC
M16	T6	0	0	0	0	TNTC	TNTC
	T1	1	4	20	79	TNTC	TNTC
B27	T6	0	0	0	0	TNTC	TNTC

TNTC = too numerous to count

Discussion

Most of the objectives in this study were achieved, demonstrating that some local isolates of the fungal control agents *Metarhizium* and *Beauveria* show good efficacy against *A. tumida* larvae and adults in laboratory assays. Generally the *Metarhizium* isolates performed best against the larvae while the *Beauveria* isolates performed best against the adult beetles. Further investigations looked at beetle age as a potential factor increasing susceptibility to fungal infection and the effect of fungal infection on fecundity. *Metarhizium* and *Beauveria* spores contaminating honey were found to lose viability quite rapidly. *Metarhizium* spores distributed inside hives were found to be efficiently cleaned up by bees in strong hives, however some bees in these hives became infected within a week of the spores being added. In the time available the research did not progress to determining the minimal spore doses of the most virulent isolates required to control both adult and larval *A. tumida*. As a result, a method of formulating and applying the spores of selected isolates to control adult *A. tumida* in hives was not trialled.

The approach taken in this research examined the life cycle of the small hive beetle and determined the optimal stages to target for control with fungal spores. These were either adults (in hive) or wandering larvae leaving the hives to pupate (surrounding soil). Methods of application contemplated were either a physical trap or refuge with added spores or spores puffed into the hive for adult beetles; the latter was considered the least preferable due to the possibility of fungal toxicity to bees. A granular spore product for spreading over and through soil was considered for larval control.

The potential methods of application influenced the refinement of assays to screen fungal isolates against both larvae and adults. The screening of isolates against larvae was relatively simple with a suspension of spores used to moisten the pupation medium to which wandering larvae were added. A suitable assay system for screening isolates against adult beetles required more investigation. Initially spores were mixed with sugar or added to a refuge to simulate how spores could be presented to beetles within a hive. With this approach it was not certain that all beetles received an equal exposure to the spores as the self contamination with spores was dependent upon beetle behaviour. The data analyses did not support any significant difference between the methods of presenting spores to beetles although there was a trend suggesting some difference. The preferred method of exposing beetles to spores in order to compare isolates was through dipping beetles in dry spore powder. This method ensured that all beetles were dosed with spores.

Initially the selection of isolates for screening was biased towards *Metarhizium.* The reasons included:

- The *Metarhizium* isolates in the ARI collection generally have higher thermal tolerances than the *Beauveria* isolates. This was considered important as the interior of bee hives is maintained at approximately 32°C - 36°C (Warhurst & Goebel, 2005).

- *Metarhizium* had been isolated from both adult and larval *A. tumida* including beetles collected from bee hives. No *Beauveria* isolates were found in any of the dead adult and larval beetles investigated.

- Currently only fungal biopesticides based on *Metarhizium* have been registered for use in Australia. Although there are a number of *Beauveria* based fungal biopesticides registered overseas, the perception is that it will be more difficult to register a new product based on *Beauveria* than on *Metarhizium.* It is inevitable that fungal biopesticides based on *Beauveria* will be registered in Australia, however in the first instance it is usually easier to try to find a suitable isolate of *Metarhizium*.

- Generally the spore production system used by both DPI&F and the commercial company producing fungal biopesticides in Australia results in much higher spore yields from *Metarhizium*

isolates than from *Beauveria* isolates. This is an important commercial consideration for a marketable product.

More than half of the *Metarhizium* isolates screened against larval SHB showed some activity. It is interesting to note that of the nine isolates originating from beetles only three (M80, M81, M82) showed good efficacy killing more than 70% of larvae by day 7. The best isolate against the larvae was an isolate provided by CSIRO which was considered to have characteristics that might prove very effective against the adult beetles. Only two *Beauveria* isolates were screened against larvae, and because both gave relatively poor results no other isolates were screened.

