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Key staff:	
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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.

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CONTENTS

SCIENCE SECTION

Introduction

Chemical insecticides are the most commonly used control method for plant pathogenic insects. Their extensive use throughout the past decades have resulted in the development of resistance against almost all types of synthetic insecticides in the peach-potato aphid, *Myzus persicae* (Bass et al., 2014; Skinner et al., 2014). The loss of effectiveness of these chemicals and the growing concern about their negative effect on human health and the environment urges scientist to work on finding new alternatives as well as improving existing ones (Chandler et al., 2011). Furthermore, European legislators are implementing strict restrictions on synthetic pesticides that fall under the newly implemented criteria for human endocrine disruptors (European Commission, 2018), which creates additional pressure on the farmers to reduce the use of chemical insecticides. This should increasingly push industry to find other alternatives for the control of pest populations.

Biopesticides include microorganisms (algae, bacteria, fungi, protozoa and viruses), pheromones and semiochemicals, macrobials and invertebrates (insects and nematodes) and plant extracts/botanicals (OECD, 1996). They represent only 5% of the global market of plant protection products but have shown a strong long-term growth compared to synthetic chemicals. The fastest growth in the near future is expected to be in Europe due to regulatory changes and the increasing demand for organic products (Marrone, 2014).

Despite the number of available formulated products, bioinsecticides based on entomopathogenic fungi (EPF) have not taken a significant share of the insecticide market. The reasons for this include poor and unreliable performance compared to chemical pesticides due to environmental factors such as temperature, humidity and UV. They exhibit a slower speed of kill and, due to the lack of residual effect, need to be applied more frequently, which complicates the logistics of use (Jaronski, 2010). Previous studies aiming to improve EPF for the control of arthropods includes genetic modification in order to overexpress enzymes (Fang et al., 2012), expression of genes encoding insect specific toxins (Lovett et al., 2019; Timofeev et al., 2019; M. Xie et al., 2015) and improving tolerance to temperature, UV and oxidative stress (X. Q. Xie et al., 2010). Strain enhancement using genetic engineering provides exceptional tools to enhance tolerance against abiotic stresses and increase virulence. The use of transgenic fungi has been showing promising results (Bilgo et al., 2017; Lovett et al., 2019; Lovett & St Leger, 2018), however field application of such GM organisms would face regulatory issues in Europe.

Using technical and non-GM biological resources to improve EPF as biological control agents might provide a commercially more acceptable alternative for the near future of pest control. In his review, Jaronski (2010) captures the considerable effort made to improve EPF strains via novel spore delivery methods, UV protectants, humidity stabilizers and different growth substrates to increase sporulation, virulence and stress resistance. Compatibility of EPF with other control agents, such as fungicides is also an important quality. Using a combination of random mutagenesis and artificial selection has been shown to increase resistance of fungal strains to broad-spectrum fungicides, without a loss of virulence (Shinohara et al., 2013; M. Xie et al., 2018).

Evolution in a controlled environment can be an effective tool for increasing virulence and identifying the genetic background of variation in virulence. Experimental evolution utilizing EPF is a relatively unexplored area compared to other biological control agents such as bacteria (Raymond & Bonsall, 2013; Raymond et al., 2007; Raymond et al., 2012) or nematodes (Shapiro-Ilan & Raymond, 2016). The currently available studies exploiting spontaneous genetic changes and selection for the improvement of virulence report mixed results. Quesada-Moraga and Vey (2003) reported increased virulence of *B. bassiana* against *Locusta migratoria* following two passages through the host. This suggests that an interruption from artificial culturing is beneficial for maintining the level of virulence. In their study Valero-Jimenez et al. (2017) did not observe any significant change in virulence of *B. bassiana* towards *Anopheles coluzzii* after 10 consecutive selection cycles through mosquitos. Social evolution theory and the production of public goods suggests that to increase virulence, the relatedness of infecting pathogen population should be kept high throughout the passaging experiment. Here, the intended method to carry out strain improvement passaging differs from previous work by applying selection methods based on social evolution such as bottlenecking the starting pathogens population.

Materials and methods

Insect stock cultures

Populations of *Myzus persicae* were reared in BugDorm-4 Polyester Mesh rearing cages (NHBS, UK) on Chinese cabbage plants at BBCH growth stage 13-15 (start). Colonies were sub-cultured onto new plants or aphid cups as required by transferring 5-15 apterous adults onto fresh plants. Cultures were maintained at 24±1°C, 14L: 10D photoperiod, which ensured the maintenance of an anholocyclic life cycle.

