Investigating the potential of an autodissemination system for managing populations of vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) with entomopathogenic fungi

by Pope, T.W., Hough, G., Arbona, C., Roberts, H., Bennison, J., Buxton, J., Prince, G. and Chandler, D.

DOI: https://doi.org/10.1016/j.jip.2018.04.002

Pope, T.W., Hough, G., Arbona, C., Roberts, H., Bennison, J., Buxton, J., Prince, G. and Chandler, D. 2018. Investigating the potential of an autodissemination system for managing populations of vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) with entomopathogenic fungi. *Journal of Invertebrate Pathology*, 154, pp.79-84.

Investigating the potential of an autodissemination system for managing populations of vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) with entomopathogenic fungi

HIGHLIGHTS

1. Simple plastic refuges for vine weevil (*Otiorhynchus sulcatus*) can be used to disseminate an entomopthogenic fungus through vine weevil populations.
2. Isolates of *Beauveria bassiana* and *Metarhizium brunneum* cause up to 100% mortality in vine weevil adults under laboratory conditions.
3. Conidial powders of a *Metarhizium brunneum* isolate placed in artificial refuges significantly increased vine weevil mortality under polytunnel conditions.

ABSTRACT

Vine weevil, also known as black vine weevil, (*Otiorhynchus sulcatus*) is an economically important pest affecting soft fruit and nursery stock in temperate regions. We used laboratory and polytunnel experiments to investigate a novel control system based on autodissemination of spores of an entomopathogenic fungus to populations of adult vine weevils. The fungus was applied as a conidial powder, used on its own or formulated with talc, to a simple plastic refuge for vine weevils. The potential for adult weevils to disseminate the fungus was investigated first in polytunnel experiments using fluorescent powders applied to the refuge in lieu of fungal conidia. In this system, 88% of adult weevils came in contact with the powder within 48 hours. When the powder was applied to five adult weevils that were then placed within a population of 35 potential recipients, it was transmitted on average to 75% of the recipient population within 7 days. Three isolates of entomopathogenic fungi (*Beauveria bassiana* isolate codes 433.99 and 1749.11 and *Metarhizium brunneum* isolate

code 275.86), selected from a laboratory virulence screen. These three isolates were then investigated for efficacy when applied as conidial powders in artificial refuges placed among populations of adult weevils held in experimental boxes in the laboratory at 20°C. Under this regime, the fungal isolates caused 70 – 90% mortality of adult weevils over 28 days. A final polytunnel experiment tested the efficacy of conidial powders of *M. brunneum* 275.86 placed in artificial refuges to increase vine weevil mortality. Overall weevil mortality was relatively low (26-41%) but was significantly higher in cages in which the conidial powders were placed in refuge traps than in cages with control traps. The lower weevil mortality recorded in the polytunnel experiment compared to the laboratory test was most likely a consequence of the greater amounts of inoculum required to kill adult weevils when conditions fluctuate between favourable and unfavourable temperatures e.g. below 15°C. The potential of an autodissemination system for entomopathogenic fungi as a means of controlling vine weevil as part of an integrated pest management programme is discussed.

Key words: *Beauveria bassiana*; *Metarhizium brunneum*; autodissemination; refuge; aggregation

INTRODUCTION

Vine weevil, also known as black vine weevil, (*Otiorhynchus sulcatus*) is an economically damaging pest affecting soft fruit and nursery stock crops

(Moorhouse *et al.*, 1992; van Tol *et al.*, 2012). It is widely distributed throughout temperate regions including northern Europe and North America (Warner & Negley, 1976; Lundmark, 2010). Damage is caused both by the adults, which feed on leaves, and larvae, which feed on plant roots, corms and tubers (Smith, 1932; Moorhouse *et al.*, 1992). As the larvae are root pests and the adult weevils are nocturnal, an infestation may pass unnoticed until leaf notching is evident or plants show signs of wilting, by which time they will have been damaged beyond recovery (van Tol *et al.*, 2012).

Biological control using entomopathogenic nematodes and fungi is used against vine weevil larvae (e.g. Willmott *et al.*, 2002; Georgis *et al.*, 2006; Shah *et al.*, 2007; Ansari *et al.*, 2008). At present, control of adult vine weevils is based on use of broad spectrum chemical insecticides (van Tol *et al.*, 2012 Insecticide sprays are often applied at dusk, when the weevils become active, which makes it difficult to effectively target applications. The broad-spectrum insecticides typically used also have a negative impact on biocontrol agents used against other pests and naturally occurring beneficial insects, such as ground beetles that prey upon vine weevil adults (Cross *et al.*, 2001). Therefore, more sustainable solutions for adult vine weevils are needed.

