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

AUTHENTICATION

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

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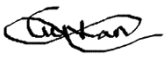
Signature  Date 21/11/2016

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GROWER SUMMARY

Headline

This is the first year of three year-PhD project with much of the time spent on developing experimental protocols. The propidium monoazide quantitative polymerase chain reaction method was developed for quantifying viable populations of *Bacillus subtilis* Strain QST 713 (Serenade) and PreStop (*Gliocladium catenulatum* strain j1446) from crops. The method was developed for advancing the research as no prior method existed for counting the viable numbers for both these biocontrol agents in a sensitive manner. In vitro dose experiments (experiments on agar plates) have suggested that both the BCAs work at suppressing *Botrytis cinerea* colonisation (the fungal pathogen responsible for grey mould) at 10^8 colony forming units or spores per droplet. In vivo studies (studies on lettuce plants) have suggested both BCAs significantly reduce disease severity and or stop infection at 10^8 colony forming units or spores a ml which is the same as 100L/hectar.

Background

Biocontrol agents are living organisms and as with every organism reproduction is critical to its survival. Understanding how environmental conditions affect survival, reproduction, dispersal and biocontrol efficacy is crucial to expanding their use. The overall aim of the study is to obtain ecological knowledge on biocontrol agents available in the UK and utilize the knowledge to produce strategies of applying biocontrol agents to improve efficacy against *B. cinerea* on lettuce and strawberry crops.

Summary

Biocontrol products such as Serenade and PreStop are formed by biological organisms. There were no appropriate ways available on how to estimate the actual numbers of these organisms on the crop after spraying. A large section of the first year focused on forming a methodology in which these organisms can be counted from the leaf surface. Knowing the amount of biocontrol agent product on the crops surface is important since a certain amount is needed to control pathogens such as botrytis the causal agent of grey mould.

Studies on agar plates have shown that a 10^8 cfu/droplet (Serenade) and 10^8 spores/droplet (PreStop) is needed to suppress the growth of botrytis. Studies on lettuce plants have shown that 10^8 cfu/ml of Serenade and 10^8 spores/ml of PreStop is needed to stop and or significantly reduce disease severity by botrytis (results will be included in second year annual report).

Now that we know how much of the biocontrol product we need, and have found a way to count/measure the amount of biocontrol agent on a crops surface; we can monitor the effect of the environment on the biocontrol agent product. Finding which environmental conditions effect the biocontrol agent number can be important for understanding when to apply the biocontrol agent product and to improve how well the biocontrol agent product works.

A large portion of the next stage in the research will be focused on finding out which environmental conditions within growing environments across the U.K such as glasshouses and polytunnels may allow the biocontrol agent product (Serenade and PreStop) to increase in number to better protect the crop.

Preliminary conclusions are drawn only from graphical representation of the data and require further statistical analyses for dose response experiments. We have developed a reliable and robust molecular based assay to quantify *B. subtilis* (Serenade) and *G. catenulatum* (PreStop) viable propagules with the PMA-qPCR technique. The threshold BCA dose for disease suppression, though temperature dependant, is estimated to be 10^8 cfu/ml for both Serenade and 10^8 spores/ml for PreStop.

The next stage of the research will focus on monitoring the number of biocontrol agents on lettuce and strawberry plants for ten days in different climatic treatments formed by different temperature and humidity combinations. The selected climatic treatments will be representative of temperature and humidity combinations which occur in glasshouse and polytunnel environments all year round, additionally the selected climatic treatments will also be representative of field environment between March to May.

The data collected will be used to form a model capable of estimating the biocontrol agent number up to ten days depending on the climate subjugation after the biocontrol agent has been sprayed onto the crop.

Once the model has been completed research effort will be focused onto how the biocontrol agent disperses on living leaf tissue and if rainfall can effectively disperse biocontrol agents from elder leaves to newly developing leaves.

Financial Benefits

Even though at this stage there are no financial benefits in year 1, forming an understanding on how the climate effects Serenade and PreStop populations may optimise the use of the biocontrol agents which could have financial benefits.

Action Points

- Identify the impact of commercial growing conditions (Temperature and relative humidity combinations) on *B. subtilis* (QST 713) and PreStop viable population's overtime (10 days).
- Identify the colonisation pattern of *B. subtilis* (QST 713) and PreStop on growing leaf tissue.
- Identify the effect of natural rainfall event on *B. subtilis* (QST 713) and PreStop viable dispersion onto newly developing leaves.

SCIENCE SECTION

Introduction

Botrytis cinerea infects around 200 plant species worldwide (Dean *et al*., 2012). The estimation in the costs of *Botrytis* damage is difficult due to its broad host range and the lack of reliable figures (Dean *et al*., 2012). Yet the global cost for chemical *Botrytis* control is estimated to be around €38/ha (Steiger, 2007). At the postharvest stage 15-50% of fruit and vegetables are lost to pathogenic decay by various pathogens; *B. cinerea* is one of the most important decay pathogens (FAO, 2011), maximum losses occur in developing countries due to the lack of essential technologies for disease management (Sharma, Singh, and Singh, 2009). Crops susceptible to *B. cinerea* include strawberry and lettuce (Droby and Lichter, 2007).

Botrytis cinerea is primarily controlled by frequent application of fungicides. The use of such agrochemicals is a blunt instrument as they are often non- or semi-selective and can damage/disrupt beneficial microbiota residing in the phyllosphere. Biological control is an eco-friendly alternative (Driesche and Bellows 1996). In comparison to agrochemicals, biocontrol efficacy and consistency is often poor, especially in outdoor 'field' conditions. Biocontrol efficacy in the field and protected crops varies greatly and can be strongly influenced by climatological (light, air, temperature, humidity, rainfall and wind) and ecological (phyllosphere, epiphytic microbiota, condition of crop surface) factors.

In the UK there are two registered BCAs for *Botrytis*; Serenade (formulated with the bacterium *Bacillus subtilis* strain QST 713) and PreStop (formulated with the fungus *Gliocladium catenulatum* strain J1446). These BCAs have specific modes of action against the target pathogen. *Bacillus subtilis* primarily uses antibiosis and competition for nutrients and space, whereas *Gliocladium catenulatum* directly parasitizes the conidia and hyphae. Depending on how these agents interact, this raises the possibility of complementary or even synergistic anti-pathogen activity.

A key aspect that affects biocontrol efficacy is the dynamics of viable BCA propagules and their dispersal. There is a lack of knowledge on how BCAs colonise and survive on crops. Without such knowledge, improvements in application strategy (timing, mixtures, concentrations as well as application technique) would be difficult. Of particular interest is the impact of climatological factors on the dynamics of BCA populations and the pathogen on crop surfaces. For effective biocontrol it is crucial to understand and be able to predict how the populations of biocontrol organisms interact with each other and how this action is influenced by application strategy and the environment. This project will primarily focus on

enumeration of biocontrol population changes in relation to external factors to improve our understanding of the ecological process.

The main research question for this project is: what are the impacts of climatic variables and the use of mixed applications on the survival, reproduction, dispersal and biocontrol efficacy of two commercial BCAs (*B. subtilis* and *G. catenulatum*) against *B. cinerea* on lettuce and strawberry crops. This knowledge can then be used to improve biocontrol strategies for the disease.

The research will focus on (1) quantifying the viable population, dispersal patterns and the efficacy of commercially available BCAs on strawberry and lettuce crops in different climatic conditions (2) assessing if the use of mixed BCAs would result in synergy and reduced variability in biocontrol efficacy, and (3) developing BCA application strategies to improve control.

In this reporting period, we have conducted several experiments to achieve the following specific objectives:

i. To develop a reliable and robust molecular-based assay to quantify viable populations of *B. subtilis* and *G. catenulatum* on crops.
ii. To identify the minimum BCA dosage required to inhibit and suppress *B. cinerea* infection in vitro (maximum expected effort).

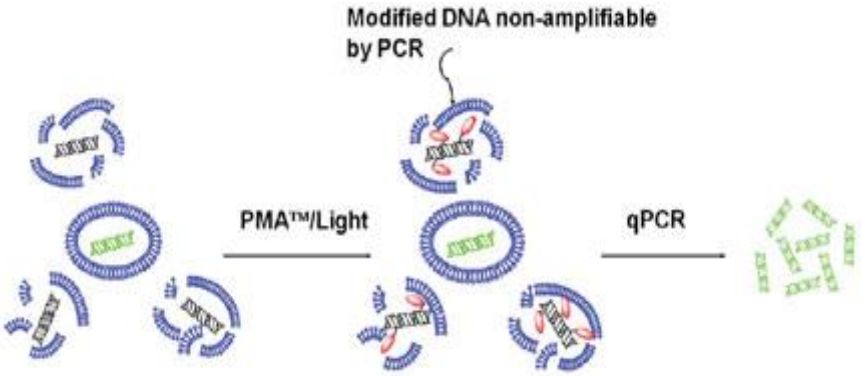
Development of reliable and robust molecular-based assays to quantify BCAs

General Methodology

We used the Propidium monoazide-based qpCR method to analyse change in viable population, and confirmed the possibility of quantifying viable biocontrol organism propagules. Propidium monoazide is a high affinity photoreactive DNA binding dye. The dye is slightly fluorescent, but becomes strongly fluorescent after binding to nucleic acids with the exposure to intense visible light. The PMA molecule specifically binds to double stranded DNA with high affinity through photolysis.

