Geographic origin may not influence vine weevil Otiorhynchus sulcatus (Fabricius) susceptibility to the entomopathogenic fungus Metarhizium brunneum (Petch)

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Geographic origin may not influence vine weevil *Otiorhynchus sulcatus* (Fabricius) susceptibility to the entomopathogenic fungus *Metarhizium brunneum* (Petch)

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Abstract

Otiorhynchus sulcatus, known as the vine weevil, is a polyphagous pest that causes economically important damage to horticultural crops worldwide. The entomopathogenic fungus *Metarhizium brunneum* is widely used to control this pest. Little research has investigated variation in susceptibility to this pathogen between vine weevil populations at different locations. This study addresses this knowledge gap by comparing survival rates of larvae from adults collected in two UK areas when treated with *M. brunneum*. Larvae from these locations did not differ in their susceptibility, suggesting that location *per se* may not affect the efficacy of *M. brunneum* against vine weevil larvae.

Introduction

The vine weevil, *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae), is a curculionid endemic to central Europe, yet its distribution has expanded to most parts of Europe, parts of North America, South America, New Zealand and Japan. The vine weevil is highly polyphagous and so threatens a wide range of horticultural crops around the world, among them economically important soft-fruit crops such as strawberry. In the UK alone, more than 2,000 ha of strawberry crops were affected in 2016 and losses were worth an estimated £14M (Wynn, 2010). In addition, ornamental crops, such as plants within the genera *Rhododendron*, *Photinia*, *Euonymus* and *Cyclamen* are also subject to damage by this pest.

Adult weevils live above-ground and feed on leaves and flowers causing mainly cosmetic damage, whereas larvae live below-ground and feed on plant roots, stems and bulbs, which reduces plant vigour and may cause plant death. Larvae are usually the greatest concern for growers because of the damage they cause affects plant health, but also because as the larvae feed below-ground infestations are sometimes only noticeable when severe damage to the plant has been caused. Hence, control strategies targeting the vine weevil often primarily focus on this

life stage.

All vine weevils analysed so far are triploid females which reproduce by thelytokous parthenogenesis. This reproductive strategy is expected to be detrimental for adaptation to new habitats, yet it has not hampered vine weevil range expansion. Little research has focused on the biological distinctiveness of populations of this species, which could be the key to understanding vine weevil adaptation ability. Lundmark (2010) compared genetic sequences of weevils collected from Germany, The Netherlands, the UK and the USA. The study inspected a partial sequence of the cytochrome oxidase III, the elongation factor-1α gene and a fragment of a non-coding nuclear sequence and reported few nucleotide substitutions within these genetic markers. These results were then extrapolated to the whole genome and it was assumed that, genetically, vine weevil populations are not significantly different. This conclusion, however, was limited by the paucity of genetic information on this insect species. This limitation remains today as there has not been any further attempt to investigate the vine weevil genetic diversity.

To identify population diversity at a microbial level, Morera-Margarit *et al.* (2019) characterised the bacterial community of vine weevils from various locations. The populations tested harboured very similar bacterial community compositions. However, the application of the newly developed bioinformatic pipeline QIIME2 could reveal greater between population variation in future investigations. Morera-Margarit *et al.* (2019) used QIIME for the analysis of the vine weevil microbiota. This is an open-source bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing. QIIME uses the Operational Taxonomic Unit or OTU approach. In this method, nucleotide sequences of a given percentage similarity, 97% in most cases, are clustered together generating what is referred to as an OTU. From the clustered sequences in an OTU, the most abundant is chosen as the representative sequence to identify the

taxonomy of the OTU. QIIME2, the newest version of the pipeline, includes statistical tools to correct for nucleotide sequencing errors. In this manner, QIIME2 generates unique sequences that are used to taxonomically identify bacteria. QIIME2 allows for a more comprehensive understanding of bacterial communities as it takes into account the biological diversity dismissed in the OTU approach (reviewed by Fricker et al., 2019).

Fitness variation between vine weevil populations inhabiting separate locations has to date been unexplored. The aim of the present study was to address this knowledge gap by investigating if vine weevils from geographically separate locations differ in their susceptibility to the fungal pathogen *Metarhizium brunneum* (Petch) (Hypocreales: Clavicipitaceae). *M. brunneum* is an entomopathogen widely used as part of integrated pest management strategies targeting the vine weevil, especially the larvae of this insect. Thus, in addition to examining vine weevil between population variation, the aim of this research was to contribute to improved control strategies targeting vine weevil larvae. To conduct our experiments, vine weevil adults were collected from two distant locations within the UK. Population variation in susceptibility to *M. brunneum* was examined in the offspring larvae of these insects. Mortality was statistically analysed using a mixed effects Cox regression model.

