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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.

Signature: Date:

Signature: Date:

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GROWER SUMMARY

Headlines

- Spray application: Most biopesticides are applied at a constant dose. There can be a perception among growers that using a high water volume gives better coverage, but this is not true. Spray water volume affects the quantity of biopesticide deposited on the crop even at a nominal constant dose, which in turn affects efficacy. The best strategy is to use a water volume that results in the highest concentration of biopesticide product on the crop. As a general rule, lower water volumes are better. They are also faster to apply and are less wasteful.
- Biopesticides and environmental conditions: The performance of biopesticides is sensitive to environmental conditions such as temperature, humidity, UV light etc. In this report, we provide detailed summaries of the effect of environmental and management conditions on the efficacy for three different biofungicides (*Bacillus amyloliquefaciens*, *Gliocladium catenulatum* and *Ampelomyces quisqualis*) using data from the scientific literature.
- Biopesticide data recording: A new biopesticides management flow chart and data recording form have been written to help growers and agronomists. It allows you to record the information needed to help understand how environmental conditions and management practice affect biopesticide performance on individual crops.
- Biopesticides and temperature: Temperature is a key environmental variable determining the performance of microbial biopesticides. Selecting the best microbial strains to use under the environmental temperatures in the crop is a difficult task for biopesticide companies. Working with fungal pathogens of insects, we have shown that strain selection can be done by measuring spore germination rate in simple petri dish tests. This is far quicker and easier than measuring the effect of temperature on efficacy against target insect pests.
- Biopesticides application model: A computer 'box car' model has been developed that predicts how different application regimes (e.g. timing and frequency of application) might affect bioinsecticide efficacy. The model has been developed first with glasshouse whitefly and peach potato aphid.

Background

AMBER (Application and Management of Biopesticides for Efficacy and Reliability) is an AHDB 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:

1) to understand the reasons why some biopesticides are giving sub-optimal results in current commercial practice;
2) to research innovations in management practices that can improve biopesticide performance;
3) to exchange information and ideas between growers, biopesticide companies and others in order to provide improved best-practice guidelines for biopesticides.

Biopesticides include a wide range of active substances, including living microbial agents and non-living natural substances. As a rule, they are less 'forgiving' than synthetic chemical pesticides and require more attention to detail to get the best out of them. In AMBER we are developing tools and practices that can be applied to a wide range of biopesticides to improve their use. In this report we give the results from four different work areas:

(1) Studying how applied water volume for nominal fixed dose spray applications influences biopesticide efficacy, to identify the best water volume strategy for different types of crops;
(2) Analysing the scientific literature on microbial biofungicides to summarise the conditions that are best for performance. This was used to develop an improved data recording sheet for growers and agronomists;
(3) Using mathematical models to give new insights on the effects of temperature on microbial agents;
(4) Developing a 'box car' model of the population growth of insect pests that is used to inform biopesticide application strategy, taking into account the different features of the pest and control agent that determine their efficacy.

Making biopesticide spray application more efficient: studying the effect of water volume on biopesticide efficacy

AMBER research on biopesticide spray application has focused on optimising spray water volumes with a range of crop types. This is essential before other methods for improving spray application can be investigated, such as new equipment or application techniques.

Biopesticide sprays are applied in one of two ways of two ways:

1) A constant dose model, in which the total amount of biopesticide applied per ha is set at a fixed amount and the volume of water used for spraying the product is chosen by the spray operator (provided that it stays within a range that has been set by the manufacturer and given on the label). In the constant dose system, the concentration of the product reduces as the water volume increases.
2) A constant concentration model. Here, the concentration is fixed, and there is a minimum and maximum water volume specified on the label, and the spray operator chooses the water volume within these limits. In the constant concentration system, the total amount of product per ha will increase as water volume increases.

Most biopesticides are used at a constant dose, although a few products are now on the market that are applied at a constant concentration.

The label recommendations for biopesticides often specify a high water volume (up to 1500 L per ha) as the upper limit. This is possibly because the labels need to cover a wide range of crop sizes and structures. Unfortunately, data supporting the recommended volumes does not appear to be available, which makes it difficult for the grower to choose the optimum volume for their crop. We have found that there can be a perception among growers that high water volumes give better coverage and penetration into the crop canopy. However, it is known from work with arable crops that lower volumes result in higher quantities of active substance on the crop, when applied at nominal constant dose, and water volume has little effect on penetration. Low volumes are a more efficient method of transferring biopesticide to a crop and result in less waste. However, in the case of biopesticides, a minimum quantity of water may be needed to ensure it performs adequately (e.g. so that it does not dry out too quickly), but there is no information available relating to this. Work in this area is technically difficult and requires specialised facilities, which may explain why it has been given insufficient attention prior to AMBER.

So far in AMBER, we have undertaken studies in two areas:

- Firstly, research was done that measured the relationship between applied volume and the quantity of spray deposited on small pot-grown plants treated with a horizontal boom. Experiments with basil, sprayed with a three-nozzle horizontal boom, indicated that, where the biopesticide is applied at a constant dose, the maximum active substance will be applied using the lowest water volume providing that the maximum label concentration is not exceeded. Where biopesticide products are used at a constant concentration, the maximum volume that should be used is less than 1000 L/ha, and there are likely to be benefits for smaller plants of reducing this down to around 500 L/ha.

- Secondly, research was done on the relationship between applied volume and the quantity of spray deposited for large plants treated with a vertical boom. This was done using a vertical boom track sprayer within an experimental tomato crop. It showed that the quantity of active substance deposited on the plant appeared to be relatively insensitive to volume, although the data had a lot of variability which may be due to the structure of the canopy, which is more complex than short, pot grown plants. The data indicated that the maximum volume that should be used is 1000 - 1500 L/ha *applied to the crop* (rather than calculated per unit floor area). For biopesticide products applied at a constant dose to tall crops, water volume can be reduced from this maximum to suit other needs (such as using a lower water volume to reduce the time needed to spray the crop).

For the current part of AMBER, we investigated the effect of spray volume on biopesticide efficacy against a target pest. A fungal biopesticide (Botanigard) was sprayed onto small tomato plants with a horizontal boom, and then spider mites (*Tetranychus urticae*) were placed on tomato leaflets and their survival was monitored over time. The biopesticide was applied at a nominal constant dose in different water volumes from 250 L per ha to 1500 L per ha with the Silsoe Spray Applications Unit track sprayer. The main findings were as follows:

- The amount of fungus deposited by the spray (measured as numbers of fungal colony forming units, CFUs, from leaves and grown on agar) was much higher on the upper leaf surface than the leaf underside. In some cases, there was clustering of colonies at the edge of the upper side of the leaflets caused by draining of suspension across the leaf. CFUs on the underside of leaflets may have been deposited as a result of drainage of spore suspension to the leaflet edge and then running underneath. This is not unexpected because of the shielding effect of the upper leaf surface, but it does illustrate the challenges of applying an effective dose of a contact-acting biopesticide to the underside of leaves, and this is obviously going to be an issue for target pests and diseases that occupy the leaf underside.
- The numbers of CFU deposited on the upper leaf surface (CFU per cm^2 leaflet surface) varied with increasing water volume although there was no evidence of an effect of canopy position. The highest deposition occurred with 500 L / ha.
- After seven days all application volumes except the untreated control resulted in 70% mortality or higher. There was no difference in the median survival times of spider mites treated with the different volumes of Botanigard, with the exception of the 500 L / ha treatment, in which median survival time was significantly lower than for all other treatments. This is consistent with our finding that 500 L / ha produced a significantly higher concentration of CFUs on the tomato leaflets.

The results suggest that water volume can have affect the quantity of active substance deposited on the crop, with concomitant impacts on efficacy, even when applied at a nominal constant dose. This experiment was technically difficult and to our knowledge this is the first study of its kind with a biopesticide. Through AMBER, we now have the ability to relate efficacy to the quantity of biopesticide active substance deposited on the leaf surface as a result of different spray conditions, which is highly encouraging.

Improving knowledge for growers and agronomists on the conditions needed for getting the best performance from microbial biopesticides

The scientific literature was analysed for three microbial biofungicides on the UK market with recommendations for foliar application to horticultural crops: Serenade ASO, Prestop and AQ10. The information was then used to summarise the conditions in which the organism or the product has been shown to perform. A generic decision tree was developed for what to consider before, during and after using these products, alongside tables which specify the environmental parameters that need to be known. In the Science section of the report, we also provide an example recording sheet to indicate the type of records that would be advantageous to keep, so that when product efficacy is either good or poor they can be referred to and utilised for future applications. Finally, some examples of AHDB reports are given to show where conditions, or the pathogen severity, may have affected the level of control achieved from either of the three products.

For Serenade ASO, Prestop and AQ10:

- There is poor understanding of the physics of spray application, e.g., the pressures, nozzle types and droplet size required to achieve optimum coverage. Efficacy would be increased by improved application techniques. Application to leaf undersides is a challenge.
- The high water volumes which can be used for application, combined with wide plant spacing necessary for particular crops, can mean significant spray waste. Further investigation should look at optimising the level of coverage achieved using lower spray volumes and more efficient methods of delivery.

Bacillus amyloliquefaciens (= *Bacillus subtilis*) used in the biofungicide Serenade ASO

- The product is registered in the UK for the control of grey mould caused by *Botrytis cinerea* in protected strawberry and under permanent protection full enclosure on tomato, pepper and aubergine and lettuce. *B. subtilis* within Serenade ASO is reported also to have some efficacy against other bacteria and powdery mildew fungus when alternated with chemical control (note however that powdery mildew is not listed on the product label). More

information is needed on the product's efficacy against other pathogens and its use within integrated crop management. There is a need for products that control bacteria and so work with Serenade ASO in this area would be particularly useful.

- For successful germination and colonisation of leaves *B. subtilis* requires humidity of around 76 – 98% RH and an optimal temperature around 25°C, but there is a good survival rate on foliage for at least two weeks in the absence of a host.

- The product has a maximum UK dose rate of 8 L / ha for foliar application, which is applied with 200 to 1500 L / ha of water. Information is lacking on the concentration of active substance on the leaf surface (i.e. spores per cm^2 of leaf) required for efficacy across the range of crops, crop situations and pathogens. Information published in scientific papers and articles rarely provides the final viable spore concentration applied.
- No information was found on specific exposure times to UV radiation and the loss in efficacy. If there is likely to be significant loss of viability over a sunny day this should be made known so that applications can where possible be done on cloudy days. Information is needed on whether crops under particular tunnel plastics or glass may benefit from greater efficacy due to UV filtration than outdoor crops. This could also be important when comparing efficacy against pathogens that tend to colonise leaf undersides rather than upper surfaces.
- *B. subtilis* can produce biofilms (where bacterial cells stick to each other and cover a surface using a sticky extracellular biochemical), but it is unclear whether biofilms are produced on foliage and then whether these may help to protect the *B. subtilis* from unfavourable environmental conditions. The relative importance of lipopeptides (which can digest pathogen cell walls) known to be produced by strain QST 713 in the product is unknown. Lipopeptide activity could be likely to be less affected by environment extremes.

Clonostachys rosea / *Gliocladium catenulatum* used as the biofungicide Prestop.

- *G. catenulatum* within Prestop can be efficacious against *Botrytis* spp. on foliage, but more information exists on its benefit as a substrate drench against root pathogens.
- Targets for spray application given on the label are restricted to Botrytis, Didymella and Mycosphaerella. A wide range of crop hosts of Botrytis are listed on UK Extensions of Application for Use and work should be carried out and published on these.
- For successful germination and colonisation of leaves *G. catenulatum* requires high humidity of around 60 – 80% RH and an optimal temperature around 25°C, but there is a good survival rate on foliage for at least two weeks in the absence of a host.

- The product is used at a constant concentration, at 0.5% for both foliar and drench applications. Research is needed to investigate the optimal water volume for the application of the product on different target crops. The number of CFU / ml necessary for the most efficacious use of Prestop remains to be elucidated.
- Although some information exists on the factors which influence both efficacy and persistence of Prestop on foliage, quantitative information on the nature of this persistence is minimal. More evidence is needed on the rate of *G. catenulatum* decline once applied to the foliage of individual crop species.
- Much of the literature on the delivery of Prestop refers to soil drenches rather than foliar sprays. There is thus a lack of knowledge on what aspects of product application are critical to improve performance for this biopesticide.
- Studies on the effects of UV radiation have been done on the related species *G. roseum*, but no information was available from the product labels or public literature on the effects of solar radiation or UV interception on the persistence of Prestop on foliar tissues and thus warrants further research.

Ampelomyces quisqualis used as AQ10

- *A. quisqualis* within AQ10 can be efficacious across multiple species of powdery mildew when the correct conditions are met, with no parasitism reported of other fungal groups.
- For successful germination and parasitism of powdery mildew, spores of *A. quisqualis* need high humidity or moisture; with efficacy decreasing rapidly below an RH of 90-95% at the site of parasitism, an optimal temperature around 25°C and the presence of a host.
- The maximum efficacious dose which should be applied to plants is no higher than $1x10^6$ CFU / ml perhaps due to *A. quisqualis*' production of an unidentified self-inhibitor above this concentration.
- *A. quisqualis* has a long latent phase, and in the presence of powdery mildew takes between 5 and 10 days to invade powdery mildew colonies on foliar tissues and complete its life cycle within the fungal host.
- *A. quisqualis* is compatible with a large number of chemical fungicides able to control powdery mildew, making it suitable for use in programmes with alternating use.
- Though the UK Registration Report for AQ10 states that without its powdery mildew host, viability of *A. quisqualis* is rapidly lost e.g., within a few days, the number of days as well as the rate of decline is not defined for particular crop situations.
- Though the maximum effective dose is known, there is no consistent information on the minimum effective dose. No public data (e.g., Registration Reports) is available on the

minimum effective concentration of CFU / ml needed and this is important given the rapid decline in viable spore counts following application to foliage.

- The method of delivery of the product was given poor attention in the literature. In particular, there was limited detail available on parameters which can affect spray application of the product to foliage, such as nozzle type, droplet sizes, tank systems and operator pressure.
- There is conflicting evidence for any change in control with the addition of adjuvants. Further research is required using individual adjuvants to ascertain the nature of their activity: when, at what concentration and how they could be used with AQ10 to perhaps boost product efficacy of the product.
- Information is given on the AQ10 label of the different weight of product per hectare to be used for different height crops, but the instruction to apply it with sufficient water to ensure coverage of both leaf surfaces needs to be clarified, as there will be dilution of the product with increasing water volume. There was no AQ10 label guidance on water volumes, and further work is needed on individual crops across a series of growth stages to determine optimum water volumes for efficacious application of AQ10.

New insights into the effect of environmental factors on biopesticides

All microorganisms used in biopesticides are ectothermic, meaning their performance rate is determined by the temperature of their environment. It is important that biopesticide companies choose strains that are able to function well under the temperature conditions within the crop, while growers and agronomists need to be given reliable information about the thermal performance of the strains used in commercial products. If a biopesticide is developed from a strain that has been selected using unrealistic, room temperature conditions rather than the more demanding conditions that the agent is exposed to in the glasshouse or field, then the strain will not perform well in commercial practice.

We investigated the use of thermal equations to give new information on biopesticide biology. A thermal equation allows the performance of an ectothermic organism to be estimated for any temperature within the performance range using data generated in an experiment. We investigated 12 different equations applied to a data set of the effect of temperature on the colony growth, spore germination and infectivity levels of 14 different species / strains of entomopathogenic fungi (EPF) that were all pathogenic to caterpillar pests and which included strains used in commercial biopesticide products. The main findings were as follows:

- The model that we are recommending as the most suitable is the CTMI (Cardinal Temperature Model with Inflection): this gave consistently good fits for all variables studied

and, in contrast to some other thermal models, all its parameters have simple biological significance. We used the model to provide estimates of the minimum, optimum and maximum temperatures for colony extension, spore germination, insect mortality and also insect development rate.

- There was a large variation between the EPF strains in the minimum temperature for activity, with the minimum germination temperature varying by up to 16 ºC. The thermal tolerance range for growth and germination also varied according to fungal strain.
- The level of virulence of fungal strains could be explained using data on spore germination rate (this explained 76% of the variance in the virulence rate of strains, in multiple linear regression analysis). This indicates that fungal pathogens of the target pest that germinate quickly are likely to be more virulent than fungal pathogens that germinate slowly.
- The results also showed that rates of germination and virulence respond to temperature in a proportionate way. This could prove highly valuable in screening programmes done by biopesticide companies. Screening of candidate strains could be done by measuring in vitro germination rate at a range of environmentally relevant temperatures, which is relatively quick and easy to do, as opposed to measuring virulence to the target pest, which is harder and takes considerably more resources. We think this is going to be particularly valuable for identifying fungal strains that work at low temperatures, as these are likely to be rare and so a large number of candidate strains will have to be screened to find them.

The data also provided new information on the thermal biology of EPF products, identifying the temperature conditions at which they are likely to work best. As stated, low outdoor temperatures are an issue, particularly if using EPF in autumn and spring, and more work is needed to devise targeted application strategies to make best use of strains during windows of favourable conditions as part of IPM.

The strains tested were all typical in that activity dropped off rapidly at temperatures that were slightly greater than optimum. This is important for glasshouse crops: in hot summers, when glasshouse daytime temperatures will be high, it would be worth spraying products in the evening when conditions are cooler so that spores are applied under the best environmental conditions for germination.

In this research, we followed the convention of estimating the minimum and maximum temperatures for activity as cardinal points. However, for best practice, we think that biopesticide companies may be better off using a different measure of thermal performance range to provide more agronomically useful information to growers. If the minimum and maximum temperatures are quoted as the thermal limits, people may mistakenly believe that the biopesticide is active at these temperatures. Instead, it could be helpful to identify an

agronomically operative temperature range. For example, this could be the temperature range at which performance is no less than 50% of that at the optimum temperature.

A microbial biopesticide should not only work under the target environmental temperature range, but it should also have a thermal performance curve that matches, or overlaps, that of the target pest (which is also ectothermic). If the thermal performance curves are different, then there is likely to be set of temperatures at which the pest can feed, grow and reproduce but the biopesticide cannot control it. If the thermal performance curves match, however, then both pest and biopesticide will respond similarly to temperature changes. In theory, this means that successful levels of crop protection can still occur at suboptimal temperatures. For example, at low temperatures, while the speed of kill of the biopesticide will be reduced, provided the pest undergoes the same rate reduction in development, feeding and reproduction, then the total amount of pest control will be maintained, albeit at a slower rate. This is something that has not been explored in any detail and requires attention in the future.

A boxcar model to get new insights on biopesticide efficacy against peach-potato aphid

Understanding the optimal way to use biopesticides is crucial to maximising efficacy and minimising cost, compared with conventional pesticides. Unlike synthetic chemical pesticides, many biopesticides do not cause instantaneous death of their insect targets – instead they can take several days before death occurs. They can also have different levels of lethality to different pest life stage, for example eggs may be less vulnerable than the adult stage. As a result, the amount of pest control is affected by a range of features associated with pest biology; these include things like pest growth rate, reproduction, the relative susceptibilities of different instars to the biopesticide, and pest population size. There are also inherent features of the biopesticide that will determine its efficacy: speed of kill, lethal concentration, persistence on the leaf surface and so forth. Until now, these issues have not been considered in any detail when people are designing an IPM programme with biopesticides, but it is important that they are thought about.

It follows that the strategy for applying a biopesticide – i.e. the timing and frequency of spraying in relation to pest population size and growth rate – will have a profound effect on efficacy. Testing out the full range of possible application strategies in the glasshouse is prohibitively expensive. Instead, a mathematical model of pest population growth could be used to investigate different strategies and pick out those that are likely to be most effective. In this way, computer models could be used for rapidly testing many hypotheses to identify those

that should be further investigated in practical experiments, thus saving time and money on laboratory and field trials. This is a novel approach and to our knowledge has not been investigated for biopesticides before now.

In this part of AMBER, a computer model was developed that predicts how biopesticide application strategy affects the level of pest control. Separate models have been developed for whiteflies and for peach potato aphid, *Myzus persicae*. Here we are reporting about the work on *Myzus*. The model was developed to predict *Myzus persicae* population increase over time using published data on aphid development rates and their susceptibility to neem-based bioprotectants. The model was then used to test and predict the efficacy of different numbers of spray applications of azadirachtin (the active ingredient in neem). An experiment was then done to test the computer model predictions. This was done using Azatin sprayed on to pansy as a model crop in a glasshouse, to compare with results from an experiment in AHDB project CP 124 which included a different azadirachtin product not yet approved in the UK. As Azatin is approved in the UK for control of thrips on protected ornamentals, the experiment aimed to provide growers with immediately applicable results.

The main findings were as follows:

- The model predicted that to eradicate an initial pest population of two adult aphids per plant with the first application after one week, it would be necessary to apply four sprays of a neem based biopesticide at weekly intervals. This prediction was then tested out in an experiment. There was a significant reduction in numbers of nymphs and winged adults where Azatin had been applied at least once, and also a reduction in the number of wingless adults where Azatin had been applied twice, indicating that application of Azatin can be used to reduce numbers of aphids as part of an IPM programme. However, the Aztin application regime did not eradicate the aphid population as predicted by the model. This is probably because the aphid development rate observed on pansy was faster than the rate used in the model, which used data published in the scientific literature for aphids on sweet pepper.
- The effect of host plant on pest population growth is probably underappreciated in IPM and it may explain why a biopesticide works against a pest species on one crop but does not give adequate control when applied against the same pest, with the same application regime, on another crop species.
- Percentage nymph mortality observed in this experiment was similar to the model parameter of 50% nymph mortality, but this level of mortality was not observed until 20 days from the first application. This suggests that Azatin acted more slowly on the nymph population than predicted by the model.

A speed of kill experiment was then set up to compare two treatments; an untreated control and Azatin applied once seven days at 1.4 L / ha in 1000 L / ha water. Azatin caused a reduction in the number of aphid nymphs and wingless adults one day after application, while winged aphids were produced nine days post infestation. The model prediction for aphid growth matched the observed numbers of aphids in the untreated control until day 27.

Accuracy of a biological model depends on the quality of information on which the parameters are based. In this case, the model overestimated efficacy of azadirachtin against *M. persicae* when compared with experimental results. Efficacy data from this trial could be used to reprogramme the model and create predictions for another research question, such as how many applications of Azatin would be necessary to control a starting population of two *M. persicae* per plant with the first application after one day rather than after one week, as in this experiment. The model could then be validated and improved and extended to other crops, with additional information.

Azadirachtin was less effective against *M. persicae* in this experiment compared with a similar trial conducted in CP 124 MOPS using a different azadirachtin product that is not yet approved in the UK. Further work is needed to determine whether observed differences in efficacy are due to formulation of azadirachtin products.

Financial benefits

Biopesticides are generally less forgiving of environmental conditions that synthetic pesticides so understanding the optimal way to use them is really important to maximising efficacy and minimising cost.

There is an assumption that using the highest water volume within the label guidance gives better coverage (and hence efficacy) on the plant. However, as a general rule this is unlikely to be the case. The best strategy is to use a water volume that results in the highest concentration of product on the crop, taking into account the potential need for water to activate the product. For nominal constant dose applications, lower water volumes are likely to be better. These reduce waste and are quicker to apply, which will save money.

Using fast track systems to screen biopesticides for response to temperature, as shown here in AMBER, will lead to more effective biopesticide products, with cost savings for growers in terms of better pest control.

The literature review of microbial biofungicides done in this report gives growers and agronomists a summary of the current ‘state of the art’ of knowledge about the conditions and management practices required for successful use of these products. The new recording

template will enable growers to systematically collect the data needed to explain the performance levels obtained with biofungicides in commercial practice. This data is vital in situations where the biopesticide does not perform as expected, as it provides the evidence to help identify the problem and develop solutions to correct it.

The box car train model developed in AMBER is the first computer system in the world that predicts the effect of application strategy on bioinsecticide performance. The model still requires some additional work, as it has shown that plant species is likely to impact on biopesticide efficacy via effects on pest population growth rate, and hence data on pest growth rate is needed on different plant species. The big advantage of the model is that it allows rapid testing of different application scenarios to flag up the most promising options, which can then be tested in the laboratory or glasshouse. Attempting to investigate all components of a spray programme in a crop would be prohibitively expensive and time-consuming. At the moment, application strategies are often developed on a trial and error basis. The AMBER model has real potential to take the guess work out of spray programme development, leading to better performance of biopesticide products. Efficacy testing is also a significant fixed cost for biopesticide companies: if it can be reduced, this should result in making biopesticide products cheaper and more price competitive with other products.

SCIENCE SECTION

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 is now in place throughout Europe which requires farmers and growers to use 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 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 project focused on biopesticides for use in three crop sectors: protected edible crops (primarily salad crops such as pepper, cucumber and tomato, as well as protected herbs, and we also included some pilot investigations on mushroom crops; however the project did not include any work on protected soft fruit crops); 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:

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

Develop and demonstrate management practices that can improve biopesticide performance.

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 focussed 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 were 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.

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

2.2.1 Better delivery of biopesticides to the target: Investigation of the effect of varying the water volume of applied biopesticide on the control of a target organism

Introduction

AMBER research to improve biopesticide spray application has focused on exploring and optimising spray water volumes with a range of crop types. It is essential to identify the optimum volume range to be used before other methods for improving spray application can be investigated, such as new equipment or application techniques.

Biopesticide sprays are applied in one of two ways of two ways: (1) a constant dose model, in which the total amount of biopesticide applied per ha is set at a fixed amount and the volume of water used for spraying the product is chosen by the spray operator (provided that it stays within a range that has been set by the manufacturer and given on the label). In the constant dose system, the concentration of the product reduces as the water volume increases; (2) a constant concentration model – here, there is a minimum and maximum water volume specified on the label, and the spray operator chooses the water volume within these limits. In the constant concentration system, the total amount of product per ha will increase as water volume increases. Most biopesticides are used at a constant dose, although a few products are now on the market that are applied at a constant concentration.

The label recommendations for biopesticides often specify relatively high water volumes (up to 1500 L per ha), possibly because such labels need to cover a wide range of crop sizes and structures. Unfortunately, data supporting the recommended volumes does not appear to be available, which makes it difficult for the grower to choose the optimum volume for their crop. We have found that there can be a perception among growers that high water volumes give better coverage and penetration into the crop canopy. This is understandable - if you can physically see that the leaves are wet after spraying, this gives reassurance that the biopesticide has been applied. However, it is known from work with a wide range of crops, particularly in arable situations, that lower volumes result in higher quantities of active substance on the crop, when applied at nominal constant dose, and water volume has little effect on penetration. Low volumes are a more efficient method of transferring biopesticide to a crop and result in less waste (for example loss of product through run off on leaves). However, in the case of biopesticides, a minimum quantity of water may be needed to ensure it performs adequately (e.g., so that it does not dry out too quickly), but there is no information

available relating to this. Work in this area is technically difficult and requires specialised facilities, which may explain why it has been given insufficient attention prior to AMBER.

To date, we have undertaken studies in two areas: (1) Laboratory research with a track sprayer and tracer dye measured the relationship between applied volume and the quantity of spray deposited on plants for small pot-grown plants that are treated typically with a horizontal boom. (2) This was followed by research to explore the relationship between applied volume and the quantity of spray on plant for large plants with a vertical structure treated with a vertical boom. This involved an experiment on a glasshouse tomato crop grown to standard commercial specification combined with a re-analysis of previously obtained data for tomato.

Experiments with basil, as a representative short plant, sprayed with a three-nozzle horizontal boom, indicated that, where the biopesticide is applied at a constant dose, the maximum active substance will be applied using the lowest water volume providing that the maximum label concentration is not exceeded. Where biopesticide products are used at a constant concentration, the maximum volume that should be used is less than 1000 L/ha, and there are likely to be benefits for smaller plants of reducing this down to around 500 L/ha. This is considerably less than the upper water volume allowed for most biopesticides on the label (which is typically 1500 L/ha).

The research done using a vertical boom track sprayer within an experimental tomato crop, showed that the quantity of active substance deposited on the plant appeared to be relatively insensitive to volume, although the data was very variable making it difficult to draw conclusions. This may be due to the structure of the canopy, which is more complex than short, pot grown plants. The data indicates that the maximum volume that should be used is 1000 - 1500 L/ha *applied to the crop* (rather than calculated per unit floor area). For biopesticide products applied at a constant dose to tall crops such as tomato, water volume can be reduced from this maximum to suit other needs (such as using a lower water volume to reduce the time needed to spray the crop).

In this section, we report on an experiment to investigate the effect of spray volume on biopesticide efficacy against a target pest, done under controlled conditions. In this case, a fungal biopesticide (Botanigard) was sprayed onto small tomato plants with a horizontal boom, and then spider mites (*Tetranychus urticae*) were placed on tomato leaflets and their survival was monitored over time. The biopesticide was applied at a nominally constant dose in different water volumes from 250 L per ha to 1500 L per ha with the SSAU track sprayer, using conventional nozzles for a horizontal boom sprayer. In this system, the mixed concentration of the biopesticide in the tank mix decreases as the volume of water sprayed increases. Based on the earlier studies using a horizontal boom, we would expect a reduced amount of

biopesticide deposited per leaf area as volume increases. If biopesticide efficacy depends only on the quantity of spores on the leaf, we would therefore expect a reduction in efficacy with increasing volume. However, water volume could have other effects on efficacy – for example, as mentioned, a minimum amount of water may be needed for the activity of the biopesticide, and water volume can influence the way the spores are distributed over a surface.

Methods

The experiment used the biopesticide Botanigard (Certis UK) which is based on the insect pathogenic fungus *Beauveria bassiana* (GHA strain). Application used the manufacturer's recommendation for pepper crops, with a dose of 0.94 Kg/ha, a range of 500 – 1,500 L/ha water volume, a recommended 100 µm droplet size (medium), and incubation post spray at 70% RH at 20-30 °C. Tomato plants were grown at Wellesbourne for 6 weeks in a glasshouse and brought to SSAU for treatment (**Figure 1**). The plants were sprayed with one of five different volumes of either water alone or water plus Botanigard. Water volumes (L per ha) were as follows: 250 (= 3.76 g per L Botanigard), 500 (= 1.88 g per L), 750 (= 1.25 g per L), 1000 (= 0.94 g per L) and 1500 (= 0.63 g per L). The spray was delivered with a three-nozzle boom mounted on a track sprayer with FF110-02 nozzles at 2.5 - 3 bar pressure spraying downwards. Track sprayer speed was varied to give the different volume rates (see **Table 1**). Eight replicate plants were sprayed per treatment.

Figure 1. Example tomato plant brought to SSAU.

Samples of Botanigard suspension were prepared in containers and then placed in canisters for spraying. Sub-samples were collected from the containers at each of the five different

volume applications. The suspensions were diluted in sterile water, plated onto selective media (Sabourand Dextrose Agar + Rose Bengal (0.05 g / L) & chloramphenicol (0.1 g per L)

Table 1: Spray conditions used to apply Botanigard to tomato plants with a track sprayer

Albuz FF110-02 @ 3bar pressure					C	
Target volume (L/ha)	Speed (Km/ h) target	Speed (Km/ h) actual	Actual volume L/ha applied	Volume as L/m^2	Concentration of Botanigard (g/L)	Concentration of Botanigard (mg/m^2)
250	3.75	3.78	254	0.0254	3.70	0.37
500	1.9	1.92	500	0.05	1.88	0.19
750	1.275	1.26	762	0.0762	1.23	0.12
1000	0.965	0.965	995	0.0995	0.94	0.09
1500	0.655	0.655	1466	0.1466	0.64	0.06

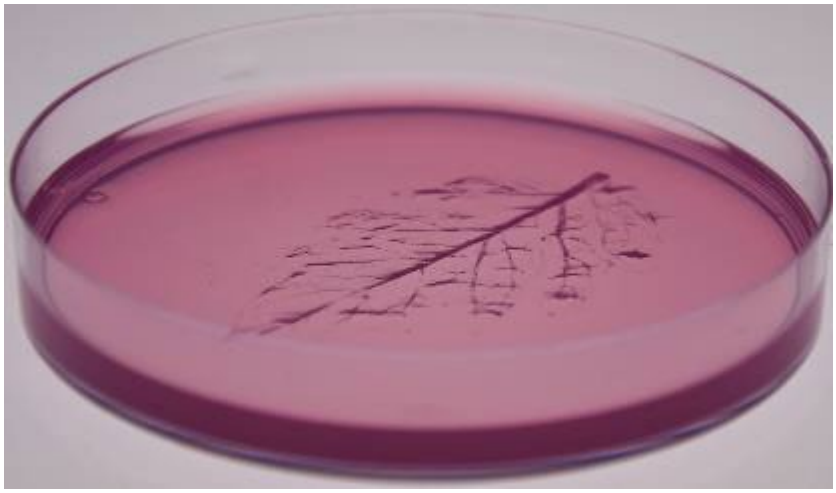

Figure 2. Leaf imprint on selective media before incubation

Oxoid UK, 10 plates per treatment). The plates were incubated at 20°C in darkness, and the number of colonies were counted after 4-5 days.

The distribution of spores on leaflets was measured from leaflets taken from the upper, middle and lower canopy from each plant after spraying (between 3 - 8 replicate leaflets depending on availability). This was done on two occasions, at one day and two days after spraying. The upper and lower leaflet surfaces were pressed onto selective media in petri dishes and CFUs were counted after 4 – 5 days incubation (**Figures 2 & 3**). Untreated controls were included to assess any background populations.

The effect of spray volume on Botanigard efficacy on tomato leaflets was evaluated in a laboratory bioassay. Spider mites (*T. urticae*) were cultured as a fixed age colony of adult females. Tomato leaflets that had been sprayed with Botanigard or water controls in the track sprayer were excised from plants within 24hrs of spraying and placed on damp filter paper, upper side facing upwards, within 9cm Petri dishes with gauze covered ventilation holes cut in the lid (one leaflet per petri dish). Five spider mites were added to each leaflet and the dishes sealed with Parafilm. The dishes were maintained in a controlled environment room at 20°C, 16:8 L:D. The number of alive and dead spider mites was assessed daily for seven days. Any dead spider mites were removed and incubated on damp filter paper within Petri dishes (20°C, darkness) for seven days and inspected for the presence of fungal mycelium on the cadavers. The presence of sporulating mycelium was taken as evidence of fungus-induced mortality. Assessments were done on 10 replicate leaflets per treatment (n = 50).

Results and Discussion

Colony forming units (CFUs) of *B. bassiana* grew on the selective media from all the sampled spray volume suspensions. The 1000 L / ha treatment had some bacterial contamination that prevented the colonies from being counted with accuracy and so we have excluded this treatment from data analysis. The measured concentration of *B. bassiana* spores ($\log_{10}$ CFUs per ml of suspension sampled from the spray reservoir) was significantly lower than the calculated concentration, and further work would be needed to explore where these losses occurred. There was a linear decrease in concentration with increasing water volume (Anova, $p < 0.01$).

When tomato leaflets were imprinted on the selective agar medium, colony forming units (CFUs) of *B. bassiana* grew and were visible after 4 – 5 days incubation (see **Figure 3**). In some cases, there was clustering of colonies at the edge of the upper side of the leaflets caused by draining of suspension across the leaf. This clustering occurred even at 250 L / ha – this is noteworthy, as we might expect this draining effect to occur only at high water volumes, which are at higher risk of run-off. Most biopesticides have contact activity and

getting an even coverage of a biopesticide over the leaf surface is often thought to be important for efficacy, although the exact effect will depend partly on the biopesticide mode of action and the biology of the target pest or disease. In the case of our experiment, the spider mites are mobile and they acquire fungal spores by walking over the leaf surface, and hence spore clumping may not be an issue provided of course that mites come into contact with the fungus by walking at the leaflet edge. However, for pests such as whitefly scales, which are immobile and require a 'direct hit' from a biopesticide, or for preventative biofungicides that work by outcompeting plant pathogens for space (e.g. Prestop, based on *Gliocladium*) then even coverage is likely to be of much higher importance.

With the exception of an application volume of 250 L / ha (where CFUs of *B. bassiana* on leaves were very low), the numbers of CFUs were higher on the upper leaflet surface than the leaflet underside (see **Figures 4 and 5,** data given as CFU counts per cm^2 of leaflet surface). CFUs on the underside of leaflets may have been deposited as a result of drainage of spore suspension to the leaflet edge and then running underneath, and hence this would be a case of indirect transfer rather than direct application. This is not unexpected because of the shielding effect of the upper leaf surface, but it does illustrate the challenges of applying an effective dose of a contact-acting biopesticide to the underside of leaves, and this is obviously going to be an issue for target pests and diseases that occupy the leaf underside.

The numbers of CFU deposited on the upper leaf surface (CFU per cm^2 leaflet surface) varied with increasing water volume (**Figures 4 and 5**) although there was no evidence of an effect of canopy position (Figure 4). A Quade nonparametric analysis of covariance, done on the data set for CFU distribution on upper leaflet surfaces sampled at 1 and 2 days after spraying, and which used spore concentration in the spray suspension as a covariate, indicated that water volume had a statistically significant effect on CFU distribution (F statistic 22.7, $p < 0.01$). In pairwise comparisons of groups (Quade nonparametric ancova), CFU numbers at 250 L / ha were lower than other treatments (df 25, $p < 0.01$), while CFU numbers at 500 L / ha were higher than 250 L / ha and 1500 L / ha (df 25, $p < 0.05$ in both cases) but not 750 L / ha (df 25, $p = 0.44$). There was no statistically significant difference in CFU numbers between 750 L / ha and 1500 L / ha (df 25, $p = 0.11$). We would, however, be cautious with the interpretation of these results because of the potential of the spore concentration to act as a confounding variable. The data in **Figure 5** indicated a trend of decreasing CFU counts on leaflets with increasing water volume from 500 L / ha to 1500 L / ha, which was supported by a linear regression analysis (Anova, $p < 0.001$).

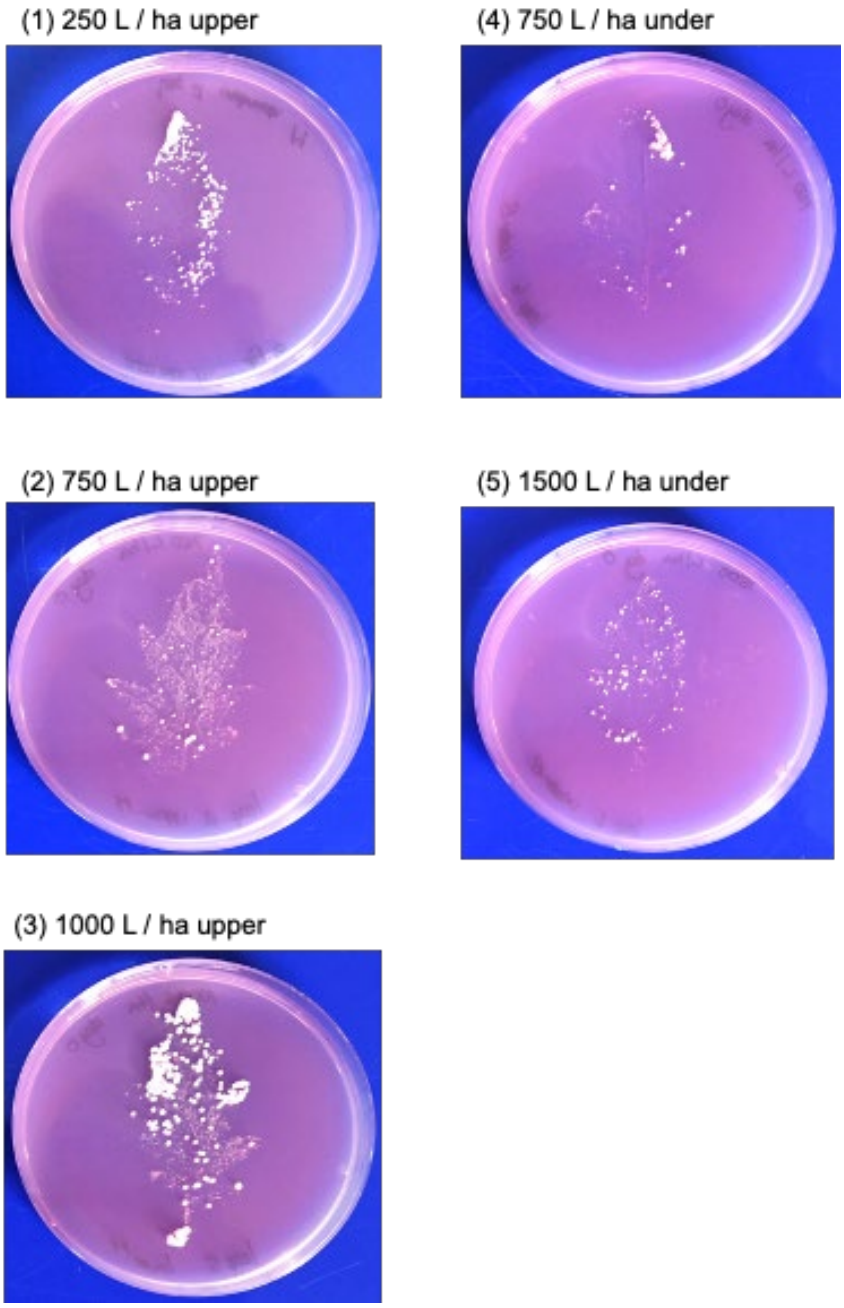

Figure 3. Example images of tomato leaflets sprayed with Botanigard and imprinted onto *B. bassiana*- selective agar. Plants were sprayed with different water volumes, and either the upper or under sides of leaflets were pressed on to the agar. Pictures (1) and (3) show clustering of colonies that have drained towards the edges of the leaflet. The deposition of fungal spores on the leaflet undersides (pictures (4) and (5)) were influenced by the upper surface application, which had run underneath at the leaf apex. This is seen clearly in picture (4).

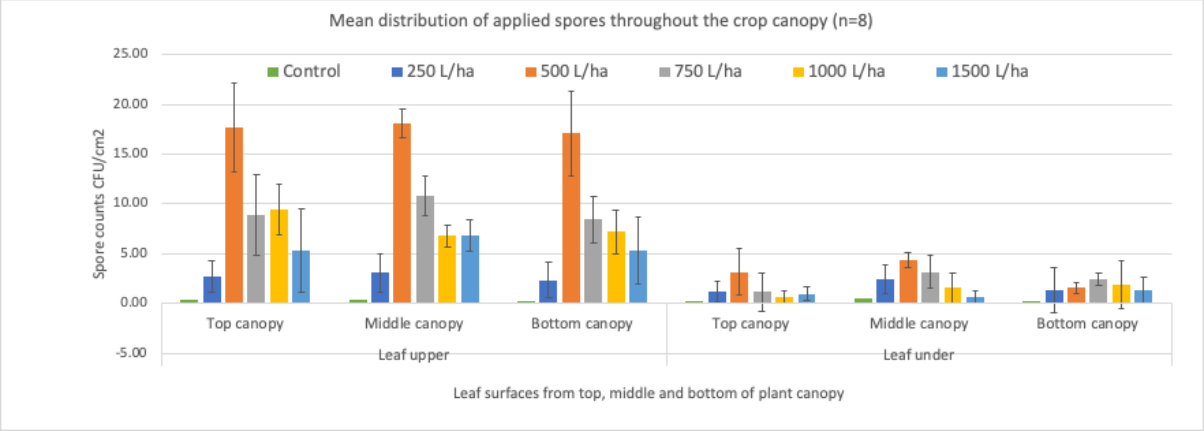

Figure 4. Mean distribution of *B. bassiana* (as CFUs per cm^2 of leaflet area) on upper and under sides of tomato leaflets sampled from the top, middle and bottom of the canopy after spraying with different water volumes at a constant nominal dose.

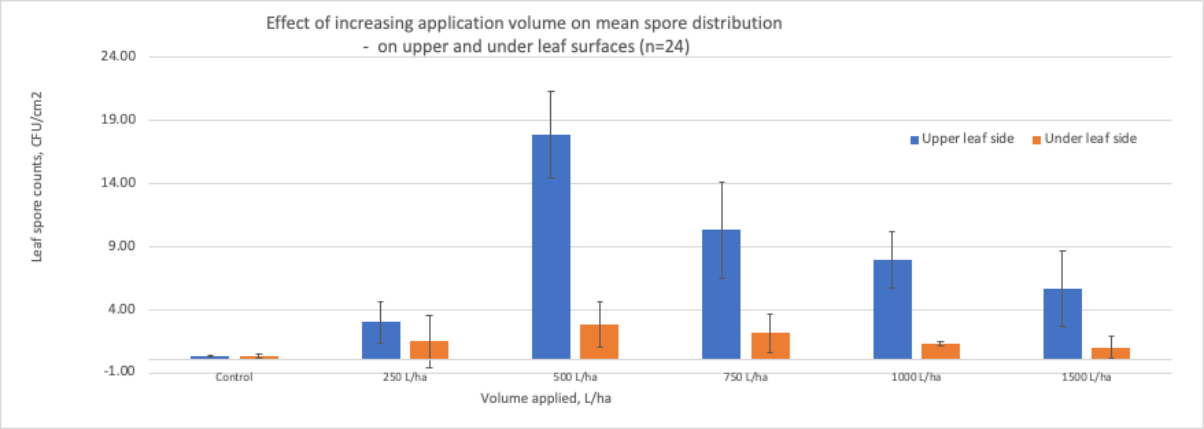

Figure 5. Effect of treatment on mean distribution of *B. bassiana* (as CFUs per cm^2 of leaflet area) on upper and under sides of tomato leaflets with data from the top, middle and bottom of the canopy combined.

