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Project leader:	Dr David Chandler University of Warwick Warwick Crop Centre, School of Life Sciences, University of Warwick, Wellesbourne, Warwick CV35 9EF UK
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Key staff:	Jude Bennison Clare Butler Ellis John Clarkson Roma Gwynn Rob Jacobson Andy Lane Aoife O' Driscoll Gill Prince David Talbot Erika Wedgewood
Location of project:	University of Warwick Warwick Crop Centre, School of Life Sciences, University of Warwick, Wellesbourne, Warwick CV35 9EF UK
Industry Representative:	Rob James, Thanet Earth Marketing Ltd, Barrow Man Road, Birchington, Kent, UK, CT7 0AX
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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.

Dr D Chandler

Principal Research Leader

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

Signature ..... Date .....

Report authorised by:

[Name]

[Position]

[Organisation]

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GROWER SUMMARY

Headlines

- Research on spray application showed that the applied water volumes currently recommended for most biopesticides (1000 L / ha or higher) are unlikely to be helpful in terms of optimizing the quantity and distribution of biopesticide active substance on the plant. Reducing the applied water volumes will be more efficient by reducing the time it takes to spray the crop.
- The persistence of *Ampelomyces*- and *Gliocladium*-based biofungicides on crop foliage was determined, and this information can be used to help schedule spray applications for disease management.
- A mathematical pest control model was developed which can be used to identify optimal biopesticide control strategies.

Background

Pests (including invertebrates, plant pathogens and weeds) have a major impact on crop production, reducing yield and quality (it is estimated that about a third of the potential global crop yield is destroyed by pests before it is harvested). The standard method for pest control has been to use synthetic chemical pesticides. However excessive use is associated with a range of problems including harm to the environment, and concerns have also been expressed about safety to pesticide spray operators. Overuse has also resulted in the evolution of resistance in many pests, which has rendered some pesticides ineffective. In recent years, environmental legislation has resulted in a lot of these pesticides being removed from the market. Alternative pest controls are needed therefore. Many growers already use Integrated Pest and Disease Management (IPDM), in which different crop protection tools are combined, including chemical, biological and cultural methods. IPM is now a required practice under the EU Sustainable Use Directive on pesticides. In order to make IPM successful, it is vital that growers have access to a full range of control agents that can be used as part of an integrated approach. One group of alternatives are 'biopesticides'. These are pest control products based on natural agents, and there are three types; living microbes, insect semiochemicals and botanical biopesticides. These types of pest control agent are based on living organisms and so it takes more knowledge and understanding to use them successfully compared to traditional pesticides.

AMBER (Application and Management of Biopesticides for Efficacy and Reliability) is a 5-year project with the aim of identifying management practices that growers can use to improve the performance of biopesticide products within IPM. The project has three main parts: (i) to understand the reasons why some biopesticides are giving sub-optimal results in current commercial practice; (ii) to develop and demonstrate new management practices that can improve biopesticide performance; (iii) to exchange information and ideas between growers, biopesticide companies and others in order to provide improved best-practice guidelines for biopesticides.

Summary

Making biopesticide spray application more efficient. The aim of this part of the project is to determine the lowest volume of water required to provide biopesticide efficacy, as this reduces waste and is more efficient, provided that it does not contravene the minimum water volume stated on the label, as this is a legal requirement. At present, the label provided to growers by biopesticide manufacturers / distributors usually contains only general recommendations about spray application. The label specifies the dose to be applied (i.e. the amount of biopesticide per ha), gives a range of water volumes that are permitted on the crop (where water acts as a carrier for the biopesticide), the frequency of applications and the maximum number of applications per crop. Research was done to investigate the effect of different water volumes on the quantity of a tracer dye (used as a proxy for a biopesticide) retained on chrysanthemum plants sprayed using a three-nozzle boom in a track sprayer. The plants were able to retain a significant volume of liquid, such that the relationship between applied volume and quantity retained had only just begun to level off at 1000 L/ha. Thus, when applying biopesticides at a constant concentration, the maximum quantity retained would be achieved at 1000 L/ha. However, when applying at a constant dose, so that concentration reduces as volume increases, there was a clear reduction in deposit as water volume increased. Therefore, the most efficient way to deliver an active substance to this kind of plant is with as low volume and as high concentration as possible. This does not compromise the distribution over the plant. However, in all cases, the quantity of spray liquid reaching the underside of leaves was low – averaging around 5% of the total deposit on a leaf, and also very variable. A similar experiment was conducted with an experimental glasshouse tomato crop and was designed to build on the results from a larger scale HDC study on tomato spray application done about 20 years ago (PC136). Data will be fully analysed in the next financial year but the current indications are that at a constant dose there was no relationship between deposit and water volume, probably because of the complex architecture of the crop and high levels of leaf shielding. In this case application at a low volume would still be preferable because of the time savings it would bring.

Understanding the persistence of biofungicides on crop foliage. There is currently a lack of information on how long microbial biofungicides survive for after they have been sprayed onto crop plants. Biofungicides are recommended for application before, or at the first signs of, disease symptoms. If they do not survive for long, then they will have to be reapplied frequently. In this part of the project, experiments were done to measure the survival of *Ampelomyces quisqualis* (the active agent in the biofungicide AQ10, used against powdery mildew), *Gliocladium catenulatum* (used in Prestop for management of botrytis) and *Bacillus subtilis* (used in Serenade for botrytis management). There was a steep drop in the number of viable propagules of *A. quisqualis* recovered from leaves sprayed with AQ10 after four days. This biofungicide is parasitic on powdery mildew and because it does not survive on the plant for long in the absence of its host, then the correct timing and frequency of application is going to be very important for its efficacy. In contrast, *Gliocladium catenulatum* reproduced on the plant and about twice as many propagules were retrieved 7, 10 and 14 days after Prestop application as on the application day. This biofungicide works as an antagonist and competitor and is applied preventatively. After 7 days from Serenade ASO application, *Bacillus subtilis* bacteria were recovered in similar numbers to within hours of application, again showing good persistence.

A pest control model to help identify optimal biopesticide control strategies. Microbial biopesticides are usually slower acting than conventional pesticides; for example, it takes 5 – 7 days for a fungal bioinsecticide to parasitize its insect host and kill it. During this time, the pest may grow and reproduce. The speed of kill is affected by a wide range of variables including the pest species, its life stage (e.g. larva versus adult), the pest population size, crop type, fungal species, and environmental conditions. This is highly complex, and it means that fungal biopesticides can give variable results depending on the particular situation in which they are used. In this part of the project, a computer model was constructed to simulate pest population dynamics over time and the impact of the biopesticide on the pest population growth. Glasshouse whitefly and entomopathogenic fungi were used as the initial model pest and biopesticide. The model includes the main factors that influence the growth of the pest population (number of eggs laid, length of time spent in each life stage, host crop, temperature and starting population size) as well as factors relating to the ability of the biopesticide to limit pest growth (life stage infected, percentage of population infected, time taken to kill the insect pest). The model allows predictions to be made about how the overall level of pest control is affected by all of these interacting factors. Using the prediction, the model can be used to make practical recommendations about the best ways for growers to use biopesticides. For example, how frequently (and at what time in the crop growing season) the biopesticide

should be used. The computer model allows us to provide guidance to growers and identify the areas where future research should be focussed.

Financial Benefits

- When spraying a biopesticide at a constant dose, the most efficient way to apply it is with a low volume and high concentration, as this reduces waste and takes less time, provided that it does not contravene the minimum water volume stated on the label, as this is a legal requirement.
- For biofungicides, savings should be possible by paying attention to the most appropriate timing of spray application. For AQ10, growers should wait to apply it until the first traces of powdery mildew are present. Earlier application is unlikely to be cost effective. Prestop was shown to have a good persistence, multiplying on foliage in the absence of a fungal host in the high humidity conditions provided, and this should give growers with crops in similar environments the confidence to use application intervals of at least a fortnight and probably longer.
- Biopesticides can be more expensive and less forgiving of environmental conditions that conventional pesticides so understanding the optimal way to use them is crucial to maximising efficacy and minimising cost. Computers models are useful for understanding systems that involve complex biological interactions where there are multiple interacting factors. They can be used for rapidly testing a large number of hypotheses to identify those hypotheses that should be further investigated. The model developed here is a valuable research tool that allows different control programmes to be tested. Once optimal control programmes are identified a subset of these will be tested experimentally to assess the accuracy of the model. Attempting to investigate all components of a spray programme in laboratory or grower experiments would be prohibitively expensive and time-consuming.

Action Points

- A good general strategy for constant dose spray applications of biopesticides is to use a low water volume with high concentration, as long as this stays within the minimum water volume on the label.
- Be aware that the survival of the beneficial fungus *Ampelomyces quisqualis* in AQ10 on healthy foliage is short, with few viable colonies after four days, so do not apply either until powdery mildew is seen or conditions are very likely to result in infestation as survival should be greater.

- Good persistence of the beneficial fungus *Gliocladium catenulatum* in Prestop WP can be expected even on healthy foliage for at least 14 days given conditions of high humidity and so reapplication following the recommended three to four week interval is likely to maintain protection of sprayed foliage.
- Applications of Serenade ASO can be expected to result in viable colonies of *Bacillus subtilis* on healthy foliage for at least seven days, but may then need reapplication.
- Remember that good coverage of the products is required and in fast growing crops or during flower production the new tissue will require protection.
- Gain experience of when best to use biofungicide products by keeping records of the environmental conditions (humidity, temperature and sunshine) in which the products were present on the crop and the level of disease control achieved.

SCIENCE SECTION

1. Project background, aims and objectives

Growers face a serious challenge to protect their crops from pests and diseases without over-relying on synthetic chemical pesticides. Synthetic chemical pesticides are important tools for crop protection, but overuse can lead to unwanted effects on non-target organisms and control failures through the evolution of resistance in pest and disease populations. Legislation (The Sustainable Use Directive) is now in place throughout Europe which requires farmers and growers to use of Integrated Pest and Disease Management (IPDM) wherever practical and effective in order to manage pesticide applications more sustainably. IPM uses combinations of crop protection tools (chemical, biological, physical and cultural controls, plant breeding) together with careful monitoring of pests, diseases and natural enemies.

Biopesticides are plant protection products based on micro-organisms, substances derived from plants and semiochemicals. Biopesticides can make a valuable contribution to pest and disease control when used as part of IPM. Most biopesticide products are recognized as posing minimal risk to people and the environment and they often have a low harvest, re-entry and handling intervals. Biopesticides are usually applied with existing spray equipment, and some microbial biopesticides may reproduce on or in close proximity to the target pest / plant pathogen, which could give an element of self-perpetuating control. Most biopesticides are residue-exempt and they are not required to be routinely monitored for by regulatory authorities or retailers. As alternatives to conventional chemical pesticides, they offer new and multiple modes of action so can help reduce the selection pressure for the evolution of pesticide resistance in pest populations and there is also evidence that some biopesticides stop the expression of pesticide resistance once it has evolved. However, there are disadvantages of biopesticides compared to conventional chemical pesticides and a balanced approach to evaluating them is required. These may include a slower rate of control and often a lower efficacy, shorter persistence, and greater susceptibility to changing environmental conditions. In particular, because biopesticides are not as “robust” as conventional chemical pesticides, and they have multiple modes of action they require a greater level of knowledge on behalf of the grower to use them effectively.

A small number of biopesticides have been available to UK growers for some time, and an increasing number will be entering the market in the next few years. Within 10 – 20 years, the number of biopesticide products available is likely to exceed the number of conventional chemical pesticides. While some biopesticides seem to be working well in IPM, UK growers have found others to give inconsistent or poor results, and the reasons for this are often not

immediately obvious. Clearly, growers need to get the best out of biopesticide products in order to support their IPM programmes.

AMBER (Application and Management of Biopesticides for Efficacy and Reliability) is a 5 year project funded by the Agriculture and Horticulture Development Board (AHDB project code CP158). The research team receives advice from an Industry Steering Group which is comprised of some of the UK's leading growers, backed up with expertise from AHDB management staff. The aim of AMBER is to have UK growers adopting new practices that have been demonstrated to improve the performance of individual biopesticide products within commercial integrated pest and disease management (IPDM) programmes. The systems will be developed and demonstrated using approved biopesticide products. Once in place, the systems can be applied to other biopesticide products that become approved in the future. The project is focused on biopesticides for use in three broad crop sectors: protected edible crops (primarily salad crops such as pepper, cucumber and tomato, as well as protected herbs, and we are also working doing targeted work on mushroom crops; however the project does not include any work on protected soft fruit crops at this stage); protected ornamental crops; and outdoor ornamental crops such as nursery stock. These industries are economically important and rely heavily on having effective systems of pest and disease management.

The project has three component objectives:

1. Identify gaps in knowledge that might be causing biopesticides to be used sub-optimally.

2. Develop and demonstrate management practices that can improve biopesticide performance.

3. Exchange knowledge and share experience with growers, biopesticide companies and other industry members in order to provide improved best-practice guidelines for optimum use of biopesticides within more robust IPM.

There are too many biopesticide products, crop types, and pest and disease problems to work on everything. Instead, we are focusing on a targeted number of commercially available biopesticides and on a selected number of pests and diseases on crops with different crop architectures. The general principles developed will then be extrapolated and tested on other crops later in the project. Once in place, these systems can then be applied to other biopesticide products that become approved in the future.

2. Research Results from Objective 2: develop and demonstrate management practices that can improve biopesticide performance.

The current phase of the project is looking at management practices that have potential to improve biopesticide performance (Objective 2 of the project). Here we report on 3 areas: (1) making biopesticide spray application more efficient; (2) understanding the persistence of biofungicides on crop foliage; (3) developing a pest control model to help identify optimal biopesticide control strategies.

2.1 Making biopesticide spray application more efficient: evaluating the relationship between applied water volume and quantity of biopesticide deposited on the crop

The objective of this work was to evaluate the relationship between applied volume and quantity deposited on the crop for up to four different crop types. The four crop types were to be two horizontal crops: bushy ornamental, small herb; and two vertical crops: tomatoes at two growth stages. It was anticipated that we would do the work on horizontal crops in 2018 (these can be done at SSAU) while we explored options for vertical crops to be done in 2019. The real costs associated with a trial in a vertical glasshouse crop was anticipated to be greater than the resources available, so we needed to identify cost-effective approaches. In practice, because a tomato crop became available at Warwick for 'free', we conducted one experiment on this, and one on a horizontal crop (bushy ornamental).

2.1.1 Experiment 1. Relationship between applied volume and deposit on the crop for a bushy ornamental crop

Materials and methods

Chrysanthemum plants were made available by Double H Nurseries. They were at a slightly later growth stage than required, with flower buds just appearing. The intention was to use these plants as a generic example of a bushy plant sprayed with a horizontal boom, rather than generating results specific to chrysanthemums, so the flower buds were removed prior to the experiment. The track sprayer at SSAU was set up with a three-nozzle boom with a conventional flat fan '02' size nozzle, operated at 2.0 bar at 0.5 m above the plants. This is a nozzle and pressure, delivering a 'fine' quality spray, which is typical of systems we have seen in commercial growers, both operated manually and automatically driven. The track sprayer was calibrated to run at 6 different speeds, thereby delivering 6 different volumes (Table 1).

Table 1. Track sprayer speed and nominal volumes delivered with a '02' nozzle at 2.0 bar

Speed, km/h	8	4	2	1.3	0.97	0.78
Volume, L/ha	98	196	392	603	808	1005

Six pots, each containing four plants, were sprayed for each treatment (Figure 1). Plastic discs were placed on the soil to determine how much spray penetrates through the vegetation to the soil surface (Figure 2). A seventh pot was also sprayed and 10 leaves sampled for quantifying deposit on upper leaf surface and underside of leaf.

Because more plants were provided than were required, a seventh treatment was added in with leftover pots using an approximate 784 L/ha volume but a much coarser spray, by using a higher flowrate nozzle (08 at 2.0 bar) and a 4 km/h speed.

Figure 1. Six plants underneath the three-nozzle boom on the track sprayer

Figure 2. Discs placed on the soil prior to application; pictured after all plant material has been sampled.

The spray liquid consisted of a wetting agent (0.1% Activator 90) and a pigment dye (0.1% Green S) as a tracer in tap water. Tank samples of the spray liquid were used to create standard curves for determining the quantity deposited on the plants.

All plant material was sampled from all six pots and separated into upper and lower levels in the pot. Each sample of plant material was weighed and the deposited washed off with a known volume of liquid. The quantity of spray liquid deposited was determined using spectrophotometry.

Results

Figure 3 shows the relationship between spray volume applied and the quantity of spray deposited on the whole plant per g plant material. The data is also presented as normalised deposit, i.e. the deposit per g plant material divided by the applied volume and multiplied by 100, i.e. ul per g plant material per 100 L/ha applied.

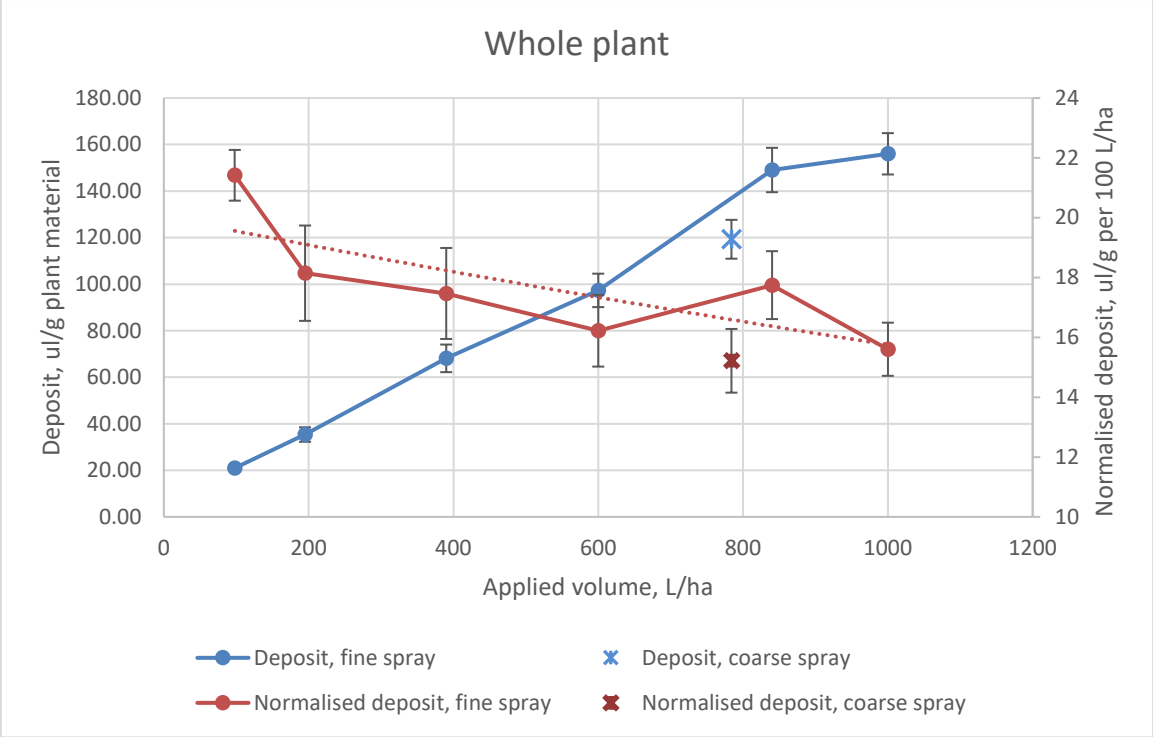

Figure 3. Relationship between applied volume and deposit on the plants.