Photolysis induces photoreactivity of the azido group on the dye and is transformed to an extremely reactive nitrene radical which promptly reacts with any hydrocarbon moiety at the binding site in order to form a stable covalent nitrogen-carbon bond, consequently resulting in permanent DNA modification (Figure 1). The dye is cell membrane-impermeable, and thus can be selectively used to solely modify the exposed DNA from dead cells, at the same time leaving DNA from viable cells intact. Only dead cells are susceptible to DNA modification due

to their compromised cell membranes exposing naked DNA. This characteristic of the dye is essential for selective detection/quantification of viable cells through quantitative real time PCR in the presence of dead cells in which the DNA becomes PMA modified and in result cannot be amplified. Optimal dye concentration and sample size may vary with strain or application (Desneux, Chemaly, and Pourcher, 2015; Lv *et al*., 2016; Barbau-Piednoir *et al*., 2014, Soto-Muñoz *et al*. 2014, Wagner *et al*., 2015; Taylor, Ross, and Bentham, 2014).



(Biotium, 2016)

Figure 1. Illustration of how the PMA dye functions. Cell membrane-impermeable PMA dye selectively and covalently modifies DNA from dead bacteria through their compromised membrane while leaving DNA from viable cells intact. Since PMA-modified DNA is non-amplifiable subsequent lysis of viable cells followed by qPCR allow s selective quantitation of viable cells.

The PMA-Lite LED photolysis device costs $1,850.00. Since the device is expensive to purchase, we thus conducted research on investigating an alternative route to activate the PMA molecules based on previous research (Desneux, Chemaly, and Pourcher, 2015; Lv *et al*., 2016; Barbau-Piednoir *et al*., 2014, Soto-Muñoz *et al*. 2014, Wagner *et al*., 2015; Taylor, Ross, and Bentham, 2014).

As the focus of the study involves the viable population amplification of biocontrol products, the developed methodology must function on the formulated version of each BCA. But during the development of BCAs into biocontrol products there is a mixture of adjuvants and additives formulated into the final product, and the presence of such compounds may interfere with the detection stage. Since the ingredients of the BCA products are not provided due to product privacy issues the research had to focus if the isolated DNA from the formulated product was being affected by these adjuvant and additives as this was specifically significant in acquiring and amplifying the viable population of each BCA.

Objectives

(1) Pre-treatment of BCAs with and without PMA in heat treated and non-heat treated conditions

In order to analyse if the PMA treatment is successful in identifying changes in the viable population of each BCA, we need to confirm changes in population when the BCA is subjected to different conditions. This specific objective focused on whether the PMA method can accurately identify change in the population.

(2) Identifying the optimum PMA concentration for each BCA

The PMA concentration can affect the amplification competence of DNA because high concentrations of PMA are toxic to cells and low concentrations of PMA may not successfully attach to all available DNA from compromised cell membranes of dead cells. Thus, we need to identify the optimum concentration for each of our two BCAs.

We tested the following four concentrations 12.5 µM, 25 µM, 50 µM, and 100 µM for the selected dosage of each organism. The effect of PMA treatment on a sample was evaluated by the ΔCq (from qPCR) values. The ΔCq of a sample is the difference among the Cq-value obtained with PMA treated sample and the Cq-value obtained with non-treated sample: $\Delta Cq = (Cq_{\text{sample w/ PMA}} - Cq_{\text{sample w/o PMA}})$ (Barbau-Piednoir *et al*., 2014).

Materials and methods

Experimental treatments and design

Experimental treatments (Table 1) were selected in to firstly identify if the PMAxx (xx relates to the newer product version defined by the manufacturer) treatments works for identifying changes in Serenade and PreStop population in the presence of a population reducer (heat treatment), and how sensitive the pre-treatment with PMA is in analysing the change in cell populations of each BCA without a population reducer.

Table 1. Treatment details which were used to evaluate the use of PMA and its sensitivity for estimating viable cell number changes in Serenade and PreStop. For each sample, the total volume for either Serenade or PreStop is 500 µl at 10^8 CFU/ml.

Sample name	PMA concentration in 500µl	Condition
A1	12.5µM	Room temperature
A2	25µM	Room temperature
A3	50µM	Room temperature
A4	100µM	Room temperature
A5	0µM	Room temperature
B1	12.5µM	Heat treated at 95°C for 5 mins
B2	25µM	Heat treated at 95°C for 5 mins
B3	50µM	Heat treated at 95°C for 5 mins
B4	100µM	Heat treated at 95°C for 5 mins
B5	0µM	Heat treated at 95°C for 5 mins
C1	12.5µM	Heat treated at 95°C for 10 mins
C2	25µM	Heat treated at 95°C for 10 mins
C3	50µM	Heat treated at 95°C for 10 mins
C4	100µM	Heat treated at 95°C for 10 mins
C5	0µM	Heat treated at 95°C for 10 mins

The research resolved both questions with a single experiment containing a single replicate for each treatment replicated three times. For confirming the function of PMA being capable of identifying changes in population the BCAs were subjected to heat treatment to reduce their viable population and consequently the ΔCq data sets of samples with PMA and heat treatment were compared with the ΔCq data sets of samples treated with PMA but not subjected to heat treatment. Simultaneously the experimental set-up allowed testing of how sensitive PMA treatment was for identifying change in population since the ΔCq data sets of samples with PMA treatment but not subjected to heat treatment was compared to ΔCq data sets of samples without PMA treatment.

PMA treatment

Through the serial dilution technique a BCA dose of 10^8 cfu/ml was produced, and 500µl of this was pipetted into a clear 1.5ml Eppendorf. From a total of 15 samples 5 were allocated into each group. The first treatment group (A1-5 Table 1) was subjected to a non-lethal temperature of 20°C (room temperature), the second treatment group (B1-5 Table 1) was subjected to 95°C for 5 minutes, and the third treatment group (C1-5 Table 1) was subjected to 95°C for 10 minutes. After an appropriate cooling period of 15-30 minutes samples were transferred into the airflow cabinet.

In the airflow cabinet from the pre-prepared PMAxx solution containing a concentration of 2.5mM the following sample concentrations were produced for each treatment group (12.5µM, 25µM, 50µM and 100µM). Immediately after the PMAxx solution was transferred into the sample the Eppendorf was placed into a matt micro centrifuge rack. Next a 50cm (length) × 45cm (width) aluminium foil layer (matt side) was used to wrap samples, while ensuring equal spacing between each sample; subsequently the foil was folded into a square shape. Samples were covered with another layer of aluminium foil (matt side). The square shaped package was placed onto a rocker for 15 minutes at 35 rev/min.

During the incubation period the time was used to prepare a set-up within a cold room with a temperature range of 4-8°C (cold room setup was necessary as samples overheat during the photo-activation stage). The set up contained an icebox filled with 5cm of ice, a rocker, and two light sources capable of producing 800w together. The rocker was transported and placed into the centre of the cold room and the icebox placed on top of the rocking platform. Prior to transporting the icebox filled with ice the surface and sides was covered with two layers of aluminium foil; the foil was placed with the shiny/reflective surface facing upwards towards the light source. The light sources (400w halogen light bulb) were positioned adjacent to each other with their screens pointing directly into the centre of the icebox.

After the incubation, the samples were transported into the cold room and placed into the icebox. All the samples were placed with equal distribution from each other and the edges of the icebox were avoided. There was two light sources placed on adjacent sides to one another with attention to ensure the light sources were fixed in a manner that the screens were 20cm away from all the samples, and pointing directly downwards onto the samples with a maximum angle of 45°.

Both the light sources were switched on and the rocker started at 35 rev/min. The PMA molecules were activated after exposure for 30 minute to the light source. The samples were subjected to 30 cycles of 1 minute light and 2 minutes cooling. A thermometer was used to monitor the temperature throughout the experiment. Under no circumstance the samples were allowed to go above 30°C. Once the photo activation stage was complete the samples were transferred into a centrifuge and centrifuged at 5,000 x g for 10 minutes to collect the cells. The supernatant of the sample was disposed prior to DNA extraction.

DNA Extraction

The following reagents were prepared for DNA extraction: 8mM NaOH, 0.1M trisodium citrate in 10% ethanol solution, absolute ethanol and 75% ethanol. The following procedure was accomplished simultaneously in the fumigation cupboard for each sample (refer to table 1 for sample details).

One ml of Trizol (TRI reagent) was suspended into each sample tube; samples were left to settle for 5 minutes at room temperature. Next 0.2ml chloroform was added to the sample. The sample was covered tightly and shaken vigorously for 30 seconds and allowed to settle for 15 minutes at room temperature. The sample was then centrifuged (SIGMA 1-15) at 12,000 × g for 15 minutes; each sample was separated into 3 phases: a red organic phase at the bottom composed of protein, the interphase composed of DNA and a colourless upper aqueous phase composed of RNA.

The aqueous phase was removed and discarded and 300µl of absolute ethanol was added to each sample, and then samples were mixed for 30-60 seconds by inversion and left to settle for 3 minutes at room temperature. Next samples were centrifuged for 5 minutes at 2,000 × g at room temperature.

