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Evidence Project Final Report

Department for Environment Food & Rural Affairs

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Project identification

1. Defra Project code: PS2140

2. Project title:
   Use of refuge traps to disseminate entomopathogenic fungi for the control of adult vine weevil

3. Contractor organisation(s):
   ADAS UK Ltd, Pendeford House, Pendeford Business Park, Wobaston Road, Wolverhampton, WV9 5AP

   Harper Adams University, Newport, Shropshire, TF10 8NB

   University of Warwick School of Life Sciences, Warwick Crop Centre, Wellesbourne, Warwick, CV35 9EF

4. Total Defra project costs (agreed fixed price): £ 150,380

5. Project: start date ..... 1 October 2012

   end date ..... 30 January 2015

6. It is Defra's intention to publish this form.

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Vine weevil is a serious pest of soft fruit and nursery stock crops. Adult weevils feed on the leaves, rendering ornamental plants unmarketable, and the larvae feed on the roots, causing plant stunting, wilting and death. Although non-chemical control methods are available for vine weevil larvae i.e. entomopathogenic nematodes and the entomopathogenic fungus (EPF) *Metarhizium brunneum* control of adult weevils is currently reliant on the use of chemical pesticides which give unreliable control and are often incompatible with Integrated Pest Management (IPM) programmes. This project is building on the CRD-funded proof of principle study (PS2134) carried out by ADAS and Warwick University, which showed that adult vine weevils enter artificial refuges containing fluorescent powder (used to mimic an EPF), pick up the powder and spread it to other weevils when foraging or aggregating together within other refuges within the crop. Project PS2134 also identified EPF isolates that effectively killed adult vine weevils within a refuge.

This project was completed to develop the principle of initiating an EPF epidemic within an adult vine weevil population using baited refuge traps in more realistic semi-field and commercial crop conditions

Objective 1: Quantify improved vine weevil adherence and efficacy of electrostatic EPF formulations when used in an artificial refuge

The susceptibility of adult vine weevils to three isolates of entomopathogenic fungi (*Beauveria bassiana* isolate 433.99 (the active ingredient in the product BotaniGard), *B. bassiana* isolate 1749.11 (isolated from an infected vine weevil) and *Metarhizium brunneum* isolate 275.86 (the active ingredient in the product Met52) was measured in a laboratory test. The fungi, combined with either talc or an electrostatic carrier powder (Entostat, Exosect Ltd) were placed in Roguard cockroach traps used as weevil refuges in a test chamber. Adult weevils entered the traps and picked up the fungus powder. All of the isolates caused greater mortality of adult weevils than the controls. Only one of the isolates (*M. brunneum* isolate 275.86) resulted in 100% mortality after 49 days post-innoculation. There was no difference in the fungal mortality for spores formulated in talc versus Entostat powder. All of the fungal isolates examined produced spores on weevil cadavers but the spore production per weevil varied between isolates. *Metarhizium brunneum* isolate 275.86 and *B. bassiana* 1749.11 produced significantly ($P<0.05$) more spores per weevil than *B. bassiana* isolate 433.99. The type of spore carrier (talc vs. Entostat) had no effect on the spore production on infected weevils. The majority of sporulation occurred between the body segments.

The transfer of fungal spores between weevils was also quantified in a laboratory test. Fungal spores of *M. brunneum* (275.86) were formulated in either talc or an electrostatic carrier powder (Entostat), and used to inoculate healthy adult weevils. One inoculated weevil ('donor weevil') was then placed in a test chamber with five healthy weevils that had not been treated with spore powder, together with a source of water and yew leaves as food.

The majority of the inoculum on the donor weevils was lost after 24h with an 82% and 91% reduction (Entostat and talc treated respectively) and after 48h this increased to 91% and 94 % (Entostat and talc treated respectively). All of the 'recipient' weevils sampled in the experiment came into contact with the inoculated donor weevil within 24h and obtained *Metarhizium* spores regardless of the type of spore carrier. The spore powder was spread very quickly between the weevils. Healthy weevils received a dose ranging from 8×10^4 and 1×10^6 spores per weevil after 24h and from 8×10^4 and 4.5×10^5 spores after 48 h. Although it was not determined if this dose was enough to cause mortality based on our knowledge of this insect and other insect species this should be a sufficient dose to cause death.

Objective 2: Improve current understanding of adult vine weevil movement in soft fruit and nursery stock crops, in order to determine the density of refuge traps needed for effective EPF spread

MiniHPT8 type radio frequency identification (RFID) tags (Biomark, Idaho, USA) were successfully used to record the dispersal behaviour of individually identifiable adult vine weevils within commercial strawberry and nursery stock crops. The tags used were HPT8 (8.4 mm 134.2 kHz FDXB) and these were read (detected) using an HRP Plus reader, fitted with the standard HRP Plus antenna.

Both cyanoacrylate and thermoplastic adhesives were evaluated as a means of attaching the RFID tags to the adult weevils. The impact of these adhesives on the survival and behaviour of tagged weevils was recorded. Thermoplastic adhesive was selected for use in subsequent experiments as it did not affect weevil survival, although it did reduce the speed of movement of weevils on horizontal or vertical surfaces and the number of eggs laid. However, effects of the thermoplastic adhesive on weevil survival and behaviour were much less than the cyanoacrylate adhesive.

When tagged weevils were released into a semi-field environment consisting of replicate gauze 'tent' cages, each containing one grow bag with ten strawberry plants, the HRP Plus reader detected 71-96% of the tagged weevils on five separate days. All of the weevils detected were alive and had their tags still attached. Weevils were found in plant crowns, under grow bags and in leaf litter within the gauze 'tent' cages.

The HRP Plus reader system was also tested in commercial strawberry and nursery stock crops after releasing tagged weevils. In the strawberry crop, the number of weevils detected was highest one day after release (73%) and declined to 28% 35 days after release. Thirty-five days after release all RFID tags were collected from the strawberry crop. This confirmed that 11 of the 40 tagged weevils released into the crop were healthy, still had the tag attached and had remained within the release site. Four weevils had died, nine tags had become detached from the weevils and 16 (40%) of the released weevils had left the crop area, indicating possible long range dispersal or effects of predation. The distance moved by a weevil along a row of strawberry grow-bags seven days after release ranged between 0.3 m and 4.3 m with an average distance moved of 1.44 m.

In the commercial nursery stock crop, 25 tagged adult weevils were released into a bed of potted *Euonymus* plants. Between 56% and 60% of the RFID tags were detected on each assessment date. Between 40% and 53% of these were live weevils with their tags still attached. Only five of the 25 original weevils were detected on every assessment date. The distance moved by these five weevils over the 14-day period ranged between 0.93 m and 2.67 m, with the average distance being 1.96 m. The longest distance travelled by a single weevil was 3.48 m and this weevil was only detected on the last assessment date, so may have moved out of the experimental area on previous assessment dates. All of the weevils detected with tags had moved from their original release point. A small number of weevils were found in the refuge traps placed amongst the crop.

Finally, the movement of adult vine weevils (in a natural infestation) and their use of artificial refuges was recorded in a commercial strawberry crop. This was achieved by placing different densities of Roguard traps containing different colours of fluorescent powder within the crop. Fluorescent weevil tracks on leaves and the polythene covering the beds were observed after dusk using UV torches 2, 9 and 15 days after setting up the traps. This confirmed that the natural population of vine weevil adults used the artificial refuge traps and that movements of naturally-occurring vine weevils could be recorded using fluorescent powder in the traps. Two days after adding the powder to the traps, 17% of weevils recorded in the field were marked with fluorescent powder and 16% of these had moved from another plot or had come into

contact with a weevil coated in fluorescent powder from another plot. Similar numbers of weevils coated with fluorescent powder were recorded on the first two assessment dates when trap densities of 0.75-6 per m^2 were used.

Objective 3: Test the efficacy of the best performing EPF formulation(s) under semi-field conditions that mimic soft fruit and nursery stock crops

A glasshouse experiment was done to test whether using fungi in refuge traps in a more realistic crop environment infected vine weevils entering the traps or coming into contact with other weevils that had entered the traps. *Beauveria bassiana* (isolate 433.99) and *M. brunneum* (isolate 275.86) spores (the best performing fungal isolates in Objective 1, Task 1.1) were mixed with fluorescent powder and placed in the centre of Roguard refuge traps. The traps were placed in a semi-field environment consisting of five replicate large gauze cages for each of the two fungal treatments and an untreated control. Each cage contained two grow bags, each with four strawberry plants. Twelve weevils were released into each cage. After two months the weevils were recovered and incubated to test for infection.

The experiment confirmed that the weevils entered the refuge traps as weevils with fluorescent powder on their bodies were found in various locations in the cages including inside the traps. Some infected weevils were found in the untreated control cages, the source of the infection probably being the commercial nursery from where the weevils were collected for the experiment. However, percentage weevil infection with *M. brunneum* (60%) in the *M. brunneum* treatment was higher than that in the untreated controls (0% by *M. brunneum* only and 8% by a mixture of *B. bassiana* and *M. brunneum*), indicating that most of the weevils became infected by entering the treated refuge traps or coming into a contact with weevils that had. Furthermore, following incubation lower proportions of live weevils were found in the *M. brunneum* treatment (13.3%) than in the other treatments (untreated 64% alive and *B. bassiana* 55.6% alive).

Percentage infection with *B. bassiana* in the *B. bassiana* treatment (22.2%) was only slightly higher than that observed in the untreated controls (12%), therefore, it could not be confirmed that the source of the fungus was from the refuge traps or from the contaminated weevil culture. The results of the experiment indicated that using refuge traps to disseminate fungi for the control of adult vine weevil is a promising strategy using *M. brunneum* (isolate 433.99) but not using *B. bassiana* (isolate 275.86).