Figure 4 shows the percentage of the total spray liquid deposited that reaches the lower half of the plant. While there appears to be a 'dip' at 600 L/ha, this is not statistically significant.

Figure 5 shows the quantity of spray reaching the soil.

Figure 6 shows the percentage of spray liquid retained on the leaf that is on the underside.

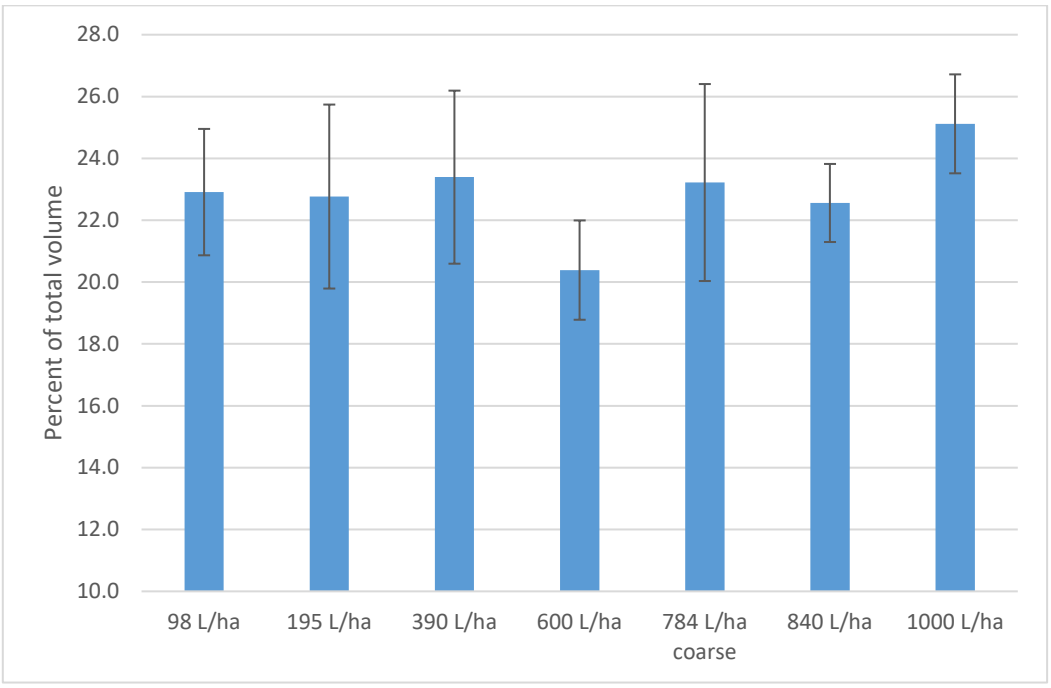

Figure 4. The percentage of spray reaching the lower half of the plant

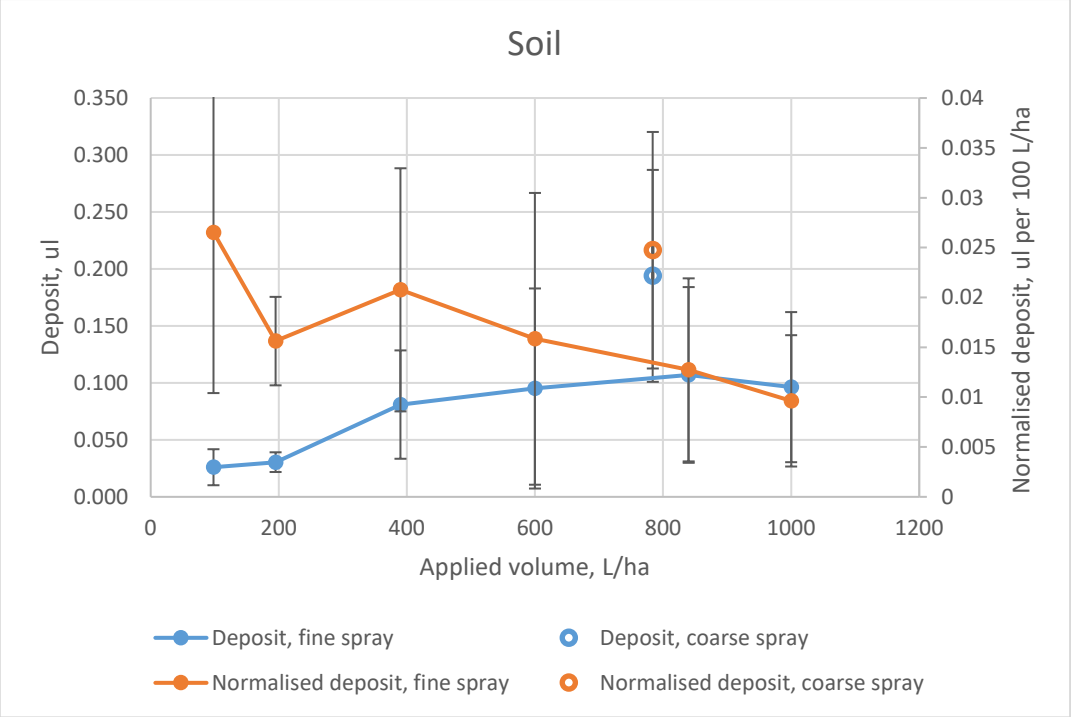

Figure 5. Relationship between applied volume and quantity of spray liquid reaching the soil.

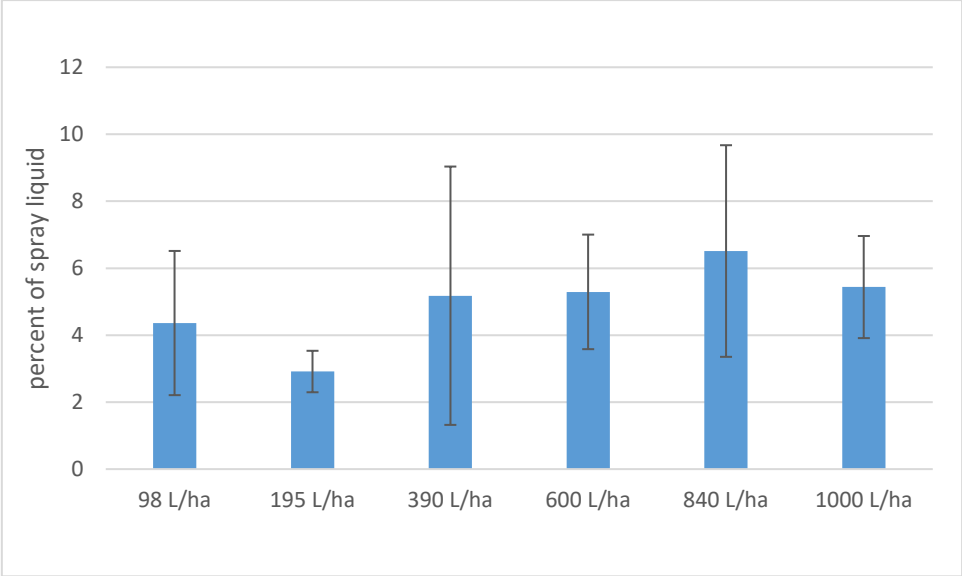

Figure 6. Percentage of spray retained on leaves that was on the underside.

Discussion

The chrysanthemum plants were able to retain a significant volume of liquid, such that the relationship between applied volume and quantity retained had only just begun to level off at 1000 L/ha. Previous work with poinsettias had suggested that the limit would be reached at 600 – 800 L/ha. Thus when applying biopesticides at a constant concentration, the maximum quantity retained would be achieved at 1000 L/ha. However, when applying at a constant dose, so that concentration reduces as volume increases, there is a clear reduction in deposit as volume increases.

The distribution of spray liquid between upper and lower levels of the plant was not changed by increasing volume. The quantity of spray reaching the soil was very variable, but the tendency was similar to the deposit on the plant, in that it increased with volume, but again, when normalised, the quantity reduced with volume.

The quantity of spray liquid reaching the underside of leaves was low – averaging around 5% of the total deposit on a leaf, and also very variable. There was no discernible effect of spray volume, although observations of leaves suggest that, at the highest volumes, some of the spray that reaches the underside may have 'leaked' round from the upper side, as shown in Figure 7. It is unlikely that this type of deposit would be helpful in targeting pests or diseases that are underneath the leaf, yet it is probably the largest component of what was measured on the underside of leaves.

Figure 7. Observed deposit on the underside of a leaf at high volume

The use of a coarse spray significantly reduced the deposit on the plants and increased the quantity on the soil.

The most efficient way to deliver an active substance to this kind of plant is with as low volume and as high concentration as possible. This does not compromise the distribution over the plant. Changing the spray quality might deliver a greater quantity to the soil, if this is required, but further work would be needed to confirm this.

2.1.2 Experiment 2. Relationship between volume and deposit for a vertical crop

A similar experiment was conducted with a glasshouse tomato crop at Wellesbourne that was grown according to commercial practice as part of other research, and which became available towards the end of the season. The size of the crop was such that two bays were available for spraying; and the nature of the experiment meant that only one treatment could be applied to each bay. Because a previous HDC-funded project (PC136) had been conducted around 20 years ago which was very much larger and was able to investigate a much wider range of variables, it was decided that the experiment we should conduct at Wellesbourne should take account of these previous findings and build on them, rather than trying to replicate a similar experimental programme.

PC136 had investigated a range of volumes to establish the optimum in terms of quantity retained on the plant, and had considered the distribution over the plant, by looking at inner and outer leaves, and upper and lower side of leaves. As well as investigating volume, a range of different nozzles, nozzle configurations and delivery systems were explored, which are well out of the scope of the current Amber project. However, since the conclusion of PC136 was that a conventional 80^{o} nozzle operated at an upwards angle of 45^{o} was optimum, we were able to use this as our starting point.

PC136 changed volume by changing nozzles and pressures but keeping velocity constant. The experiment we have undertaken explored the option of reducing volume by keeping nozzle and pressure at the optimum but increasing velocity, such as could be achieved with a simple automated system.

The experiment was conducted in October 2018, and the analysis of samples conducted immediately following the experiment.

Materials and methods

A rig was constructed to enable a section of the tomato crop to be sprayed (Figures 8 and 9), based on the design used in PC136 and the pipe-rail sprayer available at Wellesbourne. The aim was to spray only a middle section of the crop, and sample from this, so that the sprayer could be kept small and lightweight. The sprayer was moved by a rope attached to a reel, which was manually turned (Figure 10). The aim was to achieve two different speeds, around 1 m/s and 2 m/s which would deliver two volumes, one close to recommendations from PC136, and one approximately half that. The sampling area was nominally 2m long by 0.6m height, approximately in the central area of the row (Figure 11).

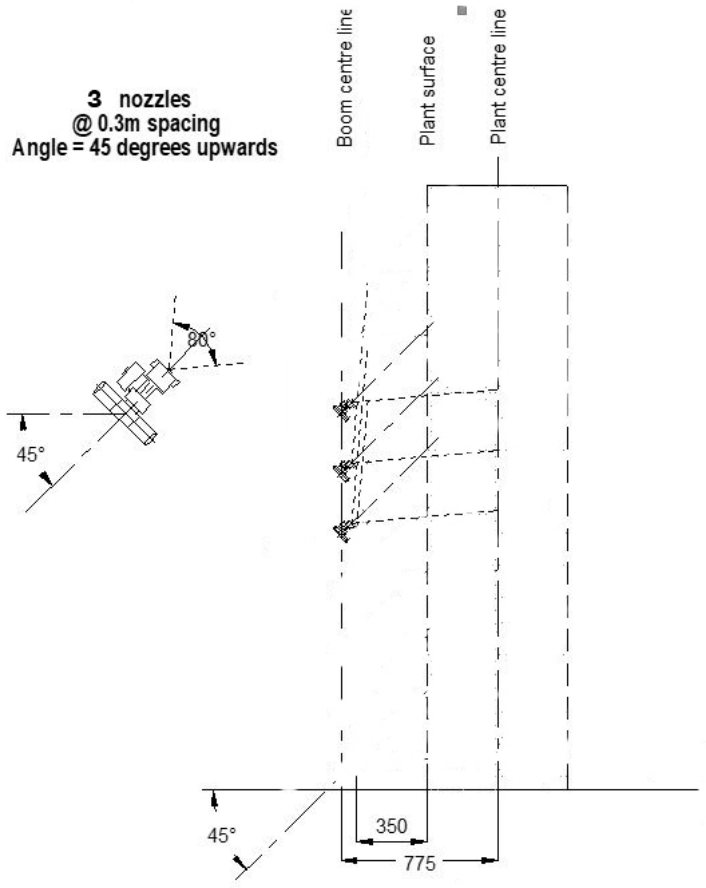

Figure 8. Vertical spray boom.

Figure 9. Vertical boom sprayer in the experimental tomato crop

Figure 10. Manual reel for moving the sprayer

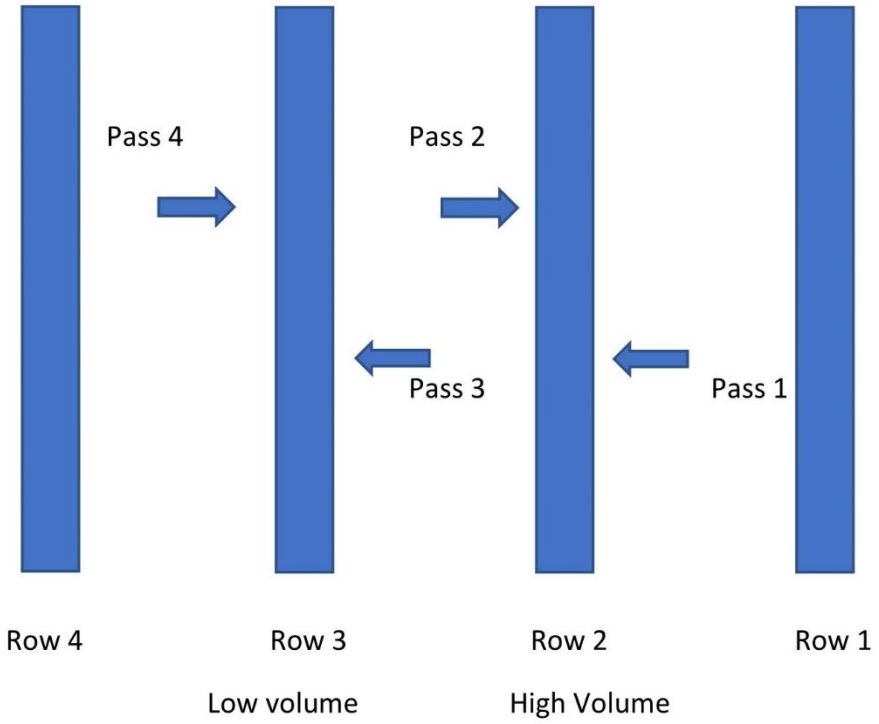

Figure 11. Layout of experimental glasshouse

Sampling. Three whole leaves were combined to make a single sample. There were 3 samples taken from each side of the row, giving 6 replicated samples for both outer and inner leaves, i.e. 12 samples per volume rate. Samples were placed into pre-weighed and labelled bags, weighed and collected dye washed off in 50ml of de-ionised water. 3 whole leaves were sampled from both sides (inner and outer) of each row. From each leaf, the terminal and 2 compound leaflets were removed and then weighed and recorded, with the upper and lower surfaces being washed separately with de-ionised water using pipettes. All leaves were allowed to dry completely before being sampled to enable any run off to take place and ensuring that all retained spray was captured. Control samples were taken to establish background readings.

Results

The data is currently in the process of being analysed, which will be completed and reported in 2019.

2.2 Understanding the persistence of biofungicides on crop foliage

The biofungicides AQ10, Prestop WP and Serenade ASO are approved for foliar application in the UK each at a recommended dose rate, with a minimum and maximum number of applications and a maximum interval between applications. For AQ10, (minimum 5.0 x 10^9 spores of *Ampelomyces quisqualis*/g) the weight of product to be applied per hectare of crop increases with crop height from a baseline of 35 g/ha for plants up to 0.5 m tall, without stating water volume but directing the need for uniform coverage. Application of AQ10 should be repeated every 7-10 days with up to 12 treatments per crop, with at least two successive applications. Prestop WP (nominal 2 x 10^8 cfu of *Gliocladium catenulatum*/g) should be made up at 500 g per 100 L (0.5%) and applied at high volume with re-application every three to four weeks on crops such as tomato for as many times as needed. Serenade ASO (*Bacillus subtilis* minimum 1.042 x 10^{12} cfu/L) is to be applied at a maximum 10 L/ha in a minimum 400 L water/ha and maximum applications have recently been reduced from 20 to six per crop with re-application up to every seven days.

The product labels stipulate that products must be applied as a preventative measure when limited or no disease is present. AQ10 only controls powdery mildew species, and both Prestop WP and Serenade ASO foliar applications are targeted at Botrytis grey mould. The beneficial micro-organisms thus need to remain viable in the interval between application and pathogen arrival. However, perceived poor efficacy in practice as well as limited information in the public domain of examples of successful use has led to the question of how long, when applied at the recommended rates and intervals, these biological control agents (BCAs) will survive in the absence of their target pathogen.

A review of the three products found that to date, limited or no *in planta* studies exist on the survival of the BCA propagules contained within these products over time. The objective of the presented research was therefore to determine the persistence of selected BCAs *in planta* in the absence of a target disease, in order to determine their relative periods of survival.

2.2.1 Persistence and survival of *Ampelomyces quisqualis* strain M10, formulated as AQ10, on tomato within a controlled environment.

Materials and methods

Tomato plants used in the study. Tomato cultivar Elegance was provided by Delflands Nurseries at the two true leaf stage. Plants were grown in plastic plant pots (9cm x 9cm x 10cm) filled with John Innes No. 1 loam based compost and these pots used for carrying out the experiments in the controlled environment (CE) chamber. Plants were acclimatised to the

conditions of the chamber (Fitotron Format 650) by growing for at least one week prior to the start of each experiment.

Experimental environmental conditions and replication. Plants were maintained at 25 ± 1°C under a 16/8 hour (h) day/night schedule (with the light period within day time). Light intensity of 800-1000µmol m^2 s^{-1} was achieved with fluorescent lamps (22 Philips 55W tubes, supplemented with four Philips 60W tubes). Humidity was maintained at ca. 95% by ensuring that the troughs which the plants were contained in had a constant, shallow level of water and transparent plastic bags were put on the plants and sealed after treatment. Temperature and humidity were recorded every 30 minutes with a logger held beside the foliage on a stake in one of the pots. Each treatment group contained twelve replicates; broken down into two experiments comprised of six replicates each (to fit in the chamber). A different plant per replicate was sampled at each of six intervals after product application (days post-inoculation, dpi). Six plants were left untreated and one taken for leaf sampling at the same time as the six treated plants. The experimental design was a randomised block, laid out with six replicate columns. Two leaves were sampled per plant and three agar plates prepared for colony counting from each leaf, with analysis carried out combining the results from the two leaves per plant per time interval per replicate block. Each continuation of the experiment began one week after the last sample had been taken from the preceding experiment.