After centrifugation the supernatant was disposed. The DNA pellet was washed thrice in 1ml of 0.1M trisodium citrate in 10% ethanol solution. For every 1ml of TRI Reagent used in sample preparation 3mls of wash solution was used. During each wash the DNA pellet was left to settle for a minimum of 60 minutes, with occasional mixing (every 5 minutes). The samples were centrifuged 2,000 × g for 5 minutes. Following centrifugation the supernatant was discarded and the DNA pellet was washed with 1.5-2ml of ethanol (75%). The sample was left to settle for 10-20 minutes at room temperature.

Following the washing stage the DNA pellet was centrifuged at 2,000 × g for 5 minutes. The pellet formed was air-dried for 5 minutes on a layer of tissue under the fume cupboard. The DNA pellet was dissolved in 8mM NaOH by shaking for 1-5 minutes (Vortex-Genie 2, at level 1 shaking). 200-500µl of 8mM NaOH was added depending on pellet size. Centrifugation was at 2,000 × g for 5 minutes for removing any insoluble material and the supernatant formed was transferred to a new tube. If some of the pellet did not dissolve it was supplemented with 8mM NaOH and stored in a freezer at -20°C.

Quantitative real time polymerase chain reaction

Before qPCR quantification, each sample was filtered with a 0.1µm filter (Millex® syringe filter units, disposable, Durapore®PVDF pore size 0.1µm, γ-irradiate), this cleansing procedure was important for DNA samples obtained from Serenade as an additive and or adjuvant within the formulation inhibits PCR. Each DNA sample was then measured with a Nano drop spectrometer thrice.

The primers were selected from a large pool of primers from various publications. The selected primers are given in Table 2 and were selected on the following criteria: the lack of presence of alternative bands on the PCR end product, and good correlation in band intensity with increasing DNA concentrations from 1ng, 10g, 100ng and 1000ng amplifications.

Table 2. The selected and used primers for qPCR

Primer name	Organism	Forward Primer (5'-3')	Reverse Primer (5'-'3)	Product size	Reference
Bs_dnaK 1154	B.subtilis (Serenade)	ACACGACGATCCCA ACAAGC	AGACATTGGGC GCTCACCT	101bp	Hertwig *et al*., 2015
Gc1-1- Gc3-2a	G. catenulatum (PreStop	CCGTCTCTTATCGA GCCAAGAT	GCCCATTCAAA GCGAGGCATTA	237bp	Huhtala et al., 2000

The target concentration of DNA for each sample in each well was 35ng. The 96 well plate contained a total of four replicate wells for each sample, 5-10 positive controls (DEPC water, SYBR green, and primers) and negative controls (DEPC water). The standard curve contained at least six standards with a minimum of three replicates for each allocated STD.

The qPCR conditions given in Table 3 were used for both the BCAs. We had to use 60°C for annealing rather than 58.8°C for Bs_dnaK1154 and 56.8°C for Gc1-1- Gc3-2a primers as the SYBR green (SensiFAST^{tm} SYBR No-ROX Kit) requires 60°C for adequate function and flourecent signalling.

Table 3. The qPCR program used for Serenade and PreStop DNA samples after pre-treatment with PMAxx and DNA extraction.

Programs

Program Name	pre incubation							
Cycles	1	Analysis Mode	None					
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)	
94	None	00:03:00	4.40		0	0	0	
Program Name	amplification							
Cycles	45	Analysis Mode	Quantification					
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)	
94	None	00:00:15	2.20		0	0	0	
60	None	00:00:30	2.20		0	0	0	
72	Single	00:00:30	4.40		0	0	0	
Program Name	melt curve							
Cycles	1	Analysis Mode	Melting Curves					
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)	
72	None	00:05:00	4.40		0	0	0	
95	None	00:00:05	4.40		0	0	0	
40	None	00:01:00	2.20		0	0	0	
97	Continuous		0.11	5	0	0	0	

The standard produced in the Serenade and PreStop PMA-qPCR experiments was aquired from the sample A1 being treated with a PMA concentration of 12.5µM but not being subjected to heat treatment. The STD curve was produced with a minimum of six STDs having three replicates each. The STDs copy number generally ranged from E11 to E6. For a detailed example of a STD produced and the quality assurance please refer to figure 2. Cq values are the cycle number in which fluorescence can be detected. For aquiring the direct concentration of a sample the Cq value allows the estimation of the copy number through the use of a STD which can be used to estimate the viable cells.

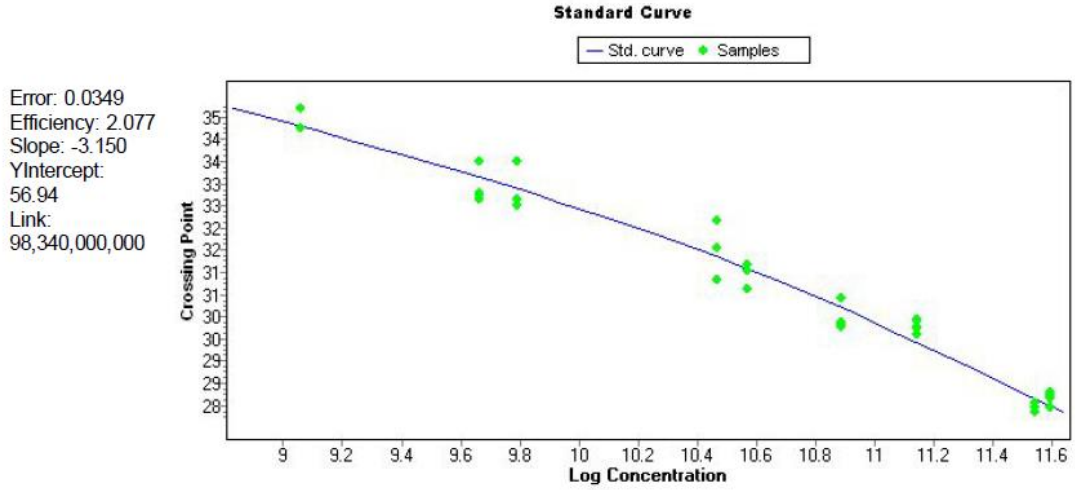


Figure 2. Example of the Standards used to determine concentration of a specific sample.

To evaluate the population change in the viable population from the original the following equation was used ($Cq_{sample\ w/\ PMA} - Cq_{sample\ w/o\ PMA}$) = ΔCq. Consequently, population changes can be quantified to evaluate if viable cell numbers increased decreased or remained stable but the exact size of the population cannot be estimated. A STD curve allows the estimation of the log concentration for each treatment which can be used to estimate the viable cells.

But in order to convert Cq into CFU another type of calibration/STD curve was required, using 10 fold dilutions of the target BCA followed by DNA extractions for each dilution was needed to produce a linear relationship between Cq and the number of BCAs. Since the first part of the research was directly focusing on the change in population, ΔCq values were sufficient to study the impact of PMA.

Statistical methods

Non-parametric tests were selected for testing differences due to the data sets being too small for testing parameters of normal distribution. Since there are more than two sets of numerical data, a multiple group comparison test was used first: one-way analysis of variance (ANOVA). After confirmation that ANOVA generated a statistically significant P-Value < 0.05 then the data sets were followed by a post hoc test to determine between which two data sets differences exist. The selected post hoc test used to test differences between each data set was the Mann-Whitney U test.

Results

The effects of PMA pre-treatment for analysing change in population of live cells in Serenade and PreStop with qPCR are represented by Figure 3 and Figure 4, respectively. The developed protocol and molecular tool successfully amplified the viable population of each BCA, and distinct differences in ΔCq-value were obtained with non-PMA treated samples.

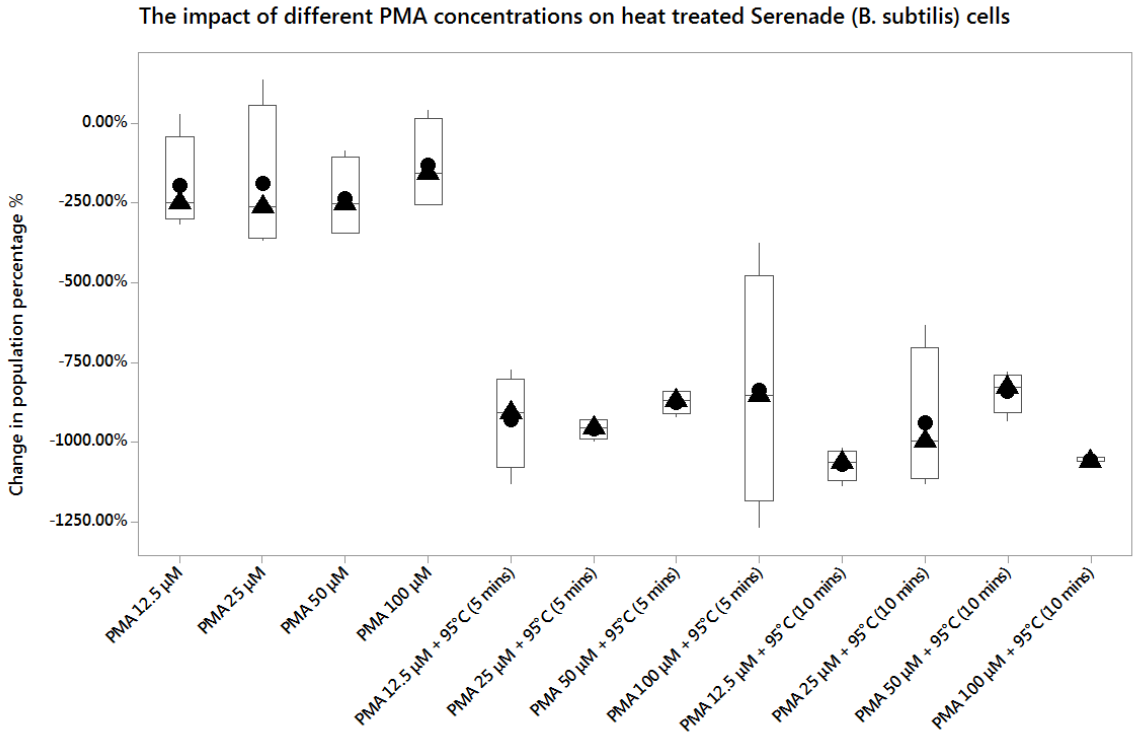


Figure 3. The effect of different PMA concentrations in amplification of *Bacillus subtilis* cells subjected to 95°C for 5 and 10 minutes represented by ΔCq values.