Production of known-age Myzus persicae

Cohorts of 5-10 adult aphids were removed from stock culture plants with a fine camel hair paintbrush and placed on detached Chinese cabbage leaves contained in aphid cups.

Alternatively, 30- 40 adult aphids were placed on whole cabbage plants contained in rearing cages. Cups and insect cages were maintained at 24±1°C, 14L: 10D photoperiod. After 48 hours all adult aphids were removed, leaving a cohort of similarly aged first instar aphids. Cultures were maintained until the aphids became adults. *M. persicae* became adults after 9 days ±1 days.

Fungal cultures

In vitro culture of strains of hypocrealean entomopathogenic fungi

Stock cultures of the different fungal strains were stored as conidia on porous plastic beads in cryotolerant plastic tubes (Pro-Lab Diagnostics, Bromborough, Wirral, UK) at minus 80 °C (Chandler, 1994). A two-stage culturing system was used to provide material for experiments. Firstly, for each strain, a culture was grown by removing a bead from cryopreservation and placing it on a Sabouraud dextrose agar (SDA) slope in a Universal tube and incubating it in darkness at 23±1 ºC for 10 days before transfer to cold storage (4±2 ºC, darkness) (this material was referred to as a "laboratory culture"). Secondly "working cultures" were grown from hyphal material taken from laboratory cultures and grown on SDA in 90mm triple vented Petri dishes (Fisher Scientific, Loughborough, UK) in darkness at 23±1 °C for 10-14 days. Laboratory culture slopes were replaced every 3-4 months in order to minimize the risk of attenuation of fungal strains caused by repeated subculture.

Preparation of hypocrealean conidial suspensions

Conidial suspensions were made by agitating the mycelium of a 10-14 day old culture with a 'L-shaped' spreader (Fisher Scientific, Loughborough, UK) in 10ml of 0.01% Triton X-100. The suspensions were then passed through sterile cheesecloth to remove any hyphal fragments. Suspensions were enumerated using Fastread102 counting chambers and adjusted to the required concentration using 0.01% Triton X-100.

Sprayer design and calibration

Abstract

The available range of laboratory spraying equipment for testing microbial agents is narrow. The spectrum comprises of expensive stationary sprayers and low-cost hand-held sprayers that lack robustness when it comes to dose-response testing. This study presents a portable, inexpensive alternative to laboratory spray equipment that can be calibrated for different pressures and doses utilizing laboratory consumables. Bioassay data was obtained by spraying *Myzus persicae* with spores of entomopathogenic fungi. Observed variation in droplet deposition within tested pressure and volume settings was low, as well as for spore

deposition within sprayed concentrations. Bioassay results show reproducible mortality for the tested doses.

Introduction

Entomopathogenic fungi (EPF) are important natural pathogens of arthropods. There are approximately 750 fungal species causing infection in insect and mite populations (Sinha et al., 2016). Fungi belonging to the order of Hypocreales, such as *Beauveria bassiana, Lecanicillium muscarium* and *Metarhizium anisoplae* have been well-studied and used widely as environmentally friendly biological control agents (Hesketh et al., 2008; Humber, 2008).

A commonly used measure of efficacy when using infective propagules of microbial agents is lethal dose (LD) or lethal concentration (LC) (Finney, 1971). In order to obtain repeatable results when studying dose-time-mortality, it is imperative to conduct bioassays with a strict control of dosing method (Inglis et al., 2012). Infection of the insect host with fungal spores initiate via the cuticle, therefore the most common application of these microbial agents is topical. Depending on the size and number of insects, and the target (habitat or insect surface), the method of application can vary greatly. The most frequently used method in applying fungal propagules on insect hosts is spraying (Inglis et al., 2012). There are a number of available devices including track- and handheld-sprayers designed for this purpose. The Potter spray tower is considered to be the standard of reference for such spraying techniques in the laboratory (Potter, 1952). However, this piece of equipment is stationary, takes up considerable laboratory space and is expensive. Here, we provide a design for an alternative to these commonly used appliances that could be readily made from inexpensive materials with relatively low cost.

