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[The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.]

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

Jude Bennison

Senior Research Entomologist, ADAS

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SCIENCE SECTION

Introduction

Vine weevil is currently the most serious pest of UK containerised hardy nursery stock. Adult damage to leaves and presence of larvae around roots can make ornamental plants unmarketable. Root damage caused by larvae leads to reduced plant vigour and if damage is severe, to plant death. Chemical control of larvae is now difficult due to the withdrawal of the most persistent products for use in growing media and to current EC restrictions on using one of the available neonicotinoid insecticides, imidacloprid (Imidasect 5GR) on flowering plants. Vine weevil populations have been increasing recently on some HNS nurseries due to these restrictions. There is now more grower interest in using methods for control of weevil adults as well as larvae, and growers need more information on the efficacy and timing of insecticide sprays that are compatible with Integrated Pest Management (IPM) programmes, linked with further knowledge on weevil activity and egg laying behaviour. Growers are under increasing pressures to reduce the use of pesticides, not only to meet retail demands but also to meet the requirements of the EC Sustainable Use Directive (SUD) which states that all growers must use IPM where practical and effective. Many growers of HNS are now adopting biological pest control methods within IPM programmes. Available biological methods for vine weevil control include the entomopathogenic fungus (Met52 Granular Bioinsecticide) for incorporation in growing media and entomopathogenic nematodes which are applied as drenches. However, growers lack confidence in the efficacy of Met52 due to its temperature requirements and view current nematode application methods using drenches in HNS as labour-intensive and thus expensive. This project will address grower needs by filling knowledge gaps in how to optimise best-practice use of available vine weevil control methods within IPM and to develop novel approaches to both monitoring and control.

Objective 1. Improve understanding of the impact of environmental conditions on vine weevil biology and behaviour in order to optimise application of plant protection products

Introduction

Overwintering vine weevil adults are not thought to start egg laying until May and June while newly emerging adults, emerging in June and July may not start to lay eggs until August (Garth and Shanks, 1978; Blackshaw, 1996). As such, spring temperatures are likely to determine the start of egg laying by overwintered adults and autumn temperatures will

determine the end of egg laying by adults completing their development during the growing season. For newly emerged weevils, host quality and temperature determine the length of the preoviposition feeding period and influences the number of eggs laid and their viability (Cram, 1965). This preoviposition period can last for 4 - 9 weeks (Garth and Shanks, 1978; Nielsen and Dunlap, 1981). Although the severity of the preceding winter will determine the numbers of weevils successfully overwintering, these individuals may contribute more than half of all the eggs laid in a season (Blackshaw, 1996) due to their faster rate of oviposition compared to newly emerged adults (Cram, 1965). Egg laying activity of vine weevil adults appears to cycle between periods of peak egg laying and periods where few or no eggs are laid. The causes of these cycles are not fully understood but are thought to relate to the nitrogen content in the host plant and to temperature (Moorhouse et al., 1992).

There is conflicting information in the literature on the minimum temperature required for egg laying. Stenseth (1979) suggests that egg laying only occurs at temperatures above 12°C while Blackshaw (1992) reports egg laying at lower temperatures. An earlier part of this project however, has shown that egg laying and feeding activity continues at temperatures above 6°C in vine weevil emerging that year. Egg development is known to continue at temperatures above 6.32°C (Masaki & Ohto, 1995). This suggests that the monitoring period for vine weevil activity should coincide with the period when temperatures rise above this threshold in the spring, summer and autumn.

The aim of this objective is to investigate the importance of temperature in determining feeding and egg laying activity of overwintered vine weevil adults. While adult weevils that emerge in the summer are known to require a preoviposition period it is not known whether overwintered adults similarly require a period of feeding before egg laying recommences in the spring. An added complication, not previously considered, is that for much of the year air temperatures fluctuate between warmer days and cooler nights (night-time temperatures below 12°C). Given that vine weevil adults typically oviposit at night when they are most active, night time temperatures are likely to be an important component in predicting the onset and continuation of egg laying. Determining the minimum temperature at which overwintered vine weevil adults feed and lay eggs is important if growers are to optimise monitoring efforts and correctly time applications of controls in outdoor, polytunnel and glasshouse grown crops.

Task 1.1.1. Determining the minimum temperature for vine weevil feeding and egg laying

Materials and methods

Site: All work was done in controlled environment cabinets in the Jean Jackson entomology laboratory at Harper Adams University.

Insects: Vine weevil adults were collected during the summer of 2016 from commercial strawberry crops grown in Shropshire and Staffordshire. A population of vine weevil adults were overwintered in strawberry pots in a polytunnel at Harper Adams University between September 2016 and March 2017 prior to being used in the experiment.

Insect conditioning: At the start of the experiment, 10 healthy weevils were selected at random from the polytunnel population and assigned to a controlled temperature cabinet (Sanyo/Panasonic) set to a constant 12, 15 or 18°C. Each weevil was placed in a ventilated Petri dish (90mm diameter) lined with damp Whatman No. 1 filter paper (Whatman Plc, UK) containing an unfurled strawberry leaf as a food source and left for 24 hours.

Feeding assessments: Each weevil was fed a single leaf disc (19 mm diameter), cut from a fully unfurled strawberry leaf (cv. Elsanta) using a cork borer. Each week, for five consecutive weeks, each weevil was allowed to consume a leaf disc for a 48 hour period as their only food source. The leaf disc was then removed from the Petri dish and photographed against a white background and the area of the leaf consumed was calculated using ImageJ software (https://imagej.nih.gov/ij/). The leaf discs were replaced with an excess of strawberry leaf material to feed the weevils until the following week's assessment.

Egg laying assessments: No egg laying was recorded during the experimental period, therefore no assessments of egg laying or egg viability were made.

Statistical analysis: Total leaf area consumed was analysed using a one-way ANOVA on log(x+1) transformed data with weevil included as an error term to account for the repeated measures. All analyses were done in R.3.2.2 (R Core Team, 2015).

Task 1.2. Estimating the period during which vine weevil egg laying may occur in outdoor, polytunnel and glasshouse grown crops

Meteorological data

Air temperature data were recorded inside a polytunnel located in Pulbourough, West Sussex, in 2014. Another set of air temperature data were recorded inside an unheated glasshouse located in Walburton, West Sussex in 2015. Additional data of external air temperatures were obtained from a nearby meteorological station (MIDAS, 2017) in 2014 and from the same site

as the glasshouse in 2015. Data for the months March to June were summarised to represent typical conditions within these systems for the period in which overwintering vine weevil are becoming active.

Results

Task 1.1.1. Determining the minimum temperature for vine weevil feeding and egg laying

Temperature and humidity conditions in each of the controlled temperature cabinets were verified using iButton (HomeChip, UK) data loggers (Table 1.1).

Table 1.1. Cabinet temperature settings and recorded mean temperatures and relative humidity for the duration to the experimental period.

Cabinet temperature set point (˚C)	Mean temperature (± SEM) (˚C)	Mean humidity (± SEM) (%RH)
12	11.88 (±0.01)	72.45 (±0.13)
15	14.91 (±0.01)	60.13 (±0.17)
18	18.05 (±0.01)	76.42 (±0.17)

Leaf area consumption data are summarised in Figure 1.1. There was no statistical difference found in average leaf area consumption at the different temperatures (P = 0.271). Similarly, by week 6, the same number of weevils remained alive at each treatment temperature (7 out of 10).

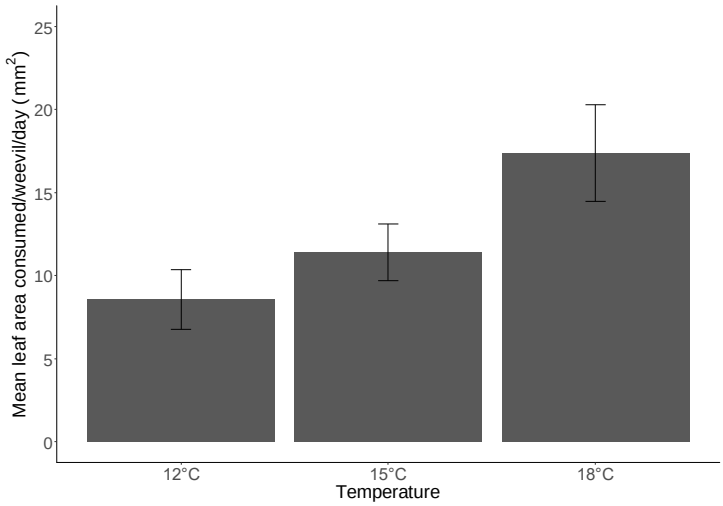
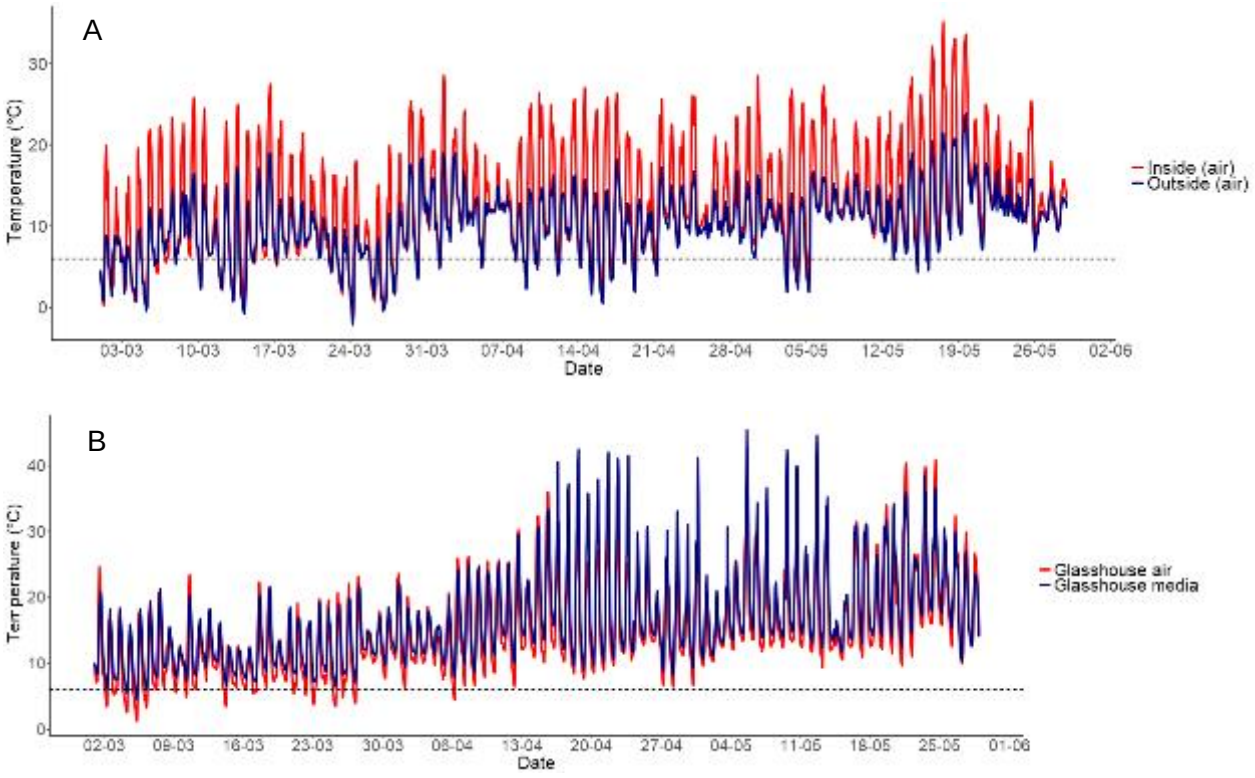


Figure 1.1. Mean leaf area consumed per weevil per day (± SEM) in mm^2 at each treatment temperature.

4.3.2. *Task 1.2. Estimating the period during which vine weevil egg laying may occur in outdoor, polytunnel and glasshouse grown crops*

From the results obtained in year 1 of this project, egg laying and feeding in vine weevils appeared to continue at temperatures as low as 6 °C. Task 1.1.1 demonstrates that overwintered weevils feed at temperatures of 12 °C at the same rate as higher temperatures. Figure 1.2. shows hourly air and media temperatures recorded at two strawberry farms in Kent early in the season. Table 1.2. summarises these data in terms of the number of nights each month (7pm – 7am) in which temperatures were above the lower egg laying and feeding threshold (6 °C), or were suitable for high levels of weevil feeding activity (12 °C) for a minimum of one hour.



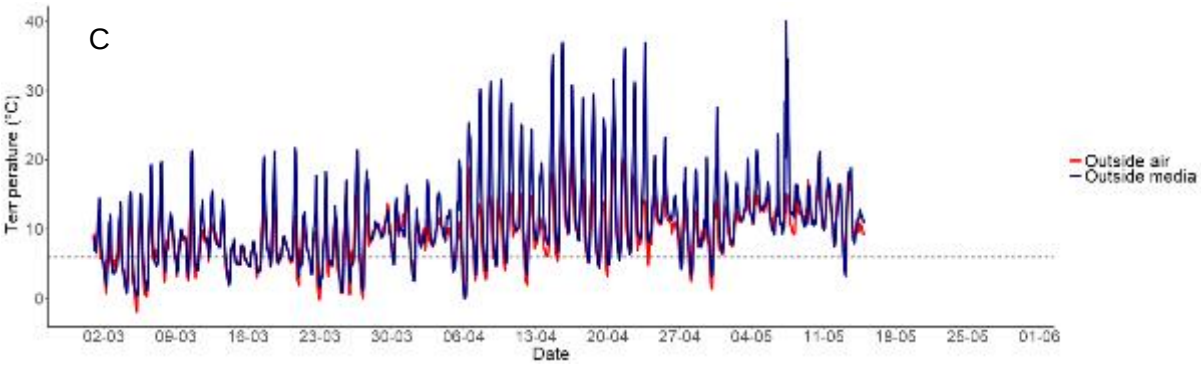


Figure 1.2. Hourly temperature data recorded from the beginning of March to the end of May at two sites in Kent. **A.** Air temperatures inside and outside a polytunnel (2014) **B.** Air and media temperatures inside an unheated glasshouse (2015) **C.** Air and media temperatures outside at the same site as the unheated glasshouse (2015). Horizontal line is at 6 °C on all plots.

Table 1.2. Number of nights per month (7pm – 7am) during which temperatures rose above stated thresholds for a minimum of 1 hour recorded at two sites in Kent in 2013-14 (polytunnel) and 2014-15 (unheated) glasshouse. Data for the glasshouse in May and September were incomplete and are therefore not presented.

	Above 6°C			**Above 12°C**		
	Sept	**Oct**	**Nov**	**Sept**	**Oct**	**Nov**
Inside polytunnel (air)	30	31	20	27	21	3
Outside polytunnel (air)	30	31	20	23	22	4
Inside glasshouse (air)	-	31	30	-	31	27
Inside glasshouse (media)	-	31	30	-	31	28
Outside glasshouse (air)	-	31	30	-	30	19
Outside glasshouse (media)	-	31	30	-	30	6
	Above 6°C			**Above 12°C**		
	Dec	**Jan**	**Feb**	**Dec**	**Jan**	**Feb**
Inside polytunnel (air)	18	19	19	0	0	0
Outside polytunnel (air)	21	22	26	1	1	1

Inside glasshouse (air)	31	31	28	10	19	18
Inside glasshouse (media)	31	31	28	5	10	15
Outside glasshouse (air)	28	25	21	4	3	1
Outside glasshouse (media)	20	18	25	0	0	6

	Above 6°C			**Above 12°C**		
	March	**April**	**May**	**March**	**April**	**May**
Inside polytunnel (air)	25	30	31	3	22	30
Outside polytunnel (air)	24	30	31	3	5	15
Inside glasshouse (air)	31	30	31	5	28	31
Inside glasshouse (media)	31	30	31	22	30	31
Outside glasshouse (air)	20	29	-	1	0	-
Outside glasshouse (media)	20	30	-	0	10	-

Discussion

The literature on vine weevil biology provides conflicting information on the minimum temperature required for egg laying. Stenseth (1979) suggests that egg laying only occurs at temperatures above 12 °C while Blackshaw (1992) reports egg laying at lower temperatures. Results reported in Year 1 of this project, however, support the work of Blackshaw (1992) by indicating that egg laying may continue at temperatures below 12 °C. Egg laying and feeding by vine weevil adults continues at temperatures as low as 6 °C but, egg laying at least, appears to stop at a temperature of 5 °C (no viable eggs laid at this temperature). In addition, egg hatch was recorded at temperatures as low as 6 °C, which appears to be in line with previous estimates of the lower temperature threshold for egg development 6.3 °C (Masaki & Ohto, 1995).

Results reported in Year 2 of this project build on results reported in Year 1 by showing that overwintered vine weevil adults feed within the range 12 to 18°C. Statistical analysis of the leaf area consumed, however, shows that temperature did not influence the amount of leaf material consumed within this temperature range. This result is similar to the results reported in Year 1 where feeding was not found to be influenced by temperatures within the range 6 to 12°C. Results in Year 2 were notable for a lack of eggs being laid during the five-week experimental period but that these weevils ate approximately 10 times as much leaf material

as weevils in Year 1, which had not been overwintered. It has previously been suggested that overwintering adults may not require a pre-oviposition period after overwintering. The results presented here, however, suggest that, like newly emerging adults, overwintering adults require a period of intense feeding activity before oviposition can recommence. Despite this, Year 1 results show that this period of intense feeding activity may commence earlier in the year than had previously been thought. Available temperature data from a range of commercial nurseries indicates that, in the years for which data is currently available, vine weevil have the potential to be active during at least some nights throughout the winter. It remains unknown, however, for how long temperatures must exceed 6°C before activity recommences. Despite this it appears likely that feeding may recommence in early spring and continue into early winter in all cropping situations.

Conclusions

- Vine weevil adults feed within the temperature range 6 - 18°C but the amount of leaf material eaten is not affected by temperature within this range.
- Overwintered vine weevil adults appear to require a period of intense feeding activity before egg laying can recommence.
- Overwintered vine weevil adults are likely to become active and start feeding, even outside, in March, although egg laying may not start for at least five weeks after feeding behaviour resumes.

Objective 2. Develop practical methods for monitoring adults in order to detect early infestations and inform control methods

Introduction

The development of an effective vine weevil lure would considerably improve existing monitoring strategies and contribute to an improved integrated pest management system. Vine weevil are polyphagous feeders and reproduce asexually (Smith 1932; Moorhouse et al. 1992). No sex pheromone exists but adult weevils display a strong aggregation behaviour and show attraction to plant odours (Pickett et al., 1996; van Tol et al., 2002; van Tol et al. 2004; Kakizaki 2001; Nakamuta et al. 2005). Several aggregation pheromones have been identified for weevil species around the world (van Tol et al., 2002), however no study has yet identified such a pheromone for vine weevil.

Vine weevil appear to be attracted by the odour of other weevils (Nakamuta et al., 2005) and specifically to the frass (droppings) produced by these weevils (van Tol et al., 2004). This is disputed however in a study by Karley et al. (2012), which found no evidence of attraction to frass, although the frass did increase weevil movement. Similarly, there is some evidence to suggest that weevils prefer refuges that have previously been used by other weevils and therefore contain weevil frass (Pickett et al., 1996), however Nakamuta et al. (2005) did not find weevils to be attracted to the odour from previously used refuges.

Several studies demonstrate evidence of vine weevil detection to plant derived odours which allows them to locate suitable host plants for feeding and oviposition but may also play a role in aggregation. For example, odours of yew (*Taxus baccata*) and *Euonymus fortunei* damaged by adult vine weevil are attractive to other adult vine weevil, but *Rhododendron* and strawberry (*Fragaria* x *ananassa*) are not (van Tol et al., 2002). It is not yet fully understood how vine weevil discriminate between the odours of potential host plants, as weevils appear to detect and respond to plant volatiles that are common to many plant species (van Tol & Visser, 2002; van Tol et al., 2012; Karley, 2012). It is, however, likely that the ratios of blends of these plant volatiles is important in host plant detection (Bruce & Pickett, 2011).

Karley (2012) found plant derived cues, such as the plant volatile E-2-hexenol, were much more attractive to vine weevil than insect-derived cues. Several volatiles derived from *Euonymus fortunei* are known to attract vine weevils (van Tol et al. 2002, 2012). A combination of two such volatiles, methyl eugenol and (*Z*)-2-pentenol (1:1 ratio), when used as an attractant in traps were responsible for increased numbers of weevils in and around the traps but did not increase trap catches (van Tol et al. 2012).

These studies suggest that vine weevil adults use olfactory cues for host plant location and aggregation. Identification of an effective attractant for vine weevil could significantly enhance existing trapping methods and improve monitoring of this pest. Such an attractant could also be exploited in a lure and kill strategy involving biopesticide formulations, which would enable IPM-compatible control options to be developed.

The aim of this objective was in Year 1 of the project to assess the relative effectiveness of a range of traps and indicator plants that may already be used or which could be easily adopted by growers to detect activity of adult vine weevil within crops. Based on results from Year 1, the aim of the work completed in Year 2 is to test the potential of lures based on host-plant volatiles to improve the reliability and sensitivity of the best performing traps. This comparison will be completed under standardised conditions using simulated crop environments and known numbers of adult weevils. Information from this work will provide growers with

information on the selection of suitable monitoring tools on which to base crop protection decisions.

Materials and methods

Task 2.2 Potential of lures to improve monitoring of vine weevil adults

Insects

Adult vine weevils were collected from soft fruit crops (mainly strawberry) in Shropshire and Staffordshire in 2017 and kept at 20 °C in a controlled environment room (Fitotron, Weiss Technik, Ebbw Vale, Wales) under long-day conditions (L:D 16:8 h). Weevils were maintained on *Taxus baccata* (Linnaeus) plants inside insect cages (47.5 x 47.5 x 47.5 cm, Bugdorm, MegaView, Taiwan), containing damp paper towels as a moisture source.

Surface hydrocarbons bioassay – weevil trail

The potential for trail following behaviour in vine weevil was tested to investigate whether surface hydrocarbons deposited by conspecifics could be exploited for monitoring purposes. Ten weevils were randomly selected and allowed to walk down a glass Y-tube olfactometer (120 mm stem, arms 90 mm, i.d. approx.18 mm and approx. 60° angle) that had one arm blocked off with aluminium foil. Following this, the aluminium foil was removed and the olfactometer was positioned so that each arm ended in individual modified plastic capture containers. The sides of the capture containers were coated with Fluon® to prevent weevils re-entering the olfactometer and wrapped in aluminium foil to create a dark interior. A further five weevils were randomly selected and placed into a release container which connected to the entrance of the olfactometer. The number of weevils present in each capture container were counted after 1 hour. The experiment was repeated eight times, alternating the treatment arm each time. Between reps, the Y-tube olfactometer was rinsed in warm water and then with acetone (25 ml/part) (Sigma-Aldrich, Dorset, UK) before being oven baked at 120 °C for 15 mins.

Surface hydrocarbons bioassay – weevil wash

Sixteen healthy weevils were randomly selected and placed in the freezer at -20°C for 24 hours. These specimens were then submerged in 4 ml of hexane for 30 minutes to extract the surface hydrocarbons. A trail was created down the treatment arm of the Y-tube olfactometer using 250 µl of the extract which was then allowed to evaporate off. The

bioassay proceeded as described in experiment 1, with ten weevils tested per rep and four reps completed in total.

Weevil and plant volatiles bioassay

A glass moving air or Y-tube olfactometer connected to a release chamber and two treatment and choice chambers, was used to conduct the bioassays (Figure 2.1). The treatment chambers comprised a 500 ml Dreschel Bottle & Bubbler Head, 6 mm o.d. arms ground flange and glass base disc which and were connected to the collecting chambers comprising of a 500ml Dreschel bottle with modified bottle head outlet ball and socket inlet, 6 mm o. d. with ground flange and glass disc base by 3-4 mm Swagelok brass fittings and PTFE tubing. The Conex tube ball and socket joints with rubber seals (straight, approx. 100 mm length) extended from the collection chambers' head to the Y-tube arms (120 mm stem, arms 90 mm, i.d. approx..18 mm and approx. 60° angle) held together by metal clamps (modified for spherical joint, with adjusting screw S35). The release chamber (100 mm dia., open topped with glass lid) connected to the stem of the Y-tube to complete the olfactometer. Airflow was purified by passage through activated charcoal and water filters, then passed through the treatment and collection chambers then into the Y-tube olfactometer and finally into the release chamber. Flow meters were used to ensure that a constant flow rate of 600 ml min^{-1} was maintained. The bioassays were carried out in darkness at a temperature of 20°C. Each test lasted for 20 mins, preliminary testing indicated that extending this period did not result in changes in choice made by weevils.

The treatments used in each experiment are listed in Table 2.1., and comprise a combination of biological specimens and plant volatiles previously identified as eliciting a response from electroantennogram analysis of vine weevil. Plant volatile compounds were prepared at 10, 100 and 500 mg ml^{-1} concentrations, diluted in liquid paraffin and kept at 5°C. Aliquots (10 µl) of the test substance were placed on Whatman glass microfiber filters (GE Healthcare Life Sciences, Buckinghamshire, UK). The control was an aliquot (10 µl) of paraffin oil (Sigma-Aldrich, Dorset, UK) on Whatman glass microfiber filter. Treatments were assigned randomly and then alternated with the control chamber to eliminate left/right bias. Each test was repeated six times with different groups of weevils. Between reps, the olfactometer was washed with warm water, then with acetone (25 ml/part) (Sigma-Aldrich, Dorset, UK) before being oven baked at 120°C for 15 mins.

Analysis by Gas Chromatography linked to Electroantennography (GC-EAG)

GC-EAG analyses were carried out with an HP 6890 instrument (Agilent) fitted with capillary GC columns (30 m x 0.32 mm i.d. x 0.25 µm film thickness) coated with polar (DBWax; Supelco) and non-polar (SPB1, Supelco) phases. The analyses were carried out on the polar column with splitless injection (220ºC), helium carrier gas (2.4 ml/min) and the oven temperature programmed from 50°C for 2 min, then at 20°C min^{-1} to 250°C. The GC column effluent was split (1:1) with low-volume connector between the FID (250°C) and through a heated outlet (250°C) into a flow of purified air (500 ml min^{-1}) through a tube (4 mm i.d.) to the EAG preparation.