The mean (circle) and median (triangle) values are overlapping indicating a normal distribution for each data set. Only application of 25 µM of PMA resulted in positive ΔCq values. After a 5 minute heat treatment of 95°C all samples presented a decline in viable population size. The best PMA concentration after heat treatment of 5 minutes appeared to 25 µM application because of its overlapping mean and median and small variability among replicates; 100µM PMA application led to the data of worst quality. The largest difference in population change was between PMA 100µM and PMA 100µM + 95°C (10 mins) which has a -890% population difference.

The difference between each group and treatment type was tested with an (One-Way Analysis of Variance) ANOVA test. With 95% confidence a P-Value of > 0.01 was generated with the Minitab statistics software suggesting that at least one mean in the data sets were different. The difference between the group's ΔCq values of just PMA treated (Samples A2 Table 1) and with PMA treatment and heat treatment of 95°C (samples B2 Table 1) was confirmed by a Mann-Whitney Test. With 95% confidence a P-Value of 0.0304 was generated strongly suggesting that a significant difference between these data sets exist, thereby confirming PMA function. Difference between the treatment groups' ΔCq values of just PMA treatment (sample A2 Table 1) and without PMA treatment (samples A5, B5, and C5 Table 1) was tested with a Mann-Whitney Test. With 95% confidence a P-Value of 0.2410 was generated suggesting that a significant difference between these data sets do not exist.

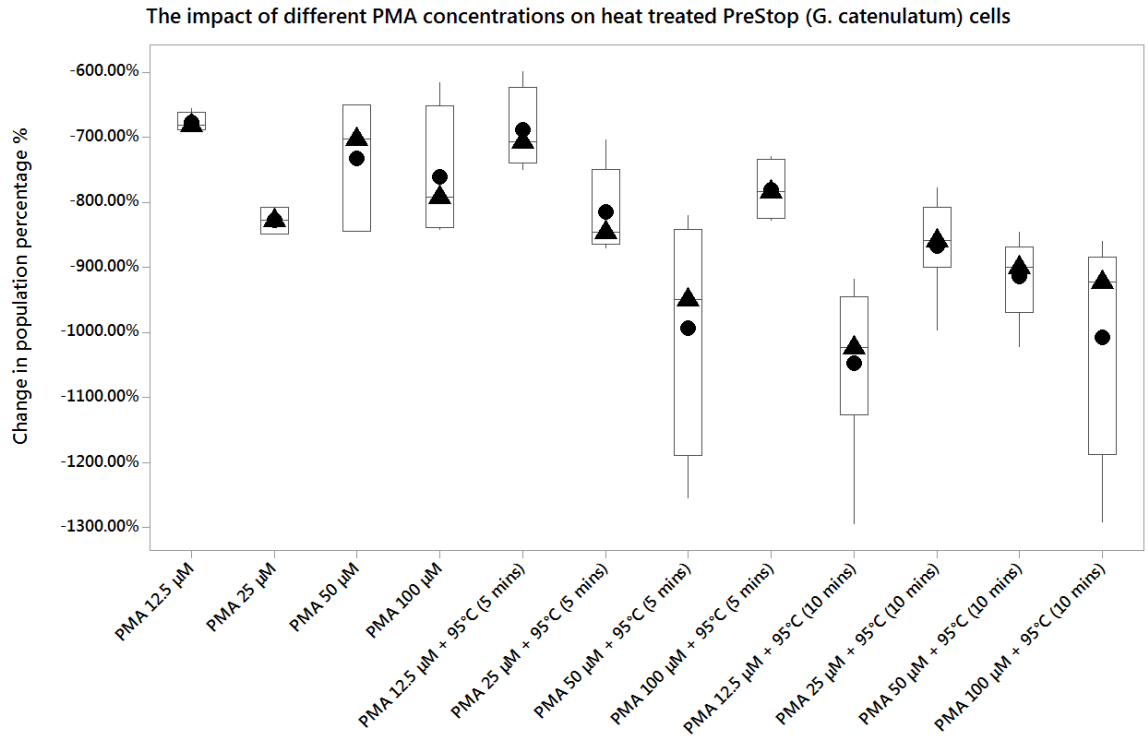


Figure 4. The effect of different PMA concentrations in amplification of *Gliocladium catenulatum* cells subjected to 95°C for 5 and 10 minutes represented by ΔCq values.

As the PMA concentration applied in non-heat treated samples was increased the variability in ΔCq value expands. The data sets for all treatment groups start with negative values with and without the heat treatment process suggesting PMA function. A difference in the impact of PMA treatment can be clearly visualised with the 12.5 µM, 50µM and 100 µM PMA application after 5 minutes of heat treatment due to the decline in the original population; with the 50 µM applications displaying the greatest decline suggested by the position of the mean

within the box plot. The largest difference in population change was between the PMA 50µM and PMA 50µM + 95°C (5 mins) which has a -350% cycle difference.

The difference between each group and treatment was tested with an (One-Way Analysis of Variance) ANOVA test. With 95% confidence a P-Value of > 0.01 was generated suggesting that at least one mean in the data sets were different. The difference between the group's ΔCq values of just PMA treated (Samples A3 Table 1) and with PMA treatment and heat treatment of 95°C (samples B3 Table 1) was tested by a Mann-Whitney Test. With 95% confidence a P-Value of 0.5083 was generated with the Minitab statistics software suggesting that a significant difference between these data sets do not exist. But difference between the treatment groups' ΔCq values of just PMA treatment (sample A3 Table 1) and without PMA treatment (samples A5, B5, and C5 Table 1) was tested with a Mann-Whitney Test. With 95% confidence a P-Value of > 0.01 was generated suggesting that significant differences between these data sets exist, thereby confirming PMA function.

Discussion

Prior to considering population analyses of BCAs and the pathogen in different climatic conditions within different environmental settings there is a need of a tool to accurately and precisely quantify their viable populations. The research developed a sensitive, reliable and robust molecular based assay for quantifying the viable populations of the BCAs in order to study the population dynamics of these organisms. A recent study by Soto-Muñoz *et al*. (2014) has successfully developed and tested this method on the BCA *Pantoea agglomerans* CPA-2. Prior to discussing which concentrations are optimum for amplifying the viable population of each BCA there is a need to question if PMA treatment displays an impact. This can be achieved through observing the difference in population of non-PMA treated and PMA treated samples. For this part of the discussion the heat treated samples was ignored as sensitivity of the developed tool was a must, and therefore without any type of subjugation of samples differences within the population should be presented with such a molecular tool.

For Serenade (*Bacillus subtilis*) Figure 1 the four left boxplots are non-heat treated samples, and theoretically should have a value of 0 for their ΔCq-value to suggest no difference in PMA and non PMA treated samples. However even within relatively new biocontrol products, as is with the case of Serenade, cells are not at a stasis, and are either increasing or decreasing in population at a particular rate. Since the formulation used was a liquid the cells/spores are kept active during storage, consequently small differences was expected from the original population which has been the case; as the population range has been between +41% to -368%. Thus, without even utilizing any means to change the population for analysis minuscular changes can be monitored with the molecular tool PMA-qPCR, indicating that

PMA pre-treatment prior to qPCR can be utilized to analyse viable population changes of the BCA *B. subtilis* Serenade in formulated form. After heat treatment the change in viable population was confirmed as comparison between ΔCq data sets of just PMA treated (A2 Table 1) and with PMA treatment and heat treatment (B2 Table 1) yielded a P-value of 0.0304 strongly suggesting that this tool can monitor changes in the population.