Preparation of the AQ10 solution and dilution. A new sachet of AQ10 provided by Fargro Ltd. was used, with storage at 4°C in the ADAS Boxworth pesticide store before use. To make up the solution of AQ10, a 500ml beaker was placed in a laminar flow cabinet and half filled with sterile distilled water (SDW). The required quantity of AQ10 wettable granules was added to the beaker and left for 30 minutes without agitation. After pre-soaking, the suspension was then continuously agitated by hand whilst adding water to make up to the final target volume. The time between mixing, soaking and use of the product was 3 hours. SDW was used, rather than tap water, for standardisation.

Pre-test and optimisation. To confirm that the spores had been released from the product's powder carrier, 10µl of the suspension was pipetted onto a sterile haemocytometer (Improved Neubauer depth 0.1mm 1/400mm^2) and the cover slip placed firmly and held for 2 minutes (Figure 12). Independent haemocytometer readings indicated that 6g of AQ10 in 1000ml of water contained 1 x 10^7 colony forming units (cfu)/ml. This solution was used as the stock solution and serial dilutions were made from this from 1x10^7 down to 1x10^3 cfu/ml. 10µl and 100µl of each dilution was then spread on PDA plates, the plates were left to dry by leaving

the lids slightly open in the laminar flow for 5 minutes. The lids were then placed back onto the petri dishes, sealed with Parafilm and placed in an incubator at 20°C.

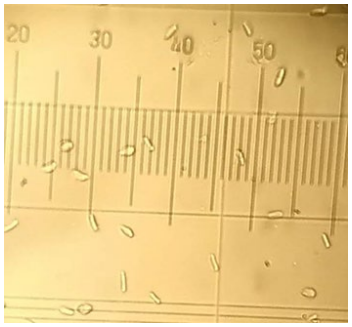

Figure 12. Checking the concentration of *A. quisqualis* spores using a haemocytometer (shown with an eye piece graticule used to measure spore size).

Spraying of plants with biocontrol agent. AQ10 was prepared as described previously. The freshly prepared solution of AQ10 at a final concentration of $1x10^6$ cfu/ml, and the SDW were transferred into clean hand sprayers, labelled and the nozzle was tightened to produce a spray quality of fine-medium droplets. The sprayer was calibrated by spraying fifty sprays of each solution into a beaker then calculating the volume of liquid in that beaker. Plants were removed from acclimatisation in the growth chamber and placed on a laboratory bench top. Plants were then randomly selected for spraying, then sprayed in an isolated location at eye level until the visible surface was at the point of run off. Negative controls were subject to application of SDW until the point of run off. 12-15ml of AQ10 solution was applied to each plant, depending on the height of the plant and size of the leaves. Application was made in the morning around 11.00h. After spraying with *A. quisqualis*, plants were placed in bags labelled with the sampling date and replicate number, sealed with elastic bands at the base of the stem then placed back into the controlled environment chamber. Bagged tomato plant pots were positioned 15 cm apart in the randomised block design.

Leaf sampling and washing. Plants were sampled at 0, 1, 4, 7 and 10 days after treatment. The sample periods were based on indications of AQ10 persistence in the literature. The first sample was taken two hours after spraying. For each time point, samples were taken at the same time of day (around 15.00h), ± 1 hour. Size variability as well as leaf age can arise between leaves on the same plant and so the two leaves for each replicate plant were sampled at the same two different heights in the canopy. A leaf disc of 0.97 cm in diameter was extracted by perforating each leaf using the top of a 1.5 ml tube. Discs were suspended individually in 1.5 ml tubes containing 1.5 ml SDW plus 0.01% Tween 80 surfactant and shaken in a vertical shaker at 300 rpm for 30 minutes to dislodge propagules. Samples were vortexed for 5 seconds to re-suspend the propagules, then aliquots of 10µl spread on plates containing potato dextrose agar (PDA) supplemented with 250 mg/L streptomycin. The

suspension was distributed evenly over the plates using a sterile plastic rod. There were three plates of eluate made per leaf disc.

Colony counts and data analysis. Colonies were counted after incubating at 20°C for 4 days, at which time colonies were visible with the naked eye and still discrete. Each plate was divided into four subsections and the number of colonies counted within each. The number of colonies per plate was then averaged for each replicate plant at each time point. The number of colonies of *A. quisqualis* was expressed as the number of cfu/cm^2 of leaf surface using a calculation from Jacob, Panchal et al. (2017). Data was log transformed and analysed using non-linear regression in Genstat Version 18.2.

Results

Preliminary experiments were conducted to determine the maximum dose rate of *A. quisqualis* which would allow for visual quantification of cfu on plate culture. At 1 $x10^7$ cfu/ml, it was found that there were too many spores on the plate to accurately quantify (Appendix 1.3). 10µl of the suspension at $1x10^6$ cfu/ml was found to be the best concentration for accurate quantification. Thus a solution containing $1x10^6$ cfu/ml of *A. quisqualis* was used for the subsequent persistence experiments.

Humidity was confirmed from the logger readings as having stayed around 95 % around the foliage sealed in a bag for 10 days. Figure 13 shows the survival of *A. quisqualis* on foliage of tomato over time, from 0 day post inoculation (dpi) (two hours after spraying) up to 10 dpi. Based on the average number of cfu detected at each time point, the number of grams of active substance/ha was then calculated (Figure 14, Table 2).

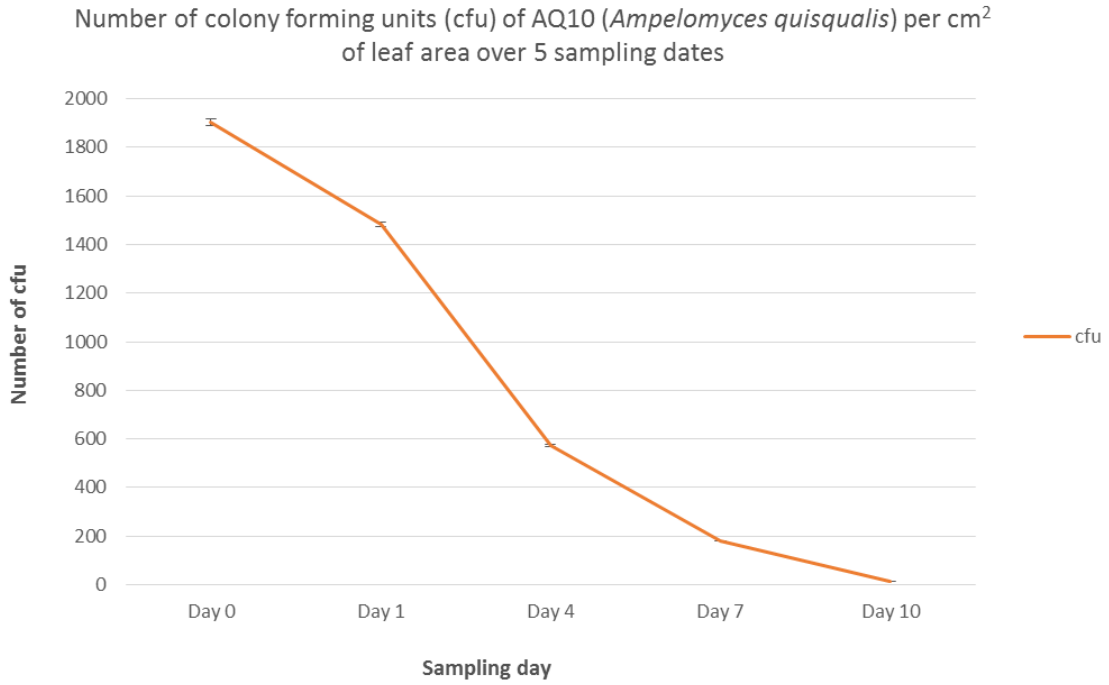

Figure 13. Time series plot of survival of *A. quisqualis* in a controlled environment chamber, expressed as cfu per cm^2 over time. Each time point is a mean of 12 leaf samples collected from six tomato plants sprayed with AQ10.

Viable populations of *A. quisqualis* declined rapidly from 1 day post inoculation (dpi) onwards (Figure 13, Figure 14, Figure 15, Table 2). At 0 dpi, 1904.07 cfu were present per cm^2 of leaf area equating to 38.08 g of active substance/ha (Table 2). Following a drop in cfu to 1483.47 cfu/cm^2 of leaf area at 1 dpi, the number of cfu rapidly decreased at the remaining sampling dates, until virtually no cfu were detected by 10 dpi (13.39 cfu/cm^2, Table 2).

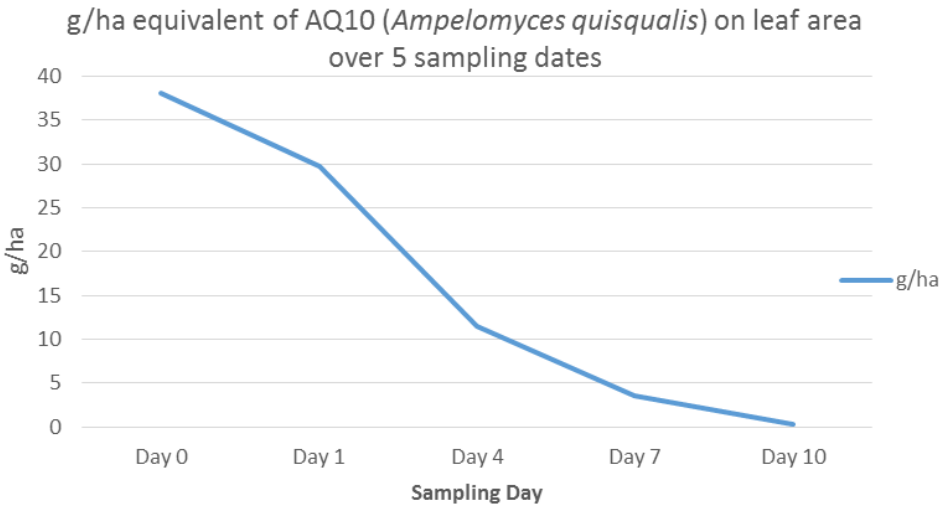

Figure 14. Time series plot of survival of *A. quisqualis* in a controlled environment chamber, expressed as g/ha over time. Each time point is a mean of 12 leaf samples collected from six tomato plants sprayed with AQ10.

Table 2. Calculation used to convert the number of cfu/ml of *A. quisqualis* detected from leaf washings to grams (g) active substance/ha. This calculation was based on the number of cfu contained in the AQ10 product as stated on the label by the manufacturer.

Sampling days after spraying	cfu per ml of leaf washing	cfu per cm^2 of leaf disc	cfu per ha of crop	grams active substance/ha
0 dpi	1308.64	1904.07	$1.90x10^{11}$	38.08
1 dpi	1019.57	1483.47	$1.48x10^{11}$	29.67
4 dpi	393.48	572.51	$5.7x10^{10}$	11.45
7 dpi	123.08	179.08	$1.8x10^{10}$	3.58
10 dpi	9.2	13.39	$1.34x10^{9}$	0.27

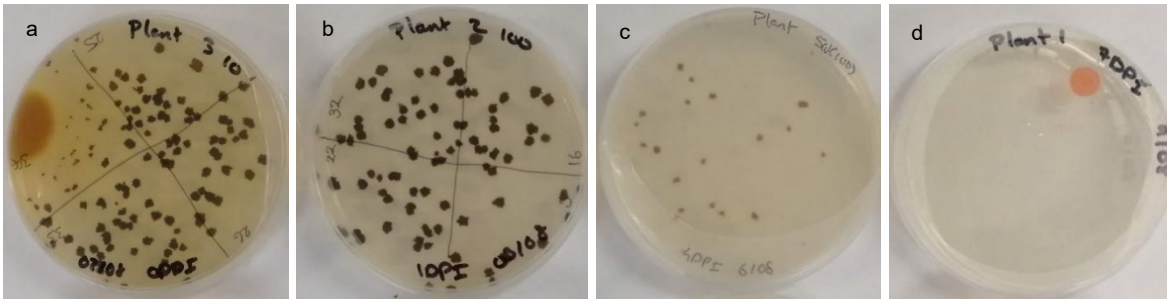

Figure 15. Visual estimation of cfu of *A. quisqualis* from AQ10 on PDA, demonstrating the decline in the number of cfu from 0 dpi (a), 1 dpi (b), 4 dpi (c) and 7 dpi (d).

The relationship between colonies and days was investigated using parallel curve analysis, looking at the data from the six replicates per batch to fit curves individually and then together. There was a small but significant improvement fitting parallel curves, compared to a single curve, but fitting the best curve for each experiment does not significantly improve the fit. Appendix 1.1 shows the parallel lines and the equations are given for both the single line and the parallel lines. Analysis was also done to fit a straight line to the data, but this was not as good as the curve (graph not shown). A further analysis was carried out with logs of the colony number and this again showed that it was worth fitting separate lines for the two batches, but that the slope could be fixed to be the same in both cases (Appendix 1.2). The fit was better than the exponential, based on percentage variance accounted for, but either method gave a reasonable estimate of fit and these can enable a calculation of values of interest (such as the number of days to 50% or 90% reduction in colony number). The estimated values for the days to 50% or 90% reduction are, however, slightly lower in the straight line fit compared to the exponential.

Discussion

The research with AQ10 achieved the primary objective of determining the persistence of *A. quisqualis* on foliage of tomato. The experiment was conducted at high humidity, the optimum condition required for spore germination to occur within the first 10-20 hours after spraying (Kiss *et al*., 2010). After an initial, steady decrease, viable populations of *A. quisqualis* declined rapidly and continuously until 10 dpi when virtually no cfu were detected on the surface of the leaves. It is not known whether the population of 572 propagules /cm^2 of leaf after 4 days would be sufficient to give control were powdery mildew be colonising at this time. To our knowledge, this is the first study to demonstrate viability decline *in planta*. Previously there was only indication in Registration Report provided in 2008 to the UK Chemical Regulations Directorate that "without the host, viability is rapidly lost e.g. within a few days". The UK label for AQ10 indicates that the product performs best if applied when the humidity is increasing or high, such as early morning or late evening. When fungal spores germinate they become susceptible to desiccation and the hyphae produced need to enter a host to survive. The Registration report (CRD 208) section on fate and behaviour in air states that it was shown that the spores of *A. quisqualis* were inactivated by UV light, however it is not known whether this was an important factor in the loss of viability in the current experiment.

The results show that the amount applied to the plants (on 0 dpi) was ~ca. 38 g/ha. The recommended rate of application of AQ10 to young tomato plants is 35 g/ha, thus the concentration applied to the plants was similar to growers' recommended practice and thus the decline in viable cfu over time on foliage should be equivalent on a crop. This is the first study to equate the results in term of g/ha, making the data relevant and understandable to growers and agronomists.

In summary, the conclusion from the study were as follows:

- A limited viable population of *A. quisqualis* was detected on tomato plants four days after application, but virtually no propagules had survived after 10 days. Further research is required to see how low humidity affects persistence.
- A sampling frequency of every two days up to ten days after treatment was sufficient to enable successful monitoring of changes in viable population. Further research could examine daily sampling up to four days post treatment to check whether the decline is steady over this period.
- *A. quisqualis* population viability reduced from 1 dpi in the absence of a food source. Further research could determine the threshold cfu, or rate of application, at which control declines and if this affected by the density of pathogen colonisation e.g. will

growers get sufficient disease control at 3.5 g/ha (10% of the recommended rate of application and as detected at 7 dpi) if a low population of powdery mildew exists?

2.2.2 Persistence and survival of *Gliocladium catenulatum*, formulated as Prestop WP on tomato within a controlled environment.

Materials and methods

Tomato plants used in the study. Tomato cultivar Elegance was provided by Delflands Nurseries at the two true leaf stage. Plants were grown in plastic plant pots (9cm x 9cm x 10cm) filled with John Innes No. 1 loam based compost and these pots used for carrying out the experiments in the controlled environment (CE) chamber. Plants were acclimatised to the conditions of the chamber (Fitotron Format 650) by growing for at least one week prior to the start of each experiment.

Experimental environmental conditions and replication. Plants were maintained at 25 ± 1°C under a 16/8 hour (h) day/night schedule (with the light period within day time). Light intensity of 800-1000µmol m^2 s^{-1} was achieved with fluorescent lamps (22 Philips 55W tubes, supplemented with four Philips 60W tubes). Humidity was maintained at ca. 95% by ensuring that the troughs which the plants were contained in had a constant, shallow level of water and transparent plastic bags were put on the plants and sealed after treatment. Temperature and humidity were recorded every 30 minutes with a logger held beside the foliage on a stake in one of the pots. Each treatment group contained twelve replicates; broken down into two experiments comprised of six replicates each (to fit in the chamber). A different plant per replicate was sampled at each of six intervals after product application (days post-inoculation, dpi). Six plants were left untreated and one taken for leaf sampling at the same time as the six treated plants. The experimental design was a randomised block, laid out with six replicate columns. Two leaves were sampled per plant and three agar plates prepared for colony counting from each leaf, with analysis carried out combining the results from the two leaves per plant per time interval per replicate block. Each continuation of the experiment began one week after the last sample had been taken from the preceding experiment.

Preparation of the Prestop WP solution and dilution. Prestop was provided in a wettable powder (WP) formulation and stored at 4°C in the ADAS Boxworth pesticide store. An unopened, new sachet, provided by ICL UK was used. A standard 0.5% dilution rate is recommended for all application. To make up a solution of Prestop WP, the powder was creamed with a small amount of water, then agitated carefully until evenly mixed, after which

it was diluted to the final concentration. The time between mixing and use of the product was 45 minutes.

Pre-test and optimisation. A colony count was performed by pipetting 10µl of the suspension onto a sterile haemocytometer (Improved Neubauer depth 0.1mm 1/400mm^2) and the cover slip placed firmly and held for two minutes. Independent haemocytometer readings indicated that 5g of Prestop WP in 1000ml of water contained 1 x 10^7 cfu/ml. This solution was used as the stock solution and serial dilutions were made from the stock at concentrations from 1x10^6 down to 1x10^3. 10µl and 100µl of each dilution was then spread on PDA plates, the plates were left to dry by leaving the lids slightly open in the laminar flow for 5 minutes. The lids were then placed back onto the petri dishes, sealed with Parafilm and placed in an incubator at 20°C.

Spraying of plants with biocontrol agents and the pathogen. Prestop WP was prepared as described previously. The freshly prepared solution of Prestop at a final concentration of 1x10^6 cfu/ml, and the SDW were transferred into clean hand sprayers, labelled and the nozzle was tightened to produce a spray quality of fine-medium droplets. The sprayer was calibrated by spraying fifty sprays of each solution into a beaker then calculating the volume of liquid in that beaker. Plants were removed from acclimatisation in the growth chamber and placed on a laboratory bench top. Plants were then randomly selected for spraying, then sprayed in an isolated location at eye level until the visible surface was at the point of run off. Negative controls were subject to application of SDW until the point of run off. 12-15ml of Prestop solution was applied to each plant, depending on the height of the plant and size of the leaves. Application was made in the morning around 11.00h. After spraying with *G. catenulatum*, plants were placed in bags labelled with the sampling date and replicate number, sealed with elastic bands at the base of the stem then placed back into the controlled environment chamber. Bagged tomato plant pots were positioned 15 cm apart in the randomised block design.