In the spider mite bioassay, all of the applied water volumes of Botanigard caused significantly greater mortality of spider mites than the untreated controls ($p < 0.001$) (**Table 2**). After five days, all application volumes except 250 L / ha resulted in greater than 50% mortality of adult spider mites, and after seven days all application volumes except the untreated control resulted in 70% mortality or higher (**Table 2; Figure 6**). All of the application volumes tested produced conidia on spider mite cadavers.

There was no difference in the median survival times (MST) of spider mites treated with the different volumes of Botanigard, with the exception of the 500 L / ha treatment, in which MST

was significantly lower than for all other treatments ($p < 0.05$, **Table 3**). This is consistent with our finding that 500 L / ha produced a significantly higher concentration of CFUs on leaflets. This is also in keeping with well-established results with synthetic chemical pesticides on arable crops, in which lower water volumes result in higher quantities of active substance deposited on the crop when applied at nominal constant dose. It is not known why a water volume of 250 L / ha gave the lowest number of CFU per unit leaf area, and this requires further investigation. It is possible that the *B. bassiana* spores require a certain amount of water on the leaf to survive, or that 250 L / ha is too low to get adequate coverage on the leaf.

This experiment was technically difficult, and to our knowledge this is the first study of this type with a biopesticide. Because of the difficulties in quantifying the actual applied dose, the effect of water application volume on biopesticide efficacy cannot yet be determined unequivocally, but the ability to relate efficacy to the quantity of biopesticide active substance on the leaf surface is highly encouraging. Further work is needed to improve the reliability of the measurements, but the refinements required would be relatively straightforward. Overall, the results support work done previously in AMBER using tracer dyes, namely that for a biopesticide used at a nominal constant dose, it is better to use a lower water volume, as this is quicker and so more efficient to apply, provided it delivers the effective amount of biopesticide to the target.

Table 2: Mean % mortality of spider mites treated with Botanigard with increasing application volume. Values in parenthesis represent the standard error of the mean

Day	Treatment					
	control	250 L/ha* (3.0 CFU/ cm^2)**	500 L/ha (17.9 CFU / cm^2)	750 L/ha (10.3 CFU / cm^2)	1000 L/ha (7.9 CFU / cm^2)	1500L/ha (5.7 CFU / cm^2)
2	4.9 (3.54)	15.8 (4.45)	17.8 (6.34)	10.0 (4.47)	16.0 (6.53)	23.5 (8.50)
3	18.8 (7.04)	30.0 (6.16)	41.4 (7.01)	32.0 (6.11)	38.0 (4.67)	33.5 (6.99)
4	18.8 (7.04)	35.4 (7.12)	47.8 (7.18)	48.0 (8.54)	42.0 (4.67)	45.5 (7.09)
5	18.8 (7.04)	42.7 (6.65)	60.6 (8.51)	58.0 (7.57)	50.0 (5.37)	60.0 (7.89)
6	27.9 (10.53)	63.9 (8.93)	75.0 (9.57)	72.0 (6.80)	63.3 (5.38)	68.0 (7.42)
7	27.9 (10.53)	73.6 (8.57)	76.7 (9.72)	76.0 (7.18)	70.0 (5.38)	74.0 (7.33)

*water volume applied
**measured amount of Botanigard on upper side of leaflets, in CFU per cm^2 of leaflet surface area

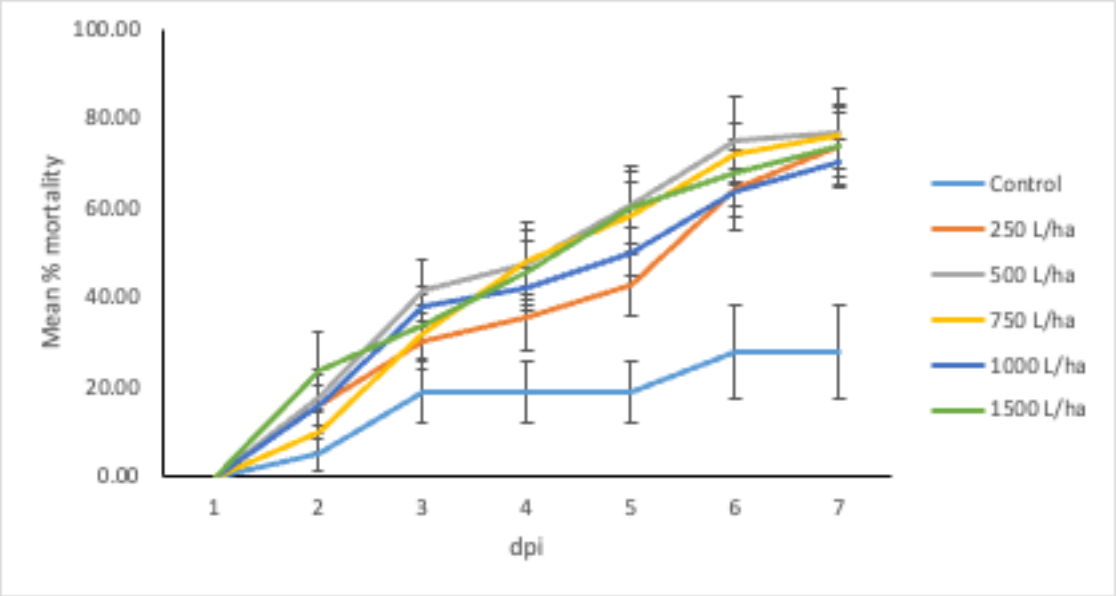

Figure 6: The cumulative daily mean % mortality of spider mites treated with Botanigard with treatment. Error bars represent the standard error of the mean.

Table 3: Survival analysis results of time-mortality responses of spider mites (N=50) treated with Botanigard at different water volumes and a nominal constant dose. Control mites were untreated.

Treatment	% mortality (7 dpi[a])	MST[b] (95% CI)	HR[c] (95% CI)	Z (HR)	P (HR)	df	n
				33.472	<0.001	5	50
Control	27.9	-	-	-	-		50
250 L/ha* (3.0 CFU/ cm^2)**	73.6	6 (5.2 - 6.7) f	3.64 (2.043 - 6.480)	19.25	<0.001	1	50
500 L/ha (17.9 CFU / cm^2)	76.7	3 (2.2 - 3.8) g	5.26 (2.970 - 9.297)	32.49	<0.001	1	50
750 L/ha (10.3 CFU / cm^2)	76	5 (4.0 - 6.0) f	3.42 (1.905 - 6.148)	16.94	<0.001	1	50
1000 L/ha (7.9 CFU / cm^2)	70	5 (3.7 - 6.30) f	2.92 (1.615 - 5.281)	12.58	<0.001	1	50
1500L/ha (5.7 CFU / cm^2)	74	5 (4.2- 5.8) f	3.42 (1.901 - 6.160)	16.82	<0.001	1	50

a dpi= days post inoculation

b MST=Median survival time, given in days. The median survival time (MST) gives the proportional cumulative survival of 50% of the populations. MST values followed by different lower-case letters (f or g) within the same column are significantly different (log rank $c2 \geq 11.070$, $p < 0.05$).

c The Hazard ratios (HR) indicate the relative average daily risk of death compared to the untreated control.

*water volume applied

**measured amount of Botanigard on upper side of leaflets, in CFU per cm2 of leaflet surface area

Objective 2 (continued): Develop and demonstrate management practices that can improve biopesticide performance.

2.2.3a Improved data recording in trials to identify biopesticide optimisation practices

Introduction

The aim of this work was to bring together published information on the effects of environmental conditions on the efficacy of microbial biopesticides used against plant diseases (i.e. biofungicides), and to use this to draw up a new data recording template for biopesticide trials. Information on the conditions in which microbial bioprotectants (aka biocontrol agents, BCAs) perform optimally is not readily available to growers. Although some indications may be given on product labels or associated technical notes, the greater the knowledge about any limitations to efficacy as a result of actions by those using the product the greater the benefits that will be obtained from use of these living organisms. Information is available across the peer review literature, project reports etc., but there is a need to bring it together into a single place for easy access.

The requirement of growers is to achieve the best efficacy of the products at any one application and to rely on the product being able to protect the crop from the pathogen each time it is used. Some of the principles for good disease management are the same for chemical plant protection products, such as timing in relation to disease appearance and good spray coverage. Such practices are even more important with bioprotectants as they need to be applied before the pathogen gets a hold and the organism needs to be placed wherever the pathogen is likely to colonise as microbial bioprotectants work by contact action. The major difference from chemical products is that the living bacteria or fungi in them have differing tolerances to environmental exposure and optimum conditions of temperature and humidity for their growth.

Methods

This section of the report thus reviews three of the first bioprotectants on the UK market with recommendations for foliar application to horticultural crops, Serenade ASO, Prestop and AQ10. The information from this review is then used to summarise the conditions in which the organism or the product has been shown to perform. A generic decision tree is provided for what to consider before, during and after using these products, alongside tables which specify the environmental parameters that need to be known. A recording sheet is provided to indicate the type of records that would be advantageous to keep, so that when product efficacy is either

good or poor they can be referred to and utilised for future applications. Finally, some examples of AHDB reports are given to show where conditions, or the pathogen severity, may have affected the level of control achieved from either of the three products.

Results

Reviews to gain an understanding how environmental factors can affect the efficacy and reliability of the bioprotectants Serenade ASO, Prestop and AQ10.

Proprietary biological control agents (BCAs) are playing an increasing role in the management of foliar plant pathogens of horticulture crops. In the UK in 2018, when these reviews were originally started, three products were on the market for foliar application:

- **Serenade ASO** (Bayer CropScience), based on the bacteria *Bacillus amyloliquefaciens* (until recently called *Bacillus subtilis*) strain QST 713, used particularly against *Botrytis* spp.

- **AQ10** (Biogard), based on the ascomycete fungus *Ampelomyces quisqualis* strain M-10, a hyperparasite of powdery mildews only.

- **Prestop** (Lallemand Plant Care / ICL) based on the ascomycete fungus *Clonostachys rosea* (until recently called *Gliocladium catenulatum*) strain J1446, used particularly against *Botrytis* spp. and root diseases.

These BCAs are all used according to a preventative strategy, i.e. the product is applied either before the plant has been infected by the target plant pathogen, or when it is present at very low disease levels. As a consequence, the effectiveness of each BCA is heavily dependent on the timing and frequency of application, and also on the length of time for which the BCA can persist in an active state on the plant surface in the absence of the target plant pathogen. In general, it can be expected that the activity of the BCA declines over time after it has been applied to the crop. Therefore, if the BCA is applied too far in advance, it may have lost activity by the time the plant has become infected with the target plant pathogen. The exact pattern of persistence varies according to the BCA species and strain, its mode of action, environmental conditions and other factors. It is important that crop protection practitioners understand the persistence characteristics of the different BCA products so that they can control their application timing and frequency as a way of maximising product effectiveness.

Aim of literature reviews

The aim of this study was to review the scientific literature on the persistence of three bioprotectants on aerial plant surfaces (leaves and stems) in order to help crop protection practitioners get the best out of this product. The principal objectives were: (i) to quantify broad patterns of persistence (ii) identify the factors that determine persistence; (iii) identify knowledge gaps.

Serenade ASO

Bacillus amyloliquefaciens / *Bacillus subtilis* strain QST 713

Bacillus amyloliquefaciens (until recently called *Bacillus subtilis*) strain QST 713 is the active component in the commercial biocontrol product Serenade ASO. This review was principally completed in 2017 before the name of the bacteria was changed on the label and so the literature search was carried out on *B. subtilis* (see Addendum). More recently, the 2020 EFSA review states that the taxonomy of the *Bacillus subtilis* group is dynamically changing due mainly to fast sequencing tools and so the species used in Serenade ASO has now been re-classified as *B. amyloliquefaciens* ssp. *plantarum* strain QST 713 with a further classification as *Bacillus velezensis* strain QST 713 being considered a synonymous designation. The earlier reports on experiments in AMBER all refer to the product as containing *B. subtilis* and so this species name has also been retained in the following review if given this name in the publications referenced. In 2021, the active ingredient was still named as *B. subtilis* on the UK HSE Pesticide database for Serenade ASO.

In December 2020, a European peer review of the pesticide risk assessment of the active substance *Bacillus amyloliquefaciens* strain QST 713 was completed (EFSA 2021). This made both information withheld in 2017 and new information publicly available and so this has been added into this review. A list of various product registration, risk assessments and decisions on crop trials evidence presented by applicants is provided in this report at the end of the main body of References for this product. Apart from minor additions or changes in phrasing, including acknowledgement of the new Latin species naming, most of the information on the *Bacillus* in Serenade ASO is repeated in the various reports down the years from that submitted by the original applicants, AgraQuest in 2001, with the risk management submission by Bayer Crop Science in 2020 referring to the AgraQuest Annex 1 inclusion documentation of 2007 as containing the latest information.

Product contents, taxonomy and mode of action of bacteria and pathogen host range

Serenade ASO is formulated as a suspension concentrate with 1.34% w/w *B. subtilis* QST 713 supplied at a minimum concentration of 1.05 x 10^{12} colony forming units / L including fermentation residues and water. According to the 2008 Registration Report, when the application rate for the product in the UK was proposed to be 10 L product per hectare this applies 139.6 g/ha active, which equates to a minimum of 1.04 x 10^{13} cfu/ha (i.e., whether 200 or 1500 L/ha is sprayed). The rate has now been reduced to 8 L / ha for all crops. However, the label says that according to crop density the water volume used can range from 200 L to 1000 L / ha on lettuce and strawberry and up to 1500 L/ha on tomato, pepper and aubergine. The label states that as the activity is by contact complete cover needs to be achieved. Onto seed potatoes pre-planting the product can be used undiluted at 1 L per tonne of potatoes or up to 2 L of water added to improve coverage.

The product is registered in the UK for the control of grey mould caused by *Botrytis cinerea* in protected strawberry and under permanent protection full enclosure on tomato, pepper and aubergine and lettuce. *Sclerotinia* spp. are also controlled in lettuce. *Helminthosporium solani* is controlled on potato tubers. The label also states limited effectiveness data indicate some control of bacterial leaf spots (*Pseudomonas syringae* and *Xanthomonas campestris*) (https://cropscience.bayer.co.uk/our-products/fungicides/serenade-aso/). There were 24 Extensions of Authorisation for Minor Use (EAMU) in September 2021, valid to October 2024, that cover most UK protected and outdoor crops and forestry, principally for foliar application against Botrytis but additionally for use as a drench against some soil borne pathogens. Product efficacy is stated on all EAMUs to be untested.

In the United States, the label for Serenade ASO, a liquid formulation with 1 x 10^{9} cfu/g, (1.34% w/w B. subtilis QST 713) also marketed by Bayer gives rates for use against a much wider variety of fungal, bacterial and oomycete plant pathogens on a wide range of different crops https://www.cropscience.bayer.us/products/fungicides/serenade-aso/labels-msds. The crops and diseases are also listed on the US Registration Action document (USEPA, 2006). These are not listed here, as in contrast to the EU and UK regulations, biofungicides in the USA do not require evidence of efficacy for mention on labels (D. Chandler, Warwick University pers. comm. January 2018). Efficacy experiments against pathogens other than Botrytis have been carried out (**Table 4**).

Serenade ASO is one of a number of *Bacillus*-based biofungicides. By 2017 at least 17 different *Bacillus* biofungicides had been marketed by nine different producers. Members of

B. subtilis sensu lato consist of Gram-positive endospore forming bacteria. The group consists of *B. subtilis sensu stricto* and a closely related sister species *B. amyloliquifaciens*. The phylogeny of the group has only recently been resolved using whole genome sequence data, with definition of a core set of genes that should ideally be found in any beneficial *Bacillus* strain (Magno-Perez-Bryan *et al*. 2015; Zhang *et al*. 2016). As a result, earlier papers may refer to these groups interchangeably. Information on strains of both species is available from *https://www.ncbi.nlm.nih.gov* the National Center for Biotechnology Information database.

Table 4. Comparison of literature on the use of *Bacillus subtilis* strains QST 713 and strains KTBS and BacB to control a number of foliar and soil-borne pathogens. The pathogen and crop being studied, crop growth stage, the spray equipment used, rate of application, frequency of application and final spore concentration of the isolate, plus the environmental conditions (temperature, environmental water and UV interception) under which *B. subtilis* was applied.

Author	Crop and pathogen	Spray equipment	Spore concentrations & application rates	Growth stage, application frequency	Temperature	Environmental Water	UV interception	Results
***Bacillus subtilis* strain QST 713**								
Abbasi & Weselowski (2015)	Tomato *Xanthomonas* spp.	Hand-held compressed-air sprayer at 30 ± 5 psi; adjustable cone nozzle	Serenade ASO applied at $1x10^8$ cfu/ml. In field applied at 4 L/ha. Under protection applied at 15-25 ml per plant depending on age	Applied at 5 to 12-day intervals, with 7 to 8 sprays applied each year. No information on growth stage.	No data provided for field conditions May- Sept in Canada	No data provided for field conditions.	No data provided for field conditions	Serenade ASO significantly reduced the severity of bacterial leaf spot in three of four years (28% - 43% reduction) but did not reduce bacterial spot incidence on fruit.
Becktell *et al*. (2005)	Petunia and tomato *Phytophthora infestans*	Hand-held, CO2-pressurized boom sprayer with a single XR11003VS flat fan nozzle	Rhapsody AS *B. subtilis* QST-713 (cfu/ml not given). Applied at 9.92 ml/L, spraying to run off	On 4 to 5-week-old plants of both species, applied twice at 7-day interval	Maintained between 25°C-30°C	Mean RH ranged 54-69% RH above canopy. Within canopy 76-98% RH	Sunlight plus 400W sodium lights 12h day :12h night	*B. subtilis* failed to suppress late blight on tomatoes (unsprayed tomato mean disease severity 84% at 7 DAI). Slightly suppressive on petunias (unsprayed petunia mean disease severity 23% at 7 DAI).
Keinath and DuBose (2004)	Watermelon *Podosphaera xanthii*	Sprayrite hydraulic boom sprayer with Teejet D4 nozzles. The sprayer delivered 698 kPa pressure, 636 L/ha	Serenade ASO applied at final concentration $1x10^8$ spores/ml. Application rate 4.5 kg/ha. Water volumes were stated.	Applied 7-8 times. 5 applications from 4 weeks after sowing. On detection of powdery mildew, 2-3 applications made	No data provided	No data provided	No data provided	Serenade ASO alone ineffective at preventing & managing powdery mildew infection. Serenade ASO alternated with azoxystrobin was best - increasing fruit weight compared with untreated when powdery mildew was severe.
Lahlali *et al*. (2012)	Oilseed rape *Plasmodiophora brassicae*	Soil drench	Serenade ASO was applied at a final concentration of ~ $5×10^7$ spores/ml, applied as a soil drench at 180 ml/pot.	Seedlings removed 7 & 14 days after sowing planted into non-infested substrate treated with Serenade ASO	No data provided	No data provided	No data provided	At both 7 and 14 DAS, Serenade ASO reduced the incidence of *P. brassicae* relative to the pathogen-inoculated untreated growing media

The EFSA 2020 review noted that this strain of *Bacillus amyloliquefaciens* was subsequently named as *Bacillus amyloliquefaciens* ssp. *plantarum* strain QST 713. However, based on more recent phylogenetic analysis from additional literature the RMS considered necessary a further reclassification as *Bacillus velezensis* strain QST 713. *Bacillus velezensis* and *Bacillus amyloliquefaciens* ssp. *plantarum* are considered synonymous taxonomic designations, and the peer reviewers favoured the latter designation.

B. subtilis sensu lato occurs naturally in a wide range of habitats and is common in soil. The species is comprised of an assemblage of different strains which are physiologically diverse and which show adaptations to different environments. Both *B. subtilis sensu stricto* and *B. amyloliquifaciens* include plant-associated strains with characteristics such as root colonisation, plant growth promotion and the production of plant growth hormones and antibiotics / antifungal compounds. Differences in the mode of action of different *B subtilis* biopesticide strains means that it may not be possible to extrapolate information from one strain to another. In this review, general trends about the mode of action of *B. subtilis* are given and reference is made to specific features of QST713 as appropriate and where information is available. *B. subtilis* and *B. amyloliquefaciens* have multiple modes of action, although the expression extent of the individual modes varies between strains according to their genomes (Earl *et al*., 2008; Niazi 2014). Strain QST713 is reported to function through a combination of antibiosis, competition (for space or nutrients), hyperparasitism and induction of host plant resistance (Bardin *et al*., 2015; Haidar *et al.,* 2016; Hinarejos *et al*., 2016; Lahlali *et al*., 2013 Mizumoto *et al.* 2007; Paulitz and Belanger, 2001; Romero *et al.,* 2004; Stein 2005).

The UK label in 2021 states “Serenade ASO prevents plant diseases by first creating a zone of inhibition on the leaf and preventing attachment and penetration of the pathogens. Biological compounds produced by *Bacillus amyloliquefaciens* (formerly *subtilis*) strain QST 713 act to destroy germ tubes and mycelia of pathogenic fungi by puncturing their cell membranes. By preventing spore germination and penetration into the plant, the infection is stopped and disease is prevented from spreading to the rest of the plant. Furthermore, compounds produced *Bacillus amyloliquefaciens* (formerly *subtilis*) strain QST 713 induce the systemic resistance response of the plant, indicated by enhanced peroxidase activity.”

Antibiosis is likely to be the main determinant of activity in QST713, and is achieved though the action of extracellular lipopeptides secreted by *B. subtilis* cells. These have a contact antifungal action and operate by causing disruption to the packing of fungal cell membrane lipids. QST713 produces three classes of lipopeptides (iturins, agrastatins, surfactins). These are deposited on the plant surface alongside the bacterial spores and prevent the germination

of fungal pathogen spores. The 2021 EFSA report says secondary metabolites detected in Serenade ASO (the cyclic lipopeptides; iturins, fengycins, surfactin, and polyketides; bacillaene, difficidin and ericins) may constitute part of the mode of action of *Bacillus amyloliquefaciens* strain QST 713.

Studies with other *B. subtilis* strains indicate that the lipopeptides are an important component of biofilm formation and will contribute to bacterial survival on the plant surface and also stimulate plant defence mechanisms (Ongena & Jacques 2007). The lipopeptides are secreted by the bacterium during factory fermentation and are formulated into Serenade ASO alongside bacterial spores. It is likely that they provide the initial fungicidal activity when sprayed onto the plant. The bacterial spores then germinate and form a biofilm on the leaf surface and compete for nutrients, water and sites on the leaf so reducing populations of other microbes. The 2021 EFSA review, undertaking a consumer risk assessment, determined that this could not be finalised because information is not available as to whether the range of metabolites that may be produced by *B. amyloliquefaciens* strain QST 713 are produced on plants under good agricultural practice (GAP) directed use and/or on the quantity of the metabolites on edible commodities at harvest.

Competition for resources is stated in the 2008 Registration Report by PSD to be principal among several ways *B. subtilis* reduces populations of pathogens. The report goes on to say there is also evidence that *B. subtilis* induces biochemical resistance mechanisms in the host plant. Reference was made to a study in which the application of Serenade ASO to sugar beet reduced disease by the bacteria *Erwinia carotovora* and resulted in “enhanced peroxidase activity being recorded in the plants, which initiates a series of biochemical events leading to processes such as lignification, cross-linking of cell wall proteins, wound healing, production of antimicrobial free radicals. Serenade also induced hydrolytic enzymes associated with direct anti-fungal activity. Further, *B. subtilis* produces secondary metabolites which bind onto bacterial membranes resulting in cell death.”

Surface cover of *B. subtilis* on foliage upon application

The UK label indicates that “Serenade ASO works preventatively and with a contact mode of action, so for optimum control it is vital to achieve complete cover when spraying. Direction is for application as a medium quality spray. Serenade ASO may be used up to a maximum of six foliar applications to the protected crops on the label, with a minimum of five days falling between applications of Serenade ASO”. To achieve coverage in crops such as tomato that grow tall and dense the water volume ranges from 200 to 1500 L/ha. The label says that for strawberries “the sprayer should be set up to spray the rows and target the flowers and fruitlets

to achieve complete coverage". However, in reality it is unlikely that complete coverage will be achieved around all sides of fruit clusters to combat external Botrytis nor inside flowers targeting the fruit receptacle pollination tubes where germinating Botrytis gains access.

Product persistence on foliage and frequency of application

The UK label for Serenade ASO recommends foliar application of the product up to every 7 days, or in rotation with another chemical. This maximum number of foliar applications per crop was originally on the UK label as up to 20, but this has since been reduced to six. In the 2008 Registration Report it was considered that since the product was also recommended for use in combination with other fungicides that in practice, by using alternation, this maximum of 20 was unlikely to be reached. Alternation in combination with the range of potential modes of action of Serenade ASO was considered to reduce the theoretically high resistance risk of multiple applications. It was stated that the nature of the product is that repeated applications will be necessary.

Long term persistence (over weeks or months) is not required for efficacy, but efficacy over a period of time from application at the approved rate could be expected to reduce if colonies die, or metabolites degrade, over time. Protection from pathogens arriving on the plant would then be lower the greater the interval from application before pathogen inoculum arrives, a situation accepted already for degradation of chemical fungicides. A key difference in persistence between BCAs and chemical fungicides will be if there is evidence that the biological control agent reproduces on the plant surface. The Pesticides Safety Directorate 2008 Registration Report for Serenade ASO (in a paragraph concerned with the fate in soil of succeeding crops rather than efficacy) seems to have conflicting statements about reproduction from the case that was made to them. They compare the dominant life stage of *B. subtilis* in soil, the dormant endospore, with the vegetative spores applied to the leaf surface that are rapidly degraded. They state "the leaf surface is a stressed environment with low water and nutrient levels and influenced by UV radiation. Therefore introduced *B. subtilis* cells are not expected to exceed the natural level after application due to the unfavourable conditions on leaf or fruit surface, including a lack of fresh organic matter. However, these unfavourable conditions do not impede the efficacy of *B. subtilis* since the preparation will be added several times in a spraying sequence and *B. subtilis* cells will reproduce as long as the pathogen and nutrient sources will be present." This indicates that *B. subtilis* could multiply if there are host exudates or nutrients such as pollen or aphid honeydew on the leaf – in effect if materials, such as sugars, as provided in agar culture plates are present, together with incubation conditions of warmth and humidity, then colonies will multiply. In the 2008 Registration Report microhabitats on the leaves such as various channels, wax platelets and

hairs are stated to provide shelter for microbes (both pathogens and beneficials), with the principal mode of action of competition for nutrients and water said to take place within the microhabitats. Organic materials are said to be leaked from germinating Botrytis spores and if these are utilised by other microbes the Botrytis germ tube can be prevented from growing.

Persistence is also a consideration for residues in the crop. The applicant (AgraQuest) did not present residue data to PSD in 2008. This was stated to be because the unfavourable environmental conditions prevailing on the leaf and fruit surfaces and the dependence of *B. subtilis* on organic matter supply for its growth make it unable to maintain persistence for long. Organic matter supply was stated to include the fungal pathogen. They referenced work showing the generally low population of saprophytic bacteria on leaf surfaces because of environmental conditions. There was however a mention that under nutrient shortage and environmental stress *B. subtilis* may form endospores. The Registration Report from Norway (Anon, 2016b) states that endospore survival in soil is likely for a few months, during which time a natural breakdown begins which gradually reduces the number of spores remaining. However, in a dry state endospores can remain viable for years.

The representative formulated product for the risk evaluation by EFSA (reported in 2020 and published in 2021) was 'Serenade ASO', a suspension concentrate (SC) containing 967 g/kg (minimum content 1.0 x 10^{12} cfu/kg, maximum content: 3.0 x 10^{13} cfu/kg) *B. amyloliquefaciens* strain QST 713. The representative uses were foliar applications by spraying up to a BBCH of 89 for the last treatment which represents the growth stage of ripe fruits at harvest on strawberries (maximally six applications with 2.52 x 10^{14} cfu/ha) outdoor, or on strawberries (6 x 3.15 x 10^{14} cfu/ha) in a greenhouse or on grapes (maximally nine applications with 2.52 x 10^{14} cfu/ha) outdoor. A preharvest interval was not reported for any of the representative uses. For *B. amyloliquefaciens* strain QST 713, studies on grapes and pepper leaves were provided to EFSA (reported in 2020) to demonstrate that viable counts were declining following application and did not multiply, however either crop and/or location and product formulation affected the decline. On grapes, it was demonstrated that following field application (up to 1.44 x 10^{14} cfu/ha), viable spore counts were declining (from initially around 9 x 10^{4} cfu/g grape berries to 7 x 10^{3} cfu/g) within 28 days. In an indoor study on pepper leaves, following foliar spray, it was demonstrated that viable counts declined within 21 days to around 1 % of the measured viable counts after treatment (from around 3.8 x 10^{5} cfu/g; AS formulation), however, for a wettable powder (WP) formulation viable counts did not decline below levels of 10^{5} CFU/g within 21 days (concentration after treatment: 4.7 x 10^{5} CFU/g). The product in use in the UK is a suspension concentrate (SC). The bacterial colonies could thus in some scenarios still be in high numbers on a crop after a month.

Experiments reported by Tut *et al*. (2021) on lettuce in a polytunnel and glasshouse to evaluate a PMAxx™-qPCR technique for showing temporal changes in viable populations utilised liquid cultures of *B. subtilis* QST 713 produced from Serenade ASO. The formulated product was found to contain qPCR inhibiting materials and additives that affected the standard curve for estimating the viable populations. The *B. subtilis* suspension was adjusted to obtain 8 log10 cfu/mL and sprayed to just before run off, with leaves then sampled on days 0 (1 h after BCA application), 2, 4, 6, 8 and 10 after spraying. Leaves were washed and the cell pellet kept for DNA extraction for qPCR. Viable population of *B. subtilis* declined in both tested environments, yet the pattern of decline was more rapid in the polytunnel than in the glasshouse (**Figure 7**). However, there was some spike in viable populations in the phyllosphere of the lettuce leaves on day 10. They stated that the reduction over eight days could have implications for the level of control achievable as BCAs such as Serenade ASO require a relatively high concentration of viable populations for efficacy. The temporal decrease in populations may thus influence the levels of control of fungal pathogens achieved.

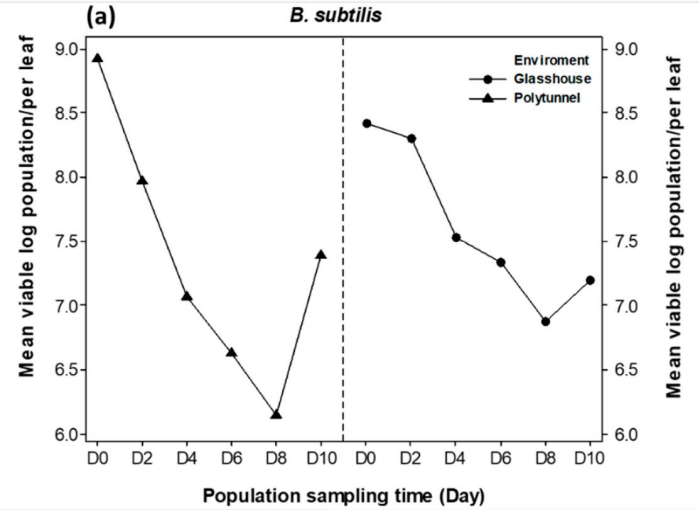

Figure 7. From Tut *et al*., (2021) showing temporal viable population of *B. subtilis* strain QST 713 applied at 8 $\log_{10}$ cfu/mL to lettuce grown in a glasshouse and polytunnel (S. errors <1.25).

The colonisation of sugar beet leaves by *B. subtilis* was investigated using a rifampicin resistant mutant (Rif+) of the *B. subtilis* isolate BacB and leaf washings and plate culture (Collins *et al*., 2003). It was applied at a concentration of $1x10^8$ cfu/ml to plants in the field in summer in the USA, but environmental conditions were not reported. The bacterial populations decreased rapidly in the days following application. Two separate experiments indicated an approximate 1000-fold decrease in the mean populations through the first 4 days. A third experiment showed a slower decline in the first few days but a more rapid decline after day 4**.** The population was around 100 cfu/cm2 after 14 days. A regression equation was produced. It would be possible to use the equation to model the population decline of the bacteria when

applied at a different concentration, however results from one strain cannot necessarily be applied to another.

Wei *et al.* (2016) separated the potential biocontrol activity of *B subtilis* and other BCAs into two components: (1) small scale colonisation of the area onto which the BCA has been sprayed and (2) spread away from this area to new parts of the plant or onto new plants entirely. The authors used Illumina MiSeq Next Generation Sequencing to quantify *B. subtilis* populations on protected vs open field strawberry plants at 4 hours and 8 days after spraying with Serenade ASO. Serenade ASO was applied to leaves at 1% (v/v) suspensions that contained bacterial cells at around 10^7 CFU ml^{-1}. There was no decline in the population on protected plants after 8 days. However, on the outdoor plants the number of *B. subtilis* reads declined by half and this was attributed to rainfall washing spores from leaves. The BCA did not spread to leaves that emerged after spraying, i.e., the bacteria did not spread away from the site of application. The environmental conditions for neither tunnel nor field were given.

In the AMBER project (Chandler *et al*., 2019), Serenade ASO was sprayed to the point of run-off onto young, healthy, tomato plants maintained in a controlled environment cabinet at a temperature of 25°C with a 16:8 light : dark cycle. The sprayed plants were placed in transparent plastic bags to maintain humidity around the foliage. Leaf discs were punched out and the spray deposit washed off at intervals after spraying of two hours, 1 day, 4 days, 10 days and 14 days, and viable colonies cultured on agar. The number of *B. subtilis* colonies recovered from leaves was similar for most of the leaf washings for 0, 1, 4 and 7 days after application, with the first washings calculated as having grown 21934 c.f.u. per cm^2 of leaf. The colonies on the 10 and 14 day washing plates could not be counted as a creamy biofilm layer was formed by the *B. subtilis* across the plates.

TaqMan-based qPCR assays for monitoring the populations of specific strains of *B. subtilis* have been developed for rapid, sensitive quantification and detection of specific strains of *B. subtilis* QST713 (Rotolo *et al*., 2016).

Serenade ASO utilises the action of its lipopeptides and so its persistence of activity will also depend largely on the ability of these compounds to remain active on the plant surface. However, research effort has mainly been directed at quantifying the viable bacteria colonies, with recent work producing a molecular method to replace plate counts (Tut, 2021), rather than studies on chemical activity either at application or following any production by the bacteria following application.

Efficacy against foliar diseases

Efficacy data was sought from the Registration Report for Serenade ASO held by the UK Pesticides Safety Directorate (PSD). However, although the November 2008 report included a section on efficacy and said results had been presented from various trials and formulations the data tables had been redacted. The assessment by PSD was given that "The data submitted from the UK trials provided evidence that Serenade ASO sufficiently reduced disease levels to result in reduced levels of fruit…". [the rest of the sentence was deleted]. "Assessing only incidence rather than severity can make interpretation of the degree of disease control difficult. This may account for why the standard product Rovral also only gave moderate results when effectiveness was assessed in this way. There was however a consistent trend of reducing the number of damaged fruit". However, PSD then states "The proposed label claim is for the control of Botrytis. This is not considered to be supported by the data, with no evidence for consistently high levels of control at the proposed rates. However, there is sufficient evidence to support a modified claim in relation both to control and reduction in fruit damage". The minimum effective dose was stated by PSD not to have been considered and the applicant was justifying a higher rate (not specified) for increased disease pressure that was not necessarily borne out by the results. The conclusion was that "overall, given the nature of the biological fungicide, accepted variability in control, and the additional evidence and argumentation provided, the proposed rate of 10 L /ha was considered justified".

Similarly, in an application for label listing in France of uses including Botrytis on cucumber and lettuce and powdery mildew on ornamentals (Anon., 2018), it was concluded "efficacy is considered variable and partial on all the requested uses. However, these levels of efficacy are considered acceptable for a product based on micro-organisms". In the EFSA 2021 review of Serenade ASO it was concluded that the representative uses of *B. amyloliquefaciens* strain QST 713 as a fungicide on field and protected strawberries and on field grapes, as proposed at EU level, result in a sufficient fungicidal efficacy against *Botrytis cinerea*. A 2009 Botrytis in strawberry factsheet by Fargo for Serenade ASO references a trial where Serenade ASO was sprayed at the then recommended rate of 10 L/ha on protected everbearer strawberry in 400 litres of water/ha with a total of six applications, made at seven-day intervals. This was stated to give a 50% reduction with a graph showing 4.9 diseased fruit rather than 10.5 in the untreated, but not what proportion these fruit counts were of the total yield.

Both chemical and microbial pesticides should be efficacious if the product is diluted by water at the volume per hectare given on the product label. Some chemical products, and microbial products such as Serenade ASO have a contact mode of action requiring complete spray coverage to give protection. The difference between chemical and microbial products is that if

spray coverage of larger canopies has to be achieved by using more water to dilute the product the chemical may still work at a lower concentration, but if microbes become spaced further apart the chance of them being where a pathogen spore will land is reduced.

Serenade ASO has the benefit of having lipopeptides in the bottles of product sold as well as colony forming units, but how diluted the lipopeptides can be and still be effective is an issue. According to a technical note produced in 2009 by Fargro for the use of Serenade ASO on strawberries, the strain QST713 was selected for use in Serenade ASO because it produces a wider range and greater volume of lipopeptides than other *B. subtilis* strains. The secondary metabolites iturins A, fengycins A and B, surfactin, bacillaene, difficidin plus ericin A and S have been detected in Serenade ASO and it is stated that these may constitute part of the mode of action (EFSA, 2021). It is thus possible that the number of colony forming units (cfu) required for good efficacy may be less than required by other strains. It should be noted that a minimum concentration of spores is given on the label, not a precise concentration, and that the label states that storage of the product above room temperature may reduce its shelf-life (although it is unclear if this is through spore death or chemical degradation).

The 2021 UK label for Serenade ASO recommends a dose of 8 L/ha and gives a water volume of a minimum 200 L/ha. However, with water volumes up to 1500 L/ha recommended (and no increase in dose rate) for bigger canopies the density of colonies on leaves will be greatly reduced. This might imply that the spore concentration from adding 8 L of Serenade ASO (containing 1.05×10^{12} spores / L) into a 200 L tank to spray a hectare of crop is much greater than necessary for control, although our work in AMBER on biopesticide application indicates that for products applied at a nominal constant dose, a good strategy is to use a lower water volume that is permissible within the label recommendations, as this gives a higher concentration of active per unit of leaf area. Another biofungicide, Prestop, instead uses a dilution rate of 0.5% (i.e. it is applied at a constant concentration) thus retaining the same density of colonies when more water is used within a hectare.

Sparse published literature was found that gave some record of the efficacy or otherwise of particular concentrations of either Serenade ASO or *B. subtilis* against diseases but did not include Botrytis control (**Table 4**). No studies tested a range of product dilutions under the same conditions. In the experiments reported the different spore concentrations and dose rates per hectare complicate efficacy comparison, and data conversion to spores per hectare is needed, but is not possible when the area sprayed with the given concentration is unreported. Even where hectarage is reported, the spray coverage on tissues may differ. Environmental conditions are not always presented in publications - Wei *et al*. (2016) did not present data but noted that several rainfall events occurred 24 h after application and probably

accounted for the 50% reduction in *B. subtilis* on leaves outdoors within eight days of Serenade ASO application.

Environmental conditions related to foliar application

Temperature

No temperature optima, or lethal temperatures are provided on the UK 2021 label nor in the 2008 PSD Registration Report. However, the 2021 EFSA review gave the growth temperature range of *B. amyloliquefaciens* strain QST 713 as between 15°C and 55°C and that it is able to grow at a pH in the range of 5.0–8.0.

A primary habitat of wild *B. subtilis* is the upper layer of the soil. Within this ecosystem, *B. subtilis* experiences a wide variety of environmental challenges and nutrient limitations that in extreme cases can induce the formation of a highly resistant endospore. Changes in temperature constitute a key factor that influences cell growth and survival in the soil. Under laboratory conditions, *B. subtilis* is able to sustain growth in a temperature range from approximately 11 °C to 52 °C. The standard temperature for laboratory culture is 35°C (Budde *et al*., 2006). In a study which examined the effect of soil temperatures on colonisation of sugar beet roots by *B. subtilis* and its biocontrol activity against *Pythium ultimatum*, it was found that population density and colonization down the root were increased in the 10 to 20°C regime compared with regimes with very low (3 to 12°C) and higher (15 to 25°C, 25 to 35°C) temperatures. However, this observation could not be reproduced when the experiment was repeated (Schmidt *et al.,* 2004). Conditions on foliage associated with higher temperatures such as sunlight and lower humidity are likely to reduce *B. subtilis* survival.

In AHDB Project CP140 (Tut, 2019) lettuce leaves were sprayed with an isolate of *B. subtilis* QST 713 and held in a controlled environment held at each of a range of temperatures; 10, 16, 22, 28 and 34°C. Viable population densities was assessed in leaf washings taken at intervals by using a molecular technique (PMAxx™-qPCR) and densities shown to be highest at temperatures of 15 to 25°C previously acknowledged to be optimum for establishment.

Environmental water and humidity

There is no mention on the UK 2020 Serenade ASO label of requiring humidity at or after application. In the Registration Report (2008) the only reference is to low water and nutrient levels on leaf surfaces creating a stressed environment. Microhabitats on leaves are said to be where both beneficial bacteria and pathogen spores will survive, rather than exposed on

the total leaf surface. Application giving good coverage can be expected to cause a transient rise in humidity. Prior to pesticide application to irrigated plants they are usually watered so that overhead irrigation can be withheld for a period. Spraying leaves with water after application would be inadvisable as evidence to the Report concerning residues says the layer built up of *B. subtilis* can easily be washed with water prior to consumption.

In the AHDB Project CP140 (Tut, 2019) experiment described above, at each temperature leaves were held at four different humidities, the humidity range starting lower and finishing lower the higher the temperature. At the lowest temperature, 10°C, the humidity ranged from 65% - 95% and at the highest, 34°C the range was 45% - 78% RH. An increase in *B. subtilis* population size occurred with increasing relative humidity, with the pattern most evident in the optimum growth temperature range of the bacteria. This supported the indications that a humidity range of 95-100% is required for population establishment in the phyllosphere.

In a growth chamber experiment a spontaneous, rifampicin resistant mutant (Rif+) of the *B. subtilis* isolate BacB was applied to sugar beet leaves at a concentration of $1x10^8$ cfu/ml (the standard concentration for application of Serenade ASO). Plants were exposed to 100% relative humidity by misting continuously for 48 hours with two ultrasonic humidifiers, then hourly during the night for an additional 48 hours. Despite the high humidity levels, there was an approximately 1000-fold decrease in mean populations through the first 4 days, followed by a stabilization of the population at around 100 cfu/cm^2 after 14 days (Collins *et al*., 2003).

In an efficacy experiment of Serenade ASO and other fungicides in controlling *Phytophthora infestans* blight infection of petunia and tomato, Becktell *et al.* (2005) raised glasshouse humidity by floor watering, using damp hay bales and pots of grass and running humidifiers to give good disease infection conditions, with temperature between 25 and 30°C. At night the plants were placed in plastic tents with humidifiers. Above the canopy, the average relative humidity ranged from 54 to 69%, whereas within the canopy it ranged from 76 to 98%. Plants were inoculated with *P. infestans* three days after receiving a second spray of the products seven days after the first. In tomatoes there was around 81% blight in the untreated seven days after inoculation, with 86% after Serenade ASO, in petunias there was some control by Serenade ASO with around 22% and 10% blight, respectively. Azoxystrobin use on other plants resulted in only a trace of blight on both hosts. It is likely the blight on tomato colonised quicker than the Bacillus could have had an effect as humidity was as high as possible for both hosts.

Pathogen spores such as botrytis and downy mildews and Phytophthora blights require high humidity to protect the germ tube from desiccation in the period before it enters the plant.

Therefore, if such conditions prevail then this is when biofungicide protection should be given and consequently they will benefit from the conditions. In-crop humidity and leaf temperature loggers that connect to decision support systems are now available so that growers can see if condensation is occurring on leaves or humidity is rising and so start ventilation or heating.

Humidity can be important other than for preventing bacteria desiccation. Spray application of *B. amyloliquefaciens* to wheat with commercial spray equipment showed that there were large, unprotected areas on the crop, and that high humidity was required for the antifungal compounds to diffuse across the leaf surface (Crane & Bergstrom, 2014).

Spray application at the higher suggested label water volumes of between 200 to 1000 L/ha (or 1500 for taller protected edibles) should provide leaf coverage by the product (at least on upper leaf surfaces) and some initial humidity. Information on the possibility of using ultra low volume application of Serenade ASO is lacking, although Fargro, a previous UK supplier of Serenade ASO, used to have a website note recommending the Nightstar Typhoon nozzle as it "generates 'tight' efficacious droplet sizes and allows difficult formulations to flow through, removing blockage problems and creating an even distribution".

Work comparing open field and protected strawberry plants sprayed with *B. subtilis* and its quantification using next generation molecular sequencing showed that its abundance reduced by around 50% in the open field over eight days. Under protection the abundance was unchanged. The difference was attributed to rain 24 hours after product application in the field (before day 8) washing the bacteria off. Rain splash was also attributed with the finding of the bacteria on unsprayed new leaves, whereas under protection only a small amount was detected perhaps following arrival by physical contact (Wei *et al.,* 2016).