Sprayer design

A portable micro-spray tower was designed following Mascarin et al. (2013) and Spence E. (University of Warwick, unpublished). The sprayer consists of an acrylic cylindrical tube, a top cap responsible for holding an artist airbrush (HP-SBS ECL3500 with standard nozzle, Iwata) securely in place with studded aluminium rods and a base cap acting as a sample tray. The airbrush is connected to a compressor (Powerjet Lite IS925, Iwata). The spray area allows space for a 90 mm Petri dish to be placed in the center (Figure 1). The top cap with the fastening, the base cap and the feet (for a stable, level contact) can be 3D printed or cut via Computer Numeric Control (CNC) machine (using the files attached as supporting material) from a material of choice depending on the intended application. Here, we used 11.7 mm thickness PVC that can withstand repeated washing with 70% ethanol. The feet, base and

top caps have 4 mm deep flanges connecting them to the acrylic tube. The parts are easily disassembled for cleaning and disinfection.

Figure 1. Sprayer assembly

Methods of calibration

Calibration of the device was carried out by spraying deionized water on 9 cm diameter filter paper (Qualitative 415, VWR). In order to understand the relationship between pressure, volume and droplet deposition, filter papers were weighted directly before and after spraying. Droplet deposition (µl/cm^2) was calculated for all pressure and volume combinations of 8, 12, 16, 20, 24 PSI and 300, 400, 500, 750, 1000 µl resulting in 150 observations. A multiple linear regression model was used to describe the relationship between sprayed volume, pressure and liquid deposition on filterpaper.

The calibration was repeated with stain solution (Methyl blue, Sigma-Aldrich), for visual inspection of droplet deposition. Observations showed considerable pressure fluctuations at 8 PSI and that aphids were flushed out of the petri dish at 24 PSI, thus these two pressure settings were excluded from further studies.

The relationship between fungal concentration and surface deposition of conidia was investigated using spore suspensions of *B.bassiana* 433.99[1], *B.bassiana* 1787.18[1] and *L.muscarium* 19.79[1] at five different concentrations ranging from $4.5x10^4$ to $7.5x10^8$ conidia/ml. Spore suspensions were sprayed on to 90 mm diameter Petri dishes containing 4-5 glass coverslips (22 x 22 mm). The applied volume was 400 µl at 12 PSI according to the previous study. All concentrations were tested at least three times. Sprayed cover slips were transferred to 45 ml centrifuge tubes containing 5 ml 0.01% triton-x-100 and vortexed for 1 minute to dislodge conidia. Recovered conidial suspension were enumerated using Fastread 102 (Biosigma) counting slides. Average number of conidia recorded from coverslips was used to quantify depositions rates (conidia/mm^2). A linear regression model was used to describe the relationship between conidial deposition and concentration.

A dose response bioassay was carried out using *L.muscarium* and even-aged apterous adult *Myzus persicae*. 18-20 aphids were treated in a 55 mm Petri dish with a concentration in the range of $1x10^5$-$1x10^8$ conidia/ml. The seven tested concentration were replicated at least 3 times. Controls were treated with sterile carrier (0.01% triton-x-100). After treatment aphids were maintained on a single leaf of chinese cabbage in plastic cups with mesh covered vents at 20±1 °C and a 14:10 L:D regime. Aphid mortality was recorded daily for 7 days post-treatment. Nymphs were removed daily. Dead insects were surface sterilized with 70 % ethanol and rinsed in sterile distilled water. Sterilized cadavers were plated separately and observed for fungal outgrowth to confirm death attributed to fungal infection.

Results

Droplet deposition showed the least spread (2.62 ± 0.01 µl) with a volume of 400 µl sprayed at 12 PSI. A significant relationship between water deposition, volume and pressure was indicated by the multiple linear model ($F_{2,147}$ = 5522, P < 0.0001; adjusted R^2=0.989) (Figure 2).