Leaf sampling and washing. Plants were sampled at 0, 1, 4, 7, 10 and 14 days after treatment. For each time point, samples were taken at the same time of day, ± 1 hour. The first sample was taken two hours after spraying. For each time point, samples were taken at the same time of day (around 15.00h), ± 1 hour. Size variability as well as leaf age can arise between leaves on the same plant and so the two leaves for each replicate plant were sampled at the same two different heights in the canopy.

A leaf disc of 0.97 cm in diameter was extracted by perforating each leaf using the top of a 1.5 ml tube. Discs were suspended individually in 1.5 ml tubes containing 1.5 ml SDW plus 0.01% Tween 80 surfactant and shaken in a vertical shaker at 300 rpm for 30 minutes to

dislodge propagules. Samples were vortexed for 5 seconds to re-suspend the propagules, then aliquots of 10µl spread on plates containing potato dextrose agar (PDA) supplemented with 250 mg/L streptomycin. The suspension was distributed evenly over the plates using a sterile plastic rod. There were three plates of eluate made per leaf disc.

Colony counts and data analysis. Colonies were counted after incubating at 20°C for 2 days, at which time colonies were visible with the naked eye and still discrete. Each plate was divided into four subsections and the number of colonies counted within each. The number of colonies per plate was then averaged for each replicate plant at each time point. The number of colonies of *G. catenulatum* was expressed as the number of cfu/cm^2 using a calculation from (Jacob, Panchal et al. 2017). Colony count data was log-transformed and analysed using non-linear regression analysis in Genstat Version 18.2

In addition to colony counts, it was seen that the fungus was fast growing and so records were taken to record the increase in colony size on agar, as it was possible similar spread might occur across on a leaf surface. The radii of 12 colonies from individual plates was measured using a digital callipers at two time points (nine and 17 dpi) to give an estimate of the rate of colony growth over time. Results were not statistically analysed and so are used in this report for drawing assumptions only.

Results

Preliminary experiments were conducted to clarify the maximum dose rate of *G. catenulatum* which would allow for visual quantification of cfu on plate culture. Prestop WP, when made up at the label recommended rate of 0.5% was found to contain 6 x10^6 cfu/ml. However at this concentration, it was found that there were too many cfu on the plate to accurately quantify them (Appendix 1.4). A 10µl suspension of 1x10^6 cfu/ml applied to plates of PDA was found to be the maximum concentration of cfu which would allow for accurate quantification. Thus a solution containing 1x10^6 cfu/ml of *G. catenulatum* was used for foliar application in the subsequent persistence experiments.

Figure 16 shows the survival of *G. catenulatum* on foliage of tomato over time, from 0 day post inoculation (dpi) (leaves sampled 2 hours after spraying) up to 14 dpi. Based on the average number of cfu detected at each time point, the number of grams of active substance/ha was then calculated (Table 3). A viable population of *G. catenulatum* was detectable up to 14 days after treatment, and increased at specific time points during the experiment. An initial population of 5249.64 cfu/cm^2 was recovered 2 hours after spraying with Prestop WP, which according to the concentration provided by the manufacturer equates to 2.62 kg/ha of the formulated product. After an initial decrease in cfu numbers at 1 dpi (3887.76 spores/cm^2), the population began to increase at 4 dpi up to 10 dpi before stabilising

at the final time point, to a cfu count that was higher than the original population detected at 0 dpi. The 9963.84 cfu/cm^2 at 0 dpi equates to 4.98 g/ha of Prestop WP.

ANOVA determined that the rate of change in colony numbers was significantly different across time points, however fitting of an exponential curve accounted for just 31% of the variability in colony numbers (Appendix 1.4) and taking logs only improved this slightly (Appendix 1.5).

Observations during the persistence experiments showed that *G. catenulatum* was a fast growing fungus on PDA (Figure 17, Figure 18). The growth of twelve individual colonies was measured at Day 9 and Day 17; the average growth rate on PDA between these two dates (after 8 days) was found to be 22.2 mm; with a minimum 17.2 mm and maximum 26.7 mm colony growth (Table 4). These observations are a rough measure of change over time of a subsample of individual colonies on plates; due to the small sample size and two dates of measurement, this result was not statistically analysed and thus can be used as observation data only.

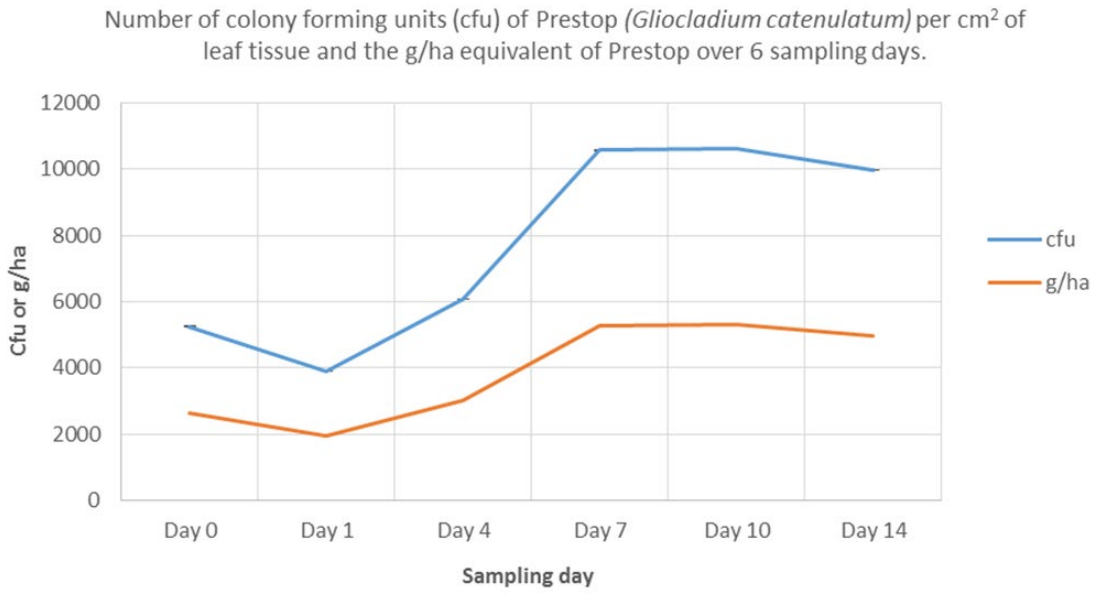

Figure 16. Time series plot of survival of *G. catenulatum* in a controlled environment chamber, expressed as cfu (blue line) and g active substance/ha (orange line) over time based on the mean viable population of *G. catenulatum* per cm^2 of leaf area recovered in leaf washings after spraying with Prestop. Each time point was a mean of twelve leaves collected from six individual tomato plants.

Table 3. Calculation used to convert the number of cfu/ml of *G. catenulatum* detected from leaf washings to grams (g) active substance/ha. This calculation was based on the number of cfu contained in the Prestop product as stated on the label by the manufacturer.

Sampling days after spraying	cfu per ml of leaf washing	cfu per cm^2 of leaf disc	cfu per ha of crop	grams active substance/ha	kg/ha
0 dpi	3608	5249.64	5.254E+11	2624.82	2.62
1 dpi	2672	3887.76	3.89E+11	1943.88	1.94
4 dpi	4172	6070.26	6.07E+11	3035.13	3.04
7 dpi	7260	10563.3	1.06E+12	5281.65	5.28
10 dpi	7300	10621.5	1.06E+12	5310.75	5.31
14 dpi	6848	9963.84	9.96E+11	4981.92	4.98

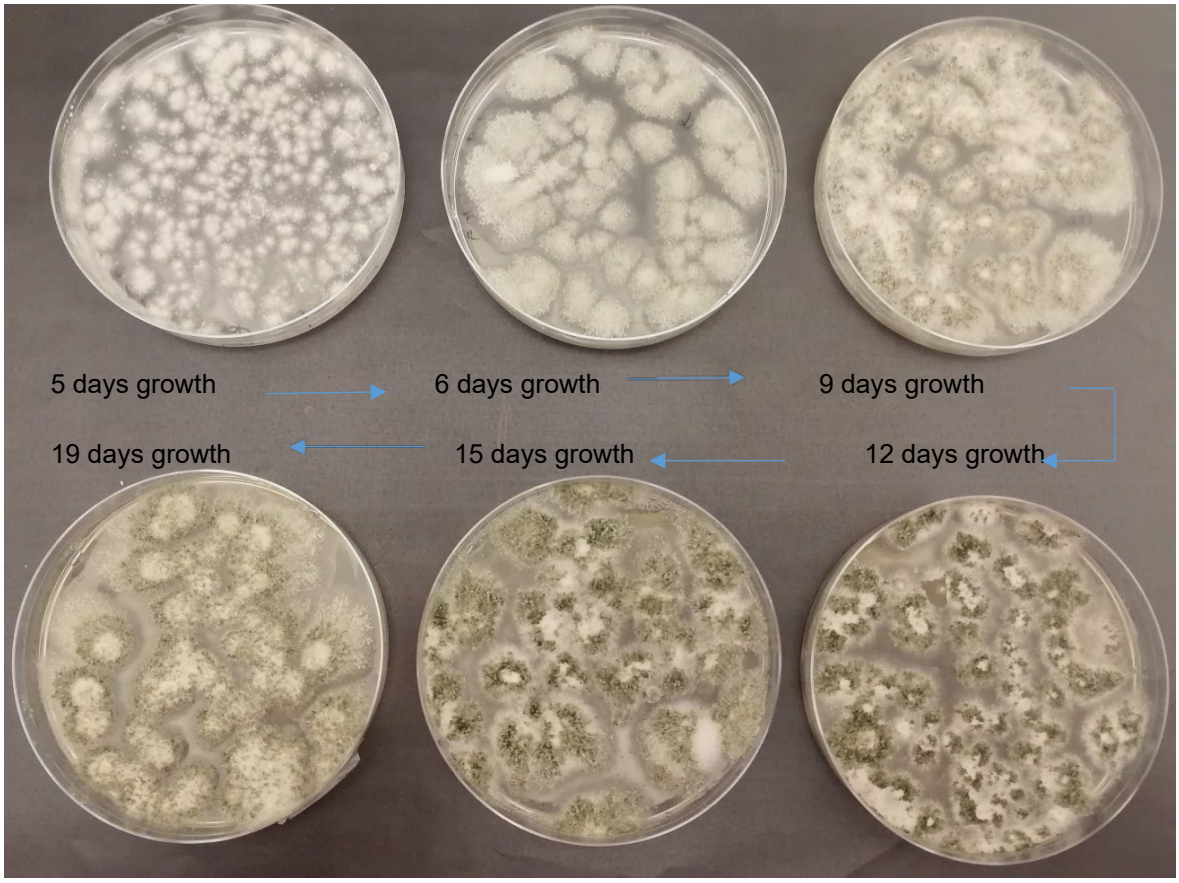

Figure 17. Visualisation of *G. catenulatum* growth from Prestop over time, from 5 days after plating on PDA up to 19 days after plating.

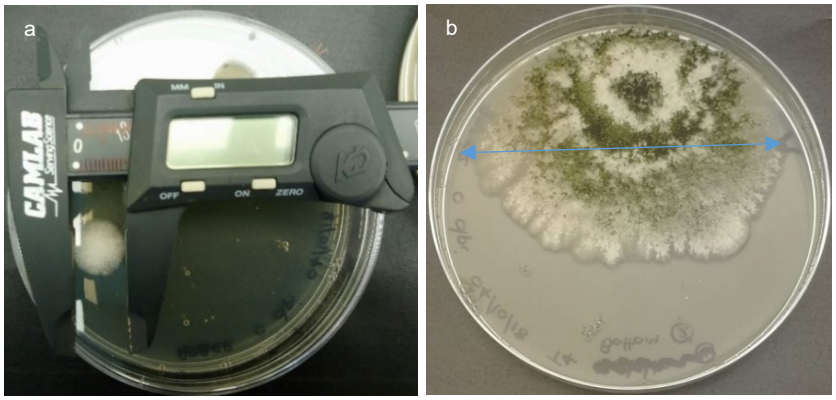

Figure 18. Measurement of radial growth of *G. catenulatum* using a digital calliper at 9 dpi (a) and 17 dpi (b).

Table 4. Measurements of *G. catenulatum* mycelial growth of individual colonies on PDA by nine days from plate inoculation and again following 17 days' growth

Day from plate inoculation	Colony diameter (mm)
Day 9	13.5
Day 17	39.4
Day 9	14.0
Day 17	35.6
Day 9	11.0
Day 17	36.1
Day 9	12.0
Day 17	29.2
Day 9	13.0
Day 17	33.0
Day 9	15.0
Day 17	39.4
Day 9	13.0
Day 17	39.7
Day 9	13.0
Day 17	38.8
Day 9	13.5
Day 17	33.3
Day 9	11.5
Day 17	31.4
Day 9	12.0
Day 17	30.2
Day 9	13.0
Day 17	35.0

Discussion

The research achieved the primary objective of determining the persistence of *G. catenulatum* on foliage. Viable populations of *G. catenulatum* were detectable on tomato foliage up to fourteen days after application of Prestop WP, with a trend of a decrease followed by an increase in the number of cfu. It is possible that sporulation occurred on the leaf surface so that additional spores were mature enough to be dislodged from sporangia in the leaf washings starting at 4 dpi, but with more spores present at 7, 10 and 14 dpi. In *G. rosea* conidia production has been reported 72 hours after inoculation onto foliage (Sutton *et al.,* 1997). Observations continued until 14 dpi, at which point there was a slight decrease in viable spore recovery from the leaves compared with 10 dpi. If new spores were to continue to be produced then in theory the cfu population would be refreshed rather than viability decline, but this may require sufficient nutrition to remain available.

Prestop WP contains mycelium and spores, but the type of spores are not defined. Thin-walled *G. catenulatum* macroconidia would be able to survive dry for some time in the environment (as probably in the product). It is also possible that chlamydospores (thick-walled asexually produced resting spores) were present in the product because these structures occur in the mycelium (European Commission 2004). Resting spores can take longer to germinate than dispersal spores (macroconidia) and could explain the delayed increase in spore concentration.

This persistence shown gives confidence for growers that at least a fortnight interval can be left between applications knowing the leaves which received the application should still have good protection. The UK label for Prestop WP recommends an interval of three to four weeks, and work is need to determine whether viability might either rapidly decline after two weeks or the proportion of viable spores gradually decrease but still be at a level to give control.

G. catenulatum activity occurs across a wide temperature range of 5°C - 34°C. Mycelial colonisation of geranium foliage has been determined using a β-glucuronidase transformed isolate of *G. catenulatum* and found to be optimum between 20-25°C (Chatterton & Punja, 2011). In the current experiments fast mycelial growth on agar was shown at 20°C and if this occurs on leaves it is possible for protection to keep up with fast growing Botrytis mycelium and possibly also expand to new tissues. This could be in addition to potential *G. catenulatum* spore production and dispersal.

Other experiments with *G. catenulatum* have shown efficacy when performed at humidity above 50% (Morandi *et al.,* 2008), but the minimum humidity possible needs to be determined because growers usually try to keep their crops at low humidity to discourage pathogen colonisation.

Since the review of biofungicide persistence and starting practical work for the current project, work has been completed on Prestop as part of an AHDB studentship (Xu and Magan, 2018). The PMA-qPCR method was used to monitor viable *G. catenulatum* propagules on lettuce in a poly tunnel and glasshouse compartment during the winter with survival found to be eight days in both locations. The range in temperature and relative humidity in the glasshouse during the experimental period was 17-22°C, and 60-95% respectively. A countable viable population existed for up to at least ten days. A mean concentration of *G. catenulatum o*f 3 × 10 ^{8} spores/ml were required for controlling a high pathogen inoculum load of *B. cinerea* macroconidia. A preliminary dose-response curve was produced of B. cinerea by counting the total number of uninfected leaf discs across both experiments out of a sample population of two hundred per treatment. The data was converted to percentages, and the mean percentage along with the error bars for each treatment was plotted and joined (Figure 19).

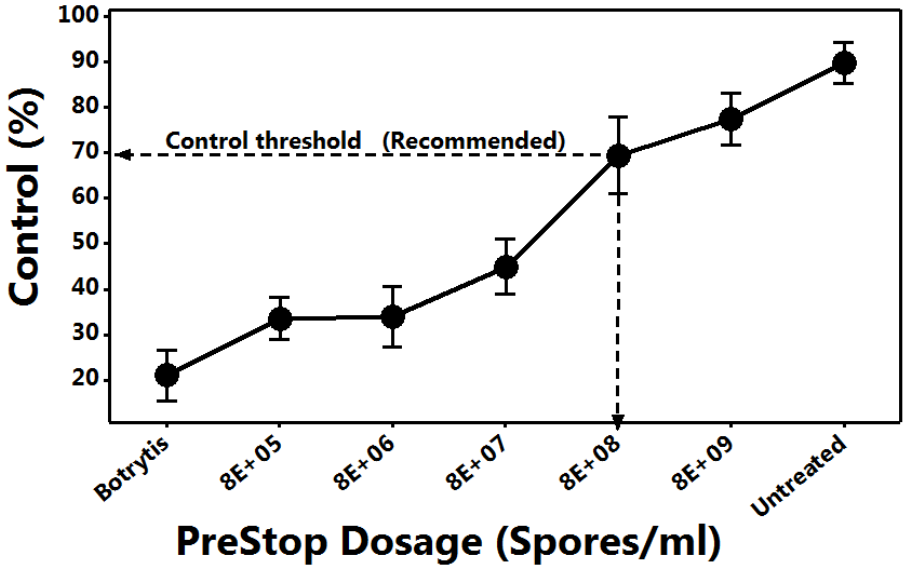

Figure 19. Dose response curve for Prestop control of Botrytis on lettuce (Xu & Magan, 2018).

Based on the findings, the following conclusions can be made:

- *G. catenulatum* remains viable on tomato leaves, in the absence of a fungal host, for at least 14 days following application in conditions of 95% relative humidity and 16 hours light per day
- After seven days the number of colony forming units had doubled from the concentration retrieved from the leaves within the first hours and had only decreased very slightly a fortnight after application to the leaves
- Prestop WP had good persistence on tomato plants grown in the controlled environment conditions tested, and further work needs to determine the density of

viable *G. catenulatum* spores required on a leaf surface to stop successful pathogen colonisation

2.2.3 Persistence and survival of *Bacillus subtilis* QST713, formulated as Serenade ASO on tomato within a controlled environment.

Materials and methods

Tomato plants used in the study. Tomato cultivar Elegance was provided by Delflands Nurseries at the two true leaf stage. Plants were grown in plastic plant pots (9cm x 9cm x 10cm) filled with John Innes No. 1 loam based compost and these pots used for carrying out the experiments in the controlled environment (CE) chamber. Plants were acclimatised to the conditions of the chamber (Fitotron Format 650) by growing for at least one week prior to the start

Experimental environmental conditions and replication. Plants were maintained at 25 ± 1°C under a 16/8 hour (h) day/night schedule (with the light period within day time). Light intensity of 800-1000µmol m^2 s^{-1} was achieved with fluorescent lamps (22 Philips 55W tubes, supplemented with four Philips 60W tubes). Humidity was maintained at ca. 95% by ensuring that the troughs which the plants were contained in had a constant, shallow level of water and transparent plastic bags were put on the plants and sealed after treatment. Temperature and humidity were recorded every 30 minutes with a logger held beside the foliage on a stake in one of the pots. Each treatment group contained twelve replicates; broken down into two experiments comprised of six replicates each (to fit in the chamber). A different plant per replicate was sampled at each of six intervals after product application (days post-inoculation, dpi). Six plants were left untreated and one taken for leaf sampling at the same time as the six treated plants. The experimental design was a randomised block, laid out with six replicate columns. Two leaves were sampled per plant and three agar plates prepared for colony counting from each leaf, with analysis carried out combining the results from the two leaves per plant per time interval per replicate block. Each continuation of the experiment began one week after the last sample had been taken from the preceding experiment.