A second experiment was done to test the transfer of *M. brunneum* spores from one weevil to another. In this experiment, one 'donor' weevil, infected with a spore + fluorescent powder mixture was released into replicate cages, each containing five healthy 'recipient' weevils, a potted strawberry plant and an empty refuge trap. Five healthy weevils were also added to similar untreated cages but only fluorescent powder was added to the 'donor' weevils. The cages were left in the same glasshouses alongside those used for the first experiment for two months, followed by a 4-week laboratory incubation period of weevils recovered from the cages.

Following incubation of the weevils recovered from the untreated cages, 3.3% were dead and infected with *M. brunneum*, 30% were dead and infected by *B. bassiana* and 3.3% were dead and infected by a mixture of *M. brunneum* and *B. bassiana*. The presence of both entomopathogenic fungi in the untreated cages is likely to be due to the weevils having come into contact with these fungi on the commercial nursery from which they were collected or in the subsequent laboratory culture.

Following incubation of the weevils recovered from the *M. brunneum* cages, 19% were dead and infected with *M. brunneum*. Although this was not statistically higher than the 3.3% weevils infected with *M. brunneum* in the untreated cages, this trend indicated that some of the weevils had become infected with *M. brunneum* through contact with the infected 'donor' weevils. As in the untreated cages, some weevils (19%) were infected with *B. bassiana* which was probably acquired in the commercial nursery from which they were collected.

Options for new work

1. Determine how many fungal spores are needed to kill an adult vine weevil.
2. Determine the sub-lethal effects of fungal infection of adult vine weevils, such as feeding, oviposition and movement rates and aggregation behaviour in natural and artificial refuges.
3. Repeat work in Objective 3 using higher numbers of weevils, if possible sourced from sites without a natural fungal infection, during the summer when temperatures are conducive to fungal development. Include the development of a more practical system for delivery of the fungus, suitable for commercial uptake.
4. Test the vine weevil control strategy using *M. brunneum* in a commercial soft fruit or nursery stock crop.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Exchange).

Background

Vine weevil is a serious pest of soft fruit and nursery stock crops. Adult weevils feed on the leaves, rendering ornamental plants unmarketable, and the larvae feed on the roots, causing plant stunting, wilting and death. Although non-chemical control methods are available for vine weevil larvae i.e. entomopathogenic nematodes and the entomopathogenic fungus (EPF) *Metarhizium brunneum*, control of adult weevils is currently reliant on the use of chemical pesticides, which give unreliable control and are often incompatible with Integrated Pest Management (IPM) programmes. This project built on the CRD-funded proof of principle study (PS2134) carried out by ADAS and Warwick University, which showed that adult vine weevils enter artificial refuges containing fluorescent powder (used to mimic an EPF), pick up the powder and spread it to other weevils when foraging or aggregating together within other refuges within the crop. Project PS2134 also identified EPF isolates that effectively killed adult vine weevils within a refuge.

Aims & Objectives

This project aimed to further develop the principle of initiating an EPF epidemic within an adult vine weevil population using baited refuge traps in more realistic semi-field and commercial crop conditions. The Objectives and tasks to meet the objectives were:

1. Quantify improved vine weevil adherence and efficacy of electrostatic EPF formulations when used in an artificial refuge.

2.Improve current understanding of adult vine weevil movement in soft fruit and nursery stock crops, in order to determine the density of refuge traps needed for effective EPF spread.

3.Test the efficacy of the best performing EPF formulation(s) under semi-field conditions that mimic soft fruit and nursery stock crops

4.Test the vine weevil control strategy in a commercial soft fruit or nursery stock crop (optional extension to the 2-year project if results of Objective 3 are promising)

Objective 1. Quantify improved vine weevil adherence and efficacy of electrostatic EPF formulations when used in an artificial refuge (Warwick)

The aim of this Objective was to measure the effectiveness of fungal spores to control adult vine weevil and to determine whether the speed of kill can be improved by combining spores with an electrostatic carrier powder.

Materials & methods

Adult weevils were obtained from the ADAS culture initiated from adult vine weevils collected from a commercial strawberry crop in July 2013. Adult vine weevils were kept in 1.5 l plastic pots. The lids of these pots were perforated in order to provide ventilation. The base of each pot was lined with tissue paper, an additional ball of damp tissue paper provided a source of moisture, a piece of corrugated cardboard provided a refuge and fresh yew leaves (*Taxus baccata*) provided a food source. Thirty to 50 weevils were placed into each pot, which in turn were placed in a controlled environment room at 20°C. Pots were cleaned on a weekly basis.

Stock cultures of the fungal isolates were stored on porous plastic beads in liquid nitrogen vapour (Chandler, 1994). Laboratory cultures were grown on Sabouraud dextrose agar (SDA) slopes and maintained in a refrigerator at 4°C for up to six months. Sub-cultures for laboratory experiments were prepared on SDA from the slope cultures and incubated at 23°C for 10-12 d in the dark and powdered spores collected from the plates. The number of spores per dry weight of powder was calculated by adding 0.1 g of spore powder to 10mls of 0.05% Triton X-100 and the suspensions enumerated using an improved Neubauer haemocytometer.

Task 1.1 Fungal efficacy

The susceptibility of adult vine weevils to three isolates of entomopathogenic fungi, combined with either talc or an electrostatic carrier powder (Entostat, Exosect Ltd), was measured in a laboratory bioassay. The isolates (Table 1) were selected from the WHRI culture collection based on their pathogenicity to adult vine weevils in previous CRD-funded research (PS2134) and their availability as commercial biopesticides.

Table 1. Fungal isolates used in Experiment 1.1.

Isolate†	Species	Original Host	Collection site
433.99[a]	*Beauveria bassiana*	-	-
1749.11	*Beauveria bassiana*	*Otiorhynchus sulcatus*	UK
275.86[b]	*Metarhizium brunneum*	*Cydia pomonella*	Germany

†Isolate number in the WHRI culture collection

(a) Isolate forms the active ingredient in the proprietary mycopesticide 'BotaniGard' (Mycotech Corporation, PO Box 4109, Butte, MT 59702, USA).

(b) Isolate forms the active ingredient in the proprietary mycopesticide 'Met52' (Novozymes, Hallas Allé 4400 Kalundborg. Denmark)

The spore powders were combined with either talc or Entostat at a ratio of 3:1 carrier: spores w/w and added to the central well of Roguard cockroach traps used as vine weevil refuges in PS2134 (0.4 g to each trap). Controls consisted of both unbaited refuge traps and refuge traps containing talc and Entostat only. Groups of five adult weevils were removed from the culture and then transferred to a bioassay chamber consisting of a ventilated plastic box (180mm long x 140mm high x 120 mm deep) lined with tissue paper and containing a Roguard trap as a refuge, and damp tissue paper and *Taxus baccata* (yew) leaves. The chambers were maintained at 20°C, 16:8 light: dark and high (> 90%) relative humidity and the *T. baccata* leaves and damp tissue replaced *ad libitum*. Numbers of living and dead weevils were counted daily for a total of 55 days. Dead weevils were removed and incubated on damp filter paper within Petri dishes at 23°C, and the production of fungal spores on these cadavers was recorded. The experiment was done according to a randomised block design. Each block comprised nine treatments, including three control chambers (refuge traps baited with talc, Entostat and untreated). There were three blocks in total. The analysis of variance was carried out separately for each day due to the fact that data between days may be correlated. The percentage mortality data was angular transformed to stabilize the variation prior to analysis of variance.

Results and Discussion

All of the isolates caused greater mortality of adult weevils than the controls (Table 2). Only one of the isolates (*M. brunneum* isolate 275.86) resulted in 100% mortality after 49 dpi (days post-inoculation). There was no difference in the fungal mortality for spores formulated in talc versus Entostat powder. In general, the weevils died more slowly than observed in previous experiments, with the LT_{50} (median lethal time) ranging from 30 dpi (fungal isolate *M. brunneum* isolate 275.86) to 46 dpi (fungal isolate *B. bassiana* isolate 433.99). It was observed that the weevils did not aggregate as much as in previous experiments, and this may have reduced the spread of fungal spores within the weevil population. The time taken for weevil death after infection may not be important if infection has sub-lethal effects such as reducing feeding or oviposition. Further research is justified on potential sub-lethal effects of entomopathogenic fungi on vine weevil behaviour.

Table 2. Pathogenicity (% mortality) of three isolates of entomopathogenic fungi applied as spore powders combined with talc or Entostat carriers in Roguard traps to adult vine weevil.

	Day						
Treatment	**7**	**14**	**21**	**28**	**35**	**42**	**49**
talc	0.0	0.0	13.3	13.3	13.3	13.3	20.0
275.86 + talc	0.0	0.0	16.7	33.3	83.3	100.0	100.0
433.99 + talc	6.7	13.3	20.0	26.7	26.7	56.7	56.7
1749.11 + talc	0.0	0.0	17.8	42.2	66.7	73.3	73.3
Entostat	0.0	0.0	6.7	6.7	6.7	6.7	13.3
275.86 + Entostat	0.0	0.0	31.7	45.0	85.0	93.3	100.0
433.99 + Entostat	0.0	0.0	6.7	26.7	33.3	46.7	60.0
1749.11 + Entostat	0.0	0.0	6.7	6.7	33.3	33.3	33.3
untreated	0.0	0.0	0.0	6.7	6.7	6.7	6.7

All of the fungal isolates examined produced spores on weevil cadavers (Table 3). Spore production per weevil varied between fungal isolates, but there was also a large amount of weevil-to-weevil variation.

Spore production for fungal isolate *M. brunneum* isolate 275.86 ranged from 5.5 x 10^7 to 4.7 x 10^8 spores per weevil; for isolate *B. bassiana* isolate 433.99 it ranged from 1.3 x 10^6 to 4.7 x 10^7; while for isolate *B. bassiana* isolate 1749.11 it ranged from 9.8 x 10^5 to 2.3 x 10^8 spores per weevil. *Metarhizium brunneum* 275.86 and *B. bassiana* isolate 1749.11 produced significantly ($P<0.05$) more spores per weevil than *B. bassiana* 433.99. The type of spore carrier (talc vs. Entostat) had no effect on the spore production on infected weevils. The majority of sporulation occurred between the body segments.