[1] Warwick Crop Centre Culture Collection strain number

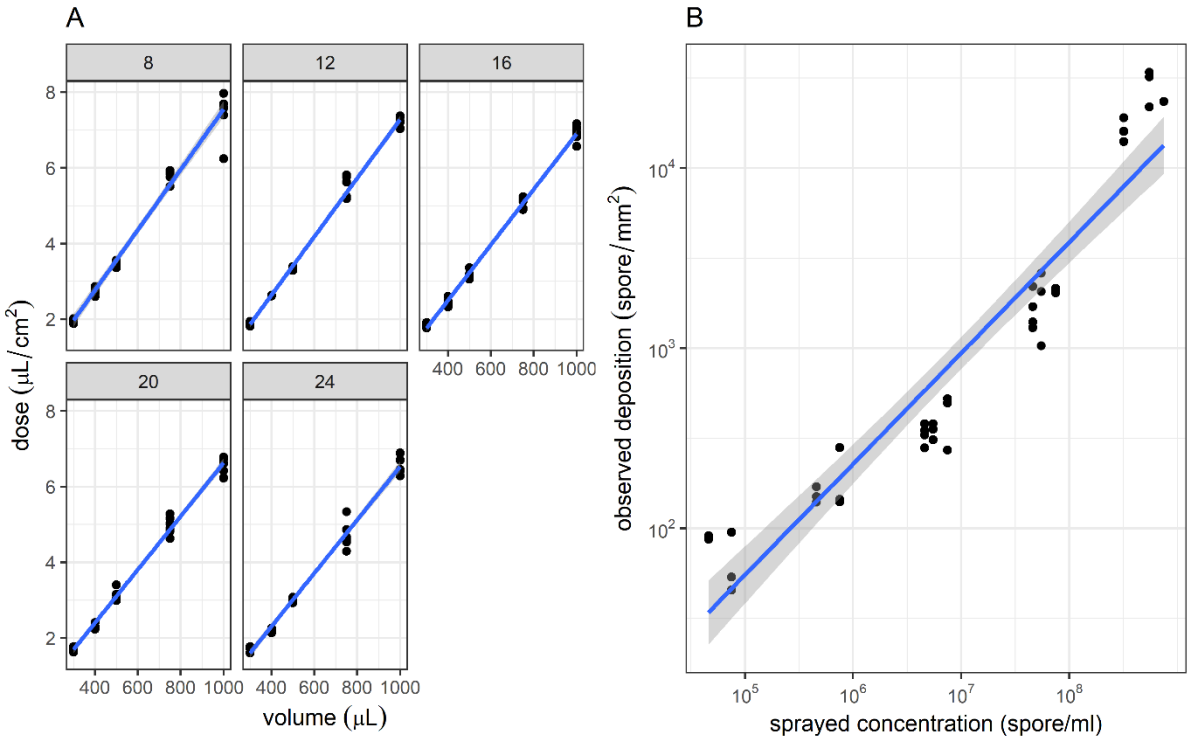

Figure 2. Water deposition at different applied pressures and sample volumes. Panel headings (8-24) refer to application pressure in PSI (A). Observed deposition of conidia after different applied concentrations (B).

Visual inspection of spray patterns on the filter papers indicated that 400 µl sprayed at a pressure of 12 PSI provided the most even coverage.

The determination of spore deposition was limited to the range where spore counts could be accurately enumerated by the counting slides. There is a significant relationship between conidia deposition and applied concentration ($F_{1,38}$ = 397.6; P<0.0001; adjusted R^2=0.911). Concentrations above $1x10^8$ conidia/ml should be taken as indicative, due to overestimation. The relationship between conidia deposition and applied concentration remains significant if concentrations above $1x10^8$ conidia/ml is omitted from the model ($F_{1,30}$ = 247.1; P<0.001; adjusted R^2=0.888). The results show that within concentration variation is low, which is key for dose-response testing of microbial propagules (Figure 2).

The dose-response bioassay resulted in repeatable mortality for a wide range of doses (Figure 3). The variation within doses could be attributed to the differences in aphid fitness