For the EAG preparation, the vine weevil was anaesthetised using carbon dioxide before excising the weevil's head. The reference electrode, containing electrolyte (0.1 M potassium chloride with 10% polyvinylpyrrolidone) was inserted into the back of the head and attached to silver electrode held in micromanipulators on a portable EAG device (INR-02; Syntech, Hilversum, The Netherlands). The circuit was completed by one antennae being inserted into the recording glass electrode attached to the EAG device. Both FID and EAG signals were collected and analyzed with EZChrom software (Elite v3.0; Agilent).

Analysis by Gas Chromatography linked to Mass Spectrometry (GC-MS)

GC-MS analyses were carried out on a CP3500 GC (Varian) coupled to a CP2200 Ion Trap Detector (Varian). The fused silica capillary column (30 mm x 0.25 mm i.d. x 0.25 µm film) was coated with DBWax (Supelco) with splitless injection (220ºC) and oven temperature programmed from 40°C for 2 min then at 10°C/min to 240°C.

Statistics

For each bioassay, the number of weevils that entered the control capture containers compared to the treatment capture containers for each experiment was analysed using a binomial test.

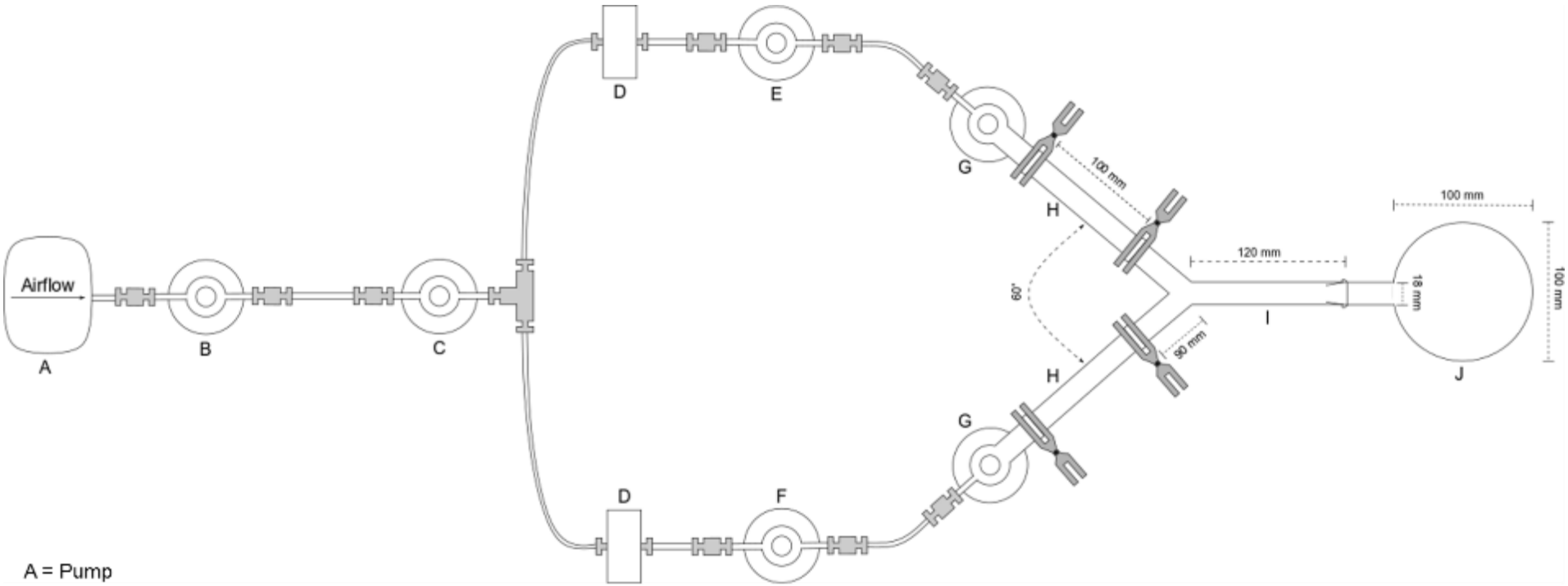


Figure 2.1. Illustration of the moving air olfactometer components, order and orientation of the olfactometer used (based on designs used by van Tol et al. (2002) and Karley et al. (2012) (illustration not drawn to scale).

Table 2.1. Biological material and plant volatiles used in vine weevil bioassays, including a description of the preparation of the material and the dilutions of the pure compounds.

Biological material			
	Description		
Euonymus fortunei	20 g, in chamber 1hr prior to experiment		
Taxus baccata	20 g, in chamber 1hr prior to experiment		
Conspecifics	40 weevils, starved 24hr, in chamber 1hr prior to experiment, responses from both starved and unstarved weevils tested		
Vine weevil frass	0.11 g, from weevils fed on *E. fortunei,* in chamber 1hr prior to experiment		
Plant volatiles from Karley *et al.* 2012*			
	10 mg/ml	**100 mg/ml**	**500 mg/ml**
Methyl salicylate	8.5 µl/ml	85 µl/ml	423 µl/ml
1-hexanol	12.3 µl/ml	123 µl/ml	614 µl/ml
(*Z*)-3-hexenol	11.8 µl/ml	118 µl/ml	590 µl/ml
(*E*)-2-hexenol	11.8 µl/ml	118 µl/ml	589 µl/ml
(*E*)-2-pentenol	11.8 µl/ml	118 µl/ml	590 µl/ml
Linalool	11.6 µl/ml	116 µl/ml	581 µl/ml
1-octen-3-ol	12 µl/ml	120 µl/ml	602 µl/ml
Blend 2	Blend prepared using 100 mg ml^{-1} of each of the compounds listed above + (*Z*)-2-pentenol.		
Plant volatiles from van Tol *et al.* 2012*			
	10 mg/ml	**100 mg/ml**	**500 mg/ml**
(*Z*)-2-pentenol	11.8 µl/ml	18 µl/ml	590 µl/ml
Methyl eugenol	9.7 µl/ml	97 µl/ml	483 µl/ml
Blend 1	Blend prepared using 100 mg ml^{-1} of each of the compounds listed above (with the exception of linalool).		

*****all compounds sourced from Sigma-Aldrich, Dorset, UK.

Cage trials – experiment 1

Six large tent cages (1.45 m x 1.45 m x 1.52 m) (Insectopia, UK) were set up within a polytunnel at Harper Adams University, each containing nine weevils to simulate a pest population. Five potted (12 cm diameter pots) strawberry plants (cv. Elstanta) were placed in each tent cage to simulate a susceptible crop. One pitfall trap (CSalomon®, Budapest, Hungary) was placed within each cage and baited with a dispenser suspended over the trap. Dispensers were supplied by NRI, University of Greenwich and comprised a 1 ml pipette tip containing a cigarette filter onto which 100 µl of the treatment compound was deposited. The pipette tip was then sealed with a crimp seal at the top, leaving the tip open to allow release of the volatile compounds. Two different lures (blends 1 and 2) were tested alongside a control treatment consisting of paraffin oil alone. Blend 1 comprised of plant volatiles from the paper by van Tol et al. (2012) and blend 2 comprised of plant volatiles from the paper by Karley et al., (2012) (Table 1). Treatments were randomly allocated to each cage and were re-randomised each day to exclude the effect of tent cage position, weevil population and/or simulated crop. The cages were assessed daily over five days and the number of weevils in the trap, cage area and plants was recorded at each assessment. Temperature in the 'tent' cages was verified using iButton (HomeChip, UK) data loggers

Cage trials – experiment 2

Four large tent cages (1.45 m x 1.45 m x 1.52 m) (Insectopia, UK) were set up within an unheated glasshouse at Harper Adams University, each containing 40 weevils to test the efficacy of the lure with a high pest population. Five potted (12cm diameter pots) strawberry plants (cv. Elstanta) were placed into two of the cages. Two trap types were used, a conical trap (Chemtica) and a pitfall trap (CSalomon®, Budapest, Hungary) which were placed into cages in pairs in a two by two factorial design. One trap of each pair was baited with plant volatile blend 1 (the best performing blend from bioassay trials) and the other with a control treatment of paraffin oil, using the same dispensers as described above. The cages were assessed daily over seven days and the number of weevils in the trap, cage area and plants was recorded at each assessment. Count data were log(x+1) transformed and a linear model was used to determine the effect of treatments with post hoc Tukey's HSD tests used to test for differences between individual treatment means. Temperature in the 'tent' cages was verified using iButton (HomeChip, UK) data loggers.

Results

Task 2.2 Potential of lures to improve monitoring of vine weevil adults

Surface hydrocarbon bioassay – Experiments 1 & 2

The results from the surface hydrocarbon bioassays are shown in Figure 2.2. Neither experiment showed a difference in weevil choice between the treatment and control arms.

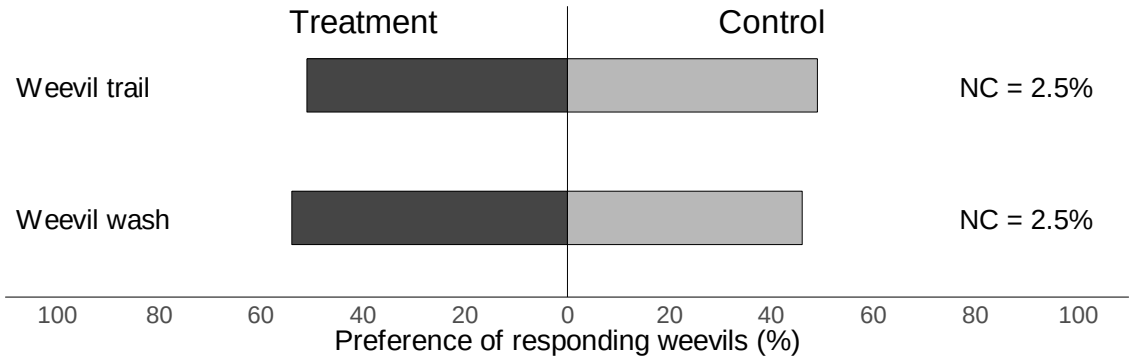


Figure 2.2. Percentage of weevils responding to either the treatment or control in a moving air olfactometer. Treatments shown are: trail made by live weevils (upper) and trail made from weevil hexane wash (lower) (n = 40 for both). NC indicates percentage of weevils that did not make a choice (no activity).

Weevil and plant volatiles bioassay

The results of the bioassays using biological samples are shown in Figure 2.3. Responding weevils showed a preference for *Euonymus fortunei*, *Taxus baccata*, conspecifics when the test weevils were not starved and weevil frass ($P < 0.001$ for all). Weevils showed no preference for conspecifics over the control when the test weevils were starved. No left/right bias was found in any of the experiments and weevils showed no response to paraffin oil over a blank control.

The results of the bioassays using plant volatiles at increasing concentrations of 10, 100 and 500 mg/ml are shown in Figure 2.4. At concentrations of 10 mg/ml, responding weevils showed no preference for any of the compounds, however 1-hexanol, (*Z*)-3-hexenol, (*E*)-2-hexenol, 1-octenol, and (*Z*)-2-pentenol all showed a significant repellent effect. No left/right bias was found in any of the experiments. At concentrations of 100 mg/ml, responding weevils

showed a preference for (*Z*)-2-pentenol and methyl eugenol. At this concentration, 1-hexanol, (*Z*)-3-hexenol, and linalool all showed a significant repellent effect. At concentrations of 500mg/ml, responding weevils showed a preference for 1-hexanol and (*Z*)-2-pentenol. At this concentration, methyl salicylate, (*E*)-2-hexenol, linalool, 1-octenol, and methyl eugenol all showed a significant repellent effect.

The results of the bioassays using blends of plant volatiles at concentrations of 10 mg/ml are shown in Figure 2.5. Responding weevils showed a preference for both blends ($P < 0.01$)

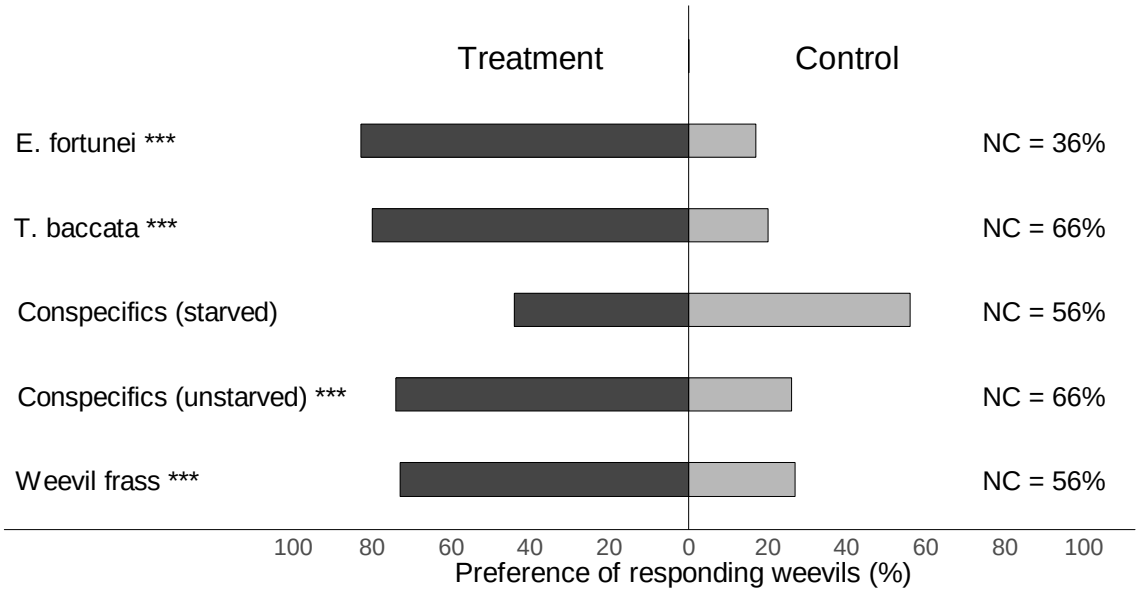
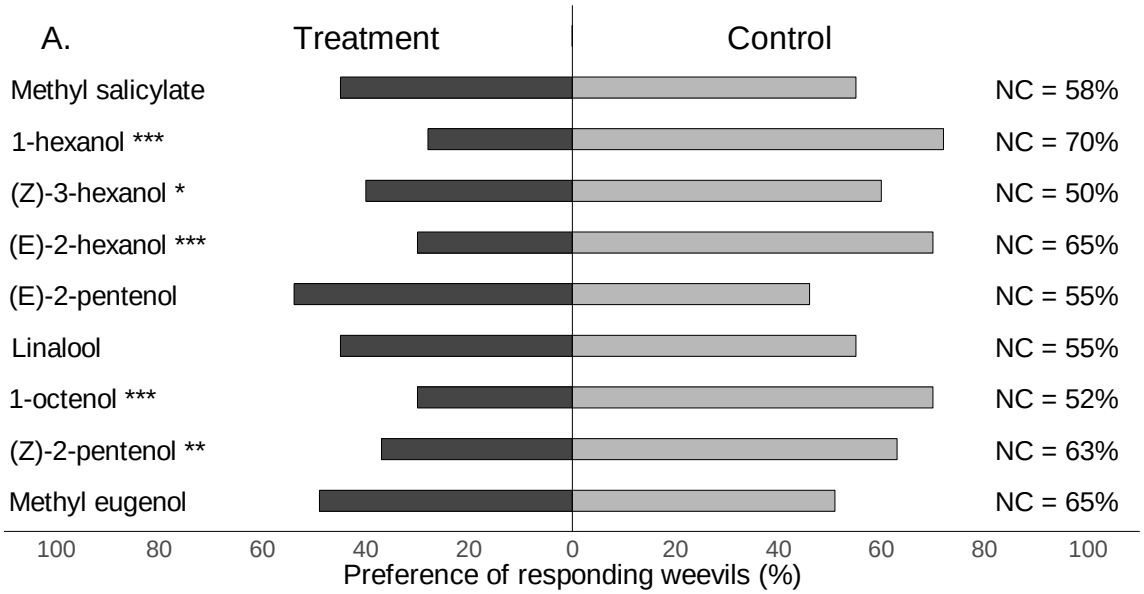
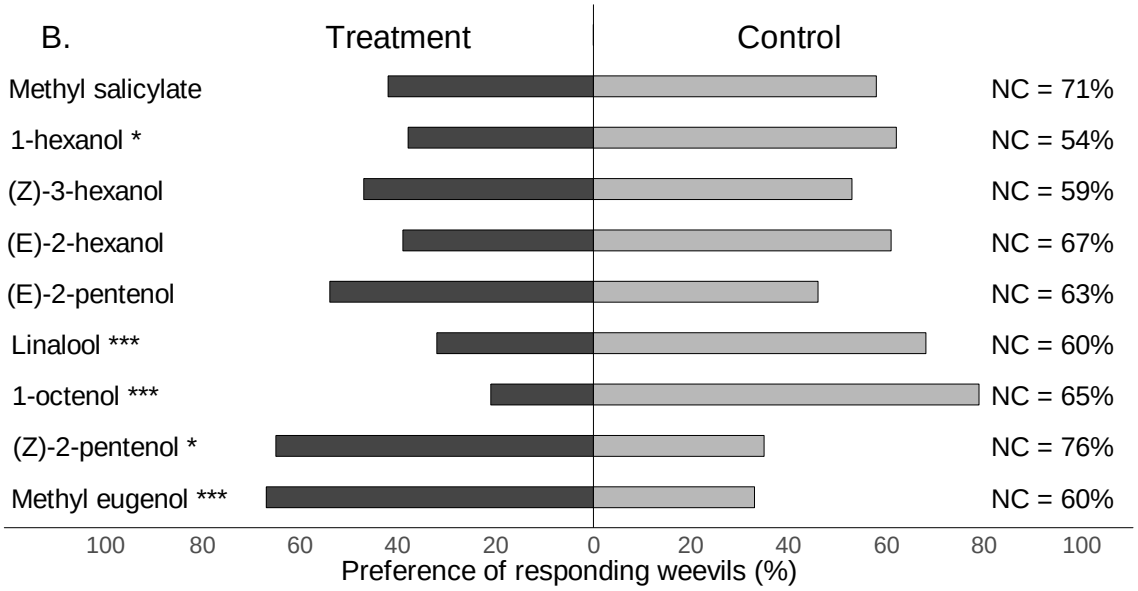


Figure 2.3. Preference and activity of vine weevils in a moving air olfactometer to odours of plant volatile compounds. Preference expressed as percentage of active weevils choosing either treatment or control arm. Treatments shown are (from top to bottom): 20 g *Euonymus fortunei*, 20 g *Taxus baccata*, 40 conspecifics (test weevils starved), 40 conspecifics (test weevils not starved) (n = 160 for all) and 0.11g weevil frass (n = 240). Asterisks indicate significant differences for each comparison at *P* = 0.05 (*), *P* = 0.01 (**) or *P* = 0.001 (***). NC indicates the percentage of weevils that did not make a choice (no activity).


A.
Treatment
Control
Methyl salicylate
1-hexanol ***
(Z)-3-hexanol *
(E)-2-hexanol ***
(E)-2-pentenol
Linalool
1-octenol ***
(Z)-2-pentenol **
Methyl eugenol
NC = 58%
NC = 70%
NC = 50%
NC = 65%
NC = 55%
NC = 55%
NC = 52%
NC = 63%
NC = 65%
100 80 60 40 20 0 20 40 60 80 100
Preference of responding weevils (%)


B.
Treatment
Control
Methyl salicylate
1-hexanol *
(Z)-3-hexanol
(E)-2-hexanol
(E)-2-pentenol
Linalool ***
1-octenol ***
(Z)-2-pentenol *
Methyl eugenol ***
NC = 71%
NC = 54%
NC = 59%
NC = 67%
NC = 63%
NC = 60%
NC = 65%
NC = 76%
NC = 60%
100 80 60 40 20 0 20 40 60 80 100
Preference of responding weevils (%)

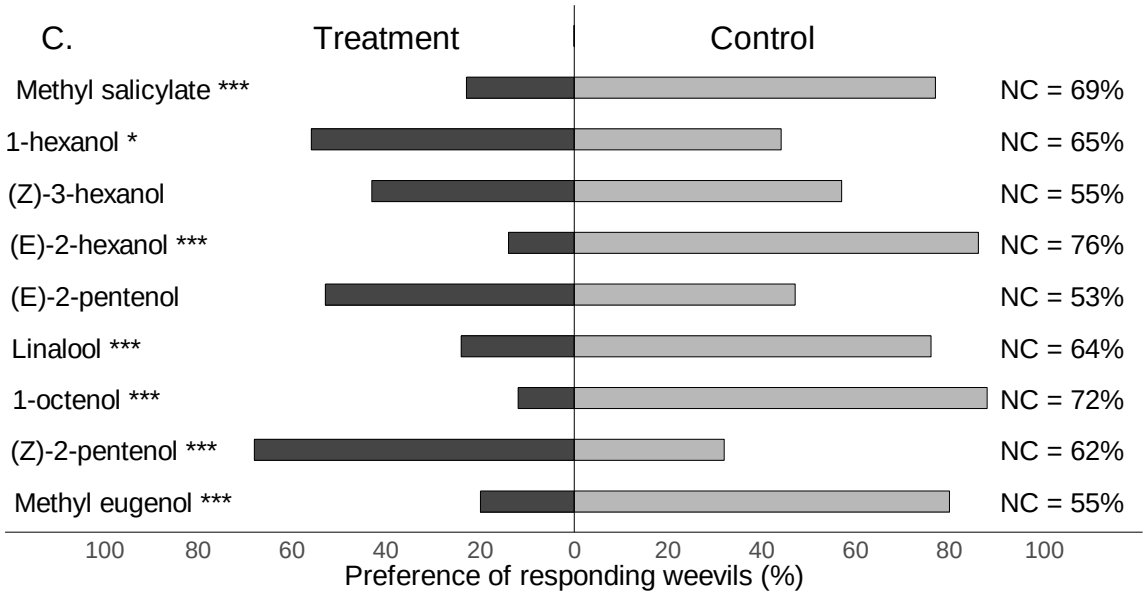


Figure 2.4. Preference and activity of vine weevils in a moving air olfactometer to odours of plant volatile compounds. Preference expressed as percentage of active weevils choosing either treatment or control arm. Treatments shown were at concentrations of **A.** 10 mg ml^{-1} **B.** 100 mg ml^{-1} and **C.** 500 mg ml^{-1} (n = 240 for all). Asterisks indicate significant differences for each comparison at P = 0.05 (*), P = 0.01 (**) or P = 0.001 (***). NC indicates the percentage of weevils that did not make a choice (no activity).

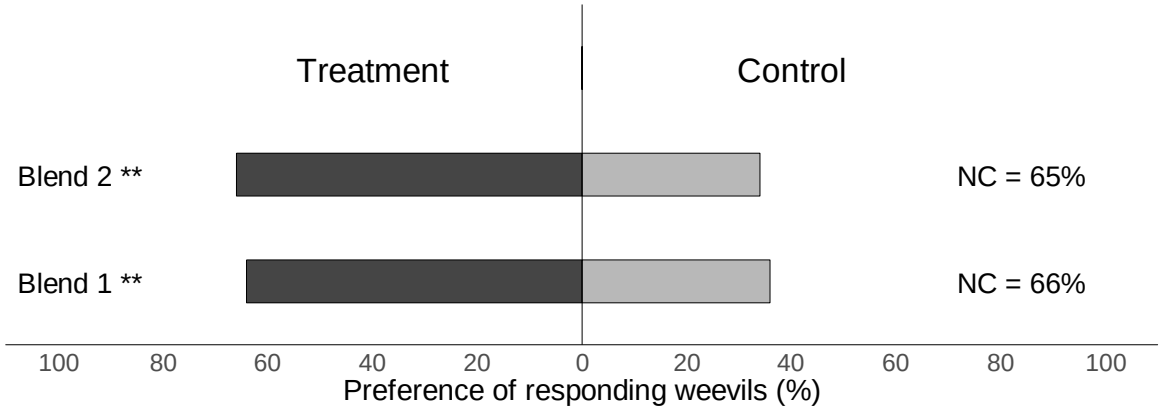


Figure 2.5. Preference and activity of vine weevils in a moving air olfactometer to odour combinations of compounds. Preference expressed as percentage of active weevils choosing either treatment or control arm. Blend 1: ((*Z*)-2-pentenol + methyl eugenol) and blend 2: ((*Z*)-2-pentenol + methyl salicylate + 1-octenol + (*E*)-2-hexenol + (*Z*)-3-hexenol + 1-hexanol + (*E*)-2-pentenol) prepared at concentrations of 100 mg ml^{-1} (n = 240 for both). Asterisks indicate significant differences from even distribution at P = 0.05 (*), P = 0.01 (**) or P = 0.001 (***). NC indicates the percentage of weevils that did not make a choice (no activity).

Analysis by Gas Chromatography linked to Electroantennography (GC-EAG) and Gas Chromatography linked to Mass Spectrometry (GC-MS)

In view of the significant attraction of vine weevils to volatiles from unstarved con-specifics (Fig. 2.3), volatiles were collected from unstarved vine weevils at Harper Adams by trapping on Porapak and elution with dichloromethane. These were analysed by GC-MS and GC-EAG at NRI.

In analyses by GC-MS of the collection with most material after concentration, the most prominent peaks were impurities from the Porapak adsorbent and phthalate plasticisers (Figure 2.6). Significant peaks of potential interest were two peaks due to alkyl chlorides, possibly from the dichloromethane solvent, and two peaks due to alkyl N,N-dimethylamines, possibly from the frass but also possibly surfactant impurities.