UV interception

No specific information was found on the effect of UV-interception levels on the persistence of *B. subtilis* on foliage. Solar radiation has been reported to significantly reduce the effectiveness of another biocontrol agent, *Bacillus thuringiensis* var. *israelensis* against mosquito (Lacey, 2007). The influence of UV radiation was included in the unfavourable environmental conditions that contribute to the rapid degradation of vegetative spores of *B. subtilis* on leaves (PSD, 2008) and the 2020 EFSA review stated that *Bacillus amyloliquefaciens* strain QST 713 is sensitive to ultra-violet light.

Storage of product

The label for Serenade ASO indicates that the product can be stored at room temperature for two years, while storage at higher temperatures will reduce its shelf life. Information on

Serenade ASO storage at 40°C for eight weeks, storage at 0°C for seven days and shelf life at 25°C in 125 ml HDPE containers in the dark was presented to PSD in 2008 but has been redacted in the Report obtained from the Directorate by ADAS. The 2021 EFSA report states the supported shelf-life of the product is 2 years at 20°C in the original packaging (high-density polyethylene; HDPE).

Evidence from the literature provides us with some information on the spore survival of *B. subtilis* and the rate of its decline over time. *Bacillus* species are able to produce spores that allow them to resist adverse environmental conditions and permit easy formulation and storage of the commercial products (Epstein *et al*., 2011). The endospore plays a dominant role in the biology and the life-cycle of *B. subtilis*. It is a dormant structure which enables the micro-organism to survive when environmental conditions turn unfavourable for vegetative growth and is a vehicle for dispersal by dust and air streams, as it is easily blown up. The endospore is the most heat tolerant bacterial life-form, enduring temperatures >80°C (McKenney *et al*., 2013). In a dry state, endospores can remain viable for several years. At room temperature, Serenade ASO is stable for 3 years. Storage at 40°C for 8 weeks has been determined not to alter physico-chemical and technical properties of this product (European Commission, 2006).

Compatibility and alternation with other chemicals and products to improve efficacy

The marketing company of Serenade ASO (Bayer) provides a tank-mix sheet via their website. However, this is stated to be a sheet showing physical compatibilities and that there has been no check on the efficacy of the individual components or for any phytotoxicity. Ten fungicides and eight insecticides and a nematicide are named. They also state that Serenade ASO is physically incompatible with fosetyl-aluminium containing products. Fungicide products are produced to be active against fungi and fungicide actives with efficacy against pathogenic bacteria are rare.

Abbasi and Weselowski (2015) found that across three years of trials, Serenade ASO alone reduced the severity of bacterial spot, *Xanthomonas* spp., on tomato foliage relative to a water control in three of four years (with reductions ranging from 28% to 43%) but in tank mix with copper hydroxide reduced disease severity on foliage in all four years. Tank mixing may not be useful because one or other sole component worked in those years - copper hydroxide alone reduced the severity of bacterial spot on tomato foliage in three of the years. Infection incidence on fruit was not significantly reduced by Serenade ASO alone and the tank mix only gave benefit in one year at the same incidence as the copper alone and was still above 50%.

Keinath and DuBose (2004) found that Serenade ASO was ineffective at preventing and managing powdery mildew infection of watermelon (caused by *Podosphaera xanthii*) when applied alone but when alternated with azoxystrobin was significantly more effective. Similar results were also reported in fungicide trials for the control of powdery mildew in other cucurbit species (Dhatt *et al*., 2001, Langston and Kelley, 2002), However, it was suggested by Keinath and DuBose (2004) that azoxystrobin was likely to be providing most of the powdery mildew control when it was alternated with *B. subtilis*. Sawant *et al*. (2011) studied the efficacy of Milastin K, a commercial formulation containing more than 10^9 cfu ml^{-1} of another strain of *B. subtilis* (KTBS) in controlling powdery mildew (*Erysiphe necator*) on grapevines. Under low to moderate disease pressure conditions, Milastin K appeared as promising as sulphur, but under high disease pressure conditions, Milastin K alone was not as effective. Its efficacy was enhanced when used in integration with flusilazole (Flusilazole 40EC + Milastin K, 0.125 ml +2.0 ml, respectively).

Key Points and Knowledge Gaps

- *B. subtilis* within Serenade ASO was reported to have some efficacy against other bacteria and, particularly when alternated with chemical control, powdery mildew fungus. However, the control of *Botrytis* spp. on foliage is the pathogen stated on the UK label, with limited activity claimed against bacteria. More information is needed on the product's efficacy against various pathogens, and pathogen levels and its use within integrated crop management. There is a need for products that control bacteria and so work with Serenade ASO in this area would be particularly useful.
- For successful germination and colonisation of leaves *B. subtilis* requires moderately high humidity or moisture of around 76 – 98% RH and an optimal temperature around 25°C, but there is a good survival rate on foliage for at least two weeks in the absence of a host.
- The product currently has a maximum UK dose rate of 8 L/ha for foliar application. However, this can be applied using between 200 to 1000 L/ha of water to achieve complete crop cover. This means that the number of colonies present per unit area of leaf surface will be much lower at the higher water volumes. Information is lacking on the colony population range required for efficacy across the range of crops, crop situations and pathogens. Information published rarely provides the final viable spore concentration applied; providing the dilution factor is not sufficient as the product just declares a minimum colony content.
- Although some information exists on the factors which influence both efficacy and persistence of Serenade ASO on foliage, quantitative information on the nature of this

persistence is minimal. It is unclear whether endospores, produced in unfavourable situations, are produced on foliage and can play a part in extended efficacy of the product when conditions improve.

- *B. subtilis* can produce biofilms, but it is unclear whether biofilms are produced on foliage and then whether these may help to protect the *B. subtilis* from unfavourable environmental conditions or chemicals and the lipopeptides assist in efficacy. The relative importance of lipopeptides (which can digest pathogen cell walls) known to be produced by strain QST 713 in the product as against the viable colonies is unknown. Lipopeptide activity could be likely to be less affected by environment extremes.
- There is poor understanding of the physics of spray application of Serenade ASO e.g., the pressures, nozzle types and droplet size required to achieve optimum coverage. For crops with overlapping leaves, where good coverage is needed to ensure the product lands with where the pathogen will colonise, then efficacy would be increased by improved application techniques. Application to leaf undersides is a challenge.
- The very high water volumes which can be used for application of Serenade ASO combined with wide plant spacing necessary for particular crops, means significant spray waste. Further investigation should look at optimising the level of coverage achieved using lower spray volumes and more efficient methods of delivery.
- No information was found on specific exposure times to UV radiation and the loss in efficacy. If there is likely to be significant loss of viability over a sunny day this should be made known so that applications can where possible be done on cloudy days. Information is needed on whether crops under particular tunnel plastics or glass may benefit from greater efficacy due to UV filtration than outdoor crops. This could also be important when comparing efficacy against pathogens that tend to colonise leaf undersides rather than upper surfaces.

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Registration Report Part B Section 5: Fate and Behavior in the environment

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Addendum

Search term logic and outputs for searches of Web of Science related to Serenade ASO and *Bacillus subtilis* QST 713 made up to October 2017. Publication types searched for included articles, reviews, proceedings papers, book chapters, editorial material and meeting abstracts. Further references, in particular the Registration Reports listed above, were reviewed to December 2021.

Search term	Results
Environment *Bacillus subtilis*	2858
Spore survival *Bacillus subtilis*	436
Persistence *Bacillus subtilis*	338
Population dynamics *Bacillus subtilis*	243
Temperature sensitivity *Bacillus subtilis*	170
Humidity *Bacillus subtilis*	141
UV sensitivity *Bacillus subtilis*	94
Foliar application *Bacillus subtilis*	57
Foliar persistence *Bacillus subtilis*	0
Bacillus subtilis QST 713	28
Serenade ASO	6

Prestop

***Clonostachys rosea* / *Gliocladium catenulatum* strain J1446.**

Approach to literature search

A Web of Science search conducted in July 2017 for literature containing the search term 'Prestop' returned 28 results, while a search for '*Gliocladium catenulatum*' returned 56 results. Other search terms used to find relevant literature are detailed in the Addendum at the end of this product section. Practical studies on *G. catenulatum* (as a strain in its own right or formulated as Prestop), particularly in the field or glasshouse, were limited. A risk assessment review on biopesticides by Mudgal *et al.* (2013) focused on scientific literature relevant for the evaluation of environmental hazards and risks posed by biopesticides, including influences on persistence of Prestop in the environment and effects of non-target organisms.

Taxonomy, mode of action and host range

The strain J1446 of *G. catenulatum* strain used in the commercial biocontrol product Prestop was originally isolated from Finnish field soil. It is a saprophytic, actively sporulating fungus, which forms aerial mycelium with conidiophores bearing asexual phialospores. The spores are widely dispersed and germinate in favourable conditions to produce new mycelium (McQuilken *et al*., 2001). Asexual chlamydospores with relatively thick cell walls, which help the fungus to survive over less favourable periods, may be present in the mycelium. (Anon, 2004). The mode of action is hyperparasitism, production of lytic enzymes and competition for living space and nutrients (McQuilken *et al.,* 2001, Utkhede & Mathur, 2002, Utkhede & Mathur, 2006). Prestop contains a nominal 2 x 10^8 cfu/g of *G. catenulatum* strain J1446 in a 32% w/w wettable powder and is registered in the UK for the control of *Didymella* spp., *Botrytis* spp., *Pythium*, *Phytophthora* and *Fusarium* spp. on protected crops and outdoor strawberry.

Literature searching using Google Scholar found little information relating to *G. catenulatum*. A second search for the *Gliocladium* species showed up many more results, particularly research conducted on *Gliocladium roseum*, a closely related species to *G. catenulatum* (Schroers *et al.,* 1999). It was found that most of this literature did not refer to *Gliocladium roseum*, but to the officially designated sexual name of the fungus, *Clonostachys rosea* **(Table 5).** The synonym for *G. catenulatum* is *Clonostachys rosea* f. *catenulata* (Paavanen-Huhtala *et al.,* 2000). The literature was not clear whether the sexual or asexual forms of the fungus were used in experiments. Often, the sexual name of a fungus is used in publications to be scientifically correct, even though the asexual phase may be being studied.

For clear reading, throughout this review and to differentiate between the two species of *Gliocladium* (*roseum* and *rosea*) discussed, in scientific papers where the sexual form of the fungus; *Clonostachys rosea* f. *catenulata* has been referenced, the organism name has been altered to the asexual form of the fungus *Gliocladium catenulatum* (**Table 5).**

Table 5. Names of the stages of *G. catenulatum*, the fungus used in the commercial formulation of Prestop and a related species referenced in the literature, *G. roseum*.

Asexual stage	Sexual stage
Gliocladium catenulatum	*Clonostachys rosea* f. *catenulata*
Gliocladium roseum	*Clonostachys rosea*

In contrast to earlier Registration Reports, a recent Registration Report names *C. rosea* strain J1446 as the active substance in Prestop (Anon, 2020).

Factors affecting persistence and efficacy of the product

Colonisation of foliage by Gliocladium after application

Application of Prestop as an aqueous suspension is through mixing the powder with the growing media, drenching, spraying or seed dressing, at final concentrations of 0.1–2.0%, from 1–12 applications per season depending on the application method (Appendix 1). Superficial development of *G. roseum* on raspberry foliage was studied by Sutton *et al*. (1997), under continuous high humidity at 21 to 23°C. Conidial germination was seen from 4 hours and abundant conidiophores and new conidia were produced up to 72 hours after inoculation (**Table 6).**

Table 6. Time course of germination of *G. roseum* conidia and infection of raspberry foliage, from 4 hours up to 72 hours after inoculation (Sutton *et al*., 1997).

Hours after inoculation	Developmental stage of *Gliocladium roseum* on leaf tissue.
4 – 12	70 to 90% of conidia had germinated and produced narrow (1 to 1.5 µm diameter) germ tubes.
12 – 24	Germ tubes had elongated slowly.
After 16	Short branches of 1 to 5 µm in length had developed on germ tubes and hyphae and penetrated host tissues directly.
32 – 72	Verticillate and penicillate conidiophores developed from hyphae on leaves.
40 – 72	Abundant conidia were produced.

A study conducted by Chatterton *et al.* (2008) to investigate the ecological requirements for the colonisation of geranium leaves by *G. catenulatum* and to characterise the extent of this colonisation found that population levels of *G. catenulatum* were highest on senescent leaves and stems, followed by fully expanded leaves, and lowest on newly emerged leaves of both cultivars. A later study conducted by Chatterton and Punja (2011) which investigated natural colonization of cucumber plants by *G. catenulatum* found that 60 days after treatment of cucumber seeds with Prestop, the crown area, shoot meristem and emerging true leaves were colonized by *G. catenulatum. G. catenulatum* was associated externally with trichomes on the stem and appeared to form a network of hyphae over the epidermis of true leaves. This work provides preliminary evidence that *G. catenulatum* can survive and persist on foliage in the absence of a target pathogen to parasitise and feed on. However, further research is required to investigate the exact nature of this persistence.

Viability on the leaf surface over time

In the current AMBER project, work in 2018 using tomato plants in a controlled environment cabinet at 25°C, 95% RH and 16 h light : 8h dark, showed Day 4 viable spore counts from leaf disc washings had risen from that on Prestop inoculation day, rose again by Day 7 and were still at that level on Day 10 and Day 14.

In work within AHDB Project CP140 an alternative technique to plate counts of viable colonies washed off treated leaves was developed; PMAxx^{TM}-qPCR, calibrated against counts. This was used to show that, when lettuce leaves sprayed with Prestop were removed from plants and spores washed off at intervals up to eight days after spraying, the population remained similar to that on inoculation day. There had been a fall by the tenth day. The pattern and population was similar whether the plants had been grown in a polytunnel or a glasshouse, but the conditions were not stated (Tut, 2021).

Low volume (LV) spraying

The product suppliers, Lallemand, provide details of trials conducted in Holland in 2011 and 2012, where LV spraying of Prestop as a fine fog was performed in a commercial tomato crop (Lallemand, 2012). Spraying was done at a pressure of 6 bars with the solution pumped through a filter nozzle combination. Excellent colonisation of *G. catenulatum* on foliage was observed after LV spraying, using a *G. catenulatum* colonisation index of 0-3, the average colonisation score was 2.8 and there was no observation of Botrytis, either *in vitro* or in the greenhouse. Details of how the average colonisation score was calculated was not provided.

Crop growth stage, schedule and frequency of application

The UK label recommends that Prestop is used preventatively, to allow *G. catenulatum* enough time to colonize plant roots or foliage prior to pathogen infection. The first treatment of Prestop to tomato, pepper and cucumber should be soon after transplanting or at the latest, immediately after de-leafing, with re-application every 3 to 4 weeks at a minimum three week interval. The U.S. Environmental Protection Agency's technical document for Prestop recommends repeat foliar sprays every 3 – 6 weeks if necessary (Anon, 2012).

Efficacy and effective concentration

Both the UK and Finnish Prestop labels recommend application of the product suspension to foliage of protected edibles at a rate of 0.5% (500 g/100 l of water). A recommended final spore concentration is not given on either of the product labels.

Sutton *et al*. (1997) suggested that the ideal inoculum concentration of *G. roseum* to control *B. cinerea* varies with the plant species, the type and age of plant tissues, pathogen concentration, microclimatic conditions, and other factors. However, inoculum concentrations of 10^6–10^8 conidia ml^{-1} of *G. roseum* normally provided good control of *B. cinerea* in field crops and in the greenhouse (Sutton *et al*., 1997). In an experiment to assess the ability of different biocontrol agents to inhibit *Botrytis aclada* on onion leaf tissue, Yohalem *et al*. (2004) found that *G. roseum* isolate 201 significantly reduced sporulation of *B. aclada* after 4 and 8 days, but not after 12 days. There was almost no *B. aclada* sporulation 4 days after inoculation. Though Prestop contains a related organism, *G. roseum* f. *catenulatum*, this study provides evidence of the antagonistic effect of the *Gliocladium* spp against *B. aclada in planta*.

Table 7 and **Table 8** provide a comparison of the published literature on effective concentrations of both *G. catenulatum* and the related organism *G. rosea* to control a variety of foliar and root based diseases. Some of the experiments were soil drench studies (Lahlali and Peng, 2014, Rose *et al*., 2003), but Morandi *et al*. (2008) used a detached leaf assay to assess the ability of *G. roseum* to suppress *B. cinerea* sporulation on rose debris and Yohalem *et al*. (2004) used young onion plants to assess the ability of different biocontrol agents, including *G. roseum* isolate 201 to inhibit *B. aclada*. It can be deduced that the effective concentration for foliar application of *G. catenulatum* is between 1 x 10^5 (Yohalem *et al*., 2004) to 1x10^7 (Costa *et al.,* 2012, Morandi *et al.*, 2008) spores/ml. The results of the present literature indicates that further study needs to be carried out to identify the optimum, efficacious spore concentration for Prestop and its biological agent *G. catenulatum* when used as a foliar application.

Table 7. A comparison of literature on *Gliocladium* spp. used to control a number of foliar and soil-borne pathogens. The pathogen and crop to which the product is applied, the spray equipment used, the final spore concentration of the product, the rate of application, the crop growth stage and frequency of application and the environmental conditions under which the product was applied is provided, where available from each source. The outcomes of the experiments are given in **Table 8**.

Reference	Pathogen and crop	Spray equipment	Spore concentrations and water volumes	Optimum growth stage, frequency of application	Temperature	Environmental Water	UV interception
***Gliocladium catenulatum*, applied as Prestop**							
McQuilken *et al.;* 2001	Pansy and snapdragon *Pythium ultimum* Alyssum and salvia *Rhizoctonia solani*	Soil drench, not a spray.	Prestop incorporated into growing medium just before sowing. 50 ml/l of growing medium, equivalent to 0.5 g of formulation /L. Final spore conc. not stated.	Soil drench pre-sowing only.	Glasshouse at 15°C ± 2°C	Trays watered as required to maintain a matric potential of approx. 20 kPa % Relative humidity not stated.	Supplementary lighting to provide 38 W/m^2 at bench level with a photoperiod of 14 hours.
Lahlali and Peng, 2014	Oilseed rape seeds *Plasmodiophora brassicae*	Soil drench, not a spray.	Prestop at a final concentration 1x10^6 cfu/ml drenched on soil at 25 ml per plant.	At sowing and 7 or 14 days afterwards.	From 18–23°C	The soil was saturated with acidified water adjusted to pH 6.3 using 2M HCl.	Photoperiod of 14 h used throughout.
Rose *et al.;* 2003	Cucumber roots *Fusarium oxysporum* f. sp. *radicis-cucumerinum*	Soil drench, not a spray.	Prestop at a final concentration 1x10^6 spores/ml drenched on soil at 15 ml per plant.	Onto seeding cavity of rock wool blocks at 1, 3, 5, 7, or 10 days following sowing.	24°C ± 2°C	No data provided	Photoperiod of 12-16 h used throughout.
Chatterton *et al.;* 2008	Cucumber roots and crown *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (Forc)	Soil drench, not a spray.	Prestop applied as a soil drench at 50 ml per pot). Final spore conc. not stated.	Applied to seeds. Assessments made on the roots.	Growth room at 21°C –24°C	No data provided	Under a 16 h photoperiod with sodium vapour lights (light intensity of 100 µmoles/m^2/s).

Reference	Pathogen and crop	Spray equipment	Spore concentrations and water volumes	Optimum growth stage, frequency of application	Temperature	Environmental Water	UV interception
Gliocladium roseum							
Costa *et al.;* 2012	Strawberry leaf discs *Botrytis cinerea*	Laboratory test with application to leaf discs in agar plates.	Using *G. roseum* strain LQC 62 (tolerant to UV-B radiation). 20 µL of *G. roseum* conidial suspension applied at 10^4, 10^5, or 10^6 conidia/ml per strawberry leaf disc.	One cm-diameter leaf discs of 30–60 day old strawberry plants used. Single application before Botrytis inoculation.	A temperature-controlled chamber at 25°C ± 2°C.	Humidity in dishes not stated.	Four UV-B 313EL lamps permitting passage of most UV-B and UV-A (290–400 nm) but preventing exposure to UV-C (>280 nm) and short-wavelength UV-B (>290 nm). Leaves exposed to UV-B radiation (irradiance 600mWm^2) for 0, 1, 2, or 3 h, corresponding to doses 0 (control), 2.1, 4.2, & 6.3 kJm^2.
Morandi *et al.;* 2008	Rose foliage *Botrytis cinerea*	Compression sprayer, at a rate of 100 mlm^2 applying 0.2 ml per leaf.	*G. roseum* applied at a final concentration of 1x10^7 conidia/ml. Each leaflet was inoculated by placing a 10 µl droplet of *G. roseum* inoculum on the surface.	Fully-expanded leaves of rose plants detached (to simulate senescence) & inoculated.	A climate-controlled glasshouse at 19–25°C in daylight & 16–21 °C at night. A plastic covered greenhouse at 20-35°C.	In a climate-controlled greenhouse, varied from 10% to 38% RH. In plastic-covered greenhouse from 50% to 95% RH	In climate-controlled greenhouse: incident solar radiation 8:00 to 16:00 h, (maximum of 225Wm^2). In plastic-covered greenhouse without climate control, incident solar radiation 6:00 to 18:00 h.
Yohalem *et al.;* 2004	Foliage of onion *Botrytis aclada*	Petri dish experiment, inoculated with a dropper	*G. roseum* strain 201 applied at final concentration 1x10^5 cfu/ml 24 h after *B. aclada* inoculation by spraying to run-off along the entire leaf.	Onion plants at three weeks old	From 17-18°C.	From 85-95% relative humidity.	Photoperiod of 16 h used throughout.

Table 8. A summary of the outcomes of the experiments on the application of *Gliocladium* spp. and its representative products as a foliar spray or soil drench under the conditions detailed in Table 7.

Reference	Crop and pathogen	Use of the beneficial fungus under particular environmental conditions and effects on disease control
***Gliocladium catenulatum*, applied as Prestop**		
McQuilken *et al.,* 2001	Pansy and snapdragon for *Pythium ultimum* damping off trials. Alyssum and salvia for *Rhizoctonia solan*i trials.	After 21 days in dual culture on PDA, *G. catenulatum* overgrew *P. ultimum* and *R. solani*, and sporulated on the pathogens' hyphae. Growing medium incorporation & drench treatments were effective in reducing pre- & post-emergence *P. ultimum* in both hosts. With product storage for 48 weeks at 5°C or 18°C in the laboratory; good *G. catenulatum* survival rates, colony counts remaining at > 10^8 cfu/g. At 25°C counts decreased with time from > 10^8 to 10^3 cfu/g. No effect on *G. catenulatum* reduction in damping-off by *P. ultimum* or *R. solani* following product storage at 5°C for 48 weeks. Compatibility testing of *G. catenulatum* showed etridiazole at 1000 g/ml inhibited mycelial growth by 76%. Tolclofos-methyl inhibited growth at all concentrations, with greatest inhibition (57%) at 1000 g/ml. Fosetyl aluminium reduced growth only at 100 and 1000 g/ml (by 13% and 24%, respectively). Furalaxyl & propamocarb HCl had no/only a minor effect on mycelial growth of *G. catenulatum* at all fungicide concentrations.
Lahlali and Peng, 2014	Oilseed rape seeds *Plasmodiophora brassicae*	Prestop applied once at sowing, 7 or 14 days after sowing, or at both sowing and 7 or 14 days after (two-application treatments) reduced clubroot compared to the control. Two applications generally more effective than one, especially when applied at sowing and 7 days after sowing. *G. rosea* conidial suspension was often slightly more effective than the product filtrate, but less effective than the biofungicide product.
Rose *et al.,*2003	Cucumber roots *Fusarium oxysporum* f. sp. *radicis-cucumerinum*	Prestop added to the sowing cavity of rock wool blocks followed 24 h later by *F. oxysporum f. sp. radicis-cucumerinum* inoculum significantly reduced the percentage of diseased plants compared with pathogen only controls. The degree of control was not significantly different from plants treated with the fungicide benomyl. In growth room trials on seedlings, only Prestop reduced disease to the same level as benomyl.
Chatterton *et al.,* 2008	Cucumber roots and crown *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (Forc)	Prestop at sowing persisted on cucumber roots for at least 50 days at above 1x10^5 cfu/g root fresh weight. By 60 days, levels *of G. catenulatum* were slightly below 1x10^5 cfu/g root fresh weight. *G. catenulatum* on rockwool blocks before inoculation with Forc gave significant decrease in Forc levels on the roots and crown when compared to plants inoculated with Forc only. At the lowest concentration of Forc (1 x 10^4 conidia/ml), treatment with *G. catenulatum* reduced pathogen levels on the roots to nil.

Reference	Crop and pathogen	Use of the beneficial fungus under particular environmental conditions and effects on disease control
Gliocladium roseum		
Costa *et al.,* 2012	Strawberry leaf discs *Botrytis cinerea*	Leaf discs on agar received *G. roseum*, then some were given UV-B before all received Botrytis inoculation. The presence and sporulation of *G. roseum* on leaf disc surfaces was reduced by the UV-B radiation and Botrytis severity was greater than without exposure. and the conidial concentration of *G. roseum*. The effects of UV-B radiation on *G. roseum* growth were more pronounced at lower spore concentrations. Without UV, at the highest concentration of 10^6 *G. roseum* conidia mL^{-1}, the incidence and severity of *B. cinerea* on strawberry leaf discs was reduced by 91% and 98% 10 days after inoculation. This confirmed the beneficial fungus is highly susceptible to UV-B, reducing its efficacy against Botrytis.
Morandi *et al.,*2008	Rose foliage *Botrytis cinerea*	In a plastic covered greenhouse, germination of *G. roseum* conidia was significantly greater when the treatments were kept out of direct light, but there was no significant difference on the suppression of Botrytis sporulation. Inside a climate controlled glasshouse, *C. rosea* suppressed sporulation of Botrytis by 93% compared to the control which received no exposure to solar radiation.
Yohalem *et al.,* 2004	Foliage of onion *Botrytis aclada*	*G. roseum* isolate 201 almost completely suppressed Botrytis sporulation on dead onion leaf pieces under constant moist conditions, however sporulation was only reduced by 20% when the periods of leaf wetness were interrupted. *G. roseum* 201 significantly reduced Botrytis biomass by 82% at day 6 and by 91% at day 8. Botrytis biomass at day 8 was reduced by 97% under interrupted wetness. There was no statistically significant effect of *G. roseum* 201 treatment on internal spread of the pathogen.

Environmental conditions at product application

Temperature

The label for Prestop does not give any specific information on the optimum temperature at which the aqueous suspension of Prestop is effective at. Studies to date have found that the concentration of *G. catenulatum* on foliage was high at 20-25°C and also at 30°C in hosts in which measurable infection was obtained, however it was considerably less at 15°C and 10°C (Sutton and Peng, 1993, Sutton *et al.,* 1997, Yu and Sutton, 1994). Chatterton and Punja (2011) found that during *G. catenulatum* colonisation of foliage in two geranium cultivars, *Pelargonium* × *hortorum* and *Pelargonium* × *domesticum*, the optimum temperature for colonisation was 20–25°C for both cultivars, as measured by mycelium progression using a β-Glucuronidase (GUS)-transformed isolate to aid visibility for assessment.

To assess the influence of application time on the establishment, survival, and ability of *G. roseum* to suppress *B. cinerea* sporulation on rose debris, Morandi *et al*. (2008) used a climate-controlled glasshouse where the temperature varied from 17 to 27°C and the relative humidity from 10% to 38%, or in a plastic-covered greenhouse without climate control where the temperature varied from 23 to 40°C and the relative humidity from 50% to 95%. Though germination of *G. roseum* was found to be positively correlated with relative humidity and negatively correlated with exposure time, no such association was made with temperature. In summary, the available literature on *G. roseum* (Morandi *et al*., 2000, Sutton *et al*., 1997, Yu and Sutton, 1994) coupled with experimental information provided in Tables 3 & 4 indicates that an optimum temperature range for application of Prestop to foliage is 20–25°C.

In the AHDB Project CP140 (Tut, 2019), the development of *G. catenulatum* J1446 on leaves following Prestop application was quantified using the novel PCR technique capable of distinguishing live from dead spore material. Spore washings were made over 10 days with leaves of lettuce and strawberry held in a controlled environment cabinet at 10°C, 16°C, 22°C, 28°C or 34°C. The 28°C and 34°C temperatures led to a reduction in the population density of *G. catenulatum* over the period. Temperature was shown to be a major abiotic factor for the establishment and development of the *G. catenulatum* populations in the phyllospheres, with viable population density highest at a previously acknowledged optimum temperature range for growth of 15°C to 25°C referenced to Helyer *et al.,* 2014.

Environmental water

Evidence from *G. roseum*, the related species to *G. catenulatum,* suggests that humidity is of paramount importance in the survival, germination and growth of the fungus on plant surfaces.

Sutton *et al.* (1997) found that a humid period is required within a few hours after application of *G. roseum* in order to optimise growth of the biological control agent, but the form of the moisture such as droplets, films and duration of wetness to biocontrol was not explored. To assess the influence of application time on the establishment, survival, and ability of *G. roseum* to suppress *B. cinerea* sporulation on rose debris, Morandi *et al*. (2008) found that relative humidity was positively correlated with germination of *G. roseum*.

In the work by Tut (2019), described above, each of the five temperatures was kept at one of three relative humidities. The higher the temperature the lower the base and upper humidity within an overall range of 45 to 95% RH, in order to replicate glasshouse conditions. An increase in the *G. catenulatum* population occurred with increasing RH, with the pattern most evident in the optimum growth temperature range, so supporting a previously acknowledged RH range of 95% to 100% required for population establishment in the phyllosphere of crops.

Rainfastness

The label for Prestop gives limited information on the rain fastness of Prestop following application to foliage. An experiment was conducted by the manufacturers, Lallemand Plant Care, to investigate the rain fastness of Prestop on strawberry plants (Lallemand, 2012), using Prestop sprayed as a 0.33% suspension. The simulated 'rain' water treatment was applied for 5 minutes either immediately after the leaf surface had dried, or two hours after the product was applied. After 24 hours, 'rain' treated leaf samples were taken to test for *G. catenulatum* colonisation. The authors found that Prestop is not sensitive to rain and that *G. catenulatum* survived well even when rain started half an hour after Prestop application. There was no clear difference between slight and heavy rain, and even if rain came within 30 minutes after Prestop spraying, reductions in *G. catenulatum* appeared minimal. The conclusion was that Prestop requires only about half an hour (or until the canopy has dried) to be rain fast.

In summary, the available literature and experimental findings summarised in Tables 3 and 4 indicate that Prestop is most effective on foliage under high humidity conditions (ca. 85-95%) and is relatively rain fast on leaves of treated crops.

Ultraviolet light

Most of the research on the effects of ultraviolet light on *Gliocladium* spp. has been performed using *G. roseum* and the sexual stage of the fungus, *C. rosea*. *C. rosea* is highly susceptible to ultraviolet radiation and has reduced ability to antagonise Botrytis in solar radiation conditions (Costa *et al.,* 2012, Morandi *et al.,* 2008). In a study by Costa *et al.* (2012), which investigated the effects of UV-B radiation on the antagonistic ability of *C. rosea* against *B.*

cinerea on strawberry leaf discs, the presence and sporulation of *C. rosea* was influenced by the UV-B radiation dose. Botrytis growth was greater in the lower *C. rosea* concentration, while increases in the UV-B dose reduced the presence and sporulation of the *C. rosea*. The deleterious effects of UV-B radiation on *C. rosea* growth were more pronounced when it had been applied at lower spore concentrations; at 10^4 conidia mL^{-1} the Area under Progress Curve (AUPC) of *C. rosea* was reduced by 60% when irradiated at 6.3 (kJ m^{-2}). At 10^6 conidia mL^{-1}, the reduction was 40% for AUPC of *C. rosea*, at the same doses of UV-B radiation. The higher dose of UV-B reduced the presence and sporulation of *C. rosea* by 20% and 42%, respectively (Costa *et al.,* 2012).

Experiments conducted by Morandi *et al.* (2008) were conducted inside glass and plastic-covered greenhouses, that filter the majority of UV radiation wavelength of 320 or 360nm or less, respectively. Here, the viability of *G. roseum* spores was significantly, albeit mildly reduced with increasing length of exposure to sunlight during the day. Some reduction in suppression of *B. cinerea* was observed with initial exposure to sunlight (from 93% to 65%, from no sunlight to one hour sunlight) in the plastic-covered greenhouse experiment, however there was no reduction in suppression with additional sunlight, but no changes from 1 to 8 hours sunlight, and only small, nonsignificant changes from 0.5 to 1 hour were observed. The authors observed that germination incidence was inversely proportional to the application time on all treatments, independent of exposure to sunlight. This indicates that other factors besides solar radiation can influence conidial germination. This result was in accordance with a previous finding by Morandi *et al*. (2006), who showed that relative humidity was the main factor that influenced the establishment of *G. roseum* on rose debris in a commercial plastic-covered greenhouse. In summary, the experiments in **Tables 7 and 8** indicate that efficacy was found with the 14 to 16 hour photoperiods used in trials of the application of the formulated product of Prestop to foliage. Further research is needed to determine the effects of different exposure times, light intensities and global radiation on *G. catenulatum* germination and efficacy against different plant pathogens.

Storage of product

The UK label for Prestop recommends storing the unopened product below 8°C. Once opened, it is recommended to use the whole product immediately or store in the freezer until next required. The Finnish label for Prestop indicates that unopened packages can be stored for up to 6 months at temperatures below 8°C and 2 weeks at room temperature. It does not mention that the package can be frozen and recommends using the entire package upon opening.

Early studies (Beagle-Ristaino and Papavizas, 1985, Caldwell, 1958, Lewis and Papavizas, 1984, Papavizas, 1985) have highlighted how chlamydospores, rather than conidia may be more important for the long-term survival of *Gliocladium* spp. in the soil. Beagle-Ristaino and Papavizas (1985) found that chlamydospores of *Gliocladium virens* germinated readily and in greater numbers in soil than conidia of the same species. No more than 22% germination of conidia had occurred within 24 hours in soil treatments, while washed chlamydospores germinated readily and in large numbers (39-99%) within 24 hours after placement in the soil.

There is some research available on the impact of culture conditions on the performance and type of *G. roseum* sporulation. Sun *et al*. (2014) investigated the effects of culture conditions on resting spore production in *G. roseum* isolate 67-1 in submerged fermentation. Chlamydospore production decreased rapidly with increased pH (88.1% at pH 3.0 to 1.0% at pH 6.5). The optimal pH for conidia production was 6.0–6.5, at which chlamydospore forming was strongly inhibited. The optimum temperature for chlamydospore production was found to be 30°C. Manipulation of culture conditions might thus be able to be used to produce products with a higher content of environmentally resistant chlamydospores of *Gliocladium* spp.

The label for Prestop indicates that the product contains ‘’dried living fungal spores and mycelium’’ but does not indicate whether the preparation contains chlamydospores, conidia or a mixture of both spore type. The EFSA Review Report for *G. catenulatum* (Anon, 2004) indicates that asexual chlamydospores may be present in the mycelium, however no further details regarding the quantity or concentration of chlamydospores in the product are provided.

Compatibility with other chemicals and products

In agar plate tests by the manufacturer, Serenade ASO (*B. subtilis* strain QST 713) has been observed to inhibit Prestop and consequently they have indicated that the safe interval to leave between Serenade ASO application and Prestop application is five days (pers. comm. Lallemand Plant Care).

The effect of chemical pesticides on the growth of *G. catenulatum* J1446 was examined in laboratory tests by Lallemand Plant Care; a list of these products can be found in Table 9. The inhibition effect was assessed using a scale from 0 to 3 where 0 = no inhibition, 1 = mild inhibition, 2 = moderate inhibition and 3 = strong inhibition. These inhibition scores were then translated into a recommended number of interval days between pesticides and foliar application of Prestop. The manufacturer provided examples of the recommended intervals between Prestop and other crop protection products **(Tables 10 - 12).** However, the rationale on which these intervals were based was not clear.

In general, it is not advised to tank-mix Prestop with any chemical pesticides or concentrated fertilizer solutions, or to apply chemical pesticides within one to four days of Prestop application, however some such as propamocarb hydrochloride, triadimefon, deltamethrin, malathion, metalaxyl, pirimicarb and pyrethrins, may be applied on the same day as Prestop (Lallemand, 2012) **(Tables 10 and 11).**

Table 9 Laboratory compatibility test results between *G. catenulatum* J1446 and a range of fungicides used against grey mould and powdery mildew. The inhibition effect was scored on a scale of 0 (no inhibition) to 3 (strong inhibition). From Lallemand: Details on compatibility, mixing & rainfastness. www.lallemandplantcare.com

Product	**Active ingredients**	**Inhibition effect (0-3) on *G. catenulatum***
Amistar	Azoxystrobin	1.5
Frupica SC	Mepanipyrim	0.5
Signum	Boscalid and pyracostrobin	2.0
Rovral	Iprodione	2.0
Scala	Pyrimethanil	1.0
Switch	Cyprodinil and fludioxonil	2.5
Stroby	Kresoxim-methyl	0 - 0.25
Fortress	Quinoxyfen	0.5
Nimrod	Bupirimate (250g/litre)	0
Systhane	Myclobutanil	0
Topas	Penconazole	1

Table 10. Recommended number of interval days between application of pesticides and Prestop as a foliar spray, based on laboratory inhibition tests in Table 9.

Inhibition rating in agar plate test (0-3)	**Number of interval days in practice**
0 - 0.75	0
1	1
1.5 - 2	2
2.5	4
3 or not tested	7

Table 11. Recommended number of days between application of Prestop as a foliar spray and some fungicide treatments. (From a Lallemand website entry: Details on Compatibility, Mixing & Rainfastness).

Active ingredients	Recommended interval (days)	Example Products
Azoxystrobin	3	Amistar
Benthiavalicarb-isopropyl + Folpet	1	Vincare
Boscalid + Kresoxim-methyl	0	Mascot, Collis
Boscalid + Pyraclostrobin	2	Signum, Pristine
Bupirimate	0	Nimrod
Chlorothalonil	0	Bravo, Daconil
Copper Oxychloride	0	Cuprokylt
Cymoxanil	0	Option
Dithianon	1	DithianonWG, Rathianon 70WG
Fenhexamid	0	Teldor
Fludioxonil + Cyprodinil	4	Switch, Reversal
Fluopyram	0 (1)*	Luna (contains this active)
Flutriafol	0	Impact
Fosetyl-Aluminium	0	Aliette 80 WG
Fosetyl-Aluminium + Propamocarb Hydrochloride	0	Previcur Energy, Avatar, Pan Cradle
Hymexazole	7	Tachigaren WP, Terrazol
Imazalil	2	Fungazil, Magnate
Iprodione	4	Rovrall
Krezoxim-methyl	0	Stroby, Beem, Fermor
Magnesium Sulphate + Orange Oil	0	Prev-Magnum
Mancozeb	4	Dithane, Mancozeb
Mepanipyrim	0	Frupica
Metalaxyl-M	0	Apron, Floreo, Fongarid
Myclobutanil	0	Systhane 20 EW
Myclobutanil + Cyclohexanone	0	Clayton Lithium
Myclobutanil + Quinoxyfen	1	Porter Super 90SC
Penconazole	1	Topenco, Topas
Prochloraz	7	Octave
Prochloraz + Propiconazole	7	Bumper P
Propamocarb Hydrochloride	0	Proplant, Promess, Edipro
Pyraclostrobin + Boscalid	2	Signum
Pyrimethanil	1	Scala, Pyrus
Quinoxyfen	0	Fortress, Apres
Sulphur	0	Kumulus
Tiophanate-methyl	4	Cercobin, Topsin WG
Toclofos-methyl	2	Basilex, Rizolex
Thiram	4	Thianosan
Triflumizole	0	Rocket EC
Trifloxystrobin + Propiconazole	4	Stratego 250 EC
Trifloxystrobin	0	Flint

(1)*. An interval of 1 day is required when Prestop and fluopyram are applied to the same part of the plant. When the root system is treated with fluopyram, Prestop can be sprayed on the foliage during the same day.

Table 12. Recommended number of interval days between application of Prestop as a foliar spray and some insecticide treatments (From Lallemand: Details on Compatibility, Mixing & Rain fastness.

Active ingredients	Recommended interval (days)	Example Products
Bacillus thuringiensis	0	Dipel, Lepinox Plus
Beauveria bassiana	0	Naturalis-L
Buprofezin	0	Applaud
Cypermethrin	2	Toppel 100, Permasect C
Deltamethrin	0	Decis, Bandu
Imidacloprid	0	Imidasect 5 GR
Malathion	0	Malathion, Carbofos
Metarhizium anisopliae	0	Met 52
Permethrin	2	Ambush
Pirimicarb	0	Aphox
Pyrethrins	0	Pyrethrum 5 EC
Rapeseed oil	0	Carbon Kick Booster

Key Points and Knowledge Gaps

- *G. catenulatum* within Prestop can be efficacious against *Botrytis* spp. on foliage, but more information exists on its benefit as a substrate drench against root pathogens. Targets for spray application given on the label are restricted to Botrytis, Didymella and Mycosphaerella. A wide range of crop hosts of Botrytis are listed on UK Extensions of Application for Use and work should be carried out and published on these.
- For successful germination and colonisation of leaves *G. catenulatum* requires moderately high humidity or moisture of around 60 – 80% RH and an optimal temperature around 25°C, but there is a good survival rate on foliage for at least two weeks in the absence of a host.
- The product is consistently instructed to be applied at 0.5% for both foliar and drench applications. This means that, unlike many bioprotectants where there can be a rate/ha, the number of colonies applied is consistent between applications to crops that grow upwards. This should make comparisons of product efficacy easier to compare between different crops and more such records are required.
- Although some information exists on the factors which influence both efficacy and persistence of Prestop on foliage, quantitative information on the nature of this persistence is minimal. More evidence is needed on the rate of *G. catenulatum* decline once applied to the foliage of individual crop species.
- Much of the literature on the delivery of Prestop refers to soil drenches rather than foliar sprays. There is thus a severe lack of knowledge on what aspects of product application are critical to improve performance for this biopesticide.

- There is poor understanding of the physics of spray application of Prestop e.g., the pressures, nozzle types and droplet size required to achieve optimum coverage. For crops with overlapping leaves, where good coverage is needed to ensure the product lands with where the pathogen will colonise, then efficacy would be increased by improved application techniques.
- The very high water volumes used for application of Prestop combined with wide plant spacing necessary for particular crops, means significant spray waste. Further investigation should look at optimising the level of coverage achieved using lower spray volumes and more efficient methods of delivery.
- Though the manufacturer of Prestop has supplied a recommended concentration to be used for foliar application across different crop types, the number of cfu/ml necessary for efficacious use of Prestop remains to be elucidated.
- Studies on the effects of UV radiation have been done on the related species *G. roseum*, but no information was available from the product labels or public literature on the effects of solar radiation or UV interception on the persistence of Prestop on foliar tissues and thus warrants further research.

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Addendum

Search term logic and outputs for a search of Web of Science publications related to Prestop and *Gliocladium catenulatum* J1446 carried out in 2017. The publication types searched for included articles, reviews, proceedings papers, book chapters, editorial material and meeting abstracts.

Search terms	Results
Gliocladium	895
Clonostachys rosea	240
Application Gliocladium	147
Clonostachys roseum	87
Gliocladium catenulatum	56
Environment Gliocladium	43
Prestop	28
Population dynamics Gliocladium	19
Application *Gliocladium catenulatum*	11
Gliocladium catenulata	10
Persistence Gliocladium	10
Population dynamics Clonostachys	7
Foliar application Gliocladium	6
Population dynamics *Gliocladium catenulatum*	2
Persistence Clonostachys	1
Foliar application Clonostachys	1
Persistence foliage Gliocladium	0
Persistence *Gliocladium catenulatum*	0

AQ10

Ampelomyces quisqualis strain M10

Approach to literature search

Internet based literature searches were conducted using the search engine facilities Web of Science, Scopus, Google Scholar and CABI. The publication types searched for included articles, reviews, proceedings papers, book chapters, editorial material and meeting abstracts. A Web of Science search conducted in July 2018 for literature containing the search term 'AQ10' returned 30 results, while a search for '*Ampelomyces quisqualis'* returned 89 results and further search terms are shown at the end of the AQ10 report in the Addendum.