Preparation of the Serenade ASO solution and dilution. Serenade ASO was provided in a suspension concentrate (SC) formulation and stored at room temperature in the ADAS Boxworth pesticide store. The recommended rate of application for Serenade is 10 L/ha in a minimum 400 L of water/ha. Therefore Serenade was made up at a 1 in 40 dilution. (12.5ml of Serenade in 500ml of SDW). 250ml (half the volume of water required) was added to a clean graduated cylinder, followed by the entire volume of Serenade ASO then agitated by hand, before adding the remaining water. The time between mixing and use of the product was 30 minutes.

Pre-test and optimisation. Colony counts of the bacteria using a haemocytometer were not possible using a compound microscope because of their size. The label states that Serenade ASO contains a minimum of 1.042 x10^{12} cfu/L. Therefore, when made up at the volume used in the experiment the final concentration applied to the plates in the optimisation tests was 2.5 x 10^7 cfu/ml. A 1 in 10 dilution (2.5x10^6) was then used for subsequent experiments with plants. Suspensions of Serenade ASO were prepared freshly on the day of foliar application.

Spraying of plants with biocontrol agents and the pathogen. Serenade ASO was prepared as described previously. The freshly prepared solution of AQ10 at a final concentration of 2.5 x 10^6 cfu/ml, and the SDW were transferred into clean hand sprayers, labelled and the nozzle was tightened to produce a spray quality of fine-medium droplets. The sprayer was calibrated by spraying fifty sprays of each solution into a beaker then calculating the volume of liquid in that beaker. Plants were removed from acclimatisation in the growth chamber and placed on a laboratory bench top. Plants were then randomly selected for spraying, then sprayed in an isolated location at eye level until the visible surface was at the point of run off. Negative controls were subject to application of SDW until the point of run off. 12-15ml of Serenade ASO suspension was applied to each plant, depending on the height of the plant and size of the leaves. Application was made in the morning around 11.00h. After spraying with *B. subtilis*, plants were placed in bags labelled with the sampling date and replicate number, sealed with elastic bands at the base of the stem then placed back into the controlled environment chamber. Bagged tomato plant pots were positioned 15 cm apart in the randomised block design.

Leaf sampling and washing. Plants were sampled at 0, 1, 4, 7, 10 and 14 days after treatment. The sample periods were based on indications of Serenade ASO persistence in the literature. The first sample was taken two hours after spraying. For each time point, samples were taken at the same time of day (around 15.00h), ± 1 hour. Size variability as well as leaf age can arise between leaves on the same plant and so the two leaves for each replicate plant were sampled at the same two different heights in the canopy. A leaf disc of 0.97 cm in diameter was extracted by perforating each leaf using the top of a 1.5 ml tube. Discs were suspended individually in 1.5 ml tubes containing 1.5 ml SDW plus 0.01% Tween 80 surfactant and shaken in a vertical shaker at 300 rpm for 30 minutes to dislodge propagules. Samples were vortexed for 5 seconds to re-suspend the bacteria, then aliquots of 10µl spread on plates of 5% TSA; a nutrient agar with 100 ppm rifampicin. The suspension was distributed evenly over the plates using a sterile plastic rod. There were three plates of eluate made per leaf disc.

Colony counts and data analysis. Plates were incubated at 25°C and colony growth was rapid so that counting was due 24 hours from plating. Due to variability in cfu and biofilm production

(Appendix 1.7), it was not possible to perform accurate colony counts for this bacteria. Hence only qualitative evaluations are provided.

Results

Preliminary experiments allowed for visual quantification of *B. subtilis* cfu on plate culture. Colonies of *B. subtilis* QST713 were found to be opaque white to cream in colour, flat, irregular and large in size with undulate margin. Samples from Day 0 showed good colony growth and it was possible to count cfu at this sampling time point. However, samples taken from 1 dpi onwards did not result in individual colony formation. Instead, the bacteria appeared to be entering into biofilm formation. This was observed across all plates which had been sampled. When plates were observed under a light box, the underside of the plates exhibited characteristic halo like colony growth of *B. subtilis*, as seen on plates sampled at Day 0 (Appendix 1.8). Untreated and water controls did not have this this type of growth. As a result of this phenomenon, it was not possible to accurately quantify the survival of *B. subtilis* on foliage of tomato over time, due to inconsistent results in cfu formation across sampling time points.

To troubleshoot and optimise the method, we looked at factors which may be causing the bacteria to enter into biofilm production. The main factors were considered to be high humidity or stagnant air while the plants were bagged, or too much humidity and not enough air movement after the bacteria had been plated onto media. To address these two factors, the experiment was set up again as previously conducted, except that half the plants were bagged and half were unbagged at each time point to test the effect of humidity *in planta*. After sampling and plating, the plates were left to dry under the laminar flow for 5 minutes, then sealed as normal. Samples from 0, 1, 4 and 7 dpi showed good results and formed colonies, however by 10dpi, *B. subtilis* entered biofilm production (Table 5). To address this, for the final sampling date (14 dpi), plating samples was performed as before but in duplicate sets, each set containing 3 replicates. One set of plates was sealed with Parafilm, while the other set was left unsealed to reduce humidity. The sealed plates entered biofilm production as seen at 10 dpi, while the unsealed plates did not (Appendix 1.8).

Table 5. Calculation used to convert the number of cfu/ml of *B. subtilis* detected from leaf washings to grams (g) active substance/ha. This calculation was based on the number of cfu contained in the Serenade ASO product as stated on the label by the manufacturer.

Sampling days after spraying	High (bagged) or low Humidity	cfu per ml of leaf washing	cfu per cm^2 of leaf disc	cfu per ha of crop	grams active substance/ ha	kg/ha
0 dpi	Bagged	15075	21934.2	2.1934E+12	2.19	0.150
0 dpi	Unbagged	16420	23891.2	2.3891E+12	2.39	0.002
1 dpi	Bagged	16875	24553.2	2.4553E+12	2.45	0.002
1 dpi	Unbagged	17750	25826.3	2.5826E+12	2.58	0.002
4 dpi	Bagged	9675	14077.2	1.4077E+12	1.41	0.003
4 dpi	Unbagged	18250	26553.8	2.6554E+12	2.66	0.001
7 dpi	Bagged	16750	24371.3	2.4371E+12	2.44	0.003
7 dpi	Unbagged	5900	8584.5	8.5845E+11	0.86	0.002

The number of *B. subtilis* colonies recovered from leaves was similar for most of the leaf washings for 0, 1, 4 and 7 dpi whether or not the Serenade ASO treated plants were bagged to maintain high humidity. Two records had fewer cfu, but a reduction over time was not seen, with viably colonies still present at seven days after application.

Discussion

Bacteria usually have a high replication rate, but it is not known whether the numbers recovered from the leaves were produced on the leaves and replaced others that had died to end up with a similar population. If the former, then drier conditions might affect multiplication.

Since the review of biofungicide persistence and starting practical work for the current project, work has been completed on Serenade ASO as part of an AHDB studentship (Xu and Magan, 2018). In this work by student Gurkan Tut the *B. subtilis* colony measurements were also impeded by the production of a biofilm on the culture plates. Tut substituted an isolate of *Bacillus subtilis* instead of working with Serenade ASO, but subsequently visual quantification was replaced by the PMA-qPCR method to monitor viable BCA propagules. *B. subtilis* viable populations necessary for controlling *B. cinerea* survived for four days on lettuce in a poly tunnel and six days in a glasshouse. The concentration of *B. subtilis* within Serenade ASO required a mean inoculum of 3×10^8 cfu/ml. A preliminary dose – response curve of *B. cinerea* to Serenade was produced (see below, Figure 20). Leaf disc assays were incubated at 20°C for 7 days, by counting the total number of uninfected leaf discs across both experiments out

of a sample population of two hundred per treatment. The data was converted to percentages, and the mean percentage along with the error bars for each treatment was plotted and joined.

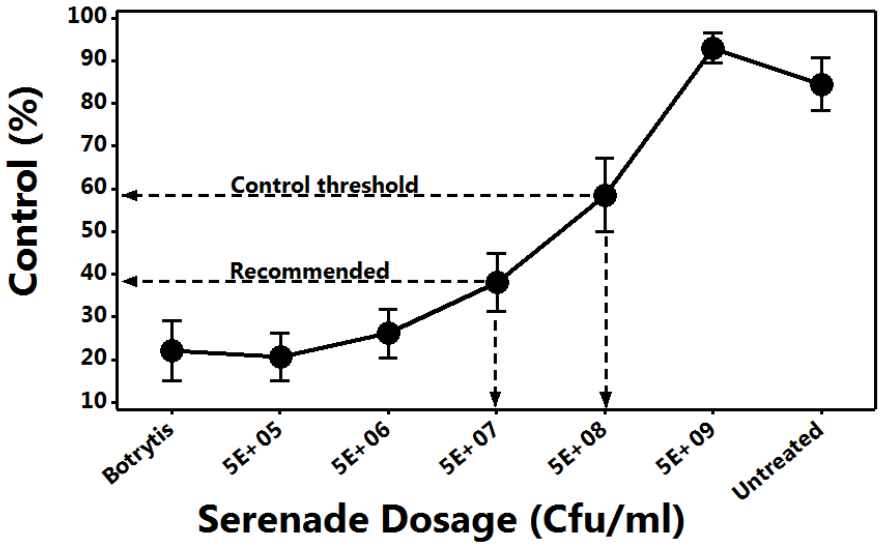

Figure 20. Dose response curve for Serenade ASO control of Botrytis on lettuce (Xu & Magan, 2018).

Molecular methods would be a way of carrying out further leaf washing assays. However, the supplier's leaflet on Serenade states that the QST 713 strain of *B. subtilis* used in the product was selected on the basis of it producing a wider range and greater volume of lipopeptides than other non-approved *B. subtilis* strains. These complex lipopeptides directly attack the pathogen, destroying spores and mycelium. The number of colonies of the bacteria are thus less likely to be important than the amount of metabolite they produce, although there may be a relationship between the two. Based on the findings, we can draw the following conclusions:

- *Bacillus subtilis* survived well on leaves for seven days after Serenade ASO application, with viable colonies persisting until records were terminated after 14 days
- High humidity around the leaves was not necessary for good bacterial survival

General conclusions

Experiments conducted on tomato plants held at over 90% humidity by enclosure in bags and growing under 16 h artificial showed that biofungicide products differed in how long the majority of beneficial microbes remained viable. The fastest decline in colony viability occurred with *A. quisqualis* with virtually none alive after 10 days, with considerable reduction having occurred from 4 days after application of AQ10. After Prestop application more

colonies of *G. catenulatum* were alive on plates after 7 days then had been found after 1 day, with good concentrations of propagules being recorded when observations terminated at 14 days. When Serenade ASO was applied to leaves *Bacillus subtilis* was recovered alive to 14 days post-application.

It is not known if maintaining plants at lower humidity would reduce biocontrol agent survival and result in fewer viable cfus being recovered from leaves. It is also not known whether the concentration of propagules recommended to be applied is required for good pathogen control or whether allowance has been made for some loss of colonies before the next recommended application.

As work on optimising the use of both Prestop and Serenade ASO against Botrytis of lettuce has been carried out during a PhD studentship, with both persistence and efficacy being studied then any further work these two products within CP158 should build on this work. However, work on optimising the use of AQ10 against powdery mildew still requires a significant amount of research.

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2.3 A pest control model to help identify optimal biopesticide control strategies: Understanding the relationship between pest population dynamics and microbial biopesticide efficacy

Pest populations can increase exponentially if left untreated. The aim of Integrated Pest Management is not to eradicate pest populations entirely, but rather to reduce them below an economic action threshold. This requires knowledge of the population dynamics of the target pest. Microbial bio-insecticides based on insect pathogenic fungi, bacteria and viruses work by infecting and then killing individuals of the target pest species. Unlike conventional chemical pesticides, death of individual pests is not instantaneous, and in some cases, it takes 5 – 7 days to cause mortality. If the target pest has a short life cycle, then the slow mortality rate of a biopesticide may not be sufficient to stop the pest population from growing (i.e. individual pests may become infected, but if it takes a long time to kill them, they may well reproduce before they die). In addition, different pest life stages can vary in their susceptibility to a biopesticide. For example, larval instars can rid themselves of a fungal biopesticide by moulting, which reduces their susceptibility. In order to make best use of a biopesticide, it is important to understand how it impacts on pest population dynamics. However, few studies have been done in this area. In particular, there is a need to determine the most suitable timing for application based on an understanding of the following key biological factors: (i) the time to development of different pest life stages and the reproductive rate of adults; (ii) the distribution of the life stages of the pest; (iii) the susceptibility of the life stages to the biopesticide; (iv) the time taken for the biopesticide to kill the life stage. Identifying optimal application programmes for biopesticides experimentally would be onerous and expensive. Using a modelling approach to identify aspects of an application programme that are likely to produce greatest improvements in control (e.g. reducing or increasing the interval between applications) and testing this experimentally would provide quicker results and be more cost effective.

The objective of this work package was to develop a deterministic model to predict pest population increase over time and the effect of biopesticides on pest population development and management. The model allows the impact of different initial pest population sizes, biopesticide attributes and spray frequency and timing on pest control to be determined. Glasshouse whitefly and entomopathogenic fungi have been modelled initially and other pests, e.g. aphids, will be added later. Data for the model have been obtained initially from the scientific literature and will be supplemented with data from our own experiments where there are gaps in knowledge or where validation is needed. By evaluating methods for making biopesticides more effective the model will increase grower confidence and the uptake of biopesticides within their IPM programmes. Further, by providing data on likely optimal

biopesticide control strategies and biopesticide research priorities the model will be a useful tool for researchers and biopesticide manufacturers. Key messages for improving the efficacy of biopesticides will be given to growers.

Materials and methods

Model structure. A discrete time, stage-structure population dynamics model was developed by ADAS. The pest population increase over time was simulated using a 'boxcar train'-type model in which each individual in a pest population transitions from one development stage to the next until it reaches adulthood and reproduces. The rate of population growth depends on the number of life stages, the development time of each life stage, the natural mortality occurring in each life stage, the adult pre-reproductive period (the time between becoming an adult and producing offspring), reproductive rate (number of offspring produced per female adult), and adult sex ratio. The efficacy of a biopesticide control programme is simulated by altering the initial pest population size, infection efficacy (the percent of the population killed by the biopesticide), the time from exposure until death, the persistence of the biopesticide (the length of time the biopesticide remains effective following application), programme start date (the time between pest arrival and the first biopesticide application), the number applications and the interval between applications. Differences between developmental stages of the pest in terms of the infection efficacy and time until death can also be included in the model. Additional details on the activity of the biopesticide can also be integrated into the model, for example biopesticide 'escape', whereby an individual exposed to an entomopathogenic fungus can avoid infection by the disease by shedding its cuticle during transition to the next developmental stage, can be included by setting infection efficacy to zero for any individuals that develop to the next stage before the time until kill period is complete. The effect of climatic conditions and host plant can also be integrated into the model, however these depend on the availability of suitable data.

Model parameterisation. A literature review was done to identify parameter values for the development time and natural mortality of each stage of the pest, the adult pre-reproductive period, reproductive rate and sex ratio, and the biopesticide infection efficacy, time until kill and persistence. The literature review included peer reviewed literature, conference papers, relevant reports, 'grey' material and relevant expert knowledge. The Web of Science and Google Scholar search engines were used to carry out the literature review. Any gaps in the literature review were then filled by carrying out bioassay experiments. To ensure maximum relevance of data taken from different sources, it was decided that, where possible, parameter values would be chosen from papers using the same host plant (tomato) and similar environmental conditions. The model was initially parameterised for the glasshouse whitefly

(*Trialeurodes vaporariorum*) and the tobacco whitefly (*Bemisia tabaci*) and control with the entomopathogenic fungi (EPF) *Lecanicillim* spp. and *Beauveria bassiana*. Once this model was complete, a further literature search to parameterize the model for peach-potato aphid (*Myzus persicae*) was started.

Identifying optimal control programmes. The completed model was used to explore the effect of altering biopesticide control programmes (e.g. changing the programme start date, number of applications and application interval) on control efficacy. This would allow the rank order (in terms of control efficacy) of different spray programmes to be determined and compared experimentally.

Results

Model parameterisation. Relevant parameter values for *T. vaporariorum* and *B. tabaci* were identified in 49 papers. The parameter values chosen for *T. vaporariorum* are shown in Table 6. Parameter values were primarily chosen from papers in which experiments used tomato as the host plant. The effect of temperature is available for the majority of parameters.

Table 6. *T. vaporariorum* parameter values (at 21°C) and whether a model is available for temperature-dependent effects.

Parameter	Value	Temperature-dependent model available?	Reference
Egg development time	8.1	Yes	Roermund & van Lenteren (1992)
1st instar development time	4.5	Yes	Roermund & van Lenteren (1992)
2nd instar development time	3.3	Yes	Roermund & van Lenteren (1992)
3rd instar development time	3.5	Yes	Roermund & van Lenteren (1992)
4 instar + prepupa + pupa development time	8.7	Yes	Roermund & van Lenteren (1992)
Adult longevity	39.2	Yes	Burnett (1949)
Egg survival (%)	96.3	No but can be adjusted for extremes	Roermund & van Lenteren (1992)
1st instar survival (%)	95.8	No but can be adjusted for extremes	Roermund & van Lenteren (1992)
2nd instar survival (%)	97.4	No but can be adjusted for extremes	Roermund & van Lenteren (1992)
3rd instar survival (%)	96.3	No but can be adjusted for extremes	Roermund & van Lenteren (1992)
4 instar + prepupa + pupa survival (%)	92.7	No but can be adjusted for extremes	Roermund & van Lenteren (1992)
Adult survival (%)	96.4	No	Burnett (1949)

♀ sex ratio	0.48	No	Roermund & van Lenteren (1992)
Pre-oviposition period (days)	1.3	Yes	Roermund & van Lenteren (1992)
Reproductive rate (eggs/day/female)	6.7	Yes	Roermund & van Lenteren (1992)

The parameter values chosen for *B. tabaci* are shown in Table 7. For *B. tabaci* the literature review focused on papers using the *B. tabaci* 'Mediterranean' species (formerly known as the 'Q biotype') as this is the sub-species most commonly imported into the UK. Parameter values were primarily chosen from papers in which experiments used tomato as the host plant.

Table 7. *B. tabaci* parameter values (at 21°C) and whether a model is available for temperature-dependent effects.