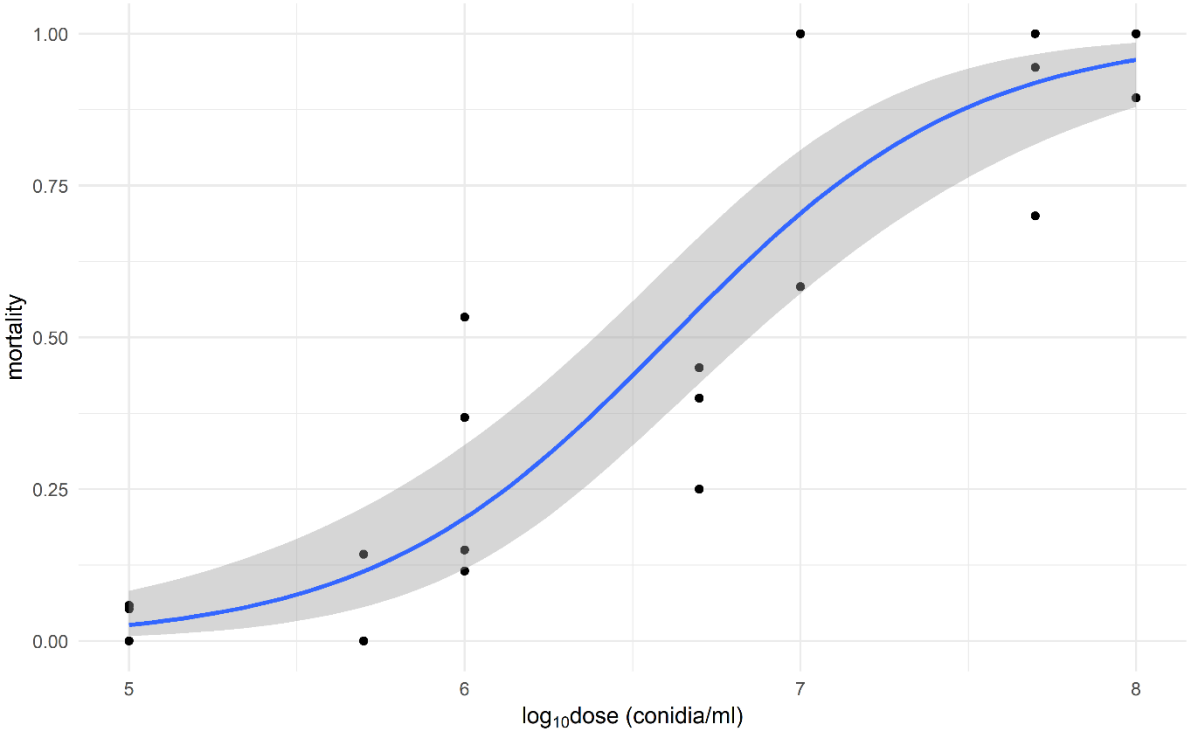

Figure 3. *Myzus persicae* mortality after 6 days of exposure to *L. muscarium* depending on tested concentrations. Points represent cumulative mortality recorded from individual aphid cups. A quasibinomial regression model (blue line) was fitted to predict the dose response. Grey area represents 95% confidence intervals.

between batches and the physiology of cabbage leafs cut from plants.

Discussion

The described device provides a relatively cheap alternative to the commonly used laboratory spraying equipment in foliar testing of microbial agents or pesticides. In comparison to the Potter spray tower, the advantages of the design described above includes inexpensive spare parts, portability, and usage in laboratories with restricted space. The most expensive parts of the presented setup are the compressor and the airbrush. The cost of the acrylic tube and the PVC sheet including the CNC machining of tower parts (four replicates) was under 600$. These costs could be further reduced by material selection. The specifications of the tower (height, diameter) could be easily changed to suit individual preferences. The airbrush used here has numerous feed and nozzle attachements that can hold higher load volumes and produce different spray patterns (https://www.iwata-airbrush.com). However, the tower parts can fit, or can be modified to fit other similar airbrushes. Limitations related to the range of applied pressure was observed due to the low weight of aphids tested in our bioassays. Such constraints should not arise with heavier targets or applications conducted on leaf.

The design described here can be adjusted to accomodate the needs of most laboratories aiming to conduct microbial agent or pesticide testing without investing in expensive kit or restricting considerable space. Further improvements, such as attaching a vacuum filter to reduce contamination when opening the chamber, can be easily realized.

Fitness cost of resistance under pathogen challenge

Fungal cultures

Fungal cultures were obtained as described above, except for *Lecanicillium muscarium.* Five grams of powder formulation of Mycotal (Koppert, NL) based on *L. muscarium* Ve6 19.79 was dissolved in 10 ml sterile water. 100 µl of the homogenized suspension was spread on Sabouraud dextrose agar (SDA) in 90 mm triple vented Petri dishes (Fisher Scientific, Loughborough, UK) and incubated in darkness at 23±1 °C for 10-14 days. Preparation of conidial suspensions from plates were carried out as detailed in section 2.2.

Table 1. Identity of fungal isolates used in the study

Fungal species	**Isolate**	**Source**
Beauveria bassiana	C1	*Myzus persicae* (Green Peach Aphid)
	347.92	*Delia radicum* (Cabbage root fly)
	365.92	*Delia radicum* (Cabbage root fly)
	432.99	*Anthonomus grandis* (Boll Weevil)
Lecanicillium muscarium	19.79	*Trialeurodes vaporariorum* (Glasshouse Whitefly)
Lecanicillium lecanii	339.92	*Brevicoryne brassicae* (Cabbage Aphid)
Metarzhizium anisopliae	Met52	Met52 Fargro Ltd.