Taxonomy, mode of action and host range

Taxonomy

A. quisqualis used in the commercial biocontrol product AQ10 is a mycoparasitic anamorphic ascomycete fungus of the order Pleosporales. *A. quisqualis* has been extensively investigated as a biological control agent of powdery mildews for over 50 years. Originally described as *Cicinnobolus cesatii* by De Bary (1870), the first description of the species was by Emmons (1930). A taxonomic revision has been proposed but not carried out for the genus Ampelomyces (Sutton (1980) to resolve the nomenclatural problems arising from the use of the binomial '*A. quisqualis'* for all strains within this group of mycoparasites. *A. quisqualis* is a slow growing fungus with an average *in vitro* radial growth rate of 0.5–1.0 mm per day on Czapek-Dox agar supplemented with 2% malt extract at 23°C (Kiss 1997; Kiss and Nakasone 1998). Angeli *et al*. (2011) showed that two colony types of *A. quisqualis* could be distinguished after 14 days on all tested media: strains that form dark brown colonies and those that form olive-green. Hyphae of *A. quisqualis* are hyaline and septate (Clare, 1964). The length and width of pycnidia ranged from 30 to 95 µm and from 25 to 55 µm, respectively, with no significant strain differences. Conidia of different strains varied in size: from 5.5 to 14.5 µm in length and 2.3 to 3.5 µm in width. Clare (1964) recorded the spore size of *A. quisqualis* Ces. as 2 x 10 µm. Angeli *et al*. (2011) concluded that these morphological characters were insufficient for accurate identification of different strains in the laboratory. Instead, the authors found that sequencing of the diverse internal transcribed spacer (ITS) regions of individual region of the nuclear ribosomal RNA gene (nrDNA) were the most appropriate method of detecting genetic diversity among *A. quisqualis* strains and to understand whether there are relationships between the origins of different strains of *A. quisqualis* and their phylogeny or other physical characteristics.

Mode of action

AQ10 is a wettable granule formulation of *A. quisqualis*, supplied at a concentration of 5 x 10^9 spores/g of product. AQ10 is registered in the UK as a biofungicide for reducing powdery mildew on a range of protected crops (peppers, tomatoes, cucurbits and strawberries). The primary mode of action is hyperparasitism where the fungus penetrates and invades hyphae, conidiophores and chasmothecia (formerly cleistothecia) of powdery mildew fungi, in the order Erysiphales. Here they absorb nutrients and degrade the cytoplasm (Parratt *et al*., 2017), leading to the collapse of pathogen hyphae and death (Gilardi *et al.,* 2008, Tollenaere *et al.,* 2014). Thus, attacked powdery mildew mycelia are averted in their sporulation by this intracellular mycoparasitism process (Almqvist, 2013, Kiss *et al*., 2004). Uninfected hyphae and conidiophores of powdery mildews are transparent, while mildew colonies which have been parasitised by *A. quisqualis* are dull, flattened, and off-white to grey in colour. Parasitised chasmothecia are typically dull, fawn coloured, flaccid, and range in size from 64 to 130 µm in diameter. Pycnidia of *A. quisqualis* vary in shape depending upon the organ in which they form. Within conidiophores they are pear-shaped, within hyphae they are spindle-shaped, and within chasmothecia they may be nearly spherical (Falk *et al*., 1995a, Falk *et al*., 1995b). Once the mycoparasite has begun to produce pycnidia, the hyphae and conidiophores swell to several times their normal diameter, and the amber colour of the pycnidial wall of the mycoparasite may be noticed through the cell walls of the host. *A. quisqualis* conidia produced in these intracellular pycnidia are unicellular and hyaline (Kiss *et al*., 2004). Dead leaf tissue may be adjacent to parasitised areas of the mildew colony, as outer leaf tissues die in response to death of the contained powdery mildew cells (Falk *et al*., 1995a, Gadoury, 1995). According to (Anon, 2004) there is no indication of any production of toxins by *A. quisqualis*.

Life cycle and epidemiology

A. quisqualis has an asexual life cycle, with pycnidiospores developing in pycnidia produced on its hosts. These pycnidia are then released and dispersed aerially or through rain splash to neighbouring mildew hosts (Kiss *et al*., 2004). *A. quisqualis* can continue its growth intracellularly if the living powdery mildew conidia land on a host plant surface, germinate and produce a new colony there (Kiss *et al*., 2010). *A. quisqualis* infects both the anamorph (asexual, conidial) and teleomorph (sexual, chasmothecia) stage of powdery mildew species. The mycoparasite has a curiously long latent phase; for approximately 7-10 days, it spreads within the hyphae of the mildew colony without killing it. Thereafter, the process of pycnidial formation begins, which is completed within 2-4 days. Infected cells generally die soon after

pycnidial formation begins. Secondary cycles of infection result from conidia released during subsequent rain events. Emmons (1930) originally presented some preliminary observations suggesting a putative sexual teleomorph of *A. quisqualis*, but this has not been subsequently reported (Kiss, 1998).

De Bary (1870) showed that the hyphae of *Ampelomyces* spp. grow internally in the mycelia of powdery mildews from cell to cell, producing their pycnidia in one or two cells of hyphae, conidiophores, conidium initials and ascocarps of their fungal hosts. Intracellular hyphae were shown to grow out from the parasitised cells when placed in water for a few hours. In addition, De Bary (1870) carried out cross-inoculation experiments demonstrating that an individual *Ampelomyces* spp. strain collected from a given powdery mildew species could also produce intracellular pycnidia in mycelia of other powdery mildew species. His pioneering work was the first detailed study published of an interfungal parasitic relationship. De Bary (1870) subsequently published an extensive cytological study describing the penetration, growth and sporulation of Ampelomyces spp. in the ascocarps of powdery mildews. The potential for biocontrol was realised around the same time and Yarwood (1932) reported the treatment of powdery mildew infected plants with a conidial suspension of *Ampelomyces* spp., thus carrying out the first biocontrol experiment against a plant pathogenic fungus.

Host range

The Ampelomyces genus contains are the most widespread and oldest known range of natural enemies of the Erysiphales (Kiss *et al*., 2004). *Ampelomyces* spp. have been recovered from over 65 species in the genera Brasilomyces, Erysiphe, Leveillula, Microsphaera, Phyllactinia, Podosphaera, Sphaerotheca, and Uncinula, as well as the anamorphic genera Oïdium and Oïdiopsis (Belsare *et al*., 1980, Clare, 1964, Emmons, 1930, Falk *et al*., 1995a, Hanlin and Tortolero, 1984, Hijwegen and Buchenauer, 1984, Kiss, 1997, Kiss, 1998, Nagy and Vajna, 1990, Puzanova, 1991, Ranković, 1997, Tsay and Tung, 1991). These reports represent powdery mildews which attack 256 plant species within 172 genera in 59 families and occur in 28 countries around the world. Despite this, all known *Ampelomyces* spp. are strictly specialized to powdery mildews. Powdery mildew species, in turn, are each specialized to one or a few host plant species (Glawe, 2008, Barnett and Binder, 1973, Schulze-Lefert and Vogel, 2000). It is not yet fully understood whether Ampelomyces strains isolated from certain species of the Erysiphales are narrowly specialized to their original mycohosts or are generalist mycoparasites of many powdery mildew fungi. Evidence from Gu and Ko (1997) suggests that strain specific variation does exist; using an *in vitro* assay on water agarose, the authors showed that conidia of *A. mougeotii*, *B. graminis*, *E. galeopsidis* and *O. euonymi-japonicae*

stimulated germination of *A. quisqualis* (isolated from *P. leucotrichamore*) more than conidia of *E. cichoracearum*, *P. leucotricha* and *E. polygoni*. Additionally, Kiss (1998) found different levels of *A. quisqualis* incidence and intensity of mycoparasitism across 9 different genera of powdery mildew spp., with *Blumeria* spp. having the lowest incidence and intensity of mycoparasitism and *Arthrocladiella* spp. having the highest. Angeli *et al*. (2016) showed that increases in the germination rate and germ tube length of *A. quisqualis* strains in the presence of powdery mildew conidia-using a spore suspension inoculation method for *P. xanthii* and a dry, physical method of inoculation method for *E. necator* was not powdery mildew species-specific. In fact, the germination rates and tube elongation of all of the examined *A. quisqualis* strains could be stimulated by all of the examined powdery mildew species and not only by the conidia of their original fungal host. Nonetheless, there was statistically significant differences among the tested strains, with strain ITA3 the most strongly stimulated and, interestingly, among all the tested strains, strain AQ10 the least by the presence of conidia of different powdery mildew species.

Co-existence with and parasitism of powdery mildew

Observations by Hashioka and Nakai (1980) and Sundheim (1982) showed that the first interactions between *Ampelomyces* spp. and parasitised powdery mildew cells were biotrophic, with invaded cells eventually destroyed. Parasitised powdery mildew colonies could continue their radial growth, albeit with decreased sporulation (Shishkoff and McGrath, 2002). *A. quisqualis* growth is dependent upon its mildew host for survival, and it can complete its life cycle within a single clonal cycle of its host (Parratt *et al*., 2017). Dik *et al*. (1998) assessed the relative efficacy of *A. quisqualis* against cucumber powdery mildew (*S. fuliginea*). The Area Under the Disease Progress Curve (AUDPC) was used to calculate efficacy and the exact days of sampling was not given, however the authors found that in most of the leaf samples from one experiment, spores of *A. quisqualis* were found but no parasitism of powdery mildew was observed on the leaf disks. In a second experiment, no parasitism of powdery mildew was observed on 69% of the leaves, while greater than 50% of the powdery mildew colonies were parasitised on only 8% of the leaves. Dilution plating on PDA was also performed to confirm that the *A. quisqualis* populations were viable. The authors attributed the lack of efficacy of *A. quisqualis* to the dry conditions in the greenhouse which were perhaps not optimal for *A. quisqualis* parasitism. Nonetheless, these results suggest that although AQ10 did not control the disease, *A. quisqualis* was able to survive on leaves with or without host parasitism.

To determine whether *A. quisqualis* could form intracellular pycnidia in conidiophores arising from a parasitised powdery mildew conidium, Kiss *et al*. (2010) conducted a detached leaf

assay with both grapevine leaves in the presence of *E. necator* and tomato leaves in the presence of *O. neolycopersici*, sprayed with conidial suspensions of *A. quisqualis* strain dDSM2222. Ten to 12 days after spraying, leaf pieces were examined for the presence of germinated conidia of *E. necator* and *O. neolycopersici*. The test was repeated several times and *A. quisqualis* was eventually detected in both *E. necator* and *O. neolycopersici*. Intracellular hyphae of *A. quisqualis*, as well as young and fully mature pycnidia were found in the conidiophores of both powdery mildew species. All the germinated powdery mildew conidia containing hyphae of *A. quisqualis* had been previously penetrated by the mycoparasite before detaching from conidiophores. In spite of the presence of *A. quisqualis* hyphae in these conidia, and *A. quisqualis* pycnidia in their conidiophores, these cells were not killed by the mycoparasite and were able to produce new parasitised conidia. Thus, mycoparasitised powdery mildew led to the rapid production of *A. quisqualis* conidia before normal asexual reproduction could occur on an established colony. This knowledge about the mechanism of *A. quisqualis* spread, indicates that it is likely that this mechanism would allow *A. quisqualis* to spread more rapidly in the field.

Long term survival

A. quisqualis may survive in parasitised chasmothecia on the bark of deciduous perennial hosts, as well as on fallen leaves and crop debris. However, reintroduction on an annual basis is likely to be necessary for commercially acceptable disease control in annual or greenhouse crops (Gadoury *et al*., 2012). Falk *et al*. (1995a) and Falk *et al*. (1995b) showed that pycnidia of *A. quisqualis* survived until the next season mainly in the parasitised ascocarps (chasmothecia) of *U. necator* produced on the bark of grapevine stocks. *In vitro* experiments by Sztejnberg *et al*. (1989) and Szentiványi and Kiss (2003) demonstrated that overwintered pycnidia of *A. quisqualis* collected from host plants in the spring, and produced in both the conidiophores and immature ascomata (within the chasmothecia) of powdery mildews during the previous season, can initiate the life cycle of *A. quisqualis*. Both *A. quisqualis* conidia found in the overwintered *A. quisqualis* pycnidia, and cells of the pycnidial walls of empty pycnidia, germinated in the spring and gave rise to new intracellular pycnidia of *A. quisqualis* when powdery mildew colonies were inoculated with them *in vitro*. Similar experiments by Szentivanyi & Kiss (2003) showed that the thick-walled, brownish resting hyphae of *A. quisqualis* can also serve as sources of primary inocula of *A. quisqualis* in the spring.

Factors affecting persistence and efficacy of the product

Persistence on foliage

The hyphal growth of *A. quisqualis* needs to be as fast as or faster than its powdery mildew host to give sufficient control. Leaf colonisation by both powdery mildew and *A. quisqualis* usually takes place within a narrower window of suitable conditions than soil-borne plant pathogenic fungi and their mycoparasites as the phyllosphere provides a much more dynamic environment than the rhizosphere (Fokkema, 1991, Kiss *et al*., 2004). *A. quisqualis* strain AQ10 is unable to grow and to proliferate outside its host in nutrient-poor situations such as found in the phylloplane. It does, however, grow on nutrient enriched growth media used in the manufacturing process. The strain colonises, germinates, penetrates host cells and forms pycnidia (fruiting bodies) from which conidia of *Ampelomyces quisqualis* strain AQ10 can be released after rupture of the pycnidial wall (EFSA, 2017).

The Registration Report for AQ10 (CRD, 2008) details information on the persistence of *A. quisqualis* in both soil and air; application of viable spores of *A. quisqualis* to soil at 35 g/ha, equivalent to 1.75 x 10^{11} spores/ha, led to a substantial decline of spores to background levels (10^2 to 10^3 cfu/g of soil) “within weeks or months”. The report stated that the biology of the spores did not justify the performing of studies aimed at evaluating their spread, mobility and persistence in water but highlighted that spores are unable to survive, grow and proliferate outside of their powdery mildew host, have a very short survival time in water and are inactivated by UV light. For successful germination the spores need high humidity or moisture, temperature around 25°C and the presence of a host. Without the host, viability is rapidly lost e.g., within a few days. This exact number of days as well as the rate of decline remains to be elucidated. While spores can survive for longer under appropriate conditions (low humidity, or lack of moisture and low temperatures) these are considered unlikely to be the prevailing conditions at the time of application or at least for any prolonged period. The 2008 Report stated that *A. quisqualis* pycnidia produced from the infected mildew are more resilient and may persist in the environment for “relatively long periods” at least into the next growing season. The registration report highlighted that at the date of reporting, no specific studies on *A. quisqualis* isolate M-10 had been carried out for the evaluation of spore spread to neighbouring tissues. However, Dik *et al*. (1998) reported that under glasshouse conditions *A. quisqualis* was only found in the leaf area compartments of cucumber foliage where the BCA had been sprayed.

Four studies (Falk *et al*., 1995a, Kiss, 2008, Sullivan & White, 2000) report that *A. quisqualis* invades powdery mildew colonies on foliar tissues and completes its life cycle within the fungal host either within 5 days, up to 7, 8 or 10 days respectively. The study by Kiss (2008) highlighted that it was dependent on temperature, relative humidity (RH) and other abiotic factors. The Sullivan and White (2000) study was conducted under laboratory conditions. These studies are in line with that of Almqvist (2013) who hypothesised that it takes 3-5 days for *A. quisqualis* to colonise its fungal host. During this 5 to 10 day period, invaded mycelium of powdery mildew can still produce fresh conidia, although these might contain intracellular hyphae of *A. quisqualis*. A consequence of this slow, intracellular growth is that powdery mildew epidemics usually reach damaging levels before their growth and sporulation can be arrested by *A. quisqualis* (Falk *et al*., 1995b, Gadoury *et al*., 2012). Dik *et al*. (1998) showed by dilution plating that *A. quisqualis* population densities on cucumber leaf tissues (expressed cfu/cm^2 of leaf tissue) were stable up to one week after spraying. This study was done in the presence of powdery mildew symptoms following inoculation with a powdery mildew spore suspension containing 100 spores ml^{-1}, but the rate of *A. quisqualis* decline were not provided.

All of the above studies were done in the presence of powdery mildew; very limited information is available on persistence of *A. quisqualis* in the absence of its powdery mildew host. Angeli *et al*. (2011) measured germination rates and germ-tube elongation of AQ10 in the presence and absence of powdery mildew conidia by incubating aqueous suspensions containing mixtures of *A. quisqualis* and powdery mildew conidia for 24 h at three different temperatures (15°, 20° and 25°C). The authors found that in the absence of powdery mildew conidia, both germination rates and elongation of *A. quisqualis* were less than 10%, regardless of temperature. In the presence of the powdery mildew species tested (*P. xanthii* and *E. necator*), germ tube elongation and germination rates increased to greater than 40% and 60% respectively, at temperatures above 20°C. In nature, *A. quisqualis* is likely to have a shorter clonal generation time than its fungal host (Kiss *et al*., 2004), however, the exact regeneration times of both the BCA and pathogen, the within-host rate of reproduction and the existence or timing of any sexual recombination remain to be elucidated.

In the current AMBER project, in 2018, young tomato plants were sprayed with AQ10 at an experimental concentration of 1 x 10^6 spore / ml and kept within a controlled environment cabinet at 25°C with 16 hours light per 24 hours. After spraying, the plants were wrapped in transparent plastic, achieving 95% RH. When leaf samples were taken and washings cultured, a rapid decline in colony numbers was shown from the first to the fourth day after inoculation, with viable spores virtually undetectable after a week. When, in 2019, tomato plants under the same conditions were AQ10 sprayed either a day before or three or seven days after

inoculation with powdery mildew, viable *A. quisqualis* spores were recovered at similarly low numbers a week later and in no greater number than plants not receiving powdery mildew spores. Small patches of grey-brown powdery mildew mycelium were visible two weeks after the AQ10 sprays and enlarged slowly, showing *A. quisqualis* survival, with pycnidia were being produced, but white (apparently healthy) mildew colonies continued expansion across leaves.

Effective concentration and minimum effective dose

Table 13 provides a comparison of the published literature on effective concentrations of *A. quisqualis* used to control a variety of foliar and root based diseases and **Table 14** summaries the results. The concentrations tested are in the range of 5 x 10^5 spores/ ml (Romero *et al*., 2007) up to 8 x 10^6 spores/ ml (Legler *et al*., 2016) and as expected the efficacy of AQ10 when applied within these concentrations was variable.

The European Commission report (Anon, 2004) indicates that AQ10 should be applied to protected crops at a rate of a minimum 35 g, up to a maximum 70 g, in 200-250 l of water/ha, this being equivalent to a minimum 1.8 x 10^{11} cfu/ha up to 3.4 x 10^{11} cfu/ha. Romero *et al*. (2007) applied a low concentration of *A. quisqualis* spores (~5 x 10^5 cfu/ ml) to greenhouse melon foliage for control of *P. fusca*; this concentration proved to be successful when applied in combination with the mineral oil ADDIT, showing disease reductions of 80–95%. Abo-Foul *et al.* (1996) used a rate of 1 x 10^6 cfu/ ml to cucumber foliage to control *S. fuliginea*. The authors found that *A. quisqualis* treated leaves were covered with a flat layer of grey-brown material indicative of degenerating powdery mildew, however the level of infection was not further quantified in this study. Legler *et al*. (2016) applied a very high concentration (from 2 up to 8 x 10^6 cfu/ ml) of different strains of Ampelomyces, isolated from *E. necator* to grapevine foliage; all eight Ampelomyces strains and the commercial product, AQ10 reduced the number of conidia produced by the parasitised powdery mildew colonies by 74.7 % to 91.5 % compared to the untreated control.

Germination of *A. quisqualis* rapidly decreases above a concentration of 10^6 cfu/ ml due to the production of an unidentified self-inhibitory substance (Gu and Ko, 1997). At 5 x 10^6 spores ml^{-1} germination of *A. quisqualis* conidia on water agarose was greatly decreased when they were incubated while separated by a polycarbonate membrane from *A. quisqualis* spores at a high concentration (200 x 10^6 spores ml^{-1}). Spores of some other fungi also germinate readily under dispersed conditions, but when crowded they do so poorly or not at all, due to the presence of a self-inhibitor (Allen, 1976; Lax *et al*., 1985). Sundheim (1982) did not detect any concentration effect on conidia of *A. quisqualis*, but only used up to 1 x 10^6 which may not have been enough to pick up any inhibitory effect.

Table 13. Comparison of literature on the *A. quisqualis* strain in AQ10, or other strains, used to control a number of pathogens. The pathogen and crop being studied, the final spore concentration of the isolate, the rate of application, the crop growth stage and frequency of application and the environmental conditions (temperature, environmental water and UV interception) under which *A. quisqualis* was applied is provided, where available from each source.

Reference	Crop and pathogen	Spore concentrations and water volumes	Optimum growth stage, frequency of application	Temperature	Environmental Water	UV interception
Abo-Foul *et al*. (1996)	Cucumber, *S. fuliginea*	*A. quisqualis* applied at 1 x 10^6 cfu/ml.	*A. quisqualis* applied 4 days after inoculation with *S. fuliginea* to plants at the 2 leaf stage.	25°C.	24h of 100% RH.	A 16h photoperiod with an 800µmol m^2 s^{-1} light intensity
Gadoury (1995)	Grape, *E. necator*	*A. quisqualis* applied at 1 x10^6 cfu/ ml.	Applied as a single 10µl drop to plants which had two true leaves.	25°C.	Not detailed.	Not detailed
Elad *et al*. (1998)	Cucumber, *S. fusca* and *B. cinerea*	AQ10 applied at 4 g/L. Final spore concentration not provided.	Applied every 3–4 weeks, from symptom appearance 20-50 days after planting for *S. fusca*, and 75 days after planting for *B. cinerea.*	No data provided.	No data provided.	No data provided
Dik *et al*. (1998)	Cucumber, *S. fuliginea*	AQ10 applied at 6 g/l in 0.05% Tween 80 in experiment 1 and in 0.3% light white oil in experiment 2. Spore conc. not stated.	Inoculation took place 6 days after planting in experiment 1 and 38 days after planting in experiment 2. Treatments were applied weekly.	19-32°C.	Glasshouse floors wetted. Inoculations done late in the day to ensure high RH. Mean RH from 30-90%.	No data provided
Shishkoff and McGrath (2002)	Pumpkin, *P. xanthii*	AQ10 applied at 0.05 mg/ml formulated product. Final spore conc. not stated.	Leaves taken from 4- to 7-week-old plants. Treatments applied to runoff. Application frequency not provided.	24± 2°C.	Leaf chambers closed immediately after spraying to eliminate evaporation of water.	16h photoperiod with 800µmol m^2 s^{-1} light intensity
Romero *et al*. (2007)	Greenhouse melon *P. fusca*	AQ10 applied at a final spore concentration of 5 × 10^5 cfu/ml. .	Plants were treated with AQ10 at the 8-leaf stage, 3 days after inoculation with *P. fusca*. 2 applications were made.	25°C.	Applied in the evenings to ensure the longest period with high RH.	16h photoperiod with an 800µmol m^2 s^{-1} light intensity
Pertot *et al*. (2008)	Strawberry, *P. aphanis*	AQ10 applied at 4 g/L. The final spore concentration not provided.	AQ10 was applied 6 hrs before artificial inoculation and again 10 days later.	16-23°C.	The average daily RH varied between 58% and 99%.	A 16h photoperiod with 800µmol m^2 s^{-1} light intensity
(Crisp *et al*., 2006)	Grapes, *E. necator*	AQ10 applied at 75 mg/L of water.	*A. quisqualis* applied to 4 of the 8 youngest leaves on the plant, every 2 weeks over 6 weeks	25°C.	50% RH.	14h photoperiod

Reference	Crop and pathogen	Spore concentrations and water volumes	Optimum growth stage, frequency of application	Temperature	Environmental Water	UV interception
Legler *et al*. (2016)	Grapes, *E. necator*	AQ10 applied at a final spore concentration of 2–8×10^6 cfu/ ml.	Leaf discs were used. The frequency of application was not provided.	15°C, 20°C and 25°C.	No data provided	16h photoperiod with 800µmol m^2 s^{-1} light intensity
Angeli *et al*. (2016)	Grape, *E. necator*	*A. quisqualis* strain ITA3 applied at 1×10^6 cfu/ ml.	8 to 10 leaves at the time of the first treatment. Treatment applied once.	25 ± 1°C.	95% RH for 48 h, reduced to 70 ± 10% for remainder of test. .	Not detailed
Angeli *et al*. (2016)	Cucumber, *P. xanthii*	*A. quisqualis* strain ITA3 applied at 1×10^6 cfu/ ml.	8 to 10 leaves at the time of the first treatment. Treatment applied once.	25 ± 1°C.	95% RH for 48 h, reduced to 70 ± 10% for remainder of test.	Not detailed

Table 14. Summary of the experimental outcomes on the application of *A. quisqualis* used to control a number of foliar and soil-borne pathogens under the environmental conditions given for each literature reference in Table 13.

Crop & powdery mildew spp.	**Efficacy and other outcomes from experiments**
Cucumber, *S. fuliginea*	Leaves of *A. quisqualis* treated plants covered with a flat layer of grey-brown material indicative of degenerating powdery mildew. Efficacy was not assessed.
Grape, *E. necator*	*A. quisqualis* produced parasitised mildew colonies within 10 days of application, but this did not always lead to destruction of mildew conidiophores. Both isolates tested infected and reproduced on all of the powdery mildew species tested. The % of collapsed conidiophores of powdery mildew ranged from 66.4% to 100% across both isolates. When plants were kept dry, *A. quisqualis* was not an effective mycoparasite, but mycoparasitism increased when plants were kept wet for 24 h following application of powdery mildew.
Cucumber *S. fusca & B. cinerea*	AQ10 and ADDQ oil mixture was superior to either alone. *S. fusca* was 40% parasitised by *A. quisqualis* on leaves with AQ10 or 60% with AQ10 + ADDQ; in untreated it was 5% or less. Efficacy declined with leaf age, but AQ10 retained about 60% –70% of its control capability.
Cucumber, *S. fuliginea*	*A. quisqualis* did not significantly reduce disease colonisation compared to the control. The effect of AQ10 was stronger on the partially resistant cultivar than on the susceptible cultivar. Dilution plating showed population densities were quite stable up to one week after spraying. However, in most of the leaf disk samples observed microscopically, spores of *A. quisqualis* were found but no parasitism was observed.
Pumpkin, *P. xanthii*	The adjuvant AddQ did not increase colonisation % *of P. xanthii* by *A. quisqualis,* but reduced mildew colony sizes when used alone or with AQ10, indicating the adjuvant had fungicidal properties. *A. quisqualis* was compatible with triforine at 10 µg/ml and myclobutanil at 100 µg/ml.
Melon, *P. fusca*	*A. quisqualis* most efficient at 90–95% RH, with disease reductions of 60 to 90%, compared to reductions of ca. 77% at 75%–80% RH. AQ10 was most effective when combined with the mineral oil ADDIT, showing disease reductions of 80–95%. In the absence of mineral oil, powdery mildew disease severity was not statistically different from untreated or water controls.
Strawberry *P. aphanis*	*A. quisqualis* reduced the incidence and severity of powdery mildew, but to a lesser extent than chemical fungicides. The level of *A. quisqualis* activity varied among locations, probably depending on environmental conditions and disease pressure throughout the season.
Grapes, *E. necator*	*A. quisqualis* was ineffective at controlling powdery mildew, possibly because of the dry conditions in the greenhouse.
Grapes, *E. necator*	All of the eight Ampelomyces strains and AQ10 reduced the number of conidia produced by the parasitised powdery mildew colonies by 74.7 % to 91.5 % compared to the untreated control.
Grape, *E. necator* Cucumber, *P. xanthii*	Application of *A. quisqualis* strain ITA3 conidia reduced powdery mildew severity (measured as AUDPC) from 672 to 483% days on grapes. Application of *A. quisqualis* strain ITA3 conidia reduced powdery mildew severity (measured as AUDPC) from 906 to 651% days on cucumbers.

Use of adjuvants and additives to enhance efficacy

To enhance its activity at lower relative humidity, *A. quisqualis* is often mixed with paraffin, mineral oils or additives such as pinolene, some of which at high rates may directly control the disease (Hijwegen and Buchenauer, 1984, Philipp and Hellstern, 1986, Verhaar *et al*., 1999). Elad *et al*. (1998) found that application of both AQ10 and ADDQ oil together at 0.004% and 0.3% respectively provided superior efficacy against *S. fusca* infection of cucumber in comparison to either agent applied alone. Observation of powdery mildew symptoms showed that *S. fusca* was either 40 or 60% parasitised by *A. quisqualis* on leaves treated with either AQ10 or with AQ10 + ADDQ, respectively, whereas in the untreated control, parasitism was 5% or less.

Dik *et al*. (1998) used AQ10 at a rate of 6 g / L suspended in 0.05% Tween 80 or 0.3% light white oil. There was a distinct inhibitory effect of Tween 80 on powdery mildew development in both cultivars tested, and powdery mildew severity was not reduced further when AQ10 had been suspended with Tween 80. The authors attributed the relative control of this treatment to the additive only. This was confirmed by the lack of powdery mildew control by *A. quisqualis* where it was applied in an oil mixture, in both cultivars tested. In additional experiments, the oil in all concentrations tested (up to 5%) did not control powdery mildew in cucumber (Dik and Bélanger, unpublished results). The authors stated that the oil and Tween 80 amendments enhance survival of AQ10 and improve homogeneous distribution of the spray solution on the leaves, thereby enhancing product efficacy.

Shishkoff and McGrath (2002) quantified powdery mildew development on squash leaves sprayed with AQ10, AddQ, or both; AddQ alone significantly reduced colony diameter and AQ10 alone did not. AQ10 and AddQ together reduced colony diameter as much as AddQ alone but the results were not consistent across experiments. Marchetti and D'Aulerio (1999) tested Ultra Fine Oil (a refined mineral oil) and Ultra Fine Oil plus AQ10 on powdery mildew–infected crape myrtle; good control was achieved with AQ10 + Ultra Fine Oil while Ultra Fine Oil on its own was least effective.

Crop growth stage, schedule and frequency of application

The UK label indicates that AQ10 should be applied preventatively at weekly intervals, starting from the very first sign of powdery mildew when conditions are conducive to powdery mildew development. The Technical Notes provided by the original UK suppliers, Fargro, indicated at low infection levels of less than 3%, AQ10 should not be used as a curative treatment. The Fargo website indicated that applications should be repeated every 7-10 days, and that it is important to use AQ10 in at least two consecutive sprays. The scientific literature

details numerous experiments where AQ10 has been applied to different growth stages of crop species at various frequencies of application.

Experiments with AQ10 at different timings are summarised in tables 13 and 14. The crop type, growth stage, range of relative humidities and temperatures, spray concentrations as well as the application timing of AQ10 in relation to any powdery mildew species inoculation and the AQ10 re-application frequency would each have had a bearing on the efficacy reported. The following paragraphs provide further detail and discussion.

To assess the effect of AQ10 on controlling *P. fusca* in greenhouse melon, Romero *et al*. (2007) applied AQ10 to plants at the 8 leaf stage, twice. The first application was done when the initial mildew colonies were observed, 3–4 days after *P. fusca* inoculation. The second application was done 10 days later. This treatment resulted in an 80-95% reduction in powdery mildew symptoms, however the efficacy could not be conclusively attributed to the use of AQ10; applying AQ10 without the mineral oil ADDIT proved unsuccessful at controlling powdery mildew symptoms. Treatments also performed better where lower oscillations in RH were present in the glasshouse.

Pertot *et al.* (2008) conducted trials to assess the ability of several fungicides and biopesticides, including AQ10 in controlling *Podosphaera aphanis* (syn. *Sphaerotheca macularis*) on strawberry plants. AQ10 was applied 6 hours before artificial inoculation and again 10 days later. The exact growth stage of the plants was not provided, however the authors state that the treatments were applied after 2 weeks growth. Two weeks after the initial inoculation, AQ10 used in a programme with azoxystrobin and sulphur on strawberry resulted in disease incidence of 38% and disease severity of 9.7%, compared with a standard fungicide programme which had a 9.3% and 1% incidence and severity respectively.

To investigate the efficacy of AQ10 against both *S. fusca* and grey mould (*B. cinerea*), Elad *et al*. (1998) applied AQ10 to cucumber foliage every 3-4 weeks, coinciding with the first appearance of symptoms on plants. *S. fusca* symptoms appeared 20-50 days after planting, whereas *B. cinerea* symptoms appeared 75 days after planting. The exact number of applications and the exact plant growth stages were not detailed in this paper. Nonetheless, AQ10 applied with the adjuvant ADDQ achieved up to 60% control against *S. fusca*. Its effectiveness declined with the progress of infection, however it retained significant control capability on older leaves; up to 70% of its original capacity. AQ10 applied with ADDQ was less effective at controlling *B. cinerea*, with only a 25–30% reduction in symptoms observed.

To evaluate the efficacy of AQ10 against *S. fusca* on cucumber foliage, Dik *et al*. (1998) applied AQ10 to leaves of powdery mildew susceptible and partially resistant cucumber varieties. The applications were made weekly. For the susceptible variety, applications were made four times; for the partially resistant variety, AQ10 was applied six and nine times in the first and second experiment respectively. Whether AQ10 was applied as a preventative or curative treatment throughout the experiment is unclear, as the date of powdery mildew inoculation is not given. AQ10 was ineffective at controlling powdery mildew in both experiments and in both cultivars; for the partially resistant cultivar the best control achieved was circa 10%. This reduction is much lower than some of the other published reports (Elad *et al*., 1998, Romero *et al*., 2007). The authors attributed the discrepancy between results in part to the drier conditions that prevailed in experiments and the possibly severe effect of climatic conditions (solar irradiation, temperature and vapour pressure deficit during daytime) which may have impeded efficacy.

To assess the effects of *E. necator* on grapevines, Legler *et al*. (2016) applied AQ10 twice, approximately 1 week before and 1 week post-harvest (from late August until mid-October depending on the vineyard). The treatment reduced both disease incidence and severity at the mid-epidemic stage in the following year by approximately 50%. The authors stated that late-season application of *A. quisqualis* can be considered a sanitation treatment to reduce overwintering inoculum which initiates the disease epidemic in the following year (Caffi *et al*., 2013, Legler *et al*., 2011). The efficacy of the sanitation treatments was confirmed by disease assessments in the treated plots in the following seasons; in 60% of the treated plots, the first disease symptoms appeared 1 to 4 weeks later than in the untreated plots.

Almqvist (2013) found that the effect of using *A. quisqualis* with an application interval of 14 days was significantly better compared to a 7 day application interval. The author found that prophylactic application with *A. quisqualis*, was only significantly better in one of the disease assessments.

Environmental conditions

Temperature

The European Food Safety Authority (EFSA) review report for *A. quisqualis* (Anon, 2004) states that the spores of this organism do not germinate at 37°C. The registration report (CRD, 2008) indicates that the optimum temperature for spore germination is 25°C; above 30°C, germination decreases and it stops at 37°C. A 2017 EFSA report gives the optimum temperature range for spore germination and pycnidia formation by *A. quisqualis* strain AQ10

as 15°C to 25°C. *A. quisqualis* strain AQ10 is able to grow at a large pH range (3–8) with a good growth and sporulation between pH 5.5 and 7.0 (EFSA, 2017).

The UK label recommends to apply AQ10 at temperatures between 12°C and 30°C, and to typically apply in early morning or late evening. Legler *et al.* (2016) showed using an *in vitro* assay on Czapek-Dox agar supplemented with 2% malt that colony growth of *A. quisqualis* decreased from 0.38 mm/day at 25°C, to 0.34 mm/day at 20°C, and to 0.26 mm/day at 15°C. At least seven studies to date (Table 1) have reported that 25°C is the optimal infection temperature for efficacious use of *A. quisqualis* against powdery mildew (Abo-Foul *et al.*, 1996, Angeli *et al.*, 2009, Crisp *et al.*, 2006, Gadoury, 1995, Legler *et al*., 2016, Romero *et al*., 2007). In studies by Romero *et al.* (2007) and Abo-Foul *et al*. (1996) inoculated plants were maintained at 25°C under a 16 hour photoperiod. Control was achieved, with an 80-95% reduction in symptoms (Romero *et al*., 2007) or demonstrated but not quantified (Abo Foul *et al*. 1996). Gadoury (1995) and Legler *et al.* (2016) found that all of the *A. quisqualis* isolates tested in their experiments against *E. necator* in grapevines were able to infect and produce parasitised mildew colonies at this temperature, with % infection of powdery mildew conidiophores ranging from 66.4% to 100%. Angeli *et al.* (2016) found that application of *A. quisqualis* strain ITA3 conidia reduced powdery mildew severity (measured as AUDPC) from 672 to 483% days on grapes from 906 to 651% days on cucumbers. Crisp *et al*. (2006) found that AQ10 was ineffective, but the authors attributed this to the dry conditions in the greenhouse as opposed to the temperature. A review of the risk of microbial organisms used in biopesticides on non-target organisms by Mudgal *et al.* (2013) did not include *A. quisqualis* in the table of organisms for which they had found information in the literature of any influence on its behaviour or efficacy of biotic and abiotic factors.

Environmental Water

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. At least 7 studies to date have reported that AQ10 was most efficient at controlling powdery mildew when RH was at 90–95%, with efficacy decreasing rapidly when below this threshold (Dik *et al*., 1998, Kiss *et al*., 2010, Legler *et al*., 2011, Philipp *et al*., 1984, Philipp and Hellstern, 1986, Romero *et al*., 2007, Verhaar *et al*., 1999). Kiss *et al*. (2010) reported that in circa. 10-20 hours under conditions of high humidity, conidia of *A. quisqualis* can germinate and hyphae of this mycoparasite can penetrate the hyphae of powdery mildew in their vicinity. To achieve high levels of humidity in their experiments, Dik *et al*. (1998) applied AQ10 during the last four hours before sunset to prevent excessive drying of plants after application and subsequent

desiccation of the biological control agent. Legler *et al*. (2016) argue that the high relative humidity requirement of *A. quisqualis* represents a limiting factor in its use in biocontrol; the dry conditions that usually favour the development of powdery mildews are not conducive to the development of *A. quisqualis* and vice versa (Paulitz and Bélanger, 2001, Verhaar *et al.,* 1999). However, raising the humidity in commercial greenhouses could encourage the growth of foliar pathogens other than powdery mildews, such as *B. cinerea.*

UV-light interception

No information was found in scientific papers on the effect of UV-light interception on the persistence of *A. quisqualis* on foliage. The Registration Report (CRD, 2008) section on fate and behaviour in air stated that it was shown that the spores of *A. quisqualis* were inactivated by UV light. No further details were provided.

Storage of product

The UK label for AQ10 indicates that the product should be stored at room temperatures when being kept for under one year, and at temperatures between 4°C and 8°C for storage periods of two years. The technical note for AQ10 states that once opened, the product should be used within 15 days. AQ10 is not suitable for freezing. The registration report for AQ10 (CRD, 2008) detailed an experiment assessing the fate of spores while in suspension in water. Only 24% of spores were still viable after immersion in water for 24 hours. Maximum recovery was achieved after 90 to 120 minutes in suspension and satisfactory levels (60-86% viable spores) were achieved over the first 6 hours only. The registration report did not provide any further details on the experiment, such as the concentration of spores used or the environmental conditions during the experiment, as the relevant study was subject to data protection.

Compatibility with other chemicals and products

A. quisqualis is compatible with many chemical fungicides (**Tables 15 and 16**) many able to control powdery mildew such as triforine, quinomethionate (Sundheim, 1982), triadimefon (Philipp *et al.,* 1984, Shishkoff & McGrath, 2002), myclobutanil (Shishkoff & McGrath, 2002) and others (Sztejnberg *et al.,* 1989). *A. quisqualis* tolerates many acaricides and insecticides (Philipp *et al*., 1984). It is not affected by sterol biosynthesis inhibitors (FRAC code 3, including triazole fungicides). Compatibility offers an opportunity to use AQ10 as part of an integrated management programme using a reduced spray regime of conventional fungicides.

Table 15. Compatibility of AQ10 in tank-mix with other fungicides. Examples of tank mixes with no loss of AQ10 where at least 5 days should be allowed between AQ10 and another fungicide (AQ10 Technical Document on Lallemand product manufacturer's website).

Active ingredients tank mixable with AQ10	Active ingredients incompatible with AQ10
B. amyloliquefaciens (D747)	Azoxystrobin
B. subtilis QST713	Captan
Benalaxyl	Chlorothalonil
Bitertanole	Captan
Boscalid	Cyprodinil / Fludioxonil
Copper hydroxide	Dithianon
Cyazofamid	Dodine
Cymoxanil	Famoxadone
Cyproconazole	Fluopyram
Cymoxanil	Fenhexamid
Dimethomorph	Folpet
Fenamidone	Kresoxim Methyl
Fenpyrazamine	Mancozeb
Fluazinam	Maneb
Fosetyl aluminium	Meptyldinocap
Iprodione	Metrafenone
Iprovalicarb	Metiram
Mandipropamid	Propineb
Mepanipyram	SB Plant Invigorator
Metalaxyl	Sulphur
Myclobutanil	Thiram
Penconazole	Trifloxystrobin
Potassium bicarbonate	
Propiconazole	
Proquinazid	
Pyrimethanil	
Quinoxyfen	
Spiroxamine	
Tebuconazole	
Thiophanate-methyl	
Triademenol	
Zoxamide	

Table 16. Compatibility of AQ10 in tank-mix with insecticides. The table shows examples of successful tank mixes and products which should not be tank mixed with AQ10 (Source: AQ10 technical document taken from UK manufacturer's website).

Active ingredients which can be tank mixed with AQ10	Active ingredients which are not compatible with AQ10
Bacillus thuringiensis (e.g., DiPel DF)	Savona
Beauveria bassiana (Naturalis L.)	SB Plant Invigorator
Pyrethrins	

Key points and Knowledge Gaps

- *A. quisqualis* within AQ10 can be efficacious across multiple species of powdery mildew, with no parasitism reported of other fungal groups.
- For successful germination and parasitism of powdery mildew, spores of *A. quisqualis* need high humidity or moisture; with efficacy decreasing rapidly below an RH of 90-95%, an optimal temperature around 25°C and the presence of a host.
- The maximum efficacious dose which should be applied to plants is no higher than $1x10^6$ cfu/ml, perhaps due to *A. quisqualis*' production of an unidentified self-inhibitor above this concentration.
- *A. quisqualis* has a long latent phase and in the presence of powdery mildew takes between 5 and 10 days to invade powdery mildew colonies on foliar tissues and complete its life cycle within the fungal host.
- *A. quisqualis* is compatible with a large number of chemical fungicides able to control powdery mildew, making it suitable for use in programmes with alternating use.
- Though the UK Registration Report for AQ10 states that without its powdery mildew host, viability of *A. quisqualis* is rapidly lost e.g., within a few days, the number of days as well as the rate of decline is not defined for particular crop situations.
- Though the maximum effective dose is known, there is no consistent information on the minimum effective dose. No public data (e.g., Registration Reports) is available on the minimum effective concentration of cfu/ml needed and this is important given the rapid decline in viable spore counts following application to foliage.
- The method of delivery of the product was given poor attention in the literature. In particular, there was limited detail available on parameters which can affect spray application of the product to foliage, such as nozzle type, droplet sizes, tank systems and operator pressure.
- There is conflicting evidence for any change in control with the addition of adjuvants. Further research is required using individual adjuvants to ascertain the nature of their activity: when, at what concentration and how they could be used with AQ10 to perhaps boost product efficacy of the product.
- Information is given on the AQ10 label of the different weight of product per hectare to be used for different height crops, but the instruction to apply it with sufficient water to ensure coverage of both leaf surfaces means there could be considerable dilution in tall crops. There was no AQ10 label guidance on water volumes, and further work is needed on individual crops across a series of growth stages to determine optimum water volumes for efficacious application of AQ10.

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Addendum

Search term logic and outputs for a search of Web of Science publications related to AQ10 and *Ampelomyces quisqualis* strain M10. The publication types searched for included articles, reviews, proceedings papers, book chapters, editorial material and meeting abstracts.

Searches	Results
Ampelomyces quisqualis	89
AQ10	30
Ampelomyces application	20
Ampelomyces quisqualis application	20
Population *Ampelomyces quisqualis*	18
Ampelomyces parasitism	13
Ampelomyces quisqualis parasitism	13
Humidity Ampelomyces	12
Temperature Ampelomyces	12
Environment Ampelomyces	9
Population dynamics *Ampelomyces quisqualis*	5
Survival *Ampelomyces quisqualis*	5
Foliar application *Ampelomyces quisqualis*	3
AQ10 application	3
Ampelomyces phyllosphere	2
Stability Ampelomyces	2
UV Ampelomyces	1

Summary tables of the environmental factors which may affect the efficacy and reliability of the bioprotectants Serenade ASO, Prestop and AQ10

The following table (**Table 17**) picks out the key environmental optimum ranges for the three bioprotectant products. Most regulatory records relate environmental conditions to soil or water (rather than foliage) as they are environmental risk assessments of survival / persistence, not efficacy related. Regulatory reports published on the web may list, but do not detail, the efficacy results from the dossiers of trials submitted to them for assessment.

Disparity between references exists with temperature and humidity ranges, usually because certain temperature points were tested and then the humidity prevailing recorded. In general, all three products grow fastest on plants in the warmer temperatures (around 25°C or a little lower) more likely to be found in heated glasshouses when not sunny, or on a sunny day outside in the UK. In such situations the humidity is unlikely to be high unless there is a mass of vegetation transpiring or water has been splashed, although warm air is capable of storing more moisture than cold. The three organisms all survive and grow better at high humidities, particularly *A. quisqualis* which needs a near-saturated atmosphere on the leaf surface while it enters its host. *G. catenulatum* and *B. subtilis* have a little greater tolerance down to around 80% RH. All three organisms lose viability over time under ultra-violet (UV-B 280-310 nm) light. This may be less important in protected crops, as polyethene tunnel covers often contain UV light-blocking components preventing transmission of 360 nm or less and the cut-off for greenhouse glass is usually 320 nm (Morandi *et al*., 2008).