Parameter	Value	Temperature-dependent model available?	Reference
Egg development time	14	No but data available to fit model.	Bonato *et al*. (2006)
1st instar development time	7.1	No but data available to fit model.	Bonato *et al*. (2006)
2nd instar development time	4.1	No but data available to fit model.	Bonato *et al*. (2006)
3rd instar development time	8.8	No but data available to fit model.	Bonato *et al*. (2006)
4 instar + prepupa + pupa development time	5.6	No but data available to fit model.	Bonato *et al*. (2006)
Adult longevity	28.8	Yes	Bonato *et al*. (2006)
Egg survival (%)	98.7	No but data available to fit model.	Bonato *et al*. (2006)
1st instar survival (%)	98.7	No but data available to fit model.	Bonato *et al*. (2006)
2nd instar survival (%)	93.4	No but data available to fit model.	Bonato *et al*. (2006)
3rd instar survival (%)	93	No but data available to fit model.	Bonato *et al*. (2006)
4 instar + prepupa + pupa survival (%)	97	No but data available to fit model.	Bonato *et al*. (2006)
Adult survival (%)	64	No but data available to fit model.	Bonato *et al*. (2006)
♀ sex ratio	0.5	No	Bonato *et al*. (2006)
Pre-oviposition period (days)	<1	No but data available to fit model.	Bonato *et al*. (2006)
Reproductive rate (eggs/day/female)	3.9	Yes	Bonato *et al*. (2006)

The literature review found 26 papers with relevant information on the effect of *Lecanicillium* spp. or *Beauveria bassiana* against *T. vaporariorum* or *B. tabaci*. This found important data on most parameter values but revealed some knowledge gaps, including infection efficacy for specific pest stages, time until kill, and the effect of temperature on EPF performance. The parameter values are still to be chosen by the project team and so will not be shown here but will be available in future project reports. Bioassays have been undertaken by Warwick to fill in knowledge gaps.

The development of the *M. persicae* model has begun with 47 papers containing relevant information identified. The parameter values are still to be selected by the project team and so will not be shown here but will be available in future project reports.

Identifying optimal control programmes. The *T. vaporariorum* and *B. tabaci* population models were used to explore control strategies using dummy data for the biopesticide. Once biopesticide parameter values are chosen for *Lecanicillim* spp. and *Beauveria bassiana* these can be included in the model. The below indicates the model output under a range of scenarios.

Effect of initial pest population size: The time taken for a pest population to reach 10 million individuals was 53% faster when the initial pest population consisted of 1000 adults (63 days) compared an initial pest population of 10 adults (133 days) (Figure 21).

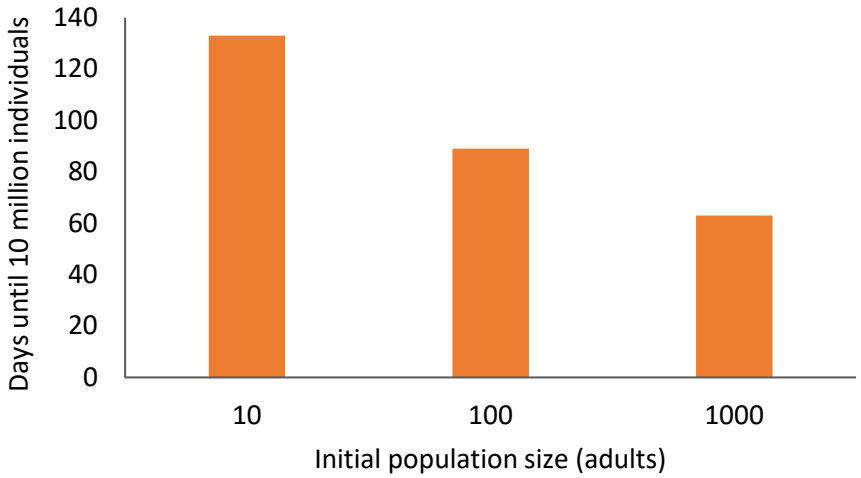

Figure 21. The time taken for a *T. vaporariorum* population to reach 10 million individuals from different starting populations at 21°C.

Effect of EPF infection efficacy on pest control: The time taken to eradicate a pest population was 37% faster when EPF infection efficacy was 90% (19 days) compared to when EPF infection efficacy was 60% (30 days). The pest population was not controlled when infection efficacy was 30% (Figure 22).

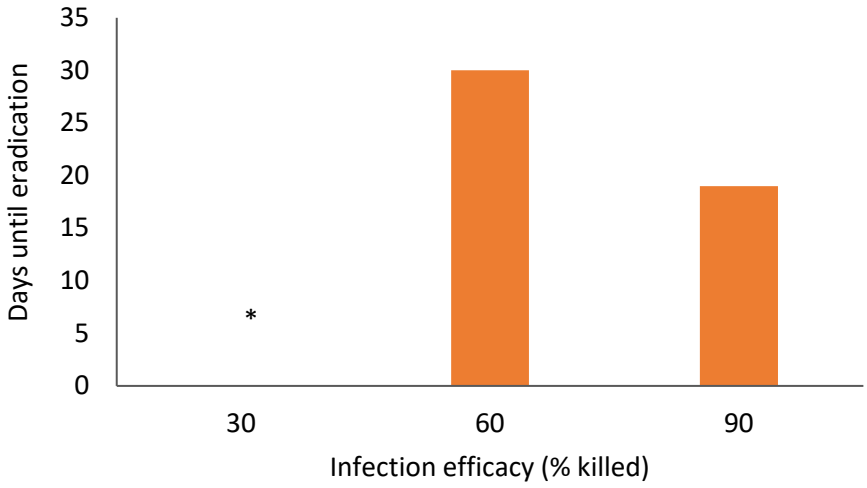

Figure 22. The time taken to control a *T. vaporariorum* population at different EPF infection efficacies at 21°C. * = no control achieved and pest population reached 10 million individuals after 92 days. Initial pest population = 1000 adults, all pest stages effected, time until kill = 3 days, persistence = 5 days, applications on days 7, 14, 21, 28 and 35.

Effect of EPF persistence on pest control: The time taken to eradicate a pest population was 10% faster when the EPF persisted for 10 days (27 days) compared to EPF persistence was five days (30 days). The pest population was not controlled when EPF persistence was two days (Figure 23).

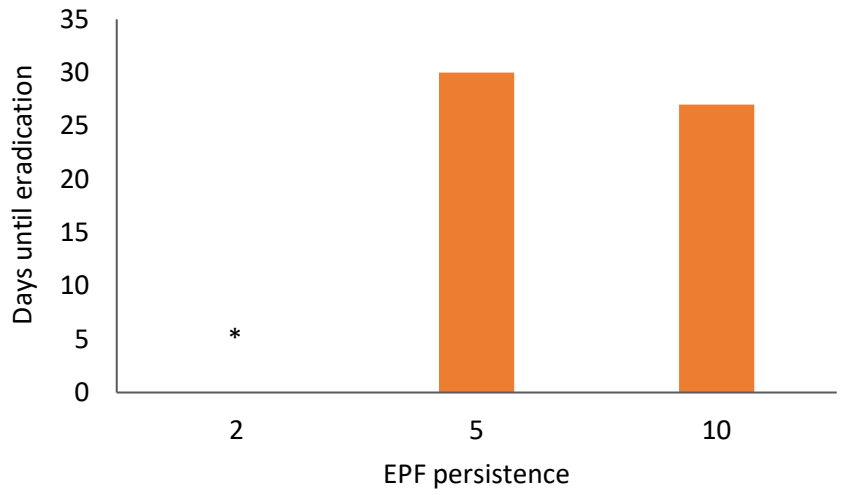

Figure 23. The time taken to control a *T. vaporariorum* population at different durations of EPF persistence at 21°C. * = no control achieved and pest population reached 10 million individuals after 97 days. Initial pest population = 1000 adults, all pest stages effected, infection efficacy = 60%, time until kill = 3 days, applications on days 7, 14, 21, 28 and 35.

Effect of EPF time until kill on pest control: The time taken to eradicate a pest population was 61% faster when the EPF took two days to kill an exposed individual (17 days) compared when the EPF took 6 days to kill an exposed individual (44 days) (Figure 24).

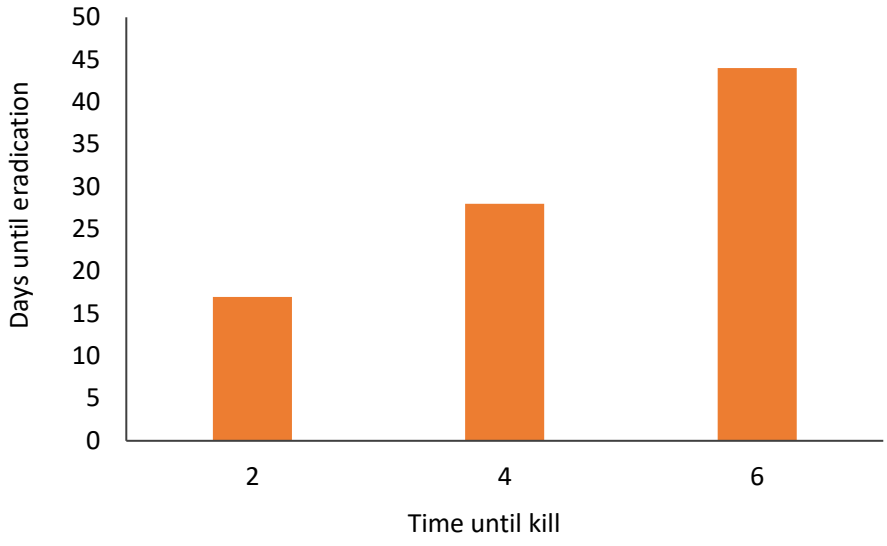

Figure 24. The time taken to control a *T. vaporariorum* population at different durations of EPF time until kill at 21°C. Initial pest population = 1000 adults, all pest stages effected, infection efficacy = 90%, persistence = 5 days, applications on days 7, 14, 21, 28 and 35.

The effect of initial pest population size on pest control: The time taken to eradicate a pest population was 57% faster when the initial pest population consisted of 10 adults (13 days) compared to an initial pest population of 1000 adults (30 days) (Figure 25).

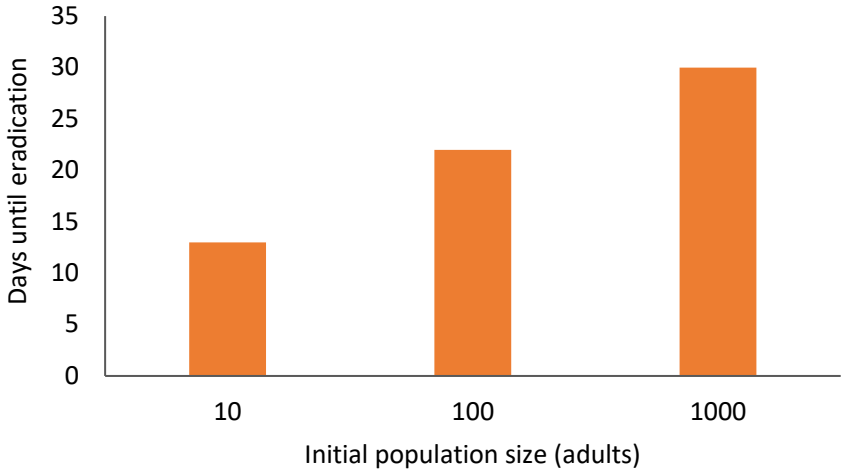

Figure 25. The time taken to control a *T. vaporariorum* population at different initial pest population sizes at 21°C. All pest stages effected, infection efficacy = 90%, persistence = 5 days, time until kill = 3 days, applications on days 7, 14 and 21.

The effect of control programme start date on pest control:The time taken to eradicate a pest population was 45% faster when the EPF was first applied five days after the pest arrived (26 days) compared when the EPF was first applied 20 days after the pest arrived (47 days) (Figure 26).

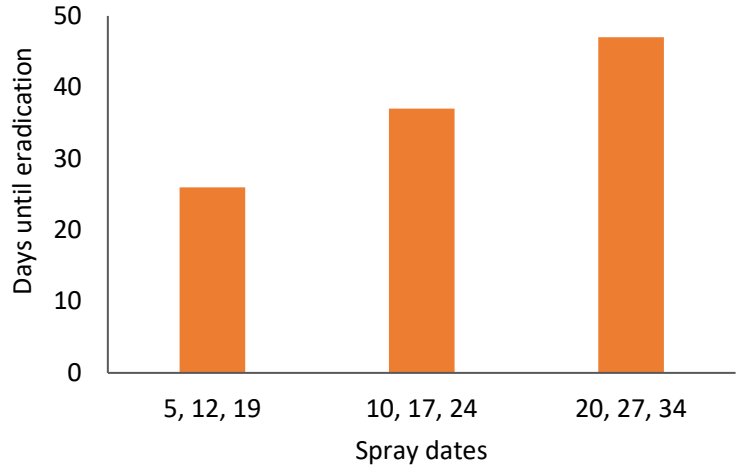

Figure 26. The time taken to control a *T. vaporariorum* population at different control programme start dates at 21°C. All pest stages effected, infection efficacy = 60%, persistence = 5 days, time until kill = 3 days, three applications at seven day intervals.

The effect of application frequency on pest control: The time taken to eradicate a pest population was 39% faster when the EPF was applied at five day intervals (23 days) compared 10 day intervals (38 days). No control was achieved when the EPF was applied at 20 day intervals (Figure 27).

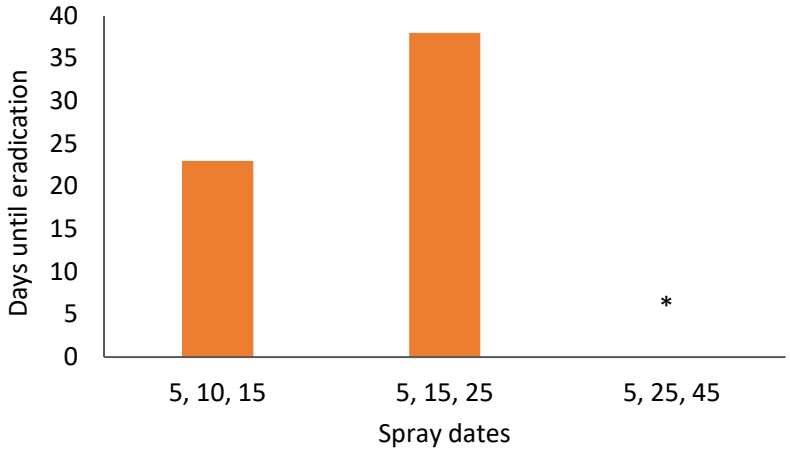

Figure 27. The time taken to control a *T. vaporariorum* population at different EPF application frequencies at 21°C. * = no control achieved and pest population reached 10 million individuals after 143 days. All pest stages effected, infection efficacy = 60%, persistence = 5 days, time until kill = 3 days, three applications starting five days after the pest arrived.

Discussion

This work developed a mathematical model to compare biopesticide control strategies. The model was constructed for both *T. vaporariorum* and *B. tabaci*, and control with *Lecanicillim* spp. and *Beauveria bassiana*. The model can be used to identify the ways in which a control programme can be improved and the aspects of the control programme that provide that greatest improvement in control when changed. Ultimately the model will be used to rank the order of biopesticide control programmes in order of control efficacy. The model will be validated experimentally in the next year of the project. A further model is currently being developed for *M. persicae* control with biopesticides.

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2.4 Assessment of compatibility of microbial and botanical pesticides in mushroom production systems

This WP focuses on the potential of biopesticides to help control the mycoparasitic fungus *Lecanicillium fungicola*, commonly known as Verticillium dry bubble. This is one of the most important diseases of cultivated mushrooms. If left untreated, it can result in an entire crop loss within 2–3 weeks of an outbreak starting. Spores of *L. fungicola* can rest in debris and reinfect following mushroom crops, meaning that long term control can be difficult once the disease has become established within a production facility. It is estimated that dry bubble causes losses to white button mushroom production equivalent to 2-4% of annual revenue.

Dry bubble infects mushroom fruiting bodies, and the infection process takes place within the casing layer. When infection occurs during casing layer application, the disease generally causes deformed mushrooms and/or splitting or peeling of the stipe tissue. If infection occurs in the late stage of the mushroom life cycle, symptoms include small necrotic lesions on the cap of the mushroom. Infection does not decrease the weight of the mushroom crop, but has the potential to decrease the total number of mushrooms produced as well as impacting on crop quality.

Laboratory experiments are being done to quantify the effects of candidate biopesticides against dry bubble and *Agaricus bisporus*. The main challenges are to develop suitable laboratory assays that can quantify the effects of the biopesticides and to identify control agents that affect dry bubble without adversely affecting its host. This is a significant

undertaking as it would require a high degree of specificity in the control agent. Candidate biopesticides were chosen by the research team in conjunction with the mushroom industry representative (Jude Wilson). Candidate selection was done on the basis of active substance type and mode of action. Targeted work with selected biopesticides will also be done to examine their effects on mushroom yield (existing conventional chemical pesticides reduce mushrooms yields, and the industry has raised the question of whether biopesticides may do the same) and on whether selected microbial biopesticides are able to proliferate within spawned mushroom compost.

Materials and Methods

A review of currently available biopesticides was undertaken and candidate biopesticides were chosen (Table 7). They included microbial control agents (Actinovate, Mycostop, T34, Amylo-X), plant extracts, aka botanicals (Regalia, an experimental product QRD460, and Prev-Am), and the secondary alcohol isopropanol (isopropyl alcohol). Selected products were obtained from the manufactures and stored according to their recommendations, before being used for experimentation. To date, five of the products / substances have been obtained and tested: Mycostop, T34, Amylo-X, Prev-Am, and isopropanol.

Table 7. Candidate biopesticides for this study

Micro-organism based products	
Actinovate	*Streptomyces lydicus* strain WYEC 108
Mycostop	*Streptomyces* sp. strain K61
T34	*Trichoderma asperellum* strain T34
Amylo-X	*Bacillus amyloliquefacien* s subsp. plantarum strain D747
Botanical based products	
Regalia	extract of *Reyonutria sacchaliensis*
-	Terpenoid Blend QRD460
Prev-Am	cold pressed orange oil – limonene
Other	
	isopropanol

The effect of the candidate biopesticides was investigated by measuring the rate of colony extension of cultures of both *L. fungicola* and *A. bisporus* on an agar growth medium treated with the biopesticides. Two different methods were used depending on the mode of action of the product. All biopesticides were evaluated at a range of concentrations. For the Prev-am and isopropanol, the biopesticides were applied to molten potato dextrose agar (PDA), mixed,

and then poured into Petri plates (90mm, triple vented). Using a flame sterilized cork borer, plugs (6mm diameter) were cut from fungal colonies of *A. bisporus* and *L. fungicola* growing on PDA, and then placed upside down in the centre of the PDA + biopesticide plates. For the microbial control agents Mycostop, T34 and Amylo-X, 1µl aliquots of liquid suspension of each biopesticide were added to 4 wells placed around the centre (ca. 2cm apart) of PDA plates (90mm, triple vented). Plugs (6mm) were cut with a sterile cork borer from cultures of either *L. fungicola* or *A. bisporus* (as described above) and placed upside down in the centre of each PDA plate. Controls consisted of PDA only (i.e. without biopesticide). Petri plates were incubated for 28 days in darkness at 22°C with two replicate Petri plates for each isolate/fungicide/concentration combination. The experiments were repeated three times. Colony diameters were measured twice weekly for four weeks. Hypal growth (calculated as colony radius) was plotted against time and colony extension rate was calculated for the linear phase.