For this study, we were interested in the potential for biological control of adult vine weevils using autodissemination of entomopathogenic fungi (EPF) as an addition to existing biological control of the larval stage of this pest. In this context, autodissemination is an application system in which pest insects are attracted to a device containing a reservoir of an entomopathogen, which they

then disseminate to other individuals within their environment (Soper, 1978). It has been developed to control a range of insect pests with EPF including emerald ash borer (Lyons *et al.*, 2012), Mediterranean fruit fly (Quesada-Moraga *et al.*, 2008), sweet potato weevil (Yasuda, 1999) and damson-hop aphid (Hartfield *et al.*, 2001). However, there is no mention in the available literature of investigations for use of this approach in the control of vine weevil. Adult vine weevils are known to be susceptible to EPF infection (Moorhouse *et al.*, 1992), although they die more slowly than infected weevil larvae (Moorhouse, 1990). They also aggregate in refuges during the day (Smith, 1932; Moorhouse *et al.*, 1992; van Tol *et al.*, 2004), which could be used as sources of fungal inoculum. Here we present results from a series of experiments testing the potential efficacy of autodisseminating an EPF through the use of artificial refuges.

EXPERIMENTAL METHODS

Vine weevil culture

Adult vine weevils were collected from commercial soft fruit crops in Staffordshire, UK, and maintained under laboratory conditions at 21°C in groups of 25-30 individuals in ventilated plastic boxes (200 L x 100 W x 95 D mm) lined with damp tissue paper (a source of moisture) and a refuge (corrugated cardboard – 70 x 50 mm). Weevils were fed leaves of yew, *Taxus baccata*, *ad libitum*.

EPF culture – storage and production

EPF isolates were taken from the Warwick Crop Centre (WCC) collection of entomopathogenic fungal cultures. Isolates were stored on porous plastic beads at minus 80°C (Chandler, 1994). Laboratory cultures were grown from these beads on Sabouraud dextrose agar (SDA) slopes and maintained in a refrigerator at 4°C for up to six months. To produce conidia for experiments, subcultures were grown from the slope cultures on SDA Petri plates at 23 ± 1°C for 10-12 days in the dark.

Experiment 1: Acquisition of fluorescent marker powders by adult weevils from artificial refuges

Based on results from preliminary experiments, Roguard (BASF plc, Cheadle Hulme, UK) crawling insect bait stations, were selected for use as simple vine weevil refuges. These bait stations have a black plastic construction (80 mm diameter x 15 mm height) with four entrances (20 mm x 5 mm) and were used without the addition of a bait in these experiments. The Roguard bait stations were otherwise not modified for use as vine weevil refuges. Any aggregation by weevils within a bait station was as a result of a strong aggregation behaviour shown by vine weevil adults (e.g. Smith, 1932; Moorhouse *et al.*, 1992). The artificial refuges were tested in gauze 'tent' cages (145 x 145 x 152 cm) placed in a ventilated polytunnel (mean temperatures were 22-26°C (daytime) and 11-13°C (night time). Sixteen *Euonymus fortunei* (cv. Emerald Gaiety) plants grown in 1.5 L pots using John Innes No. 2 compost (William Sinclair Horticulture Ltd., Lincoln, UK) were placed on the floor of the cage. Forty adult weevils were then released into each cage and left to acclimatise for 24 hours, after which 12 Roguard refuges were placed into each cage. Six refuges were

spread evenly across the floor of the cage while the other six were placed on the surface of the compost of six pots. Each refuge contained 0.2 g of a hydrophobic fluorescent powder (Swada, Stalybridge, UK) placed in the central well. The fluorescent powder was used to quantify the numbers of weevils entering the refuge. Adult weevils were collected seven days after placing the refuges in the cages and scored for the presence / absence of fluorescent powder by examining them under a UV light (Lighting Ever, Birmingham, UK). There were eight replicate cages.

Experiment 2: Dissemination of florescent powders among adult weevils

Gauze 'tent' cages were prepared as previously described. Thirty-five weevils were released into each cage and left to acclimatise for 24 hrs, after which 12 Roguard refuges were placed into each cage and arranged as previously described but with no fluorescent powder. Five adult vine weevils, marked with water-based paint and then coated in yellow fluorescent powder by placing the weevils into a 20 ml specimen tube containing approximately 1 g of fluorescent powder. The lid of the tube was secured in place before gently rotating the tube for 30 s to ensure that all of the weevils had become coated in the powder. Each group of five powder coated weevils were placed into a ventilated plastic box lined with tissue paper for 30 minutes to allow excess powder to be dislodged before the weevils were released into each cage. All adult weevils were collected seven days after the powder coated weevils were released into the cages. Collected weevils were scored for the presence of fluorescent powder, excluding those that were coated with powder at the start. There were eight replicate cages.