A. quisqualis has the lowest persistence as a living colony on foliage of the three organisms reviewed; this fungus needs to enter a powdery mildew to feed, although it can then produce pycnidia that can survive without a host. Both *G. catenulatum* and *B. subtilis* can survive more widely in the environment, competing with the phylloplane microbial flora for nutrients, while also directly attacking by *G. catenulatum* penetrating fungal hyphae or *B. subtilis* using lipopeptides against microbe cell walls. In the current project in 2018 it was confirmed that at 25°C at high humidity, in the absence of a pathogen, viable colonies of *A. quisqualis* had declined rapidly by four days after leaf spraying, whereas *G. catenulatum* was still at a high level after a fortnight. *B. subtilis* remained at a similar level between the first and seventh day and a biofilm incorporating the bacteria then formed from colonies retrieved after ten and 14 days. When *A. quisqualis* and powdery mildew were inoculated on leaves parasitism was rare and slow to develop.

Growers need to use the information available to select a plant protection product most likely to manage disease in the conditions present in their crop.

Table 17 Parameters of importance for the survival and efficacy of Prestop, Serenade ASO and AQ10. References for each product are given in their individual review sections of this AMBER report

Parameters	**Prestop (fungus)** *Gliocladium catenulatum* strain J1446, now called *Clonostachys rosea*	**Serenade ASO (bacteria)** *Bacillus subtilis* QST 713 now called *Bacillus amyloliquefaciens*.	**AQ 10 (fungus)** *Ampelomyces quisqualis* strain AQ10
Temperature optima	20°C to 25°C. Helyer *et al.* 2014 give 15-25°C. Optimum for growth 25-28°C, efficacy in peat similar at 5-15 °C, 20°C & 25°C (2008 Registration Report)	25°C +/- 5°C. No negative impacts on product efficacy in controlling diseases anticipated under normal European growing season conditions (around 10°C to 30°C) (2001 Monograph)	25°C for germination & infecting (Registration Report 2008). Spores do not germinate at 37°C (EFSA 2004). The optimum temperature for spore germination and pycnidia formation by *A. quisqualis* strain AQ10 ranged from 15°C to 25°C. (EFSA Peer Review, 2017).
Period of optimum conditions needed for successful colonisation	Not noted as a factor. 70-90% of conidia germinate within 4-12 hours of foliage inoculation, penetrating the host after 16 hours (Sutton *et al.,* 1997).	Not noted as a factor. The bacteria remain on the leaf surface, they do not enter a host fungus to colonise.	High humidity needed while spore germination and hyphal penetration of the pathogen occurs over a period of 10 to 20 hours (Kiss *et al*., 2010)
Temperature range at which active i.e., colonisation spread.	6°C to 30°C. Helyer *et al.* 2014 give 5-34°C. Populations on leaves declined over 10 days at either 28°C & 34°C (Tut, 2019)	11°C to 52°C for laboratory culture of *B. subtilis in vitro.* Maximum 55°C for growth of QST 713. Grew at lowest temperature tested of 15°C (2001 Monograph, 2020 EFSA review).	Application recommended between 12°C – 30°C, as typically present early mornings / late evenings (Label information).
Lethal temperatures for survival	Survival noted below 6°C and above 30°C, but limits were not defined.	Lethal temperature not given for vegetative cells. Endospores can be produced when nutrients lack and endure temperatures over 80°C (2006 SANCO)	AQ10 packeted product not suitable for freezing (Technical Note). Lethal temperatures for inoculum not reported. Pycnidia formed in the host are more resilient and persist in the environment at least a year (Registration Report 2008)
pH	Optimum for growth pH 5.6, efficacy in soil similar at pH5.3-7.4. (2008 Registration Report). Conidia production by another strain greatest at pH 6.0-6.5 (Sun *et al*., 2014)	Growth within range pH 5.5 to 8.5 (2001 Monograph)	*A. quisqualis* strain AQ10 can grow within pH range 3–8, good growth & sporulation pH 5.5-7.0 (EFSA Peer Review, 2017).
Surface wetness	Not noted as a factor.	Not noted as a factor.	Not noted as a factor in efficacy

Parameters	**Prestop (fungus)** *Gliocladium catenulatum* strain J1446, now called *Clonostachys rosea*	**Serenade ASO (bacteria)** *Bacillus subtilis* QST 713 now called *Bacillus amyloliquefaciens*.	**AQ 10 (fungus)** *Ampelomyces quisqualis* strain AQ10
Wetness period tolerated before death	Survived 7 months in sea, lake, tap & distilled water at 8°C & 22°C (Registration Report 2008). In suspension viable up to 7 days at under 8°C, 24 hours at 20°C. 30 minutes soak of spores pre-use recommended by Lallemand (but Fargro Tech01/11 says no benefit from soaking).	Used via irrigation in USA, & in UK as a drench, so likely tolerant of being in water for long periods. The product is supplied in liquid formulation.	Only 24% of spores viable after immersion in water for 24 hours, 60-86% within first 6 hours (Registration Report 2008). The product is for foliar application, not drenching. Pre-spray soak 30-60 mins in water recommended (Fargro Tech 01/11 notes); the spores in the product are in a powder formulation.
Humidity optima for initial colonisation & further growth	85%-95% (60-80% in AHDB Factsheet). 95-100% stated, increasing population with increasing RH (Tut, 2019).	76% to 98% RH shown to result in good efficacy.	90%-95% needed for 48 h, with efficacy decreasing rapidly below this. 70% acceptable later. Susceptible to drying.
Humidity minimum for survival after spraying	Not defined. Lower humidity ranges usually in glasshouses at lower temperatures so viability inter-related (Tut, 2019).	Not mentioned in reports for vegetative spores. If endospores form, they survive in dry conditions.	Hyphae enter powdery mildew mycelium & depend on the host hyphae not drying out. Produces resting spores when the host is exhausted which do not require humidity.
UV	Increasing UV-B reduced presence & sporulation of *C. rosea* (Costa *et al*., 2012; Morandi *et al*., 2008).	Growing spores sensitive to ultra-violet light (2020 EFSA review)	Inactivated (2008 Regulatory Report, stated in relation to environmental risk).
CFU/ml giving efficacy	Product contains a nominal $2x10^8$ cfu/g product for use at a 0.5% concentration (5g/L water). 10^6 - 10^8 effective against onion Botrytis (Sutton *et al.,* 1997).	Product contains a minimum 1.05 x 10^{12} cfu/L. Serenade ASO at $1x10^8$ ineffective v watermelon powdery mildew (Becktell *et al.,* 2005), but reduced Xanthomonas on tomato (Abbasi & Weselowski, 2015)	Product contains 5 x 10^9 spores/g/. 2 up to 8 x 10^6 cfu/ml reduced grape powdery mildew conidia production (Legler *et al.,* 2016) Above 5 x 10^6 cfu/ml self-inhibition of germination in agar plates (Gu & Ko,1997).
Need for the presence of fungi or bacteria as food	It is a hyperparasite on fungi & competes for nourishment with other fungi (Product label).	Organic matter needed for growth, which can be from other bacteria, or fungi (Regulatory Report, 2008)	Spore germination requires host presence. Without host, viability rapidly lost within a few days (Registration Report, 2008).
Need for sugars or other nutrients	*G. catenulatum* isolated originally from Finnish field soil. It colonises plant leaves and roots, and so may utilize exudates.	*B. subtilis* reproduces under aerobic conditions. In the presence of glucose and nitrate anaerobic growth occurs (2001 Monograph).	No specific needs reported, other than requiring a host. Mycelium is grown on potato dextrose agar, but very slowly.

Parameters	**Prestop (fungus)** *Gliocladium catenulatum* strain J1446, now called *Clonostachys rosea*	**Serenade ASO (bacteria)** *Bacillus subtilis* QST 713 now called *Bacillus amyloliquefaciens*.	**AQ 10 (fungus)** *Ampelomyces quisqualis* strain AQ10
Persistence	4 weeks on foliage (EFSA). Decline to background levels over 3 weeks. AMBER project 2018 at optimum environmental conditions, showed viable spore counts on a tomato leaf increased up to a week after application and remained high to the last count after two weeks. A similar population was recovered from strawberry and lettuce leaves over eight days from application, with a decrease at the last measure at ten days (Tut, 2021).	Viability on foliage declines in pathogen absence. On pepper leaves colonies increased for 5 days, then declined sharply. Survival for 2 weeks in a glasshouse (Monograph 2001). Population decreased on lettuce over 10 days, then increased slightly. Poorer Botrytis control by Day 4 in tunnel, and by Day 6 in glasshouse (Tut *et al.,* 2021). AMBER project 2018 showed similar spore counts Days 1 to 7, with biofilm produced Days 10 & 14. Endospores form under nutrient shortage & environmental stress (Regulatory Report 2008). In a dry state, endospores can remain viable for years (2001 Monograph).	Unable to survive, grow & proliferate outside a powdery mildew host. Less than 10% germination in host absence. In host presence spore viability declines over a week. Lifecycle of 5-10 days. However, pycnidia in parasitized chasmothecia and thickened hyphae can overwinter. AMBER project 2018 showed viable spores on a tomato leaf declined to near zero within a week at optimum environmental conditions.
Storage	12 months below 4°C. 6 months below 8°C and 2 weeks at room temperature in unopened packet. Can be stored in a freezer once opened. 6 months at +4°C, 4 weeks room temperature (2008 Reg. Report)	Storage at room temperature for 2 years at 20°C, period is reduced by higher storage temperatures (label statement). Not to be stored above 25°C (2018 French label Regs.).	The product label says: Unopened packets need to be kept in a fridge 4°C - 8°C and can be stored for 2 years. 1 year storage at room temperature. Use by date on packet. Use up within 15 days once packet opened and put in fridge. Do not freeze.
Activity of other components in the product	Secondary metabolite production uncommon (Registration Report 2008). Product label says the fungus produces enzymes to break down fungal cell walls during its hyperparasitism process.	Contains a range of lipopeptides, from *B. subtilis* metabolism. Biofilms can be produced on surfaces, involving extra-cellular polymeric substances including lipopeptides with anti-fungal action (VKM Report, 2016).	Secondary metabolites with anti-bacterial activity produced by *A. quisqualis,* but not characterised (Mudgal *et al.,* 2013).

It is clear from the preceding literature that there are environmental and other conditions that growers, and those carrying out efficacy testing, need to be aware of as these affect the level of control achieved from the product. Growers, whether testing out bioprotectants or using them as an established part of their plant protection schemes, should keep records of both the conditions in the crop and how the incidence and severity of the target diseases change. The diagrams below give an outline of when and what records should be taken (**Figure 8).**

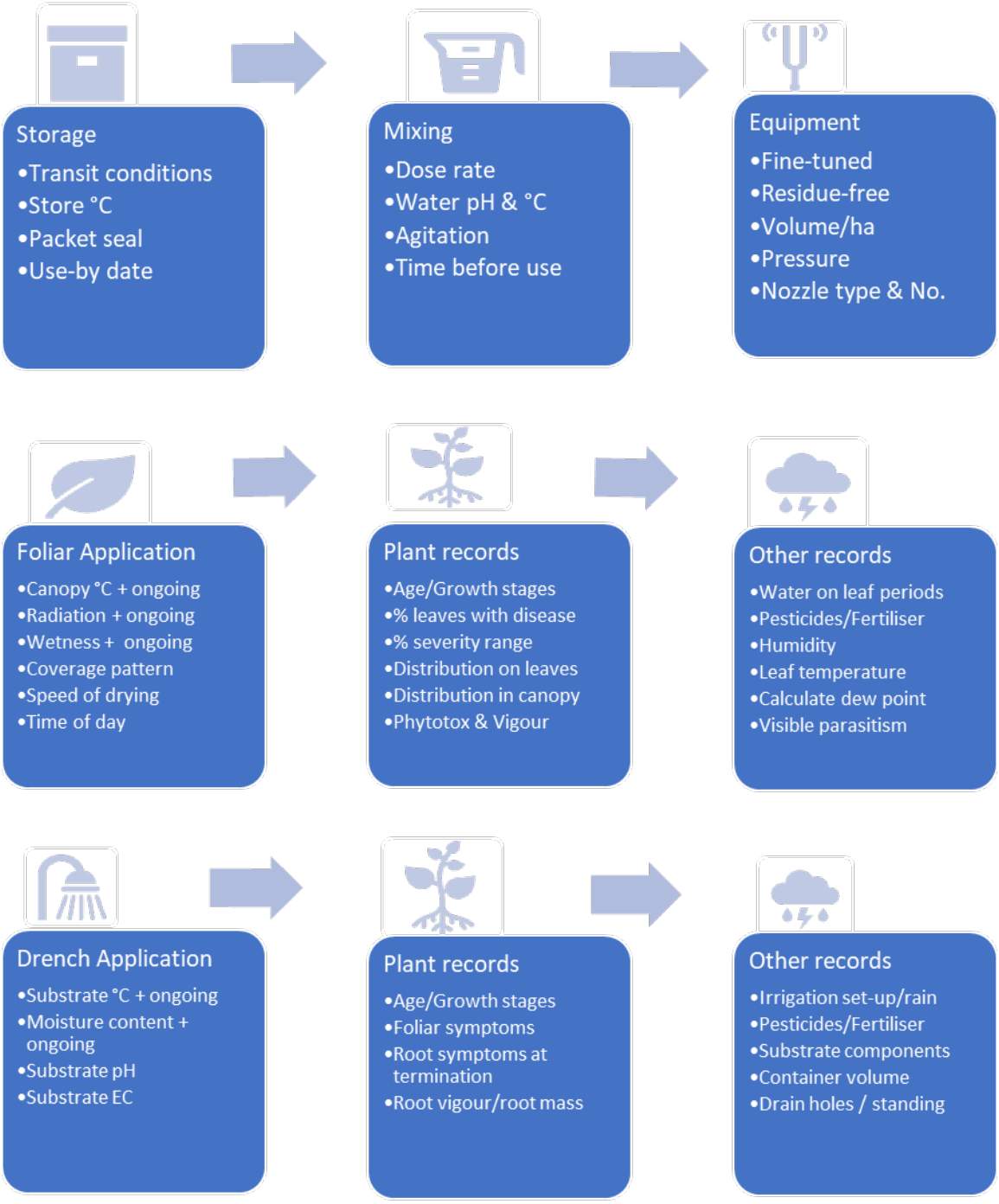

Figure 8. Summary of basic records to be kept to aid product use and monitor efficacy.

The specific environmental measurements which could be recorded at various stages in the crop are given in **Table 18** based on the information in **Table 17**. Recording is facilitated by digital devices and so loggers can be set up within the canopy and are able to send data wirelessly to a receiver station and thence into "The Cloud" to be picked up on computer or smart phone in "real-time" and then stored for later analysis. Such information can be used to determine the conditions before spraying, and potentially change conditions in a glasshouse via the environment control computer. Records related to product mixing and spraying equipment should also be kept. Details on the crop are also needed as given in **Table 19**.

On the next pages a recording sheet has been devised as a template of what should be recorded when bioprotectants are used. This is based on the details required for ORETO standard efficacy trials and is a modification of that used for the AHDB Horticulture SCEPTRE Plus experiments that were contributing to the granting of Extensions of Authorisation for Minor Use (EAMUs) by the Pesticides branch of the UK Health and Safety Executive (formerly known as PSD or CRD). Some of the details, for example on spray equipment or time of day, may also be recorded elsewhere, but having the information in one place will aid evaluations.

In contrast to label approvals, where use is registered for particular crops for named diseases, EAMUs often list a number of crops and do not require each of these to have been tested to check either for phytotoxicity or product efficacy. It is thus important that growers using bioprotectants (and chemical products) under an EAMU have a procedure in place for recording how they used the product and in what conditions, plus make (and keep records of) observations on both crop and variety safety and the level of disease control achieved.

After the recording template, two examples are given of experiments for AHDB that included foliar application of either AQ10, Prestop or Serenade ASO. The AQ10 and Serenade ASO against hawthorn powdery mildew, were originally under codes because they were being trialed under Experimental Approvals. Prestop tested against grey mould on cyclamen was an on-label use. These are included to highlight that when receiving information on product efficacy from suppliers or advisers that it is particularly important with bioprotectants to know the details of application procedure, what the environmental conditions were and the starting level of disease. Efficacy trials usually include chemical plant protection products applied to other plots concurrently to compare efficacies without any consideration given to carrying out the test in more-optimum environmental conditions for the bioprotectant. The efficacy of the bioprotectants may thus be recorded as not as good as the chemical product, whereas in a commercial situation using the bioprotectant alone it could be possible to alter the time of day

the spraying is done or to manipulate the environment to favour the survival and growth of the beneficial bacterial or fungal colonies that the product contains.

Table 18. Environmental parameters to be recorded before, during and after the use of bioprotectants that will have a bearing on their efficacy.

What to measure & units	In product storage	Application day	Immediately post-application	Ongoing
Air Temperature °C	Record from beside product from reception and check records from fridge, freezer, or shelf for issues before product use. Temperature threshold registering labels on purchase products would be beneficial.	Check weather forecast pre-spraying. Check temperature in glasshouse ideally from a logger at canopy height in a shaded screen - Wi-Fi linking allows real-time checking from elsewhere. Ensure tank mixing water is within optimum temperature range.	Continue canopy & leaf measurements, potentially every 30 minutes for first 3 days (72 hours) after any biofungicide application	Canopy & leaf measurements hourly for rest of production cycle thereafter.
Substrate Temperature °C	n.a.	Bury a logger & have a probe with a read-out visible to check temperature before drenching.	Hourly logger records. Pots in differing sun exposure may need separate loggers.	Hourly records to continue.
Radiation [PAR] (µmol $m^{-2}s^{-1}$)	n.a. unless products are not stored away from the sun.	% Cloud cover at start and finish and note sunshine or light quality.	GH computer roof records may be adequate ongoing.	GH computer roof records may be adequate ongoing.
Photoperiod (h)	n.a.	Standard tables OK for daylength. Record the time of day at the start and end of application.	Standard tables can be used for daylight hours.	Standard tables can be used for daylight hours.
Atmospheric moisture, relative humidity, or vapour pressure deficit (VPD) (% or kPa)	Powders may instruct not to store once opened (as spores hydrate). Check part-used packets are well sealed/in a bag/box	Note any condensation/dew. Note pattern across canopy – water volume should be calculated to give good coverage. Install a RH logger in the canopy.	Ongoing hourly. On exiting crop, note speed of drying. If outdoors, note any rainfall within next 12 hours. Indoors record next irrigation time.	Ongoing hourly. In fast-growing crops add another logger in newer growth. Check records for the duration of low humidities.
pH	n.a.	Mixing water should be tested & source noted.	n.a.	n.a.
Electrical conductivity [EC] (S m^{-1})	n.a.	Relevant when drenching. Record in containers or hydroponics.	n.a.	Determine a final measurement.
Nutrient – liquid (mmol L^{-1}) or solid (mol kg^{-1})	n.a.	Dose rate and content of feed should be recorded.	n.a.	Ensure any changes are recorded
Watering (Litre)	n.a.	Substrate should be moist if drenching. Note time of last watering or rainfall.	Record irrigation re-start & volume per pot. Record wetness period length. Ideally use a soil and a leaf wetness logger in the crop.	Note automatic set timings and volumes. Check irrigation is even across area. Continue records throughout the crop life.

Table 19 Records to complete on plants & growing situation when plant protection products are used to be able to assess & compare results

What to measure	What to include
Glasshouse/tunnel/cabinet or outdoor site details	Latitude & longitude. Orientation of long axis relative to compass North. Area measurement. Gutter and peak heights of glasshouse. Manufacturer. Glazing material. Shade/night break curtains. Vent type & mode of operation. Heating system. Watering system. Flooring. Surrounding crops or vegetation. Lighting type & if daylight sensing or timed.
Number of plants per unit	Plant spacing (centre to centre, or gap between pots) and any re-spacing.
Pot volume (L) & width (mm)	Report manufacturer's name and name of the product. If a multi-celled tray, note number of cells per tray.
Container construction	Type of plastic, clay, coconut-fibre. Note container colour, as this could affect heating by sunshine.
Pot drainage	Hole arrangement in base, any legs. If stood in a saucer.
Soil / substrate or hydroponics	Give soil type. Substrate should have % components.
Species and variety of plant	Note why this was selected e.g., susceptible to disease, leaf texture or canopy architecture.
Source	Seed or vegetatively propagated & location. If not propagated on-site aim to obtain husbandry history especially PPP.
Plant passport details	Original source grower and any in between and arrival date on current nursery.
Potting on date	Note also previous container type/size if any e.g., plug, module, or bare root.
Age of plant	If not known give date arrived on nursery.
Growth stage at start and at each recording	Use BBCH keys or utilize one for a similar (stated) species. Record both leaf growth and flower development stages. If there is variation, state extremes and most common. Photograph regularly. For height/spread have a measure in view.
Deficiencies or phytotoxicity	Try to identify cause and assess incidence & severity. Photograph.
Pests	Identify. Use ORETO guidelines to record incidence and severity of each life stage. Distribution on plant (height, under leaves, in centre of plant) and distribution within crop (edges, centre, patchy, isolated pots).
Pest survival	Note dead/parasitized pest incidence and proportion. Distribution on plant.
Other e.g., biocontrol agents	Identify & note if natural colonisation or released (and density and date/s of release).
Disease	Use ORETO guidelines. Record incidence and severity. Distribution on plant (height, under leaves, in centre of plant) and distribution within crop (edges, centre, patchy, isolated pots).
Pathogen parasitism	Measure area of any parasitism e.g., by *A. quisqualis* and record % of total disease area affected.

AMBER Recording sheet for monitoring of bioprotectant efficacy

Site:

Item	Details
Location	
Bed number*	
Crop	
Cultivar	
Container size (width & volume)	
Soil / substrate type	
Irrigation type & timing details	
Row spacing	
Area sprayed (width x length)	
Number of plants sprayed	
Note any area left untreated	

* There should be a map of the bed position & a record of the type of structure & flooring in place

Observations:

Disease name:			
Score date*	**Proportion of plants diseased %**	**Severity range on affected plants % x – x%**	**Commonest disease severity %**

* Records to commence before disease is seen and to be repeated prior to each spray application and to continue after the last application.

Phytotoxicity:			
Score date*	**Proportion of plants affected %**	**Describe symptom e.g., yellowing / stunting**	**Is it getting worse Y/N?**

Date	Comments

Products:

Product	Active ingredient	Batch number	Purchase date	Expiry date

Record all products of any type applied to the crop. Include a separate listing for any adjuvant added, or any other products used in a tank mix

Product	Used Application Day A Y / N ?	Used Application Day B Y / N ?	Used Application Day E Y / N ?	Used Application Day D Y / N ?

Application details:

Details at each timing	Application A	Application B	Application C	Application D
Application date				
Initials of spray operator				
Spray tank & hoses cleaned Y/N				
Product dose				
Application water volume / ha				
Minutes product left to soak				
Application type (spray/drench)				
Total coverage intended Y/N				
Application equipment				
Nozzle pressure				
Nozzle type				
Nozzle size				
Nozzle number				
Boom height from canopy				
Any spray run-off Y/N				
Poor coverage noted Y/N				
Any other problems?				

Crop Environment at Application:

Parameters	Application Day A	Application Day B	Application Day C	Application Day D
Application date				
Crop growth stage (max BBCH)				
Crop growth stage (min BBCH)				
Crop growth stage (mean BBCH)				
Crop height (cm)				
Hours since plant was watered				
Time at start (24 h clock)				
Air Temperature at start (°C)				
Humidity at start (% RH)				
Time at finish (24 h clock)				
Air Temperature at finish (°C)				
Humidity at finish (% RH)				
Dew / water presence (Y/N)				
Wind speed range (m/s)				
Cloud cover (%)				
Sunshine (Strong / Weak / None)				
Soil Temperature 5 cm (°C) or n/a				
Soil moist Y/N or n/a				
Temperature of air - shade (°C)				
Relative humidity (%)				

Crop Environment later on Application Day:

Parameters	Application Day A	Application Day B	Application Day C	Application Day D
Date				
Time of day (24 h clock)				
Air Temperature				
Sunshine (Strong / Weak / None)				
Humidity (% RH)				
Dew / water presence (Y/N)				
Soil moist Y/N				

Crop Environment the day after Application:

Parameters	Application Day A	Application Day B	Application Day C	Application Day D
Date				
Time of day (24 h clock)				
Air Temperature				
Sunshine (Strong / Weak / None)				
Humidity (% RH)				
Dew / water presence (Y/N)				
Soil moist Y/N				

Evaluation of nursery site experiments with AQ10, Prestop or Serenade ASO

Examples of how methodology & conditions may affect control levels reported

The decision support flow diagrams shown earlier highlighted aspects that should be checked on to get the best results from bioprotectants. This information can be used when assessing the validity of reports of either efficacy or control failure of products. Reviewing full trial reports is good training for anyone using bioprotectants as it increases awareness of what should be noted, so that when efficacy has been high in one's own nursery situation then it is possible to aim to duplicate the "set-up" so that the efficacy can more-reliably be repeatedly achieved.

The focus of this AMBER project has been foliar application. Prestop and Serenade ASO testing in most AHDB funded trials has been as drenches against root pathogens. Two examples of foliar applications have been found on the AHDB website for evaluation here. Their conclusions have been brought to the front and a series of challenges/questions tabulated as a guide to what should be looked for in the following extracts of the results. This should help to determine whether the products have had a "fair trial" and to check the validity of the conclusions by understanding what was or was not done to get the efficacy reported.

CP 124 Managing ornamental plants sustainably (MOPS). Annual report, Dec. 2014.

https://ahdb.org.uk/cp-124-managing-ornamental-plants-sustainably-mops-developing-integrated-plant-protection-strategies

Aim

To identify novel biological and conventional products with activity against powdery mildew (*Podosphaera clandestina*) on hawthorn (*Crataegus monogyna)* and define their performance in relation to standard treatments.

This trial included AQ10 (coded 11) and Serenade ASO (coded 38) as "straight" treatments in comparison with water sprayed untreated plants and a chemical standard (Signum).

Conclusion to be considered from Report

Powdery mildew was not observed until the day of the fifth bioprotectant applications on 26 June 2014 and increased during July while applications continued, and beyond.

Prior to the completion of treatment applications, all treatments showed significantly less powdery mildew cover than the untreated control, with the experimental chemical treatments showing equivalent levels of mildew to Signum. The four biological / alternative products showed good efficacy at low disease levels but were unable to maintain control as the infection progressed. Ten days after their applications ceased, Serenade ASO treated hawthorn had slightly less mildew than AQ10 but both infestations were severe.

Method

The trial was carried out on naturally infected field-grown rows of first-year hawthorn seedlings at a nursery (J & A Growers Ltd). Each plot consisted of a foliar sprayed 5-row 4 m bed length of seedlings, with the central 2 m assessed.

Products were applied over a period of eight weeks to a randomised block design with six fold replication. Each product was re-applied to the same plots, i.e., there was no product alternation. The chemical fungicides were sprayed four times at fortnightly intervals and the other products were applied eight times at one week intervals, all at 400 L water/ha.

AQ10 was applied at 0.07 kg/ha and Serenade ASO was applied at 8 L/ha. The producer of AQ10 requested it was tested without the use of a wetter. However, at the supplier's request Silwett 77 at 0.2 L/ha was added to the tank with each Serenade ASO application and so the 10 L/ha rate of Serenade ASO (standard at that time) was lowered.

Applications started on 30 May 2014 at the two true leaf stage prior to visible infection being observed with an air-assisted back-pack sprayer operating at 2 bar with 1 m spray boom width for Application 1, but thereafter 1.5 m (03-F110 nozzles at 0.5 m spacing). Assessments of % powdery mildew cover and phytotoxicity were made weekly and continued after the final spray to determine contact and persistence attributes and any effects on vigour.

Results

Records made at each application day and up to three weeks later are given in **Table 20.**

Discussion on validity of efficacy Conclusion

Table 21 picks out actions and conditions that could have affected the trial outcome. The products were used as intended; preventatively. There was a gradual natural build-up of powdery mildew, rather than a swamping by inoculation, and AQ10 was re-applied once there was the host mycelium that it needs available. The warm temperature for the *A. quisqualis* and *B. subtilis* colonisation was good. Humidity at the time of application was well below optimum so this is likely to have limited their success, with AQ10 label guidance to spray early or late evening not followed. Two more applications than permitted were used for Serenade ASO and there was added wetter. Both products significantly reduced powdery mildew, statistically similar to the chemical standard, however Signum left only a trace of mildew.

Table 21 Some suggested challenges to assess the validity of the Conclusions on product efficacy within AHDB project CP124 (MOPS).

Challenges/queries on conditions in CP124	Information related to AQ10	Information related to Serenade ASO
Product has label use against p. mildew?	Yes	Botrytis & Sclerotinia on UK label
Use outdoor stated on UK label?	Protected crops only on label.	Protected crops only on label.
Dose used of 0.07 kg/ha; as label?	Yes, 0.07kg strawberry (0.035kg tomatoes).	8 L /ha currently - should lower with wetter.
Water of 400 L/ha used; as label?	Good coverage, no volume range given.	200 -1000 L possible to give good coverage.
Same spray volume/ha applied throughout?	Yes, but plants grew from 0.5 to 2.0 m tall over the period suggesting poorer coverage.	
Application interval used of 7 days; as label?	7-10 days, 2 successive, max 12 times/crop.	Yes, a min. of 5 days, but max. 6 times/crop.
Wetter used?	No, but 2017 label mentioned Nu Film P.	Yes, but not mentioned on label.
Time of day applied?	Either mid-morning or mid-afternoon, so drying likely to have been quick but not recorded.	
Air Temp. at application; optimums are 25°C	20-26°C at later applications when mildew was present, so within AQ10 germination range.	
Good high humidity at application ?	All but first spray was at humidity below 60%, so not conducive to colony germination.	
Rain or irrigation within first 24 hours?	Dry at application, but only monthly figures subsequently.	
Mildew present at application?	First trace prior to 5th spray, so early AQ10 could have had poor survival without a host.	
Natural infection or inoculation?	Natural, gradual increase some weeks after first product use, allowing protectant effect.	
Max. mildew % in untreated damaging?	10%, which would be obvious, but vigour differences only reported a month after sprays.	
Max. mildew % compared to standard?	AQ10 and Serenade held mildew at 5% and 3% respectively when 0.5% by Signum.	

Table 20 CP124: Conditions at Application and powdery mildew severity. AQ10 and Serenade ASO were applied to hawthorn on all dates, chemicals on Days A1, A3, A5 and A7.

	Application Days				
	A1	**A2**	**A3**	**A4**	**A5**
Date	30.05.14	06.06.14	13.06.14	20.06.14	26.06.2014
Growth stage (leaves)	2	4 - 6	8	6 - 8	8
Height (mm)	50 – 100	50 – 150	120 – 150	150	150 mm
Time of day (24 h)	08:15 – 11:15	08:50 – 9:30	10:30 - 12:00	15:45 – 16:45	14:05 – 15:20
Air Temp. range (°C)	14.8 – 17.1	15.9 – 16.1	19.1 - 20.7	21.6 – 22.1	20.6 – 20.8
Humidity range (% RH)	80.1 – 80.2	45.1 - 46.0	45.6 – 45.8	37.1 – 37.2	46.9 – 47.1
Cloud cover (%)	cloudy	50%	20%	50%	75
Crop comments	Dry leaves	Dry leaves	Dry leaves	Dry leaves	Dry leaves
UT* Mildew %	0.00	0.00	0.00	0.00	1.00
Signum Mildew %	0.00	0.00	0.00	0.00	0.00
AQ10 Mildew %	0.00	0.00	0.00	0.00	0.33
Serenade Mildew %	0.00	0.00	0.00	0.00	0.00
F value (40 df)	-	-	-	-	n.sig.
Least sig. difference	-	-	--	-	0.717

*UT = Untreated with any product

	Application Days				
Application No.	**A6**	**A7**	**A8**	**Post-spray**	**Post-spray**
Application date	04.07.2014	11.07.2014	18.07.2014	01.08.14	08.08.14
Growth stage (leaves)	8 - 9	10 - 12	12 - 16	12 - 30	15 - 40
Height (mm)	150 mm	200 mm	200 mm	-	-
Time of day (24 h)	10:30 - 11:00	9:30 - 10:45	16:00 - 16:50	-	-
Air Temp. range (°C)	21.1 – 22.2	22.1 – 22.3	26.0 – 26.0	-	-
Humidity range (% RH)	59.1 - 61.2	48.2 – 49.1	53.0 – 53.0	-	-
Cloud cover (%)	50	50 - 80	30	-	-
Crop comments	Dry leaves	Dry leaves	Dry leaves	-	-
UT Mildew %	6.33	10.50	10.50	74.17	81.67
Signum Mildew %	0.33	0.50	0.50	23.33	52.50
AQ10 Mildew %	1.17	4.50	4.50	60.83	70.83
Serenade Mildew %	1.17	2.83	2.83	49.17	64.17
F value (40 df)	<0.001	<0.001	<0.001	<0.001	<0.001
Least sig. difference	1.970	3.255	3.255	11.650	13.770

CP 158 AMBER project. Annual report dated 2017.

https://ahdb.org.uk/cp-158-application-and-management-of-biopesticides-for-efficacy-and-reliability-amber

https://projectblue.blob.core.windows.net/media/Default/Research%20Papers/Horticulture/CP%2015 8_Report_Annual_2016.pdf

Aim

To carry out a benchmarking study to evaluate the effect of Prestop (*Gliocladium catenulatum)* on Grey mould (*Botrytis cinerea*) on cyclamen in commercial production, using nursery spray equipment and comparing against the standard nursery chemical programme.

Conclusion to be considered from Report

Application of Prestop at three-week intervals reduced the incidence and severity of Botrytis on the leaves of cyclamen more than an alternating spray programme of Amistar and Rovral WG at the same application intervals. Neither treatment programme stopped Botrytis appearing, and sporulating, on over half of the plants.

Method

The trial was carried out on a naturally infected polythene tunnel grown crop of cyclamen cv. Picasso Verandi potted at the end of May 2016. Pots were on the ground on capillary matting and watered onto the matting. They were held in position by six-hole carry-trays 390 mm x 280 mm, with three plants arranged in alternate holes (so that pots were about 100 mm apart).

Beds of 20 x 72 pots (3 m x 10m) were either side of a pathway, one bed for chemical treatment, the other for Prestop (**Table 22**). The minimum 21 day interval specified for Prestop was used, and the nursery's standard programme of an alternation of Amistar and Rovral WG at 21 day intervals was adopted. There were no untreated plants. Replicate blocks of the treatments were not set up because the spray equipment was not suitable for small plots. Application was made by nursery staff using the nursery's Brinkman 250 sprayer which had RIPA adjusted to deliver 7 L / minute at 15 bar pressure.

Prestop was stored in a fridge at the nursery before use. The powder was left to soak in tap water (pH 7.4) for 30 minutes before use. Its application rate followed label instructions to apply at high volume to just before run-off, ensuring thorough coverage of the crop. Before using the sprayer for Prestop the grower was asked to clean out his spray tank. Once the Prestop had been sprayed, the tank was washed out again and another area of crop sprayed with the chemical fungicide to flush out any residues before spraying the monitored bed.

Symptoms were not visible looking from above the crop so 25 plants were selected at random from along each bed and picked up to examine the leaves and petioles for any Botrytis visible

(soft brown rot and/or sporulation), in particular those leaves resting on the growing-media.

Table 22 Treatments, rates and spray dates in AMBER nursery trial on cyclamen

T	Products	Active ingredient	Rate / 100 L water	Application dates			
1	Prestop	*Gliocladium catenulatum*	500 g	not used	12.07.16	02.08.16	24.08.16
2	Amistar	azoxystrobin	100 ml	not used	12.07.16	not used	24.08.16
2	Rovral WG	iprodione	67 g	28.06.16	not used	02.08.16	not used

Results

Records made at each application day and up to three weeks later are given in **Table 23**. It was noted that, with the standard walking speed used by the nursery spray operative, puddles of the spray solution were held in the cups of some leaves for at least an hour.

At the assessment before the second Prestop spray on 2 August, the Prestop sprayed had half the number of affected plants, with both treatments having on average one leaf with symptoms. By 21 days after the last applications, more plants had Botrytis following chemical use (mean 2.5 out of around 45 leaves/plant affected), whereas Prestop plants had 1.4. On the affected plants only four of the 25 Prestop treated plants had Botrytis rot progressing back from the leaves into the petioles, whereas 13 chemically treated plants had softened petioles.

Discussion on validity of efficacy Conclusion

Table 24 picks out actions and conditions that could have affected the trial outcome. Prestop was used preventatively as the label, with natural Botrytis infection. Both programmes used the same spray intervals. The temperature was favourable at applications (*G. catenulatum* germinates well between 15-25°C). The crop canopy was above the minimum optimum humidity of 60% for its growth for the five hours after application (germination takes place within the first four to 12 hours). As temperatures rose the humidity fell below optimum in the afternoon on the 12 July and 24 August (but remained ideal for 24 hours after 2 August spray (**Figure 9**). The report noted that on 12 July the optimum humidity would have been held for longer if applications had taken place after 18:00h.

The report conclusion correctly stated that neither programme stopped Botrytis appearing, but there was no statistical evidence to support better efficacy of the bioprotectants. The 7 and 15 affected plants out of 25 for Prestop and chemicals, respectively could have arisen by chance, however the second assessment using a different 25 plants also showed fewer plants with Botrytis after Prestop than the chemical programme. Replicated testing is needed.

Table 23 CP124: Conditions at application and Botrytis incidence. Prestop was applied to cyclamen on all three dates. Chemical treatment given on these dates and 21 days before.

	Prestop Application Days			
Crop records	**A1**	**A2**	**A3**	**Post-spray**
Application & Score Dates	12.07.16	02.08.16	24.08.16	08.09.16
Growth stage	Pre-flower	Pre-flower	Pre-flower	In flower
Application start time	08:45	08:39	Not noted	-
Daily Air Temperature °C*	12 - 25	15 - 25	15 - 37	15 - 30
Daily % Relative Humidity	53 - 90	77- 99	55 - 95	35 - 98
Cloud cover	overcast	not noted	not noted	-
UT, Botrytis %**	No UT	No UT	No UT	No UT
Chemical: Botrytis %incidence	0	60	No record	84
Prestop: Botrytis %incidence	0	28	No record	56

* approximate reading from logger chart in the report for 12.07.16 to 07.09.16
**UT = untreated with any product

Table 24. Example challenges to the validity of the Conclusions on Prestop efficacy

Challenges/queries on conditions in AMBER	**Information related to Prestop**
Product has label use against Botrytis?	Yes.
Use indoors stated on UK label?	Yes, protected edibles & ornamentals.
Dose used of 0.5% as label?	Yes, so dilution of colony count was constant.
Water as label, to just before run-off?	Yes, at high volume, but pooling & run-off.
Same spray volume/ha applied throughout?	Yes. Wastage between early rosettes noted.
Application interval used of 21 days; as label?	Yes, repeat at 3-4 week intervals directed
Time of day applied?	First task in the morning.
Air Temp. at application; optimum 25°C	Yes, good although warmer later one day.
Good high humidity at application?	Yes, but “fell off” except for one application day
Rain or irrigation within first 24 hours?	Bottom irrigation was used.
Botrytis present at application?	No, but expected as crop canopy closes.
Natural infection or inoculation?	Natural.
Max. Botrytis % in untreated damaging?	No untreated. Any incidence a problem.
Max. Botrytis % compared to standard?	Prestop 28% & 56% v 60% & 84% chemical

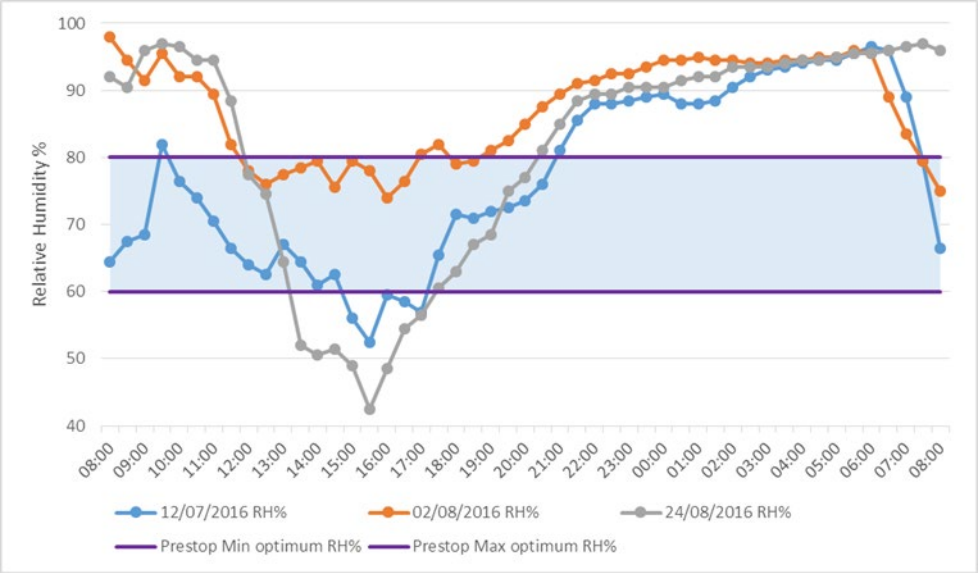

Figure 9. Relative humidity in the cyclamen canopy on spray dates within the AMBER benchmark trial, showing a fall from around midday to 16.00h. Spraying between 08:00h to 09:00h was within the 60% to 80% humidity optimum for Prestop colony germination.

Discussion

The reviews carried out on the factors affecting the efficacy of the three microbial bioprotectant products show that the conditions at application will affect the level of control achieved to a much greater extent than any chemical plant protection product. This reflects the fact that the bioprotectants are living organisms with particular requirements for, and a range of tolerances of, physical conditions for their growth and reproduction. Good spray coverage becomes a greater issue as all of the agents work through contact action, There is a lack of publicly available efficacy information from commercial crops. The modes of action of bioprotectants are not the same as chemical products and so comparisons of efficacy will rarely rank bioprotectants highly. More work is needed to ensure the bioprotectants are only put on in the conditions that they need. Their use needs to be integrated with other measures such as variety selection and decision support based on environment and pathogen monitoring. Assistance to growers on bioprotectant positioning within chemical spray programmes is also needed, based on product compatibilities and the levels of various diseases that they can applied to with the confidence that the pathogen will be adequately managed.

Conclusions

- More attention should be given to environmental conditions before deciding to apply bioprotectants, with monitoring and any adjustments continuing subsequently.
- Efforts need to be made to ensure spray coverage is as good as possible.

- More information is needed on the colony counts/unit of leaf area required for control.
- Awareness of the conditions favouring disease and frequent monitoring for disease is required to ensure bioprotectants are applied in good time.

Objective 2 (continued): Develop and demonstrate management practices that can improve biopesticide performance.

2.2.3b. Determine the effect of environmental factors on activity of biopesticides

Introduction

Nearly all plants, invertebrates and microorganisms (including the microbes used as biopesticides) are ectothermic, meaning they do not generate internal heat through metabolism, and therefore their internal body temperature is determined by the temperature of their environment. As a result, the rate of their physiological and biological processes is also determined by environmental temperature. For microbial biopesticides, this includes characteristics such as the rate of spore germination, growth, infection and reproduction – all of which may be important to performance as a microbial control agent (Chandler, 2016). Any change in environmental temperature causes an equal change in internal temperature with concomitant effects on performance. This contrasts with endotherms, which generate internal heat through metabolism (e.g., birds and mammals), and whose body temperature is largely independent of environmental temperature.

Environmental temperature has a profound effect on microbial biopesticide activity. It is important that biopesticide companies choose strains that are able to function well under the temperature conditions within the crop, while growers and agronomists need to be given reliable information about the thermal performance of the strains used in commercial products. If a biopesticide is developed by a company using a strain that has been selected using unrealistic, room temperature conditions rather than the more demanding conditions that the agent is exposed to in the glasshouse or field, then the strain will not perform well in commercial practice. However, most biopesticide companies do not provide detailed information about the thermal biology of their products.

For this part of AMBER, we investigated the thermal biology of different species and strains of entomopathogenic fungi, EPF, with the aim of providing new insights and helpful information that could improve their use. Researchers conduct experiments on the thermal biology of microbial biopesticides by measuring the performance of the agent at different constant temperatures and then analysing the data using statistical techniques. Performance measures include variables such as in vitro growth or spore germination rate: these can be done in petri dish experiments in the laboratory and have the advantage of being relatively quick and easy. Measuring the effect of temperature on efficacy is more complex: it is usually done as a bioassay, in which the biopesticide is applied at a defined dose against the target

pest / disease on a host plant under controlled conditions in a laboratory incubator or a controlled environment room and its effects on the target are measured over time. Bioassays are more representative of the biological and environmental complexity that occurs in the glasshouse or field, but they require more resources and take longer.

Performance metrics can be presented in different ways. The simplest method is to display the performance variable at the discrete temperatures used in the experiment, in a table or bar graph (Fargues et al., 1997; Vandenberg et al., 1998) (see **Figure 10**). In this case, temperature is treated as a categorical variable, and analysis of variance tests can be done to look for significant differences between the performance metrics. This is straightforward and can provide useful information, for example you can identify which discrete temperature produced the highest performance level.