Materials and methods

Vine weevil adults were collected from strawberry crops at two UK sites separated by 524 km: Stafford, Staffordshire, and Invergowrie, Perth and Kinross (collection site = population). These vine weevils were kept in Petri dishes lined with moist paper (Kleenex, Kimberly-Clark professional, Kent, UK) and provided with strawberry leaves (*Fragaria* x *ananasa* Duchesne, mixed varieties) in controlled conditions (18°C, 16:8 h L:D).

Strawberry (*Fragaria x ananasa*, var. Elsanta) plants used for the experiment were grown in 1 L pots with a 3:1 mixture of compost (peat-sand-perlite 6N: 3P: 1K; Everris Ltd, Ipswich, UK): grit sand (Arthur Bower's Ltd, Lincoln, UK). Vine weevil eggs were collected from Petri dishes in which the collected adults had fed on strawberry leaves for a week. To infest the plants, 20 eggs were gently washed into a small indentation in the surface of the compost 2 cm deep and 1 cm wide at 2 cm from the main plant stem. Compost temperature was measured with thermocrons (DS1921G-F5 thermocrons, Homechip Ltd, Milton Keynes, UK), placed 5 cm deep in the compost, and the software OneWireViewer.exe v. 0.3.19.47. Average substrate temperature was 15°C ± 6°C. Plants were arranged in a randomised block design, each block comprising two strawberry plants representing a replicate of each population. Three blocks were infested each week for 14 weeks. Experiments were completed in a glasshouse (14-20°C, 16:8 h L:D).

Larvae were collected four to six months after the plants had been infested with eggs by removing the plants from the pots and hand searching the compost and roots for larvae. Only larvae that were between 0.045 g and 0.09 g in weight were used in this experiment. Plants from blocks infested during three consecutive weeks were grouped to ensure enough larvae were collected for a single experiment.

Metarhizium brunneum isolate 275.86, strain commercialised as Met52® (Novozymes, Denmark), was provided by Warwick Crop Centre at Warwick University, UK. Cultures of this isolate were grown for 14 days in Sabouraud dextrose agar media (20 g glucose, BDH, Lutterworth, UK; 5 g mycological peptone, Oxoid, Basingstoke, UK; 10 g technical agar no.3, Oxoid, Basingstoke, UK; 500 ml deionised water) in the dark at 23°C ± 1°C. Conidia were harvested by gentle agitation in sterile 0.01% Triton X-100 solution (BDH, Lutterworth, UK).

Conidia concentration was enumerated using a Neubauer improved haemacytometer. Conidia were then spun down and re-diluted with sterile water to a final concentration of 10^7 conidia/mL. This conidia suspension was diluted with a Triton X-100 solution to achieve a working concentration of 10^6 conidia/mL in 0.05% Triton.

Vine weevil larvae treatment consisted of pipetting 25 µL of conidia suspension onto the thoracic segments close to the head of the larva (Klingen et al., 2015). Negative controls were treated with the same volume of 0.05% Triton X-100 solution. *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae) larvae (Big larvae, UK Waxworms Ltd) were used as positive controls and were treated with 25 µL of conidia suspension in a similar way. Larvae were placed in Petri dishes (92 mm diameter) with a 1 cm deep layer of moist compost (insecticide-free peat-sand-perlite 6N: 3P: 1K; Everris Ltd, Ipswich, UK) and thin carrot slices as a food source at 18°C, in the dark (Klingen et al., 2015).

Each experimental replicate (4 experimental replicates in total) was arranged as a randomised block design comprising four blocks. Each block contained at least one *M. brunneum* treatment dish per vine weevil population, two negative control dishes for each vine weevil population and one positive control dish consisting of *Galleria mellonella* treated with *M. brunneum*. Given that natural mortality of vine weevil larvae can be variable, for example as a result of a natural infections or disturbance occasionally killing all weevils within a Petri dish, two negative control dishes were used per population replicate in order to accurately record control mortality in this experiment. The position of the dishes within the block was randomised.

Petri dishes with the same treatment, i.e. conidia-treatment or control, within the same experiment replicate always contained the same number of larvae. Petri dishes were assessed once each week in a four-week period by removing and counting dead larvae, and replacing the

carrot slices. Mortality caused by the fungal pathogen was manually assessed by applying a slight pressure to identify mummified larvae, as well as visually by observing white mycelia and/or green conidia on the larval surface. Initial numbers of larvae per population and treatment were: Stafford-control = 28, Stafford-conidia treatment = 61, Invergowrie-control = 43 and Invergowrie-conidia treatment = 154.