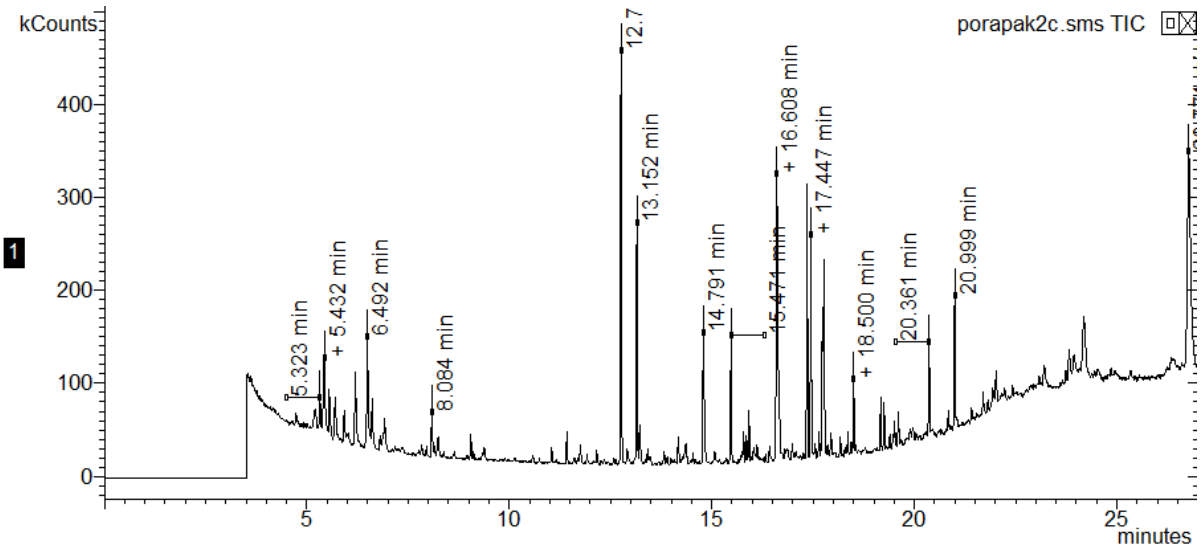


Figure 2.6. GC-MS Analysis of volatiles from unstarved vine weevils (polar GC column: Porapak impurities at 12.76, 13.15, 17.40 and 17.73 min; phthalates at 17.70 and 20.36 min; dodecyl and tetradecyl chlorides at 15.47 and 17.35 min; hexadecyl and octadecyl N,N-dimethylamines at 14.79 and 16.61 min)

In analyses by GC-EAG, preparations seemed to be more 'noisy' than in previous work. Nevertheless, good responses were obtained to 1-hexanol, (*E*)-2-hexenol as previously (Figure 2.5). Subsequent analyses of the collections of volatiles from unstarved weevils failed to show any consistent EAG responses (Figure 2.6). In some runs, 1-hexanol (10 ng) was added to confirm the EAG preparation was responding.

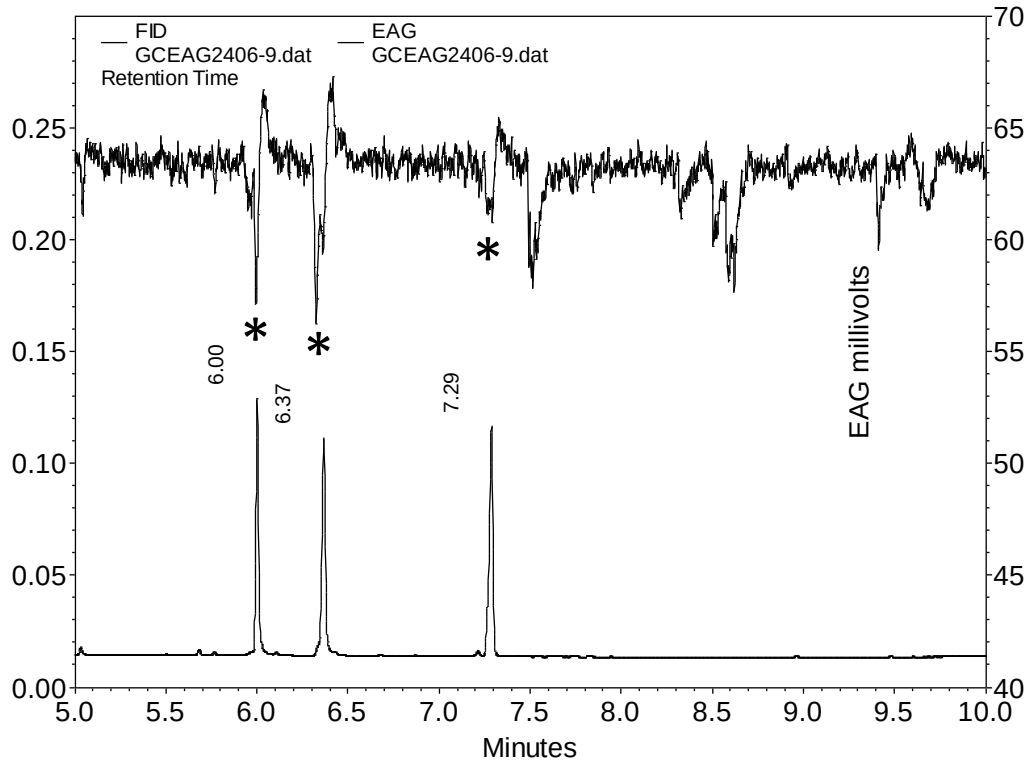


Figure 2.7. GC-EAG Analysis of showing EAG responses (*) to hexanol (6.00 min), (*E*)-2-hexenol (6.37 min), linalool (7.29 min) (polar GC column; 20 ng injected, 10 ng to EAG preparation; upper EAG trace, lower GC FID trace).

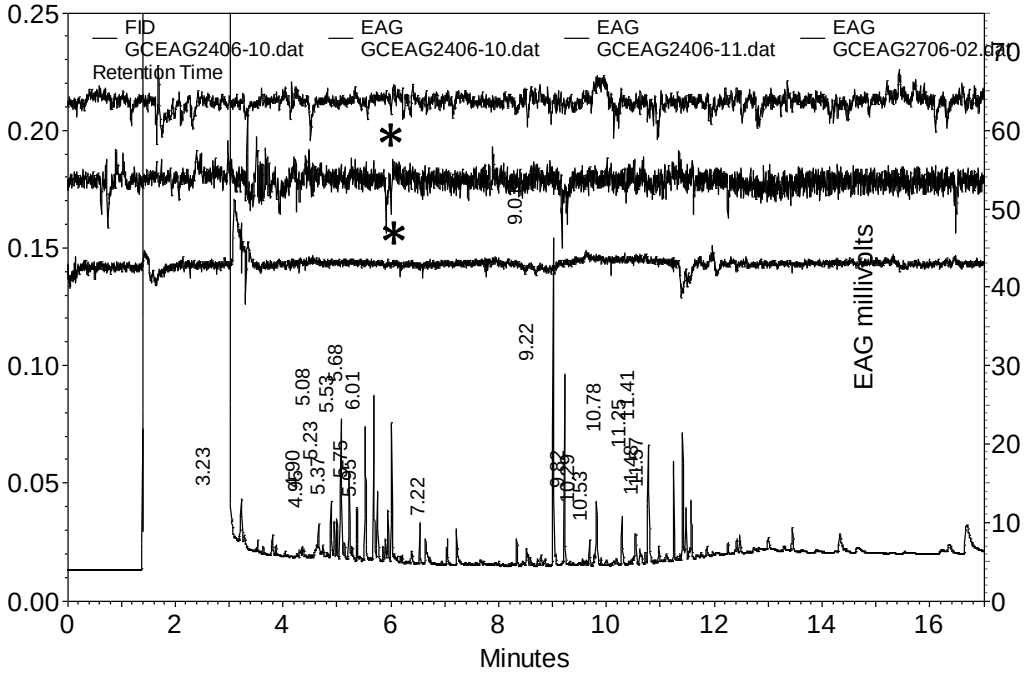


Figure 2.8. GC-EAG Analyses of volatiles from unstarved vine weevils (polar GC column; lower trace GC FID, upper traces three EAG traces from different weevils; * EAG response to 1-hexanol at 6.01 min added in first two runs)

Cage trials – experiment 1

Apart from one occasion, no weevils were found in any of the traps during this experiment therefore no statistical analysis was done with regards to the relative efficacy of the volatile compound blends as lure in a pitfall trap. Most weevils were found in or around the plants at the time of each count (Figure 2.9). The mean distribution of weevils within the cage environment for each treatment for the duration of the experiment is shown in Figure 2.10. The mean daytime (0630 to 1930 BST) temperature between 12th September and 17th September was 24.8°C (max = 28.3 °C, min = 23.1 °C) and the mean night-time temperature was 10.4°C (max = 13.0 °C, min = 8.5 °C).

Figure 2.9. Vine weevil adults aggregating underneath a plant pot (seen after moving the pot).

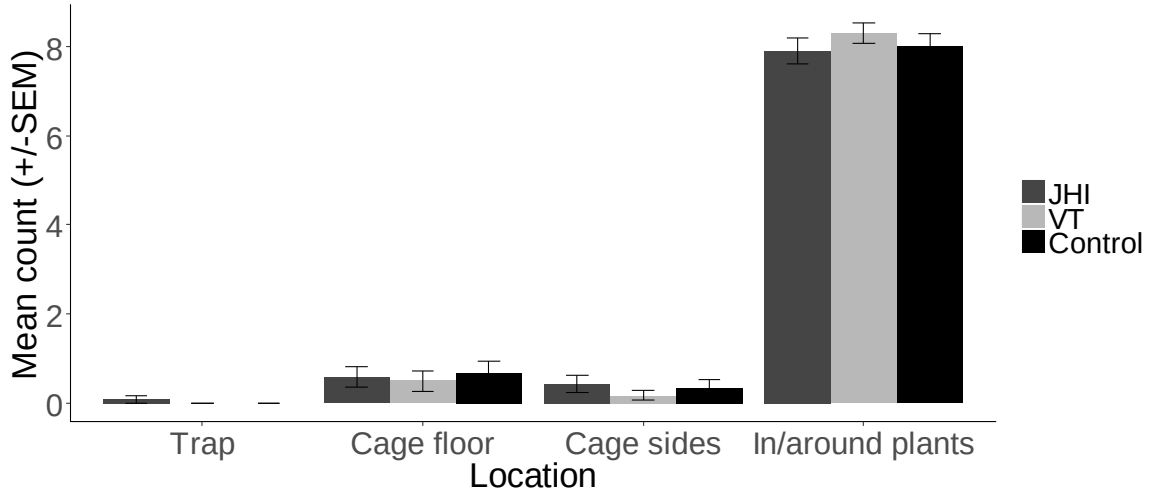


Figure 2.10. Mean count of weevils found in different cage locations for each lure treatment (± SEM) (nine weevils per cage). Lure treatments comprised of JHI (100 µl Blend 2), VT (100 µl Blend 1) and Control (100 µl Paraffin oil).

Cage trials – experiment 2

The results from the second cage trial are shown in Figure 2.11. More weevils were found in Chemtica traps compared to pitfall traps across all treatments ($F_{1, 116} = 6.46$, $P = 0.01$). There was no difference in numbers of weevils caught in traps baited with a lure compared to control traps in either trap type. The presence of plants had a significant effect of weevil location ($F_{3, 116} = 7.68$, $P < 0.001$) and reduced the numbers of weevils found in every location ($P < 0.001$ for all) as the majority of weevils were found in or around plants in these cages. The mean daytime (0800 to 1600 BST) temperature between 7th December and 13th December was 12.2°C (max = 17.3°C, min = 8.0°C) and the mean night-time temperature was 8.1°C (max = 10.2°C, min = 5.2°C).

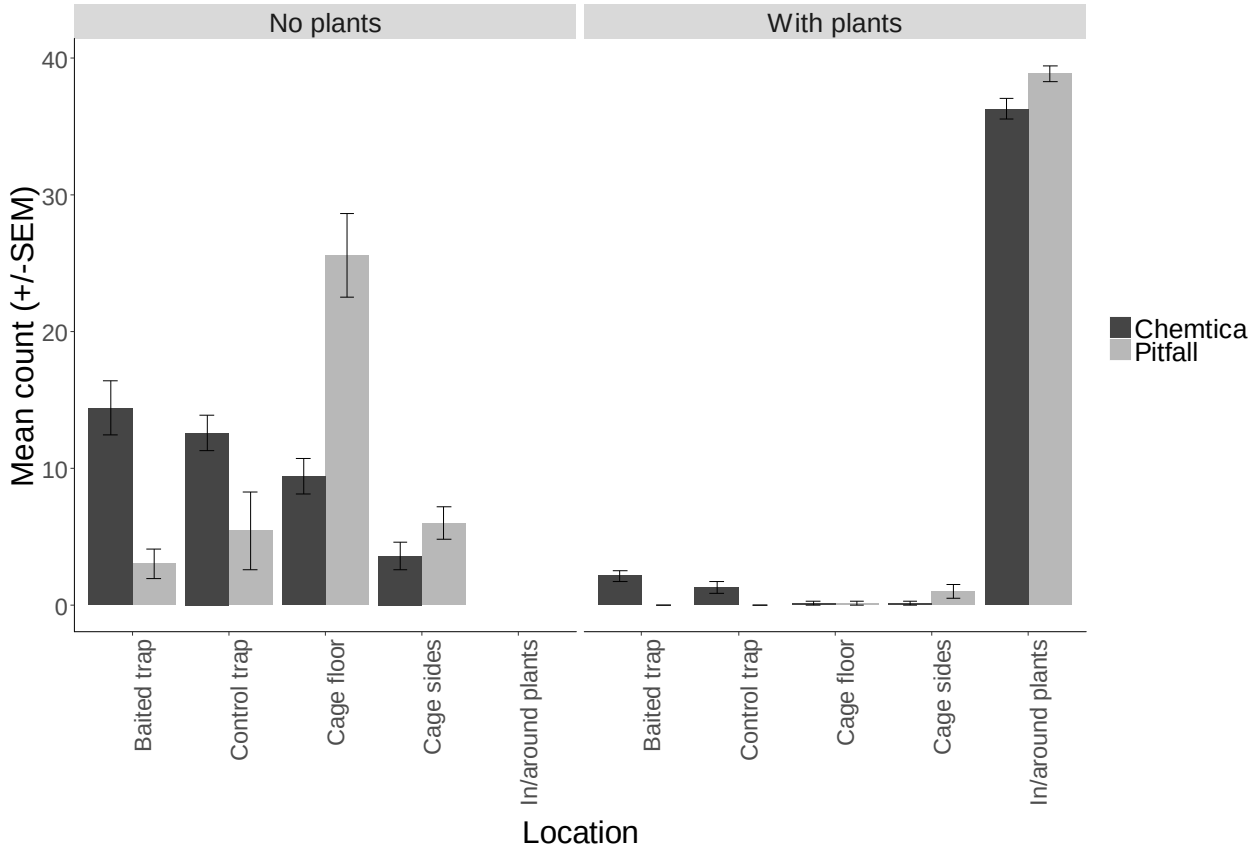


Figure 2.11. Mean count of weevils found in different cage locations for each trap type and plant treatment (± SEM) (40 weevils per cage). Trap types were Chemtica or pitfall traps, each cage contained one baited (with 100 µl Blend 1 lure) and one control trap of the same type. Cages either contained five strawberry plants or no strawberry plants.

Discussion

Given the aggregation behaviour of vine weevil, it is perhaps surprising that their cuticular lipids have apparently not been studied previously. Across many insect taxa, cuticular lipids, particularly hydrocarbons, have evolved to become part of their communication system acting as short-range/contact pheromones involved in species and sex recognition (Blomquist et al., 1998; Howard and Blomquist, 2005; Blomquist and Bagnères 2010; Prestwich and Blomquist 2014). Despite this, the results presented here found no evidence that cuticular hydrocarbons are important in trail-following behaviour by adult vine weevils. This is despite the fact that results from work completed at NRI, University of Greenwich in Year 1 of this project indicated that vine weevil cuticular hydrocarbons may be species specific.

Research on attractants for vine weevil adults has focused on potential aggregation pheromones produced by the live weevils, volatiles produced by host plants and weevil frass. Results presented here largely confirm previous research (see van Tol et al., 2002) by showing that vine weevil adults are strongly attracted to the odour of yew (*Taxus baccata*) and *Euonymus fortune*. Similarly, work presented here confirmed that vine weevil adults are attracted by the odour of weevil frass (see van Tol et al., 2004). Interestingly, van Tol et al. (2004) did not find vine weevil adults to be attracted to the odour of other vine weevil adults. In the present study, vine weevil adults were attracted by the odour of other vine weevil adults but only when the responding weevils had not been starved, suggesting that aggregation behaviour may be dependent on the physiological state of the weevils.

In HDC project SF/HNS 127 tests using a moving-air olfactometer showed that vine weevil adults are attracted by the odour of damaged strawberry leaves. Volatiles from *Euonymus fortune* and strawberry leaves were found to be dominated by "green-leaf" volatiles. A range of synthetic volatiles was tested by GC-EAG and reproducible EAG responses were recorded to (*E*)-2-pentenol, 1-hexanol, (*Z*)-2-pentenol, (*E*)-2-hexenol, (*Z*)-3-hexenol, 1-octen-3-ol, linalool and methyl salicylate. Occasional responses were also recorded to α-farnesene and to methyl eugenol. When candidate attractants were tested in the moving air olfactometer at a concentration of 100 mg ml^{-1} (*E*)-2-hexenol was attractive but 1-hexanol and (*Z*)-3-hexenol were repellent. In the present, however, (*E*)-2-hexenol was found to be repellent at concentrations 10 mg ml^{-1} and 500 mg ml^{-1} while at 100 mg ml^{-1} this compound did not appear to affect weevil behaviour. There was, however, agreement between these two projects with (*Z*)-3-hexenol repelling weevils at both 10 mg ml^{-1} and 100 mg ml^{-1}. For 1-hexanol, this compound repelled weevils at both 10 mg ml^{-1} and 100 mg ml^{-1}, but at 500 mg ml^{-1} weevils were attracted to the same compound, clearly indicating the importance of concentration in determining insect responses. Of the other compounds highlighted in SF/HNS 127, weevils either did not respond to or were repelled by the plant volatiles presented. The exception to this were (Z)-2-pentenol and methyl eugenol, which both attracted weevils when presented at a concentration of 100 mg ml^{-1} and (Z)-2-pentenol, which was attractive at 500mg/ml.

The olfactometry results for (Z)-2-pentenol and methyl eugenol build on the results reported by van Tol et al. (2012). In their work, van Tol et al. (2012) collected volatiles and extracts from a preferred host-plant, *Euonymus fortunei*, and analysed these by gas chromatography (GC) coupled to EAG recording from the weevil antenna. Eight compounds which elicited EAG responses were identified. The three most active compounds were not tested in an olfactometer by van Tol et al. (2012) but were tested in field trapping experiments using boll weevil traps. No weevils were caught in the traps, but more weevils were found within a radius

of 60 cm of traps containing (Z)-2-pentenol with or without methyl eugenol than round traps containing (*E*)-2-hexenol alone or mixed with the other two compounds. These results formed the basis of a patent application.

The two blends of plant volatiles tested in this study demonstrate the importance of combinations of plant volatiles in determining whether insects are attracted or repelled (Bruce & Pickett, 2011). This may include both the combination of volatiles in a blend as well as the ratios between these constituent parts. The blend of (Z)-2-pentenol and methyl eugenol was found, like the individual components, to be attractive to weevil adults. The blend of plant volatiles identified in SF/HNS 127 was also found to be attractive to vine weevil adults, despite the fact that 1-hexanol and (*Z*)-3-hexenol repelled vine weevil adults when presented individually and the other components of the blend, elicited no behavioural response.

Results from the two cage experiments found that the addition of either blend to the Chemtica or pitfall traps did not increase trap catches. Indeed, in cages where plant pots were present, most weevils were found beneath these pots. This result is similar to that reported by van Tol et al. 2012, who similarly failed to increase trap catches with the addition of a lure based on plant volatiles. These results indicate that further work is required to optimise the composition of a lure to attract vine weevil adults, particularly in the presence of attractive crop plants. In addition, additional work is required to more effectively combine a lure with a trap or some other crop protection tool.

Conclusions

- Vine weevil adults are attracted to host plant volatiles and to the odour of frass produced by other weevils.
- Vine weevil adults are attracted by the odour produced by other vine weevils but only when not starved.
- Vine weevil adults are attracted by the plant volatiles (*Z*)-2-pentenol and methyl eugenol as well as to two simple blends of plant volatiles.
- There is no evidence that the addition of a lure based on plant volatiles increases catches of vine weevil adults.

Objective 3. Improve best-practice IPM approaches including the use of entomopathogenic nematodes, fungi and IPM-compatible insecticides

Task 3.1. Alternative application method for entomopathogenic nematodes (ADAS, years 1 and 2)

Objective

The aim of this task was to provide growers with an alternative, less time-consuming and more cost-effective application method than using drenches for reliable control of vine weevil larvae with entomopathogenic nematodes. The method was be based on a 'little and often' approach for maintaining control of vine weevil larvae through the season, applying reduced rates of entomopathogenic nematodes through overhead irrigation. The work was be done over two years, in the first year the method was tested and nematode dose rates compared in a research polytunnel at ADAS Boxworth and the results are reported in the first annual report. In the second year the method and results were validated on a commercial nursery.

Task 3.1.2 Experiment testing little and often nematodes on commercial nursery (year 2).

Materials and methods

Site

Polytunnel at Darby Nursery Stock, Methwold Hythe, Thetford, Norfolk.

Experimental plants

Young fuchsia plants were potted into 2 litre pots using untreated growing media at Darby Nursery Stock on 15 May 2017. Sixty pots of each of four fuchsia varieties were used in the trial. The varieties were Riccartonii, Mrs Popple, Hawkshead and Tom Thumb. Additional pots of var. Snowdrop were also potted up for use as indicator plants to monitor vine weevil development and as 'sacrificial' plants to measure nematode application to the growing media.

Treatments

The treatments are shown in Table 3.1. Nematode rates, timings and application methods were the same as in the pilot experiment at ADAS Boxworth in year 1. However, unlike in the pilot experiment when *Steinernema kraussei* (Nemasys L) was used for all the nematode applications, *Heterorhabditis bacteriophora* (Nemasys H) was used for the first four applications and Nemasys L was used only for the final applications in October. This is consistent with commercial practice as *S. kraussei* (recommended temperatures 5-30°C) is more expensive than *H. bacteriophora* products (recommended temperature range 12-33°C depending on product e.g. Nemasys H 12-30°C).

Table 3.1. Treatments

Treatment number	Treatment	Application method and water volume	Timing	Total number nematodes applied
1	Nemasys L full rate (500,000 per m^2)	Drench in 200 ml per 2L pot	2 applications (14 Sep, 11 Oct)	1,000,000 per m^2
2	Nemasys L full rate (500,000 per m^2)	Overhead irrigation in 4L water per m^2	2 applications (14 Sep, 11 Oct)	1,000,000 per m^2
3	Nemasys H 40% rate (x 4) and Nemasys L 40% (x 1) (200,000 per m^2)	Overhead irrigation in 4L water per m^2	5 applications (21 June, 19 July, 16 August, 14 Sep, 11 Oct)	1,000,000 per m^2
4	Nemasys H 20% rate (X 4) and Nemasys L 20% (X 1) (100,000 per m^2)	Overhead irrigation in 4L water per m^2	5 applications (21 June, 19 July, 16 August, 14 Sep, 11 Oct)	500,000 per m^2
5	Water control	Drench 200 ml per pot	2 applications (14 Sep, 11 Oct)	0
6	Water control	Overhead irrigation in 4L water per m^2	5 applications (21 June, 19 July, 16 August, 14 Sep, 11 Oct)	0

Experimental design

After potting into 2 L pots, the 240 Fuchsia plants (60 of each of the four varieties) were laid out in the 'holding bay' in a randomised block design using two plants of the same variety per

plot (plants A and B) and 20 replicate plots (five plots for each variety) per treatment (Fig. 3.1 and Appendix 1). In each of the 120 plots, in addition to the two plants, there was an empty 2 L pot with a plastic plant pot saucer glued to the inside of the base of the pot to block off the drainage holes. These pots were used to measure water and nematode application rates in each plot.

In addition to the 240 experimental plants, 12 additional 'spare' plants (three of each of the four varieties) were infested with vine weevil eggs at the same time as the trial plants and used to monitor vine weevil larval development during the experiment period in order to help decide on numbers of eggs to infest the plants with and when to carry out the final destructive assessment. An additional eight plants (two of each of the four varieties) were not infested with vine weevil eggs and were used as a comparison to calculate percentage root damage in the trial plants. All these additional plants were kept in the holding bay alongside the trial plants.

Figure 3.1. Experiment layout in holding bay in polytunnel

Plant husbandry

The pots were watered twice per day for five minutes on each occasion through the overhead irrigation. No other pests other than fuchsia flea beetle were observed on the trial plants throughout the trial. The grower applied deltamethrin (Decis) for control of flea beetle on 1 August.

Vine weevil egg infestation

Vine weevil adults were collected from the control fuchsia plants used in the pilot experiment in year 1. These plants were kept in insect-proof cages in a polytunnel at ADAS Boxworth over the winter of 2016/2017. Freshly emerged vine weevil adults were collected during April after collecting pupae from the growing media and transferring these into plastic boxes of growing media kept in a controlled temperature laboratory at 21°C. The adults were maintained in plastic boxes on damp tissue with sprigs of yew as food in the same controlled temperature laboratory.

Each experimental plant and the 12 extra plants to monitor vine weevil larval development were infested with 10 brown (embryonated) eggs per plant on 6 June, 5 July, 2 and 30 August to mimic the natural vine weevil egg laying period. Therefore each plant was infested with a total of 40 eggs during the experimental period. On each infestation date, the required number of brown eggs were collected from the vine weevil culture using a fine paintbrush and transferred to pieces of damp filter paper, using ten eggs per filter paper and one filter paper per plant. A small area of the topmost layer of growing media next to each plant was removed and the eggs were washed onto the growing media. The eggs were then covered lightly with damp growing media. Eggs were applied to all the control treatments first in order to avoid transferring any nematodes to untreated pots.

Vine weevil egg viability

Percentage egg viability was determined by collecting 100 additional brown eggs from the culture on each of the four infestation dates and assessing how many hatched in the laboratory.

Calibration of water application through sprinklers

Nematode application was done in the ‘treatment bay’ opposite the ‘holding bay’ for the experimental plants in the polytunnel. Prior to nematode application, the volume of water applied through the overhead sprinklers was calculated in the area below the overhead irrigation. The test area was laid out in a grid with 81 plastic plant pot saucers (27.5 cm diameter) spaced 45 cm from centre to centre in nine rows of nine (Fig. 3.2). Each of the 81 locations in the grid were labelled 1-9 from left to right and A-I from top to bottom. The

Figure 3.2. Plant pot saucers used to measure water application through the overhead sprinklers in the 'treatment bay'. Area outlined in red is the selected treatment area. C4, F5, and I6 are three randomly selected pots to assess nematode delivery

overhead irrigation was run for ten minutes in order to apply 4 L per m^2 water. The water in each saucer was then collected and the volume measured. This was replicated three times. The results demonstrated a high variability in water volume applied throughout the bay especially towards the edges. Thus it was decided to select a representative treatment area in the treatment bay rather than using the most areas where water volumes were most accurate as in the pilot trial in year 1, so that the area represented what a grower would do in commercial practice. The treatment area selected was a 3 x 7 area in the centre of the bay (Fig. 3.2).