The promising results with the *Metarhizium* isolates and larvae did not correlate with the adult screens. The most virulent *Metarhizium* isolate for adults (M10) only killed 56% of adult beetles after 14 days. This isolate killed only 13% of larvae after 7 days, but killed all larvae after 14 days. As well as a lower virulence to the adult beetles there was a high degree of variation in mortality both within and between assays using the *Metarhizium* isolates. One factor considered as a source of this variability was beetle age, however, investigations refuted this for the range of ages of beetles used in this study.

Of the seven *Beauveria* isolates screened against adult beetles all but one (B4) were more virulent than any of the *Metarhizium* isolates. Muerrle *et al*, (2006) also reported a higher adult mortality with *B. bassiana* than with *M. anisopliae.* The best isolates of *Beauveria*, B28 and B26, respectively killed 80% and 63% by 7 days, and 99% and 100% of beetles by 14 days. The variation within assay and between assays was also much lower than that seen with the *Metarhizium* isolates. The results with *Beauveria* are very promising, but further investigation is necessary, especially in respect to the thermal characteristics of these isolates and their virulence and viability under hive conditions.

The effect of *M. anisopliae* infection on beetle fecundity was investigated and while surviving treated beetles produced far fewer offspring than control beetles the result was not significant due to the degree of variation between replicates. It was hoped that using eight replicates would compensate for not knowing the sex ratio of the beetles. Some of the variation between replicates might be corrected if the sex ratio of the beetles was determined before exposure to the spores, then again for the breeding survivors. It would be valuable to know the breeding potential of beetles between the time of fungal infection and death, particularly as this can take between 7 and 14 days. Further investigations with the better *Metarhizium* isolates and some of the *Beauveria* isolates should be considered.

One method of spore application contemplated for SHB control was through puffing spore powder into hives. Before such a method could be seriously considered the toxicity of the fungal spores to bees in the hive needed to be investigated. Observations on the infection of a range of insects with the ARI *Metarhizium* isolates suggested that evidence of infection in bees could be expected to be detectable from approximately 4 to 7 days after exposure to *Metarhizium* spores. A significant increase in dead adult bees and undeveloped bees from the treated hives was observed between days 4 and 7. Dead adult bees were collected from the two strong hives while mostly dead undeveloped bees were collected from the weak hive. This effect of increased dead bees from the treated hives did not continue. Within 10 days the numbers of dead bees from treated hives was not significantly different to that from the control hives. Bees in the two strong hives showed robust hygienic behaviour and cleaned up the spores within a short period of time. Bees in the weak hive did not appear to have cleaned up many spores. *Metarhizium* was isolated from dead bees collected from the two strong hives during this time. The observations in the strong hives suggest that the bees cleaning up the spores were exposed to a lethal dose of spores but the hygienic behaviour of the bees in that colony resulted in removal of dead bees from the hive.

Adding loose spores to strong hives with good hygienic behaviour had only a short term negative effect. However the weak hive did not clean up the spores but was still overrun with *A. tumida* larvae three weeks after the spores were added. The investigations indicate that introducing dry spore powder into hives will be ineffective for beetle control because a hive with good hygienic behaviour will quickly clean up the spores while a weak or queen-less hive will still succumb to the beetle larvae. An

effective fungal control will need spores of an isolate virulent to adult beetles provided inside a beetle refuge so that bees cannot access them.

A. tumida larvae are killed relatively quickly when they move into and through soil treated with fungal inoculum. However the “in-hive” testing against larval *A. tumida* raised concerns about the economics of using a fungal biopesticide treatment of the soil to kill *A. tumida* larvae. During the investigation time no larvae or pupae were recovered from the large containers of sand placed under the hives. This indicated that the number of larvae wandering from the hives and pupating in the nearby soil was either very low or not occurring for the hives investigated. This may be a result of bee behaviour with bees attacking beetle larvae within the hives or it could have been influenced by the time of year. Fungal spores applied to the soil will need to remain viable in the soil to control larvae entering the soil. To justify the cost of applying spores to the soil around the hives there must be certainty that large numbers of larvae are entering the soil. For this type of application it is difficult to appreciate what advantage a fungal biopesticide could have over the careful use of a chemical soil drench. A chemical will stay active in the soil killing both wandering larvae entering the soil or emerging beetles leaving the soil.