Table 3. Mean number of spores recovered from infected weevils in experiment 1.1.

Treatment	Mean no of spores per weevil
talc	0
275.86 + talc	2.02 x 10^8
433.99 + talc	7.07 x 10^6
1749.11 + talc	9.45 x 10^7
Entostat	0
275.86 + entostat	2.38 x 10^8
433.99 + entostat	1.74 x 10^7
1749.11 + entostat	4.77 x 10^7
untreated	0

Conclusions

- Weevils entered the Roguard traps.
- All of the fungal isolates produced spores on weevil cadavers.
- The type of carrier (talc or Entostat) had no effect on the spore production on infected weevils.
- The majority of sporulation occurred between the body segments.

Task 1.2 Transfer of fungus between weevils

Materials and methods

The transfer of fungal spores between weevils was quantified in a laboratory bioassay. Fungal spores of *M. brunneum* (275.86) were formulated in either talc or an electrostatic carrier powder (Entostat, Exosect Ltd). The formulated spore powders were prepared and used to inoculate healthy adult weevils as described previously. One inoculated weevil (hereafter referred to as a "donor weevil") was then placed in a bioassay chamber (Figure 1) consisting of a ventilated plastic box (150mm long x 125mm high x 80 mm deep) lined with dry tissue paper plus a piece of damp tissue paper (as a source of water) and *Taxus baccata* (yew) leaves (as a food source). In addition, five living, healthy weevils that had not been treated with spore powder were placed inside the same box (these are referred to as "recipient weevils"). The bioassay chambers were maintained at 20°C, 16:8 light: dark and high (> 90%) relative humidity. At 0h, 24h and 48h, weevils were removed from replicate chambers and were washed individually in 1 ml of 0.05% Triton X-100 in 1 ml microcentrifuge tubes. Tubes were vortex-mixed for approximately 20 seconds to dislodge spores from the integument of the weevils. Spores in the suspension were enumerated using an improved Neubauer haemacytometer. Spore counts were $\log_{10}$ transformed before analysis of variance (Genstat, 2010).

A)  B) 

Figure 1. Bioassay chamber (A) and *Metarhizium* inoculated weevil (B)

Results and Discussion

There was no significant difference between the number of *Metarhizium* spores received by weevils when the spores were formulated with talc or Entostat, however the dose received by weevils treated with *Metarhizium* formulated with Entostat was less variable (SEM of Log_{10} transformed data =0.0216 and 0.1265 (Entostat treated and talc treated respectively) (Figure 2). The majority of the inoculum on the donor weevils was lost after 24h with an 82% and 91% reduction (Entostat and talc treated respectively) and after 48h this increased to 91% and 94 % (Entostat and talc treated respectively) (Figure 2).

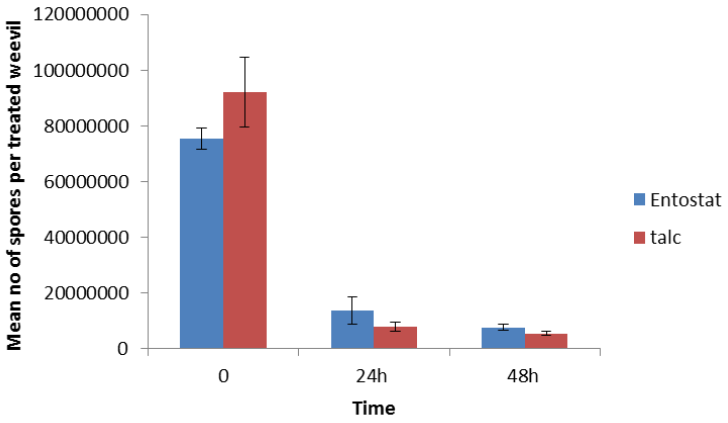

Figure 2. Mean number of spores per inoculated weevil at 0h, 24h, and 48h post inoculation.

All of the "recipient" weevils sampled in the experiment came into contact with the inoculated donor weevil within 24h and obtained *Metarhizium* spores regardless of the type of spore carrier (Figure 3). There was no significant difference between carriers after 24h or 48h. After 24h recipient weevils had received between 8 x 10^4 and 1 x 10^6 spores per weevil (Entostat formulation) and 9 x 10^4 and 4.8 x 10^5 spores per weevil (talc formulation). After 48 h recipient weevils had received between 9 x 10^4 and 4.8 x 10^5 spores per weevil (Entostat) and 8 x 10^4 and 4.5 x 10^5 spores per weevil (talc). From observing the movement of the weevils during the experiment it was clear that the spore powder was spread very quickly throughout the weevil population. Weevils were seen to have fungal spores within two hours of setting up the experiment and fungal spores were seen deposited in all parts of the bioassay chamber.

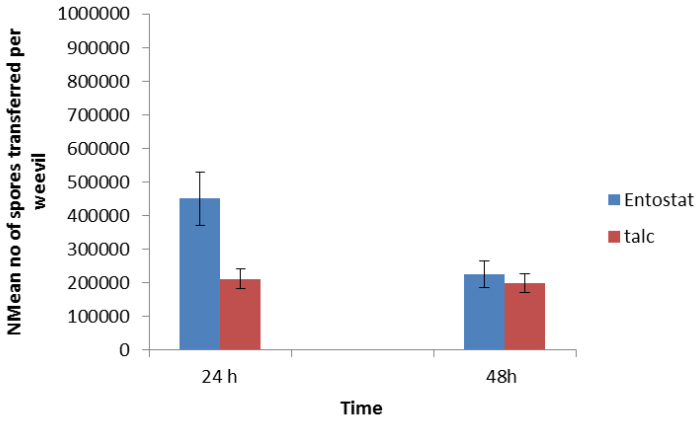

Figure 3. Mean number of spores transferred per weevil after 24h and 48h.

Conclusions

- One infected weevil was able to transfer spores to all of the weevils in the experiment within 24 hours.
- Healthy weevils received a dose ranging from 8 x 10^4 and 1 x 10^6 spores per weevil after 24h and from 8 x 10^4 and 4.5 x 10^5 spores after 48 h. Although not confirmed here, this dose, based on our knowledge of this insect and other insect species, should be a sufficient to cause death.

Objective 2. Improve current understanding of adult vine weevil movement in soft fruit and nursery stock crops (Harper Adams and ADAS)

This objective was completed as a proof of principle study testing the potential of MiniHPT8 type radio frequency identification (RFID) tags (Biomark, Idaho, USA) to record the dispersal behaviour of individually identifiable adult vine weevils within commercial strawberry and nursery stock crops. Information gained using this approach will help to inform decisions such as the density of artificial refuges required to provide effective control of this pest and the potential of infected weevils to disperse the EPF through the crop. In addition, work has been undertaken using fluorescent powders (developed in PS2134) to determine use of artificial refuges in a commercial strawberry crop.

Task 2.1. Laboratory and semi-field testing of RFID tags to mark adult vine weevil

Materials and methods

Read range of RFID tags

RFID equipment used in the study was supplied by Biomark. The tags used were HPT8 (8.4 mm 134.2 kHz FDXB) and these were read using an HRP Plus reader, fitted either with the standard HRP Plus antenna or a larger BP Portable antenna. Read range was recorded when the antenna was held directly above the tag (90°), to one side of the tag (0°) as well as a number of intermediate angles between the antenna and tag (45°, 22.5° and 11.25°). As the tags used in this study are passive, the detection range is dependent on the strength of the signal emitted by the reader. Obstacles that can be found in the cropping environment were placed between the tag and the reader to determine their effect on read range. The read range was tested when tags were covered with growing media with different levels of moisture content (0, 30 and 60% v/v) and/or covered with a polythene mulch.

The HRP Plus reader fitted with a BP Portable antenna had a longer read range than the HRP Plus reader fitted with a standard HRP Plus antenna. The relative angle between the antenna and the tag affected read range. For the BP Portable antenna read ranges were 20 cm (90°), 17 cm (45°), 10 cm (22.5°), 6 cm (11.25°) and 0 cm (0°). For the standard HRP Plus antenna read ranges were 14 cm (90°), 13 cm (45°), 9 cm (22.5°), 4 cm (11.25°) and 0 cm (0°). Placing obstacles between the antenna and tag had no recordable effect on read range at any of the angles tested.

While the BP Portable antenna offers a slightly greater read range compared to the standard HRP Plus antenna this benefit has to be balanced against the greater size and weight of the BP Portable antenna. The standard HRP Plus antenna was, therefore, selected for use in subsequent experiments as it was found to be more manoeuvrable and could be used comfortably for extended periods.

Effect of RFID tagging on survival and movement of adult vine weevils

Adult vine weevil were collected from commercial strawberry crops in Shropshire and Staffordshire in May, June and July 2013. Additional weevils were supplied by ADAS from a culture established from larvae originally collected from a commercial nursery. Adult vine weevil were maintained as previously described in project PS2134, however, the food source was strawberry leaves rather than yew (*Taxus baccata*).

An RFID tag was attached to a weevil by first restraining the weevil by gently pressing it onto a softened roll of putty (Blue-tack®). Next a small droplet of adhesive was applied to the dorsal surface of the weevil abdomen. Immediately after applying the adhesive a tag was gently pushed into the adhesive. Once the adhesive had set and the tag was securely fastened the weevil was removed from the putty and was ready for use in the experiment (Figure 4). Two types of adhesive were used in this work:

1. Thermoplastic adhesive applied using a low melt (130°C) hot glue gun (TEC250LT, Basildon, Essex, UK).
2. Cyanoacrylate adhesive (Loctite Power Easy Gel, Ohio, USA).