Calibration of nematode application through sprinklers

A pack of 50 million Nemasys H was used to assess how many nematodes were applied to the treatment area below the overhead irrigation. A 'stock' suspension of nematodes was mixed using one pack mixed with 4 L water to give the full rate to use in the feeder bucket for the Dosatron®. This stock suspension was then diluted with water to give the 40% and 20%

rates for use in the feeder bucket for the application of the reduced rates of Nemasys H or L (Table 3.2).

Table 3.2. Numbers of nematodes and water volumes added to feeder bucket for Dosatron® to make up the stock suspensions of Nemasys H or L at the three dose rates applied.

Nemasys L or H rate	Numbers of nematodes added	Volume of water added	Nematode concentration in feeder bucket
100%	1 pack of Nemasys L or H	4 L water	50 million (12,500 nematodes per ml)
40%	1600ml of the 100% rate	2400 ml water	20 million (5,000 nematodes per ml)
20%	800ml of the 100% rate	3200 ml water	10 million (2,500 nematodes per ml)

Each rate of Nemasys H or L was delivered through the sprinklers at 4 L/ha using a Dosatron® set at a 1% feed intake rate. There were no filters in the sprinkler nozzles but the filter on the outside of the Dosatron® was removed to avoid nematode blockages and to allow the nematodes to move freely through the system. Before assessing the numbers of nematodes applied to the treatment area, each of the three rates were applied through the Dosatron® and overhead irrigation for five minutes to allow the nematode suspension to travel through the irrigation line and to start being applied through the sprinklers. Nematodes were shown to travel from the stock suspension to the sprinklers in a minimum of two minutes but the system was ‘over primed’ for 5-8 minutes as irrigation on use elsewhere on site on the treatment dates could have an impact on water pressure and in turn affect nematode flow rate through the system. The nematode suspension was kept agitated during application to prevent settling out.

After the system had been primed, the irrigation was turned off and plastic pots (85 mm diameter) were placed into the treatment area (Fig. 3.3). The pots had been fitted with plastic plant pot saucers glued to the inside of the base to seal off the drainage holes. The sprinkler system was then run for 10 minutes for each of the three nematode rates, using fresh

collection pots for each rate. Samples (25ml) were taken from three randomly selected pots (C4, F5 and I6) in the top, middle and bottom rows. Application of each nematode rate was replicated three times as the numbers of nematodes delivered were found to be very inaccurate. The grower's Dosatron® was replaced with an ADAS Dosatron® and this led to much more accurate numbers of nematodes being delivered. The reason for this difference in nematode delivery between different Dosatrons® is unknown but the grower's was a newer model than the ADAS one.

The collection pots were taken to the laboratory for assessment of nematode numbers per ml applied through the system. One ml of the nematode suspension was taken from each pot using an Eppendorf pipette and transferred to a 'Doncaster' counting dish and examined under a low power binocular microscope. Numbers of active nematodes per ml were recorded. This procedure was replicated three times for each collection pot.

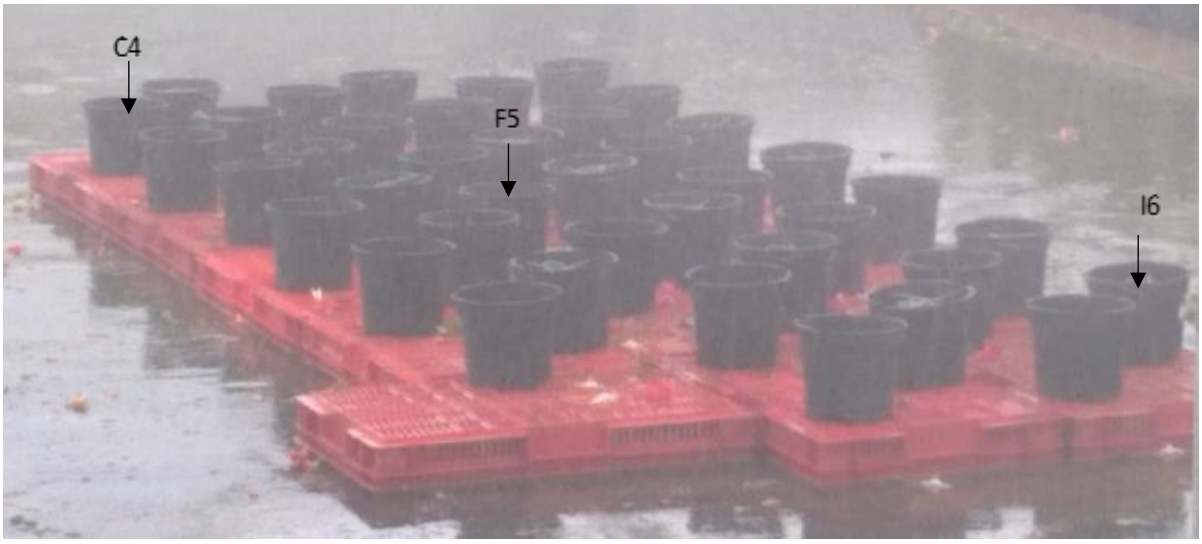


Fig. 3.3. Plastic pots used to assess numbers of nematodes applied through overhead irrigation. C4, F5, and I6 are three randomly selected pots.

Treatment application to experimental plants

Treatment application dates are shown in Table 3.1. The plants to be treated with each of the treatments applied through the overhead irrigation (Treatments 2-4 and 6) were moved to the treatment area into the bay opposite the holding bay for the experimental plants. One treatment was applied at a time after placing the plants into the same randomised design as in the holding area, below the sprinklers. Treatments were applied between 9 am and 4 pm on each treatment date.

A fresh 50 million pack of Nemasys H or Nemasys L was used to apply all nematode rates (Treatments 1-4) on each application date. The nematodes were made up according to the manufacturer's recommendations. For the nematode treatments applied through the sprinkler (Treatments 2-4), the same procedure was used as that used to calibrate the nematode application through the sprinkler. The pack contents were first added to a small amount of water and mixed to a paste, then the required amount of water was added to make the nematode suspension in the feeder bucket for the Dosatron®. To confirm the numbers and viability of the nematodes, three replicate 1 ml samples of the nematode suspension for each of Treatments 1-4 were taken and transferred to a Hauxley haemocytometer slide in the laboratory. The samples were examined under a binocular microscope and numbers of active nematodes per ml were recorded. All other manufacturer's recommendations were followed during application.

All nematode applications made through the overhead irrigation (Treatments 2-4) and the water control (Treatment 6) were applied to the plants at 4 L/ha in sessions ranging from 8-11 minutes depending on water pressure at the site on the day. The water pressure was calculated by collecting water, using a measuring cylinder, from one of the overhead sprinklers for 60 seconds. From this it could be calculated how long the system needed to be run for to achieve the intended 83.5 ml per pot (equivalent to 4 L/ha) for the application rate. Following application, as recommended by the nematode supplier, water was applied using a backpack sprayer and lance at 750 L/ha to wash any nematodes remaining on the leaves into the growing media.

During nematode application of each treatment through the overhead irrigation, empty pots (with a plastic plant pot saucer glued to the inside of the base to seal off the drainage holes) with a 'sacrificial' fuchsia (cv. Snowdrop) plant placed inside were placed in four randomised locations (in plots 1, 10, 15 and 20) in the treatment area to collect nematodes in order to check how many reached the growing media (Figure 3.4). Ideally replicate plants of each of the same four varieties used in the trial would have been used for this purpose but insufficient were available so cv. Snowdrop was used instead. The sacrificial plants had been cut at the base of the stem and secured inside the pot using wire so that the plant was in a similar position as those in the trial pots. Following nematode application a sub sample of 25 ml was taken from each nematode collection pot and the samples were taken to the laboratory at ADAS Boxworth where the volume of nematode suspension and numbers of nematodes per ml were assessed, compared with the numbers expected in the collection pots for each of the three application rates.

Fig. 3.4 ‘Sacrificial’ plant (cv. Snowdrop) secured in collection pot used to check nematode numbers and water volumes that would have reaching the growing media when applied through overhead irrigation.

The nematode treatment and water control applied as a drench (Treatments 1 and 5) were applied in 200 ml water per 2 L pot as this was consistent with the volume most growers apply drenches (10% of pot volume). The drench was applied to each pot using a backpack sprayer and lance held close above the crown of each plant after checking that the growing media was moist to enable the drench to be absorbed.

Destructive plant assessments

The experimental plants were destructively assessed between 27 November and 7 December. The growing media in each pot was searched for live vine weevil larvae and numbers per pot were recorded. The roots of each plant were then washed over a sieve to collect and remaining vine weevil larvae and the roots were assessed for percentage root area damaged by vine weevil larvae, using uninfested plants of each variety as examples of no root damage for comparison (Fig. 3.5).

Figure 3.5. Uninfested fuchsia plants with no vine weevil damage to roots (right) were used to score infested plants for percentage root damage (30% damaged, left).

Growing media temperatures

Growing media temperatures were monitored during the experiment period using two data loggers buried in the growing media in two of the spare Fuchsia pots. The loggers were removed for downloading and replaced on 2 August.

Statistical analysis

The data on vine weevil larvae numbers and root damage were subjected to analysis of variance (ANOVA) using Genstat 14th Edition. The data on collected nematode suspension volumes was analysed using a t-test. The correlation between numbers of larvae and root damage scores was done using Regression Analysis using Genstat.

Results

Vine weevil egg viability

Vine weevil egg viability tests showed that 92%, 98%, 91% and 93% of the eggs successfully hatched on the four infestation dates respectively.

Calibration of water application through sprinkler

As explained in the methods, following measurement of the water volumes collected in the replicate saucers, it was decided to use a central treatment area in the treatment bay (Fig. 3.2) as the representative area of a typical commercial setting. It was calculated that for accurate delivery of 4 litres/m^2 238 ml should be collected in each saucer during the time period the sprinkler was used. When a nematode suspension was delivered at 4 litres/m^2 the greatest recorded variation in volume in this area was +/- 18.59% over three replicate irrigation runs (Table 3.3).

Table 3.3. Mean volumes of water (ml) applied to three replicate pots in each of the 20 grids in the treatment area over an 8.5-minute period of the 10-minute irrigation run. Grids coloured green delivered the least variable water volumes and within 5 %(+/-) of the expected 238 ml, grids coloured yellow delivered slightly more variable water volumes (+/- 10 % of 238 ml), grids coloured orange delivered +/- 15 % and grids coloured red delivering the most variable water volume (+/-25%). The blue grid never correctly delivered the 238 ml during the test applications.

		4	5	6
C	Ml Measured (Mean of 3 runs)	229.67	244.87	241.33
	Variability (+/-) on Ml	17.00	40.00	44.00
D	Ml Measured (Mean of 3 runs)	234.40	214.27	247.20
	Variability (+/-) on Ml	6.80	24.00	11.60
E	Ml Measured (Mean of 3 runs)	255.33	239.80	267.20
	Variability (+/-) on Ml	19.00	3.40	28.40
F	Ml Measured (Mean of 3 runs)	250.40	221.67	243.27
	Variability (+/-) on Ml	38.80	1.00	25.00
G	Ml Measured (Mean of 3 runs)	258.07	229.20	262.13
	Variability (+/-) on Ml	34.00	20.40	38.60
H	Ml Measured (Mean of 3 runs)	242.27	246.00	267.20
	Variability (+/-) on Ml	37.20	54.00	33.40
I	Ml Measured (Mean of 3 runs)		261.40	263.80
	Variability (+/-) on Ml		40.20	38.60

Calibration of nematode application through sprinklers

As explained in the methods, initial mean numbers of nematodes per ml collected in the pots placed in the three locations in the treatment area (C4, F5 and I6) were all below the desired amount when the grower's Dosatron® was used. With Nemasys H and Nemasys L, means of only 46% and 33% of the expected number were delivered respectively. After changing the dosing unit, numbers were much closer to expected (Table 3.4). Delivery rate was then highly accurate at all application rates and in all locations, with mean delivery rates being at least 99% of the expected.

Table 3.4. Mean numbers of Nemasys H per ml collected in the pots in the three selected grid locations and mean numbers of expected nematodes if they were delivered accurately in 4L/ha water.

Location	20% rate	40% rate	100% rate
	mean of 3 counts per ml	Mean of 3 counts per ml	Mean of 3 counts per ml
C4	23	50.7	128.3
F5	23	49	129
I6	23.7	52.3	122
Mean	23.23	50.7	126.4
Expected	**25**	**50**	**125**

Nematode application to the growing media

Mean numbers of nematodes per ml collected in the empty pots placed next to the treated pots and in the 'sacrificial plant' pots at each of the three rates applied through the overhead irrigation are presented in Figure 3.6. The data presented are means of all the applications during the trial (five for the 20% and 40% rates and two for the 100% rate).

The observed numbers of nematodes per ml were very close to expected in the 20% and 40% rates. In the 20% rate, the expected mean numbers were 25 per ml and the observed mean numbers were 24 and 23.6 per ml in the empty pots and 'sacrificial plant' pots respectively. In the 40% rate, the expected mean numbers were 50 per ml and the observed mean numbers were 49.1 and 48.3 per ml in the empty pots and 'sacrificial plant' pots respectively. The observed mean numbers of nematodes per ml (98.7 and 96.9 in the empty pots and 'sacrificial plant' pots) were less close to those expected (125 per ml) in the 100% rate, which equated to 79% and 77% of the expected nematodes were delivered to the pots respectively.

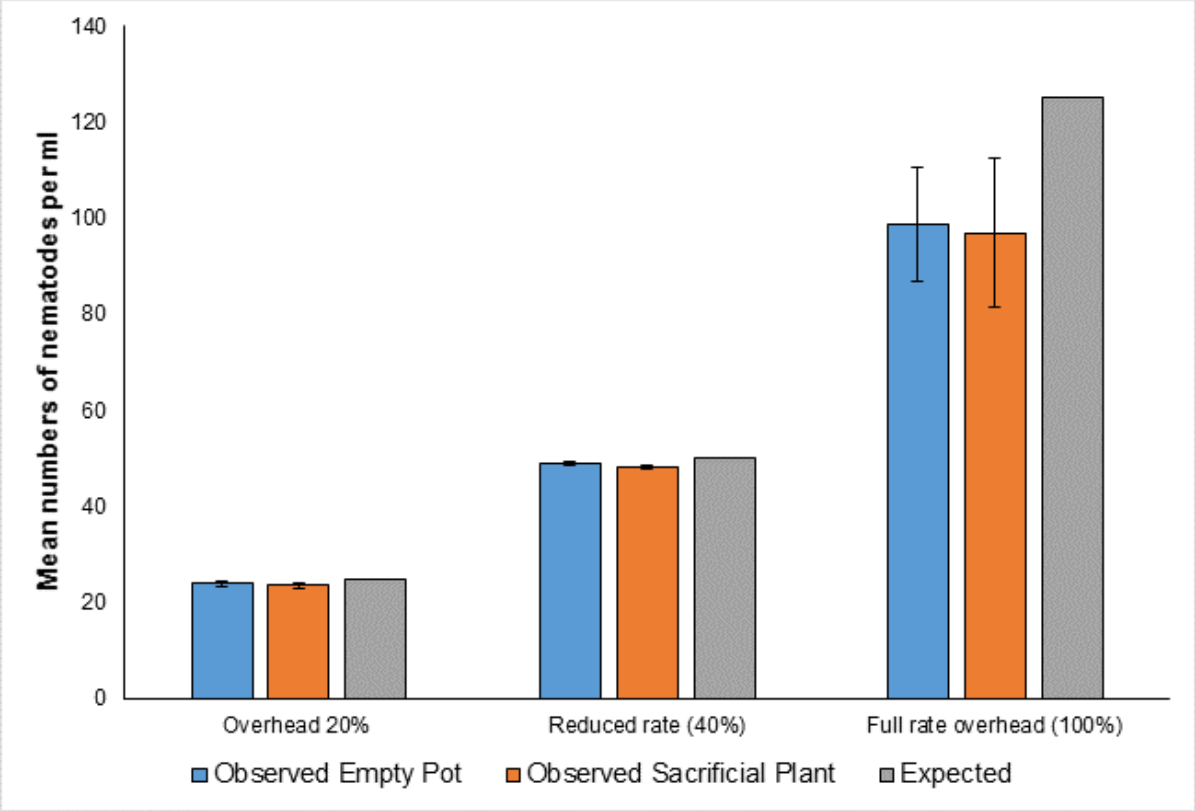


Figure 3.6. Mean numbers of observed and expected nematodes per ml collected in the empty pots and pots with 'sacrifical plants' during application through the overhead irrigation. Means of all applications (five for the 20% and 40% rates and two for the 100% rate) +/- SE.

The observed mean nematode suspension volumes collected in the empty pots placed next to the treated pots and in the 'sacrificial plant' pots at each of the three rates applied through the overhead irrigation, are presented in Figure 3.7. The data presented are means of all the applications during the trial (five for the 20% and 40% rates and two for the 100% rate).

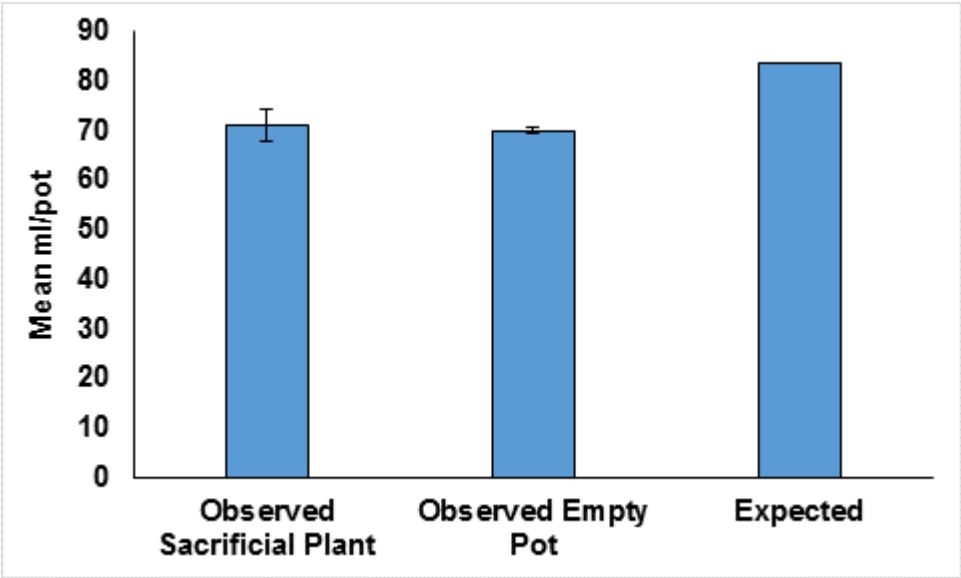


Figure 3.7. Mean volumes of nematode suspension (ml) expected and observed in the empty and 'sacrificial plant' pots during application through the overhead irrigation. Means of all applications (five for the 20% and 40% rates and two for the 100% rate) +/- SE.

The expected mean volume was 83.6 ml. The observed volume for the empty and 'sacrifical plant' pots were 70.1 and 71.3 ml respectively, which equated to means of 85% and 84% of the expected volumes being delivered.

The mean nematode nematode suspension volumes collected in the empty pots during overhead application of the three nematode rates to each Fuchsia species are shown in Table 3.5. The only volumes that were statistically equal to the expected numbers were those for the 100% rate applied to Mrs Popple, Hawkshead and Tom Thumb. Volumes applied by all other nematode rates to all varieties were significantly less than those expected.

Table 3.5. Mean volumes of nematode suspension collected in the empty pots for each species compared to the expected volume. * significantly equal to expected volume (P<0.05).

Treatment	Expected ml of nematode suspension	Riccartonii	Mrs Popple	Hawkshead	Tom Thumb
20% rate overhead	83.56	68.76	63.36	67.84	74.68
40% rate overhead	83.56	66.24	71.12	68.16	71.24
100% rate overhead	83.56	66.9	77.8*	79.2*	75.5*

Numbers of vine weevil larvae – analysis of the four species combined

Despite mean volumes of nematode suspensions delivered to the empty pots and the pots with 'sacrificial plants' being significantly lower than those expected for the 40% and 20% rates on all varieties and for the 100% rate on Riccartonii, all the nematode treatments gave significant reductions of vine weevil larvae compared with both the water controls (P<0.001) (Figure 3.8 and Table 3.6). The full rate drench (mean 1.9 larvae) and the 40% rate overhead (mean 2.8) were equally effective. Mean numbers of larvae in the full rate drench (1.9) were significantly lower than in both the full rate overhead (4.0) and the 20% rate overhead (4.0) (P<0.05). Mean numbers of larvae in the 40% rate overhead were not significantly different to those in both the full rate overhead and the 20% rate overhead.

Mean numbers of larvae per pot in the water controls applied as a drench and through the overhead were 7.3 and 6.7 respectively and were not significantly different from each other.

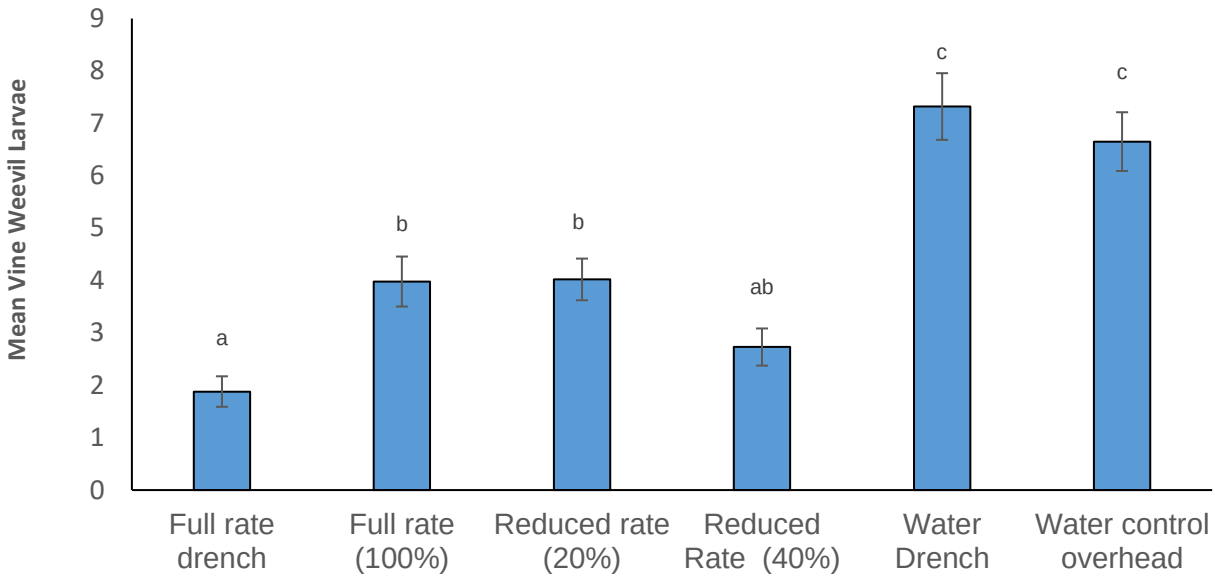


Figure 3.8. Mean numbers of vine weevil larvae per treatment +/- SE. Bars sharing none of the same letters are significantly different (P<0.001)

Numbers of vine weevil larvae - analysis of the four separate species

Mean numbers of vine weevil larvae per pot in each of the four species and the four species combined are shown in Table 3.6 and Figure 3.9.

- The full rate drench and the 40% rate overhead gave significantly fewer mean larvae per pot than in both water controls in all four individual varieties and in the variety combination and these two treatments were equally effective.
- The 20% rate overhead gave significantly fewer mean larvae per pot than the water overhead control and was equally effective as the full rate drench and 40% rate overhead in Riccartoni, Tom Thumb and the variety combination.
- On Hawkshead, the 20% rate overhead gave equal control of larvae per pot as the full rate drench and the 40% rate overhead but mean numbers of larvae were also not significantly different to those in the water overhead control.

- On Mrs Popple, the 20% rate overhead gave similar numbers of larvae per pot to the water overhead control and was less effective than the full rate drench and the 40% rate overhead.
- The full rate overhead gave significantly fewer mean larvae per pot than the water overhead control and was as effective as the full rate drench on Mrs Popple, Tom Thumb and the variety combination. However on Riccartoni and Hawkshead it gave similar mean larvae per pot as the water overhead control.

Table 3.6. Mean numbers of vine weevil larvae per pot in individual and combined Fuchsia species. Figures sharing the same letters are not significantly different, those with no common letters are significantly different from each other.