Statistical analysis and graphical representation were performed using R software v. 3.3.3 and the packages ggplot2 survival (T. M. Therneau & Grambsch, 2000), coxme (T. C. Therneau, 2018), survminer (Kassambara & Kosinski, 2018), car and plyr. To test for significant differences a survival object was created and was tested using a mixed effect Cox model for interactions. The model included the interactions between the fixed factors vine weevil population-conidia treatment and the random factors block and experiment replicate. A post-hoc log-rank test for multiple comparisons with Benjamini-Hochberg p-value adjustment method was performed.

Results

The Mixed effects Cox regression to test for the effect of *M. brunneum* on larval survival revealed a significant effect of the treatment although not for the population of origin ($\chi^2 = 10.15$, d.f. = 1, p-value for treatment = 0.001). The Cox proportional hazard, or hazard ratio (HR), calculated for treatment indicated that treating larvae with conidia increased the mortality rate by a factor of 2.2 (HR=2.2). The hazard ratio for population also indicated that the origin of the vine weevil population did not affect larvae survival (HR=1). The post-hoc analysis revealed a significant decrease in survival of conidia-treated larvae over the experimental period within population but no differences were found between the two populations (Stafford control-conidia treatment p-value = 0.003, Invergowrie control-conidia treatment p-value = 0.003; Figure 1).

Closer inspection at the descriptive statistics revealed that there was background mortality in the negative control larvae. Mortality was higher for the Invergowrie population, although it showed greater variation for the Stafford population (median values for survival: Stafford-control = 2 weeks 95% CI [2, 4], Stafford-conidia treatment = 2 weeks 95% CI [2,2], Invergowrie-control = 2 weeks 95% CI [2, 3], Invergowrie-conidia treatment = 2 weeks 95% CI [2, 2]). We attribute this mortality to stress induced during collection and handling of the larvae when setting-up the experiment, as well as due to the artificial experimental conditions.

Background mortality did not affect the efficacy of the entomopathogenic fungus under the conditions used for our experiments. In the 1st treatment week, probability of survival was similar between control and conidia-treated larvae (Table 1). After the 1st week, however, the probability of survival in conidia-treated larvae decreased to a much higher level than the probability of survival in the control larvae (Table 1). Additionally, both conidia treatments had 100% mortality at the end of the experiment (Table 1). Hence, despite having background mortality in control larvae throughout the experimental period, a mortality rise in conidia-treated larvae was attributed to the fungal pathogen.

The difference in number of larvae from each weevil population used reflects differences in larval mortality during the rearing of these insects on pot grown plants. High mortality when rearing vine weevil larvae on potted plants has been previously reported, with levels of mortality ranging from 80 to 99%. The reason(s) for this high mortality is as yet unknown, although it has been suggested that cannibalism or lack of nutrients could be the underlying cause (LaLone & Clarke, 1981).

Discussion & Conclusion

This study is the first to investigate differences in susceptibility of vine weevil larvae collected at different geographic locations to the entomopathogenic fungus *M. brunneum*. It adds to the limited literature employing survival analysis to examine vine weevil susceptibility to *M. brunneum* (there is only one other study: Klingen et al., (2015)). Our experiments confirm that *M. brunneum* can infect vine weevil larvae despite using a temperature close to the lower fungal growth threshold following the experiments carried out by Klingen et al. (2015). However, we did not detect differences in susceptibility to *M. brunneum* associated with vine weevil population.

Mortality values on larvae treated with conidia obtained in our experiments were similar to values reported by Klingen et al., (2015), despite the fact that Klingen et al., (2015) applied a 10-fold higher conidia concentration. This may suggest that the conidia concentration used for our experiments saturated the immune response capacity of the insect, which could have masked location-associated variation in susceptibility to this natural enemy. It would therefore be useful to test lower conidia concentrations in future research to reveal the existence of geographic differences in resistance to this entomopathogenic fungus.

Vine weevil larvae are known to be subject to high levels of mortality when reared under artificial conditions. Rearing vine weevil larvae on pot grown plants presents a more natural environment but despite this, larval mortality remains high and has previously been reported to be above 80% under such conditions (LaLone & Clarke, 1981). Klingen et al., (2015) tested survival of vine weevil larvae following exposure to different entomopathogenic fungi. These experiments were carried out in controlled environment rooms with set temperature, humidity and light regime. However, mortality was still high reaching approximately 50% for control

larvae after four weeks in these experiments. In our experiments, mortality of larvae not exposed to *M. brunneum* conidia was similarly high at the end of the experimental period.