Figure 4. RFID tag attached to a vine weevil adult.

In preliminary work, fine nylon cord used to attach the tag so that it was pulled behind the weevil, similar to that used by Vinatier *et al*. (2010), was found to be impractical for use with adult vine weevil and was not investigated further in this study.

Survival bioassays were completed using adult vine weevil carrying an RFID tag attached using the thermoplastic adhesive or the cyanoacrylate adhesive. Untagged weevils were used as the control in this experiment. Thirty weevils were tagged, half using the thermoplastic adhesive and the other half using the cyanoacrylate adhesive. A further 15 weevils were left untagged. Each weevil was maintained individually in a ventilated Petri dish lined with damp tissue paper placed in a controlled environment chamber set to 18°C and 65% relative humidity. Each weevil was provided with a fresh 1 cm diameter disc of *Primula* leaf as a food source every 2-3 days. Survival was recorded over a period of 21 days. In addition to recording insect mortality, the number of eggs laid by each weevil was recorded together with leaf area consumed.

Movement bioassays were completed using adult vine weevil carrying an RFID tag attached using the thermoplastic adhesive or the cyanoacrylate adhesive. Untagged weevils were again used as the control in this experiment. As in the weevil survival bioassay each adhesive was used to attach RFID tags to 15 weevils while a further 15 weevils were left untagged. The movement of each weevil was recorded before the tag had been attached and 24 hours after tag attachment. To record weevil movement on a flat (horizontal) surface, weevils were placed in the centre of a well-lit arena. Individuals were placed directly underneath a light held 10 cm above a sheet of white paper in order to trigger a dispersal response away from the centre of the arena. The time taken for an individual to cover a distance of 10 cm was recorded. To record weevil movement on a vertical surface (similar to the side of a plastic plant pot), weevils were placed inside a transparent plastic tube with a 3 cm diameter. The tube was placed in a vertical position and the time taken for the weevil to climb to a height of 10 cm was recorded. For both the horizontal and vertical bioassays each weevil was recorded over three replicated runs and the mean of these taken.

Movement of RFID tagged weevils within a semi-field environment

Semi-field environments similar to those used in PS2134 were established. Large gauze ‘tent’ cages (145 x 145 x 152 cm) containing a single one-metre strawberry grow-bag were placed in a ventilated polytunnel at Harper Adams University. Ten one year old strawberry (cv. Elsanta) plants were growing in each grow-bag. Six weevils carrying an RFID tag attached using the thermoplastic adhesive were released into a 'tent' cage and allowed to acclimatise for 24 hours. The position of each weevil was then determined using the HRP Plus reader fitted with the standard HRP Plus antenna. Once a tag had been detected a careful search by hand was completed to find the weevil, confirm that the weevil was alive and that the tag was still attached. Four 'tent' cages were prepared in this way and the positions of weevils within each cage recorded on five consecutive days.

Results and Discussion

No mortality was recorded for untagged weevils or weevils carrying an RFID tag attached using the thermoplastic adhesive. Eight (53%) of the weevils carrying an RFID tag attached using the cyanoacrylate adhesive died during the course of the experiment. RFID tag attachment significantly affected weevil egg laying ($F = 6.28$, $P < 0.004$). Individual comparisons were made between the treatments using Least Significant Difference (LSD). From these individual comparisons untagged weevils were found to lay significantly more eggs (2.9 eggs/weevil/day) than weevils tagged using the thermoplastic adhesive (1.2 eggs/weevil/day) or weevils tagged using the cyanoacrylate adhesive (0.3 eggs/weevil/day). Tagged weevils did not differ significantly in the number of eggs laid. Although survival and egg laying of weevils carrying an RFID tag attached using the cyanoacrylate adhesive was reduced, these individuals consumed more leaf material (90-99% of leaf offered). In comparison, untagged weevils consumed 80-89% of leaf offered while weevils tagged using the thermoplastic adhesive consumed 70-79% of leaf offered.

Mean horizontal speed of movement did not differ significantly ($F = 0.56$, $P =$ n.s.) between each group of weevils before RFID tags had been attached (means of 1.44-1.59 cm/s). Attaching RFID tags to weevils significantly ($F = 74.37$, $P < 0.001$) affected horizontal speed of movement. Individual contrasts using LSD found that the speed of movement of untagged weevils (1.83 cm/s) was significantly faster than weevils tagged using the thermoplastic adhesive (1.01 cm/s). Weevils tagged using the thermoplastic adhesive were in turn significantly faster than weevils tagged using the cyanoacrylate adhesive (0.29 cm/s). Mean vertical speed of movement did not differ significantly ($F = 0.25$, $P =$ n.s.) between each group of weevils before RFID tags had been attached (means of 0.45-0.46 cm/s). After attaching the RFID tags using the cyanoacrylate adhesive none of the weevils were able to climb up the walls of the tube. Of the 15 weevils tagged using the thermoplastic adhesive eight were still able to climb the vertical surface. However, speed of movement was significantly ($t = 5.11$, $P < 0.001$) slower (0.18 cm/s) than untagged weevils (0.37 cm/s).

These results indicate that attaching RFID tags to adult vine weevils using the thermoplastic adhesive does not affect weevil mortality. However, the use of the cyanoacrylate adhesive resulted in a marked reduction in weevil survival. Similarly, the thermoplastic adhesive had a reduced effect on speed of movement on horizontal surfaces compared to the cyanoacrylate adhesive. Speed of movement on vertical surfaces was significantly slower when weevils were tagged using the thermoplastic adhesive but still possible. The number of eggs laid by weevils tagged using the thermoplastic adhesive was significantly reduced compared to untagged weevils. While attaching RFID tags to weevils using the thermoplastic adhesive affected some aspects of weevil behaviour these affects were much less than when tags were attached using the cyanoacrylate adhesive. Based on these results the thermoplastic adhesive was used in subsequent experiments.

Movement of RFID tagged weevils within a semi-field environment

Detection rates of released weevils using the HRP Plus reader were high on each day; Day 1 = 96%, Day 2 = 96%, Day 3 = 71%, Day 4 = 75 and Day 5 = 75%. Of the weevils detected 57% were found in the crown of the plant, 12% under the grow-bag, 15% within the leaf litter around the plants, while the remaining weevils were detected using the HRP Plus reader but their position could not be confirmed through searching by hand non-destructively. All of the weevils relocated were alive and had their tag securely attached.

Conclusions

- Thermoplastic adhesive was an appropriate adhesive to attach the tags to the adult weevils.
- Thermoplastic adhesive does not affect weevil survival but does reduce the speed of movement on horizontal and vertical surfaces. The number of eggs laid was affected by tagging. Effects of the thermoplastic adhesive were, however, much less than when tags were attached using the cyanoacrylate adhesive.
- The HRP Plus reader detected 71-96% of the tagged weevils in a semi-field environment on five assessment dates.

Task 2.2. Record movement of adult vine weevils within commercial soft fruit and/or nursery stock sites

2.2.1. Record movement of adult vine weevils within a commercial soft fruit site at Harper Adams University using the RFID tagging system

Materials and methods

A ‘commercial’ strawberry crop was established at Harper Adams University, Shropshire. Strawberry plants

(cv. Elsanta) were grown in one metre grow-bags, 10 plants in each bag. A total of 76 grow-bags were arranged in four rows of 19 on a woven geotextile ground-cover membrane. The crop was grown outdoors. The distance between each row was one metre (measured from the centre of each row). Weevil movement within the strawberry crop was recorded during July and August 2013. A total of 40 tagged weevils were released during the day into the strawberry crop on 3 July. Weevils were released after scanning the attached tags to record their unique identification numbers before placing single weevils in the centre of a strawberry grow-bag. Alternate bags in each row were infested with a single weevil in this way. Twenty-four hours after releasing the weevils, the position of each weevil within the strawberry crop was determined using the HRP Plus reader with the standard HRP Plus antenna. Each grow-bag was scanned along both sides as well as over the top of the bag (Figure 5). In addition, the area between each row of bags was scanned as well as the area immediately around the strawberry crop (5 m) for the presence of tagged weevils. After recording the position of the tagged weevils on 4 July the position of weevils was subsequently recorded on 5, 6, 7, 8, 10, 12, 16, 20 and 28 July as well as 7 August.

Figure 5. Detecting RFID tagged weevils within a commercial strawberry crop at Harper Adams University.

Results and Discussion

The positions of RFID tagged weevils were successfully recorded. The numbers of weevils detected on each recording date remained high throughout the experiment. The number of weevils detected was highest one day after release (73%) and declined to 28% 35 days (7 August) after release. If a weevil was not recorded to have moved on three consecutive assessments a hand search was completed in order to confirm that the weevil was alive and that the tag was securely attached. In the final assessment completed on 7 August, all tags were collected, confirming whether the weevil was alive and the tag was still attached. This confirmed that 11 of the 40 tagged weevils released into the crop were healthy, still had the tag attached and had remained within the release site. Eight of these weevils were found within the crown of the strawberry plant. The other weevils were recovered from within or under a grow-bag. Four other tagged weevils were recovered but had died during the course of the experiment. Nine tags, which had become detached from the weevil, were also recovered. Throughout this experiment, no weevils were found between the rows of strawberry grow-bags or in the area immediately around the crop. Despite this 16 (40%) of the released weevils had left the crop area, indicating possible long range dispersal or effects of predation.

Results from PS2134 indicated that weevils take at least seven days to die after coming into contact with EPF spores. In this experiment, seven days after releasing the weevils the positions of 21 weevils were recorded. Of these individuals only two had not moved from their release points. Of those weevils that had moved, 12 remained in the same row in which they were released while seven weevils had moved between rows. The distance moved by a weevil along a row of strawberry grow-bags ranged between 0.3 m and 4.3 m with an average distance moved of 1.44 m.