Treatment	Riccartonii	Mrs Popple	Hawkshead	Tom Thumb	All species combined
Full rate drench	2.9 a	1.4 a	1.7 a	1.5 a	1.9 a
Full rate overhead	6.3 bc	2.5 a	4.1 ab	3.0 ab	4.0 a
20% rate overhead	4.9 ab	5.5 b	3.6 ab	2.1 a	4.0 a
40% rate overhead	2.8 a	3.4 a	2.4 a	2.3 a	2.8 ab
water drench	7.5 c	6.5 b	9.6 b	5.7 bc	7.3 c
water overhead	7.8 c	6.0 b	6.1 b	6.7 c	6.7 c
P value	P<0.001	P<0.001	P<0.001	P<0.05	P<0.001

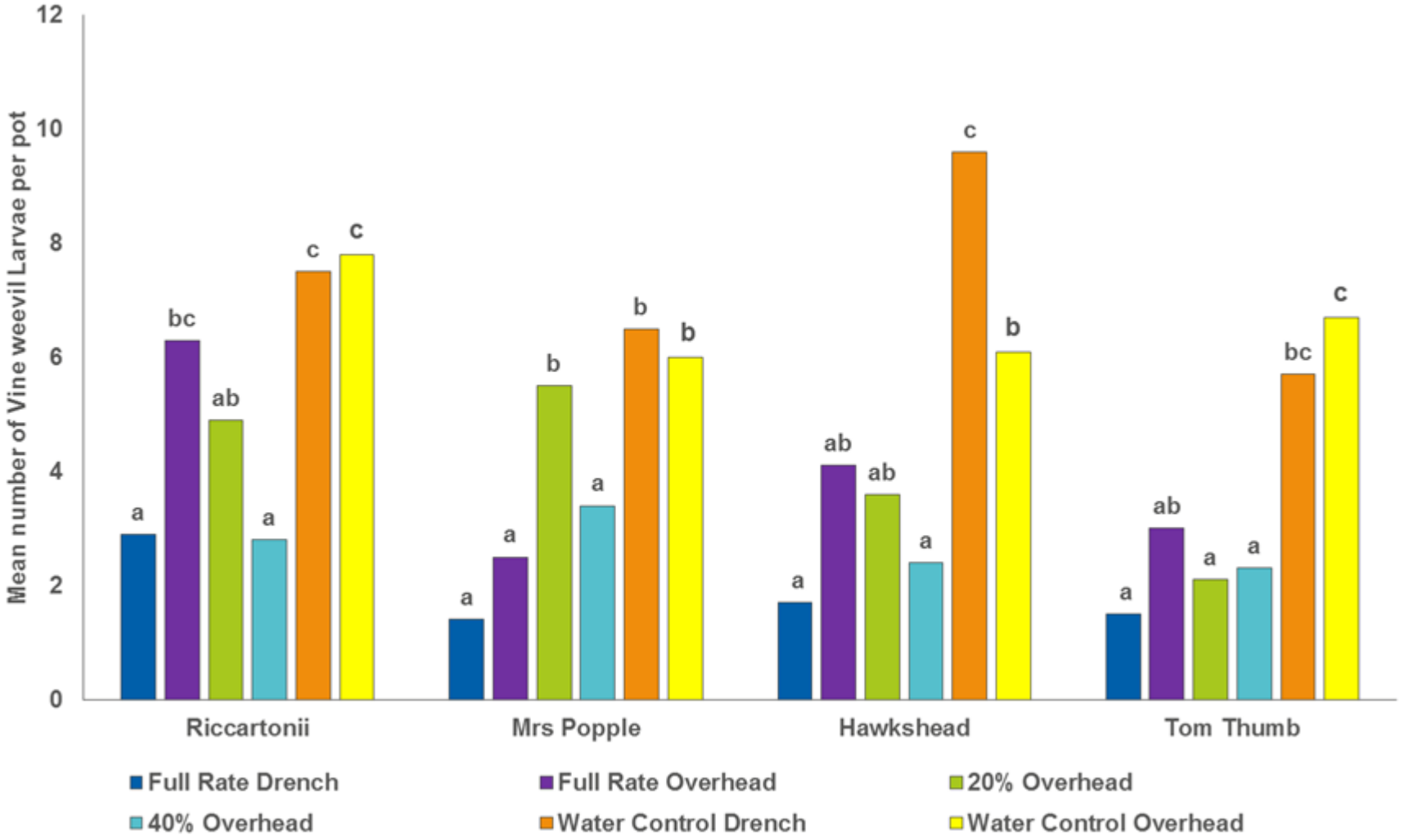


Figure 3.9. Mean numbers of vine weevil larvae per species and treatment. Bars sharing none of the same letters are significantly different (P<0.001 for species 1, 2 and 3, P<0.05 for species 4)

Percentage root damage - analysis of the four species combined

The mean percentage root damage scores for each treatment are shown in Figure 3.10 and Table 3.6. The water controls applied as a drench and through the overhead irrigation resulted in 36.4% and 32.1% damaged roots systems respectively. All nematode tratments significantly reduced the percentage root damage compaired with in the water controls. The 40% rate applied five times through the irrigation system was the most effective treatment with a mean percentage root damage of 6.1%.

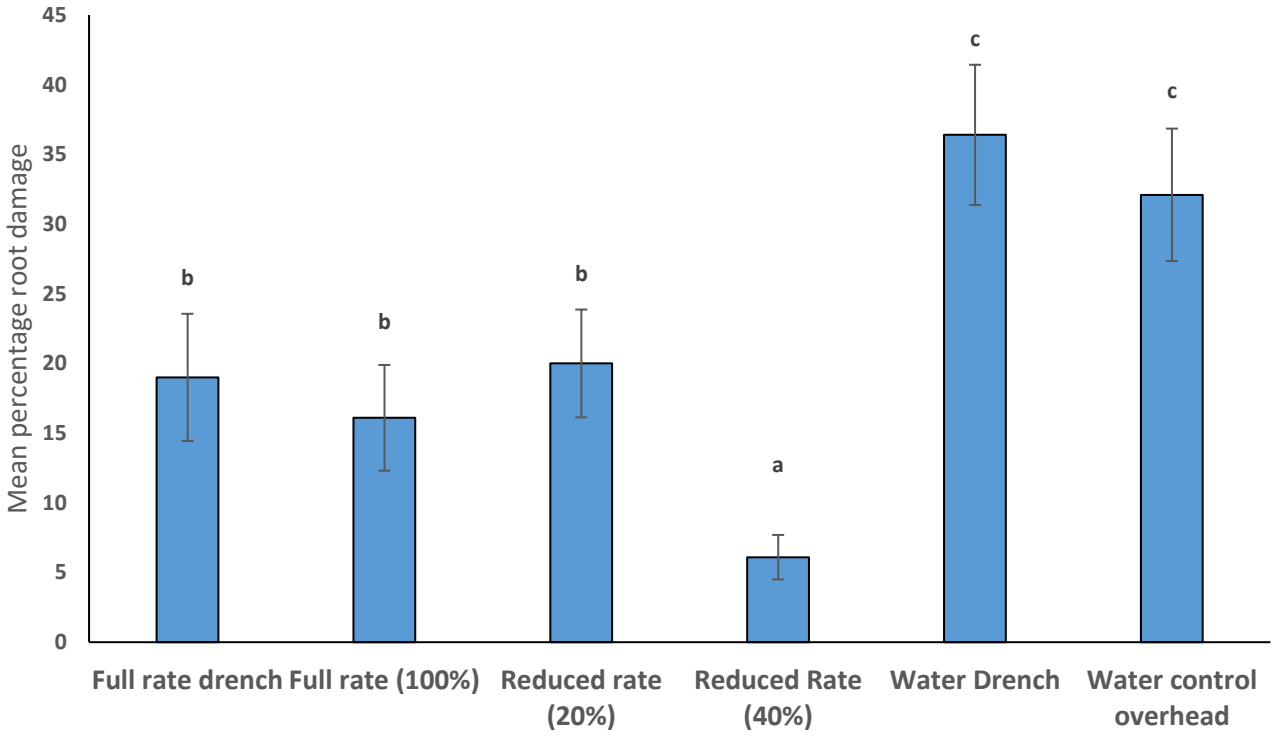


Figure 3.10. Mean percentage root damage per treatment for all Fuchsia varieties combined +/- SE. Bars with different letters are significantly different (P<0.001)

Percentage root damage - analysis of separate species

Mean percentage root damage in each treatment are presented in Table 3.7 and Figure 3.11.

Table 3.7. Percentage root damage in individual and combined Fuchsia species. Figures sharing the same letters are not significantly different, those with no common letters are significantly different from each other. N.S. = not significant.

Treatment	Riccartonii	Mrs Popple	Hawkshead	Tom Thumb	All species combined
Full rate drench	17.5% a	7.5% a	0.5% a	50.5% bc	19.0% b
Full rate overhead	17.5% a	9.5% a	2.5% a	34.8% ab	16.1% b
20% rate overhead	16.0% a	8.0% a	6.5% a	49.5% bc	20.0% b
40% rate overhead	6.0% a	5.5% a	1.0% a	12.0% a	6.1% a
water drench	34.5% a	27.0% b	20.5% b	63.8% c	36.5% c
water overhead	19.5% a	32.5% b	17.0% b	59.5% bc	32.1% c
P value	N.S.	P<0.05	P<0.001	P<0.05	P<0.001

- On Riccartoni, there was no significant difference between any of the treatments in percentage root damage compared with the respective water controls.
- On Mrs Popple, all the nematode treatments led to significantly lower percentage root damage than in the respective water controls (P<0.05).
- On Hawkshead, all the nematode treatments led to significantly lower percentage root damage than the water controls (P<0.001).
- On Tom Thumb, only the 40% rate applied five times through the overhead irrigation led to significantly lower percentage root damage than in the respective water controls (P<0.05). The percentage root damage in this variety in the water controls was much higher than in the other varieties, with means of 63.8% and 59.5% for the water drench and overhead applications respectively, compared with a maximum mean of 34.5% (water drench on Riccartoni) in the other varieties.

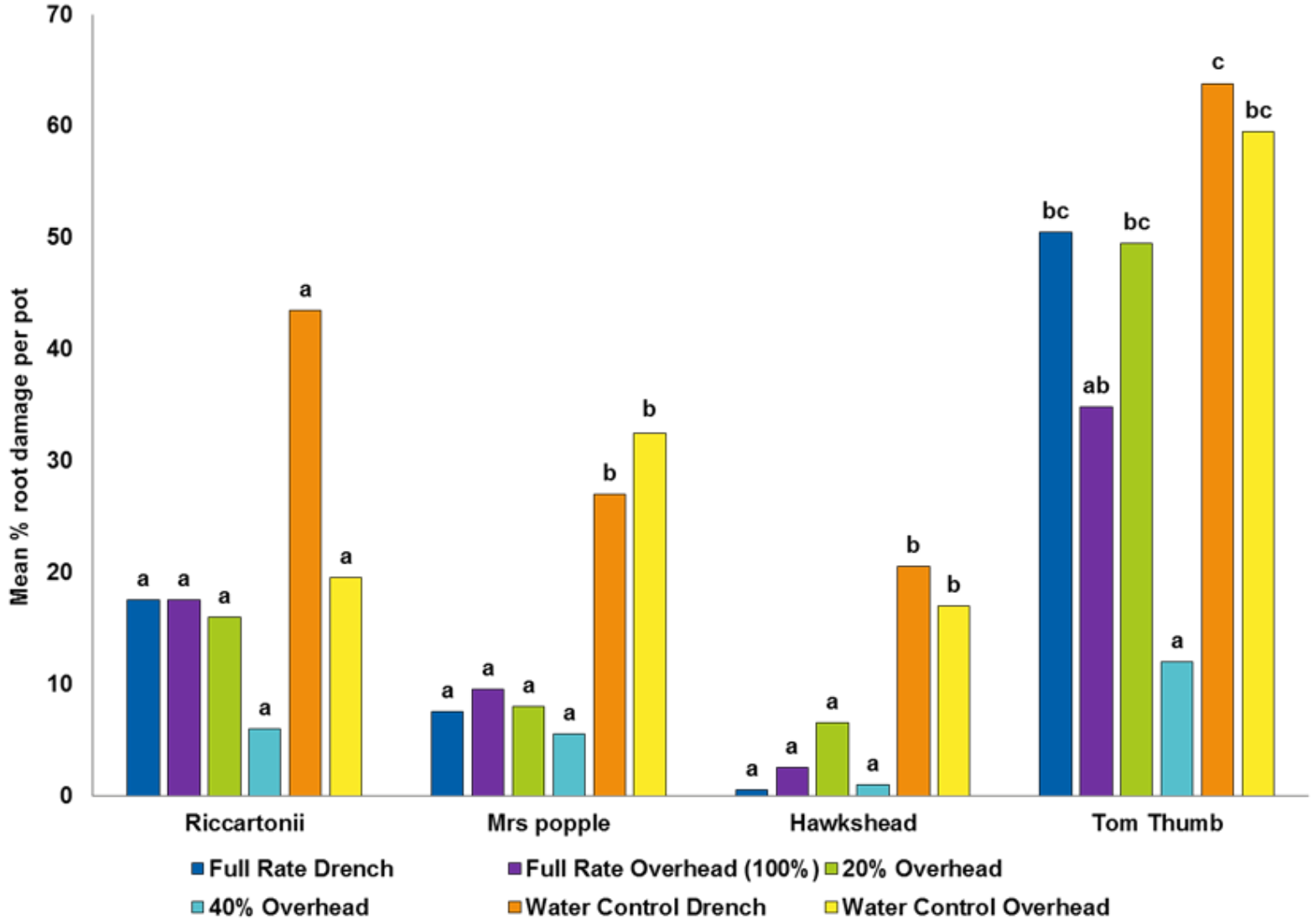


Figure 3.11. Mean percentage root damage on each Fuchsia species. Bars not sharing any of the same letters are significantly different (P<0.05 for species 2. P<0.001 for species 3 and P<0.005 for species 4)

Growing media temperatures

Although two loggers were used and they were replaced halfway through the trial on 2 August the batteries in both loggers failed after the replacement date, one on 4 August twelve days before the third application of Nemasys H and the other on 16 September, two days after the fourth application of Nemasys H. Unfortunately no further growing media temperatures are available after this date.

Mean, maximum and minimum growing media temperatures during the experiment recorded by the datalogger which ran until 16 September are shown in Figure 3.12.

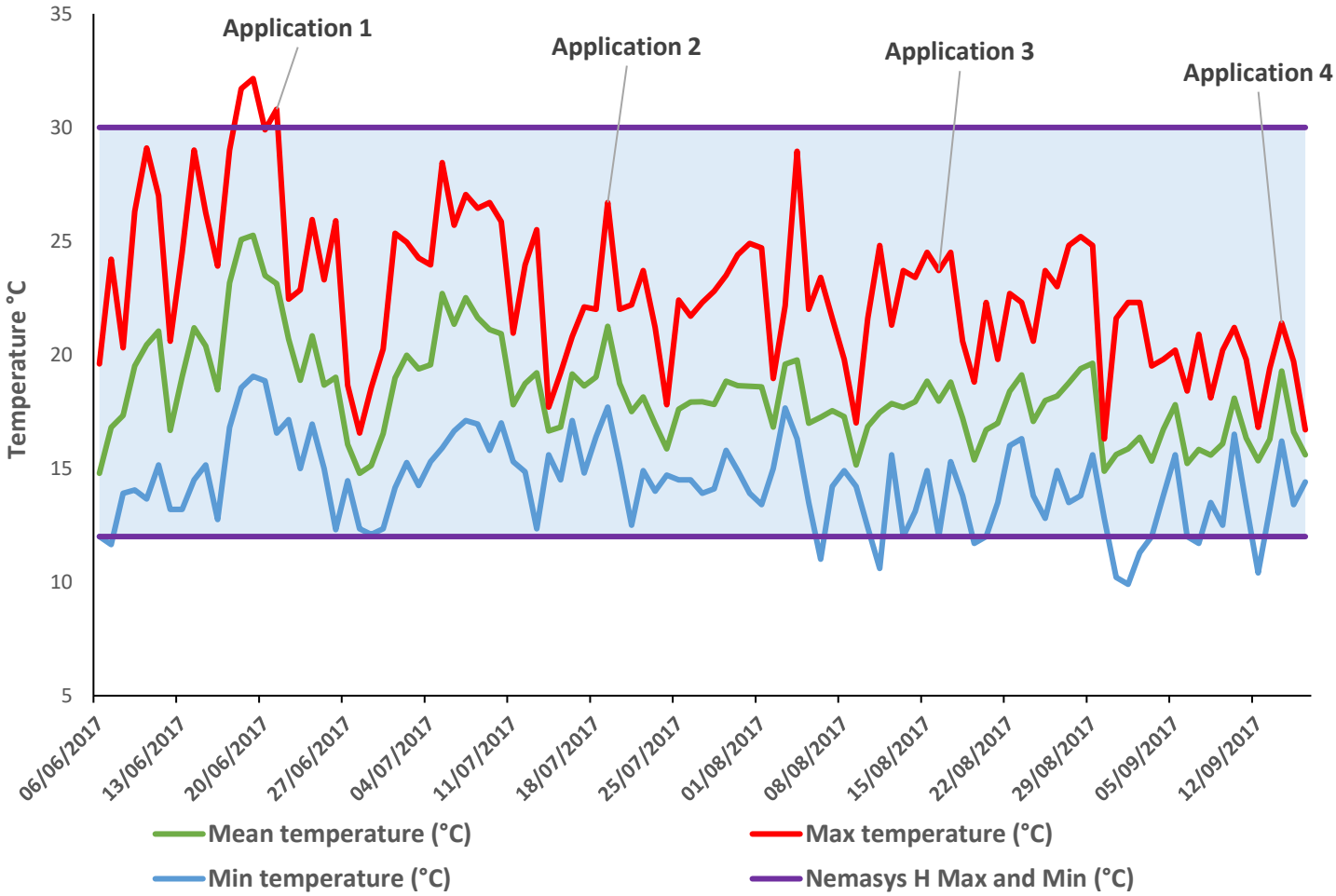


Figure 3.12. Mean, maximum and minimum growing media temperatures up to 16 September during the experiment. The horizontal lines indicate the recommended temperature range for Nemasys H (12-30°C)

On the date of the first Nemasys H applications on 21 June, maximum growing media temperatures rose to 30.8°C for two hours which was 0.8°C above the recommended temperature for this product. Minimum growing media temperatures fell to below the recommended minimum temperature for Nemasys H (12°C) on the following dates:

- 6 August (for three hours down to 11°C)
- 11 August (for four hours down to 10.6°C)
- 19 August (for one hour down to 11.7°C)
- 31 August (for 5.5 hours down to 10.2°C)
- 1 September (for 6.5 hours down to 9.9°C)
- 2 September (for 2.5 hours down to 11.3°C)
- 7 September (for two hours down to 11.7°C)
- 12 September (for two hours down to 10.4°C)

Vine weevil larvae damage correlation

The regression analysis indicated that there was a small positive correlation between numbers of vine weevil larvae and percentage root damage (Figure 3.13). The best line for each individual species was fitted and this accounted for 28% of the percentage variance. The slopes for species 2, 3 and 4 are similar, indicating that the rate of increase of percentage root damage as numbers of larvae increase are consistent. The slope for species 1 is steeper than those of the other species, indicating that the rate of increase of percentage root damage is faster than that of the other species as numbers of larvae increase. The line for species 4 sustained the highest percentage root damage in the presence of the same number of larvae as the other three species. The equations of the four lines are:

Species 1 (Riccartonii) Damage = 2.84*Larvae Number + 2.9

Species 2 (Mrs Popple) Damage = 1.36 *Larvae number+9.59

Species 3 (Hawkshead) Damage = 1.28*Larvae Number +2.13

Species 4 (Tom Thumb) Damage =1.54*Larvae number +38.7

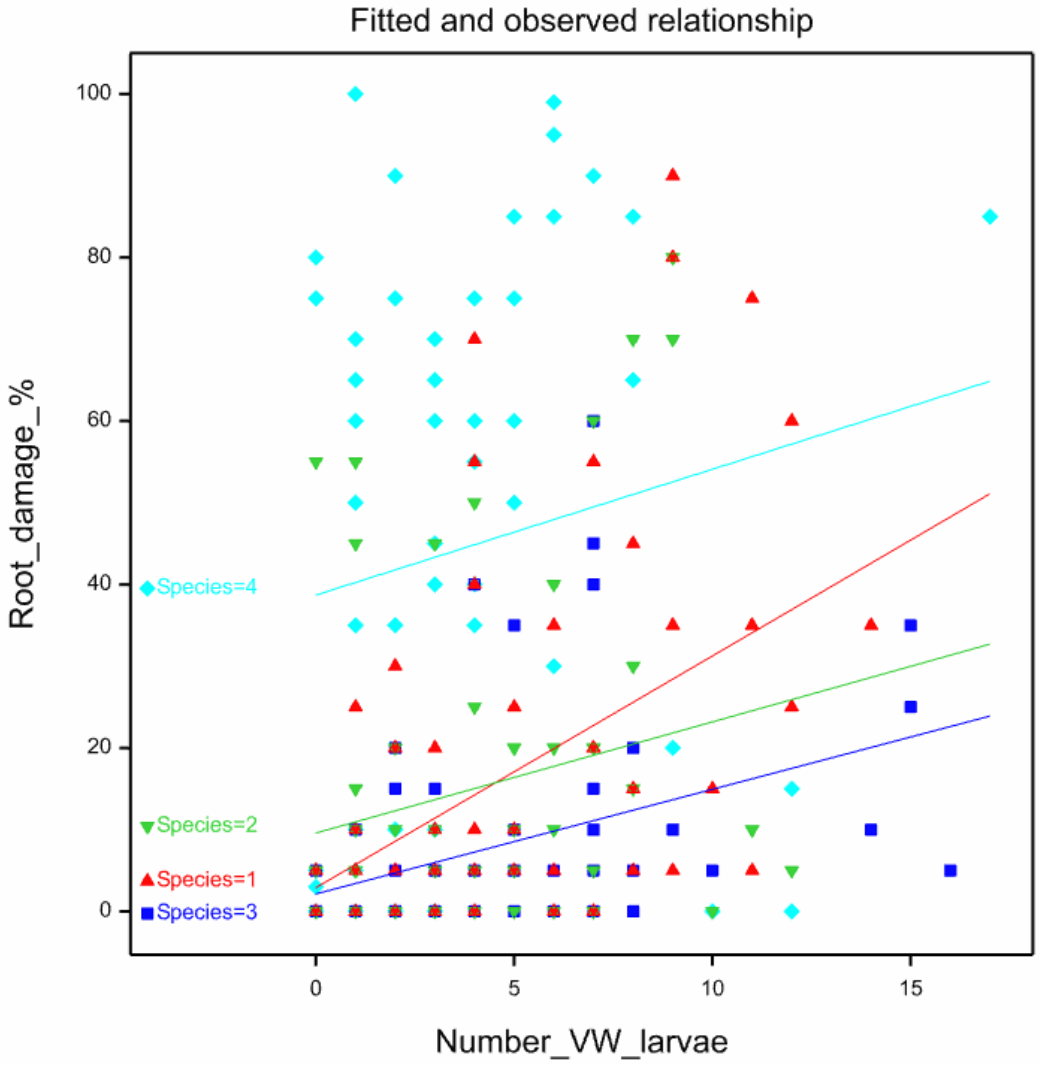


Figure 3.13 Correlation between mean numbers of vine weevil (vw) larvae and percentage root damage, using the individual lines of best fit for each of the four Fuchsia species. Species 1= Riccartonii, 2= Mrs Popple, 3= Hawkshead and 4= Tom Thumb.

Discussion

Calibration of water and nematode application through sprinklers

As in the pilot experiment in year 1, the results of the calibration of water volumes applied to different grids in the selected treatment area before application to the trial plants demonstrated how variable the delivered water volumes were.

Once the Dosatron® had been changed, mean numbers of nematodes per ml were very accurate during calibration of the three nematode rates before application to the trial plants and this is consistent with the results of nematode calibration in the pilot experiment in the

first year of the project. The inaccurate dosing of the grower's Dosatron needs to be investigated further as models newer than the ADAS one may have an internal filter that causes nematode blockages.

Nematode volumes applied to growing media through overhead irrigation

The mean volumes of nematode suspension collected in both the empty collection pots and the pots with the 'sacrificial plants' across all the applications and nematode rates were 85% and 84% of the expected volumes delivered respectively. These results were different from those in the pilot experiment in year one where specimen tubes were pushed into the growing media to collect nematode suspensions during application in order to estimate nematode delivery to the growing media under the plant canopy. The results in the pilot experiment indicated much lower volumes of nematode suspension reached the growing media than expected when applied at all rates than in the trial reported here. As the volumes collected in the empty pots and pots with the 'sacrificial plants' were so similar in the trial on the commercial nursery this indicates that the Fuchsia plants did not impede nematode delivery to the growing medium and that the use of pots with 'sacrificial plants' was a much more accurate method of measuring volumes of nematode suspension delivered than the use of specimen tubes which represented only a portion of the growing media area. However, as only one Fuchsia variety (cv. Snowdrop) was used as the 'sacrificial plant' and this was a different variety to the other four used in the trial, this result should only be used as an indication of nematode volumes reaching the growing media.

The mean volumes of nematode suspension collected in the empty collection pots in each individual Fuchsia variety, for each nematode rate, showed that although numbers of nematodes per ml were very similar to those expected, the volumes of nematode suspension were statistically lower than those expected for both the 40% and 20% rates on all varieties and for the 100% rate on Riccartonii and this may have negatively impacted the control of vine weevil larvae especially with the 20% rate. The under-delivery of nematode suspension volumes is likely to have been due to the variability in water volume delivery over the treatment area by the sprinklers that was demonstrated in the pre-application calibration.

Control of numbers of vine weevil larvae

The results demonstrated that when the four Fuchsia varieties were combined for analysis, all nematode treatments significantly reduced numbers of vine weevil larvae compared with

both the water controls. This result was consistent with those in the pilot experiment in year 1 when only one Fuchsia variety was used (Alice Hoffman). In the pilot experiment *Steinernema kraussei* (Nemasys L) was the only nematode species used whereas in the trial reported here *Heterorhabditis bacteriophora* (Nemasys H) was used for the first four application dates and the more cold-tolerant but more expensive Nemasys L was only used on the final date in October when temperatures were expected to be cooler. The use of *H. bacteriophora* products when growing media temperatures are 12°C or above and use of *S. kraussei* only when temperatures fall below 12°C is consistent with grower practice and the results of this trial indicate that use of the two nematode species in this way should give similar levels of vine weevil control as using Nemasys L for all applications.

When the data for the four Fuchsia varieties were combined for analysis, the full rate nematode drench applied twice in September and October and the 40% rate applied five times through the overhead irrigation between July and October were equally effective in reducing mean numbers of vine weevil larvae. The full rate drenches were more effective than the full rate and the 20% rate applied twice in September and October through the overhead irrigation. However, control of larvae was equally effective in the 40% rate, full rate and 20% rates applied five times through the overhead irrigation. These results are different from in the pilot experiment in year 1 when all nematode rates were equally effective.

When the data from the four Fuchsia varieties were analysed separately, slightly different results were given than when the data from the four varieties of Fuchsia were combined for analysis. The full rate drenches and the 40% rate applied through the overhead irrigation were equally effective in reducing mean numbers of larvae on all four varieties. However, the 20% rate applied through the overhead irrigation was only as effective as the full rate drenches and the 40% rate applied through the overhead irrigation on three of the varieties, Riccartoni, Hawkshead and Tom Thumb. On Hawkshead, the 20% rate was also equally effective as the water control applied through the overhead irrigation. It is not clear why the 20% rate did not significantly reduce numbers of larvae compared with the water overhead controls on Mrs Popple and Hawkshead. This result could have been partly due to mean volumes of nematode suspension being significantly lower than expected for both 20% and 40% rates, although this occurred on all Fuchsia varieties. The lower volumes of nematode suspension than expected could have been due to the variability in delivery of water by the overhead sprinklers in the treatment area and to the trial plants being placed in all grids of the treatment area during application (consistent with potential grower uptake of the system), unlike in the pilot experiment where the trial plants were placed only in grids known to deliver accurate amounts of water during application. If growers are interested in trying the ‘little and

often' system through the overhead irrigation, using the results available to date, the results to date indicate that it would be safer to use the 40% rate than the 20% rate.