Insect rearing

Table 2. Clones of *Myzus persicae* selected for screening from Prof. Chris Bass' collection

Clone	**Colour**	**Resistance mechanism[2]**	**Country of origin**
4106A	Green	Full Susceptible	UK
4255A	Green	Full Susceptible	UK

[2] Resistance mechanisms described in Bass et al. (2014)

5557(NS)	Green	Full Susceptible	Germany
1X	Green	Full Susceptible	Italy
US1L	Green	Full Susceptible	UK
92H6	Green	Kdr, sKdr, CYPCy3	Italy
5191A	Red	MACE, CYP6Cy3	Greece
O	Green	sKdr, CYPCy3	UK
FRC	Green	R81T, Kdr, Kdr, CYPC6y3	France
5444B	Green	R81T, Kdr, Kdr, CYPC6y3	Italy

Insect stock cultures and known-age adults were obtained as detailed under the methods section. Ten distinct clonal lineages of *M. persicae* with different resistance mechanisms to chemical insecticides have been screened for potential differences in susceptibility to fungal infection (Table 3).

Screening of fungal isolates for virulence against M. Persicae

A total of 7 isolates (4 isolates of *B. bassiana*, one of *L. muscariuam*, one of *L. lecanii* and one of *M. anisoplae,* Table 2) were evaluated for their pathogenicity against *M. persicae* clone „O". Eighteen to twenty adult apterous aphids were treated with a high dose ($1x10^7$- $1x10^8$ conidia/ml) of conidial suspensions in a 55 mm Petri dish. Treatments were replicated at least 3 times, controls were treated with sterile carrier (0.01% Triton-x-100). After treatment aphids were maintained on a single leaf of chinese cabbage in plastic cups with mesh covered vents at 20±1 °C and a 14:10 L:D regime. Aphid mortality was recorded daily for 7 days post-treatment. Nymphs were removed daily. Dead insects were surface sterilized with 70 % ethanol and rinsed in sterile distilled water. Sterilized cadavers were plated separately and observed for fungal outgrowth to confirm death attributed to fungal infection. Known age adult apterous aphids and conidial suspensions were prepared as described in section 2.

Screening of M. persicae clones for difference in susceptibility to L. muscarium

The median lethal dose (LD_{50}) for *L. muscarium* was calculated as $4x10^6$ conidia/ml for the aphid clone „O" based on a dose response bioassay carried out as described in section 2.3.

Twenty to twenty-two adult apterous aphids were treated in a 55 mm Petri dish with the LD_{50}. The experiment was repeated with a concentration of 10x LD_{50} to see if there are any clonal lineages with an extreme tolerance to fungal infection. Treatments were replicated at least 3

times. Controls were treated with sterile carrier (0.01% Triton-x-100). The bioassays were carried out as described above.

Statistical analysis

Data analysis was carried out in R version 3.6.0 (R Core Team, 2019). Lethal time values were obtained using probit analysis. Generalized linear models (GLM) with binomial error structure were used to analyse mortality data after 7 days of exposure. Survivorship analysis used Cox proportional hazard models.

Results

Screening of fungal isolates for virulence against M. Persicae

All three fungal species showed pathogenicity to *M. persicae.* Calculated mortality after 7 days of exposure ranged between 82.0 % for the least virulent isolate (*L. lecanii* 339.92) to 98.2 % for the most virulent isolate (*B. bassiana* C1) with no significant difference between any of the isolates (Figure 4). Median lethal time (LT_{50}) values were calculated from all individual replicates that caused > 50% mortality. Values ranged between 3.2 and 6.49 days (Figure 4). Based on short LT_{50} value (4.415, second fastest on average), ease of conidia propagation, culturing (radial growth experiment excluded from current report) and availability of commercial products to compare, we selected (19.79) *L. muscarium* for further studies.