Conclusions

- The number of weevils detected was highest one day after release (73%) and declined to 28% 35 days (7 August) after release.
- After 35 days 11 of the 40 tagged weevils released into the crop were healthy, still had the tag attached and had remained within the release site, four weevils had died, nine tags had become detached from the weevil and 16 (40%) of the released weevils had left the crop area, indicating possible long range dispersal or effects of predation.
- The distance moved by a weevil along a row of strawberry grow-bags ranged between 0.3 m and 4.3 m with an average distance moved of 1.44m.

2.2.2 Record movement of adult vine weevils within a commercial nursery stock site using the RFID tagging system

Materials and methods

The movement of adult vine weevils was monitored using the RFID tagging system at Darby Nursery Stock, Methwold Hythe, Thetford, Norfolk.

An ornamental crop of potted *Euonymus* plants (cv. Silver Queen) with a pre-existing vine weevil infestation was selected. Within the crop, 400 plants (in 2 l pots) were marked out into a grid (3.62 m x 3.62 m) consisting of 25 squares (20 x 20 plants). Each square was made up of 16 plants (4 x 4 plants), where the distance between each pot was approximately 2 cm and the diameter of each pot was 16.3 cm. A refuge trap was placed on the sand bed on which the pots were stood in the centre of each square (density of 1.91 traps per m^2) to monitor whether they were used as a refuge by the weevils in a commercial nursery. Twenty-five refuge traps were also distributed in an adjacent *Euonymus* crop in pots of the same size for additional monitoring.

On 27 August 2013, 25 weevils were collected from within the *Euonymus* crop and RFID tags were attached using a thermoplastic adhesive applied using a low melt hot glue gun. One tagged weevil was then released in each of the 25 squares of the grid. Weevils were released by placing a single weevil on the compost surface in each pot then lightly covering with more compost. The release point of each weevil on the grid and the unique identification number of each tag was recorded.

Weevil movement in the *Euonymus* plants was recorded on day three, seven and 14. Their position was recorded by carefully scanning the crop area using the HRP Plus reader to detect the tags (Figure 6). Once the tag had been detected attempts were made to locate the weevil or the tag (if it had become detached from the weevil). When tags were found without a weevil (detached) the tag was attached to a new weevil and released from the original release point used for that tag. The refuge traps placed in each grid were also checked on each assessment date and the number of weevils present were recorded.

Figure 6. Using the HRP Plus reader to detect the tags in a commercial *Euonymus* crop.

Results and Discussion

On the first assessment on day three (29 August), 15 (60%) of the 25 tags were detected. Of the 15 tags detected, eight were live tagged weevils, one was a dead tagged weevil and six were tags that had become detached from the weevils. One tagged weevil was found on the side of a pot, three were under the pots,

one had entered a refuge trap, two were within the plant foliage and one was within the compost in the pot. The average distance moved by the eight tagged weevils by day three was 0.26 m.

On the second assessment on day seven (3 September), 15 (60%) tags were detected. Of the 15 tags detected, six were live tagged weevils, eight were tags that had become detached from the weevils and one tag could not be found to determine whether it was a tagged weevil or a detached tag. Three tagged weevils were found under the plant pots, one was within the compost in the pot, one was trying to enter a refuge trap but was restricted by the tag and one was on the sand bed between the pots. The average distance moved by the six tagged weevils on day seven was 1.09 m (including data for weevils which were not detected on the first assessment and therefore had less movement data available). This average distance moved was less than the average 1.44 m moved over a seven day period in the strawberry crop at Harper Adams University.

On the third assessment on day 14 (10 September), 14 (56%) tags were detected. Of the 14 tags detected, seven were live tagged weevils, five were tags that had become detached from the weevil and two could not be found. Four weevils were found within the compost in the pots, one was within the plant foliage and two were on the sand bed between the pots. The average distance moved by the tagged weevils (including data for weevils which had not been detected on previous assessments and excluding data for a weevil which had previously been re-released) on day 14 was 1.87 m.

Only five of the 25 original weevils were detected on every assessment date. The distance moved by these five weevils over the 14-day period ranged between 0.93 m and 2.67 m, with the average distance being 1.96 m. Three days following weevil release the average distance of movement was 0.27 m and by day seven they had moved on average 0.99 m. The longest distance travelled by a single weevil was 3.48 m and this weevil was only detected on the last assessment date, so may have moved out of the experimental area on previous assessment dates. Overall nine individual tagged weevils were detected during the 14-day period showing that the same weevil was detected on more than one assessment date. All of the weevils detected with tags had moved from their original release point.

On each assessment date the refuge traps placed in the adjacent *Euonymus* crop (1.91 traps per m^2) were also checked for untagged resident weevils (i.e. a natural infestation). On the first assessment date a weevil was found in each of two traps. On the second assessment date one weevil was found in a trap and on the final assessment date no weevils were found in traps. Tagged weevils from the adjacent experimental crop were not detected on any assessment date.

Conclusions

- Between 56 and 60% of the RFID tags were detected on each assessment. Between 40% and 53% of these were live weevils with their tags still attached.
- Only five of the 25 original weevils were detected on every assessment date. The distance moved by these five weevils over the 14-day period ranged between 0.93 m and 2.67 m, with the average distance being 1.96 m.
- The longest distance travelled by a single weevil was 3.48 m and this weevil was only detected on the last assessment date, so may have moved out of the experimental area on previous assessment dates.
- All of the weevils detected with tags had moved from their original release point.
- A small number of untagged weevils were found in the refuge traps.

2.2.3 Record the movement of adult vine weevils and the use of artificial refuges using fluorescent powder within a commercial soft fruit site

Materials and methods

The movement of adult vine weevils (in a natural infestation) and their use of artificial refuges was recorded during 2013 in a commercial strawberry crop (cv. Fenella planted March 2010) at H&H Duncalfe, Maltmas Farm, Wisbech, Cambridge.

The trial consisted of four treatments (Table 4) replicated five times. Twenty plots measuring 2 x 2 m^2 were marked out representing each treatment. Each plot contained two beds of strawberries with an alleyway in between. There were approximately 12 strawberry plants in each bed section and the beds were covered in black polythene.

Table 4. Treatments.

Trap density	Total number of traps	Location	Powder colour
6 per m^2	24 per plot	8 in alley way, 8 in each bed	Pink
3.75 per m^2	15 per plot	5 in alleyway, 5 in each bed	Orange
2.25 per m^2	9 per plot	3 in alleyway, 3 in each bed	Yellow
0.75 per m^2	3 per plot	1 in alley way, 1 in each bed	Green

On 6 August, 0.2 g of powder was added to the central well of each Roguard trap used as an artificial refuge and a piece of fluorescent sticky tape corresponding to the powder colour was added to the lid of each refuge trap so they could be identified easily in the field without the need to open the refuge trap. Refuge traps were placed in the alleyways and beds as per Table 4. Refuge traps in the bed were placed partly under the plastic in the planting hole to shelter them from the wind.

After two, nine and 15 days, UV torches were used after dusk to check for coloured weevil tracks in each plot. The colour observed in each plot was recorded. UV torches and normal torches were then used to look for weevils with and without coloured powder. The number of weevils was recorded along with a record of any colour markings and their location (leaves, soil, polythene, and alleyway). On the final assessment, all the traps were collected and brought back to ADAS Boxworth where the number of weevils present in each plot was recorded.

Results and Discussion

Fluorescent weevil tracks were identified in the beds of each plot on every assessment day, but tracks were not always observed in the alleyways. On the first assessment, 10 plots contained other colours in addition to the powder colour in the trap within that plot. On the second and third assessment date, additional colours were found in seven and nine of the plots respectively.

On the first assessment date two days after set-up a total of 178 weevils were recorded; 170 were on the leaves and eight were on the polythene. Of these 178 weevils, 31 (17%) were detected with fluorescent powder on their body confirming that they had entered an artificial refuge or come into contact with another weevil which had. Of these 31 fluorescent powder-coated weevils, 12 were pink (24 traps per plot), eight were orange (15 traps per plot), eight were yellow (9 traps per plot) and three were green (3 traps per plot). Eight weevils (26%) were coated in a fluorescent powder which was a different colour to the plot they were found in, confirming they had moved between plots or had come into contact with another weevil from a different coloured plot. There were no significant differences between the total number of weevils ($F = 0.28$, P = n.s.) or number of coloured weevils between treatments ($F = 1.62$, P = n.s).

On the second assessment date a total of 54 weevils were recorded; 31 were on the leaves and 23 were on the polythene. Of these 54 weevils, six (11%) were detected with fluorescent powder on their body with three having orange powder, one pink, one green and one yellow. Two weevils out of the six had coloured powder which was a different colour to the plot they were found in. There were no significant differences between the total number of weevils ($F = 1.76$, P = n.s) or number of coloured weevils between treatments ($F = 1.21$, P =n.s).

On the final assessment date a total of 13 weevils were counted with nine on the leaves and four on the polythene. No fluorescent powder was detected on the weevils. When the traps were collected and brought back to ADAS, Boxworth, no weevils were found inside. There were no significant differences between treatments in the total number of weevils ($F = 0.87$, P = n.s.). Numbers of marked and unmarked weevils declined over the 15-day recording period and this was thought to be due to the rapidly senescing crop leading to weevil emigration from the experimental plots. Although only 17% and 11% of the weevils were detected with fluorescent powder on their bodies two and nine days after adding the refuge traps respectively, higher levels of pick-up may have been recorded over a longer period if the traps had been placed in the crop earlier in the season before the crop started to senesce. Continuous exposure of insects to entomopathogenic fungi over long periods is unlikely to encourage the development of resistance to the fungi in the same way as occurs with chemical pesticides. When 15 successive generations of *Drosophila melanogaster* were exposed to infection with *Beauveria bassiana*, flies from selected lines did not live longer than control lines (Kraaijeveld & Godfray, 2008). However there was evidence of increased fecundity

in selected lines which may indicate the development of tolerance of fungal infection.