The full rate applied through the overhead irrigation five times between July and October was only included to test whether the efficacy of nematode application through the overhead irrigation was affected by nematode rate and/or due to the system itself. This treatment was only effective in reducing numbers of vine weevil larvae on two varieties, Mrs Popple and Tom Thumb, where it was equally effective as the full rate drench. On Ricccartonii and Hawskhead it gave similar numbers of larvae per pot as the water overhead control. The nematode calibration showed that volumes of nematode suspension applied in the 100% overhead rate were similar to those expected in all varieties except Riccartonii. However, volumes applied to Riccartonii were similar to those applied in the 40% rate which was effective on all varieties. Similarly, numbers of nematodes per ml in the empty collection pots and the pots with the 'sacrificial plants' during application were 79% and 77% lower than lower than expected for the 100% rate, but numbers per ml were still higher than in the 40% rate which was effective on all varieties. The poorer control with the 100% overhead rate could possibly have been due to the high rate of nematodes causing nematode clumping in the droplets applied through the overhead irrigation which may have prevented them dispersing when they reached the growing media, although this will not explain why control was not given on only two of the varieties. In addition, water was applied immediately after each overhead application as recommended by the supplier to wash any nematodes that may have remained on the foliage and to help disperse nematodes into the growing media.

Control of percentage root area damaged

The results demonstrated that when the four Fuchsia varieties were combined for analysis, all nematode treatments significantly reduced percentage root area damaged compared with both the water controls in addition to reducing mean numbers of vine weevil larvae. The 40% rate applied five times through the overhead irrigation was the most effective treatment, whereas the most effective treatments in reducing numbers of larvae were both the 40% rate and the full rate drenches.

When the data from the four Fuchsia varieties were analysed separately, all nematode treatments significantly reduced percentage root area damaged on Mrs Popple and Hawkshead compared with the water controls. This result was similar to that in the pilot experiment in year 1 when all nematode treatments reduced the percentage of Fuchsia cv. Alice Hoffman with severe root damage. Mean numbers of larvae had been significantly reduced on Mrs Popple by all nematode treatments except for the 20% rate overhead and on

Hawkshead by all treatments except for the full rate and 20% rates overhead. In contrast, none of the treatments reduced percentage root damage compared with the water controls on Riccartonii despite all of them except for the 100% rate overhead reducing numbers of vine weevil larvae. The correlation between numbers of larvae and percentage root damage indicated that percentage root damage increased faster on Ricartonii than on the other three varieties as numbers of larvae increased, but it is not known why this variety might be more susceptible to damage than the others as it is a species variety with a strong root system. Similarly, only one of the treatments, the 40% rate overhead reduced percentage root damage on Tom Thumb despite all of the treatments except for the 100% rate overhead reducing numbers of vine weevil larvae. The correlation between numbers of larvae and root damage on Tom Thumb showed that percentage root area damaged was higher on this variety than on the other three in relation to numbers of larvae. It is likely that this dwarf variety had a smaller root or less robust root system than the other three varieties and was thus more susceptible to vine weevil damage.

Effect of growing media temperatures on nematodes

Unfortunately the batteries in the two dataloggers placed in the growing media failed on 4 August and 16 September respectively so no data on growing media temperatures are available after these dates. Therefore it is not possible to discuss the potential effect of temperature on performance of the final application of Nemasys H on 14 September or on the application of Nemasys L on 11 October. Prior to the batteries failing, the data showed that maximum growing media temperatures rose to above the recommended 30°C for Nemasys H on only one date, 21 June when they rose to 30.8°C for two hours. This was the date when the first nematode applications were made and it is possible that the nematodes could have been slightly adversely affected on that date. Minimum growing media temperatures fell to below the recommended 12°C for Nemasys H on eight dates between 6 August and 12 September, down to between 9.9 and 11.7°C for up to 6.5 hours. These temperatures might have adversely affected Nemasys H activity on these dates, although they are unlikely to have affected viability as the product is recommended to be stored in the fridge until use before the use-by date. Nemasys L may have been a better choice for the 14 September application instead of Nemasys H as well as on the final application on 11 October.

Conclusions

- Application of nematodes at 40% rate five times between 21 June and 11 October was equally as effective in reducing mean numbers of vine weevil larvae per plant on all four Fuchsia varieties as two conventional full rate drench applications on 22 September and 21 October
- Application of nematodes at 20% rate five times between 21 June and 11 October was equally as effective in reducing mean numbers of larvae on three of the Fuchsia varieties (Riccartoni, Hawkshead and Tom Thumb) as the 40% rate and as two conventional drench applications of the full rate on 14 September and 11 October. However, on Hawkshead, mean numbers of larvae were also statistically similar to those in the water overhead control.
- All nematode treatments were equally effective in reducing percentage root damage on two Fuchsia varieties (Mrs Popple and Hawkshead) compared with the respective water controls. None of the nematode treatments reduced root damage on Riccartonii. Only the 40% rate applied five times between 21 June and 11 October reduced root damage on Tom Thumb, despite the other nematode treatments being as effective as the 40% rate in reducing numbers of vine weevil larvae.
- Numbers of nematodes per ml applied through the overhead irrigation were very similar to those expected. However, the volumes of nematode suspension were statistically lower than those expected for both the 40% and 20% rates on all varieties and for the 100% rate on Riccartonii and this may have negatively impacted the control of vine weevil larvae especially with the 20% rate.
- The under-delivery of nematode suspension volumes is likely to have been due to the variability in water volume delivery over the treatment area by the sprinklers as demonstrated in the pre-application calibration. This variability is likely to occur in all commercial overhead irrigation systems.
- Mean volumes of nematode suspension reaching the growing media did not seem to be reduced by the crop canopy of cv. Snowdrop used as the ‘sacrificial plant’ in this trial.
- The grower’s Dosatron led to under-delivery of nematode numbers per ml of nematode suspension but using the (older) ADAS Dosatron led to very accurate numbers per ml. The innacurate dosing of the grower’s Dosatron needs investigation to avoid similar problems on other nurseries if growers adopt the system.
- Nematode application through the overhead irrigation five times at 40% of the label rate (four applications of *Heterorhabditis bacteriophora* and one of *Steinernema*

feltiae) would save 31% of the cost of applying two nematode drenches at full recommended rates (one of *H. bacteriophora* and one of *S. kraussei*) including labour costs. If using three applications of 40% rate *H. bacteriophora* and two applications of *S. kraussei* (in a cold autumn) this would save 26% of the cost of two full rate drenches (one of *H. bacteriophora* and one of *S. kraussei)*.

- Cost savings of applying the 40% rate of nematodes five times through the overhead irrigation would be even greater if growers currently apply three consecutive drenches of nematodes at recommended rates (two of *H. bacteriophora* and one of *S. kraussei*) i.e. a saving of 52% if using four applications of *H. bacteriophora* and one of *S. kraussei* and a saving of 49% if using three applications of *H. bacteriophora* and two of *S. kraussei*.
- Cost savings would be even greater if using 20% rates of nematodes but using 40% rates is considered a safer option.

Task 3.3. Effect of fluctuating temperatures on Met52 performance (Warwick, years 1 and 2)

Objective

The aim of this task was to provide growers with practical information in order to improve control of vine weevil larvae with the Met52 granular formulation. This was done by quantifying and analysing the effect of fluctuating temperatures on the infectivity of the fungus in a laboratory bioassay, coupled with pot-based experiments.

Materials and methods

Adult vine weevil culture

Field-collected adult vine weevils were collected from ornamental crops in May 2016 and 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. Twenty-five to 30 weevils were placed into each pot, and maintained within a controlled environment room at 20°C; 16:8 L: D. Pots were cleaned once a week taking care to remove any dead or dying weevils.

3.3.1: *Laboratory bioassay*

Eggs were collected from the adult culture and larvae reared up to second / third instar (larval stage) on strawberry plants within a controlled environment room at 20°C; 16:8 L:D. Met52 grains (batch number: 1314SG 07BR) were incorporated into Levington M2 compost (Scotts) using a St Moritz paddle compost mixer with a 300 litre capacity according to the manufacturers recommendations (0.5 kg /m^3). Treated or untreated substrate was then placed in plastic food boxes (11 x 8 x 7cm), along with carrot slices, to which 20 third instar weevil larvae were added (Figure 3.14). The boxes were maintained at a range of constant temperatures, from 12.5°C to 30°C. The survival of the larvae was evaluated over time and the numbers of living and dead weevils counted. Dead weevils were removed and placed on damp filter paper in Petri dishes and incubated at 20°C and observed for the appearance of sporulating mycelium. All temperatures were assessed simultaneously on each occasion and the experiment was repeated on three occasions.

Figure 3.14 Laboratory bioassay set up

3.3.2: *Nursery experiments*

Sedum cauticola 'Coca cola' was obtained from Darby Nursery Stock Ltd, Norfolk UK in July 2016. The roots of the plug plants were washed to remove any compost and the plants potted up into either untreated Levington M2 compost (Scotts) or treated Levington M2 compost (Scotts) which had Met52 grains (batch number: 1314SG 07BR) incorporated as described in 3.3.1 according to the manufacturers recommendations (0.5 kg /m^3) and maintained within a polytunnel prior to inoculation. Replicate pots were infested with 10 weevil eggs, collected from the ADAS laboratory adult weevil culture. This was done on four separate occasions: (i) Replicate 1, start date = 4th August; (ii) Replicate 2, start date = 25th August; (iii) Replicate 3, start date = 15th September; (iv) Replicate 4, start date = 6th October 2016. Both treated and untreated (= control) pots were maintained either under protection (i.e. within a polytunnel),

or outdoors on hardstanding (Figure 3.15). After four weeks, the pots of the first three inoculations were destructively sampled and the numbers of live and dead larvae per pot counted. Any dead larvae were removed and placed on damp filter paper within Petri dishes and incubated at 20°C for seven days and the production of *Metarhizium* conidia on cadavers used to confirm fungal-induced mortality. Plants were weighed and root damage ranked on a scale of zero (no damage) to five (plant dead with no roots remaining). Pots inoculated on the 6th October 2016 were destructively sampled in May 2017. Temperature was recorded using Tiny Tag data loggers (Gemini Data loggers, Sussex, UK) within pots and local air temperature.

Additional tests were set up to determine the presence of Met52 in the compost at the end of each experiment. Replicate samples of compost from each replicate (1 g) were added to 100 ml of 0.05% Tween 80. The suspension was thoroughly vortex mixed for 30s and further diluted 1:10 and vortex mixed again. 100 µl was plated evenly over the surface of a selective agar plate (39 g Potato dextrose agar, 1 g yeast extract, 0.5 g chloramphenicol, 0.25 g cyclohexamide, 0.004 g, thiabendazole, 0.01 g rose bengal) and maintained at 23 ± 2°C, in the dark. Three aliquots per sample were plated out. After 4 – 5 days, the number of colonies grown on the plate were counted and adjusted to determine the approximate number of colony forming units per gram of soil. Met52 was also isolated from samples (10 g) by baiting with waxmoth larvae, *Galleria mellonella* (Lepidoptera: Pyralidae). Coarse debris was removed from each sample, placed within a Petri dish (9 cm), and ten larva of *G. mellonella* (Charlcote Pet Shop, Warwick, UK) was placed in each dish. The dishes were held at 20°C, in darkness, and larvae were inspected daily for two weeks. Dead, intact larvae were removed and surface sterilised in 1.0% sodium hypochlorite for three minutes, then washed three times in sterile distilled water and placed on damp filter paper within a sealed Petri dish (90 mm diameter) and incubated at 20°C for 7-14 days prior to identification.

Figure 3.15 Pot experiment set up within a protected polytunnel or outside on hardstanding

Results

3.3.1: *Laboratory bioassay*

Control (i.e. untreated) mortality varied with temperature. At day 28 the mortality in controls ranged from 27% (at 12.5°C) to 55% (at 27.5°C) (Figure 3.16a). The mean control mortality was 44%. The highest control mortality was observed at 17.5°C and dead larvae appeared black and soft and it is suspected that they died from a bacterial infection. At temperatures lower than 15°C there was no difference in mortality observed between those in the Met52-treated plots and untreated (= control) plots. At temperatures of 20°C and above there was a divergence between the two groups with mortality occurring faster with the Met52 treated group.

The mortality of Met52 treated vine weevils also varied with temperature, showing an overall trend for increased mortality with increasing temperature. At day 14, mean mortality in the Met52 treated pots was 27% at 12.5°C, 25% at 15°C, 28% at 17.5°C, 55% at 20°C, 28% at 25°C, 67% at 27.5°C and 80% at 30°C (Figure 3.16b). By day 21, mean mortality in the Met52 treated pots was 30% at 12.5°C, 52% at 15°C, 41% at 17.5, 71% at 20°C, 89% at 25°C, 90% at 27.5°C and 97% at 30°C. By day 28, 100% mean mortality was observed in the Met52 treated pots held at 25°C, 27°C and 30°C. Of the dead weevils recovered, 27% had obvious signs of *Metarhizium* infection but when incubated on damp filter paper this increased to 67% confirming death was due to Met52. There was no obvious sign of Met52 infection on weevils recovered from the control pots.

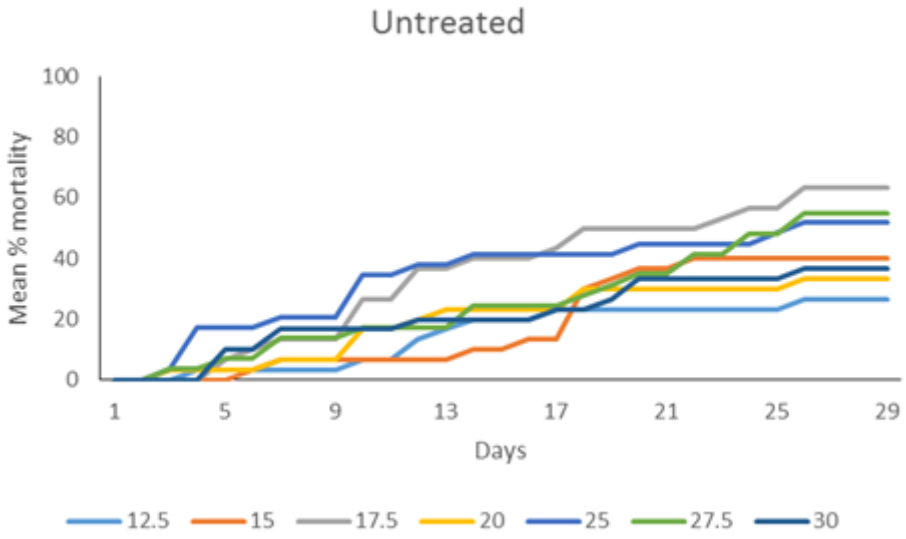


Figure 3.16a Mean % mortality of weevil larvae over 28 days at seven temperatures in untreated compost.

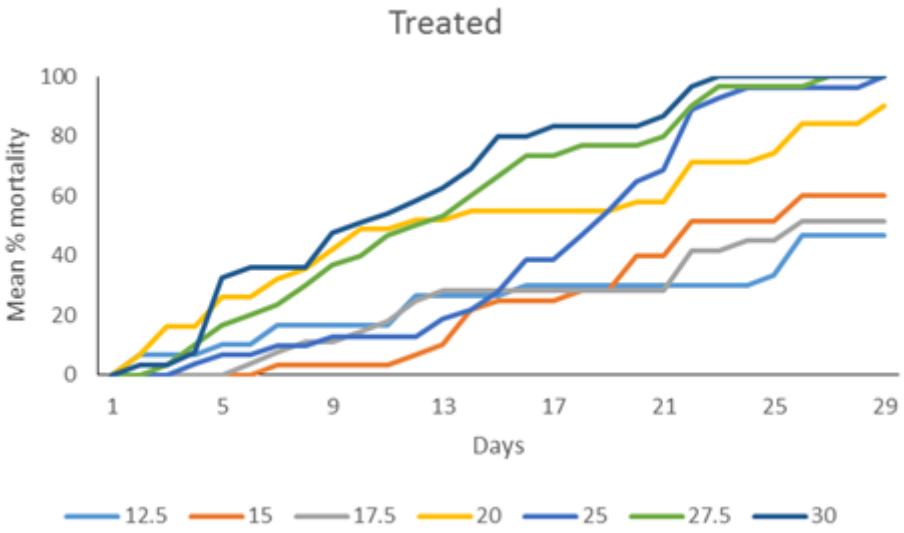


Figure 3.16b Mean % mortality of weevil larvae over 28 days at seven temperatures in Met52—treated compost.

The data was adjusted to correct for the control mortality using Schneider-Orelli's formulae, as follows:

$$\text{Corrected mortality (\%)} = ((a - b)/(100 - b)) * 100$$

Where *a* is the percentage mortality data from the treated group and *b* is the percentage mortality from control group.

Corrected mortality increased with temperature (Figure 3.17). At temperatures below 15°C mortality was remained low, reaching 26% and 35% at 12.5°C and 15°C at day 28 respectively. At day 14, mortality was 39%, 0%, 55% and 76% at 20°C, 25°C, 27.5°C and 30°C respectively. By day 21, mean mortality was 56%, 78%, 55% and 83% at 20°C, 25°C, 27.5°C and 30°C respectively. By day 28, 100% mean mortality was observed at 25°C, 27°C and 30°C.

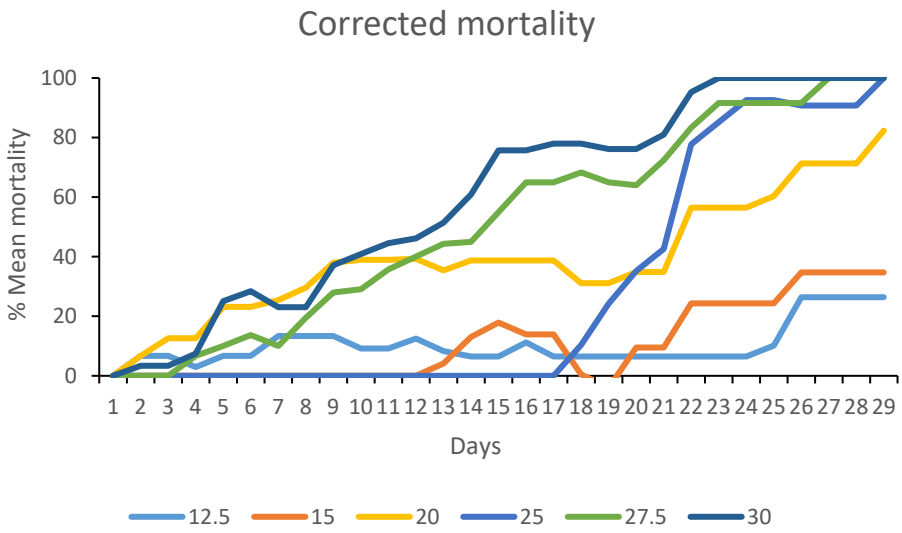


Figure 3.17 Mean % corrected mortality of weevil larvae over 28 days at six temperatures in Met52 treated compost.

A day degree approach was used to develop a predictive model of the virulence of Met52 against third instar weevil larvae. A polynomial regression was fitted to the relationship between temperature and the corrected % mortality at 21 days (Figure 3.18). The minimum (= threshold) temperature for Met52 infection estimated from the regression was 11.6°C. The optimum and maximum temperatures were >30°C and could not be estimated from the data available. However it is unlikely that growing media temperatures experienced by vine weevil larvae on nurseries in the UK will exceed 30°C for more than a short time and hence this should not impact on any day degree forecasts of Met52 activity under fluctuating temperature conditions. The threshold temperature was used to reconfigure the relationship between % weevil mortality and temperature in terms of day degrees accumulated above the threshold; this was done for each temperature in the bioassay (accumulated day degrees were calculated using Met. Office Formulae at https://www.metoffice.gov.uk/climatechange/science/monitoring/ukcp09/faq.html#faq1.8.

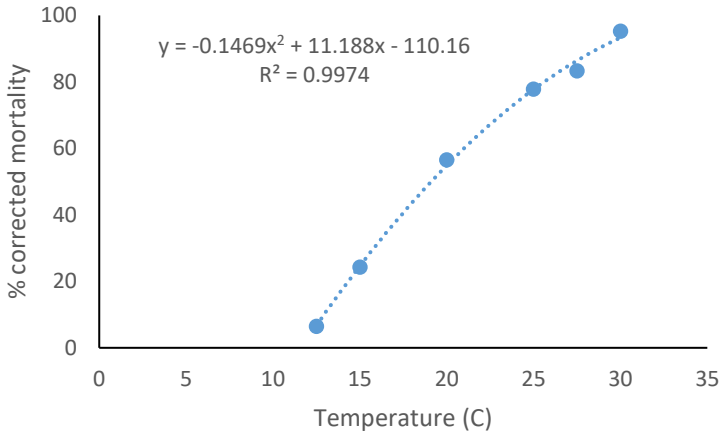


Figure 3.18 Polynomial model fitted to mean % corrected mortality of weevils at 21 days at six temperatures in Met52 treated compost.

The relationship between percentage mortality and accumulated day degrees could be described using a polynomial regression (Figure 6). This simple relationship enables percentage mortality to be estimated on any given accumulation of day degrees above the threshold temperature (Figure 3.19; Table 3.8).

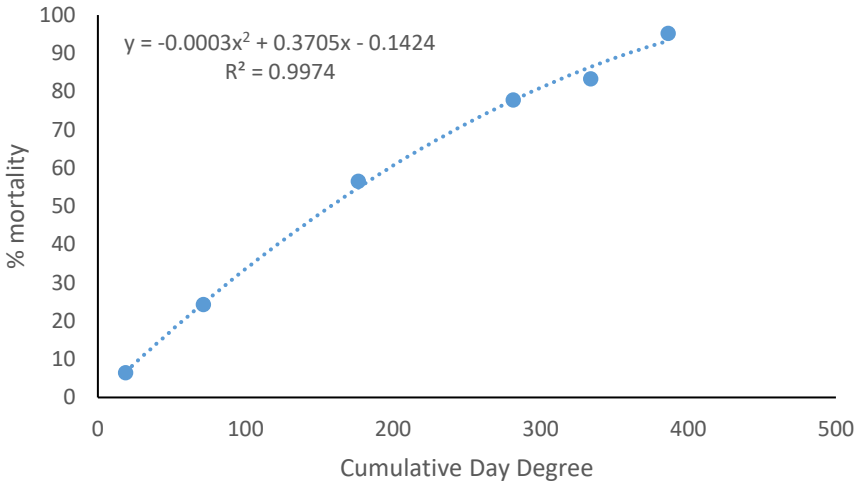


Figure 3.19 Regression model fitted to mean % mortality of weevils at 21 days in treated Met52 compost against cumulative day degrees.

Table 3.8: Predicted weevil mortality based on cumulative day degrees (CDD)

% mortality	Estimated CDD
25	72
50	155
75	256
90	333

100	400

Discussion

Weevil mortality was lower than expected at the lower temperatures. There are a number of possible reasons why mortality may have been low: (i) the larvae did not move throughout the compost to come into contact with the *Metarhizium* conidia; or (ii) the environment was not suitable for infection. Analysis of the data suggests that no control will occur at temperatures below 11.6°C. However, the Met52 spores will remain viable until temperatures conducive to infection re-occur. Development of a day degree model estimates that for 75% control 256 CDD has to be achieved. Analysis of historical temperature data recorded from UK nurseries suggests that this could be reached in the months of June, July and August in some years and locations.

Conclusions

- Mortality of weevil larvae was temperature related.
- Low levels of weevil control were observed at temperatures below 15°C.
- A predictive model estimated that no weevil mortality would occur at temperatures below 11.6°C.
- A day degree model was developed to predict the mortality that could be achieved at given cumulative day degrees.

3.3.2: *Nursery experiments*

Hatch rate of the eggs added to the pots varied between 57% and 92% (mean 75.2%) (Table 3.9). Overall 53.6% of the weevil eggs added to the untreated pots and 39.7% from the treated pots were recovered as larvae in the experiment.

Table 3.9 Weevil egg hatch rate

	Rep 1 04/08/2016	**Rep 2 25/08/2016**	**Rep 3 15/09/2016**	**Rep 4 06/10/2016**	**Overall**
Mean % hatch rate (+ SE)	56.7 (6.667)	91.5 (2.796)	86.7 (3.309)	68.7 (3.742)	75.2 (3.234)

Replicate 1 (start date 4th August 2016). For the untreated control, pot location (i.e. inside the polytunnel versus outdoors) had no significant effect on the mean number of weevil larvae recovered per pot (polytunnel = 5.8, outdoors = 6.6; P = 0.05). Within the polytunnel, treatment with Met52 had no significant effect on numbers of larvae recovered per pot (mean 5.4 larvae per pot) compared to the untreated control (5.8 per pot). In contrast, outside of the polytunnel, treatment with Met52 caused a statistically significant ($P < 0.05$) reduction in the mean number of larvae recovered per pot (mean 4.3 larvae per pot) compared to the untreated control (6.4 per pot), equivalent to a 33% reduction in weevil population size (Figure 3.20). However, none of the larvae recovered from Met52 treated pots exhibited any symptoms of Met52 infection. At the end of the trial, living larvae that were recovered were maintained at 20°C in boxes containing untreated compost and fresh carrot. After fourteen days, 25% of the larvae recovered from the pots maintained in the polytunnel were dead and infected with *Metarhizium*. The equivalent figure for larvae recovered from the pots maintained outside was 23%.