For PreStop (*G. catenulatum)* Figure 2 the four left boxes are just PMA treated samples (A1-4 Table 1) and theoretically should have a value of 0 for their ΔCq-value to suggest no difference with PMA treatment. As observed in figure 2 this was far from the case due to the population starting at -600% for all treatment groups. Comparison between the data sets in ΔCq values of just PMA treated (A3 Table 1) and non-PMA treated (A5, B5 and C5) generated a P-Value of > 0.01 strongly suggesting the function of the PMA treatment without the need of a population decliner. The biocontrol product PreStop is prepared as a dry formulation, and the act of dehydration and rehydration reduces the initial concentrations (Abadias *et al*., 2003). Downstream processing embeds drying, addition of volume materials (inert ingredients), adhesives, emulsifiers and adjuvants, which can adversely affect the original population of *G. catenulatum (*Abadias *et al*. 2000, 2001, 2003*).* The drastic difference in the original population and the viable one was most probably due to these circumstances as dehydration and rehydration cause high mortality (Abadias *et al*., 2003). For *G. catenulatum* this packaging procedure has significantly reduced the original population as presented in figure 2, indicating that even before heat treatment there was a mass amount of dead cells in turn justifying why a statistical difference was not reached after heat treatment (P-value 0.5083). For the purpose of the molecular tool PMA-qPCR this strongly suggests that the use of PMA as a pre-treatment before qPCR can be utilized to analyse the viable population change of this BCA *G. catenulatum* PreStop in formulated form.

The concentration of PMA used can be fundamental on the outcome of viable cells amplified (Desneux, Chemaly, and Pourcher, 2015*;* Lv *et al*., 2016). Preliminary research suggested that 10^8 cfu/ml for each BCA was the threshold for control and consequently the research focused on identifying the optimum PMA concertation for Serenade and PreStop at this dosage. The type of cell affects the amount of PMA required. The cell wall properties and components can cause difficulty for the PMA molecule to access to the exposed DNA and consequently the DNA modification stage. The simpler less protective peptidoglycan cell walls of *B. subtilis* post cell death can allow easier access of PMA molecules to the exposed DNA through larger opening, while the complex highly protective chitin, glucans, mannans and glycoproteins of spores and mycelium cell walls may physically obstruct by containing smaller/limited openings for the PMA molecule to gain access or the DNA to be swept out. In

result *B. subtilis* required a lower PMA concentration of 25µM, and *G. catenulatum* required a higher PMA concentration of 50µM for reliable viable population analyses.

1. In vitro studies on the effect of BCA dosage on *B. cinerea* mycelial colonization and macro conidia production

Background for research

Prior to in vivo studies in vitro studies allow the comprehension on how each BCA functions without being limited by other factors such as the plants natural biota and stressful conditions. In vitro studies allow the research to focus on the maximum potential of Serenade and PreStop against botrytis as the environment of an agar plate is non-hostile. Most importantly the applied dose can be relied upon due to the lack of adverse conditions affecting the initially applied dose. Identifying how much of the acquired control efficacy depends on the BCA and how much depends on the formulation is important for comprehending what might be affecting the inconsistency of both these BCAs.

Objectives

(1) Identify the dose response and threshold of control of *B. cinerea* to Serenade (both in formulated and pure isolate form).

(2) Identify the dose response and threshold of control of *B. cinerea* to PreStop (both in formulated and pure isolate form).

Identifying the dose response of *B. cinerea* to Serenade and PreStop and developing dose response models are the key in determining the consistent and effective dose required for controlling *B. cinerea*. The control threshold and or economic threshold refers to the minimum required dose needed to control *B. cinerea* in order to avoid economic loss or aesthetic damage to the crop.

Materials and methods

Experimental treatments

We conducted in vitro studies to determine the dose response curves. BCAs were applied at different dosages in plates co-inoculated with a *B. cinerea* mycelial plug (Figure 5). For each BCA, there were 18 treatments: six BCA doses (10^5, 10^6, 10^7, 10^8, 10^9 and 10^{10}cfu/ml) three temperatures (4°C, 10°C and 20°C). For each treatment, 15 replicates were used; in addition,

three replicates with only *B. cinerea* mycelial plug were also used as control. The experiment was repeated twice.

Model description

The experiment for testing the dose effect of PreStop and Serenade in formulated and pure isolate form was opted for a simple model in which only the pathogen (*Botrytis cinerea*) and BCA (Serenade or PreStop) were present in the environment. Such a model allowed the perception on how well each BCA treatment type and dose controls botrytis and if the control was dependant on dose, temperature, formulation or all.

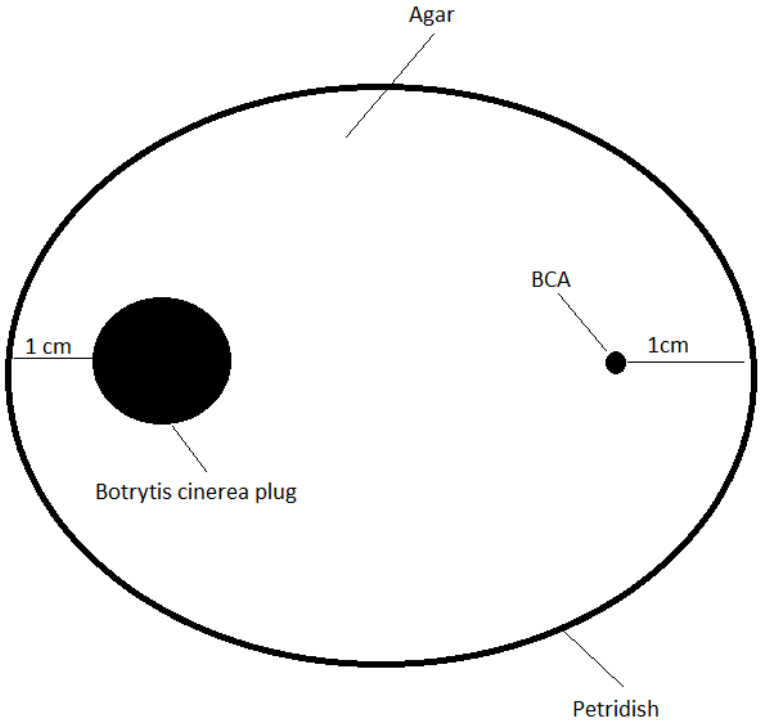


Figure 5. Plate design of in vitro BCA dosage testing on mycelial colonisation and macro conidia production (not to scale).

For ensuring both the BCA and the pathogen did not have an advantage for nutrient acquisition from the agar they were placed simultaneously. After 7 days an image of the plate was acquired and analysed with image j to calculate the area gained by the pathogen. After image acquisition the plate was washed with a solution composed of maximum recovery diluent/tween20, and the contents passed through filter paper to acquire a solution containing macro conidia of *B. cinerea*. A haemocytometer was utilised to calculate the concentration of *B. cinerea* conidia in each solution. There was rational in repeating the in vitro dosage testing experiments with pure isolates of the BCAs, in order to further our understanding on what extent the effect of formulation has on control efficacy. Since formulations can contain additives, adjuvants and etc., which can assist in improving the BCAs efficacy.

Experimental design

Each formulated BCA treatment group contained thirty replicates broken down into two experiments containing fifteen replicates each. Each pure isolate BCA treatment group contained a total six replicates broken down into two experiments containing three replicates each. For macroconidia spore release experiments each treatment group contained a total of six replicates broken down into two experiments containing three replicates each.

The plate treatment for each BCA was prepared in the order represented by table 4.

Table 4. Order of plate treatment preparation for each BCA.

10^6	10^5	10^6	10^9	10^8	10^6	10^6	10^9	10^6	10^{10}
10^8	10^9	10^8	10^{10}	10^9	10^9	10^{10}	10^6	10^9	10^5
10^7	10^8	10^9	10^8	10^{10}	10^8	10^7	10^5	10^5	10^9
10^{10}	10^{10}	10^{10}	10^6	10^5	10^7	10^8	10^{10}	10^{10}	10^7
10^5	10^6	10^7	10^5	10^6	10^{10}	10^9	10^7	10^7	10^8
10^9	10^7	10^5	10^7	10^7	10^5	10^5	10	10^8	10^6

The order in table was followed to an extent to produce 15 replicates for each treatment group.

BCA dose on botrytis colonization

The nutrient agar (50%) / potato dextrose agar (PDA) (50%) plates were used for Serenade dose experiments, while malt extract agar (50%) / PDA (50%) were used for PreStop dose experiments. The commercially available formulated version of each BCA product (Serenade or PreStop) was prepared to contain 10^{10} cfu/ml.

Next an agar plug with an area of 908.89 mm^2 was obtained with the use of a vial cap and disposed. The plug was obtained 1cm away from the edge of the plate with the radius of the plug being at the centre of the plate. The cap was heat sterilized for 30 seconds and then placed into a 70% ethanol solution for 60 seconds. Next a new vial cap with the same area was acquired and used to obtain and transfer a botrytis plug (10 day old *B. cinerea* culture grown at 20°C) into the empty agar space. Following on the cap was heat sterilized for 30 seconds and then placed into a 70% ethanol solution for 60 seconds.

Subsequently the appropriate treatment, (Serenade or PreStop) and type (formulated form, or pure isolate) was obtained with the correct pipette, and the required BCA dose was transferred as a single droplet onto the adjacent side of the botrytis plug 1cm away from the

edge of the plate, ensuring that the droplet's radius was at the centre of the plate (represented by Figure 5). Succeeding this stage the plate lid was placed onto the petri dish and the plate was sealed with parafilm.

The plates were stored for 7 days at 4°C, 10°C, and 20°C. After 7 days the plates were transferred into a class 1 cabinet unsealed and placed into a dark background, and a high resolution image (16.1-megapixel high-resolution photos) of the plate without the lid was acquired with a camera. Within each image a standard of 0.25cm square graph paper was included. The images were analysed for botrytis colonization by Image J.