Experiment 3: Susceptibility of adult vine weevils to EPF isolates in a laboratory bioassay

The susceptibility of adult vine weevils to eight isolates of EPF was measured in a single dose laboratory bioassay. The isolates (Table 1) were selected based on their availability as commercial biopesticides and / or their virulence to vine weevil larvae reported in previous research (Moorhouse,1990). Fungal conidia were applied as an aqueous suspension at a constant volume and concentration to ensure that weevils received a comparable dose, allowing the virulence of different isolates to be compared. Conidia were grown as described previously, harvested from SDA plates in sterile 0.05% Triton X-100 and filtered through sintered glass thimbles (40-100 µm pore). Conidia were then enumerated using an improved Neubauer haemacytometer and aliquots (10 ml) were prepared at a concentration of 10^8 conidia ml^{-1}. Groups of five adult weevils were inoculated by immersion in suspensions of conidia for 10 seconds. Controls were treated with sterile 0.05% Triton X-100. Excess suspension was removed by filtration through filter paper under vacuum. The weevils were left to air dry on the filter paper for one hour, transferred to a ventilated plastic box (200 L x 100 W x 95 D mm), and maintained at 20°C, 16:8 light:dark with yew leaves and damp tissue (to maintain > 90% relative humidity) replaced *ad libitum*. Numbers of living and dead weevils were counted daily for 28 days. Dead weevils were removed and incubated on damp filter paper within Petri dishes at 23°C, and the production of fungal conidia on these cadavers was scored. The viabilities of conidia of the fungal isolates were measured following incubation for 24 h on SDA at 23°C (Goettel & Inglis, 1997). All

isolates exhibited >87% germination. The experiment was done according to a block design. Each block comprised of the eight fungal isolates plus a control. There were three blocks in total, each done on a separate occasion.

Experiment 4: Quantifying efficacy of the autodissemination technique in a laboratory bioassay

The susceptibility of adult weevils to EPF applied as a conidial powder within the Roguard refuge was measured in a replicated laboratory bioassay. The isolates (*B. bassiana* isolate 433.99, *B. bassiana* 1749.11, *M. brunneum* 275.86; see Table 1) were selected on the basis of their virulence to adult vine weevils in the previous experiment. Conidia were grown on SDA as described previously, harvested as a powder using a spatula, and the number of conidia per g of powder was calculated by counting conidia in suspensions (0.1g conidia in 10ml of 0.05% Triton X-100) using a haemacytometer The viabilities of conidia of the fungal isolates were measured following incubation for 24 h on SDA at 23°C (Goettel & Inglis, 1997). All isolates exhibited > 91% germination. The conidia powders were added to Roguard refuges (0.4 g to each trap). Groups of five adult weevils were placed in ventilated plastic boxes (200 L x 100 W x 95 D mm) together with a single, fungus treated Roguard refuge. Boxes were maintained at 20°C, 16:8 light:dark with yew leaves and damp tissue (to maintain > 90% relative humidity) and numbers of living and dead weevils were counted daily for a total of 28 days. Dead weevils were removed and incubated on damp filter paper in Petri dishes at 23°C, and the production of fungal conidia on these cadavers was again scored as presence or absence. The experiment was done according to a randomised block design. Each block comprised three

fungal isolates, with three blocks in total. Each block contained two control chambers (refuge containing talc).

Experiment 5: Efficacy evaluation of M. brunneum *applied against adult vine weevil under polytunnel conditions*

This experiment evaluated the efficacy of of *M. brunneum* 275.86 against adult vine weevils when applied in Roguard refuges under polytunnel conditions, similar to those found on commercial ornamental nurseries. Treatments were established within gauze 'tent' cages (see Experiment 1) contained within a ventilated polytunnel. Twelve strawberry plants (cv. Malling Centenary) grown in 1.5 L pots using John Innes No. 2 compost were placed in the centre of each cage. A conidia powder of *M. brunneum* 275.86 was prepared as described in Experiment 4. This was then added to a 50:50 (w/w) mixture of talc (Sigma, UK) and fluorescent powder (see Experiment 1) at a ratio of 0.3 g of conidia powder: 0.1g talc / fluorescent powder. Aliquots of 0.4g of this mixture were then placed in the central well of Roguard refuges. Mean conidia germination was 84% (SE = 2.69). Six refuges, each containing the conidia powder of *M. brunneum* 275.86, were placed in each cage equally distributed by placing between every other of the 12 plant pots. Controls consisted of 0.4 g of the talc / fluorescent powder mixture added to each Roguard refuge. There were five replicate cages. Groups of 40 adult vine weevils were placed into each cage on the foliage of the plants. The weevils were marked on their backs with bright yellow nail varnish before release so that they were easier to find in subsequent assessments. After five weeks, the numbers of dead and live adult weevils in each cage were counted, including the number of weevils coated in fluorescent

powder. The presence of sporulating mycelia on weevil cadavers, visualised by incubating dead weevils on damp filter paper within Petri dishes at 23°C for approximately three weeks, was used as an indication of fungus-induced mortality. Samples of powder were collected from refuge traps to evaluate conidia viability.