Conclusions

- A natural population of vine weevil adults used artificial refuge traps in a commercial strawberry crop and movements of naturally-occurring vine weevils could be recorded using fluorescent powder placed in the refuge traps.
- Two days after adding the powder to the refuges traps, 17% of weevils recorded were marked with fluorescent powder and 16% of these had moved from one plot to another or had come into contact with a powder coated weevil from another plot.
- On each assessment date similar numbers of weevils were marked with fluorescent powder when refuge trap densities of 0.75-6 per m^2 were used.

Objective 3 Test the efficacy of the best performing EPF formulation(s) under semi-field conditions that mimic soft fruit and nursery stock crops

This objective was completed to bring together results from Objectives 1 and 2 in order to test the efficacy of an EPF spore formulation to control adult vine weevil. Experiments completed as part of Objective 3 tested the efficacy of the EPF under semi-field conditions simulating those of soft fruit and nursery stock crops.

Task 3.1 Under semi-field conditions test the efficacy of the most effective EPF spore formulation to control adult vine weevil

This experiment was planned to be done in summer 2014 but owing to the shortage of adult vine weevils despite several nocturnal collection trips to known infested commercial soft fruit and ornamental crops, it had to be delayed until autumn 2014. Another problem was that some of the weevils collected and maintained in culture at ADAS were subsequently found to be infected with entomopathogenic fungi, which depleted weevil numbers further, therefore lower numbers of weevils than planned were available for the experiment. This infection likely came from the weevils coming into contact with Naturalis or Met52 in the nursery from which they were collected or in the lab. The experiment was done in experimental glasshouses instead of a polythene tunnel as originally planned, in order to maintain suitable temperatures for the fungi to develop. However, the glasshouses at ADAS all have to run at the same temperature regime and due to constraints imposed by other experiments in adjacent glasshouses, ideal temperatures could not be maintained throughout experiment, thus at the end of the glasshouse experiment, the weevils were incubated in the laboratory to assess any fungal infection.

Large gauze 'tent' cages (1.45 m x 1.45 m) were prepared to simulate a soft fruit crop. Each cage contained two grow bags, each with four strawberry plants (cv. Calypso). The experiment had an untreated control and two fungal treatments (Table 5) and five replicates of each treatment. *Metarhizium brunneum* (isolate 275.86) and *B. bassiana* (isolate 433.99) were selected for the experiment as they were the best performing fungi in Objective 1, Task 1.1. The experiment was carried out in five glasshouse compartments at ADAS Boxworth, with one cage of each treatment in each compartment (three cages per compartment). Roguard traps used as artificial refuges were filled with 0.4 g of the appropriate treatment on 13 November (Figure 7). Fungal spores were cultured by Warwick Crop Centre and sent to ADAS where the appropriate amount of spores and fluorescent powder+talcum powder (mixed) were weighed, combined and shaken together to mix. Once the fungus and the powder had been mixed, 0.1 g was added to a universal tube containing Triton and sent to the University of Warwick to determine the spore count. Warwick Crop centre confirmed that the fluorescent powder did not affect the spores. The glasshouses were set to maintain a temperature of 25°C where possible but this often had to be set at a lower temperature due to other researchers' requirements in adjacent glasshouses. A datalogger was placed in a cage in two of the compartments to monitor the temperatures.

On 14 November, six refuge traps of the appropriate treatment were added to each cage. Two refuge traps were placed on the floor between the grow bags and two were placed on each grow bag (Figure 8). Adult weevils were taken from the ADAS culture and 12 weevils were placed into each of 15 plastic boxes. One box was then opened in the centre of each cage and the weevils were left to leave the container (the containers were removed after two days when all the weevils had left).

After two months (13-16 January 2015), the number of dead and alive adult weevils in each cage was assessed. The cage floor, cage roof, plants, refuge traps, under grow bags, around roots and in the surrounding substrate were searched for weevils. When a weevil was found a note was made of whether it had orange fluorescent powder on its body (indicating it had entered a refuge trap or had come into contact with another weevil which had), whether it was clearly infected with fungus (white or grey-green

hyphae/spores), whether it was alive or dead and finally where it was found.

Any live and dead adult weevils recovered from the cages were kept individually on damp filter paper in sealed Petri dishes containing yew leaves as food and incubated for two weeks at 22°C and a further two weeks at ambient temperature in the laboratory to check for any additional mortality through development of a pathogen and for evidence of sporulation. After the incubation period, any dead adult weevils clearly infected with fungus were placed individually in universal tubes of Triton and sent to Warwick Crop Centre to confirm the species of fungus responsible for the infection. A refuge trap from each treatment was also sent to Warwick Crop Centre to determine whether the spores in the refuge traps were still viable after two months.

Table 5. Treatments.

Treatment number	Treatment	Amount of talc and fluorescent powder mix (50:50) per trap (g)	Amount of fungus per trap (g)	Total powder per trap (g)
1	Untreated	0.4	0	0.4
2	*Metarhizium brunneum* (isolate 275.86) 1 x 10^8	0.1	0.3	0.4
3	*Beauveria bassiana* (isolate 433.99) 1 x 10^8	0.033	0.367	0.4

Figure 7. Refuge traps containing 0.4 g of powder in each central well.

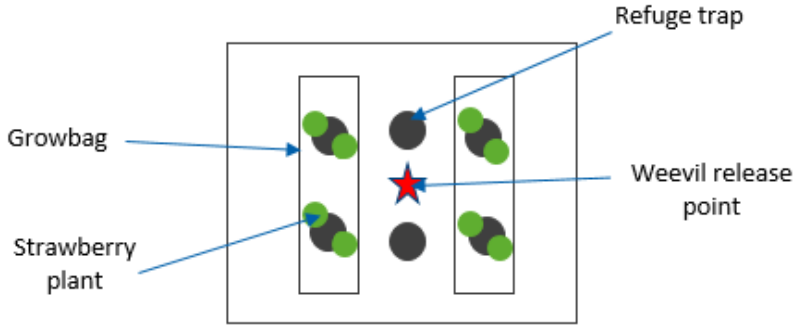

Figure 8. Layout of the grow bags and strawberry plants used to simulate a strawberry crop and the position of the refuge traps.

Results and Discussion

Total weevils recovered from the cages

A total of 60 weevils (12 in each of five replicate cages) were released for each treatment and a total of 25 (41.7%), 30 (50%) and 18 (30%) of these 60 weevils were recovered in the untreated, *M. brunneum* and *B. bassiana* treatments, respectively (Table 6). The number of weevils recovered from each cage ranged between one and nine. In the untreated cages, five of the 60 released (8.3 %) were found dead, 20 (33.3%)

were found alive and 35 (58.3%) were missing. In the *M. brunneum* treatment, 16 of the 60 released (26.7%) were found dead, 14 (23.3%) were found alive and 30 (50%) were missing. In the *B. bassiana* treatment, three of the 60 released (5%) were found dead, 15 (25%) were found alive and 42 (70%) were missing (Table 6).

Weevils were found on the floor of the cage, under the grow bags, within the substrate, on the surface of substrate within the grow bag, on or near to the strawberry plant, in the refuge traps and around the roots of the plants. The weevils which were not recovered may have been missed during the assessment or may have died and disintegrated during the experiment.

Infection status of weevils recovered from the cages

None of the weevils found at the end of the glasshouse phase of the experiment had obvious signs of mycosis in any of the treatments. Temperatures in the glasshouses remained above 15°C for the first two weeks of the experiment (Figure 10) but lower temperatures following this period are likely to have reduced the effect of the entomopathogenic fungi (spores will remain viable but infectivity may be reduced). In addition, as infection is dose-dependent it is possible that some weevils picked up only a small number of spores, which would increase the time needed for infection to develop. Therefore, all the weevils found at the end of the glasshouse experiment were incubated at warmer temperatures in the laboratory for four weeks, during which time obvious signs of mycosis subsequently developed.

The results of the infection assessments following incubation are summarised below. Statistical analysis of the data was not appropriate due to the high numbers of missing weevils.

Infected weevils in untreated cages

In the untreated cages 32% (eight) of the 25 weevils found had orange fluorescent powder on their body indicating they had entered a refuge trap or had come into contact with another weevil which had. One weevil (4% of those recovered) was found in a refuge trap during the assessment.

Following incubation of the 25 weevils found from the untreated cages, 16 (64%) were still alive and healthy, three (12%) were dead and infected by *B. bassiana*, two (8%) were dead and infected by a mixture of *M. brunneum* and *B. bassiana*, three (12%) were dead and infected by saprobes and one (4%) weevil was dead with no evidence of mycosis (Table 7, Figure 9). The presence of *B. bassiana* and *M. brunneum* in the untreated cages is likely to be due to the weevils having coming into contact with these fungi either on the nursery from which they were collected or in the laboratory culture which subsequently became contaminated with fungi.

Infected weevils in cages treated with Metarhizium brunneum

In the *M. brunneum* treatment, 12 (40%) of the 30 weevils recovered had orange fluorescent powder on their bodies indicating they had entered a refuge trap or come into contact with another weevil which had. Six (20%) adult weevils were found in the refuge traps during the assessment.

Following incubation of the 30 weevils found from the *M. brunneum* treatment; four (13.3%) were still alive and healthy, one (3.3%) was dead and infected by *B. bassiana*, 18 (60%) were dead and infected by *M. brunneum* and three (10%) were dead and infected by a mixture of *M. brunneum* and *B. bassiana*, (Table 7, Figure 9). In addition, two weevils (6.7% of those recovered) were dead and infected by secondary saprophytic fungi (saprobes) and two weevils (6.7%) were dead with no evidence of mycosis (Table 7). As in the untreated cages, the presence of *B. bassiana* in the cages treated with *M. brunneum* is likely to be due to the weevils having to come into contact with this fungus either on the nursery from which they were collected or in the subsequent laboratory culture.