BCA dose on botrytis macroconidia release

From each treatment group and level of dose within each temperature 6 plates were acquired at random and put through the following stages for botrytis macroconidia release per mm^2 of botrytis colonized regions. Initially a maximum recovery diluent solution was prepared and within a class 1 cabinet 10µl of tween 20 was added for every litre.

With the use of a pipette 5mls of the MRD/T20 solution was transferred twice slowly onto the plate of interest, a total of 10mls of MRD/T20 was transferred into each plate. The lid was closed, and the plate was placed onto a set at 15 rev/min for 5 minutes. After 5 minutes of slow agitation the contents of the plate was poured through a plastic funnel containing a micro cloth filter into a vial.

After adequate mixing of the spore solution 10µl of the conidia solution from the vial was transferred onto a sterile Haemocytometer (IMPROVED NEUBAUER depth 0.1mm 1/400mm^2) and covered with the cover glass. The macroconidia was counted under a light microscope. Subsequently the total macroconidia released per mm^2 was acquired by: amount of macroconidia in 1ml × total volume of sample/ total area gained by botrytis.

Results

The data on figures 6-13 are of graphical representation of the results and are pending statistical analysis. For the biocontrol product Serenade increasing the dose declined botrytis colonization both in formulated and pure isolate form (Figure 6 and 7), and for the biocontrol product PreStop increasing the dose declined botrytis colonization both in formulated and pure isolate form (Figure 10 and 11), excluding the 10°C sample set for the pure isolate. For Serenade increasing the dose in pure isolate form reduced the macroconidia release from botrytis (Figure 9), while the latter was observed in formulated form (Figure 8). As PreStop dose was increased both in formulated and pure isolate form a decreasing trend in macroconidia spore release transpired (Figure 12 and 13).

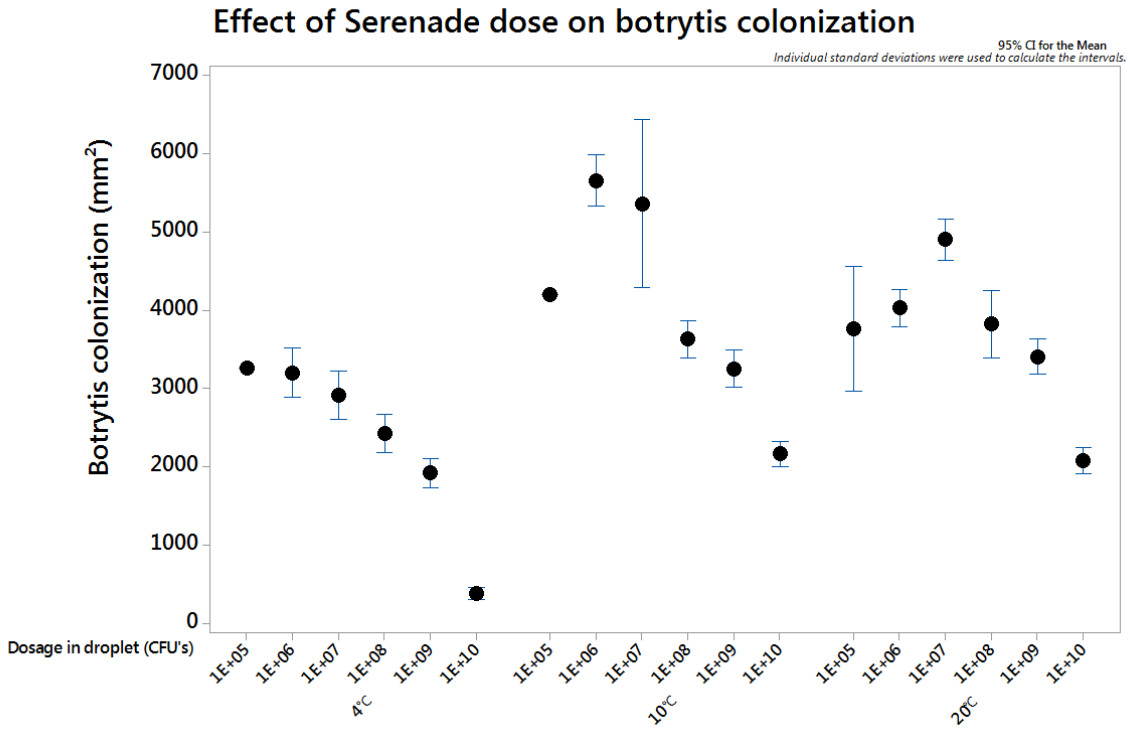


Figure 6. The effect of Serenade dose on botrytis colonization

The trend for dose response appears to be reduction in the area gained by botrytis as dose was increased, the highest dose (10^{10} cfu/droplet) appears to reduce and or suppress the maximum amount of botrytis colonization. For 4°C the initial drop off point in botrytis colonization occurs at 10^{9} cfu/droplet, while at 10°C and 20°C the initial drop off point in botrytis colonization occurs at 10^{8} cfu/droplet.

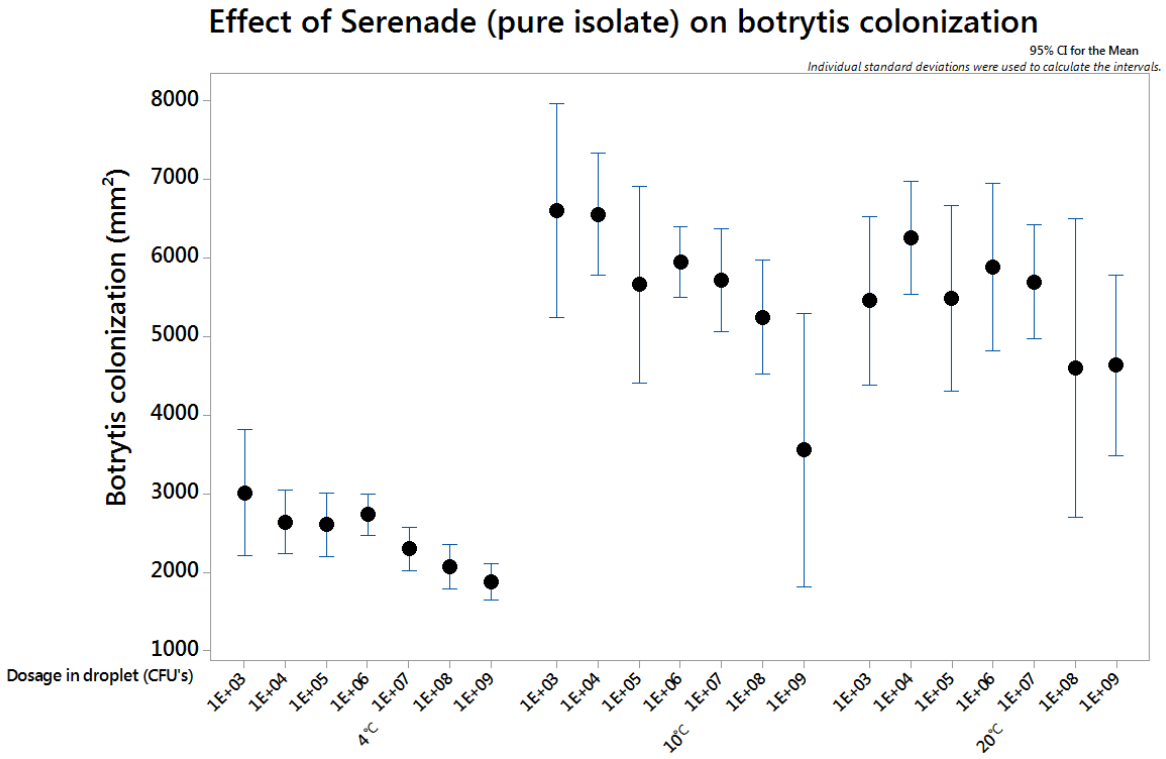


Figure 7. The effect of Serenade in pure isolate form on botrytis colonization

The trend for dose response appears to be reduction of botrytis colonization as the pure isolate dose was increased. For 4°C and 10°C the highest doses (10^9 cfu/droplet) have been the most effective for reducing the botrytis colonization. The initial drop off point in botrytis colonization at 4°C occurs at the 10^7 cfu/droplet dose, at 10°C occurs at 10^9 cfu/droplet and at 20°C occurs at 10^8 cfu/droplet.