Results and Discussion

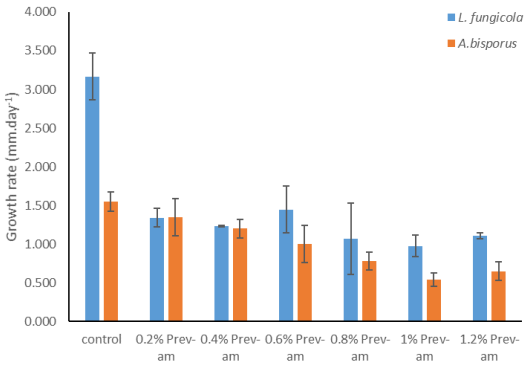
- All of the biopestcides examined were detrimental to the development of *L. fungicola* in vitro, with the exception of Amylo-X which had very little effect regardless of concentration tested (Figure 28, Table 8).
- The botanical Prev-am reduced *L. fungicola* growth by > 50% at all of the concentrations examined. This was the only biopesticide to appreciably reduce *L. fungicola* growth without also significantly impairing the growth of *A. bisporus*. At concentrations of 0.2%, Prev-Am reduced *L. fungicola* growth by about 50% with a concomitant reduction in *A. bisoprus* growth of c. 5%.
- Isopropanol completely inhibited growth of *L. fungicola* and *A. bisporus* at 50 and 100ppm. Overall it appeared to have equal potency to both *L. fungicola* and A. bisporus, for example it caused c. 30% reduction in *L. fungicola* and *A. bisporus* at 1 ppm.
- Mycostop and T34 also did not show evidence of differential activity against *L. fungicola* and *A. bisporus.* For example 1% Mycostop caused a 40% reduction in *L. fungicola* growth and c. 30% reduction in *A. bisporus*. Similarly, 1.2% T34 caused c. 50% reduction in growth of both *L. fungicola* and *A. bisporus*.

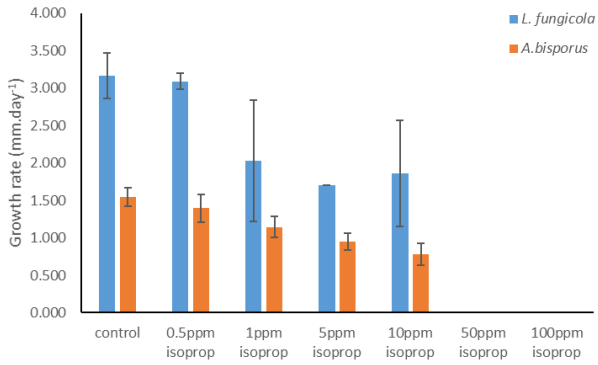
Overall the work has identified Prev-Am as the lead product. Mycostop and T34 appear not to differentiate between *L. fungicola* and *A. bisporus*, although scale up effects could mean that impacts on mushroom production could be different at the crop scale (the

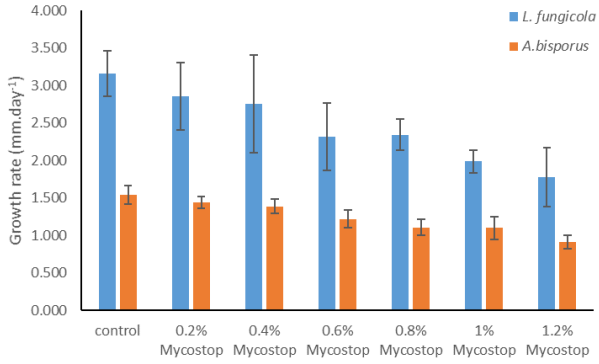
corollary of this is that biopesticides that are harmless in vitro are likely to remain so in the field (Loria et al. , 1983; Majchrowicz & Poprawski, 1993)).

Table 8. Effects of the selected biopesticides at varying concentrations, on extension rate of *L. fungicola* and *A. bisporus*. Values are the mean extension rates of three replicates expressed as percentage of growth on PDA (Control).

Treatment	***L. fungicola***	***A.bisporus***
0.2% Prev-am	42.4	87.1
0.4% Prev-am	39.1	77.7
0.6% Prev-am	45.7	64.7
0.8% Prev-am	33.7	50.5
1% Prev-am	30.8	35.0
1.2% Prev-am	35.1	42.1
0.5ppm isopropanol	97.6	90.3
1ppm isopropanol	64.1	74.1
5ppm isopropanol	53.7	61.5
10ppm isopropanol	58.7	50.5
50ppm isopropanol	0.0	0.0
100ppm isopropanol	0.0	0.0
0.2% Amylo-X	96.2	107.1
0.4% Amylo-X	94.8	103.4
0.6% Amylo-X	93.0	94.2
0.8% Amylo-X	87.0	87.4
1.0% Amylo-X	84.8	81.9
1.2% Amylo-X	80.6	64.1
0.2% Mycostop	90.2	93.5
0.4% Mycostop	87.0	90.0
0.6% Mycostop	73.3	79.0
0.8% Mycostop	74.1	71.8
1% Mycostop	62.8	71.2
1.2% Mycostop	56.2	58.9
0.2% T34	105.3	95.7
0.4% T34	91.3	81.3
0.6% T34	87.8	74.8
0.8% T34	77.4	72.4
1.0% T34	62.6	57.9
1.2% T34	52.1	49.1







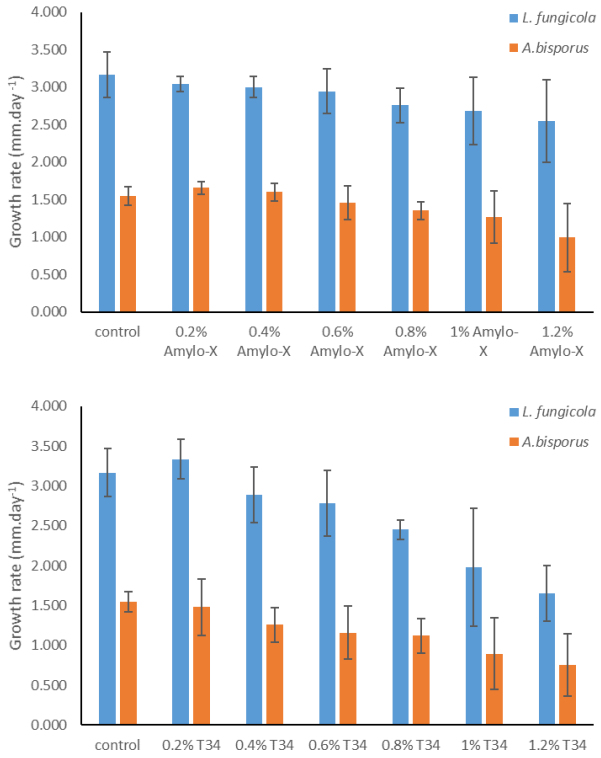


Figure 28. Effects of the selected biopesticides at varying concentrations, on extension rate of *L. fungicola* and *A. bisporus*. . Values are the mean extension rates of three replicates. Error bars are the standard error of the mean.

Conclusions

None of the candidate biopesticides showed high efficacy against *L. fungicola* without affecting the growth of *A. bisporus*. This is not unexpected; a similar issue occurs with conventional fungicides, where only a small number of products have activity against *L. fungicola* but are not harmful to *A. bisporus*. Of the products tested, 0.2% Prev-Am showed the best differential activity and would be worth taking forward for further investigation. It would also be worth investigating other botanical products. Many of the botanical products have a volatile action which might require us to adapt the laboratory bioassay used here in order to investigate this further. From the results of the initial screen, it would be useful to test a further range of concentrations of isopropanol in in vitro tests.

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3. OVERALL CONCLUSIONS AND FUTURE WORK

A key task for the AMBER project has been to identify those grower management practices that result, unintentionally, in a biopesticide giving inconsistent results, or which prevent the biopesticide from achieving its highest level of efficacy. We can then develop improved practices, either through making specific recommendations or by developing tools that will help growers and the biopesticides industry to help themselves. This process has to start with grower management practices that are relevant to a wide range of biopesticides, crops, and pests and diseases, as these are likely to have the biggest impact on the horticultural industry as a whole. The area that stands out the most is effective biopesticide delivery, i.e. the ability of the grower to deliver the right biopesticide to the right place, at the right time, and at the right dose, in an efficient way, and which works as part of IPM. Our observations have shown that, currently, this process is not happening as effectively as it should. We are now working on solutions to the 'delivery problem' in three different ways:

(1) Improving spray application. There are a range of straightforward measures that growers need to put in place now so that they comply with best management guidelines. These include things such as checking and regularly replacing spray nozzles, calibrating spray equipment, and cleaning spray tanks correctly. We have covered these basic points in our AMBER biopesticide application workshops (see below). We are also investigating a strategic issue about optimising water volumes for spray application. Biopesticide product labels specify the dose to be applied (i.e. the amount of biopesticide per ha), gives a range of water volumes that are permitted on the crop (where water acts as a carrier for the biopesticide), the frequency of applications and the maximum number of applications per crop. Our results show that, when applying at a constant dose (where the biopesticide concentration reduces as volume increases), the most efficient way to deliver a biopesticide to the plant is with as low a volume and as high a concentration as possible. This does not compromise the distribution over the plant, it will reduce loss of product through run off, and take less time to apply.

Our plan to take this part of the work forward is as follows. Work on the 'volume optimisation' experiments will be completed by studying the relationship between applied water volume and quantity of biopesticide deposited on the plant using a herb crop as a representative of a sparse horizontal crop. So far, these experiments have been done using tracer dyes to represent biopesticide. Therefore, we plan to run experiments comparing contrasting spray regimes (high water volume versus low water volume, at a constant dose) on biopesticide activity (probably a microbial biopesticide against spider mite on a PE). Having gained a good data set now on the relationship between water volume and agent deposition, we will also talk with biopesticide manufacturers about reducing water volumes for spray application and seek to find a common way forward.

(2) Understanding the persistence of biofungicides on crop foliage. The three microbial biofungicides studied in the project differed markedly in their persistence on plant leaves in the absence of a plant pathogen target. As stated previously, biofungicides are recommended for application before, or at the first signs of, disease symptoms. If they do not survive for long, then they will have to be reapplied frequently. Both *Gliocladium catenulatum* and *Bacillus subtilis* showed good persistence on tomato leaves, with no decline at 7 days after application, indeed *Gliocladium* showed an increase over 14 days as it reproduced on the plant. Therefore, on protected crops at least (where there is no chance of losing biofungicide by being washed off leaves by rain) then growers can schedule applications knowing that the biofungicides will persist on the crop for at least a week and probably at least two weeks. In contrast, the mycoparasite *Ampelomyces quisqalis* survived for no more than 4 days in the absence of its powdery mildew host. In all cases, the grower faces a critical decision about when to start biofungicide applications for disease management. For a preventative product such as *Gliocladium*, which works partly by outcompeting plant pathogens for space on the leaf surface, then it is important that the biofungicide is applied sufficiently in advance of its target plant pathogen to allow it to colonize plant surfaces. In this case, a reliable strategy would be preventative sprays done regularly through the season: there are often limits placed on the maximum number of sprays permitted, and the data generated here on persistence is helpful in deciding how far apart these sprays should be spaced. For a biopesticide based on a mycoparasite such as *A. quisqualis*, timing of application is particularly critical. This biofungicide will not survive on the plant in the absence of its powdery mildew host, and hence routine sprays when powdery mildew is not present will lead to wasted product. Equally, because biofungicide efficacy depends on the population size of both the biofungicide and its target plant pathogen, then applications that are made after powdery mildew has established

and grown in population size may well result in control failure. The decision to apply a biofungicide such as AQ10 is often done by crop walking and scouting for the first visible signs of powdery mildew, but given that this disease can grow quickly then by this stage it may be too late. Hence an advance warning system to help decide about the timing of the first application would be helpful. The plan going forward for this part of AMBER is as follows. Firstly, the persistence and disease-control ability of *A. quisqualis* will be quantified on plant leaves in the presence of different levels of powdery mildew. This will enable us to determine the powdery mildew threshold where action is needed to start applications of AQ10. Secondly, a proprietary, automated environmental monitoring system will be investigated that can alert the grower to conditions likely to result in a powdery mildew outbreak and help inform the application of AQ10. The system uses an array of humidity and temperature sensors placed throughout the crop, which combined with a cloud-based data analysis algorithm, sends alerts and summary information to the grower's smartphone. The idea is to identify the 'hotspots' within the glasshouse where powdery mildew is likely to develop first, as a consequence of localised fluctuations in temperature and humidity. This will then provide a prompt for application of AQ10, either as a targeted spray within the hotspot area or across the crop as a whole.

(3) A pest control model to help identify optimal biopesticide control strategies. Biopesticides are increasingly being used to fill the gap created by the loss of conventional synthetic chemical pesticides. However, the majority of biopesticides are slower acting than conventional pesticides. At the moment, the relationship between biopesticide speed of kill and virulence to different life stages, the growth rate of the pest, and its reproductive rate, are not considered to any great extent when planning how to use a biopesticide in IPM. This is probably because the interactions involved create a complex situation that is very difficult to understand using purely empirical experiments. Indeed, our model simulations show that these variables can have a major effect on predicted levels of pest control. With further development, the model will allow us to identify which biopesticide application strategies are likely to be most effective for particular groups of pests. The plan going forward is to complete the development of a box car train model for *Myzus persicae*, and to run a series of laboratory bioassays with *M. persicae* and *T. vaporariorum* to fill in data gaps about the susceptibility of different life stages of these pests to biopesticides and their speed of kill. We can then make some specific predictions ahead of experimental validation. After this, the model could be made available to IPM practitioners, biopesticide developers and other researchers.

4. Knowledge and Technology Transfer

Presentations

Chandler, D. Optimising biopesticide performance on nurseries. Presentation at the AHDB Ornamentals Conference, *'Developing holistic control programmes', 20 February 2018, Kenilworth, Warwickshire*

Chandler, D. The use of biopesticides in IPM: the UK AMBER project. Presentation at the Integrated Pest and Disease Management biosecurity & biopesticides Workshop 12 June 2018, Stockbridge Technology Centre, Cawood, Selby

Ramsden, M. Monitoring & biocontrol. Presentation at the Integrated Pest and Disease Management biosecurity & biopesticides Workshop 12 June 2018, Stockbridge Technology Centre, Cawood, Selby

Chandler, D. Helping growers get the best from biopesticides: the UK AMBER project... Presentation at the Sceptre plus meeting, 4th June 2018, Wellesbourne, Warwickshire

Chandler, D. Getting the best from biopesticides – output from the AMBER project. Presentation at the National Cut Flower Centre Open Evening, 8th August 2018, Spalding, Lincolnshire

Chandler, D. The use of biopesticides in IPM: the UK AMBER project, Presentation at the IBMA meeting, 18Th September 2018, Wellesbourne, Warwickshire

Chandler, D. The use of biopesticides in IPM: the UK AMBER project. Presentation at the AHDB monitor conference 20th November 2018

Posters

D Chandler, J Bennison, C Butler Ellis, R Gwynn, G Prince, R Jacobson, M Ramsden, E Wedgwood. Application & Management of Biopesticides for Efficacy and Reliability. Poster presentation at the NFU IPM summit, 5th June 2018, Stoneleigh, Warwickshire

Articles

A changing Landscape. The Grower, Issue 237, Dec/Jan 2018

Make practice perfect. The Grower, Issue 238, Feb/March 2018

Biopesticides: your questions. The grower, Issue 239, April/May 2018

AHDB News: Time for a biopesticide revolution? October 2018

Time for a biopesticide revolution? The Grower, Issue 243, Dec/Jan 2019

Workshop

Biopesticides application workshop 23rd October 2018, Bordon Hill, Warwickshire

During the two years that the AMBER project has been running, there have been a number of key areas explored by the project team aiming to improve the efficacy and understanding of biopesticides for horticultural businesses. Application practice and integration into current programmes were two of the areas of importance where there were information gaps, and that further work and knowledge exchange within industry was required. A workshop at Bordon Hill Nursery was held, in October 2018, and attended by growers, agronomists, spray operators and biopesticide manufacturers. Through a series of practical demonstrations, presentations and discussions the workshop highlighted the importance of equipment cleaning, maintenance of equipment and the potential influence of applied volume on performance to maximise the efficacy of biopesticdes through application. The workshop was a great opportunity for attendees to learn more about application best practice, there was plenty of opportunity for discussion between growers and manufacturers. Biopesticide manufacturers are aware of the importance of application accuracy, and are becoming increasingly aware of the need to liaise with industry through work such as the AMBER project, to ensure the maximum potential of biopesticides is achieved on commercial sites.

Website

The website went live in June 2017.

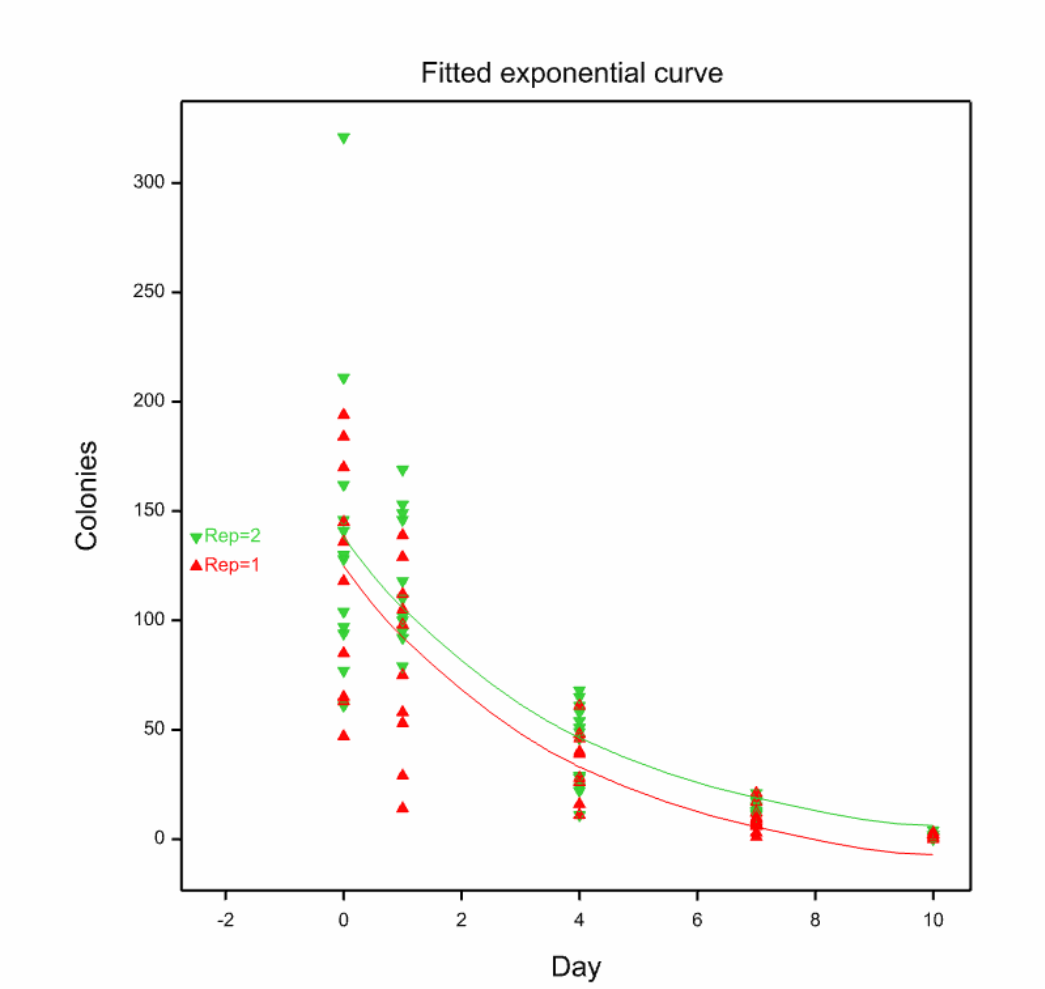
Table 8: Website summary statistics

Page	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec	Total
Amber project	172	103	120	68	78	88	65	100	164	`156	128	68	1154
What are biopesticides?	53	57	70	47	81	83	153	196	332	256	428	441	2197
Biopesticides- pros & cons	109	133	152	109	155	72	109	114	216	217	409	327	2122
Project details	36	32	35	24	22	25	27	17	40	26	45	25	354
Research plan	23	17	27	15	17	12	19	19	42	25	28	23	267
Project team	43	43	35	52	41	35	40	29	73	48	43	32	514
Links	29	29	31	22	18	16	21	16	32	17	34	21	286
Total	465	414	470	337	412	331	434	491	899	589	1115	937	6894

5. Appendices

Appendix 1.1 Fitted exponential curve of the number of colony forming units on agar plates of *A. quisqualis* from AQ10 washed from different plants after increasing time periods.