Infected weevils in cages treated with Beauveria bassiana

In the cages treated with *B. bassiana*, four (22.2%) of the 18 weevils recovered had orange fluorescent powder on their body indicating they had entered a refuge trap or come into contact with another weevil which had. One weevil (5.6%of those recovered) was found in a refuge trap during the assessment.

Following incubation of the 18 weevils recovered, 10 (55.6%) were still alive and healthy, four (22.2%) were dead and infected by *B. bassiana*, and four (22.2%) were dead and infected by saprobes (Table 7, Figure 9)

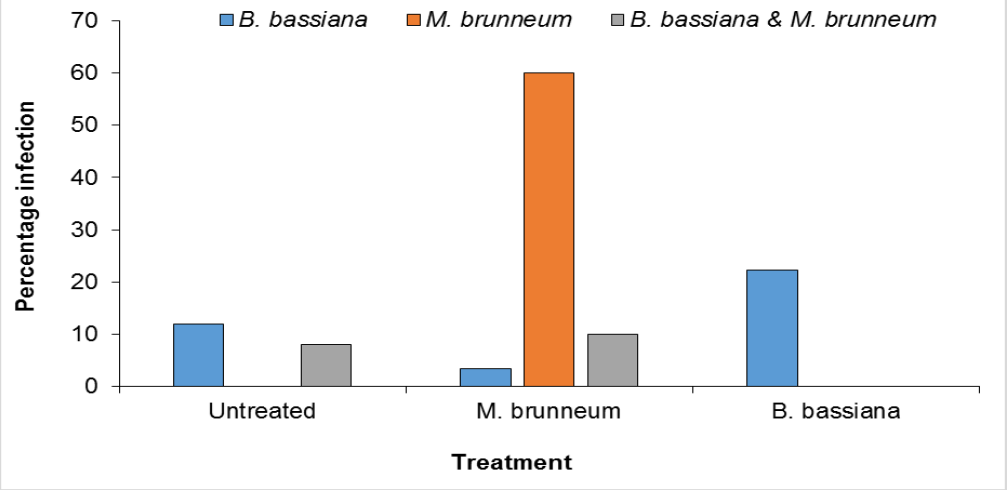

Figure 9. Percentage infection by *B. bassiana, M. brunneum* or are mixture of the two in the untreated, *M. brunneum* and *B. bassiana* treatments.

Table 6. Numbers of live, dead and missing weevils recovered out of the 60 weevils released per treatment.		**Total weevils found out of 60 (range per replicate cage)**	**Numbers of live weevils found out of 60**	**Numbers of dead weevils found out of 60**	**Numbers of missing weevils out of 60**
Untreated	**Number (Range)**	25 (1-9)	20 (1-7)	5 (0-2)	35 (3-11)
	% of 60	41.7%	33.3%	8.3%	58.3%
***M. brunneum* (isolate 275.86)**	**Number (Range)**	30 (3-9)	14 (1-5)	16 (2-4)	30 (3-9)
	% of 60	50%	23.3%	26.7%	50%
***B. bassiana* (isolate 433.99)**	**Number (Range)**	18 (1-7)	15 (1-5)	3 (0-2)	42 (5-11)
	% of 60	30%	25%	5%	70%

Table 7. Numbers and percentage of live, dead and infected weevils (of the weevils recovered per treatment) following incubation

		Alive after incubation	Dead and infected with *B. bassiana*	Dead and infected with *M. brunneum*	Dead and infected with *B. bassiana* and *M. brunneum*	Dead and infected with saprobes	Dead with no evidence of infection
Untreated	**Number**	16	3	0	2	3	1
	%	64	12	0	8	12	4
***M. brunneum* (isolate 275.86)**	**Number**	4	1	18	3	2	2
	%	13.3	3.3	60	10	6.7	6.7
***B. bassiana* (isolate 433.99)**	**Number**	10	4	0	0	4	0
	%	55.6	22.2	0	0	22.2	0

Glasshouse temperatures

The temperatures recorded in the glasshouses between 14 November 2014 and 15 January 2015 are shown in Figure 10. During the experiment period the mean daily temperatures ranged between 11.3 and 16.9°C. The maximum daily temperatures ranged between 13 and 22.5°C on 15 January and 17 November respectively. The minimum daily temperatures ranged between 8.5 and 15.8°C on 14 January and 28 November respectively. Mean temperatures remained above 15°C for most of the first two weeks of the experiment but low temperatures following this period are likely to have reduced the effect of both entomopathogenic fungi. The minimum temperature for *M. brunneum* activity is 15°C (shown as the dashed line in Figure 10). The lower than optimum temperatures during the glasshouse experiment explain why no obviously infected weevils were observed at the end of the glasshouse phase of the experiment and why subsequent incubation in the laboratory was required to allow sporulation.

Figure 10. Mean, maximum and minimum temperatures in the glasshouse compartments. The dashed line at 15°C is the minimum temperature for *M. brunneum* activity.

Numbers of fungal spores in powder used to infect weevils

When the spores were mixed with the fluorescent powder and talc at the start of the experiment it was confirmed that the powder with *M. brunneum* contained 8.78 x10^7 spores and the powder with *B. bassiana* contained 1.37 x 10^7 spores. The spore rates for *B. bassiana* were lower than expected and this may have

been due to the uneven distribution within the powder due to the clumping nature of this isolate. This may have affected the infection rates recorded with *B. bassiana* in this experiment.

The spores were tested for germination at the beginning and end of the experiment. At the start of the experiment mean germination of the spore powder was higher than at the end of the experiment for both isolates (Table 8). By the end of the experiment spore germination rate had decreased to 48% with *B. bassiana* and 61.4% with *M. brunneum*. The results at the end of the experiment may have been an underestimate as some of the powder had emptied out of the traps and stuck to the plastic bag during transport to Warwick Crop Centre.

Table 8. Mean percentage germination of spores in powder at the start and end of the experiment.

	Percentage mean spore germination	
	Start	**End**
M. brunneum (isolate 275.86)	91	61.4
B. bassiana (isolate 433.99)	99	48

Conclusions

- Adult weevils entered the refuge traps or came into contact with weevils that had. This was demonstrated by the 32%, 40% and 22% of the weevils recovered from untreated, *M. brunneum* and *B. bassiana*-treated cages having orange powder on their bodies.
- Some weevils died of natural causes and were infected by saprobes
- The presence of weevils infected with both entomopathogenic fungi in the untreated controls made interpretation of the results more difficult.
- Percentage weevil infection with *B. bassiana* was only slightly higher than that observed in the untreated controls, therefore, it could not be confirmed that the source of the fungus was from the refuge traps or from the contaminated culture.
- The higher percentage of weevil infection with *M. brunneum* (60%) in the *M. brunneum* treatment compared with in the untreated controls (0% by *M. brunneum* only and 8% by a mixture of *B. bassiana* and *M. brunneum*) indicated that most of the weevils became infected by entering the treated refuge traps or coming into a contact with a weevil that had.
- Percentage live weevils was lower in the *M. brunneum* treatment (13.3%) than in the untreated (64%) and the *B. bassiana* treatment (55.6%).
- Both fungal spore powders were still viable at the end of the 2-month period in the glasshouse, although percentage germination had decreased over time. This indicates that if the experiment had been continued for a longer period, further weevils may have become infected.
- The experiment indicated that using refuge traps to disseminate entomopathogenic fungi for the control of adult vine weevil is a promising strategy using *M. brunneum* (isolate 275.86) but not using *B. bassiana* (isolate 433.99).

Task 3.2: Under semi-field conditions, test the transfer of the EPF between weevils

Materials and methods

Small mesh cages (0.5 m x 0.5 m x 0.5 m) with the floor of the cages lined with capillary matting were prepared. One potted strawberry plant and an empty refuge trap (to test if it was used by the adult weevils) were placed on the floor of each cage (Figure 11).

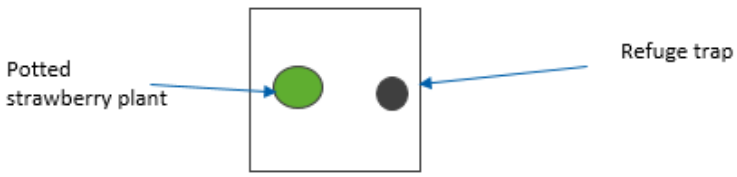

Figure 11. Layout of the potted strawberry plant and refuge trap in each small cage.

There were two treatments (Table 9) and five replicate cages per treatment. The experiment was carried out in the same five glasshouse compartments as Task 3.1 (at the same time) with one of each treatment in each compartment (two cages per glasshouse compartment).

Table 9. Treatments.

Treatment number	Treatment
1	Untreated
2	*Metarhizium brunneum* (isolate 275.86) 1 x 10^8

In each cage, five healthy weevils were placed onto each plant. In the untreated cages one additional weevil (the 'donor weevil') was added, which had been marked with white clay paint and covered in yellow fluorescent powder (Figure 12). The powder was added to the marked weevils by placing the weevils in a universal tube containing 0.4 g of the fluorescent powder and gently rolling them for 15 seconds.

Figure 12. Adult weevils covered in yellow fluorescent powder.

In the treated cages one additional weevil ('donor weevil') was added, which had been marked with clay paint and covered in yellow fluorescent powder mixed with *M. brunneum* (1 x 10^8 spores). To infect the adult weevils with *M. brunneum*, the donor weevils were placed into a universal tube containing 0.4 g of spore + yellow fluorescent powder mixture (0.1 g spore powder and 0.3 g fluorescent powder) and gently rolled for 15 seconds. An additional three weevils were also infected in this manner and sent to Warwick Crop Centre in universal tubes of Triton in order to calculate the spore dose.