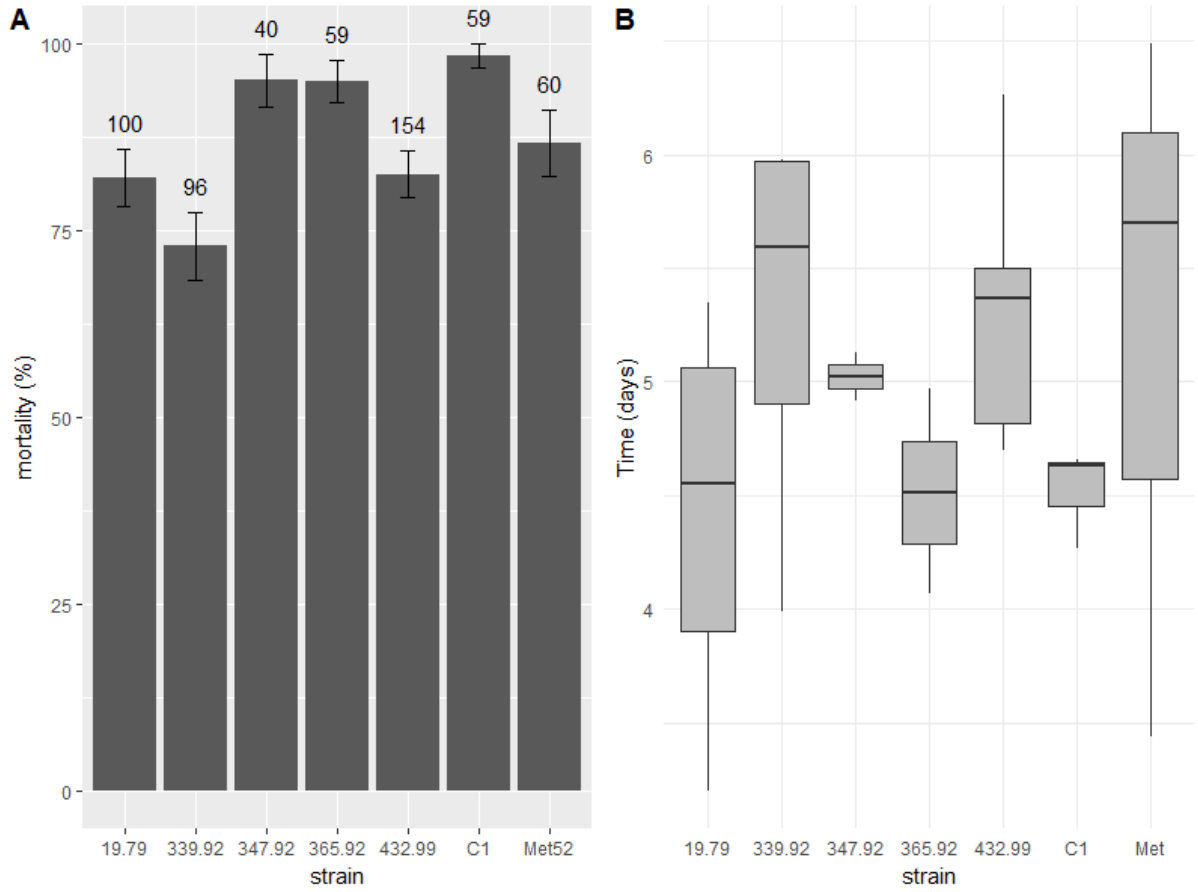

Figure 4. (A) Cumulative mortality recorded after 7 days of exposure to a high dose ($1x10^7$-$1x10^8$ conidia/ml) of fungal suspensions. Number over bars represent the number of individual aphids tested. (B) Lethal times for tested isolates obtained with probit analysis.

Screening of M. persicae clones for difference in susceptibility to L. muscarium

Contrary to our expectation that insects carrying resistance genes would be more likely to succumb to fungal infection, we observed that resistant insects were slightly more tolerant to entomopathogenic fungi (EPF). Susceptible aphids showed a higher proportion of mortality after 7 days of exposure to *L. muscarium* (Figure 5). The effect of resistance status on mortality is only marginally significant (generalized linear model with dose and resistance status as fixed effect; residual deviance 56.06 on 71 df; P =0.0294).

The effect of clonal lineage on mortality is not significant with the exception of clone “O” showing marginal significance (generalized linear model with clone, dose and resistance status as fixed effect; P = 0.049, Figure 5).

Similarly, survivorship analysis revealed a marginally significant relationship between survival and resistance status (likelihood ratio test 388.5, df= 2, p = 0.016). Testing whether aphid genotypes have a different effect on survival, we found clone “O” to have a significant relationship (hazard ratio (HR) = 0.52, p<0.001). This correlates with our observation that

clone “O” showed longer survival with a sudden increase in mortality during bioassay (6th- 7th day) compared to majority of clones which followed a more gradual pattern of mortality with first deaths occurring earlier (3rd-4th day).