The viability of fungal spores added to honey was assessed to give a measure of what could happen if spores applied to hives contaminated the honey. Given the results of the in-hive spore toxicity testing reported above it is unlikely that loose spore powder would be applied through hives. Regardless, the honey investigation confirmed that fungal spores rapidly became non-viable. It is presumed that the various factors in honey which confer antibacterial properties (Molan, 1992) also affect fungal spores.

Conclusions

This research identified a number of local isolates of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* that show good efficacy against adult and larval small hive beetles. The *B. bassiana* isolates were more effective against the adult beetles, while the *M. anisopliae* isolates were more effective against the larval beetles. The susceptibility of beetles to fungal infection did not appear to increase with age. Although isolates of *M. anisopliae* did not kill high numbers of adult beetles there was a trend of lowered fecundity in the beetles surviving the treatment that is worthy of further investigation. Spores applied as a dry loose powder to hives were rapidly cleaned up by bees in strong hives, although the spores did not appear to have any long term effect on the bees. Fungal spores contaminating honey lose their viability rapidly.

Recommendations

Based on the results of the current project a fungal biopesticide for the in-hive control of small hive beetles may be feasible. However, further research needs to be conducted with isolates of *Beauveria bassiana* to first develop a formulation and application strategy for adult small hive beetle control, then to test this in hives in different locations.

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Feasibility Study into In-Hive Fungal Bio-Control of Small Hive Beetle

By Diana Leemon and Jacinta McMahon

RIRDC Publication No. 09/090

The small hive beetle (*Aethina tumida*), a native to South Africa where is it only a minor pest, was discovered in Australia in 2002. Since this time small hive beetles have become an increasing problem in bee hives in the eastern states of Australia and appear to be spreading at a rapid rate. There is concern for even greater damage from this pest to the beekeeping industry in the warm moist regions of Australia where it can reproduce faster.

This research identified a number of local isolates of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* that show good efficacy against adult and larval small hive beetles.

The report is targeted at the beekeeping industry in Australia, particularly those in the warmer regions affected by small hive beetles.

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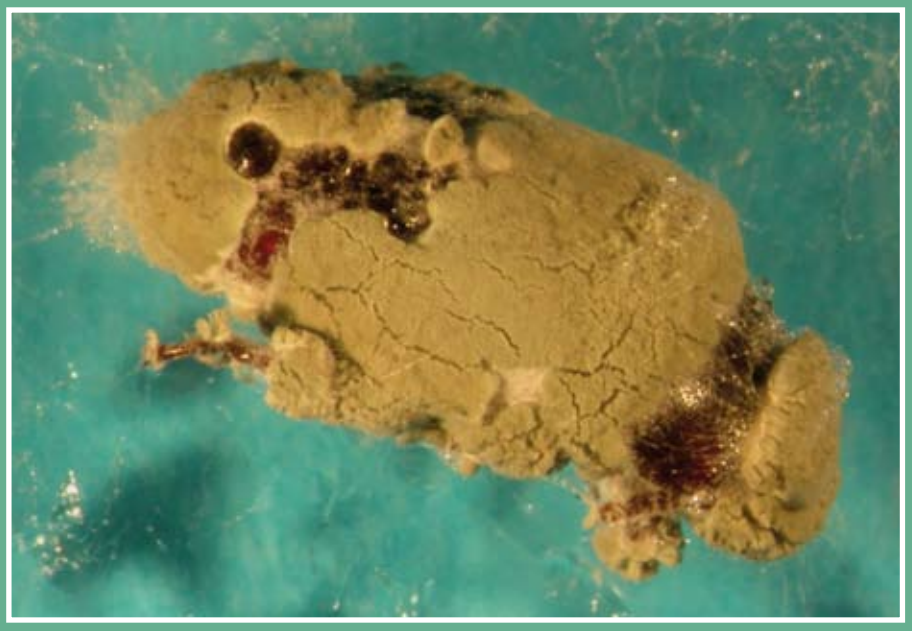

Cover photo: *Metarhizium* growing from *A. tumida* adult exposed to spores

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