Analysis

Data from experiments 3 and 4 were analysed using SPSS Statistics Version 24.0 (IBM Corp., 2016). A Cox proportional-hazards regression model (Cox, 1972) was used for analysing the time-mortality responses (i.e. survival) of vine weevils in all treatments compared to the control over 28 days. The Cox proportional hazard is expressed as the hazard ratio (relative average daily risk of death), which is assumed to remain constant over time. The event was death. Factors were replicate and treatment. The proportional cumulative survival of 50% of the population (i.e. median survival time (MST)), of the weevil populations of each treatment and their 95% confidence intervals were calculated and pairwise comparisons were done using a log-rank χ^2 test (Bewick *et al*. 2004). Data from experiment 5 were analysed using a Generalised Linear Model (GLM) with a log link function and negative binomial error distribution for over dispersed count data (R-3.2.2, R Core Team, 2015). Wald tests were used to determine the significance of predictor variables.

RESULTS

Experiment 1: Acquisition of fluorescent marker powders by adult weevils from artificial refuges

Seven days after introducing the Roguard refuges containing fluorescent powder, a mean of 37 (range of 32 to 40) of the 40 adult vine weevils were recovered from each cage. Of these, a mean of 88% (range of 83 to 95%) of the recovered weevils had come into contact with fluorescent powder. Weevils that had contacted the fluorescent powder were typically heavily coated in powder, with more than 50% of the body area covered.

Experiment 2: Horizontal transmission of florescent powders among adult weevils

Seven days after introduction of fluorescent powder–coated weevils to a recipient population, a mean of 33 (range of 30 to 35) of the 35 unmarked weevils were recorded from the cages. Of these, a mean of 75% (range of 66 to 93%) of the recipient population had fluorescent powder on their cuticle.

Experiment 3: Susceptibility of adult vine weevils to EPF isolates in a laboratory bioassay

All of the EPF isolates caused significantly greater mortality of adult weevils than controls ($P < 0.001$) (Table 2). After 28 days all except two (*B. bassiana* isolates 342.92 and 432.99) of the eight isolates tested resulted in 100% mortality of adult weevils. The median survival time (MST) of weevils treated with two of the isolates (*B. bassiana* isolates 433.99 and 1749.11) were significantly ($P < 0.05$) less than the other isolates at 7 and 8 days respectively. All of the isolates tested produced conidia on adult cadavers. The majority of sporulation occurred between the body segments and leg joints.

Experiment 4: Quantifying efficacy of the autodissemination technique in a laboratory bioassay

There was significantly ($P < 0.001$) greater mortality of adult weevils in all of the treatments with refuges containing EPF than in the controls after 28 days (Table 3). The refuges inoculated with isolates *M. brunneum* 275.86 and *B. bassiana* 433.99 resulted in more than 66% weevil mortality after 28 days. The MST of weevils exposed to *M. brunneum* 275.86 and *B. bassiana* 433.99 inoculated refuges was 15 and 17 days respectively. Weevils had visible amounts of fungal conidia on their cuticles within four hours of starting the experiment and fungal conidia were seen to be carried out of the traps by weevils leaving. *Beauveria bassiana* 1749.11 was not as effective in the refuges as had been expected, based on the data from the previous experiment. There was little evidence that weevils visited refuges containing this isolate, as indicated by the amount of weevil frass in the refuges and the amount of conidial powder on the floor of the bioassay chamber outside the refuge.

Experiment 5: Efficacy evaluation of M. brunneum *applied against adult vine weevil under polytunnel conditions*

Daily average temperatures and daily maximum temperatures in the polytunnel remained above 15°C during the experiment, while daily minimum temperatures fell below 15°C on 33 of the 40 days. Results from this experiment are summarised in Table 4. For both the control treatment and the *M. brunneum* treatment, over 95% of the weevils were recovered. Of the recovered weevils, mortality in the *M. brunneum* cages was significantly higher ($z = 3.00$, $P = 0.003$) than in the control cages. None of the dead weevils

recovered from control cages were infected with *M. brunneum*, while 34% of the dead weevils recovered from the *M. brunneum* treated cages had fungal mycelium emerging through the cuticle. Similarly numbers of recovered weevils had fluorescent powder on the cuticle in both the control and *M. brunneum* treated cages.