An alternative is to use the data to construct a mathematical model (i.e., an equation) of the effect of temperature on the performance variable. The results can be visualised as a thermal performance curve, TPC (**Figure 11**, taken from Krenek *et al*., 2012). In this case temperature is analysed as a numerical variable.

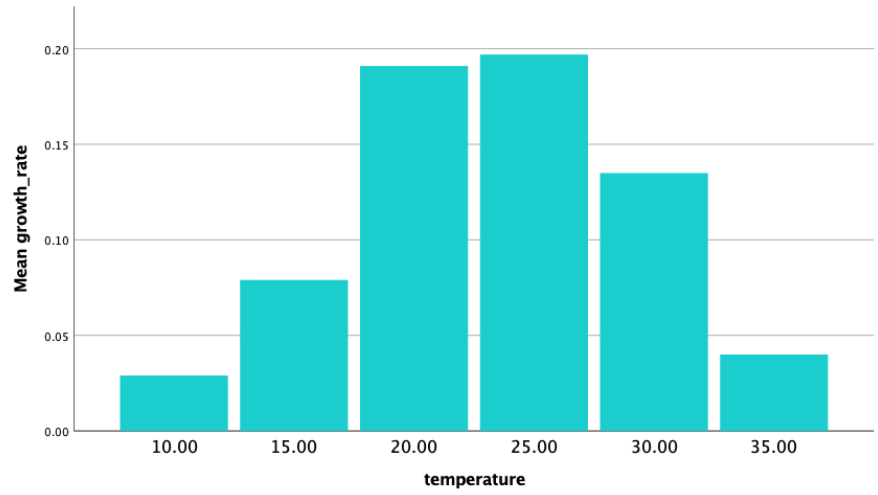

Figure 10. Effect of temperature (discrete temperatures) on mean in vitro growth rate of the entomopathogenic fungus *Metarhizium brunneum* strain 275.86, represented as a simple bar graph. This fungus is used in the commercial biopesticide Met52.

TPCs are a step on from treating temperature as a categorical variable, as they have greater predictive power. A thermal equation allows performance to be estimated for any temperature within the performance range, not just the temperatures used in the experiment. By convention, the main estimates used are the lower and upper critical thermal limits (T_{min} and T_{max}), which represent the minimum and maximum temperatures respectively under which

the organism is able to perform, and the temperature for the highest performance level, the optimum temperature, T_{opt}. These three values (T_{min}, T_{max}, T_{opt}) are taken as cardinal temperatures. TPCs for ectotherms typically take the form of a normal (bell-shaped) distribution skewed to the left, in which performance increases in a curvilinear way from T_{min} to T_{opt}, and then drops steeply as temperature increases from T_{opt} to T_{max}. (**Figure 11**) (Golizadeh et al., 2007, Marchioro and Foerster, 2011) (you can actually see this skewness in the bar graph of **Figure 10**). This means that small increases in temperature past the optimum can result in relatively large decreases in performance, which has obvious implications when thinking about using biopesticides at high temperatures. The thermal tolerance breadth (T_{max} - T_{min}) gives the temperature range over which the organism can function.

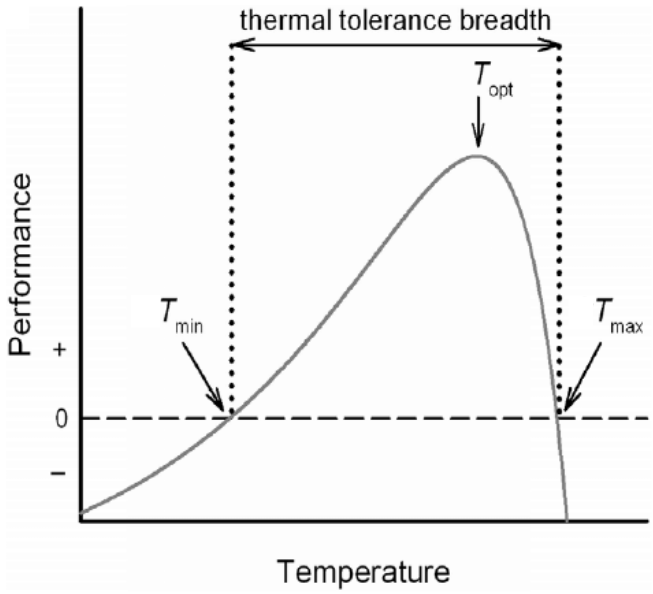

Figure 11. Generalised thermal performance curve (TPC) for an ectothermic organism (taken from Krenek et al., 2012). The graph shows the relationship between organism performance (Y axis) and environmental temperature (X axis). Performance includes life history traits such as survival, growth and reproduction and the biochemical / physiological processes that underpin them. The curve itself is derived from a mathematical equation of the effect of temperature on organism performance. Details in the text.

Because the TPC is skewed, it's not appropriate to use a normal distribution model to describe the curve, as this will give a poor fit and will underestimate T_{opt}. Fitting data to a skewed distribution is technically difficult, but several different mathematical models are available, developed originally to describe the effect of temperature on the growth of bacteria (Ratowsky

et al., 1983) or arthropods (Logan *et al*., 1976; Lactin *et al*., 1995; Briere *et al*., 1999). In principle these could have general application for other types of ecothermic organism although they are not used yet to any extent for microbial biopesticides. The data generated from an experiment (e.g. measurements of organism growth rate at different temperatures) are fitted to the model using statistical techniques, and different models can be compared using goodness-of-fit criteria.

Although fitting these models can be difficult, they are worth investigating, since a good understanding of the effect of temperature on microbial biopesticides is clearly important to getting the best out of them for plant pest / disease control. Having accurate, precise values for T_{min}, T_{max} and T_{opt} (and knowing the thermal tolerance breadth T_{max} -CT_{min}) is helpful for choosing the right strains of microbial biopesticide that can perform at the environmental temperatures under which the crop is grown. A difference in the TPCs of the biopesticide and its target pests / diseases (which, of course, are also ectothermic) might explain why a biopesticide can work well under one set of temperature conditions, but not perform under another (Thomas & Blanford, 2003).

Only a small number of investigations of TPC models have been published for microbial biopesticides (Davidson *et al*., 2003; Smits *et al*., 2003) and hence we felt it was something that needed to be addressed in AMBER. For this part of the project, we evaluated different TPC models to see how useful they could be for understanding biopesticide performance at different temperatures. We used a data set on the thermal biology of different fungal pathogens of the diamondback moth, *Plutella xylostella*, that was produced as part of a PhD studentship at Warwick. We studied 14 EPF strains in total, including 6 strains used in commercial biopesticides for protected edible and ornamental crops. The data consisted of measurements of the growth and germination of the fungal strains at different temperatures, and the virulence (i.e. the lethality, or amount of kill) of 5 strains to moth larvae at different temperatures. We also had data on the effect of temperature on development rate of the pest from egg to adulthood. We tested different thermal models to see how well they fitted the data and looked at their predictions of the cardinal temperatures. In principle, the thermal biology equations are not specific to any particular group of ectothermic organisms, and hence can be applied to different types of microbial biopesticide. However, it is likely that some models will be better than others depending on the individual situation, for example different EPF species or strains, or for different biological dependent variables (growth, germination, lethality etc.). We also wanted to see if there was a statistically significant relationship between the lethality of fungal strains and their growth and germination, all tested across different temperatures.

Our motivation for starting this work was to provide benefits for crop protection practitioners and the biopesticides industry:

- A good mathematical model would enable biopesticide performance to be predicted at any environmental temperature within the performance range, and would enable a more precise determination of T_{min}, T_{max} and T_{opt}. Companies that are developing microbial biopesticides could use the model for targeted selection of control agents capable of performing well across the range of environmental temperatures experienced in the field or greenhouse. As stated previously, these models can be difficult to work with, but there may be ways of using the models to validate simpler forms of data analysis to identify the cardinal temperatures.
- In principle, a mathematical model could be used to predict the effect of fluctuating temperatures on biopesticide performance using an integration function. It could also be used to investigate the potential effects of climate change on the performance of biopesticides.
- As stated above, biopesticide efficacy is measured using bioassays, and to do these across a range of temperatures can take a lot of time and resources (typically replicate measures are needed at five or six different temperatures). This restricts the number of microbial strains that can be tested in a screening programme. In contrast, microbial physiological processes such as germination or growth are measured in simple petri dish set ups that are much faster and easier to do, allowing large numbers of treatments to be done quickly. As part of this project, we looked to see whether these physiological measures can be used to predict the effect of temperature on the pest control activity of microbial biopesticides. We did this by looking for relationships between fungal growth and germination with lethality across a range of temperatures using multiple regression statistical techniques. If growth and or germination rate can help explain the level of virulence of pathogenic fungal strains, it would be possible to fast-track biopesticide development by screening candidate microbial strains for thermal performance using simple petri dish tests.

Table 25 Strains of entomopathogenic fungi used in the study.

Fungal species	Strain code	Host insect / source	Origin
Beauveria bassiana	11.98[a]	Click beetle (Coleoptera: Elateridae	USA
	432.99 [b]	*Anthomonus grandis* (Coleoptera: Curculionidae	USA
	433.99[c]	*Bemisia* sp. (Hemiptera: Aleyrodidae)	USA
	1730.08[d]	-	UK
	1757.15 (1850)[e]	*Choristoneura sp.* (Lepidoptera: Tortricidae	Canada
	1758.15 (3404	*Lymantria dispar* (Lepidoptera: Lymantriidae	USA
	1759.15 (3530)[e]	*L. dispar* (Lepidoptera: Lymantriidae)	USA
Isaria fumosoroseus	1761.15 (6799) [e]	*Plutella xylostella* (Lepidoptera: Plutellidae)	Australia
	1762.15 (6800) [e]	*P. xylostella* (Lepidoptera: Plutellidae)	Australia
Lecanicillium longisporum	1.72[f]	*Macrosiphoniella sanborni* (Homoptera: Aphididae)	UK
Lecanicillium muscarium	19.79[g]	*Trialeurodes vaporariorum* (Hemiptera : Aleyrodidae	UK
Metarhizium brunneum	275.86[h]	*Cydia pomonella* (Lepidoptera : Tortricidae)	Germany
	445.99[i]	-	-
	1760.15 (4522)[e]	*P. xylostella* (Lepidoptera: Plutellidae)	USA

[†]Fungal strain number in Warwick Crop Centre culture collection (No. from culture collection of origin).

a. Kindly supplied by B. Ownley, University of Tennessee, 2505 E.J. Chapman Drive, 370 Plant Biotechnology Building, Knoxville, TN 37996-4560, USA.
b. Active ingredient in 'Naturalis' (Troy Biosciences Inc., 113 South 47th Avenue, Phoenix, AZ 850433, USA).
c. Active ingredient in 'BotaniGard' (Mycotech Corporation, PO Box 4109, Butte, MT 59702, USA).
d. Taken from the Warwick Crop Centre culture collection, after being identified during an MSc research project.
e. From the USDA ARSEF collection and kindly supplied by Dr Richard A. Humber of The USDA-ARS Biological Integrated Pest Management Research Unit, Robert W. Holley Center for Agriculture and Health, 538 Tower Road, Ithica, USA
f. Active ingredient in Vertalec (Koppert Biological Systems, PO Box 155, 2650 AD Berkel en Rodenrijs, The Netherlands).
g. Active ingredient in 'Mycotal' (Koppert Biological Systems, PO Box 155, 2650 AD Berkel en Rodenrijs, Netherlands).
h. Active ingredient in 'Met52' (Novozymes, Krogshoejvej 36, 2880 Bagsvaerd, Denmark).
i. Active ingredient in Bio-Blast (Eco-Science Corporation, 17 Christopher Way, Eatontown, NJ 07724, USA).

Methods

Fungal Cultures. Fourteen strains of hypocrealean EPF from four genera were used in the study (Table 25): *Beauveria, Isaria, Lecanicillium and Metarhizium.* The strains are referred to by the accession number used in the culture collection at Warwick Crop Centre. All the strains were pathogenic to DBM (Perry, 2017). Laboratory cultures were grown on Sabouraud dextrose agar (SDA) slopes from cryopreserved stocks (Chandler, 1994) and maintained at 5˚C. For laboratory experiments, subcultures were prepared on SDA from the slope cultures and incubated at 23°C for 10 d in the dark. Conidia were harvested in sterile 0.05% Triton X-100 and filtered through sterile milk filters (Goat Nutrition Ltd, Kent, UK). Conidia were enumerated using a haemacytometer and aliquots were prepared at concentrations of 1 x 10^6 or 1 x 10^7 ml^{-1}.

Insect Rearing. We used a stock of DBM collected originally in 1995 from Wellesbourne, Warwickshire, UK. DBM were reared on Brussels sprouts plants, *Brassica oleracea* cv 'Doric' (Elsoms Seeds Ltd, Lincoln, UK) grown in Levington F2 compost (Scotts UK, Ipswich, UK) and maintained in mesh rearing cages (30 x 30 x 30 cm) (Watkins and Doncaster Ltd, Herefordshire, UK) at 20°C, 18:6 LD, 40% RH in a controlled environment room. Experiments were done with fixed age populations of eggs or larvae. These were produced by allowing adult moths to oviposit on 3-4 week old Brussels sprouts plants (3rd true leaf stage) for 24h before being removed, and plants incubated as above. Mature plants (5 – 10 weeks old) were added to cages twice each week to feed developing larvae.

In vitro fungal colony extension rates. Colony extension rates were measured on SDA for all 14 EPF strains at six different temperatures (10, 15, 20, 25, 30 and 33°C). The rate of extension of the colony radius was used as an indicator of the specific biomass accumulation rate (Trinci 1971; Cooke and Whipps 1993). For each isolate, 100µl of conidial suspension (1 x 10^7 ml^{-1}) was spread evenly over SDA (15 ml) in Petri dishes (90 mm diameter), incubated at 25°C for 48 h, and then 7 mm diameter plugs were cut with a flame-sterilised cork borer and placed upside down in the centre of fresh SDA (15 ml) in Petri dishes (90 mm diameter), marked with an x/y axis on the base. Dishes were sealed in polyethylene bags and incubated in controlled environment cabinets at each temperature (incubator temperatures were recorded throughout using data loggers). Colony diameters were measured with a ruler using two cardinal diameters every 7 d for 3 wks. Mean colony radius was plotted against time for each isolate, temperature and replicate, and colony extension rate was calculated during the linear phase (Fargues *et al*. 1992). The experiment was done as a simple block design with one block comprising of 84 Petri dishes (14 EPF strains x six temperatures). Three blocks were done in total with each block being completed on a separate occasion.

In vitro spore (conidia) germination. The germination of conidia of all fungal isolates was measured on SDA at six different temperatures as above. Aliquots (20 µl) of conidia suspension (1 x 10^7 ml^{-1}) were pipetted onto a marked area on SDA within a 4.5 cm diameter Petri dish and incubated in a controlled environment cabinet for 12 h, after which germination was terminated by pipetting a drop of lactophenol methylene blue inside each marked area. Numbers of germinated and ungerminated conidia were counted from a group of no less that 100 conidia observed under a microscope. A conidium was considered germinated if the length of the germ tube exceeded twice the width of the spore. The experiment was done as a simple block design, The experiment was done as a simple block design with one block comprising of 252 Petri dishes (14 EPF strains x three technical replicates x six temperatures). This was done on three separate occasions in total.

Quantifying the development rate of DBM (egg to adulthood). The development rate and mortality of DBM was assessed, from egg to adult, over seven temperatures (12.5, 15, 20, 25, 27.5, 30 and 35°C) based on the method of Golizadeh et al. (2007) and Marchioro and Foerster (2011). Approximately 200 DBM adults were allowed to oviposit overnight (c. 15 h) on a 3 – 4 wk old Brussels sprout plant (cv 'Doric') within a rearing cage (20°C, 16:8 LD, as described previously). Batches of 100 eggs were transferred onto 3cm diameter leaf discs on 1.4% water agar in 9 cm Petri dishes (the lid of each dish contained a circular air hole covered with fine plastic mesh). One batch of eggs was then maintained in a controlled environment cabinet at each temperature (18:8 L:D). Eggs were checked for viability under a microscope every 24 h for 3 – 5 d depending on the temperature; if neonatal larval could be seen within the egg then it was assumed to be viable. When neonate larvae were observed, 30 viable eggs per temperature were transferred to a 3 cm leaf disc on water agar within a 3 cm Petri dish (lid modified for aeration as above). Leaf discs were changed every three to five days for the duration of the experiment. Petri dishes were observed daily and the numbers of each life stage (egg to adult) were recorded, allowing the average timespan for each stage to be estimated. Head capsule size, appearance of exuvia upon the leaf surface, and the visible darkening of the larvae cuticle directly after moult were used to indicate transition from one instar to the next (Golizadeh et al., 2007, Marchioro and Foerster, 2011). Mortality was also recorded daily for the duration of the experiment.

Laboratory bioassays of fungal virulence against DBM larvae. Laboratory bioassays with EPF strains were done using newly emerged 2nd instar larvae which were reared as described previously. Batches of 10 – 15 larvae were placed on dampened filter paper within the lid of a 9 cm Petri dish and refrigerated at 5°C for 30 min to immobilize them. They were then sprayed with 4 ml of a suspension of fungal conidia (concentration 1x10^6 conidia ml^{-1}) using a Potter tower (Potter, 1952) with an 'intermediate' atomiser and a spray pressure of 50 kPa.

Controls consisted of 0.05 % Triton X-100. Immediately after spraying, a 3 cm diameter leaf disc (*B. oleracea*, cauliflower, cv 'Skywalker' (Elsoms Seeds Ltd., UK)) was placed within each Petri dish and these were then sealed with Parafilm and incubated in a controlled environment cabinet (Panasonic MLR-352; 16:8 LD, 800W fluorescent lamps for 24 h). After this time, larvae were transferred with a fine paintbrush to a 6 cm leaf disc on 15 ml 1.4% water agar in a separate 9 cm Petri dish in which the lid was modified with two 3 cm circular air holes covered with fine plastic mesh. Leaf discs were changed every three days for the duration of the experiment. Larval mortality was assessed once every 24 hours, at the same time each day after treatment, for 7 d. Dead larvae were removed and incubated on damp filter paper within Petri dishes (20 ± 1°C, darkness) for 7 d, and inspected for the presence of sporulating mycelium on cadavers. The dose of conidia sprayed from the Potter tower was estimated for each treatment using a colony forming unit (CFU) methods as follows: A glass coverslip (18 x 18 mm) was placed in each Petri dish of DBM larvae and, immediately after spraying, it was transferred to a 30ml Universal tube containing 1 ml 0.05% Triton X-100 and vortex mixed for 2 min. The suspension was serially diluted and aliquots of 100ul were spread onto SDA supplemented with 0.06% N-dodecyl-guanidine (Inglis et al., 2012), then incubated in darkness at 20°C for 6 d, after which numbers of CFUs per plate were counted.

To measure the effect of temperature on EPF virulence, laboratory bioassays were done at six different temperatures (10, 15, 20, 25, 30 and 35^{o}C) with five different EPF strains: *B. bassiana* strains 433.99 and 1757.15, and *M. brunneum* strains 275.86, 445.99 and 1760.15. The strains had been shown previously to be pathogenic to DBM (Perry, 2017) and had different thermal performance curves for growth and germination. The experiment was done according to a randomized block design with six blocks, each block comprising of all five EPF strains at three different temperatures, such that each temperature / strain combination was done three times in total (n = 30 – 45 larvae). Each block was done on a separate occasion. However, after this was done, extra bioassays were conducted for *M. brunneum* 445.99 and 1760.15 at 36.5^{o}C (three true replicates per strain plus controls) in order to provide additional data points for modelling.

Data analysis

Thirteen non-linear models which had previously been used in modelling rate responses to temperature in biological systems, together with a polynomial with a comparable number of parameters, were fitted to the data using GenStat. Each isolate was modelled separately. Non-linear modelling requires that initial values of the parameters be supplied. These were calculated from the data: for example, an initial minimum temperature would be estimated from a linear fit of the rates at the two lowest temperatures, and an initial optimal temperature

from the temperature which achieved the observed maximum. The colony extension rate data were modelled directly. Conidia germination, and virulence were measured once after a fixed time. Percentage data (% spore germination at 12h, and % of insects dead at 7 days after treatment) were transformed with a logit transformation, modified to allow values of 0 and 100%. A constant was then added to make all values positive. This gives a rate of development proportional to the logit scale, assuming an arbitrary starting point. In addition, the same models were fitted to diamond back moth development rate data, measured as the reciprocal of the number of days from egg hatch to emergence of the adult life stage from pupae, was modelled directly.

The models used in the study are as follows:

1. Briere ('Brie'): The first model given Briere et al. (1999), developed to analyse temperature-dependent development in arthropods.
2. Briere 2 ('Bri2'): second model given in Briere et al. (1999).
3. Cardinal Temperature Model with Inflection ('CTMI'): model developed by Rosso et al. (1993; 1995) of the effect of temperature on microbial growth.
4. Generalised Beta Function ('GenB'): the generalized beta function of Bassanezi et al. (1998) used to describe development of rust and leaf spot in *Phaseolus* plants.
5. Lactin ('Lact'): A simplification of the Logan model, omitting a redundant parameter, taken from Lactin et al., (1995).
6. Lactin1 ('Lac1'): a second model from Lactin et al. (1995). This model adds a constant to the first Lactin model, so that it can reach a rate of zero.
7. Logan ('Loga'): Logan model, used to describe temperature dependent development in arthropods, from Logan et al. (1976).
8. Polynomial ('Poly'): A (cubic) polynomial
9. Ratkowsky 2 ('Rat2'): model proposed for temperature dependent growth of bacteria, in Ratkowsky et al. (1983).
10. Ratkowsky 3 ('Rat3'): modified Ratkowsky model proposed in Zwietering et al. (1991).
11. Schoolfield ('Scho'): Schoolfield & Sharpe model of ectotherm development based on enzyme kinetics (Schoolfield et al., 1981) as modified for high temperatures by Davidson et al. (2003).
12. Taylor ('Tayl'): Taylor model of temperature-dependent growth of insects, given in Taylor (1981).
13. Van der Heide ('VDH'): a simple third order polynomial developed to measure temperature-dependent growth of freshwater plants in Van der Heide et al. (2006).

Results and Discussion

Goodness of fit (adjusted R^2 and AIC values) metrics were generated for all 12 models for the effect of temperature on: (1) fungal spore germination (14 fungal strains, % germination after 12 h) (2) fungal colony growth (14 fungal strains, rate of colony extension, cm per day) (3) virulence / lethality (5 fungal strains, % insect death at 7 days after treatment with fungi) and (4) insect development (rate of DBM development from egg hatch to adult emergence). These goodness of fit metrics are used to identify the best fitting model. The model fits varied with the type of model, the fungal strain and the type of dependent variable (growth, germination, lethality, insect development) (**see Appendix Tables A1 – A4**). The model that we are recommending as the most suitable is the CTMI (Cardinal Temperature Model with Inflection) (Rosso et al., 1993, 1995). This model gave consistently good fits for fungal growth, germination and virulence across the different fungal strains tested, and also gave a good fit for insect development rate. Furthermore, in contrast to some other thermal models, all its parameters have simple biological significance (Rosso et al., 1995) which makes interpretation much simpler.

The CTMI was developed to model bacterial growth rate μ and is given as

$\mu_{max} = \gamma \cdot \mu_{opt.}$ where μ_{max} represents specific growth rate at temperature T, and $\mu_{opt.}$ is the maximum specific growth rate at T_{opt}.

with

$T \leq T_{min} \qquad \gamma = 0$

$T_{min} < T < T_{max} \qquad \gamma = \frac{(T - T_{min})^2 \ (T - T_{max})}{(T_{opt} - T_{min}) \ ((T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{min} + T_{opt} - 2T)}$

$T \geq T_{max} \qquad \gamma = 0$

(Rosso et al., 1993).

In our case, μ represented the different measures of performance (colony extension rate, germination rate, lethality, insect development) with temperature.

The estimates of the cardinal temperatures for fungal growth and germination, produced using the CTMI, are given in **Table 26 (see also Appendix Figures A1 & A2)**. There was generally good model fit, with adjusted R^2 values ranging from 0.60 (*B. bassiana* 1758.15) to 0.95 (*M. brunneum* 445.99) for colony growth and 0.74 (*L. muscarium* 19.79) to 0.98 (*B. bassiana* 1758.15) for germination.

The estimated minimum temperatures for growth T_{min}, ranged from – 5.0 ºC (*B. bassiana* 432.99 and 433.99) to +13 ºC (*M. brunneum* 1760.15). Optimum temperatures for growth

(T_{opt}) showed quite large variation and ranged from 20.2 ºC (*L. muscarium* 19.79) to 32.0ºC (*B. bassiana* 11.98). Estimates were also made for maximum temperatures for growth. These showed less variation than for the optimum temperatures, with 12 / 14 fungal strains having a T_{max} of 33 or 34 ºC; of the remaining two strains, *L. longisporum* 1.72 produced the lowest T_{max} at 31.8 ºC while *M. brunneum* 1760.15 produced the highest at 40.1ºC.

The estimated minimum temperatures for germination T_{min} ranged from 4.2 ºC (*B. bassiana* 11.98) to 12.8 ºC (*B. bassiana* 1757.5). Optimum temperatures for germination (T_{opt}) ranged from 23.7 ºC (*B. bassiana* 1757.15) to 33.0 ºC (*M. brunneum* 1760.15), while the maximum germination temperatures (T_{max}) ranged from 33.0 ºC (*M. brunneum* 1760.15) to 40.0 ºC (*M. brunneum* 445.99).

Table 26; Goodness of fit, and estimated cardinal temperatures, for 14 strains of entomopathogenic fungi using the Cardinal Temperature Model with Inflection (CTMI).

	colony growth					spore germination			
Species	strain	adj R2	Tmin	Tmax	Topt	adj R2	Tmin	Tmax	Topt
B. bassiana	11.98	0.89*	-3.1	33.0	32.0	0.93	4.2	34.6	28.1
	432.99	0.78	-5.0	33.2	28.5	0.81	5.4	33.5	28.3
	433.99	0.79	-5.0	33.2	28.5	0.89	8.1	33.4	28.0
	1730.08	0.87*	0.6	33.2	30.3	0.89	6.0	33.5	28.3
	1757.15	0.97	0.0	33.1	26.4	0.79	12.8	34.5	23.7
	1758.15	0.98	-0.7	33.1	25.7	0.60	8.5	34.1	26.7
	1759.15	0.86	-3.5	33.5	26.5	0.72	12.7	33.2	26.2
I. fumosoroseus	1761.15	0.96	6.5	34.3	23.9	0.87	7.6	34.0	27.6
	1762.15	0.91	8.1	34.3	23.2	0.93	10.8	34.8	26.9
L. longisporum	1.72	0.98	6.5	31.8	21.0	0.81	7.6	33.6	24.7
L. muscarium	19.79	0.74	6.3	34.0	20.2	0.86	12.2	33.1	25.3
M. brunneum	275.86	0.96	7.0	33.9	24.3	0.93	8.1	35.4	30.0
	445.99	0.93	3.3	34.1	26.8	0.95	10.9	40.0	30.9
	1760.15	0.94*	13.0	40.1	26.5	0.93*	5.8	33.0	33.0

*Fit has not converged

For all 14 fungal strains, T_{min} for germination > T_{min} for growth. Some of the differences were large, including 13ºC for *B. bassiana* strains 433.99 and 1757.5, and 16ºC for *B. bassiana* 1759.15. The comparison of T_{opt} values gave a different picture: for 6 fungal strains T_{opt} for growth > T_{opt} for germination, while for 8 fungal strains T_{opt} for growth < T_{opt} for germination. The largest difference was for *M. brunneum* 1760.15, in which T_{opt} for growth was 6.5 ºC higher than T_{opt} for germination. There was less variation between T_{max} values for growth and germination; for 8 fungal strains, the difference was <1.0 ºC, and for 4 fungal strains it was <2.0 ºC. The largest differences in T_{max} values were for *M. brunneum*: for *M. brunneum* 445.99, T_{max} germination was 4.1 ºC higher than T_{max} growth, while for *M. brunneum* 1760.15, T_{max} growth was 7.1 ºC higher than T_{max} germination.

The thermal tolerance range ($T_{max} - T_{min}$) for growth and germination varied according to fungal strain. For 11 of the fungal strains ($T_{max} - T_{min}$) was larger for fungal growth compared to germination. The largest tolerance range for growth was with fungal strains *B. bassiana* 432.99 and 433.99 (38.2 ºC). The largest tolerance range for germination was with *B. bassiana* 11.98. (30.4ºC).

The estimates of the cardinal temperatures for lethal infection of diamondback moth larvae by 5 EPF strains, using the CTMI, are given in **Table 27**. Estimated values of T_{min}, T_{opt} and T_{max} varied according to fungal strain. Values for T_{min} ranged from -2.5ºC (*M. brunneum* 1760.15) to 9.1 ºC (*M. brunneum* 275.86). Values for T_{opt} ranged from 22.3ºC (*B. bassiana* 1757.15) to 34.2ºC (*M. brunneum* 1760.15), while values for T_{max} ranged from 37.7 ºC (B. bassiana 1757.15) to 44.6ºC (*B. bassiana* 432.99).

Table 27; Goodness of fit, and estimated cardinal temperatures, for lethality of 5 strains of EPF tested against diamondback moth larvae using the Cardinal Temperature Model with Inflection (CTMI). Lethality was measured as % death of larvae 7 days after treatment.

	Lethality				
Species	strain	adj R^2	T_{min}	T_{max}	T_{opt}
B. bassiana	432.99	0.7	8.0	44.6	27.5
	1757.15	0.8	6.5	38.0	22.3
M. brunneum	275.86	0.83	9.1	44.2	27.6
	445.99	0.85	2.3	37.7	33.0
	1760.15	0.83	-2.5	36.8	34.2

For the five EPF strains whose virulence had been assessed, the mean virulence at each temperature for each strain was regressed on the mean extension rate, germination rate, and the larval development rate for diamond back moth at each temperature using multiple linear regression. Each trait was defined as in the model fitting. The diamondback moth development rate data was repeated for each fungal strain. Because the temperatures studied in the various experiments had differed some adjustments had to be made to the data. Conidia germination and tube extension rates at 35 ºC were taken to be equal to the measured values at 33 ºC. Insect growth rate at 10 ºC was taken to be equal to that measured at 12.5 ºC. For fungal strains *M. brunneum* 445.99 and 1760.15, conidia germination rate at 36.5 ºC was taken to be the average of that measured at 33 ºC and 40 ºC. Initially each trait, and one that allowed the different fungal strains to vary were fitted separately, and then the most significant, conidia germination rate, was combined with the fungal strain trait. We then tried adding an interaction between these terms and the insect development rate and fungal extension rates to the model, one term at a time. Finally, we added the insect development rate and fungal extension rates jointly to the model.

The only traits that gave a significant regression individually were spore germination rate and colony extension rate, both of which were highly significant ($p < 0.001$), however conidia germination gave a better fit, explaining 76% of the variance in the virulence rate. Adding the term for the different fungal strains to the model gave a significant improvement to the fit ($p < 0.05$) and increased the percentage variance explained to 81%. None of the other terms added individually improved the fit of this model ($p > 0.05$), however adding both the insect development rate and fungal colony extension rate to the model gave a small improvement in fit, with the percentage of the variance explained rising to 87% ($p < 0.01$). In this model, the terms for conidia germination rate and colony extension rate are both positive, and that for insect development rate is negative, so that as expected rapidly germinating and growing fungal strains and slowly developing insects are good for virulence.

The germination of spores is the critical, initial phase in the activity of all fungal biocontrol agents. For EPF, infection of pest insects is started when spores attach to, and then germinate on, the insect cuticle. The finding that fungal germination can explain variance in virulence is potentially important. Our results suggest that fungal pathogens of the target pest that germinate quickly are likely to be more virulent than fungal pathogens that germinate slowly. The results also indicate that rates of germination and virulence respond to temperature in a proportionate way. The relationship is illustrated in **Figure 12** where T_{opt} for germination is plotted against T_{opt} for virulence (linear regression, $R^2 = 0.898$, $p = 0.014$ (anova)). This could prove valuable in screening programmes to identify virulent strains that are able to operate well under the temperature conditions of the crop environment. Thermal

response screening of virulent strains could be done by measuring in vitro germination rate at a range of environmentally relevant temperatures and determining T_{opt} (plus T_{min} and T_{max}) This is relatively quick and easy to do, as opposed to measuring the effect of temperature on fungal virulence to the target pest, which is harder and takes considerably more resources.

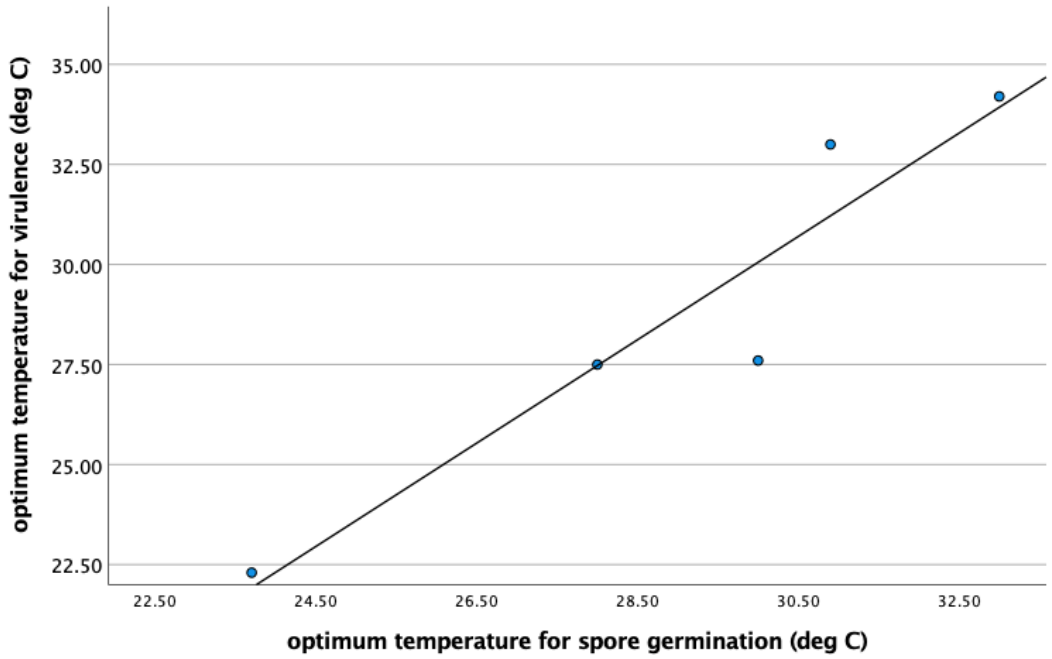

Figure 12. Relationship between optimum temperature for fungal germination, and optimal temperature for virulence to diamondback moth larvae, for 5 fungal strains.

The data for germination generated in this project provides new insights into the thermal performance of the commercial biopesticides included in the study.

- EPF strain *M. brunneum* 275.86 is used in the product Met52 for the control of vine weevil larvae in the root zone of ornamentals. It had a relatively high estimated T_{min} for germination of 8.1ºC, and a T_{opt} of 30.0ºC. This suggests that control of vine weevil larvae on outdoor ornamentals could be affected by low temperatures during late summer to spring, when vine weevil larvae are feeding on plant roots and need to be controlled. **Figure 13** shows the long term average maximum and minimum temperatures for the UK Midlands as an example. The average maximum is always below the T_{opt} for *M. brunneum* 275.86, while it is close to, or below, T_{min} from November to March. The time window for getting this fungal strain to work well on outdoor crops is likely to be quite short, probably from July (when vine weevil egg laying starts) to September. Use of the fungus will need to be targeted around this time.

- *Lecanicillium muscarium* 19.79, is used for whitefly control (but it will also infect aphids and thrips) as the product Mycotal. It also had a relatively high T_{min} for germination, at 12.2ºC. This is unlikely to be an issue for protected crops (its main area of use) but could be important if considering the product in future for outdoor crops. T_{opt} for this fungal strain is 25 ºC and T_{max} is 33 ºC. Growers need to be aware that performance of all EPF strains is likely to drop off rapidly if environmental temperatures rise above T_{opt}, and hence in hot summers, when glasshouse daytime temperatures will be high, it would be worth spraying this product in the evening when conditions are cooler. Similar arguments apply for two *B. bassiana* products used in glasshouses for whitefly, thrips, spider mites and aphids: Naturalis (which uses *B. bassiana* strain 432.99) and Botanigard (*B. bassiana* strain 433.99). These products both have an estimated germination T_{opt} at 28 ºC and a T_{max} at 33 ºC.

Microbial biopesticides are being used more widely, and new products are coming on to the market at an accelerating rate. Historically, they have been used in protected crops, but they will start to be used more in outdoor crops in future. It is important to use microbial strains that perform well under the temperature conditions of the crop environment. One of the challenges for biopesticide companies is to be able to identify 'winning' strains efficiently. For EPF, the use of simple petri dish tests of spore germination at different temperatures (rather than doing more complicated, and resource consuming virulence tests) means that large numbers of candidate fungal strains can be assessed rapidly. This will increase the chances of finding good strains – this could be particularly important for identifying low temperature strains for use outdoors, and which are likely to be rare, meaning that large numbers of candidates will have to be screened to find them. The ideal situation would be to use a non-linear mathematical model for data analysis, but if necessary visual inspection of bar graphs (**Figure 10**) could still be useful for identifying the cardinal temperatures. It would also be valuable to see if use of spore germination data as a substitute for virulence applies also to fungal biopesticides of plant diseases.

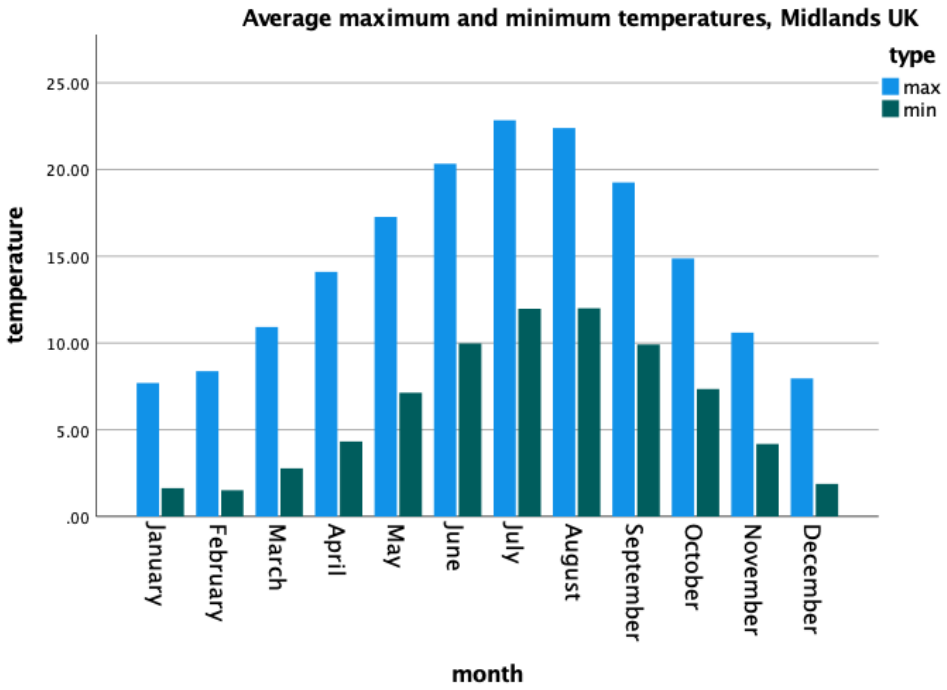

Figure 13. Temperature averages (1991 – 2020) for the Midlands, UK (source, Met Office).

Researchers use T_{min} and T_{max}, as cardinal points, as they represent the theoretical limits of performance, and (T_{max} - T_{min}) gives the thermal tolerance breadth. However, biopesticide companies may be better off using a different measure of thermal performance range in order to provide more agronomically useful information to growers. If T_{min} and T_{max} are quoted as the thermal limits, people may mistakenly believe that the biopesticide is active at these temperatures. Instead, it could be helpful to identify an agronomically operative temperature range. For example, this could be the temperature range at which performance is no less than 50% of that at T_{opt}.

A microbial biopesticide should not only work under the target environmental temperature range, it should also have a thermal performance curve that matches, or overlaps, that of the target pest (which, remember, is also ectothermic). If the TPCs are different, then there is likely to be set of temperatures at which the pest can feed, grow and reproduce but the biopesticide cannot control it (as illustrated in **Figure 14**). If the TPCs match, however, then both pest and biopesticide will respond similarly to temperature changes. This means that successful levels of crop protection can still occur at suboptimal temperatures. For example, at low temperatures, while the speed of kill of the biopesticide will be reduced, provided the pest undergoes the same rate reduction in development, feeding and reproduction, then the total amount of pest control will be maintained, albeit at a slower rate.

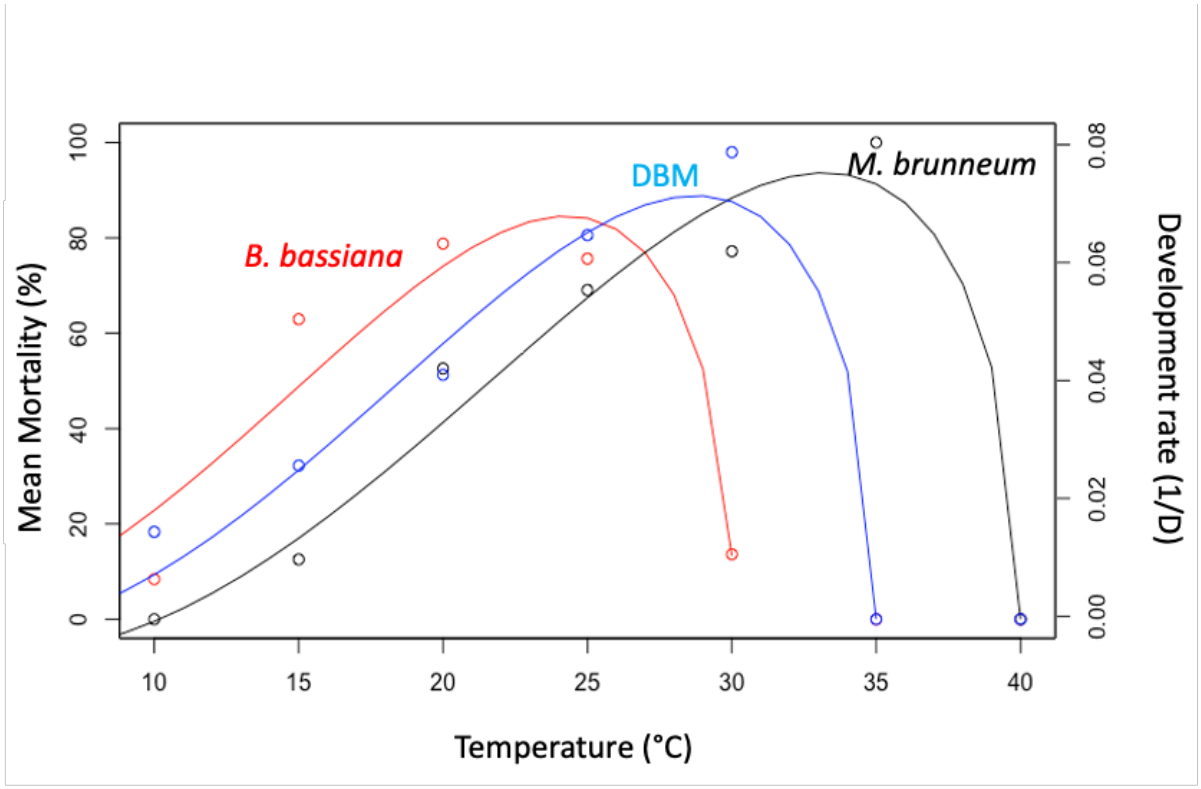

Figure 14. Thermal performance curves (TPC) for the virulence (% mortality after 7 days) of *B. bassiana* and *M. brunneum*, compared to the equivalent curve for the development rate of diamondback moth (DBM) larvae. The TPC for *B. bassiana* is in red, *M. brunneum* is black, and DBM is blue. The curves show separation along the Temperature axis. It illustrates how different bioprotectants may be required in different temperature conditions: (1) There is a temperature range (c. 28 – 33 ºC) at which *B. bassiana* causes < 50% mortality but DBM is able to grow and develop at or close to its maximum rate. In contrast, *M. brunneum* causes c. 75% mortality at this temperature range. Therefore, in regions where temperatures are optimum for DBM growth, *M. brunneum* would be the best bioprotectant. (2) At high temperature conditions (> 33 ºC) *M. brunneum* would still be the best choice. (3) However at lower temperatures (< 25 ºC), *B. bassiana* would give better control of DBM than *M. brunneum*.