The application of a Cox regression model to analyse our data allows a more robust analysis of the entomopathogen action irrespective of larval mortality in the control group. This survival analysis determines the probability of survival at each time point and the hazard ratios for each of the variables. Statistical methods for survival analysis are superior to linear regressions to test pesticide susceptibility. This is because survival regressions take into account the time passed until an *event* occurs (i.e. the speed of entomopathogen action), while linear regressions only take into account the number of individuals that experienced the *event* by the end of the experimental period (George et al., 2014).

This study is limited by the small number of populations tested, yet, it establishes the foundations for future research focusing on testing the existence of niche-associated changes in vine weevil resistance to *M. brunneum*. Results presented here suggest that location-specific strategies for vine weevil control using *M. brunneum* may not be necessary. Nonetheless, additional vine weevil populations collected from a wider range of locations but also from a greater diversity of cropped and uncropped habitats should still be studied in this way. These experiments will contribute to confirming whether susceptibility to *M. brunneum* remains consistent regardless of the geographic origin or crop environment.

Metarhizium brunneum is commercialised as a product for which application is currently standardised, hence the same procedure is applied irrespective of the geographic area or the crop affected. This initial study indicates that *M. brunneum* is equally effective regardless of the geographic origin of the vine weevil population, however, additional work is still required to confirm these initial findings.

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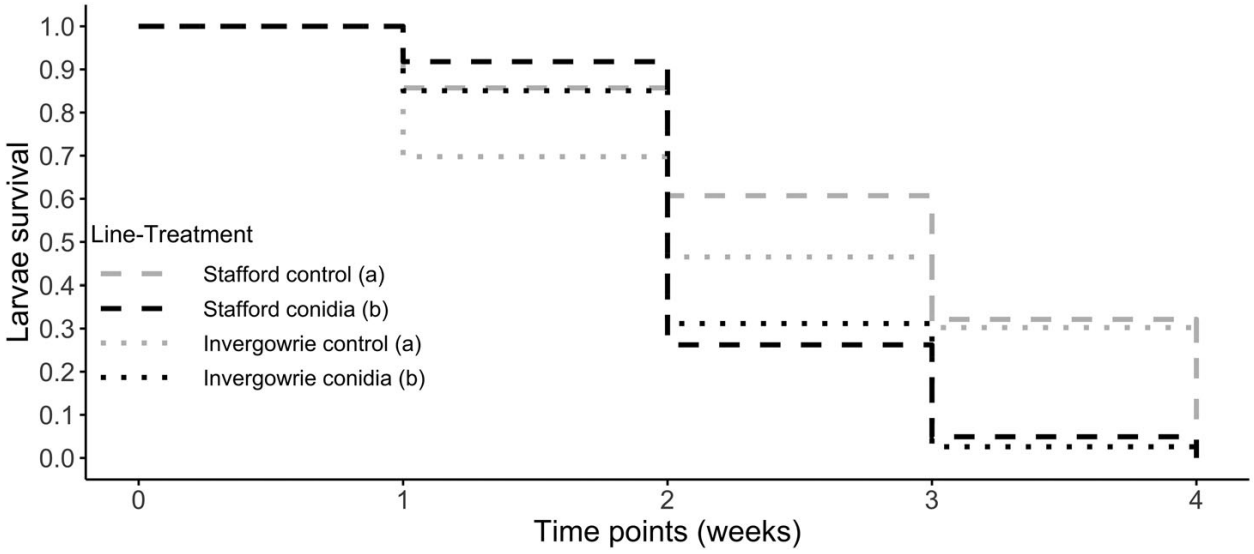

Figure 1. Cox regression for survival of vine weevil larvae from two different populations treated with *Metarhizium brunneum*. Y-axis represents larvae survival while x-axis represents the time points considered for the study (weeks). Line-treatment combinations sharing the same letters were not significantly different (log-rank test: p-value< 0.05).

289 Table 1. Probability of survival for larvae from both populations, Stafford and Invergowrie. The
290 table shows the probability of survival as a percentage of the total number of larvae throughout the
291 experimental period given by the Cox regression model. The time points at which mortality was
292 recorded are represented as 1st to 4th week..
293

Probability of survival	Stafford control	Stafford conidia	Invergowrie control	Invergowrie conidia
1st week	86%	92%	70%	85%
2nd week	61%	26%	47%	31%
3rd week	32%	5%	30%	3%
4th week	6%	0%	24%	0%

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