DISCUSSION

Simple plastic crawling insect bait stations were readily used as refuges by vine weevil adults and in cage experiments there was effective dissemination of a hydrophobic fluorescent powder. This was apparent even when weevils had access to a range of refuges known to be exploited in crop habitats (e.g. Smith, 1932; Moorhouse *et al.*, 1992). This effective dissemination of powders is likely to be due, at least in part, to the strong aggregation behaviour shown by vine weevil adults (e.g. Smith, 1932; Moorhouse *et al.*, 1992). Through this aggregation behaviour weevils are likely to come into contact with the fluorescent powder either by themselves entering one of the artificial refuges or by coming into contact with a weevil that has. Indeed, the experiment investigating the horizontal spread of the fluorescent powder shows that large numbers of weevils using artificial refuges may not be required for spores of an EPF to be spread through the weevil population. Further work is required to investigate the effect of refuge position and density on the spread of EPF spores throughout weevil populations. Finally, there is considerable scope to optimise the design of artificial refuges. Olfactory lures based on sex (Hartfield *et al.*, 2001) and aggregation (Tinzaara *et al.*, 2007) pheromones as well as plant volatiles (Klein & Lacey (1999; Lyons *et al.*, 2012) have, for example,

previously been used to promote the autodissemination of an EPF in damson-hop aphid and emerald ash borer populations respectively. For vine weevil, it is already known that responses of weevils to refuges may be enhanced through the addition of plant volatiles such as (Z)-2-pentenol and methyl eugenol (van Tol *et al.*, 2012).

Several studies have shown *M. brunneum* to be an effective control of vine weevil larvae (e.g. Bruck & Donahue, 2007; Moorhouse *et al.*, 1993; Shah *et al.*, 2007). In contrast, few studies have investigated the potential of *B. bassiana* for control of vine weevil (e.g. Prado, 1980; Bruck, 2004) or the use of EPFs to control adults. Moorhouse (1990) does, however, report an LT_{50} of 13 days for *M. brunneum* isolate 275.86 when weevil adults were maintained at 20°C. In Experiment 3, we tested the same isolate under the same set of conditions as Moorhouse (1990) and recorded a slightly faster, LT_{50}. The LT_{50} for isolate *M. brunneum* 275.86 was comparable to the other *Metarhizium* isolates tested but two *B. bassiana* isolates, 433.99 and 1749.11, killed 50% and 90% of the weevil population significantly ($P<0.05$) faster than the other isolates tested.

In Experiment 4, conidia powders of *M. brunneum* isolate 275.86 and *B. bassiana* isolate 433.99 placed within refuges significantly increased weevil mortality under laboratory conditions. However, *B. bassiana* isolate 1749.11 had no effect on weevil mortality, despite this isolate being virulent to vine weevil adults when applied as a conidia suspension in the bioassay for Experiment 3. There was little evidence (e.g. frass inside the refuge or disturbance of the conidia powder) of weevils entering refuges containing a

conidia powder of this isolate. It is, therefore, possible that weevils avoided conidia of isolate 1749.11. Insect avoidance of pathogenic fungi has been described in several other systems e.g. the anthocorid bug, *Anthocoris nemorum*, and the ladybird, *Coccinella septempunctata*, avoiding isolates of *B. bassiana* (Meyling and Pell, 2006; Ormond *et al.*, 2011) as well as the termite, *Macrotermes michaelseni*, avoiding both *B. bassiana* and *M. brunneum* (Mburu *et al.*, 2009). If this hypothesis is true, it suggests the possibility of developing a fungus-based chemical repellent.

Under polytunnel conditions, conidia powders of *M. brunneum* isolate 275.86 placed within refuges had a small but statistically significant effect on weevil mortality. This result is similar to previous studies e.g. field testing autodissemination as a means of controlling emerald ash borer. In a previous study only 1% of emerald ash borers in the field site area were recorded as being infected with the EPF isolate placed in the inoculation chambers after a six week trapping period (Lyons *et al.*, 2012). However, as inoculation chambers may remain within crops throughout the season they are likely to have a cumulative effective on pest populations.

A feature of the results from the polytunnel experiment (Experiment 5) - in which we tested the efficacy of conidia powders of *M. brunneum* placed within refuges - was that far more weevils (144 weevils) came into contact with the conidial powder than subsequently died from infection due to this pathogen (26 weevils). This suggests that within the 40 days of this experiment around 18% of weevils that came into contact with the conidial powders placed in the refuges acquired

a lethal dose under these experimental conditions. This is much lower than results for the laboratory condition, which resulted in over 66% weevil mortality after 28 days. This may reflect the importance of temperature in determining the efficacy of *M. brunneum* in control of vine weevil (Bruck, 2007), although the time between infection and the end of this experiment was unknown. A minimum temperature of around 15°C is required for effective control of larvae and a similar temperature requirement is likely to apply for control of adults. However, in the present study temperatures fluctuated between being below (night-time) and above (daytime) 15°C. How these temperature fluctuations affect the efficacy of *M. brunneum* is not known but 10-20 times more inoculum Is known to be required to maintain the efficacy of *B. bassiana* when conditions fluctuate between unfavourable high and low temperatures (Fargues & Luz, 2000).