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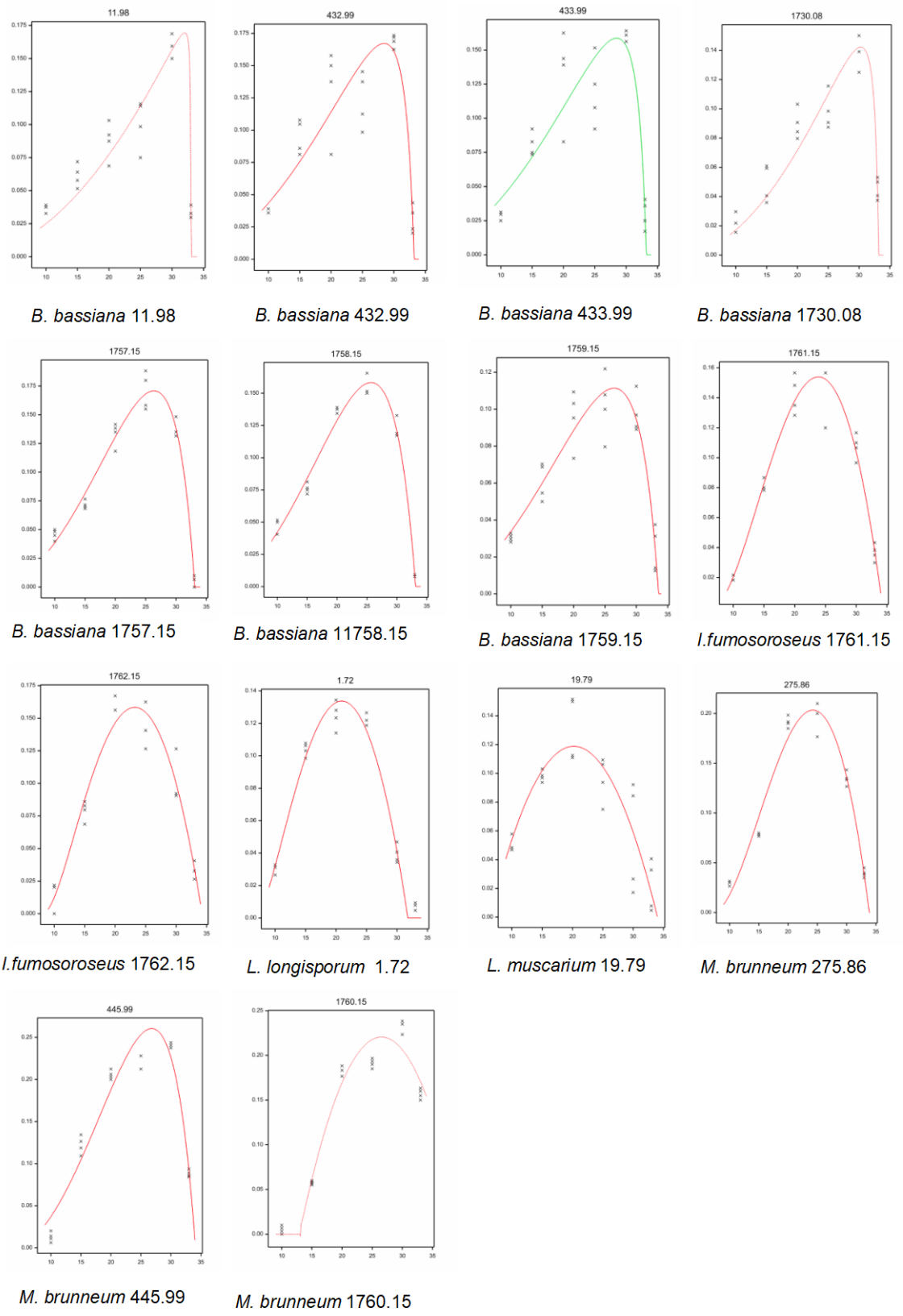
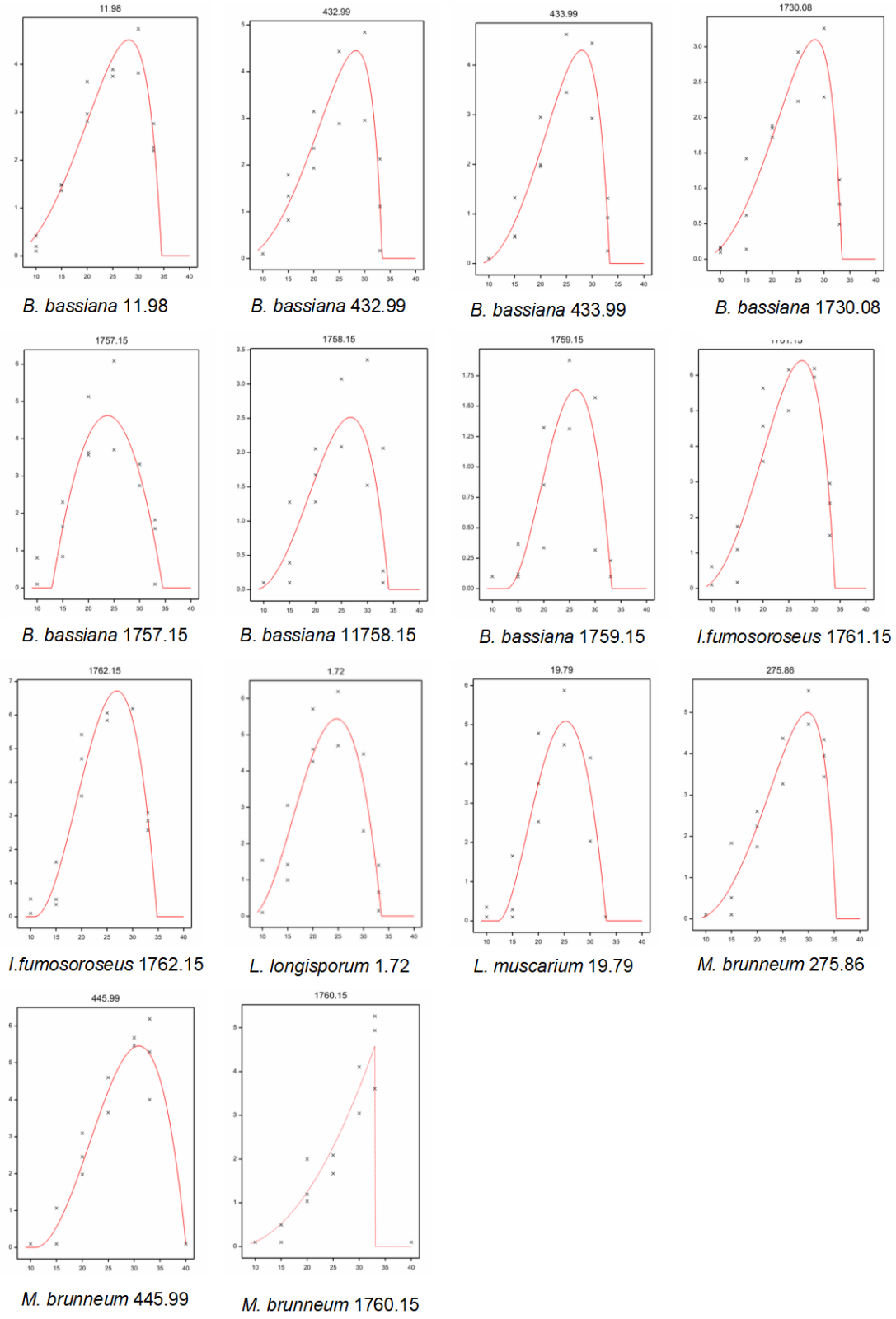


Appendix Figure A1. CTMI temperature dependent model describing colony extension of 14 strains of entomopathogenic fungi.



Appendix Figure A2. CTMI temperature dependent model describing spore germination of 14 strains of entomopathogenic fungi.

Appendix Table A1: Goodness of fit for 13 nonlinear models describing the effect of temperature on colony extension rate of 14 EPF strains

		Brie[1]		Bri2		CTMI		GenB		Lact		Lac1		Loga	
Species	strain	AIC[2]	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj. R^2
B. bassiana	11.98	-98	0.70	-103	0.76	-120	0.89	-111	0.84	-104	0.77	-101	0.75	-115	0.86
	432.99	-96	0.76	-98	0.80	-97	0.78	-55	*	-92	0.73	-90	0.71	-95	0.77
	433.99	-98	0.78	-97	0.78	-98	0.79	-99	0.80	-94	0.74	-92	0.73	-95	0.76
	1730.08	-114	0.81	-127	0.89	-122	0.87	-121	0.87	-119	0.85	-117	0.84	-125	0.88
	1757.15	-137	0.96	-139	0.97	-140	0.97	-139	0.97	-143	0.97	-143	0.97	-141	0.97
	1758.15	-152	0.98	-154	0.98	-156	0.98	-156	0.98	-149	0.97	-155	0.98	-147	0.97
	1759.15	-133	0.88	-131	0.87	-129	0.86	-130	0.87	-129	0.86	-128	0.85	-117	0.77
I. fumosorosea	1761.15	-107	0.83	-139	0.96	-140	0.96	-138	0.96	-107	0.83	-95	0.73	-91	0.68
	1762.15	-97	0.79	-113	0.90	-117	0.91	-115	0.9	-92	0.74	-78	0.55	-80	0.59
L. longisporum	1.72	-86	0.56	-148	0.97	-155	0.98	-150	0.97	-100	0.75	-122	0.91	-90	0.63
L. muscarium	19.79	-95	0.58	-101	0.69	-105	0.74	-105	0.74	-97	0.62	-102	0.7	-91	0.52
M. brunneum	275.86	-101	0.89	-123	0.95	-126	0.96	-128	0.96	-92	0.83	-49	0.02	-75	0.67
	445.99	-110	0.95	-109	0.95	-103	0.93	-96	0.91	-92	0.89	-106	0.94	-78	0.8
	1760.15	-107	0.94	-107	0.94	-108	0.94	-35	*	-90	0.87	-106	0.93	-75	0.76

		Poly		Rat2		Rat3		Scho		Tayl		VDH			
Species	strain	AIC[2]	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2		
B. bassiana	11.98	-88	0.57	-106	0.80	-66	*	-127	0.91	-81	0.4	-84	0.47		
	432.99	-83	0.60	-99	0.80	-99	0.80	-97	0.78	-77	0.49	-84	0.60		
	433.99	-86	0.65	-97	0.78	-97	0.78	-95	0.76	-80	0.54	-87	0.65		
	1730.08	-103	0.71	-130	0.91	-96	0.62	-127	0.89	-97	0.61	-100	0.65		
	1757.15	-134	0.96	-142	0.97	-141	0.97	-133	0.96	-88	0.73	-104	0.86		
	1758.15	-147	0.97	-149	0.97	-156	0.98	-131	0.95	-97	0.77	-121	0.91		
	1759.15	-123	0.82	-127	0.85	-128	0.86	-109	0.68	-111	0.70	-121	0.80		
I. fumosorosea	1761.15	-140	0.96	-136	0.95	-118	0.9	-117	0.89	-127	0.93	-141	0.96		
	1762.15	-116	0.91	-115	0.9	-111	0.89	-107	0.86	-112	0.89	-115	0.9		
L. longisporum	1.72	-143	0.96	-151	0.97	-108	0.83	-126	0.92	-133	0.94	-150	0.97		
L. muscarium	19.79	-106	0.75	-101	0.69	-93	0.56	-107	0.76	-109	0.77	-104	0.71		
M. brunneum	275.86	-125	0.96	-128	0.96	-107	0.91	-112	0.93	-122	0.95	-125	0.96		
	445.99	-98	0.92	-97	0.91	-101	0.92	-83	0.84	-84	0.84	-100	0.92		
	1760.15	-106	0.94	-105	0.93	-100	0.92	-101	0.92	-100	0.92	-109	0.94		

[1] Full model names are given in the text. [2] Akaike Information Criterion. AIC is used to determine model quality – it takes into account the goodness of fit of the model versus its simplicity. Lower scores indicate higher quality. [3] Adjusted R square value. It identifies the % of variance explained by the model inputs. Higher values indicate better goodness of fit.

Appendix Table A2: Goodness of fit for 13 nonlinear models describing the effect of temperature on spore germination rate of 14 strains of entomopathogenic fungi

		Brie[1]		Bri2		CTMI		GenB		Lact		Lac1		Loga	
Species	strain	AIC[2]	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj. R^2
B. bassiana	11.98	17	0.94	19	0.94	21	0.93	19	0.94	27	0.9	21	0.93	40	0.8
	432.99	43	0.81	43	0.82	44	0.81	46	0.79	43	0.81	44	0.81	54	0.68
	433.99	34	0.88	41	0.83	33	0.89	35	0.88	35	0.88	33	0.89	51	0.7
	1730.08	20	0.88	21	0.89	21	0.89	35	0.76	21	0.88	21	0.88	36	0.73
	1757.15	54	0.69	49	0.77	48	0.79	50	0.77	56	0.65	89	*	64	0.48
	1758.15	44	0.5	42	0.59	41	0.6	43	0.57	41	0.58	41	0.59	47	0.44
	1759.15	22	0.66	24	0.65	20	0.72	21	0.71	22	0.65	22	0.67	30	0.49
I. fumosorosea	1761.15	47	0.88	48	0.88	49	0.87	48	0.88	52	0.84	50	0.86	65	0.69
	1762.15	40	0.92	42	0.92	38	0.93	38	0.94	50	0.87	48	0.89	65	0.7
L. longisporum	1.72	53	0.79	53	0.8	52	0.81	54	0.79	58	0.72	98	*	67	0.57
L. muscarium	19.79	49	0.83	52	0.81	47	0.86	49	0.85	56	0.76	92	*	66	0.6
M. brunneum	275.86	34	0.91	32	0.92	31	0.93	33	0.92	47	0.81	32	0.93	35	0.91
	445.99	45	0.94	47	0.93	41	0.95	43	0.94	54	0.9	54	0.9	67	0.82
	1760.15	50	0.86	37	0.93	36	0.93	112	*	34	0.93	36	0.93	56	0.82

		Poly		Rat2		Rat3		Scho		Tayl		VDH			
Species	strain	AIC[2]	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2		
B. bassiana	11.98	26	0.91	23	0.92	22	0.93	63	0.33	30	0.89	26	0.91		
	432.99	49	0.75	44	0.81	44	0.81	73	0.03	52	0.69	49	0.73		
	433.99	39	0.85	33	0.89	33	0.89	73	0.02	45	0.77	42	0.82		
	1730.08	28	0.83	21	0.88	21	0.88	59	0.05	33	0.76	29	0.81		
	1757.15	49	0.77	48	0.78	49	0.77	78	*	47	0.79	47	0.79		
	1758.15	41	0.6	41	0.6	41	0.6	58	*	41	0.6	40	0.62		
	1759.15	19	0.72	19	0.72	20	0.7	19	0.72	19	0.71	18	0.73		
I. fumosorosea	1761.15	50	0.87	50	0.86	49	0.87	83	0.13	54	0.82	48	0.87		
	1762.15	38	0.94	40	0.93	39	0.93	83	0.22	45	0.9	36	0.94		
L. longisporum	1.72	52	0.81	52	0.8	52	0.81	85	*	53	0.78	51	0.81		
L. muscarium	19.79	47	0.86	48	0.85	48	0.85	99	*	53	0.8	44	0.87		
M. brunneum	275.86	34	0.92	31	0.93	31	0.93	59	0.67	31	0.93	34	0.91		
	445.99	41	0.95	42	0.95	42	0.95	104	*	55	0.89	43	0.94		
	1760.15	50	0.86	39	0.92	38	0.92	92	*	54	0.82	51	0.85		

[1] Full model names are given in the text. [2] Akaike Information Criterion. [3] Adjusted R square value.

Appendix Table A3: Goodness of fit for 13 nonlinear models describing the effect of temperature on the virulence (lethality) of 5 strains of entomopathogenic fungi

		Brie[1]		Bri2		CTMI		GenB		Lact		Lac1		Loga	
Species	strain	AIC[2]	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj. R^2
B. bassiana	433.99	46	0.65	46	0.67	44	0.7	47	0.67	50	0.58	97	*	55	0.47
	1757.15	60	0.27	45	0.7	38	0.8	33	0.85	59	0.32	104	*	58	0.39
M. brunneum	275.86	43	0.76	40	0.8	38	0.83	40	0.81	48	0.68	100	*	55	0.56
	445.99	54	0.86	54	0.87	56	0.85	156	*	58	0.83	60	0.82	60	0.82
	1760.15	62	0.8	58	0.84	60	0.83	131	*	65	0.77	67	0.76	67	0.76
		Poly		Rat2		Rat3		Scho		Tayl		VDH			
Species	strain	AIC[2]	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2		
B. bassiana	433.99	44	0.7	49	0.61	48	0.64	48	0.62	44	0.69	44	0.69		
	1757.15	31	0.87	51	0.59	47	0.67	71	*	31	0.86	43	0.72		
M. brunneum	275.86	38	0.83	52	0.62	43	0.78	62	0.35	40	0.8	38	0.81		
	445.99	57	0.85	58	0.84	57	0.84	1596	*	57	0.84	55	0.85		
	1760.15	65	0.78	66	0.77	65	0.78	152	*	65	0.77	63	0.79		

[1] Full model names are given in the text. [2] Akaike Information Criterion.. [3] Adjusted R square value.

Appendix Table A4: Goodness of fit for 13 nonlinear models describing the effect of temperature on the development rate of *P. xylostella*

Brie[1]		Bri2		CTMI		GenB		Lact		Lac1		Loga	
AIC[2]	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj. R^2
-635	0.96	-697	0.98	-704	0.98	-704	*	-673	0.97	-353	0.46	-135	0.97
Poly		Rat2		Rat3		Scho		Tayl		VDH			
AIC[2]	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2	AIC	Adj.R^2		
-572	0.92	-693	0.97	-697	0.9	-135	*	-430	0.73	-432	0.98		

[1] Full model names are given in the text. [2] Akaike Information Criterion. [3] Adjusted R square value.

Objective 2 (continued): Develop and demonstrate management practices that can improve biopesticide performance.

2.2.7 Insights into biopesticide performance using pest population modelling

Introduction

Myzus persicae is a serious pest of many horticultural crops and has become increasingly difficult to control due to its propensity to develop pesticide resistance and a lack of available plant protection products. This aphid species has also developed resistance to carbamates, organophsphates, pythrethoids and neonicotinoids (IRAC sucking pest WG, 2018). Enhanced expression of esterases causes resistance to organophosphates and carbamates. MACE (modified acetylcholinesterase) leads to target site resistance to dimethylcarbamantes, and nAChR target-site resistance confers resistance to IRAC mode of action group 4 insecticides (IRAC sucking pest WG, 2018). Knock-down and Super knock-down resistance has caused high level pyrethroid resistance in *M. persicae*. Elevated levels of cytochrome P450 has been shown to metabolise neonicotinoids and nicotine but recommended rates are still effective (IRAC sucking pest WG, 2018).

Recent withdrawal of the active thiacloprid further limits the available control options and there are few novel conventional actives being granted approval. Many growers now use IPM programmes, as required under the EU Sustainable Use of Pesticides Directive, to control aphids. Bioprotectants can play an integral role in IPM programmes, providing alternative options where biocontrols may be struggling or applications of conventional insecticides are limited, or use is restricted. Bioprotectants are defined as pest control products based on natural agents, and there are four types; invertebrate biocontrol agents, microbials, semiochemicals, and natural substances (IBMA, 2020). More bioprotectants are gaining approval and coming onto the market. These types of pest control agent are based on living organisms and therefore understanding the optimal way to use them is crucial to maximising efficacy and minimising cost, compared with conventional pesticides.

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

bioprotectant companies and others in order to provide improved best-practice guidelines for bioprotectants.

A model was developed as part of the AMBER project as a research tool that allows different bioprotectant control programmes to be tested. Attempting to investigate all components of a spray programme in laboratory or grower experiments would be prohibitively expensive and time-consuming. Computer 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 was developed to predict *Myzus persicae* population increase over time on sweet pepper and the effect of neem-based bioprotectants on pest population development and management. The model results were compared with published experimental results with formulated commercial azadirachtin products and were found to be within a sensible range. The model was used to test the efficacy of the number of spray applications of azadirachtin. The model predicted that to eradicate an initial pest population of two adults per plant with the first application after one week, it would be necessary to apply four sprays of a neem based bioprotectant at weekly intervals.

This experiment aimed to validate the computer model predictions for the efficacy of the number of spray applications of azadirachtin against *Myzus persicae*. Currently the only azadirachtin product approved for use in the UK is Azatin, which is approved and has a label recommendation for control of thrips on ornamentals with permanent protection and full enclosure. Azatin must be applied in blocks of four applications with seven-day intervals between applications. If the model predictions are correct this will be sufficient to eradicate a starting population of 10 aphids per plant or less. Azatin also has EAMUs for use against thrips and whitefly on protected aubergine and tomato (EAMU 3056/22, 2226/21) and against thrips and whitefly on protected courgette and cucumber (EAMU 2227/21, 3057/22). In this experiment Azatin was tested on pansy as a model crop in a glasshouse to provide growers with immediately applicable results and to compare the results with those given in an experiment on pansy in the AHDB project CP 124 (Pope *et al.*, 2015; Smith *et al*., 2018).

Materials and methods

Pansy plugs, var. Matrix were potted into 9cm pots with M2 Pot and Bedding compost on 30 June. Plants were stored under insect proof mesh in a polytunnel until large enough to begin the trial on 18 August.

Aphid culture set-up

Myzus persicae clone 2169G (exhibiting the Super-kdr mutation conferring resistance to pyrethroids, typical of populations found on commercial nurseries) was cultured on Pak Choi. The aphids were allowed to condition to pansy var. Matrix from 20 July, to allow for at least two generations prior to infesting the trial (**Figure 15**). The culture was kept in a 'BugDorm' to prevent other pests or aphid parasitoids or predators gaining entry to the culture. The culture was checked for presence of any parasitised aphid 'mummies' or aphids infected with entomopathogenic fungi, which were removed from the culture if found.

On 12 August, six days prior to the start of the trial, 300 adult *Myzus persicae* were added to pansy leaves and placed in Blackman boxes with damp cotton wool. This followed a pilot experiment on Pak Choi, where five adult *M. persicae* produced three nymphs in 24 hours. The adults were removed after 48 hours, leaving ~714 fixed-age nymphs behind, which became adults for the start of the trial, on 18 August.

Figure 15 Fixed age *M. persicae* culture on pansy leaves in Blackman boxes.

Pansy plants were checked for presence of aphids immediately prior to infestation and rejected if an aphid was found. The plants were then each artificially infested with two fixed age adult aphids from the fixed age culture and transferred carefully with a paintbrush to a cupped, central leaf to prevent falling.

Spray calibration

The track sprayer was calibrated by measuring the spray deposition of water in Petri dishes across the spray area. The results from this calibration showed that spray deposition was most uniform in the area between nozzles and advice from Silsoe Spray Application Unit corroborated this.

Trial design

Six treatments were applied to pansy var. Matrix in a replicated block design with six treatments replicated six times (**Table 28**). Plots consisted of four 9 cm pots stood on capillary matting in large saucers inside 'BugDorm' cages with 10 cm spacing between pots.

The first treatments were applied seven days post infestation and subsequent treatments were applied at 7-day intervals. Applications were made with a track sprayer fitted with three flat fan 02 nozzles. Plants were arranged in two lines centred between nozzles for spraying, since calibration results indicated this area was the most uniform (**Figure 16**).

Figure 16 Plants arranged in two lines in the centre of the three nozzles in use by the precision track sprayer

Before treatment application spray deposition was tested with water sensitive paper attached to pansy plants to assess spray coverage on the upper and lower leaf surfaces.

Treatments were applied according to **Table 28.** Treatments 2 – 5 were applied with the same pass of the track sprayer since the rate and water volume was the same for each treatment. Treatment 6 was applied with a separate pass of the track sprayer.

Table 28 Treatment application rates and dates, days post infestation.

Treatment number	**Treatment Name**	**Rate (L / ha)**	**Timing (DPI days post infestation)**
T1	Untreated		
T2	Azatin	0.14% (140 ml per 100L water) 1.4 L / ha (1000 L /ha water)	7
T3	Azatin	0.14% (140 ml per 100L water) 1.4 L / ha (1000 L /ha water)	7, 14
T4	Azatin	0.14% (140 ml per 100L water) 1.4 L / ha (1000 L /ha water)	7, 14, 21
T5	Azatin	0.14% (140 ml per 100L water) 1.4 L / ha (1000 L /ha water)	7, 14, 21, 28
T6	Azatin	0.14% (140 ml per 100L water) 0.84 L / ha (600 L /ha water)	7, 14, 21, 28

Speed of kill trial design

Two additional plots were set up with five plants in each cage in order to assess speed of kill on untreated plants and those treated with T5 (Azatin application four times in 1000L / ha water).

Assessments

Aphid counts were carried out weekly on the day before treatment applications. Counts were carried out in situ using a headband magnifier or hand lens to aid distinction between adults and nymphs. The cauda is well developed and visible on adult *Myzus persicae* but not on the nymphs. Plants were carefully inspected on the underside and topside of leaves, stems and flowers. Numbers of nymphs, apterous adults and alate adults were recorded separately per plant. The number of alate adults found in the cage was also recorded. Phytotoxicity was also assessed on a scale of 1-9 where 1 represents no damage and 9 represents complete crop kill. Aphid count and phytotoxicity assessments were completed 6, 10, 13, 20, 27 and 34 days after infestation.

Speed of kill assessments

The ten plants set aside for speed of kill assessment were assessed daily from eight days after infestation to 14 days after infestation. Numbers of *Myzus persicae* nymphs, apterous adults and alate adults were recorded.

Meteorologic records and crop husbandry.

Two data loggers were placed in the trial, one inside a 'BugDorm' and one hung in the glasshouse. Two-spotted spider mite was observed on trial plants on 25/08/21. *Phytoseiulus persimilis* were released on 3/08/21 and 17/09/21 at a rate of approximately 13 predatory mites per plant, with more directed to the hotspots.

Statistical analysis

Data was analysed with ANOVA in Genstat 18th edition. Data were transformed with log base 10 (x+1) to account for variation.

Running the model

The model was run with a starting population of two adult *M. persicae* to match the initial population in the experiment. Sublethal effects on adults were included in this version of the model. The sublethal effect on adult *M. persicae* for this experiment was a reduction in modelled fecundity from two to zero nymphs per day, persisting for seven days after treatment application.

Statistical analysis

ANOVA (Genstat edition 18.2) was used to analyse the data. Numbers of aphids per plant were transformed by $\log_{10}$ (x+1) to remove dependency of the variances on the means. Transformed values were subjected to analysis of variance (ANOVA) with a significance level of $P<0.05$. Separation of the means was determined by Duncan's Multiple Range Test. Graphs are presented with back-transformed means in order to have relevance for growers. Abbott's formula was used to calculate percentage reduction in numbers of aphids compared with the untreated control.

On the day 34 assessment some plants were excluded from the analysis as they had died either from stem rot, spider mite damage or aphid damage, since the numbers of aphids on these plants were far lower than on the other healthy plants. However, this made no difference to the statistical analysis.

Results

Spray efficacy

Good spray coverage was achieved on the upper surface of leaves however there was no spray coverage seen on the underside of the leaves (**Figure 17**).

A)  B) 

Figure 17 Water sensitive paper spray deposition on the upper leaf surface (A) and the lower leaf surface (B) of pansy.

Aphid nymphs

There was no significant difference in the mean number of aphid nymphs between treatments on days 6, 10, 13, 20 and 35. On day 27 there were significantly fewer nymphs in treatments T2, T4 and T5 compared with the untreated control (**Table 29**) with reductions of 63.56%, 46.20% and 52.19% respectively (**Table 30, Figure 18**).

Table 19. Transformed mean number of *M. persicae* nymphs per plant on each assessment date. Transformation: Log base 10 (x+1) (back-transformed means are shown in brackets). Values not sharing the same letter are statistically different (P<0.05).

Treatment	**Days Post Infestation**					
	6	**10**	**13**	**20**	**27**	**34**
T1	0.9157 (7.24)	1.393 (23.71)	1.439 (26.5)	2.236 (171.06)	2.219 c (164.39)	2.568 a (369.04)
T2	1.1127 (11.96)	1.340 (20.87)	1.430 (25.88)	2.146 (139.03)	1.785 a (59.90)	2.461 a (288.24)
T3	1.0204 (9.48)	1.145 (12.97)	1.430 (25.90)	2.039 (108.48)	2.109 bc (127.45)	2.666 a (462.42)
T4	1.0306 (9.48)	1.219 (15.55)	1.440 (26.57)	2.063 (114.68)	1.952 ab (88.44)	2.439 a (273.66)
T5	0.9670 (8.27)	1.098 (11.52)	1.158 (13.40)	1.869 (73.01)	1.901 ab (78.60)	2.427 a (266.35)
T6	0.9361 (7.63)	1.205 (15.04)	1.410 (24.70)	1.960 (90.14)	1.986 abc (95.80)	2.410 a (256.11)
d.f.	25	25	25	25	25	25
s.e.d	0.1433	0.1608	0.1922	0.1283	0.1053	0.1468
l.s.d	0.2952	0.3311	0.3958	0.2643	0.2245	0.3023
P value	0.765	0.444	0.649	0.104	0.013	0.472
	Significantly different from untreated control (P<0.05)					

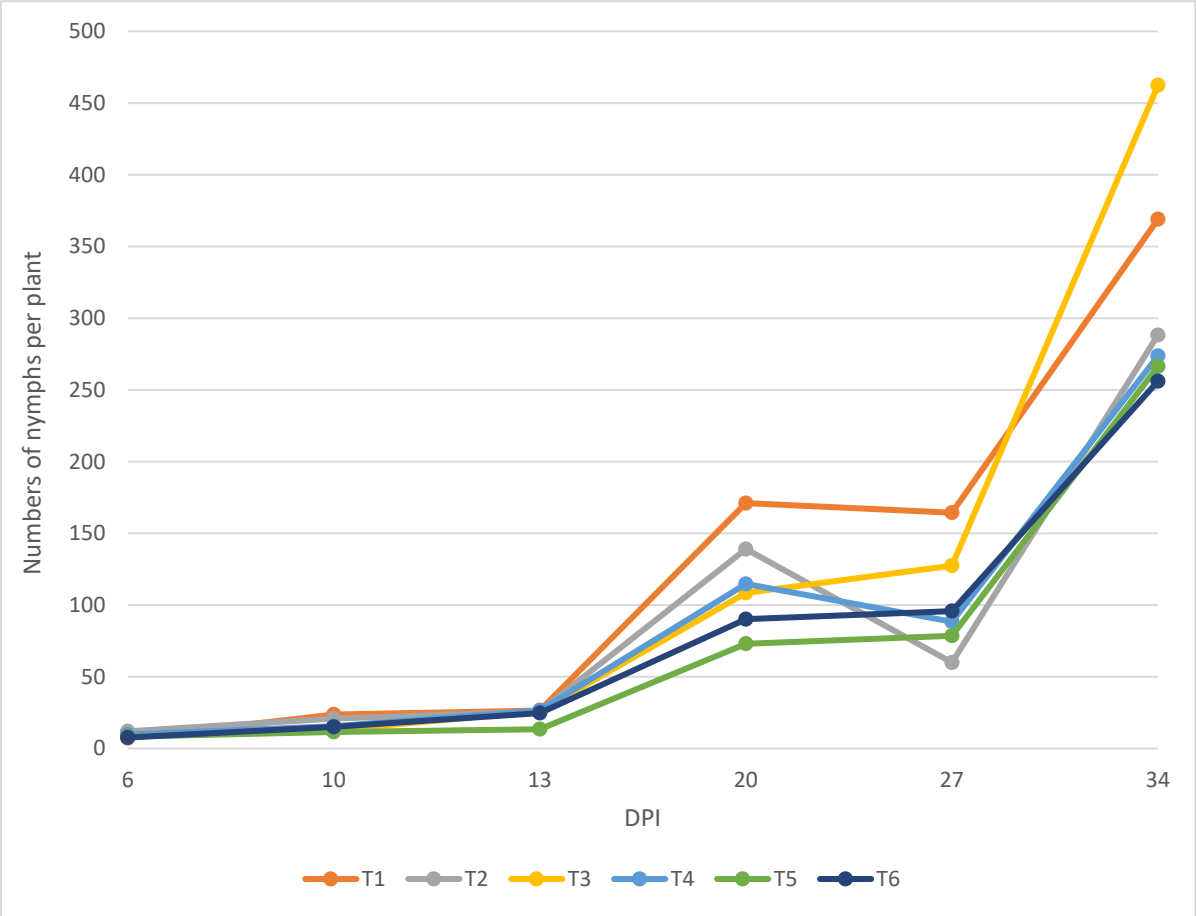


Figure 18 Back-transformed mean numbers of *M. persicae* nymphs recorded 6, 10, 13, 20, 27, and 34 days post infestation (DPI).

Table 20 Abbott's formula values for percentage reduction in numbers of *M. persicae* nymphs per plant compared with the untreated control, based on back transformed means.

Treatment	Days Post Infestation (DPI)					
	6	**10**	**13**	**20**	**27**	**34**
T1	-	-	-	-	-	-
T2	-65.19	11.98	2.34	18.72	63.56	21.89
T3	-30.94	45.30	2.26	36.58	22.47	-25.30
T4	-30.94	34.42	-0.26	32.96	46.20	25.85
T5	-14.23	51.41	49.43	57.32	52.19	27.83
T6	-5.39	36.57	6.79	47.31	41.72	30.60

Apterous adult aphids

There were no significant differences between treatments on days 6, 10, 13, 27 and 34. However on day 20 there were fewer adult wingless aphids in T5 and T6 compared with the untreated control (P=0.062, P<0.1) (**Table 31, Table 32, Figure 19**)

Table 31 Transformed mean number of *M. persicae* apterous adults per plant on each assessment date. Transformation: Log base 10 (x+1) (back-transformed means are shown in brackets). Values not sharing the same letter are statistically different (P<0.1).

Treatment	Days Post Infestation (DPI)					
	6	**10**	**13**	**20**	**27**	**34**
T1	0.3706 (1.35)	0.7716 (4.91)	1.0230 (9.54)	1.627 a (41.36)	1.637 (42.34)	2.061 a (113.97)
T2	0.4683 (1.94)	0.7418 (4.52)	0.9804 (8.56)	1.520 ab (32.11)	1.390 (23.54)	1.889 a (76.51)
T3	0.4077 (1.56)	0.6324 (3.29)	0.9863 (8.69)	1.384 ab (23.23)	1.452 (27.28)	1.865 a (72.34)
T4	0.3439 (1.21)	0.7753 (4.96)	0.9568 (8.05)	1.414 ab (24.94)	1.502 (30.79)	1.831 a (66.77)
T5	0.3460 (1.22)	0.5803 (2.80)	0.9002 (6.95)	1.246 b (16.63)	1.472 (28.65)	1.941 a (85.07)
T6	0.3460 (1.25)	0.6994 (4.00)	0.9591 (8.10)	1.344 b (21.07)	1.485 (29.53)	1.935 a (85.07)
d.f.	25	25	25	25	25	25
s.e.d	0.1066	0.1277	0.1558	0.1215	0.1201	0.1332
l.s.d	0.2196	0.2631	0.3208	0.2503	0.2559	0.2744
P value	0.831	0.580	0.982	0.062	0.488	0.609
	Significantly different from untreated control (P<0.1).					

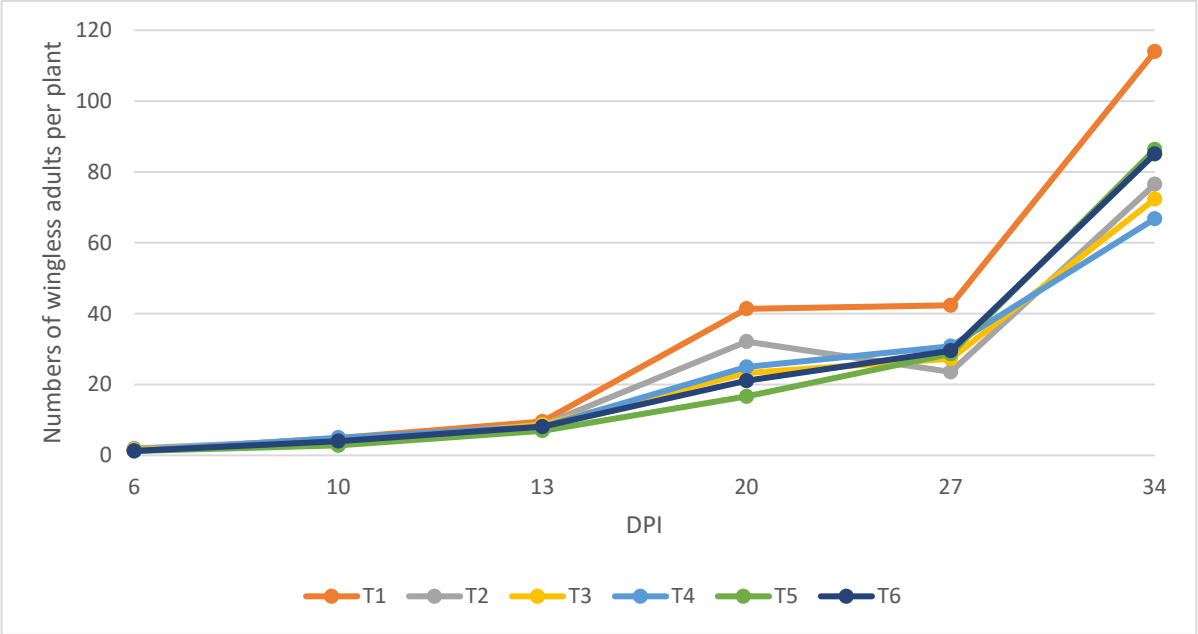


Figure 19. Back-transformed mean numbers of *M. persicae* apterous adults recorded 6, 10, 13, 20, 27, and 34 days post infestation (DPI).

Table 32 Abbott's formula values for percentage reduction in numbers of *M. persicae* apterous adults per plant compared with the untreated control, based on back transformed means.

	Days Post Infestation (DPI)					
Treatment	**6**	**10**	**13**	**20**	**27**	**34**
T1	-	-	-	-	-	-
T2	-43.70	7.94	10.27	22.36	44.40	32.87
T3	-15.56	32.99	8.91	43.83	35.57	36.53
T4	10.37	-1.02	15.62	39.70	27.28	41.41
T5	9.63	42.97	27.15	59.79	32.33	25.36
T6	7.41	18.53	15.09	49.06	30.26	25.36

Alate adult aphids

There were no significant differences in numbers of alate aphids per plant between treatments on any date, although numbers of winged aphids were highest in the untreated control from day 10 (**Table 33**). Alate aphids which had left the plant were recorded as numbers in the cage from 20 days post infestation. There were more alates found in the untreated plot cages compared with treatments 2 - 6 on day 27 (P=0.006) (**Table 34**). On day 34 there were more alates found in the untreated plot cages compared with T4, T5 and T6 (P = 0.023) (**Figure 20, 21**).

Table 33 Transformed mean number of *M. persicae* alate adults per plant on each assessment date. Transformation: Log base 10 (x+1) (back-transformed means are shown in brackets).

Treatment	**Days Post Infestation (DPI)**					
	6	**10**	**13**	**20**	**27**	**34**
T1	0.05230 (0.13)	0.17202 (0.49)	0.3164 (1.07)	0.9876 (8.72)	1.467 (28.32)	1.897 (77.92)
T2	0.13967 (0.38)	0.14744 (0.40)	0.2466 (0.76)	0.7329 (4.41)	0.930 (7.51)	1.676 (46.43)
T3	0.00000 (0.00)	0.03763 (0.09)	0.1401 (0.38)	0.8673 (6.37)	1.346 (21.16)	1.735 (53.34)
T4	0.01988 (0.05)	0.06675 (0.17)	0.2036 (0.60)	0.8060 (5.40)	1.162 (13.53)	1.602 (39.02)
T5	0.01988 (0.05)	0.03763 (0.09)	0.0575 (0.14)	0.6427 (3.39)	1.081 (11.05)	1.602 (39.03)
T6	0.00000 (0.00)	0.08018 (0.20)	0.1787 (0.51)	0.7546 (4.68)	1.156 (13.33)	1.565 (35.76)
d.f.						
s.e.d	0.06995	0.0897	0.1309	0.2115	0.2062	0.1960
l.s.d	0.14407	0.1846	0.2695	0.4357	0.4395	0.4036
P value	0.360	0.558	0.482	0.675	0.192	0.570

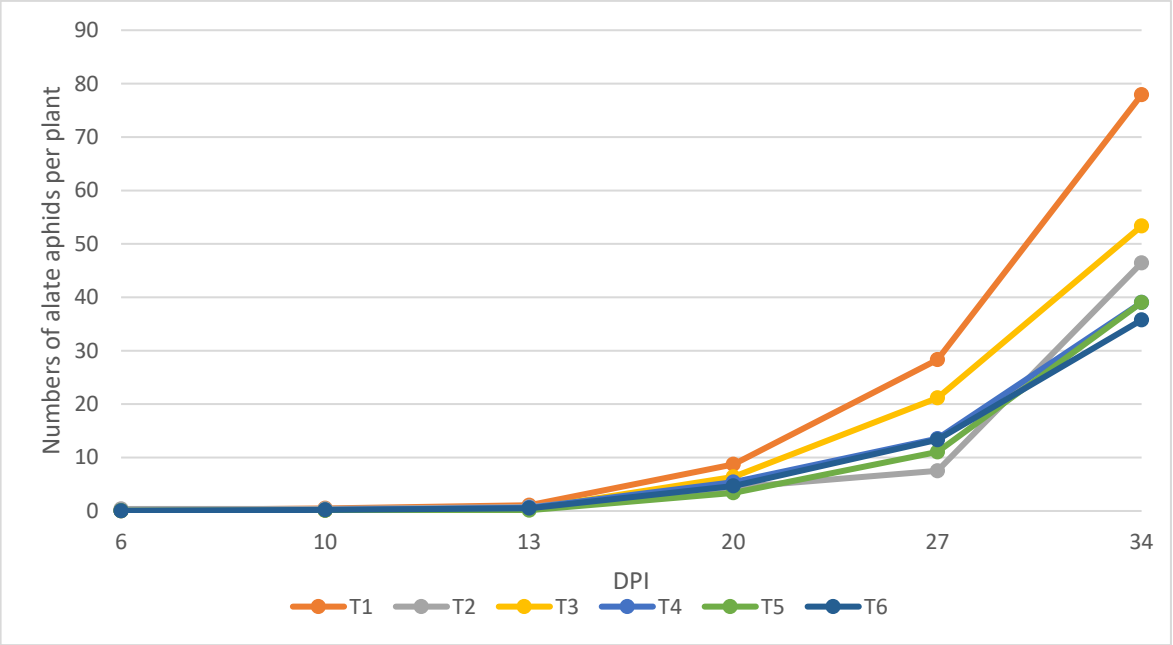


Figure 20. Back-transformed mean numbers of *M. persicae* alate adults recorded 6, 10, 13, 20, 27, and 34 days post infestation (DPI).

Table 34 Transformed mean number of *M. persicae* alate adults per 'BugDorm' cage on each assessment date from 20 days after infestation. Transformation: Log base 10 (x+1) (back-transformed means are shown in brackets). Values not sharing the same letter are statistically different (P<0.05).

Treatment	Days Post Infestation (DPI)		
	20	**27**	**34**
T1	1.1538 (13.25)	1.892 b (76.96)	2.143 b (137.85)
T2	0.9877 (8.72)	1.421 a (25.37)	1.849 ab (69.65)
T3	0.9583 (8.08)	1.324 a (20.10)	1.898 ab (77.99)
T4	1.1896 (14.47)	1.183 a (14.25)	1.683 a (47.19)
T5	0.8352 (5.84)	1.124 a (12.30)	1.584 a (37.34)
T6	0.7180 (4.22)	1.233 a (16.09)	1.676 a (46.37)
d.f.	25	25	25
s.e.d	0.2841	0.1700	0.1600
l.s.d	0.5851	0.3647	0.3295
P value	0.552	0.006	0.023
	Significantly different from untreated control (P<0.05).		

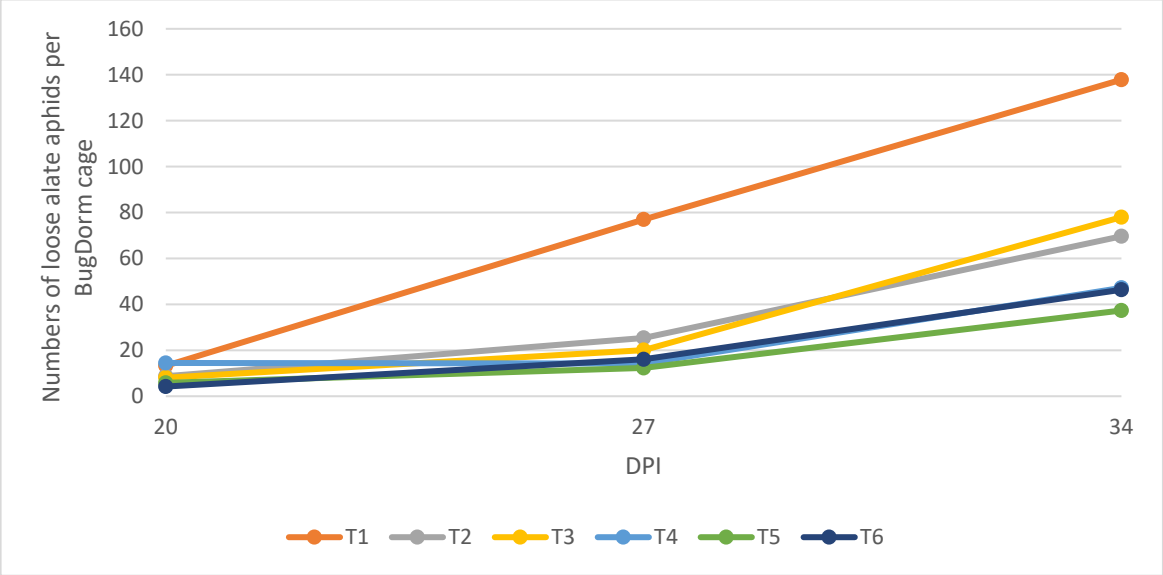


Figure 21 Back-transformed mean numbers of loose *M. persicae* alate adults per 'BugDorm' cage, recorded 6, 10, 13, 20, 27, and 34 days post infestation (DPI).