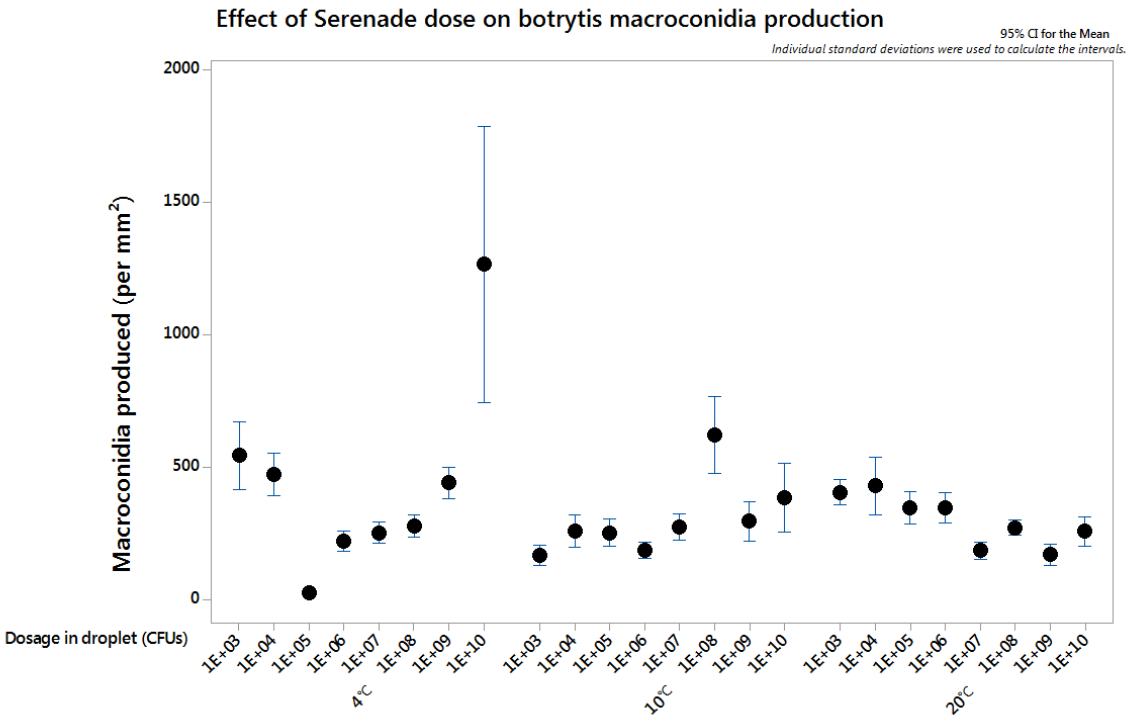


Figure 8. The effect of Serenade dose on botrytis macroconidia release

The trend for effect of Serenade dose on botrytis macroconida spore release at 4°C and 10°C suggest that increasing Serenade dose proliferates macroconidia release by botrytis, and a decreasing trend in macroconidia release only occurs at 20°C with the drop off point being 10^7 cfu/droplet.

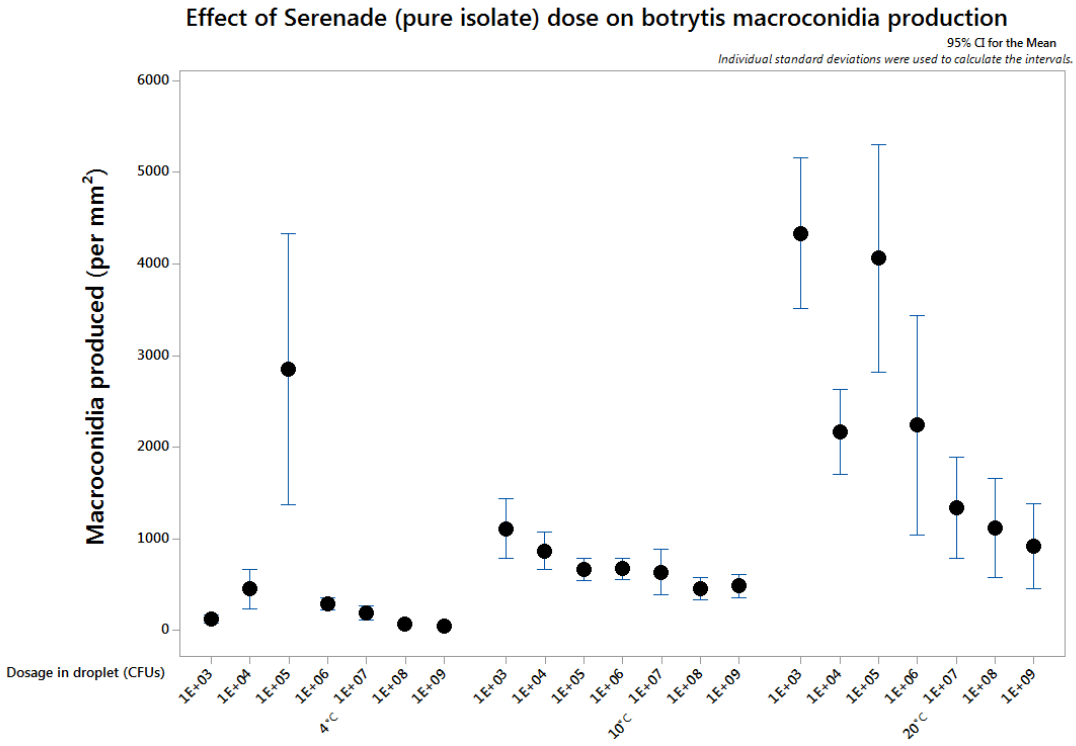


Figure 9. The effect of Serenade dose in pure isolate form on botrytis macrocondia release

Increasing the dose of Serenade within pure isolate form reduces the macroconidia spore release. Yet the steepness of the drop appears to be minimal at 4°C and 10°C, while at 20°C steeper decline transpires, the drop off point occurs at 10^8 cfu/droplet at 20°C.

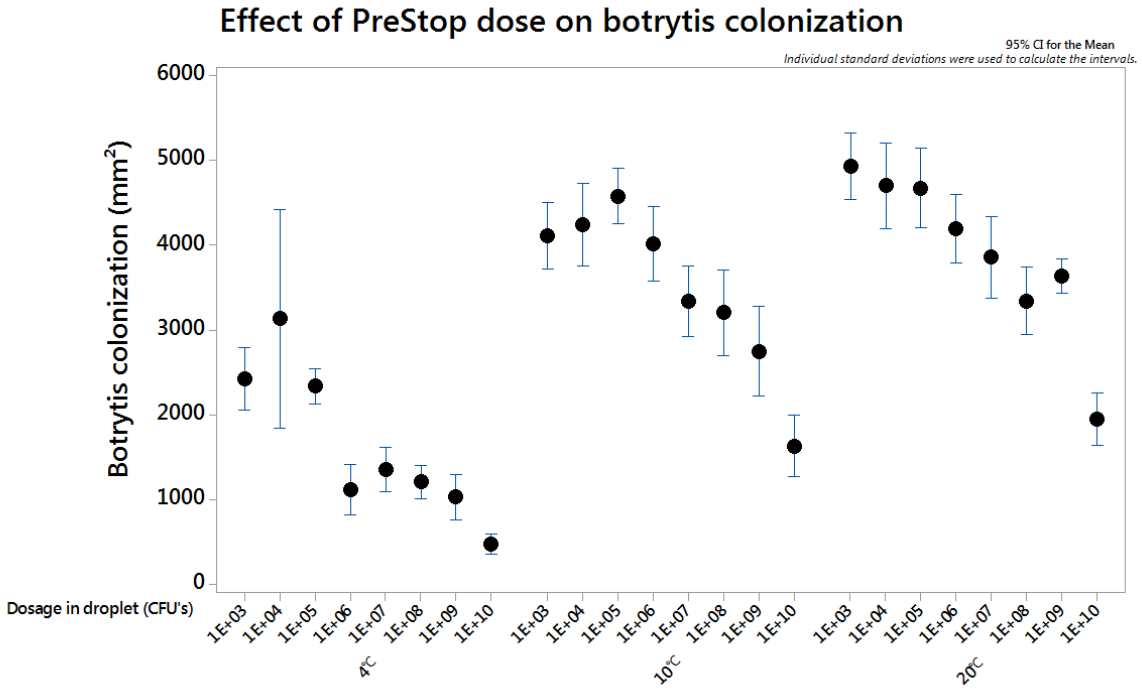


Figure 10. Effect of PreStop dose on botrytis colonization

The trend in figure 10 suggests increasing the dose in each temperature reduces the botrytis colonization, with the most effective dose being a 10^{10} cfu/droplet for each temperature. The initial drop off point at 4°C occurs at 10^{6} cfu/droplets, while at 10°C occurs at 10^{7} cfu/droplet and at 20°C occurs at 10^{8} cfu/droplet.