Autodissemination of an EPF through the use of artificial refuges as inoculation chambers offers promise for controlling vine weevil as a component of an IPM programme. For example, EPF targeted against adult weevils could be deployed alongside entomopathogenic nematodes and fungi used against vine weevil larvae. This might reduce the need for the use of broad spectrum chemical insecticides to control vine weevil adults (van Tol *et al.*, 2012), which may disrupt biological control programmes for other pests.

ACKNOWLEDGEMENTS

This study was funded by the Chemicals Regulations Directorate (CRD) on behalf of Defra (project PS2134).

REFERENCES

Ansari MA, Shah FA, Butt TM (2008) Combined use of entomopathogenic nematodes and *Metarhizium anisopliae* as a new approach for black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) control. *Entomologia Experimentalis et Applicata*. 129: 340–347.

Bewick V, Cheek L, Ball J (2004) Statistics review 12: Survival analysis. Critical Care, 8:389-394.

Bruck D. (2004) Natural occurrence of entomopathogens in Pacific Northwest nursery soils and their virulence to the black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera : Curculionidae). *Environmental Entomology*. 33:1335-1343.

Bruck DJ. (2007) Efficacy of *Metarhizium anisopliae* as a curative application for Black Vine Weevil (*Otiorhynchus sulcatus*) infesting container-grown nursery crops. *Journal of Environmental Horticulture*. 25:150–156.

Bruck DJ, Donahue KM (2007) Persistence of *Metarhizium anisopliae* incorporated into soilless potting media for control of the black vine weevil, *Otiorhynchus sulcatus* in container-grown ornamentals. *Journal of Invertebrate Pathology*. 95:146-150.

Chandler D (1994) Cryopreservation of fungal spores using porous beads. *Mycological Research*. 98:525-526.

Cox DR (1972) Regression models and life-tables. *Journal of the Royal Statistical Society B*. 34:187-220.

Cross JV, Easterbrook MA, Crook AM, Fitzgerald JD, Innocenzi PJ, Jay CN, Solomon MG (2001) Review: natural enemies and biocontrol of pests of strawberry in northern and central Europe. *Biocontrol Science and Technology*. 22:165-216.

Fargues J, Luz C (2000) Effects of fluctuating moisture and temperature regimes on the infection potential of *Beauveria bassiana* for *Rhodnius prolixus*. *Journal of Invertebrate Pathology*. 75:202-211.

Georgis R, Koppenhöfer AM, Lacey LA, Bélair G, Duncan LW, Grewal PS, Samish M, Tan L, Torr P, van Tol RWHM (2006) Successes and failures in the use of parasitic nematodes for pest control. *Biological Control*. 38:103-123.

Goettel MS, Inglis GD (1997) Ch. V-3 Fungi: Hyphomycetes. *In* "Manual of Techniques in Insect Pathology", (L. A. Lacey, Ed.), pp.213-248, Academic Press.

Hartfield CM, Campbell CAM, Hardie J, Pickett JA, Wadhams LJ (2001) Pheromone traps for the dissemination of an entomopathogen by the damson-hop aphid *Phorodon humuli*. *Biocontrol Science and Technology*. 11:401-410.

Klein MG, Lacey LA (1999) An attractant trap for autodissemination of entomopathogenic fungi into populations of the Japanese beetle *Popillia*

japonica (Coleoptera:Scarabaeidae). *Biocontrol Science and Technology*. 9:151–158.

Lundmark M (2010) *Otiorhynchus sulcatus*, an autopolyploid general-purpose genotype species? *Hereditas*. 147:278–282.

Lyons DB, Lavallee R, George K-P, van Frankenhuyzen K, Johny S, Guertin C, Francese JA, Jones GC, Blais M (2012) Towards the development of an autocontamination trap system to manage populations of emerald ash borer (Coleoptera: Buprestidae) with the native entomopathogenic fungus, *Beauveria bassiana*. *Journal of Economic Entomology*. 105:1929-1939.

Meyling N, Pell JK (2006) Detection and avoidance of an entomopathogenic fungus by a generalist insect predator. *Ecological Entomology*. 31:162–171.