After two months, the numbers of dead and live adult weevils in each cage were recorded, followed by laboratory incubation for four weeks to allow fungal sporulation and identification of entomopathogenic fungi, using the same methods as in Task 3.1.

Results and Discussion

Total weevils recovered from cages

Of the 30 weevils released (six in each of the five replicate cages for each treatment), all 30 were recovered in the untreated cages, with 16 (53.3%) being alive and 14 (46.7%) dead (Table 10). The weevils were found on the substrate of the strawberry plant, the leaves, under the capillary matting, in the refuge traps, the side of the pot, the wall or floor of the cage.

In the cages treated with *M. brunneum*, 21 (70%) were recovered, with the numbers found per cage ranging between 0 and six (Table 10). In the *M. brunneum*-treated cages, 11 (36.7%) were found alive, 10 (33.3%) were dead and nine (30%) were missing. In one of the five cages treated with *M. brunneum*, all six weevils were missing and these are likely to have escaped from the cage as a hole was found in the mesh, which was likely to have been caused by mice. The remaining three missing weevils in the other four cages treated with *M. brunneum* could have been missed during the assessment or died and disintegrated during the experiment.

Only a small number of weevils were found with obvious signs of mycosis during the glasshouse phase of the experiment in both treatments. Temperatures in the glasshouses remained above 15°C for the first two weeks of the experiment (Figure 9) but low temperatures following this period are likely to have reduced the effect of the entomopathogenic fungi (spores will remain viable but infectivity may be reduced). In addition, as infection is dose-dependent it is possible that some weevils only picked up a small number of spores, which would increase the time needed for infection to develop.

The results of the infection assessments following laboratory incubation to allow fungal sporulation and

mycosis are summarised below.

Infected weevils in untreated cages

In this experiment, one weevil was released into each cage after being covered with yellow fluorescent powder and marked with white clay paint. In the untreated cages, only three weevils were recovered with fluorescent powder on their bodies, two from one cage and one from another. Two of these yellow weevils were marked with white paint (from separate cages) and were thus 'donor' weevils which had been pre-exposed to the powder. One yellow weevil was not a donor weevil, so must have come into contact with a 'donor' weevil. As three of the five 'donor weevils' did not have fluorescent powder on their bodies when recovered at the end of the experiment, this suggests that the powder is lost over time and thus is not a reliable indicator of powder transfer over extended periods.

Following incubation of the 30 weevils found in the untreated cages, 11 (36.7%) were alive and healthy, one (3.3%) was dead and infected with *M. brunneum*, nine (30%) were dead and infected by *B. bassiana*, one (3.3%) was dead and infected by a mixture of *M. brunneum* and *B. bassiana*, four (13.3%) were dead and infected by saprobes (saprophytic fungi) and four (13.3%) had died with no obvious signs of mycosis (Table 11, Figure 13). It should be noted that lack of mycosis does not necessarily mean that the weevil did not die from fungal infection but rather the conditions may not have been suitable for sporulation after death. The presence of both entomopathogenic fungi in the untreated cages is likely to be due to the weevils having come into contact with these fungi on the commercial nursery from which they were collected or in the subsequent laboratory culture.

Infected weevils in cages treated with M. brunneum

In the cages treated with *M. brunneum* one 'donor' weevil had been released per cage, which had been exposed to *M. brunneum* spores mixed in a yellow fluorescent powder. Only three of the five marked 'donor weevils' were found and all three had traces of yellow florescent powder. Only one additional weevil which was not marked at the start of the experiment was found with fluorescent powder, suggesting it had come into contact with a 'donor weevil'. The three additional 'donor weevils' which had been sent to Warwick Crop Centre were confirmed to have picked up 8.2×10^6, 8.6×10^6 and 5.9×10^6 spores. Literature suggests that the LD50 at 10 days for adult vine weevils is between 10×10^6 and 1×10^7 spores at 20°C suggesting that the spore doses these adult weevils picked up should have been sufficient to cause infection.

Following incubation of the 21 weevils found in the *M. brunneum* cages, 9 (42.9%) were alive and healthy, four (19%) were dead and infected with *M. brunneum* (one of these was a donor weevil), four (19%) were dead and infected by *B. bassiana*, three (14.3%) were dead and infected by saprobes and one (4.8%) had died with no obvious signs of mycosis (Table 11, Figure 13). It should be noted that lack of mycosis does not necessarily mean that the weevil did not die from fungal infection but rather the conditions may not have been suitable for sporulation after death. The presence of *B. bassiana* in the *M. brunneum* treatment is likely to be due to the weevils having come into contact with the fungus in the nursery from which they were collected or in the subsequent laboratory culture.

Statistical analysis of data on infected weevils

A logistic regression analysis was carried out on the percentage of weevils infected with each or both entomopathogenic fungi. The data from the *M. brunneum*-treated cage where all weevils had escaped were omitted from the analysis. No significant effect of treatment was observed on the total percentage of infected weevils ($F = 0.03$, P = n.s). Furthermore, no significant differences were observed in the percentage of infection by *M. brunneum* ($F = 3.50$, P = n.s.), *B. bassiana* ($F = 0.80$, P = n.s.) and the mix of the *M. brunneum* and *B. bassiana* ($F = 1.08$, P = n.s.) between the untreated and *M. brunneum* treatments. However, the trend for a higher percentage of *M. brunneum*-infected weevils in the *M. brunneum*-treated cages (19%) than in the untreated cages (3.3%) indicated that *M. brunneum* was transferred to some of the infected weevils through contact with the donor weevils.

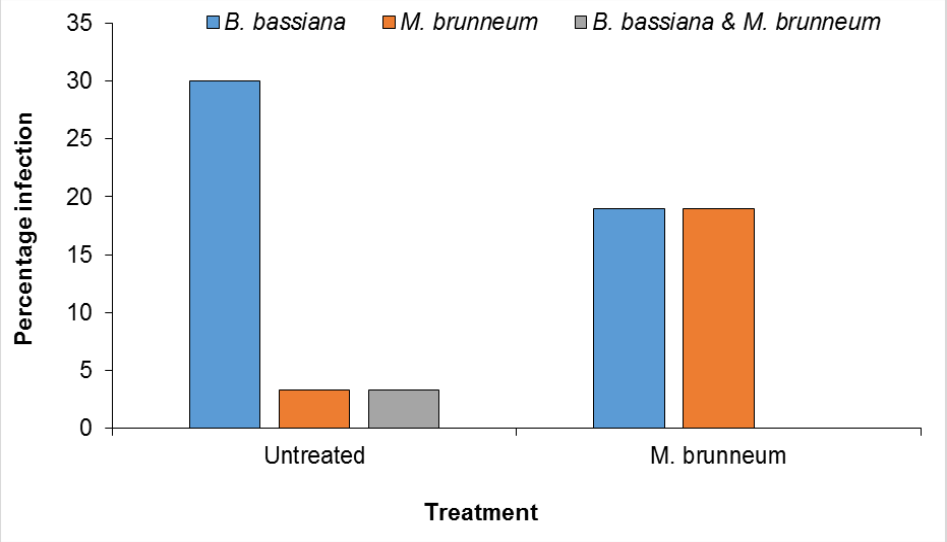

Figure 13. Percentage infection by *B. bassiana, M. brunneum* or are mixture of the two in the untreated and *M. brunneum* treatments.

Table 10. Number of weevils found (live and dead) and missing out of 30 weevils released per treatment.

		Weevils found out of 30	**Weevils found alive out of 30**	**Weevils found dead out of 30**	**Weevils missing out of 30**
Untreated	**Number** **(Range per cage)**	30 (6)	16 (2-5)	14 (1-4)	0 (0)
	%	100	53.3	46.7	0
M. brunneum **(isolate 275.86)**	**Number** **(Range per cage)**	21 (0-6)	11 (0-5)	10 (0-5)	9 (0-6)
	%	70	36.7	33.3	30

Table 11. Number and percentage of live or dead and infected weevils (of the weevils found per treatment) following incubation

		Weevils alive after incubation	Dead and infected with *B. bassiana*	Dead and infected with *M. brunneum*	Dead and infected with *B. bassiana* and *M. brunneum*	Dead and infected with saprobes	Dead no evidence of infection
Untreated	**Number**	11	9	1	1	4	4
	%	36.7	30	3.3	3.3	13.3	13.3
***M. brunneum* (isolate 275.86)**	**Number**	9	4	4	0	3	1
	%	42.9	19	19	0	14.3	4.8

Conclusions

- The presence of both *M. brunneum* and *B. bassiana* in the untreated controls and in *M. brunneum*-treated cages made interpretation of the results more difficult.
- Percentage weevil infection by *M. brunneum* in the *M. brunneum* treatment was higher (19%) than the untreated control (3.3%). Although this result was not statistically significant, it indicated that some of the infected weevils became infected through contact with the infected donor weevils.

Future work

- Determine how many spores an adult weevil requires to cause mortality.
- Determine the sub-lethal effects of fungal infection of adult vine weevils, such as feeding, oviposition and movement rates and aggregation behaviour in natural and artificial refuges.
- Repeat work in Objective 3 during the summer when temperatures are more suitable for fungal infection, using higher numbers of weevils that have been maintained in laboratory conditions for one to two months in order to minimise the risk of using weevils naturally-infected with an entomopathogenic fungus.
- Test the vine weevil control strategy using *M. brunneum* in a commercial soft fruit or nursery stock crop (This was originally suggested when the project was commissioned, as an optional extension to the 2-year project if results in Objective 3 were promising). Include the development of a more practical system for delivery of the fungus, suitable for commercial uptake.

Acknowledgements

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Thanks to Selchuk Kurtev, Darby Nursery Stock and all colleagues from ADAS, Harper Adams University and Warwick Crop Centre for late night adult weevil collections.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

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