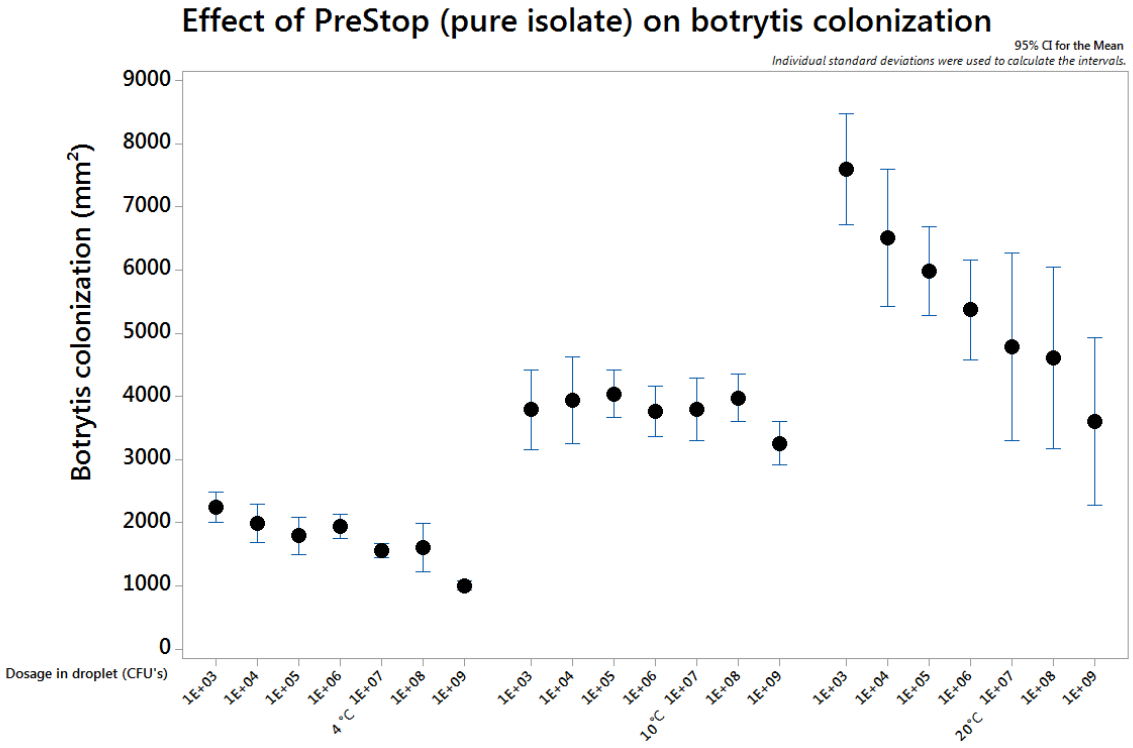


Figure 11. The effect of PreStop in pure isolate form on botrytis colonization.

Increasing the dose of PreStop within pure isolate form at 4°C and 20°C reduced botrytis colonization. A reduction trend of botrytis colonization was absent at 10°C. At 4°C the intial drop off point occurs at 10^6 cfu/droplet, and at 20°C occurs at 10^7 cfu/droplet.

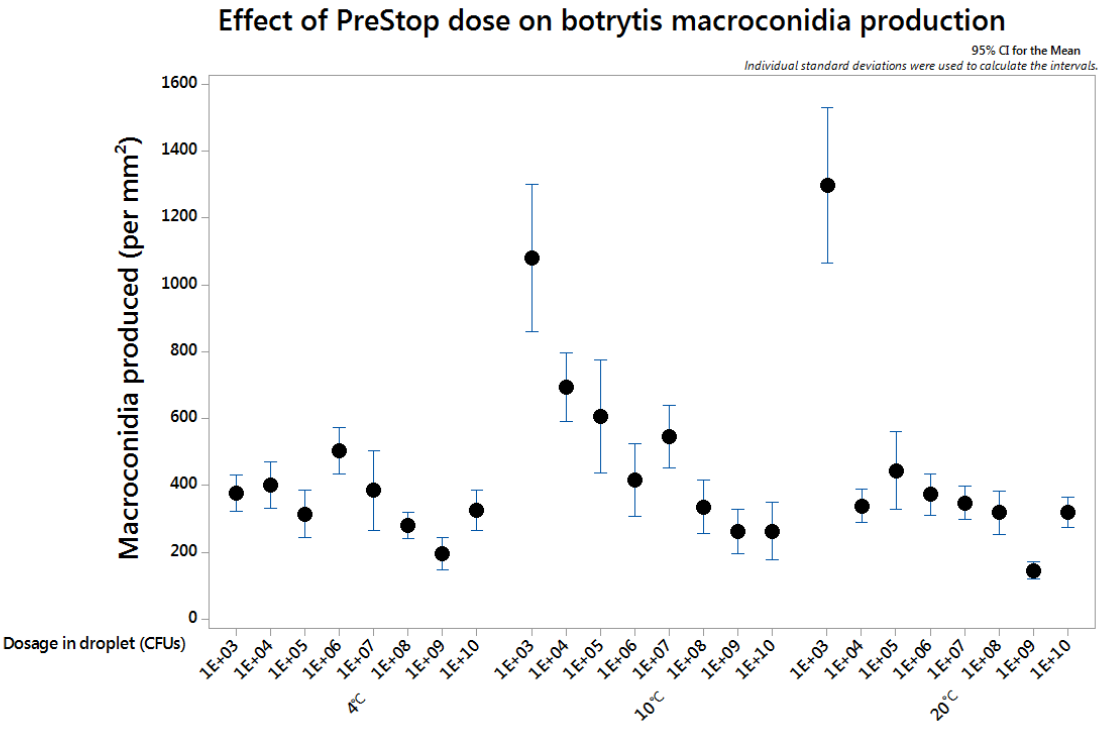


Figure 12. The effect of PreStop dose on botrytis macroconidia release

Increasing the PreStop dose induced a reduction in macroconidia release in all temperatures. The drop off point for 4°C and 10°C occurs at 10^8 cfu/droplet while at 20°C occurs at 10^9 cfu/droplet. In all temperatures the highest dose 10^{10} cfu/droplet was not the most effective dose for reducing macroconida release by botrytis.

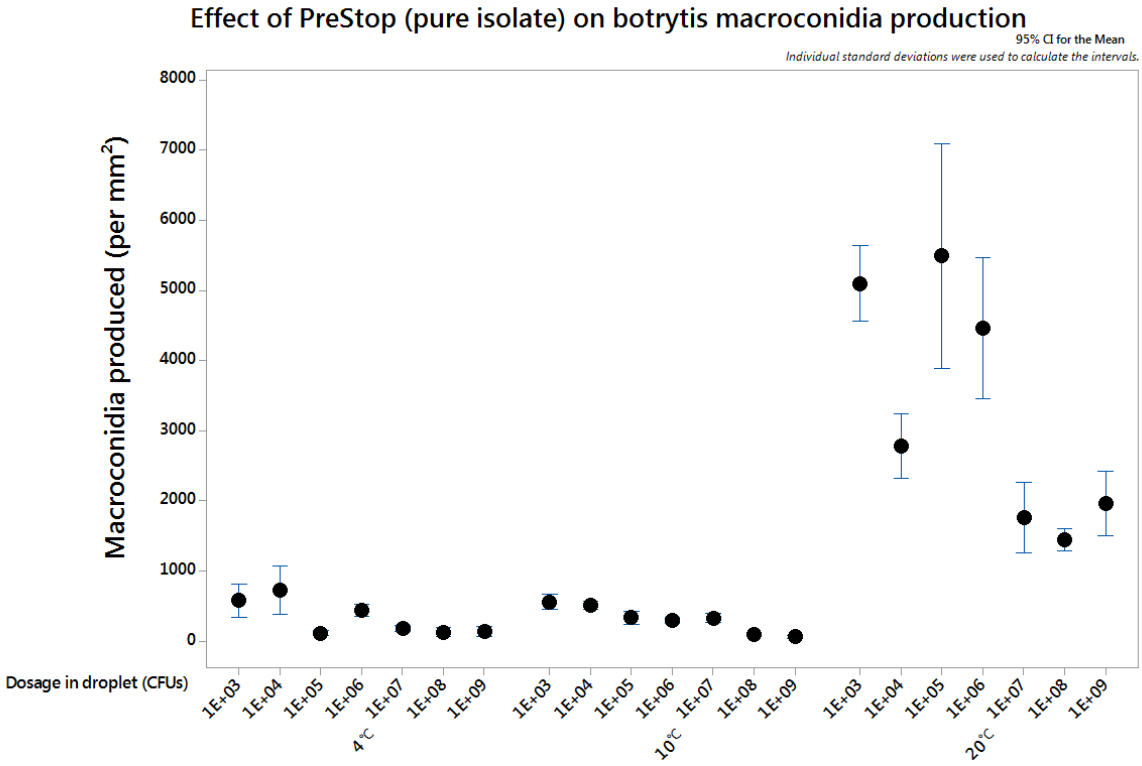


Figure 13. The effect of PreStop dose in pure isolate form on botrytis macroconidia release

Increasing the dose of PreStop within pure isolate form induced a slight reduction in botrytis macroconidia release at 4°C and 10°C, and a stable reduction of macroconidia in 20°C. The drop off point for 4°C occurs at 10^7 cfu/droplet, at 10°C occurs at 10^8 cfu/droplet and at 20°C occurs at 10^7 cfu/droplet.

Discussion

In order to discuss in vitro studies they must be replicated in an in vivo setting.
Pending further in vivo experiments.

Conclusions

The development of the molecular assay for amplifying the viable population change of Serenade (*B. subtilis*) and PreStop (*G. catenulatum*) was successful and the sensitivity of the tool was confirmed. Increasing the dose for both BCAs suppresses and reduces botrytis mycelial colonization.

Knowledge and Technology Transfer

- 2016 AHDB Crops PhD Studentship Conference poster presentation
- Visit to Laurence J Betts Ltd and knowledge transfer with their agronomist as well as field inspections for botrytis.
- Visit to an Anglia salads Ltd and JEPCO for commercial glasshouse and hydroponic growing of lettuce, and knowledge transfer with their agronomist as well as inspections for botrytis.
- 2016 EMR Association/AHDB Soft Fruit Day poster presentation

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Appendices

N/A