Mburu DM, Ochola L, Maniania NK, Njagi PGN, Gitonga LM, Ndung'u MW, Wanjoya AK, Hassanali A (2009) Relationship between virulence and repellency of entomopathogenic isolates of *Metarhizium anisopliae* and *Beauveria bassiana* to the termite *Macrotermes michaelseni*. *Journal of Insect Physiology*. 55:774–780.

Moorhouse ER (1990) The potential of the entomopathogenic fungus *Metarhizium anisopliae* as a microbial control agent of the black vine weevil *Otiorhynchus sulcatus*. *Phd Thesis, University of Bath*. 533pp.

Moorhouse ER, Charnley AK, Gillespie AT (1992) A review of the biology and control of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). *Annals of Applied Biology*. 121:431-454.

Moorhouse ER, Gillespie AT, Charnley AK (1993) The development of *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae) larvae on a range of ornamental pot-plant species and the potential for control using *Metarhizium anisopliae*. *Journal of Horticultural Science*. 68:627-635.

Ormond EL, Thomas APM, Pell JK, Freeman SN, Roy HE (2011) Avoidance of a generalist entomopathogenic fungus by the ladybird, *Coccinella septempunctata*. *FEMS Microbiology Ecology*. 77:229–237.

Prado E (1980) Control of black vine weevil larvae (*Otiorrhynchus sulcatus*) using the insect pathogenic fungi *Beauveria bassiana*, *Metarrhizium anisopliae* and *Metarrhizium flavoviride*. *Vaxtskyddsnotiser*. 44:160-167.

Quesada-Moraga E, Martin-Carballo I, Garrido-Jurado I, Santiago-Álvarez C (2008) Horizontal transmission of *Metarhizium anisopliae* among laboratory populations of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). *Biological Control*. 47:115-124.

Shah FA, Ansari MA, Prasas M, Butt TM (2007) Evaluation of black vine weevil (*Otiorhynchus sulcatus*) control strategies using *Metarhizium anisopliae* with sublethal doses of insecticides in disparate horticultural growing media. *Biological Control*. 40:246-252.

Smith FF (1932) Biology and control of the black vine weevil. *U.S. Department of Agriculture Technical Bulletin*. 325.

Soper R (1978) Autodissemination of entomopathogens: fungi. In: Allen GE, Ignoffo CM, Jaques RP (eds) Future strategies in pest management systems.

Proceedings of the National Science Foundation, United States Department of Agriculture and the University of Florida Workshop on Microbial Control of Insect Pests, Gainesville, FL, pp 63–65.

Tinzaara W, Gold CS, Dicke M, Van Huis A, Nankinga CM, Kagezi GH, Ragamaet RE (2007) The use of aggregation pheromone to enhance dissemination of *Beauveria bassiana* for the control of the banana weevil in Uganda. *Biocontrol Science and Technology*. 17:111–124.

van Tol RWHM, Visser JH, Sabelis MW (2004) Behavioural responses of the vine weevil, *Otiorhynchus sulcatus*, to semiochemicals from conspecific, *Otiorhynchus salicicola*, and host plants. *Entomologia Experimentalis et Applicata*. 110:145-150.

van Tol RWHM, Bruck DJ, Griepink FC, De Kogel WJ (2012) Field attraction of the vine weevil Otiorhynchus sulcatus to kairomones. *Journal of Economic Entomology*. 105:169-175.

Warner RE, Negley FB (1976) The genus *Otiorhynchus* in America north of Mexico (Coleoptera: Curculionidae). *Proceedings of the Entomological Society of Washington*. 78:240-262.

Willmott DM, Hart AJ, Long SJ, Edmondson RN, Richardson PN (2002) Use of a cold-active entomopathogenic nematode *Steinernema kraussei* to control overwintering larvae of the black vine weevil *Otiorhynchus sulcatus* (Coleoptera: Cucurlionidae) in outdoor strawberry plants. *Nematology*. 4:925-932.

Yasuda K. (1999) Auto-infection system for the sweet potato weevil, *Cylas formicarius* (Fabricius) (Coleoptera: Curculionidae) with entomopathogenic fungi, *Beauveria bassiana* using a modified sex pheromone trap in the field. *Applied Entomology and Zoology*. 34:501-505.

1 Table 1. Fungal isolates used in the initial screen.

2

3 Table 2. Survival analysis results of time-mortality responses of adult black

4 vine weevil treated directly with EPF isolates 28 days post inoculation.

5

6 Table 3. Survival analysis results of time-mortality responses of adult black

7 vine weevil to EPF inoculated Roguard refuges 28 days post inoculation.

8

9 Table 4. Results from efficacy evaluation of *M. brunneum* applied against

10 adult vine weevil under polytunnel conditions.