Data points are the number of colonies counted on a 9 cm diameter agar plate at 0, 2, 4, 7 and 10 days post inoculation of tomato plants. Two leaves providing a leaf disc each/plant with six replicate plants per batch. Washings from each disc were put onto three plates, but where colonies became overgrown by saprophytes from the leaves these were excluded.

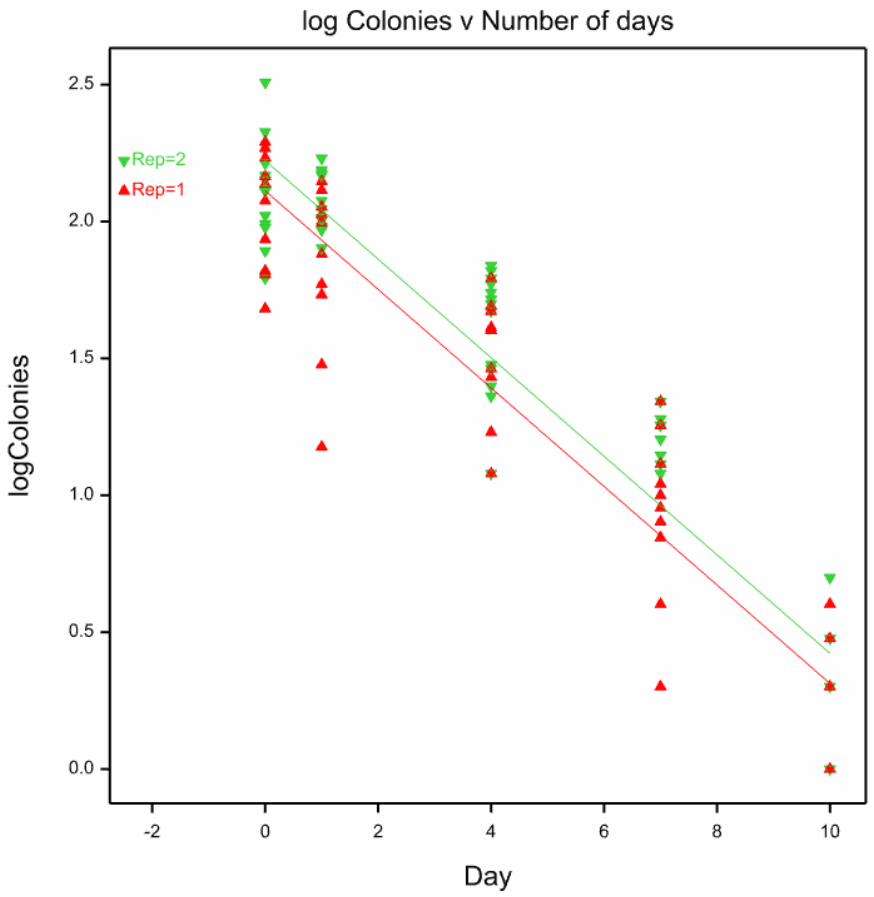


Equation of single curve is
Colonies=-10.8+143*0.7736**Days
Percentage variance accounted for 70.8

Equation of the 2 curves are
1Colonies=-17.99+142.7*0.7729**Days
2Colonies =-4.663+142.7*0.7729**Days)
Percentage variance accounted for 71.8
There is a small but significant improvement in the fit. ANOVA F ratio for the improvement is 0.025

Fitting separate curves for each replicate does not significantly improve the fit. ANOVA F ratio for the improvement is 0.085

Appendix 1.2 Fitted exponential curve of the logged number of colony forming units on agar plates of *A. quisqualis* from AQ10 washed from different plants after increasing time periods.



Fitting a single line to the logged data gives a higher percentage variance accounted for than the exponential fit
Equation is logcolonies=2.173-0.17996*Days

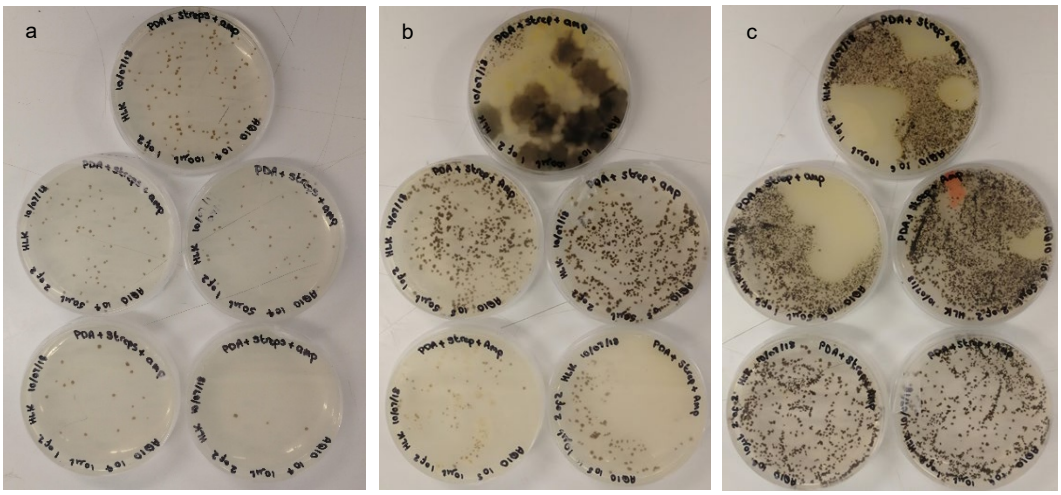
Equations of the fitted lines are
Rep1 Log colonies =2.1117-0.17993*Days
Rep2 Log colonies =2.2219-0.17993* Days.
Fitting 2 parallel lines improves the fit by a small but significant amount

Fitting the best lines for each replicate does not significantly improve the overall percentage variance accounted for.

Appendix 1.3 Serial dilution of *A. quisqualis* on PDA to determine the optimum concentration for effective quantification of cfu.

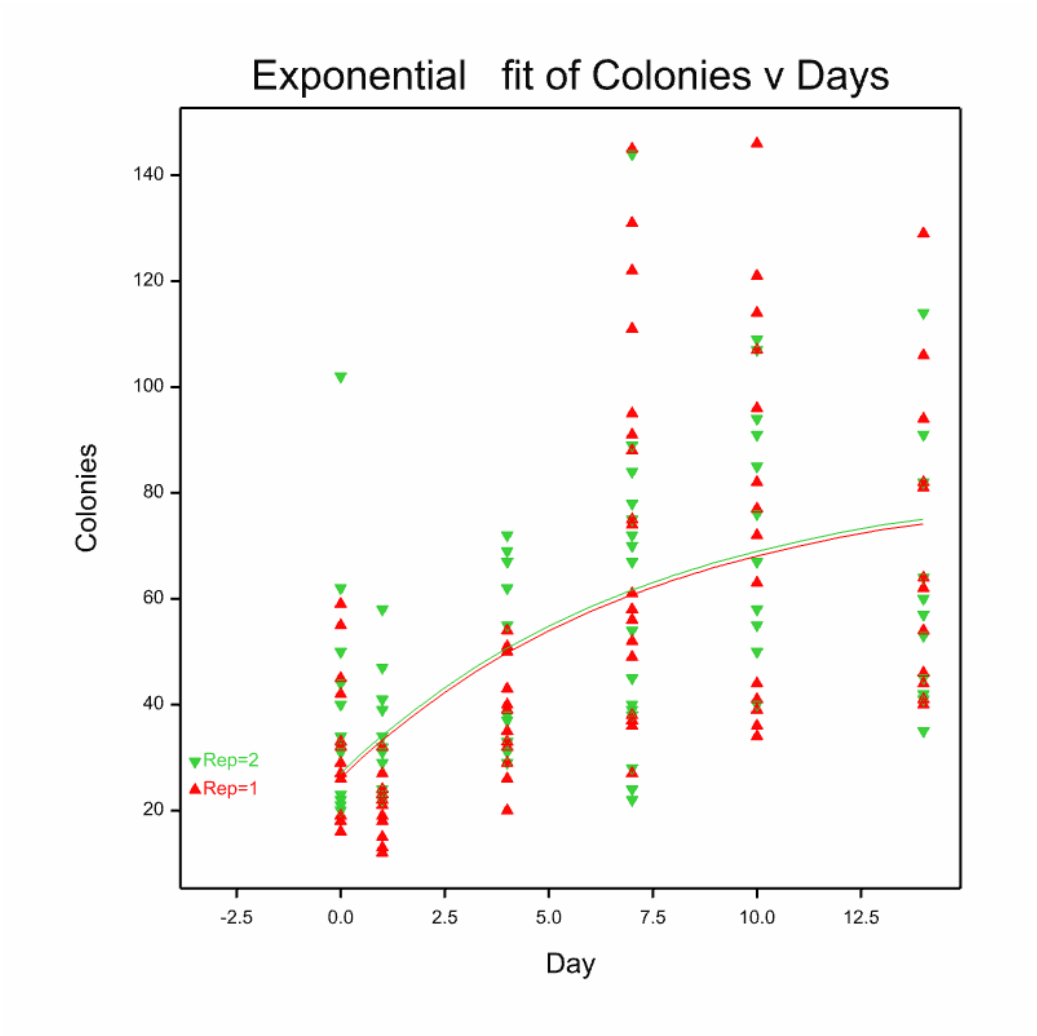
The concentrations shown are 1 x10^4 (a), 1 x 10^5 (b) and 1 x 10^6 cfu/ml (c).

100µl (top plate), 50 µl (middle two plates) and 10µl (bottom two plates) of each concentration plated onto PDA and spread evenly with a sterile plastic rod.

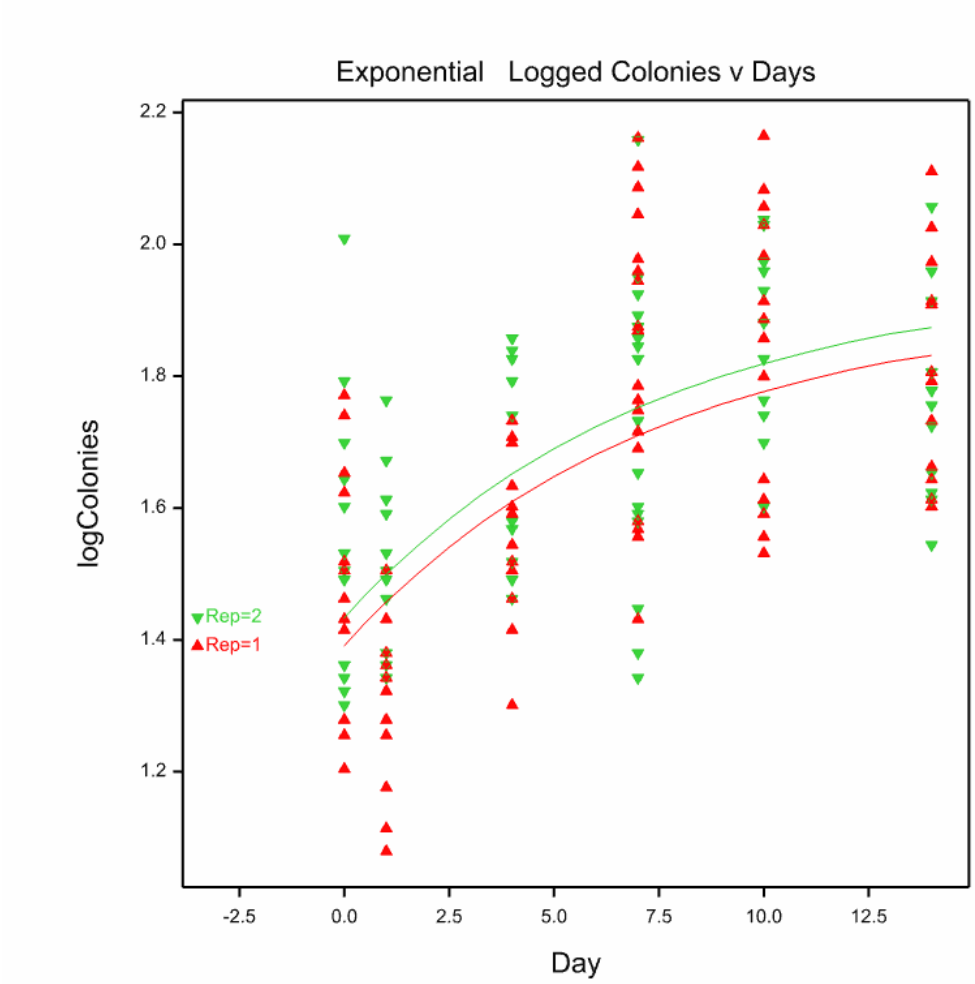


Appendix 1.4 Attempted fitting of an exponential curve of the number of colony forming units on agar plates of *G. catenulatum* from Prestop washed from different plants after increasing time periods. The curve only explains 30% of the variance and so a regression equation has not been produced.

Data points are the number of colonies counted on a 9 cm diameter agar plate at 0, 2, 4, 7, and 10 days post inoculation of tomato plants. Two leaves providing a leaf disc each/plant with six replicate plants per batch. Washings from each disc were put onto three plates, but where colonies became overgrown by saprophytes from the leaves these were excluded.



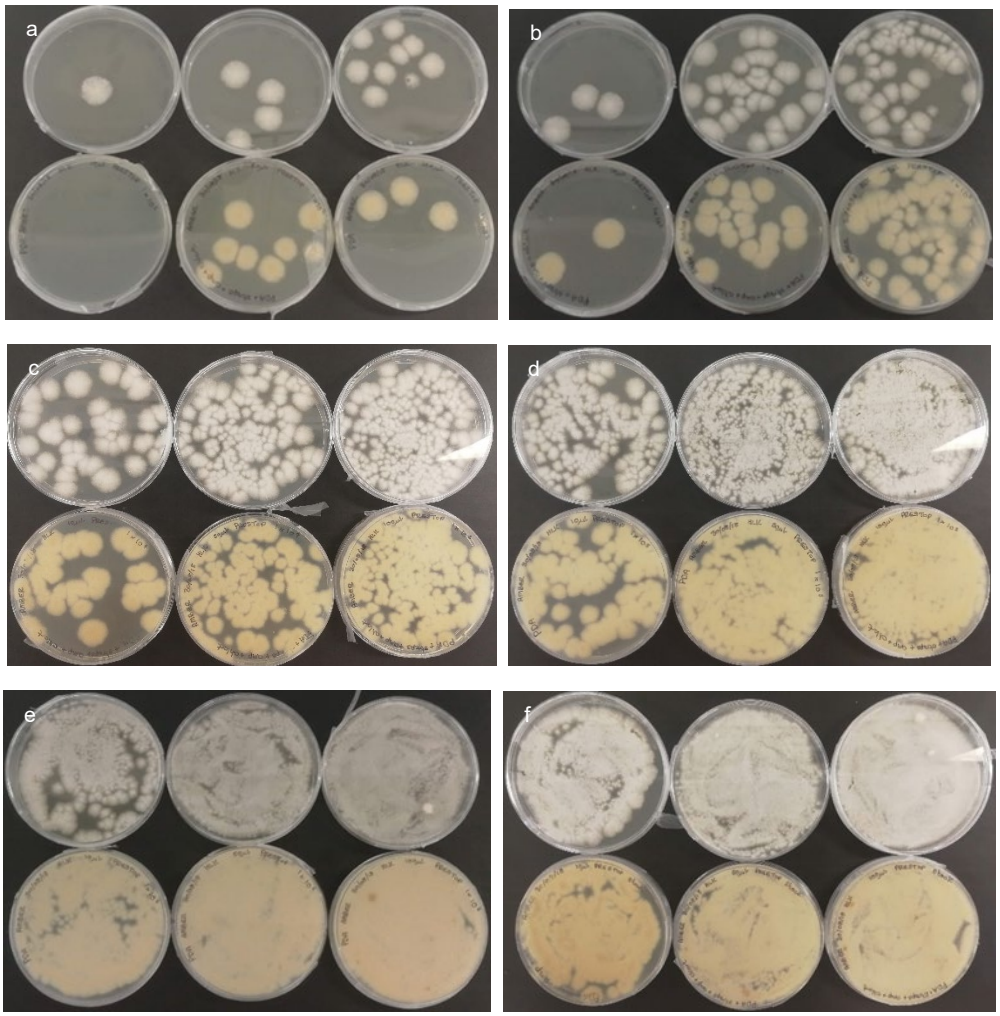
Appendix 1.5 Attempted fitting of an exponential curve of the log number of colony forming units on agar plates of *G. catenulatum* from Prestop washed from different plants after increasing time periods. The curve using logged data only explains only a little more of the variance seen without logging and so a regression equation has not been produced.



Appendix 1.6 Serial dilution of *G. catenulatum* on PDA to determine the optimum concentration for effective quantification of cfu.

The concentrations shown are 1 x10^2 (a), 1 x 10^3 (b), 1 x 10^4 (c), 1 x10^5 (d), 1 x10^6 (e) and 1 x10^7 cfu/ml (f).

10µl (left two plates), 50 µl (middle two plates) and 100µl (right two plates) of each concentration plated onto PDA and spread evenly with a sterile plastic rod.



Appendix 1.7 Biofilm formation of *B. subtilis* on 5% TSA; a nutrient agar with 100 ppm rifampicin agar plate culture, preventing counting of individual colonies.

Appendix 1.8 Visualisation of *B. subtilis* QST 713 growth over days from plant inoculation at 0 dpi (a), 1 dpi (b), 4 dpi (c), 7 dpi (d), 10 dpi (e) and 14 dpi (f) on 5% TSA; a nutrient agar with 100 ppm rifampicin.