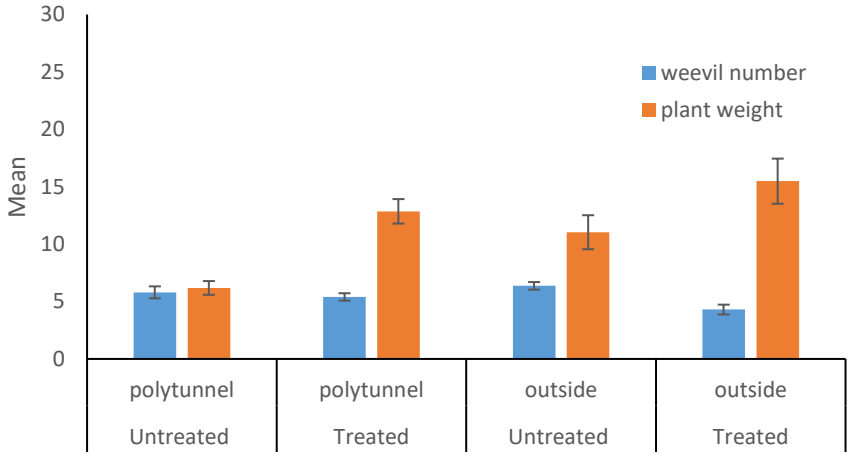


Figure 3.20 The mean number of weevil larvae recovered per plant and the plant weight in August. The error bars represent the standard error of the mean.

Plants which were maintained in Met52 treated compost had a greater above ground plant weight than those plants maintained in untreated compost. This occurred both for pots maintained outside and for pots maintained inside the polytunnel. Pots that had been treated with Met52 were 6.7g heavier and 4.4g heavier than controls when grown in the polytunnel or outside respectively (Figure 3.20).

Mean compost temperatures during the trial ranged from 13.8 to 20.1°C (mean 17.5°C) for pots maintained within the polytunnel, and 12.7 to 21.8°C (mean 17.2°C) for pots maintained

outside (Figure 3.21). The minimum compost temperature for pots maintained within the polytunnel ranged from 7.4 to 17.8°C (mean 12.9°C), while for pots maintained outside it ranged from 6.6 to 17.5°C (mean 12.6°C). The maximum compost temperature for pots maintained within the polytunnel ranged from 19.3 to 27.6°C (mean 23.2°C), while for pots maintained outside it ranged from 17.3 to 34.6°C (mean 23.7°C).

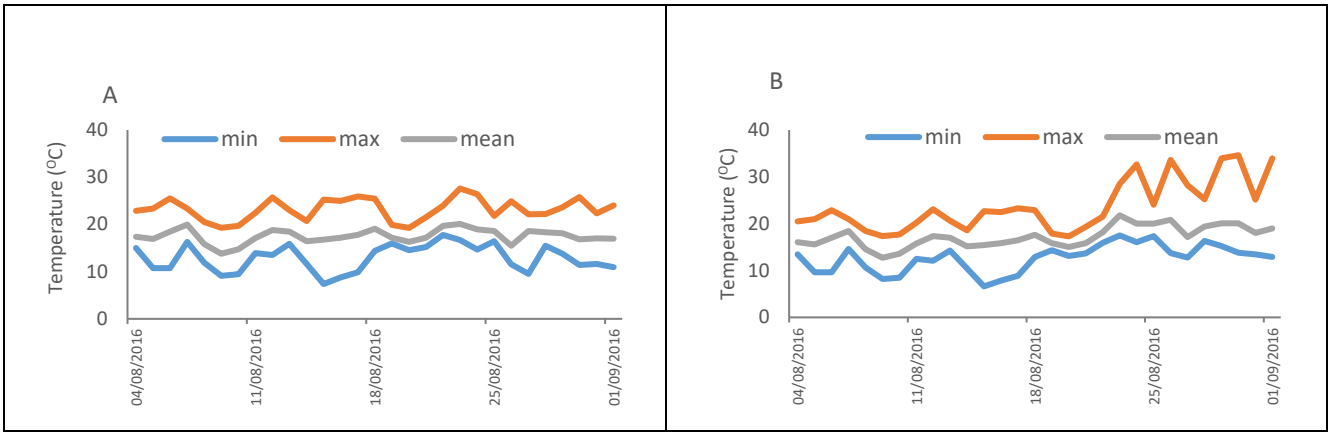


Figure 3.21 The mean, minimum and maximum compost temperature in pots maintained in either A) polytunnel or B) outside in August.

Replicate 2, start date - 25 August 2016. For the untreated control, pot location (i.e inside the polytunnel versus outdoors) had no significant effect on the mean number of weevil larvae recovered per pot (polytunnel = 6.5, outdoors = 5.8; *P*= 0.05). Within the polytunnel treatment with Met52 had no significant effect on numbers of larvae recovered per pot (mean 6.3 larvae per pot) compared to the untreated control (6.5 larvae per pot). Similarly no significant effect on numbers of larvae recovered was observed with Met52 treatment outdoors (5.1 larvae per pot) compared to the untreated control (5.8 larvae per pot) (Figure 3.22). No larvae recovered exhibited any symptoms of Met52 infection but after fourteen days incubation at 20^{O}C, 30% of the lavae recovered from the pots maintained in the polytunnel and 29% of the larvae recovered from the pots maintained outside were dead and heavily infected with *Metarhizium.*

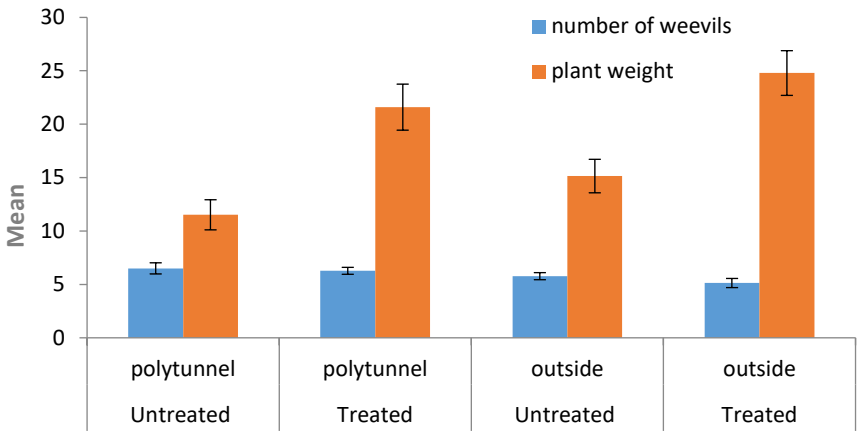


Figure 3.22 The mean number of weevil larvae recovered per plant and the plant weight in late- August to late-September. The error bars represent the standard error of the mean.

Plants which were maintained in Met52 treated compost during mid-August to late-September had a greater above ground plant weight than those plants maintained in untreated compost. This occurred both for pots maintained outside and for pots maintained inside the polytunnel. Pots that had been treated with Met52 were 10.1 g heavier and 9.6 g heavier when grown in the polytunnel or outside respectively (Figure 3.22).

Mean compost temperatures in late-August to mid-September ranged from 13.5 to 21.1°C (mean 17.2°C) for pots maintained within the polytunnel, and 14 to 34.6°C (mean 18°C) for pots maintained outside (Figure 3.23). The minimum compost temperature for pots maintained within the polytunnel ranged from 8.2 to 17.9°C (mean 13°C), while pots maintained outside ranged from 8.1 to 18°C (mean 13.4°C). The maximum compost temperature for pots maintained within the polytunnel ranged from 15.7 to 26.7°C (mean 21.6°C), while for pots maintained outside it ranged from 16.4 to 34.6°C (mean 25.4°C).

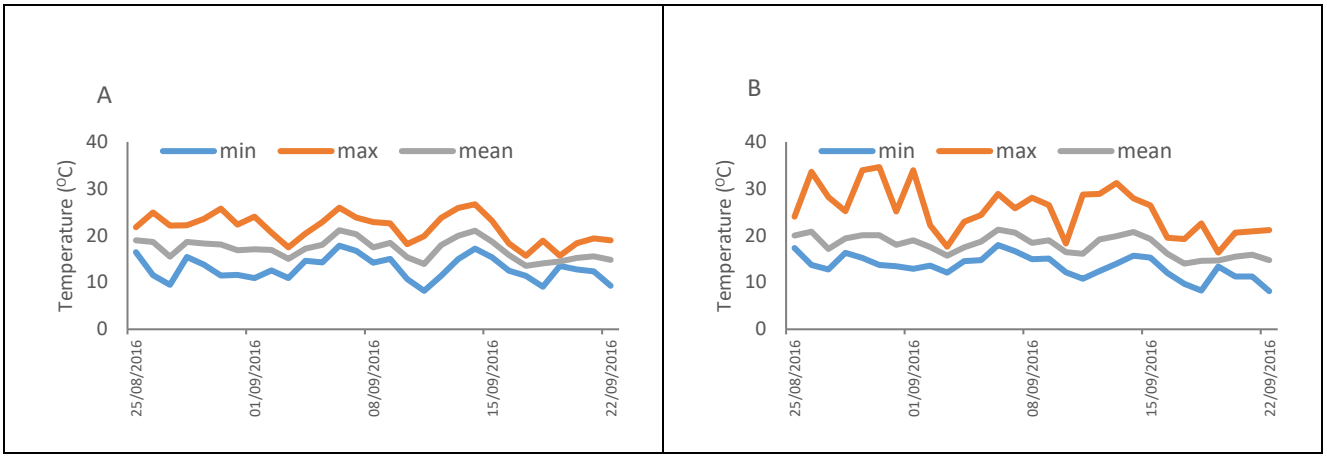


Figure 3.23: The mean, minimum and maximum compost temperature in pots maintained in either A) polytunnel or B) outside in late- August to late-September.

Replicate 3, start date = 15 September 2016. Fewer weevils were recovered from all pots on this occasion. For the untreated control, pot location had a significant effect on the mean number of weevil larvae recovered per pot (polytunnel = 4.6, outdoors = 3.1; $P < 0.05$). Within the polytunnel, treatment with Met52 had no significant effect on numbers of larvae recovered per pot (mean 3.9 larvae per pot) compared to the untreated control (4.6 per pot). Similarly no significant effect on numbers of larvae recovered was observed with Met52 treatment outdoors (2.7 larvae per pot) compared to the untreated control (3.1 larvae per pot) (Figure 3.24). All larvae recovered were alive and did not exhibit any symptoms of Met52 infection. After fourteen days incubation at 20^{O}C, 18% of the lavae recovered from the pots maintained in the polytunnel and 24% of the larvae recovered from the pots maintained outside were dead and heavily infected with *Metarhizium.*

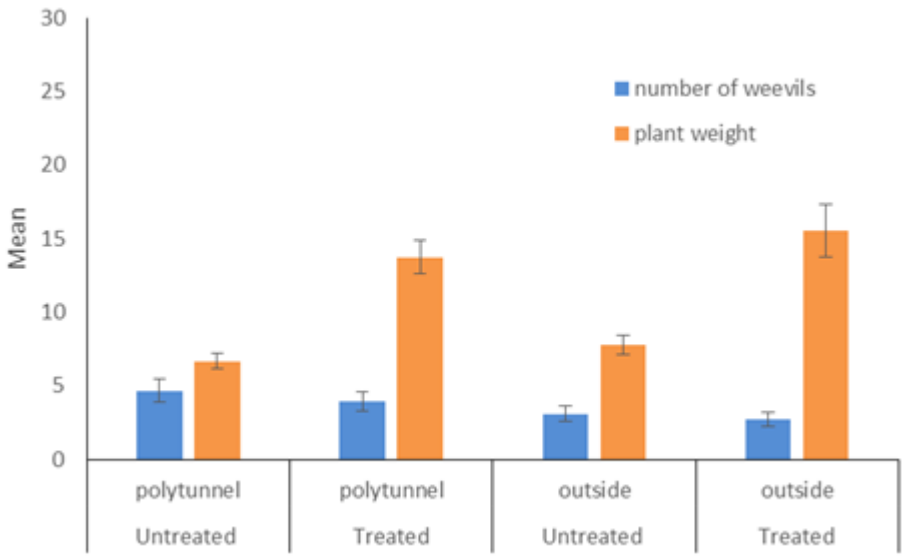


Figure 3.24 The mean number of weevil larvae recovered per plant and the plant weight in mid-September to mid-October. The error bars represent the standard error of the mean.

Plants which were maintained in Met52 treated compost had a greater above ground plant weight than those plants maintained in untreated compost. This occurred both for pots maintained outside and for pots maintained inside the polytunnel. Pots that had been treated with Met52 were 7.1 g heavier and 7.8 g heavier when grown in the polytunnel or outside respectively (Figure 3.24).

Mean compost temperatures during the trial ranged from 8.2 to 18.7°C (mean 13.2°C) for pots maintained within the polytunnel, and 8.2 to 19.3°C (mean 13.2°C) for pots maintained outside (Figure 3.25). The minimum compost temperature for pots maintained within the polytunnel ranged from 1.7 to 15.4°C (mean 9.2°C) while for pots maintained outside it ranged from 2.5 to 15.3°C (mean 8.5°C). The maximum compost temperature for pots maintained within the polytunnel ranged from 12.7 to 23.3°C (mean 17.7°C), while for pots maintained outside it ranged from 14.4 to 26.4°C (mean 18.6°C).

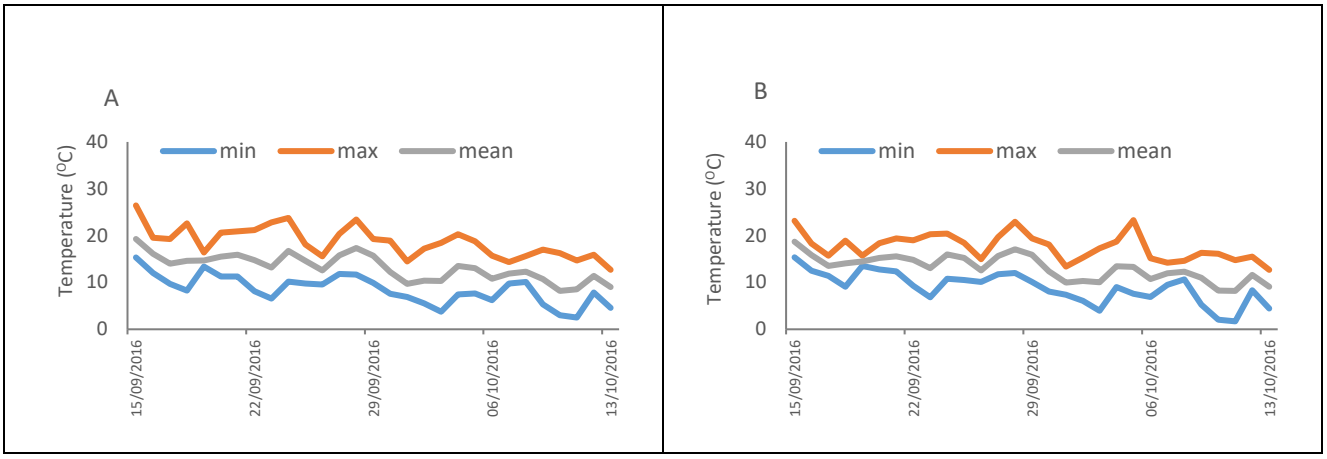


Figure 3.25 The mean, minimum and maximum compost temperature in pots maintained in either A) polytunnel or B) outside in mid-September to mid-October.

Replicate 4, start date = 1 October 2016. For the untreated control, pot location had a significant effect on the mean number of weevil larvae recovered per pot (polytunnel = 5, outdoors = 2.9; $p < 0.05$). Treatment with Met52 significant reduced the numbers of larvae recovered per pot (mean 3.9 larvae per pot) compared to the untreated control (4.6 per pot) within the polytunnel. No significant effect on numbers of larvae recovered was observed with Met52 treatment outdoors (1.8 larvae per pot) compared to the untreated control (2.9 larvae per pot) (Figure 3.26). All larvae recovered were alive and did not exhibit any symptoms of Met52 infection. After fourteen days incubation at 20^{O}C, 15% of the lavae recovered from the pots maintained in the polytunnel and 22% of the larvae recovered from the pots maintained outside were dead and heavily infected with *Metarhizium.*

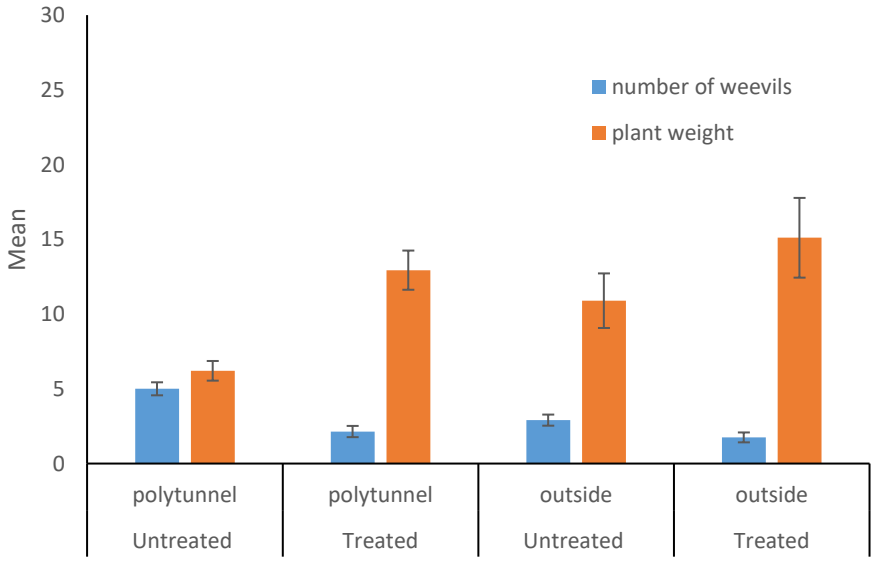


Figure 3.26 The mean number of weevil larvae recovered per plant and the plant weight in

October to May. The error bars represent the standard error of the mean.

Plants which were maintained in Met52 treated compost had a greater above ground plant weight than those plants maintained in untreated compost. This occurred both for pots maintained outside and for pots maintained inside the polytunnel. Pots that had been treated with Met52 were 6.7 g heavier and 4.2 g heavier when grown in the polytunnel or outside respectively (Figure 3.26).

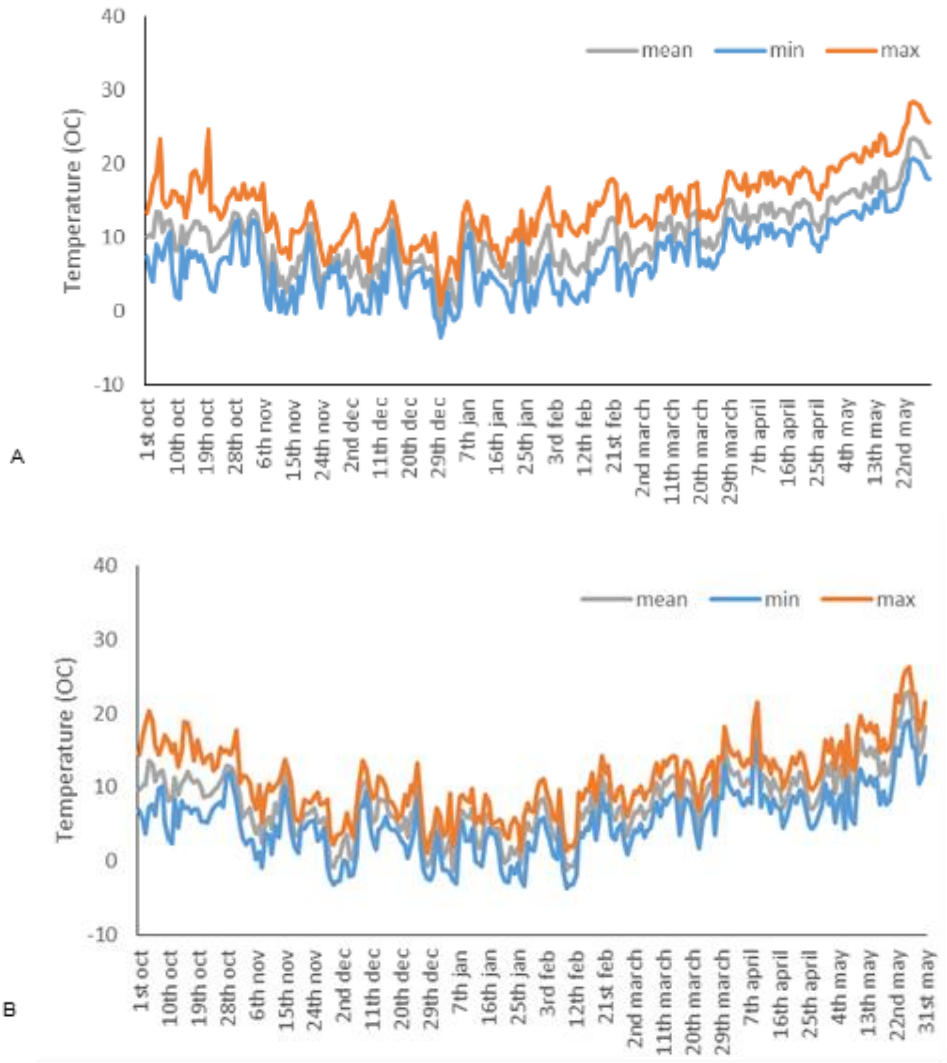


Figure 3.27 The mean, minimum and maximum compost temperature in pots maintained in either A) polytunnel or B) outside in October to May.

Mean compost temperatures during the trial ranged from -1.0 to 23.5°C (mean 10.1°C) for pots maintained within the polytunnel, and -1.3 to 22.9°C (mean 8.1°C) for pots maintained outside (Figure 3.27). The minimum compost temperature for pots maintained within the polytunnel ranged from -3.6 to 20.7°C (mean 6.9°C) while for pots maintained outside it

ranged from -3.7 to 19.0°C (mean 5.2°C). The maximum compost temperature for pots maintained within the polytunnel ranged from 0.9 to 28.4°C (mean 14.4°C), while for pots maintained outside it ranged from 1.1 to 26.2°C (mean 11.2°C).

Met 52 was present in all of the samples tested at the end of each replicate test. The number of colonies recovered from 1 g of compost ranged from 1.39 x 10^5 to 1.48 x 10^6 colonies. No significant differences in the number of colonies recovered from the compost were observed over time or between the locations of the pots. 100% waxmoth mortality was observed within five days of incubation within the treated compost at 20°C and Met52 mycosis observed on all of the cadavers (Figure 3.28).

Figure 3.28 Met52 colonies and Met52 infected waxmoth isolated from treated compost at the end of each experiment.

Discussion

Lower weevil control was observed in these experiments than expected. In previous research work for example, 66% weevil control was observed in strawberry plants treated with the same rate of Met52 and maintained at 20°C. There are a number of possible reasons why control may have been low: (i) the larvae did not move throughout the pot to come into contact with the *Metarhizium* conidia; or (ii) the environment was not suitable for infection. The larvae

that were recovered from the trials were smaller than expected . At the end of the trial, living larvae that were recovered were maintained at 20°C in boxes containing untreated compost and fresh carrot. After fourteen days, about 30% of the lavae were dead and heavily infected with *Metarhizium*, suggesting that the larvae had come into contact with Met52 but that the temperatures were not conducive to the development of a lethal infection. There are indications from the results that as temperature inceases the level of control increase (Figure 3.29) but further work is needed to validate this at further times in the growing season.

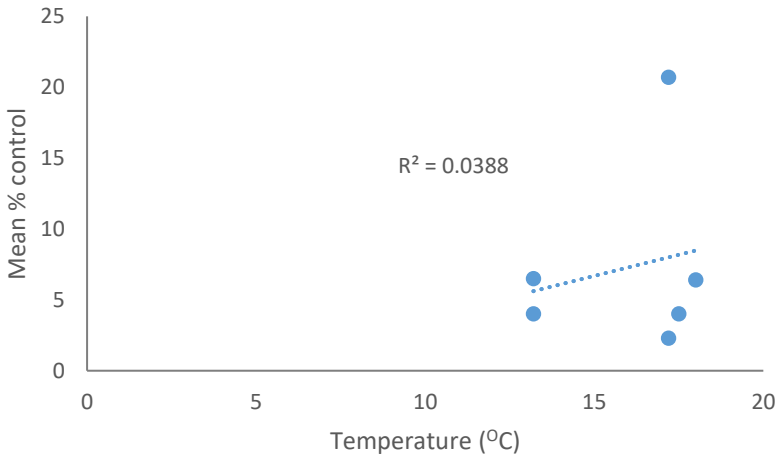


Figure 3.29 The mean % weevil control versus mean temperature.

Conclusions

- Low levels of control of weevil larvae were observed in comparison with an untreated control.
- An increase in plant weight was observed in Met52 treated plants.
- *Metarhizium* conidia remained viable within the compost.

Acknowledgements

Thanks to Fargro for providing the Met52 and to Alastair Hazell, Darby Nursery Stock for providing the plants.

Revision: Task 3.3.2a cold active entomopathogenic fungi (Warwick, years 2 and 3)

Laboratory experiments to quantify the effect of temperature on the virulence of Met52 to vine weevil larvae indicated strongly that this fungus has an optimum of about 27°C and has low infectivity at temperatures below 15°C. This is likely to be a problem when using the product on outdoor plants, as the soil temperatures in the autumn and spring when larvae are active and damaging plants are likely to be below 15°C. Fungal biopesticides can work very well against vine weevil larvae when the temperature is favourable, and they fit in well with IPM programmes. Hence there would be potential for a fungal strain that works well at the lower temperatures that typically occur in soil in the autumn and spring in the UK and other northern temperate countries where vine weevil is a problem. The aim of this new piece of work is to investigate the potential of 'cold active' EPF strains against vine weevil larvae. The target temperatures for fungal activity will be between 5 - 15°C. The work will be done as follows:

- An analysis of the scientific literature suggests that there are a number of EPF strains which are able to germinate and grow adequately at between 5 - 15°C and hence could have potential against vine weevil at these temperatures, although their infectivity to the pest has not yet been studied.
- We can get access to some of these strains from our research network and from 'open access' culture collections such as the US Dept. Agriculture collection of entomopathogenic fungal cultures (ARSEF). We will also seek to obtain cultures of other low temperature strains by directly contacting the authors of scientific papers where apparently cold active strains have been identified. Finally, we have some EPF strains in our own culture collection from northern European areas and other 'cold' regions of the world that may be worth investigating. However to our knowledge none of these strains have been tested against vine weevil. We will look to obtain up to ten different strains.
- Laboratory experiments will be done to measure the rate of spore germination and the rate of fungal colony extension on agar-based media at a range of temperatures from 5 - 15°C. The cardinal (i.e. optimum, minimum and maximum) temperatures for growth and germination will be estimated using non-linear statistical models that have been used elsewhere to investigate the physiology of ectothermic organisms.
- Fungal strains will then be evaluated for their virulence against vine weevil larvae at low temperatures. The exact temperatures will be decided once the results of the growth / germination experiments have been analysed, but will be within the 5 - 15°C range and will be compared against a 'high' temperature of 20°C. Met52 will be included as a standard and a positive control at 20°C. larval mortality over time will be analysed as described previously.