Model results

The total number of aphids, as predicted by the model with one, two, three and four applications of a neem-based product has been compared with the total number of aphids per plant recorded during the trial with one, two, three and four weekly applications of Azatin (day 7, 14, 21, 28) (**Table 35, Figure 22**). The total number of aphids recorded per plant in the untreated control (T1) was lower, but similar to the number predicted by the model until day 27 when the model prediction far exceeded the experimental results. The number of aphids per plant exceeded the model prediction in the treated plots (T2-T6) on all assessment dates, except for day 6.

Table 35 Total numbers of aphids recorded on each assessment date in the trial compared with the model prediction.

DPI	**6**		**10**		**13**		**20**		**27**		**34**	
	Trial	Model	Trial	Model	Trial	Model	Trial	Model	Trial	Model	Trial	Model
T1	9	18	29	33	37	46	224	242	254	846	595	3712
T2	14	18	26	3	35	2	178	26	97	62	429	352
T3	11	18	16	3	35	2	140	2	181	26	608	44
T4	11	18	21	3	35	2	149	2	136	2	391	8
T5	10	18	14	3	20	2	94	2	121	2	401	0
T6	9	18	19	3	33	2	117	2	143	2	389	0

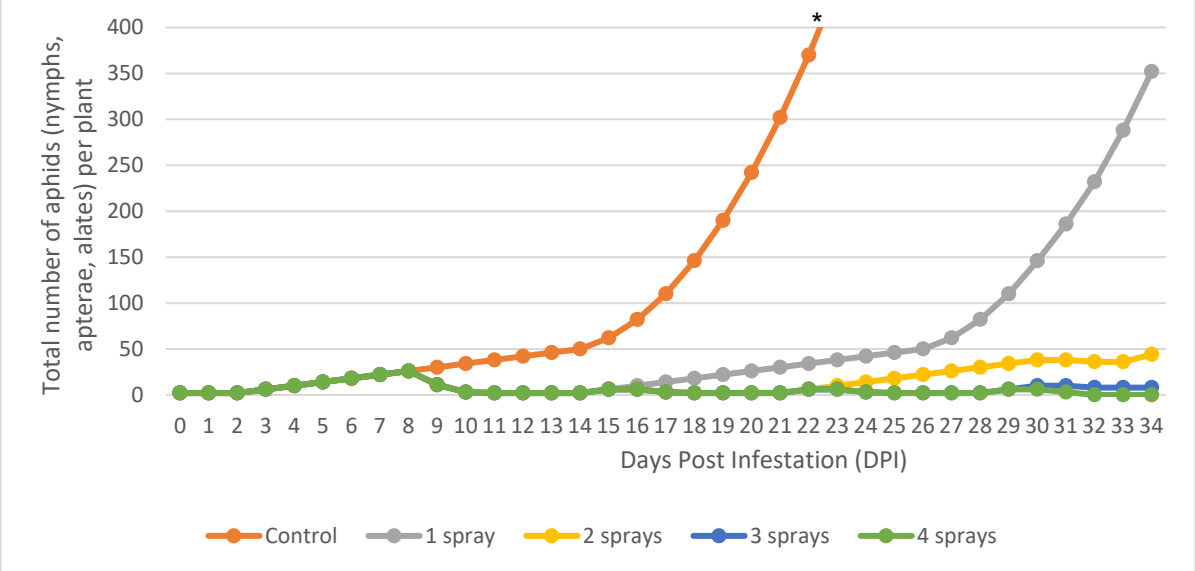


Figure 22 Model results predicting total aphid population after 34 days from a starting population of two adult *M. persicae* per plant after one, two, three and four applications of Azatin. *Modelled numbers in the untreated control increase to a value of 3,712.

Speed of kill

Speed of kill assessments showed that the number of nymphs increased by 682% from day 6 to day 14 in the untreated plot and by 232% in the treated plot (**Figure 23**). There was a sudden decrease in the number of nymphs and wingless adults nine days after infestation (**Figures 23 & 24**). The reduction in aphid nymphs and wingless aphids on day 9 is almost equal to the increase in the number of winged aphids found on day 9 in the untreated and treated plots (**Figure 25**). The number of alates reduced to less than five after day 9.

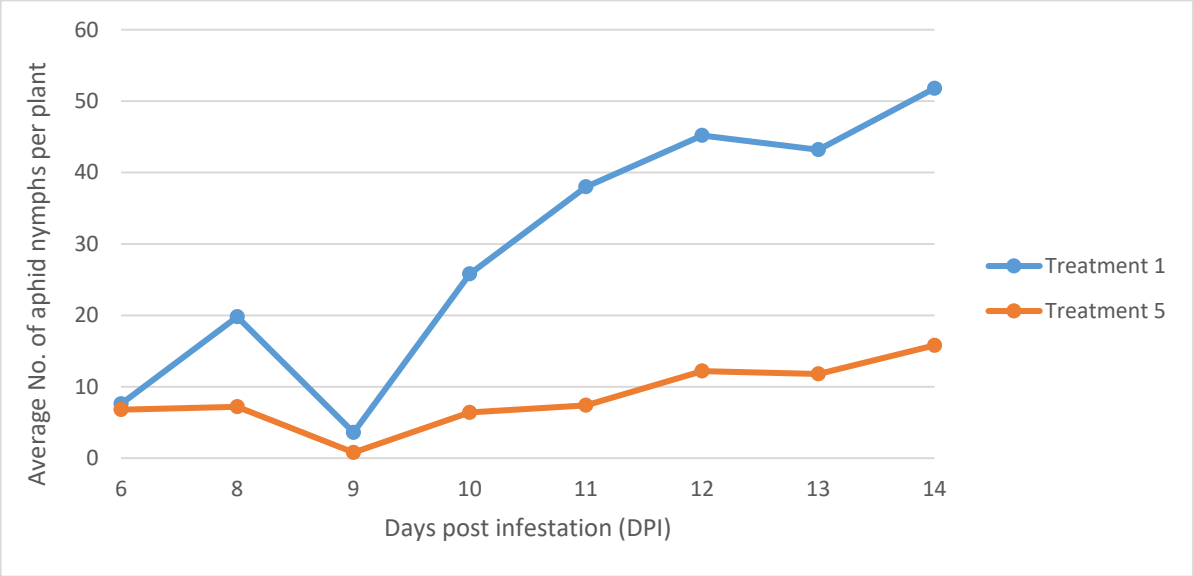


Figure 23 Numbers of *M. persicae* nymphs per plant recorded in a non-replicated speed of kill assessment from 6 to 14 DPI. In treatment 5, one application of Azatin had been made 7 DPI at 1.4 L / ha in 1000 L / ha water volume. Treatment 1 is the untreated control.

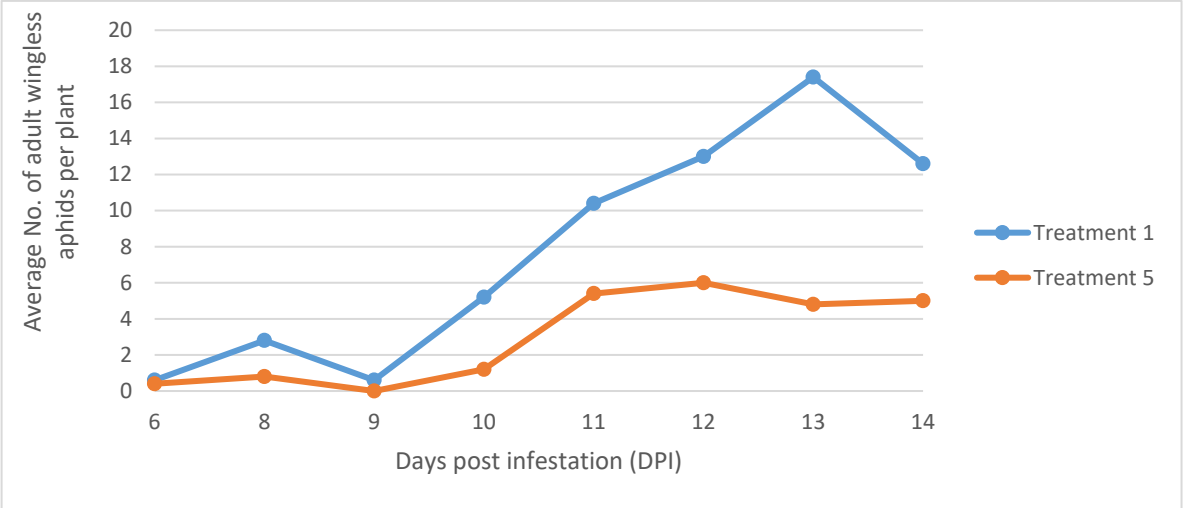


Figure 24. Numbers of *M. persicae* wingless adults recorded in a non-replicated speed of kill assessment from 6 to 14 DPI. In treatment 5, one application of Azatin was made 7 DPI at 1.4 L / ha in 1000 L / ha water volume. Treatment 1 is the untreated control.

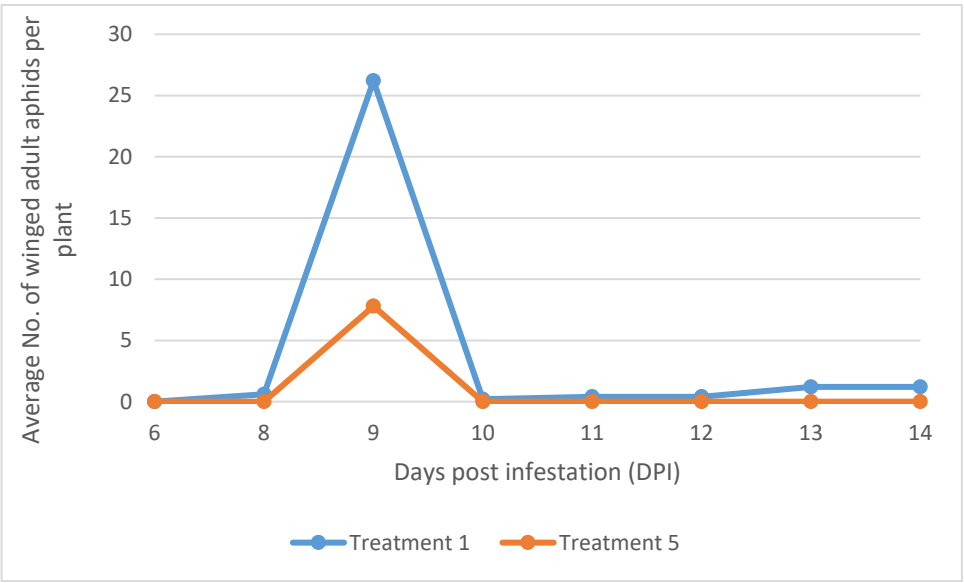


Figure 25.1 Numbers of *M. persicae* winged adults recorded in a non-replicated speed of kill assessment from 6 to 14 DPI. In treatment 5, one application of Azatin was made 7 DPI at 1.4 L / ha in 1000 L / ha water volume. Treatment 1 is the untreated control.

The number of aphid nymphs were equal on day 6 between the untreated and treated plots but there were more aphids in the untreated plot on day 8 (Figure 23: treatment 1 is the untreated control, treatment 5 is applications of Azatin every 7 days). (**Table 36**). The number of alates reduced after day 9 in both the treated and untreated plots (**Figure 25**). A single spray of Azatin caused large reductions in aphid numbers within 24 – 48 h of application, but this was not enough to eradicate the aphid population (**Table 36**), which then slowly recovered (e.g. **Figure 23**). *Myzus persicae* nymphs became adults after six days when

generating fixed age adults for this experiment, at 20ºC on pansy (data not shown). The number of nymphs in the treated plot increased throughout the experiment, whereas the number of treated wingless adults showed a slight decrease after 12 DPI (**Figure 24**).

Table 36 Percentage reduction in numbers of nymph and adult *M. persicae* after one treatment application of Azatin seven days post infestation, compared with the untreated control from 6 to 14 days post infestation.

Days post infestation	Percentage aphid reduction compared with untreated control (%)		
	Apterous (wingless) aphids	Aphid nymphs	Alate (winged) aphids
6	33.33	10.53	0.00
7	Spray applied	Spray applied	Spray applied
8	71.43	63.64	100.00
9	100.00	77.78	70.23
10	76.92	75.19	100.00
11	48.08	80.53	100.00
12	53.85	73.01	100.00
13	72.41	72.69	100.00
14	60.32	69.50	100.00

Aphid development

In this experiment pansies were infested with two fixed-age *M. persicae* adults on day 0, having been acclimated to pansy. On day 6, before any treatment applications were made there was an average of 2.7 apterous adults per plant (**Table 37**). Mean numbers of apterous adults per plant greater than two show that some of the produced nymphs had moulted into adults when the assessment was carried out, on day 6. There was an average of 6.1 nymphs produced per adult, equivalent to one nymph produced per adult, per day.

Table 37. Average number of apterous adult aphids and nymphs six days after infestation, before any treatments were applied.

Treatment	Mean no. of apterous adult aphids per plant	Mean no. of aphid nymphs per plant	Nymphs per adult	Nymphs per adult per day
T1	2.0	12.2	6.2	1.0
T2	5.8	23.3	4.0	0.7
T3	2.1	13.9	6.7	1.1
T4	2.3	14.6	6.3	1.0

T5	1.8	13.0	7.1	1.2
T6	2.2	14.6	6.6	1.1
Mean	2.7	15.3	6.1	1.0

Phytotoxicity

There was no phytotoxicity observed on pansy in this experiment. Some growth distortion was observed prior to the first spray application (6 DPI) and this has been attributed to Pansy Mottle Syndrome **(Figure 26)**.

A) B)

Figure 26. Pansy mottle symptoms observed six days post infestation. A) bleached, thickened growing points. B) flower distortion (left) compared with an undistorted flower (right).

Meteorological data

Average temperature was maintained around 20ºC, however the maximum temperature reached 38.5ºC and the minimum reached was 13ºC, giving a maximum temperature range of 25.5ºC (**Figure 27)**. Average relative humidity was maintained between 62% and 87%. The maximum relative humidity did not exceed 90% and 38% was the minimum humidity recorded (**Figure 28)**.

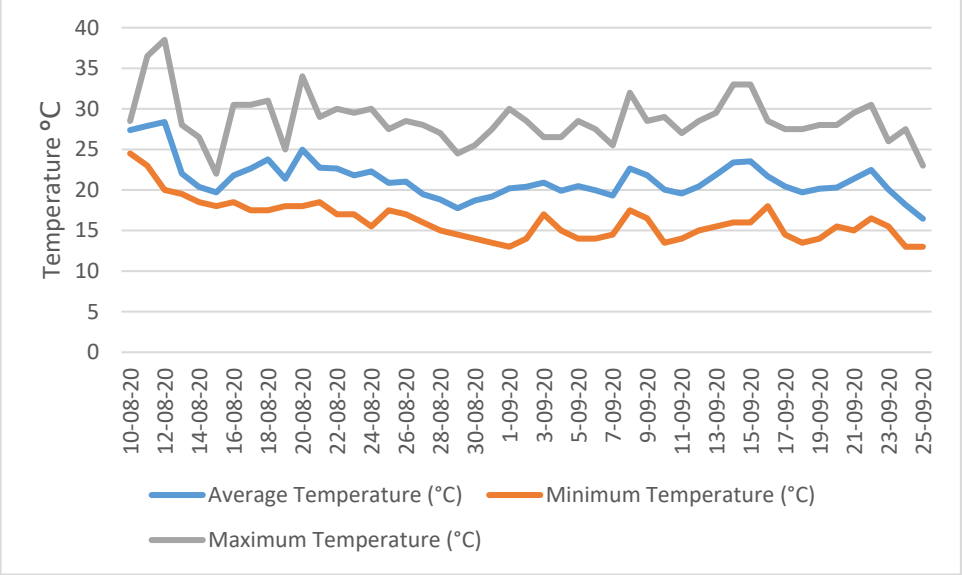


Figure 27. Mean, maximum and minimum temperature recorded during the experiment inside a central plot cage.

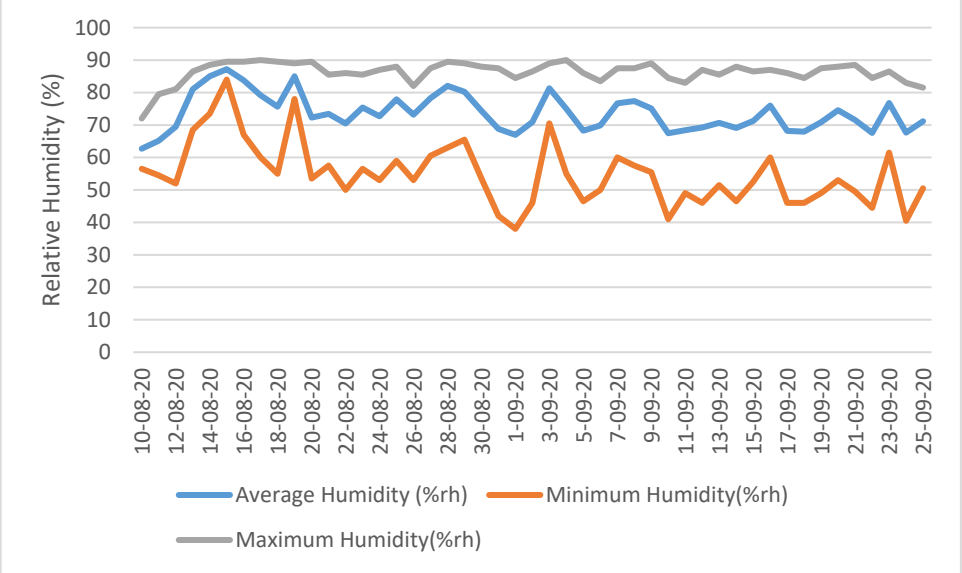


Figure 28. Mean, maximum and minimum temperature recorded during the experiment inside a central plot cage.

Discussion

Experiment results summary

Results from the validation experiment differed from the model predictions for one, two, three and four applications of a neem-based biopesticide. Four applications of Azatin did not eradicate the starting population of two adult *M. persicae* per plant as predicted by the model.

M. persicae performance on pansy

Myzus persicae parameter values were taken from La Rossa *et al.,* 2013 at 20-21ºC on Argentinean *M. persicae* on sweet pepper. Parameter values were compared to other studies using European *M. persicae* with good similarity but there was no available UK data (Pickering *et al*., 2020). Temperature will have influenced aphid developmental time, survival rate and fecundity; *M. persicae* population growth increases between 5 and 25ºC with an upper temperature threshold for increase between 25 and 30ºC (Barlow, 1962). Average temperature was maintained around 20ºC however this was not a constant temperature and the fluctuations above and below 20ºC may have influenced deviation from the model predictions. The model did not take account of the impact of humidity on aphid development. Data for azadirachtin efficacy parameters was taken from papers with experiments conducted between 17 and 23ºC, similar to this experiment.

On pansy in this experiment one nymph was produced per adult per day, which is less than the model parameter of two nymphs per day, which could account for the numbers of aphids in the untreated control being slightly lower than the model predictions up until day 27, after which numbers in the untreated control were much lower than the model prediction. The developmental time from birth to adult was six days on pansy, which is less than the programmed model parameter of nine days nymph development time, taken from experiments on sweet pepper and tobacco (La Rossa *et al.*, 2013; Barbosa *et al.*, 2011; Nikolakakis *et al.*, 2003). In an experiment on the life history and population growth parameters of *M. persicae* Hong *et al.*, 2019 found that the solanaceous host *Capsicum annuum* (sweet and chili pepper) was a less suitable host for *M. persicae* than *Nicotiana tabacum* (tobacco)*, Raphanus sativus* (radish) *and Vicia faba* (broad bean)*.* Fecundity, reproductive period, total longevity and total pre-reproductive period were more favourable on the other plant species compared with *C. annuum*. Life history traits of aphids are affected by the host plant species (Hong *et al.*, 2019). This experiment suggests that pansy is also a more favourable host for *M. persicae* than *C. annum,* on which the model was based. Since Azatin is claimed to be more effective against nymphs than adults and the nymphs were developing faster than predicted in this experiment, this could account for reduced efficacy of Azatin.

From 27 DPI the population in the untreated control in the experiment did not reach the model prediction, suggesting that pansy is a less favourable host than sweet pepper after 27 days. This could have been due to limited resource availability, since pansy growth was limited by the small pots.

Efficacy of Azatin

The efficacy of Azatin in this experiment was far lower than the model predications for efficacy of a neem-based bioprotectant. The pest population was not eradicated after four applications, as predicted by the model. The number of aphid nymphs was significantly reduced compared with the control when Azatin had been applied once (T2), three times (T4) and four times (T5), 27 DPI. The model predicted that multiple applications of a neem-based bioprotectant would be necessary to control *M. persicae* since it is most effective against nymphs. Multiple applications would be necessary to target the nymphs before they become adults.

Application

The model was programmed with information on the effect of neem-based bioprotectants against *M. persicae* with parameters based on results from spray application where available or from leaf dip experiments, where unavailable. Lowery and Isman, 1994 conducted a leaf dip experiment on sweet pepper against *M. persicae*. This experiment provided the model parameters for estimated adult mortality (10%), instar 1-3 nymph mortality (78%), instar 4 nymph mortality (50%). Nymph speed of kill (two days) was based on a different leaf dip experiment on sweet pepper (Lowry & Isman, 1994). In this experiment there was no significant reduction in the number of nymphs until day 27, when the number of nymphs was reduced by 63.56%, 27 days after one spray application (T2) and by 46.20% and 52.19% six days after three and four applications (T4 and T5, respectively). The results align with the model parameter for instar 4 nymph mortality of 50%. The model parameters for nymph mortality were validated in this experiment on only one assessment date (day 27) when Azatin was effective, however it was slower to take effect than the model predicted.

The discrepancy between the model prediction and the experimental results could be due to difference caused by using spray application in the experiment and data from leaf dip experiments in the model. Lowry *et al.*, (1993a) found that neem seed oil reduced *M. persicae* numbers on pepper by 95.4% in the laboratory and 80.9% in the field. Reduced efficacy in the field compared with the laboratory could be due to UV light, which can breakdown azadirachtin in water (Lowry *et al.*, 1993a). Azadirachtin is degraded by sunlight with a half-life of less than 24 hours on plant foliage (Boonsoong & Bullangpoti, 2009). Azatin may have persisted in the crop for less time than the model parameter of three days, reducing efficacy.

Azadirachtin solutions in water are most stable between pH 4 and 6 (Jarvis *et al.*, 1998), whereas the mains water used to spray Azatin in our experiment was pH 7.4 when tested on 10 May 2019, which could have reduced efficacy of the product. Efficacy of azadirachtin can be affected by application method. Aphids were found on the inside of the flowers, between the petals and around the calyx. In these locations it would be difficult to achieve spray coverage, even with the precision of a track sprayer. However commercial pansy growers would sell their plants before flowering, when they may be able to achieve greater contact with the pest. Lowry *et al.,* (1993) state that neem products must be ingested to be effective having shown that contact toxicity of neem does not significantly contribute to a reduction in aphid numbers, since there was no difference in efficacy between applications made immediately before infestation and 48 hours after infestation. Whereas Kaul (1999) found that the lethal concentration of azadirachtin was 80% less when applied topically to the aphids compared with introducing aphids to treated leaf discs against *Macrosiphum rosae* and *Macrosiphoneiella sanbornii.* Azadirachtin has shown to be effective systemically in some plants, such as cabbage (Arpaia and van Loon, 1993).

Azadirachtin may have multiple modes of action involving the neurosecretory-neuroendocrine axis and particular stages in cell division (Mordue *et al.*, 2005) causing an antifeedant effect in some species and a growth regulation and sterility effect in most species tested (Mordue (Luntz) *et al.*, 1998). Azadiractin interferes with insect growth regulation by affecting ecdysone biosynthesis and catabolism resulting in symptoms such as abnormal moults, larval-adult intermediates, mortality at ecdysis, delayed moults and extended instar lengths (Mordue *et al.,* 2005). Apterous adult mortality was greater (P=0.062) than the model parameter of 10% (taken from Lowry & Isman 1994) on day 20 when numbers of adults were 59.79% and 49.06% lower than the control, six days after two applications of Azatin in 1000 L water (T5) and 600 L / ha water (T6) respectively. Adults can also be affected by growth regulatory disruption by azadirachtin, passing growth regulatory effects to the offspring, causing mortality, along with effects on fertility and fecundity (Kaul, 1999).

Host plant

Data was included in the model parameters from non-sweet pepper hosts where information on sweet pepper was unavailable. Instar 1-3 nymph mortality (78%) was based on a neem kernel water extract spray application on cabbage (Basedow, 2003). Persistence of neem extract (three days) was estimated based on a spray application of Biosal 10 EC on outdoor cabbage (Akbar, 2021). Lowery *et al*, (1993) found that effectiveness of neem is influenced by the host plant since control of *M. persicae* was better on pepper than rutabaga. Therefore, using pansy as a host may have impacted the efficacy of Azatin in this experiment. Pansy

plants were used to provide results comparable with an experiment done in AHDB project CP 124 MOPS (Managing Ornamental Plants Sustainably) project. The MOPS experiment was on pansy and showed a similar percentage reduction in *M. persicae* numbers compared with the control to the AMBER model prediction (Pickering *et al*., 2020; Pope *et al*., 2015). This suggested that the model parameters based on sweet pepper would be applicable to *M. persicae* population development on pansy.

Rate and water volume

Where possible model data was taken for azadirachtin rates of 30 azadirachtin ppm based on the label rate for Azatin (217 g azadirachtin / L; 1.68 L product / ha) (Certis Europe, 2021). Therefore, it is unlikely that the rate caused a difference between the model predictions and the experiment results. The results from this experiment suggest that the higher water volume (T5) was effective against nymphs on day 27 whereas the lower water volume (T6) was not. Azadirachtin content in formulated products has been shown to correlate with efficacy against many species (Kaul, 1999; Lowery & Isman 1993). Therefore, using a lower water volume would not be recommended for this product. This could be because the product rate is a concentration (0.14%), therefore applications with a lower water volume also apply less product.

Product

The model parameters were taken from experiments using neem-based products, but not the product Azatin, used in this experiment. The efficacy of Azatin against aphids may differ from that of other azadirachtin products. Jukes and Collier (2019) applied two azadirachtin products on day 0 and day 7 to a starting population of 10 *M. persicae* on Brussels sprouts. Azatin was not effective against *M. persicae* in their study but another azadirachtin product, applied with adjuvant Phase II significantly reduced aphid numbers compared with the control (Jukes & Collier, 2019). Azadirachtin has been shown to have efficacy against pests: whiteflies, leaf miner, fungus gnats (sciarid flies), thrips, aphids and caterpillars (Mordue *et al*., 2005). However, Azatin is only recommended on label for control of thrips with EAMUs for control of whitefly (1265/20 and 1266/20). Azadirachtin content required for aphid control is higher than for control of lepidopteran species (Kaul, 1999). Efficacy of Azatin against other species could be lower compared with thrips and whitefly control. Azatin is an emulsion concentrate, containing 217 g / L azadirachtin (formulated to achieve a concentration of 26 g / L Azadirachtin-A) (Certis Europe, 2021). The main biological effects of neem seed extracts have been shown to be due to azadirachtin. However other components of neem seed extracts have been linked to activity against insects (Kaul 1999; Mordue *et al*., 2005). *Myzus persicae* developed resistance to azadirachtin over 40 generations but did not develop

resistance to neem seed extract over the same number of generations (Feng & Isman (1995). This suggests that azadirachtin products containing other components of neem seed extract could have greater efficacy against *M. persicae* compared with pure azadirachtin products. It is also possible that the aphids used for our experiment could have some resistance to azadirachtin as the resistant clone 2169G was used for this experiment. Results from our experiment differ from the MOPS experiment, which also used a clone of *M. persicae* resistant to pyrethroid and carbamate insecticides (Smith *et al*., 2018). The MOPS experiment showed a significant reduction in aphid numbers from the water control 6, 13, and 21 days after the first spray application of a different biopesticide based on azadirachtin A (Pope *et al*., 2015; Smith *et al*., 2018). Difference in efficacy between the two experiments could be due to differences in formulated azadirachtin products.

Phytotoxicity

No phytotoxicity was seen on pansy in this experiment. Some phytotoxic effects have been recorded on seedlings of other plant species at high concentrations of 500 ppm pure azadirachtin (Mordue *et al.*, 2005). Saintpaulia and Cyclamen can experience temporary spotting from spray deposits of Azatin (Certis Europe, 2021).

Speed of kill

The number of nymphs in the treated plot continued to increase after 12 days, whereas the number of treated wingless adults started to decline. This suggests that nymphs exposed to the application on day 7 died trying to moult into an adult on day 12, five days after exposure. whereas the model was programmed with two days before mortality of nymphs.

The decrease in aphid nymphs recorded on day 9 in the speed of kill assessment was likely caused by the development of fixed age nymphs into alates instead of mortality caused by Azatin since the number of alates rose simultaneously. The number of apterous adults also decreased slightly on day 9, this could be due to misidentification of nymphs or mortality due to application of Azatin. The difference in number of alates found on day 9 between the treated and untreated plots could be attributed to application of Azatin, preventing moulting and development into the alate form. The number of alates reduced after day 9 in both the treated and untreated plots, which suggests that this reduction could be due to the alates flying from the plant; alates loose in the cage were not recorded in the daily assessments. *Myzus persicae* nymphs became adults after six days in this experiment, at 20ºC on pansy. The non-replicated speed of kill assessment showed a reduction in nymphs of 78% after two days, in line with the model parameter for instar 1-3 mortality and speed of kill.

Trial limitations

Aphids were predominantly found on the pansy flowers rather than on the leaves, which may have enabled a faster development time compared with that on sweet pepper. Several studies have shown that *M. persicae* prefer senescing leaves to mature leaves (Kennedy, 1958; Aldamen and Gerowitt, 2009). As the pansy flowers were senescing faster than the leaves this could explain the preference of the *M. persicae* for the flowers. In an experiment by Assefh *et al.,* (2014) induced senescence of potato improved plant acceptance by *M. persicae* through an increase in sap ingestion and consequent reduction in nymph development time. Therefore, it is possible that *M. persicae* benefited from a reduced development time by locating on the flowers, which developed from bud to senescence in a matter of days.

In order to use the track sprayer, the plants needed to be moved from their cages in the glasshouse to the spray polytunnel. The pansy petals were fragile and some of them fell off during transferral to the track sprayer or during assessments. Aphids tended to be found on the petals and may have been lost from the experiment. However, since the efficacy of the product was lower than expected any missing aphids would have contributed to an even lower efficacy compared with the untreated control plants which were not moved from the cages to the spray tunnel

The trial plants died when heavily infested with aphids, limiting their potential. *Phytoseiulus persimilis* was released to control two-spotted spider mite, however this was not enough to control them on a few plants and insecticides could not be used. These plants were excluded from the analysis.

A biological model is inherently limited by its parameters as many biological parameters cannot be directly measured, over parameterised models are also limited if the measured data cannot provide accurate estimates for all model parameters (Zaffaroni, *et al.,* 2020). Therefore, it is reasonable to expect limitations in the model, which can be further enhanced with the addition of more data to base the parameters on.

Conclusions

The strategy by which a biopesticide is applied (i.e. the timing and frequency of application) is fundamental to its efficacy. Biopesticides are much less ‘forgiving’ that conventional chemical pesticides and so it is important to identify the best application strategies in order to get the best out of them. Most biopesticides have contact activity, and they are generally slower to act compared to conventional pesticides (which often have an instantaneous mode of action). A slower speed of kill means that the target pest may grow, feed and reproduce after the biopesticide has been applied. They can also have different levels of lethality to

different pest life stage, for example eggs may be less vulnerable than the adult stage. As a result, the amount of pest control is affected by a range of features associated with pest biology; these include things like pest growth rate, reproduction, the relative susceptibilities of different instars to the biopesticide, and pest population size. There are also inherent features of the biopesticide that will determine its efficacy: speed of kill, lethal concentration, persistence on the leaf surface and so forth. Until now, these issues have not been considered in any detail when people are designing an IPM programme with biopesticides, but it is important that they are thought about. At the moment, application strategies for biopesticides are often developed on a trial and error basis rather than by using detailed information on their population dynamics.

The box car train model developed in AMBER is the first computer system in the world designed to predict the effects of different application strategies on bioinsecticide performance. The big advantage of a model is that it allows rapid testing of different application scenarios to identify the most promising options. These can then be tested on a crop, either in the glasshouse or the field. Attempting to investigate all the different options for a spray programme in a crop at the outset would be prohibitively expensive and time-consuming. The model is not intended to replace glasshouse / field experiments, but rather to guide experimenters and IPM practitioners in designing effective application strategies more quickly.

The model has been developed for whitefly and – as studied here – for *Myzus persicae*. Now that the basic principles have been developed, they can be applied to any pest species where there is suitable data for modelling. Of course, the accuracy of a biological model depends on the quality of information on which the parameters are based and, unfortunately, there is a paucity of information needed for model construction for many pests. Now that we have demonstrated the potential of the approach, the next stage would be to generate the baseline data that is missing for a range of pest species.

The model still requires additional work. The results of the study suggest that Azatin acted more slowly on the nymph population than predicted by the model. The results also show that plant species has potential to significantly impact on biopesticide efficacy via effects on the pest population growth rate. In this case, our experiments showed that aphid development rate observed on pansy was faster than the rate used in the model, which used data published in the scientific literature for aphids on sweet pepper. The effect of host plant on pest population growth is probably underappreciated in IPM and it may explain why a biopesticide works against a pest species on one crop but does not give adequate control when applied against the same pest, with the same application regime, on another crop species. This is

something that needs more attention. Earlier in this report, we made recommendations for a new trials data recording template for biofungicides, but the basic principles (e.g. the importance of collecting good quality data in a systematic way) clearly also apply to bioinsecticides. Growers and their advisors should be collecting information on the population growth rate for pest species on different crop species and varieties, and then relate that to the speed of kill of the biopesticides they are using. As a rule of thumb, pests with short life cycles will need more frequent applications than those with long life cycles. If the biopesticide has low persistence, and insect nymphs can 'escape' contact acting biopesticides by moulting, then it may also be worth considering a 'little and often' application strategy in which, rather than apply a high dose in one go, a lower dose is applied frequently. However, this would need to be investigated using carefully designed experiments, so that the effect of the different parameters that impact on efficacy can be studied in a reliable way.

When biopesticide companies develop a biopesticide product, they are required to perform efficacy studies for registration, but in reality more detailed information is required than this that links biopesticide performance to pest population biology and crop type in order to 'fine tune' the use of the biopesticide within IPM. The modelling approach that we have developed here would be the best way to do this. Going forward, for this part of the research, the logical next step would be to record the development rate of *M. persicae* on pansy, and also to test the efficacy of Azatin against *M. persicae* on pansy in leaf dip experiments and with spray application on different plant growth stages (this would help us determine whether aphid location and spray application are the cause of variable results in this trial). It would also be useful to compare bioassay results for Azatin with the azadirachtin A product used in AHDB project CP 124 MOPS to test for differences in efficacy due to formulation (Pope *et al*., 2015; Smith *et al*., 2018). The new data could be used to reprogramme the model and refine its predictions for testing in the glasshouse.

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Final conclusions and recommendations

The availability of synthetic pesticides is under severe pressure because of product withdrawals on environmental and safety grounds, as well as control failures caused by the evolution of heritable resistance in target pest and disease populations. Biopesticides / bioprotectants have now entered the mainstream and have potential to fill some of the gaps if used as part of an integrated pest and disease management (IPDM) system. (As an aside, the term 'bioprotectant' is becoming increasingly popular within the industry, while the term 'biopesticide' is falling out of favour. In AMBER, we have tended to stick with 'biopesticide' for consistency but in any future programme we will probably use 'bioprotectant').

The original task set for the AMBER team was to identify improvements to management practice to make biopesticides more reliable and effective. Biopesticides encompass a very wide range of entities, including living microorganisms and natural products, each with their own specific characteristics and requirements. Identifying generic improvements to management practice that apply across the board, for the range of biopesticide products, crops and growing systems used in modern commercial horticulture, is not simple. Our approach was to put a lot of effort into observing how growers used biopesticides in commercial practice, within their existing IPM and growing systems, for a range of crops, pest and diseases, and biopesticide products. This allowed us to identify where the common weak points were in management practice and to come up with ways forward.

The central, unifying issue is that biopesticides require precision application systems to work effectively. This contrasts with many synthetic chemical pesticides, which are more 'forgiving' and enable high levels of pest and disease control even when application is not that good. Biopesticides are attractive because of their human and environmental safety, but if they are

to realise their potential, we need precision application so that biopesticide products can be delivered at their effective dose at the right time and right place in the crop. Our strategy has been to: (i) bring together the scientific knowledge that underpins precision application – which we have done through experiments and also by critically analysing the available literature: (ii) use this knowledge to develop new tools that enable precision application; and (iii) translate information over to growers, agronomists, biopesticide companies and others.

Our approach is well illustrated by the work that we report on in this annual report.

We have shown that, in nominal constant dose applications, spray water volume affects the amount of biopesticide on the leaf surface. The best strategy is to identify water volumes that result in the highest concentration of product on the crop. In many cases, this is likely to mean using a lower water volume, which is also quicker to apply and reduces waste. There has been a long-held misconception that higher water volumes give better coverage, which is probably encouraged by the fact that labels often specify high water volumes, up to 1500 L / ha (contrast this with pesticide applications to arable crops which are around 60 L / ha). Unfortunately, data supporting the recommended volumes for commercial biopesticides does not appear to be available from the companies themselves. Moving forward, it is important that the best water volumes are identified and the information is sent out to end users.

The activity levels of all microbial biopesticides are determined by environmental temperature. However, not enough work has been done to identify the microbial strains with the best thermal performance curves for the target crop environment. Using fast track systems to screen biopesticides for response to temperature, as shown here, will lead to more effective biopesticide products, with cost savings for growers in terms of better pest control.

The literature review of microbial biofungicides done in this report gives growers and agronomists a summary of the current ‘state of the art’ of knowledge about the conditions and management practices required for successful use of these products. The new recording template will enable growers to systematically collect the data needed to explain the performance levels obtained with biofungicides in commercial practice. This data is vital in situations where the biopesticide does not perform as expected, as it provides the evidence to help identify the problem and develop solutions to correct it.

The box car train model developed in AMBER is the first computer system in the world that predicts the effect of application strategy on bioinsecticide performance. The model still requires some additional work, as it has shown that plant species is likely to impact on biopesticide efficacy via effects on pest population growth rate, and hence data on pest growth rate is needed on different plant species. The big advantage of the model is that it allows rapid testing of different application scenarios to flag up the most promising options,

which can then be tested in the laboratory or glasshouse. Attempting to investigate all components of a spray programme in a crop would be prohibitively expensive and time-consuming. At the moment, application strategies are often developed on a trial-and-error basis. The AMBER model has real potential to take the guess work out of spray programme development, leading to better performance of biopesticide products. Efficacy testing is also a significant fixed cost for biopesticide companies: if it can be reduced, this should result in making biopesticide products cheaper and more price competitive with other products.

To the best of our knowledge, AMBER is the only project in the world to have taken this systematic approach for biopesticides, and the research has generated a lot of interest from researchers overseas who have been keen to learn from the work and to see how our strategy can be replicated elsewhere.

Looking forward, a set of emerging technologies looks set to transform biopesticide use (and IPM more generally) in horticultural crops in the coming years (**Figure 29)**. They include digital technologies (big data, the Internet of Things, machine learning, blockchain), physical technologies (robotics, autonomous vehicles, machine vision, advanced materials) and a wider range of bioprotectants (aka biopesticides). Advances in these areas have potential to enable significant improvements in the precision application of biopesticides. This will be done by integrating pest and disease detection, analytics, and automated spray application.

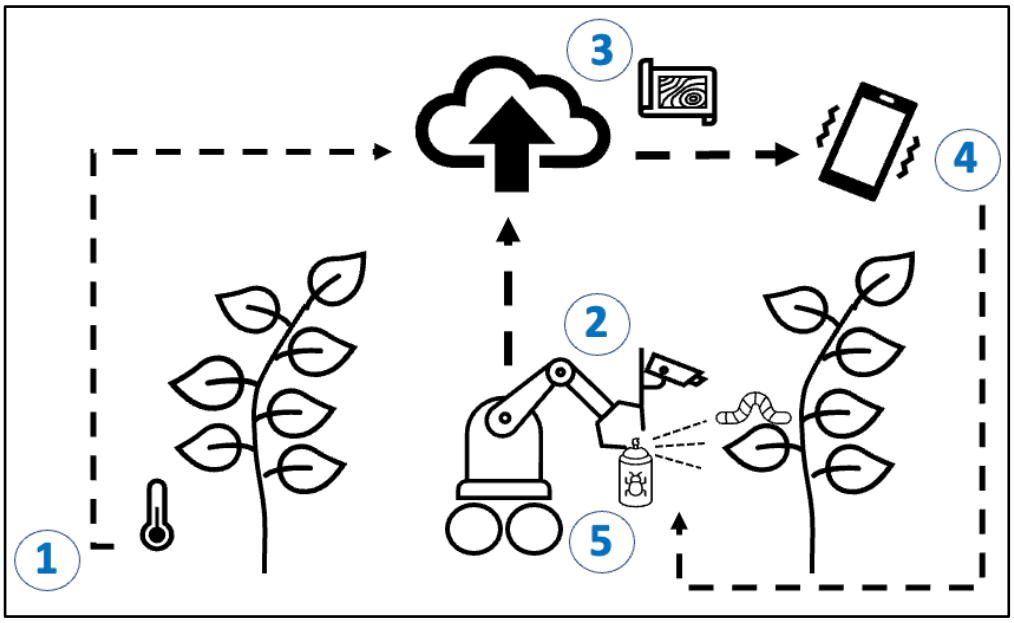

Figure 29 Schematic of an automated, 'smart' decision support system for precision application of biopesticides in IPM. This example is for a biopesticide that acts as a curative, but the system will essentially be the same for preventative treatments. (1) An array of wifi enabled sensors relay data on

environmental conditions and plant health metrics to the cloud via a data hub; (2) GPS enabled autonomous vehicles use machine vision and other onboard sensors for P&D scouting and relay data to the cloud; (3) Cloud based algorithms construct a 3D crop map and identify P&D hotspots. Epidemiological models for pests & diseases (based on machine learning from big data sets achieved by pooling data from multiple locations) are combined with in-crop environmental data, plus information on the performance and compatibility of biopesticides, natural enemies and other IPM agents, to make decisions about targeted application of biopesticides. (4) Real-time data, 'action alerts' and guidance sent to dashboard on grower's smartphone. Grower makes informed decision about biopesticide application. (5) Targeted, low volume application of biopesticide to pest / disease hotspot by autonomous robot sprayer.

A number of 'smart' technology platforms are already starting to come on to the market. Some companies offer multiple components and are working to develop an integrated complete package, while others provide individual components that the grower would put into a system they develop themselves. In some cases, companies selling complementary technologies have formed partnerships. As we see it, the big advances will be made by fully integrating the different components into a total precision application system, supported by detailed scientific knowledge on the interactions between pest / disease, biopesticide, crop and environmental conditions.

Knowledge and Technology Transfer (2020/2022)

- The team held a 2 hour webinar on the 30th June 2020 entitled “From AMBER to Green - the next phase in using bioprotectants for Integrated Pest Management in horticultural crops’. The presenters explained the progress of the project and gave recommendations for growers, agronomists and the biopesticides industry. There were > 50 participants on the day, and the webinar had an additional 70 views on YouTube https://youtu.be/Cd1Hh_C_KoA

  Presentations were as follows:

  - Webinar introduction – (Dave Chandler, Warwick University) .
  - Incorporating Bioprotectants into IPM Programmes: Theory into Practice (Rob Jacobson, RJ Consultancy).
  - A systematic approach to improving spray application of bioprotectants (Clare Butler Ellis, Silsoe Spray Applications Unit).
  - How a new ‘boxcar’ model of insect pest population growth will help inform bioprotectant application strategies (Elysia Bartel, ADAS) .
  - Two heads are better than one: why improved recording of trials data will lead to better results with bioprotectants (Erika Wedgwood, ADAS).
  - The view from the bioprotectants industry (Roma Gwynn, Biorationale).
- Presentation to OECD Expert Group on BioPesticides, Seminar on “Different aspects of efficacy evaluation of biopesticides” 28th June 2021.
- Presentation to Plant Biologicals Network, Copenhagen, 11 November 2021
- Presentation to Pesticides Action Network 16th February 2022.
- Presentation to John Deere 5th January 2022.
- Presentation on AMBER at AAB Conference Bringing Biocontrol and IPM to Market, Marston, 17 November 2022.
- Presentation on AMBER at BCPC Pests and Beneficials IPM meeting 26 January 2023.