11

12

Species	Isolate†	Host/Substrate	Collection site
Beauveria bassiana	342.92	*Otiorhynchus sulcatus*	UK
	432.99[a] (ATCC 74040)	*Anthonomus grandis*	USA
	433.99[b] (strain GHA)	*Diabrotica undecimpunctata*	USA
	1749.11	*O. sulcatus*	UK
Metarhizium anisopliae s.l.	35.79	*O. sulcatus*	UK
.	189.83	*O. sulcatus*	UK
.	276.86	*O. sulcatus*	Germany
Metarhizium brunneum	275.86[c] (F52 / BIPESCO5)	*Cydia pomonella*	Germany

†Isolate number in the WCC culture collection (isolate number from culture collection of origin)

Isolate forms the active ingredient in the proprietary mycopesticide; (a) 'Naturalis' (Troy Biosciences Inc., 113 South 47th Ave., Phoenix, AZ 850433, USA). ATCC = American Type Culture Collection; (b) 'Botanigard' (Mycotech Corporation, PO Box 4109, Butte, MT 59702, USA); (c) 'Met52' (Novozymes, Hallas Allé 4400 Kalundborg. Denmark).

Species	Isolate[†]	% mortality		Factors	MST[b] (95% CI)		HR[c] (95% CI)	Z (HR)	P (HR)	df	n
		14 dpi[a]	28 dpi	Rep				0.002	0.961	1	2
				Treatment				72.86	<0.001	8	9
Control		3	13		-	a					30
Beauveria bassiana	342.92	47	67		14 (3.9 - 24.1)	bd	10.45 (3.31 - 32.98)	16.02	<0.001	1	15
	432.99	47	67		22 (1.8 - 42.2)	b	8.74 (2.73 -27.96)	13.35	<0.001	1	15
	433.99	100	100		7 (6.4 - 7.6)	e	104.74 (31.42 - 349.09)	57.35	<0.001	1	15
	1749.11	100	100		8 (6.8 - 9.2)	e	79.15 (23.83 - 262.89)	50.95	<0.001	1	15
Metarhizium anisopliae	35.79	80	100		12 (10.1 – 13.9)	c	32.01 (9.95 – 103.03)	33.78	<0.001	1	15
	189.83	67	100		10 (8.8 - 11.2)	c	27.54 (8.74 - 86.79)	32.05	<0.001	1	15
	276.86	60	100		13 (11.8 - 14.2)	cd	23.29 (7.33 - 73.99)	28.49	<0.001	1	15
Metarhizium brunneum	275.86	87	100		10 (9.1 - 10.9)	c	31.54 (9.78 - 101.76)	33.36	<0.001	1	15

[†]Isolate number in the WCC culture collection

The Hazard ratios (HR) indicate the relative average daily risk of death compared to the 0.05% Triton-X treated control. The median survival time (MST) gives the proportional cumulative survival of 50% of the populations. MST values followed by different lower case letters within the column are significantly different (log rank $\chi^2 \geq 3.841$, $P < 0.05$).

a dpi = days post inoculation

[b] MST = median survival time, given in days

[c]HR = hazard ratio, compared to the 0.05% Triton-X treated control

1

2

Species	Isolate[†]	% Mortality		Factors	MST[b] (95% CI)		HR[c] (95% CI)	Z (HR)	P (HR)	df	n
		14 dpi[a]	28 dpi	Rep				0.161	0.689	1	2
				Treatment				35.82	<0.001	3	4
Control		0	3		-	a					30
Beauveria bassiana	433.99	27	67		17 (15.5 - 18.5)	c	17.74 (4.474 - 70.322)	16.74	<0.001	1	15
	1749.11	13	27		-	a	3.81(0.845 - 17.170)	3.03	<0.082	1	15
Metarhizium brunneum	275.86	47	93		15 (8.8 - 21.2)	b	34.3 (9.469 - 124.262)	28.97	<0.001	1	15

[†]Isolate number in the WCC culture collection

The Hazard ratios (HR) indicate the relative average daily risk of death compared to the 0.05% Triton-X treated control. The median survival time (MST) gives the proportional cumulative survival of 50% of the populations. MST values followed by different lower case letters within the column are significantly different (log rank $\chi^2 \geq 3.841$, $P < 0.05$).

[a] dpi = days post inoculation

[b] MST = median survival time, given in days

[c] HR = hazard ratio, compared to the talc control

	Control treatment	***Metarhizium brunneum* treatment**
Mean number of weevils recovered (+/- SE)	38.20 (1.06)	37.80 (0.86)
Mean number of dead weevils recovered (+/- SE)	10.00 (2.10)	15.40 (1.99)
Mean numbers of *M. brunneum* infected weevils (+/- SE)	0.00 (0.00)	5.20 (1.39)
Mean numbers of weevils coated in fluorescent powder (+/- SE)	27.60 (2.32)	29.00 (2.02)

1