Methods

A literature review of cold tolerant entomopathogenic fungal isolates which have potential for control of vine weevil identified potential candidate isolates. Eleven isolates were acquired for experimentation from a variety of sources and were catalogued and placed in cryostorage in the Warwick Crop Centre collection of entomopathogenic fungal cultures (Table 1).

Table 1: Fungal isolates obtained for this study.

Species	Isolate	Host	Geographic origin
Beauveria bassiana	1789.17 (ARSEF 252)	*Hypothenemus hampei* (Coleoptera : Hyponomeutidae)	Brazil
	1790.17 (ARSEF 7552)	*Galleria mellonella* (Lepidoptera: Pyralidae)	Norway
	1791.17 (ARSEF 7554)	*Galleria mellonella (*Lepidoptera: Pyralidae)	Norway
Metarhizium anisopliae	108.82	*Wisena* sp. (Lepidoptera: Hepialidae)	New Zealand
	135.82	Oryctes rhinoceros (Coleoptera: Scarabaeidae)	France
	159.83	(Coleoptera: Scarabaeidae)	New Zealand
	1791.17 (ARSEF 4343)	-	Australia
	1792.17 (ARSEF 11661)	*Galleria mellonella* (Lepidoptera: Pyralidae)	Norway
Metarhizium brunneum	1793.17 (ARSEF 6477)	*Phyllopertha horticola* (Coleoptera: Scarabaeidae)	Norway
	1794.17 (ARSEF 5626)	*Tenebrio molitor* (Coleoptera: Tenebrionidae)	Finland

Metarhizium frigidum	1795.17 (ARSEF 4561) -		Australia

A series of experiments is underway to investigate the thermal biology of these fungal isolates. All experiments were ran at eight temperatures (4°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C) and each experiment was replicated three times.

The effect of temperature on fungal growth is being studied by measuring the rate of colony extension on a solid agar medium. Conidial suspension (100 µl) was spread evenly over SDA in Petri dishes and incubated in the dark at 23^{O}C for 48 h. Plugs (6 mm) cut from these plates with a flame-sterilised cork borer were then placed upside down in the centre of fresh SDA in Petri dishes, one plug per plate. The plates were incubated for 28 days (d) in darkness at 4°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C with two plates for each isolate / temperature combination. Colony diameters were measured with a ruler using two cardinal diameters every 7d for the duration of the experiment. The radial colony extension rate was calculated by plotting colony radius against time and a linear regression model was used to obtain the radial rate for each temperature.

The effect of temperature on fungal germination was assessed using the following method: 20 µl of conidial suspension was pipetted onto three previously-marked circles (approx. 2cm diameter) on plates of SDA. The plates were incubated at 4°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C in darkness for 24h. The germination assessment was carried out destructively by pipetting a drop of lactophenol methylene blue inside each circle. Treated plates were stored at 4°C before examination under a compound light microscope (Olympus BH-2, Tokyo, Japan) magnification x200. The numbers of germinated and ungerminated spores (conidia) were counted from a total sample of approximately 100 conidia per circle. Germination was defined as the point when an emerging germ tube was equal to, or longer than the width of the conidium.

The data, for both colony extension and germination, will be fitted to five non-linear models (a fourth order polynomial model, a model proposed by Briere et al (1999), a model proposed by Logan et al. (1976), a model proposed by Taylor (1981) and a model proposed by Lactin et al. (1995)) in RStudio (version 0.99.903 – © 2009-2016 RStudio, Inc) using the package Minpack.lm (version 1.2-0). The suitability of each model will be compared using AIC values and r2 and predictions made of the isolates thermal profile.

Objective 4. Develop novel approaches to control including the use of attractants, traps, botanical and microbial biopesticides

Introduction

Growers currently have limited IPM compatible options with which to control vine weevil adults. There is an urgent need to develop effective alternatives to the use of broad spectrum insecticides. In this respect the project team has recently completed two CRD-funded projects (PS2134 and PS2140) investigating the potential of refuge traps for infecting adult weevils with an entomopathogenic fungus. Results from these projects clearly demonstrate the potential of a lure and infect or lure and kill approach based on the use of artificial refuges. However, further development of this approach would require the approval of a fungal formulation for use in the refuges.

E-nema have recently developed the nematop® Käfer-Stopp traps, which are a modification of the grooved boards used for monitoring. However, here the grooves are filled with a gel containing insect-pathogenic nematodes of the species *Steinernema carpocapsae* so that adult weevils seeking refuge in the grooves become infected with the nematodes. Currently the nematop® Käfer-Stopp traps are sold for home garden use and are likely to be too expensive to be used under most commercial situations but the development of this product provides a model on which a cost-effective lure and kill approach could be developed.

The aim of this task is to assess the efficacy of a gel formulation of *Steinernema carpocapsae* used in the e-nema nematop® Käfer-Stopp traps (grooved boards similar to those used for monitoring but in which the grooves contain the gel. The purpose of this work is to determine the efficacy of the nematode gel formulation in controlling vine weevil adults under semi-field conditions.

Materials and methods

Task 4.1 Efficacy of lure and kill traps containing gel with insect-pathogenic nematodes of the species Steinernema carpocapsae

Insects

Insects were obtained from cultures maintained at Harper Adams University as described in Task 2.2.

Cage trial 1

Six large tent cages (1.45 m x 1.45 m x 1.52 m) (Insectopia, UK) were set up within an unheated glasshouse at Harper Adams University. Five potted (12 cm diameter pots) strawberry plants (cv. Elstanta) were placed in each tent cage to simulate a susceptible crop. These plants were watered from above as required, every three to four days. This ensured that the floor of the cage underneath each pot was damp but that there was no free water in which the weevils could drown. In half of these cages, 5 ml of the gel formulation of *Steinernema carpocapsae* was applied to the base of one of the plant pots (positioned in the centre of the cage (Figure 4.1) using a large syringe (Figure 4.2). Treatments were randomly allocated to each cage and control cages contained only untreated plant pots. Thirty weevils were then released into each cage. The cages were assessed after 28 days and the number of dead weevils in each cage was counted. Dead weevils from the treatment cages were dissected under a stereo microscope to confirm the presence of juvenile nematodes as the cause of death using the same method as that describe in project CP 089. Here each weevil was dissected in a watch glass in a droplet of water, paying particular attention to the head of the weevil. The number of dead weevils was compared between treatment and control cages using a one-way ANOVA. Temperature in the 'tent' cages was verified using iButton (HomeChip, UK) data loggers.

Figure 4.1. Five millilitres of the gel formulation of *Steinernema carpocapsae* applied to the base of a plant pot.

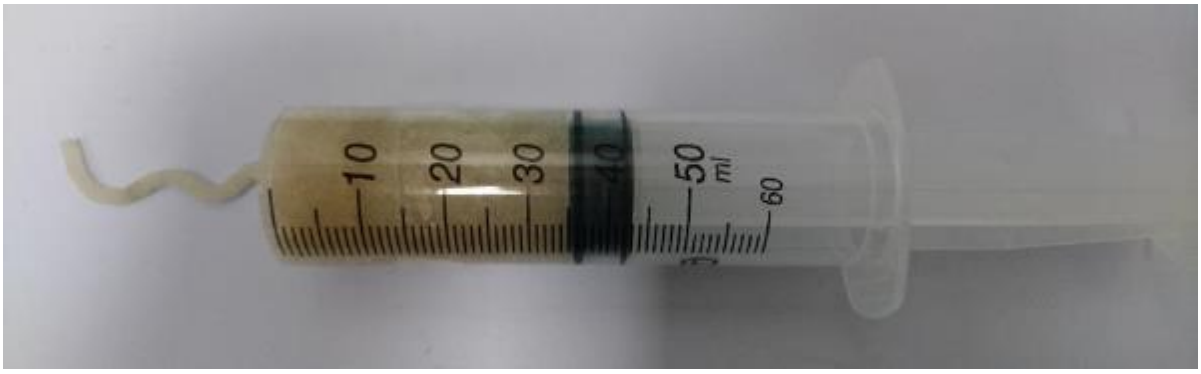


Figure 4.2. Large (60 ml) syringe used to apply the gel formulation of *Steinernema carpocapsae* to the base of a plant pot.

Cage trial 2

Six insect proof cages (47.5 cm x 47.5 cm x 47.5 cm) (BugDorm, MegaView, Taiwan) were set up within a controlled environment room (Fitotron, Weiss Technik, Ebbw Vale, Wales) set to 20°C, 60% RH and long-day conditions (L:D 16:8 h). Four potted (12cm diameter pots) strawberry plants (cv. Elstanta) were placed in each tent cage to simulate a susceptible crop. In half of these cages, 5ml of the gel formulation of *Steinernema carpocapsae* was applied to the central well of a Roguard crawling insect bait station (see Figure 4.3). The Roguard bait station was then placed on the floor in the centre of each of three cages. Approximately five millilitres of water was applied to the gel formulation every three to four days using a wash bottle to prevent the gel from drying out. In the three remaining cages an untreated Roguard crawling insect bait station was placed on the floor in the centre of each cage. Treatments were randomly allocated to each cage. The cages were assessed after 26 days and the number of dead weevils in each cage was counted. Dead weevils from the treatment cages were dissected to confirm the presence of juvenile nematodes as the cause of death.

Figure 4.3. Five millilitres of the gel formulation of *Steinernema carpocapsae* applied to the central well of a Roguard crawling insect bait station.

Results

Cage trial 1

Of the weevils that were released, 87.8% were recovered from the nematode treatment cages and 93% from the control cages. There was no significant effect (P = 0.056) of the nematode treatment on weevil mortality (Figure 4.4) although it is possible that this borderline result would show a difference between treatments with increased numbers of weevils. Of the dead weevils however, only one individual was shown to be infected with nematodes. The mean daytime (0800 to 1600 BST) temperature between 13th December and 10th January was 17.0 °C (max = 22.1 °C, min = 10.9 °C) and the mean night-time temperature was 10.4 °C (max = 13.5 °C, min = 6.3 °C). Taking a sample of gel containing *Steinernema carpocapsae* from

each pot and re-suspending this it was water it was possible to confirm viability of nematodes in each replicate (> 90 % viability based on a count of 100 nematodes).

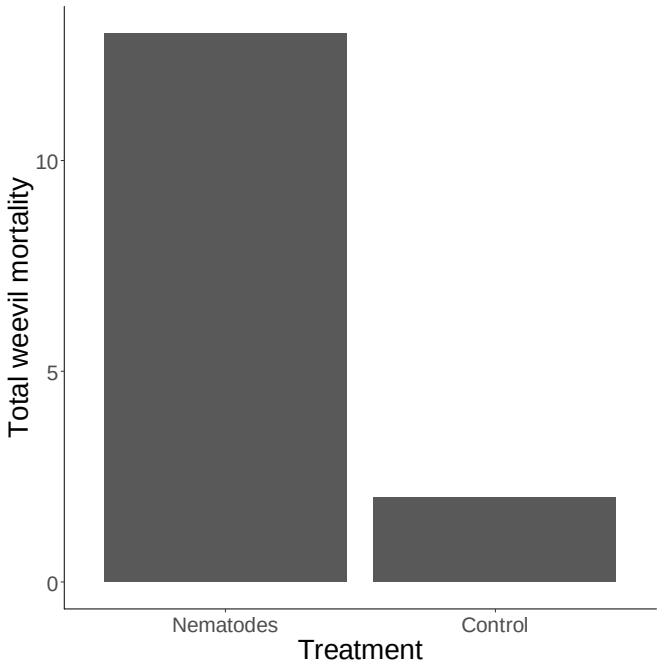


Figure 4.4. Number of dead weevils recovered from cages in which 5 ml of the gel formulation of *Steinernema carpocapsae* was applied to the base of one of the plant pot in each cage (n = 90 weevils).

Cage trial 2

Of the weevils that were released, 78% were recovered from the nematode treatment cages and 73% from the control cages. There was no significant effect (P = 0.078) of the nematode treatment on weevil mortality (Figure 4.5). Of the dead weevils, 42.9% were shown to be infected with nematodes. Taking a sample of gel containing *Steinernema carpocapsae* from

each pot and re-suspending this it was water it was possible to confirm viability of nematodes in each replicate (> 90 % viability based on a count of 100 nematodes).

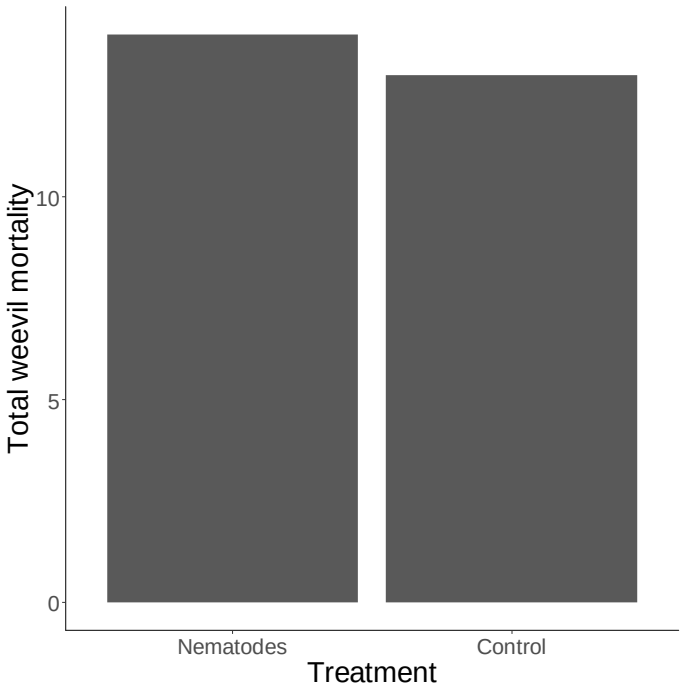


Figure 4.5. Number of dead weevils recovered from cages in which 5ml of the gel formulation of *Steinernema carpocapsae* was applied to the central well of a Roguard crawling insect bait station (n = 60 weevils).

Discussion

The efficacy of Nematop® Käfer-Stopp traps containing the *S. carpocapsae* gel formulation was tested in project CP 089. Results from this previous work showed that in insect proof cages containing one Käfer-Stopp trap placed on a coir substrate vine weevil mortality was 92% after 30 days. This compared with just 8% mortality in control cages. Furthermore, of the weevils that had died 83% were confirmed to contain nematodes. By comparison, in the present study using just the *S. carpocapsae* gel formulation placed either under plant pots or within a Roguard crawling insect bait station this treatment did not significantly increase vine weevil mortality after similar periods of time. Indeed, weevil mortality in treated cages was low in both trials, at 14% in Cage trial 1 and 23% in Cage trial 2. This is despite the fact that weevils were found to be in direct contact with the gel formulation in both trials and the *S. carpocapsae* were confirmed to be viable at the end of each trial.

There are several possible explanations for these contradictory results. Firstly, in the work completed in CP 089 alternative refuges, other than the Käfer-Stopp traps, were restricted to

a single seed tray with compost and sprigs of yew. As a result it is possible that there was a high probability that the weevils would have come into contact with the *S. carpocapsae* gel formulation in this study than in the work presented here. In the trials reported here, untreated potted strawberry plants were placed in each cage in addition to the treated pot in Cage trial 1 and the Roguard crawling insect bait station in Cage trial 2. As noted from the results from the cage trials completed in Objective 2, many of the weevils were found under or around plant pots during the day. In addition, both trials completed in this project used much larger numbers, 30 in Cage trial 1 and 20 in Cage trial 2, of weevils in each cage than in the work completed in project CP 089, which used five weevils in each cage. As such, even though up to two weevils in each cage were seen to be touching the *S. carpocapsae* gel formulation (numbers not recorded on each day of trials) in both trials, these weevils represented just a small proportion of the total number of weevils in each cage.

Temperature during Cage trial 1 may at first sight have been an important factor in explaining the results recorded. Grower advice is that *S. carpocapsae* requires temperatures of at least 12°C for several hours to be effective (see: https://www.e-nema.de). While this temperature was exceeded during daylight hours on most days of the trial this was rarely the case at night where the mean temperature was just 10.4°C. Nonetheless, weevils would have been more likely to have come into contact with the *S. carpocapsae* gel formulation during the day than during the night given that vine weevil adults seek refuge during the day (Moorhouse et al., 1992). It is also known that *S. carpocapsae* is capable of invading greater wax moth, *Galleria mellonella*, larvae at temperatures between 8 and 16°C (Saunders & Webster, 1999), although similar information is not available for vine weevil adults. Reproduction is, however, rare at low temperatures and development often does not progress beyond the infective juvenile stage (Saunders & Webster, 1999), which may explain the fact that only one of the dead weevils recorded from Cage trial 1 was found to be infected with nematodes.

Finally, it is recommended that the ground beneath the Käfer-Stopp traps is irrigated before placing the traps on the ground and that the traps themselves should be kept moist when in use (see: https://www.e-nema.de/assets/Uploads/Downloads/instruction_nematop-vine-weevil-stop-en.pdf). In the current study, the *S. carpocapsae* gel formulation was kept moist throughout both trials by watering the strawberry plants regularly (every 3-4 days) (Cage trial 1) and by applying approximately 5 ml of water to the central well of the Roguard crawling insect bait station every 3-4 days (Cage trial 2). Even though the nematodes were confirmed to be viable at the end of both trials it remains possible that the conditions in each trial were not suitable for the nematodes to invade the weevils.

Conclusions

- Vine weevil adults come into contact with a gel formulation of *Steinernema carpocapsae* when this is placed under plant pots or within Roguard crawling insect bait stations.
- No evidence that the presence of the gel formulation of *Steinernema carpocapsae* increases the mortality of vine weevil adults under the conditions tested.
- Further work with the Käfer-Stopp traps is required to fully evaluate the potential of these devices and the gel formulation of *Steinernema carpocapsae.*

Acknowledgements

We would like to thank the funders of the research AHDB Horticulture for their support. We would also like to thank:

- Berry Gardens, growers and ADAS fruit consultants for continued support in sourcing sites for collecting vine weevils.
- Darby Nursery Stock for hosting and helping with the 'little and often' nematode trial.
- BASF for supplying Nemasys H and Nemasys L for the 'little and often' nematode trial and for technical discussions.
- E-nema for the supply of the gel formulation of *Steinernema carpocapsae.*

Conclusions from all the work in year 2

Objective 1. Improve understanding of the impact of environmental conditions on vine weevil biology and behaviour in order to optimise application of plant protection products

- Vine weevil adults feed within the temperature range 6 - 18°C but the amount of leaf material eaten is not affected by temperature within this range.
- Overwintered vine weevil adults appear to require a period of intense feeding activity before egg laying can recommence.

- Overwintered vine weevil adults are likely to become active and start feeding, even outside, in March, although egg laying may not start for at least five weeks after feeding behaviour resumes.

Objective 2. Develop practical methods for monitoring adults in order to detect early infestations and inform control methods

- Vine weevil adults are attracted to host plant volatiles and to the odour of frass produced by other weevils.
- Vine weevil adults are attracted by the odour produced by other vine weevils but only when not starved.
- Vine weevil adults are attracted by the plant volatiles (*Z*)-2-pentenol and methyl eugenol as well as to two simple blends of plant volatiles.
- There is no evidence that the addition of a lure based on plant volatiles increases catches of vine weevil adults.

Objective 3. Improve best-practice IPM approaches including the use of entomopathogenic nematodes, fungi and IPM-compatible insecticides

- A 'little and often' system for applying reduced rates of entomopathogenic nematodes through the overhead irrigation was validated on a commercial nursery. Application of nematodes at 40% rate five times between 21 June and 11 October was equally as effective in reducing mean numbers of vine weevil larvae per plant on all four Fuchsia varieties as two conventional full rate drench applications on 22 September and 21 October. Using 40% rates five times between June and October offers up to 52% cost savings compared with using standard high volume drenches without compromising on efficacy.
- A predictive day degree model was developed to predict Met52 infection and kill of vine weevil larvae. The model estimates that no kill will occur at temperatures below 11.6°C and that for 75% control 256 cumulative day degrees are needed. Analysis of historical temperature data recorded from UK nurseries suggests that this could be reached during June, July and August in some years and locations. A cold-tolerant fungal strain would be useful for use in autumn pottings and investigations into the activity of cold tolerant strains are underway.

Objective 4. Develop novel approaches to control including the use of attractants, traps, botanical and microbial biopesticides

- Vine weevil adults came into contact with a gel formulation of *Steinernema carpocapsae* when this was placed under plant pots or within Roguard crawling insect bait stations.
- There was no evidence that the presence of the gel formulation of *Steinernema carpocapsae* increased the mortality of vine weevil adults under the conditions tested.
- Further work with the Käfer-Stopp traps is required to fully evaluate the potential of these devices and the gel formulation of *Steinernema carpocapsae.*

Knowledge and Technology Transfer

Presentations

- 5-8 June 2017 – Jude Bennison and Tom Pope, IOBC conference 'Integrated Control in Protected Crops, Temperate climate, Niagara, Ontario, Canada.
- 25-26 September 2017– Sam Brown, Joe Roberts and Tom Pope, AAB conference 'Advances in IPM, Harper Adams University, Newport, Shropshire.
- 7 December 2017 – Jude Bennison, AHDB growing media conference, Fareham, Hanmpshire.
- 20 February 2018 – Jude Bennison, AHDB Horticulture ornamentals conference, Kenilworth, Warwickshire.
- Video of 'little and often' nematode trial at Darby Nursery Stock – Alastair Hazell, Jude Bennison, Sam Brown and Kerry Boardman. https://horticulture.ahdb.org.uk/video/vine-weevil-control-%E2%80%93-overhead-nematode-application

Publications

- Bennison, Jude; Brown, Sam & Boardman, Kerry (2017). A 'little and often' system for application of entomopathogenic nematodes for vine weevil control in hardy ornamental nursery stock. *IOBC/wprs Bulletin* **124**, 88-94.
- Pope, Tom; Graham, Juliane; Rowley, Charlotte; Bennison, Jude; Prince, Gill; Chandler, Dave and Hall, David (2017). Improved monitoring of vine weevil (*Otiorhynchus sulcatus*) adults. *IOBC/wprs Bulletin* **124**, 81-87.

Glossary

Entomopathogenic – capable of causing disease or death in insects

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Appendix 1

Trial plan in holding bay at Darby Nursery Stock

PLOT	1	2	3	4	5	6
BLOCK	1	1	1	1	1	1
TREATMENT	6	1	2	4	1	4
SPECIES	2	3	3	3	2	1
PLOT	7	8	9	10	11	12
BLOCK	1	1	1	1	1	1
TREATMENT	2	6	1	2	3	5
SPECIES	2	3	1	1	1	3
PLOT	13	14	15	16	17	18
BLOCK	1	1	1	1	1	1
TREATMENT	5	6	4	2	5	5
SPECIES	1	1	4	4	2	4
PLOT	19	20	21	22	23	24
BLOCK	1	1	1	1	1	1
TREATMENT	3	3	4	1	3	6
SPECIES	2	3	2	4	4	4

PLOT	25	26	27	28	29	30
BLOCK	2	2	2	2	2	2
TREATMENT	3	1	2	4	1	3
SPECIES	4	4	2	4	3	3
PLOT	31	32	33	34	35	36
BLOCK	2	2	2	2	2	2
TREATMENT	2	2	4	6	6	5
SPECIES	4	3	1	2	3	3
PLOT	37	38	39	40	41	42
BLOCK	2	2	2	2	2	2
TREATMENT	4	5	3	1	6	1
SPECIES	2	1	1	2	1	1
PLOT	43	44	45	46	47	48
BLOCK	2	2	2	2	2	2
TREATMENT	5	3	5	4	2	6
SPECIES	2	2	4	3	1	4
PLOT	49	50	51	52	53	54
BLOCK	3	3	3	3	3	3
TREATMENT	1	5	4	1	5	2
SPECIES	2	4	3	4	2	1
PLOT	55	56	57	58	59	60
BLOCK	3	3	3	3	3	3
TREATMENT	4	3	3	6	2	5
SPECIES	2	1	4	2	3	3
PLOT	61	62	63	64	65	66
BLOCK	3	3	3	3	3	3
TREATMENT	5	1	3	2	6	4
SPECIES	1	3	2	4	3	4

PLOT	67	68	69	70	71	72
BLOCK	3	3	3	3	3	3
TREATMENT	1	6	2	6	4	3
SPECIES	1	1	2	4	1	3
PLOT	73	74	75	76	77	78
BLOCK	4	4	4	4	4	4
TREATMENT	6	3	1	2	2	1
SPECIES	1	2	4	2	4	2
PLOT	79	80	81	82	83	84
BLOCK	4	4	4	4	4	4
TREATMENT	2	5	2	3	5	1
SPECIES	3	3	1	3	1	3
PLOT	85	86	87	88	89	90
BLOCK	4	4	4	4	4	4
TREATMENT	6	5	3	4	4	6
SPECIES	4	4	1	3	1	2
PLOT	91	92	93	94	95	96
BLOCK	4	4	4	4	4	4
TREATMENT	4	5	4	6	1	3
SPECIES	2	2	4	3	1	4
PLOT	97	98	99	100	101	102
BLOCK	5	5	5	5	5	5
TREATMENT	6	3	3	2	5	1
SPECIES	1	3	4	1	1	1
PLOT	103	104	105	106	107	108
BLOCK	5	5	5	5	5	5
TREATMENT	3	4	2	6	6	1
SPECIES	1	1	3	4	2	4
PLOT	109	110	111	112	113	114

BLOCK	5	5	5	5	5	5
TREATMENT	1	4	2	5	4	6
SPECIES	2	2	2	3	3	3
PLOT	115	116	117	118	119	120
BLOCK	5	5	5	5	5	5
TREATMENT	2	5	4	3	1	5
SPECIES	4	4	4	2	3	2

Treatment Number	Treatment
1	Nemasys H/L Full rate drench**
2	Full rate Nemasys H/L
3	Reduced rate Nemasys H/L (20%)
4	Reduced Rate Nemasys H/L (40%)
5	Water Drench**
6	Water control overhead

Species number	Species
1	Fuchsia Riccartonii
2	Fuchsia Mrs popple
3	Fuchsia Hawkshead
4	Fuchsia Tom Thumb