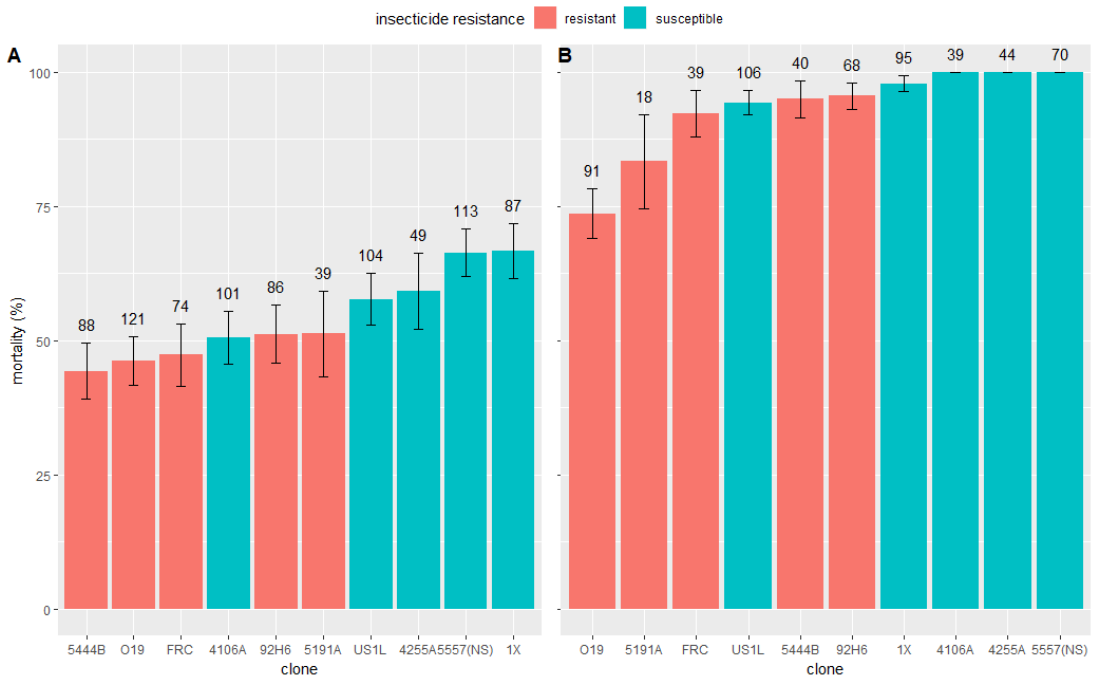

Figure 5. Effect of clonal lineage on mortality after 7 days of exposure to *L. muscarium*. Bioassay conducted with median lethal dose (LD_{50}). (A) and 10x LD_{50} (B).

Conclusions

The effect of chemical resistance on susceptibility to fungal infection in aphids is only marginally significant, yet there is a pattern that suggests cross resistance between chemicals and entomopathogenic fungi. Therefore further studies are required to conclude.

A fitness cost experiment will be carried out in insect cages with whole Chinese cabbage plants. A resistant (clone “O”) and a fully susceptible (1X or NS) clone will be used. Cages will be set up with initial 15-15 even-aged apterous adults of each genotype. Treatment with *L. muscarium* will be delivered with a hand-held sprayer 24-48 hours after adults have been placed. This ensures that some of the progeny will be directly exposed to EPF. The experiment will be run until a second aphid generation has laid nymphs (approx. 24 days.) with a second treatment after 10-12 days. After 22 days nymphs will be picked randomly from the plant and subjected to a high-throughput Taqman assay (Bass et al., 2007) to identify the ratio of resistant and susceptible aphid clones. We hypothesize that resistance carries a fitness cost which will result in susceptible clones being present in higher frequency at the end of the experiment.

Knowledge and Technology Transfer

- Attended the AHDB PhD stundentship visit in Dundee where I managed to get in touch with farmers using products based on entomopathogenic fungi (EPF). This provided insight in to the qualities users are looking for in products based on EPF.

- I will be attending CLESCon 2019 in November, which is a student-led, one-day conference designed to bring together researchers from across Geography, Biological Sciences, Psychology, and Sports & Health Sciences. It is a great opportunity to showcase research to a college-wide audience, and spark ideas and collaborations.

- Paper submitted on mini spray tower design

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