Project title:	OPTIMISING THE USE OF BIOCONTROL AGENTS TO IMPROVE THE CONTROL OF *B. CINEREA* IN KEY VEGETABLE AND FRUIT CROPS
Project number:	
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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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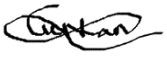
Signature  Date 30th October, 2017

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CONTENTS

GROWER SUMMARY

Headline

- Studying the effect of climatic conditions on the efficacy of Serenade and Prestop.

Background and expected deliverables

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

Summary of the project and main conclusions

The second year of the PhD project focused on characterizing the dose response curves of *B. cinerea* to Serenade and Prestop on lettuce (*Lactuca sativa*) leaves. Characterisation of the relationship allowed determination of the LD_{50} values, and guided testing on how long *Bacillus subtilis* (biologically active ingredient in Serenade) and *Gliocladium catenulatum* (biologically active ingredient in Prestop) propagules survive in polytunnel and glasshouse systems during the winter. The research primarily focused on testing the effect of climatic conditions on the temporal dynamics of *B. subtilis* and *G. catenulatum* viable populations.

Determining the dose response curve for control of *B. cinerea* by Serenade and Prestop on lettuce leaves was critical for identifying the LD_{50} dose. This was important because a relationship between the BCA and the target pathogen is often very complicated. The concentration of *B. subtilis* within Serenade required a mean inoculum of 3×10^8 cfu/ml, and for *G. catenulatum* within Prestop a mean of 3×10^8 spores/ml for controlling a high pathogen inoculum load of *B. cinerea* macroconidia.

The PMA-qPCR method was used to monitor viable BCA propagules in a polytunnel and glasshouse compartment during the winter. *B. subtilis* viable populations necessary for controlling *B. cinerea* survived for four days in the polytunnel and six days in the glasshouse. For *G. catenulatum,* survival was eight days in the polytunnel and glasshouse. A countable viable population existed for up to at least ten days for both BCAs.

Gaining ecological knowledge on how commercial growing climates affect the BCA viable populations is critical for understanding the timing of the BCA application for successful

control of *B. cinerea*, and the reliability period of the application. A total of eighteen climatic treatments were tested for each BCA. Currently all samples have been collected and the DNA extracted and the samples stored at -20°C. qPCR will be applied to determine viable population sizes for each climatic treatment.

The data from these analyses will form the basis for the construction of a leaflet to inform growers of the conditions under which Serenade and PreStop can be applied for consistent control of *B. cinerea*. In addition, the data will have potential use in development of models to predict the efficacy under different climatic conditions in relation to the efficacy of the biocontrol treatment used. This should provide more confidence for growers in terms of expected and actual control achieved.

Financial benefits

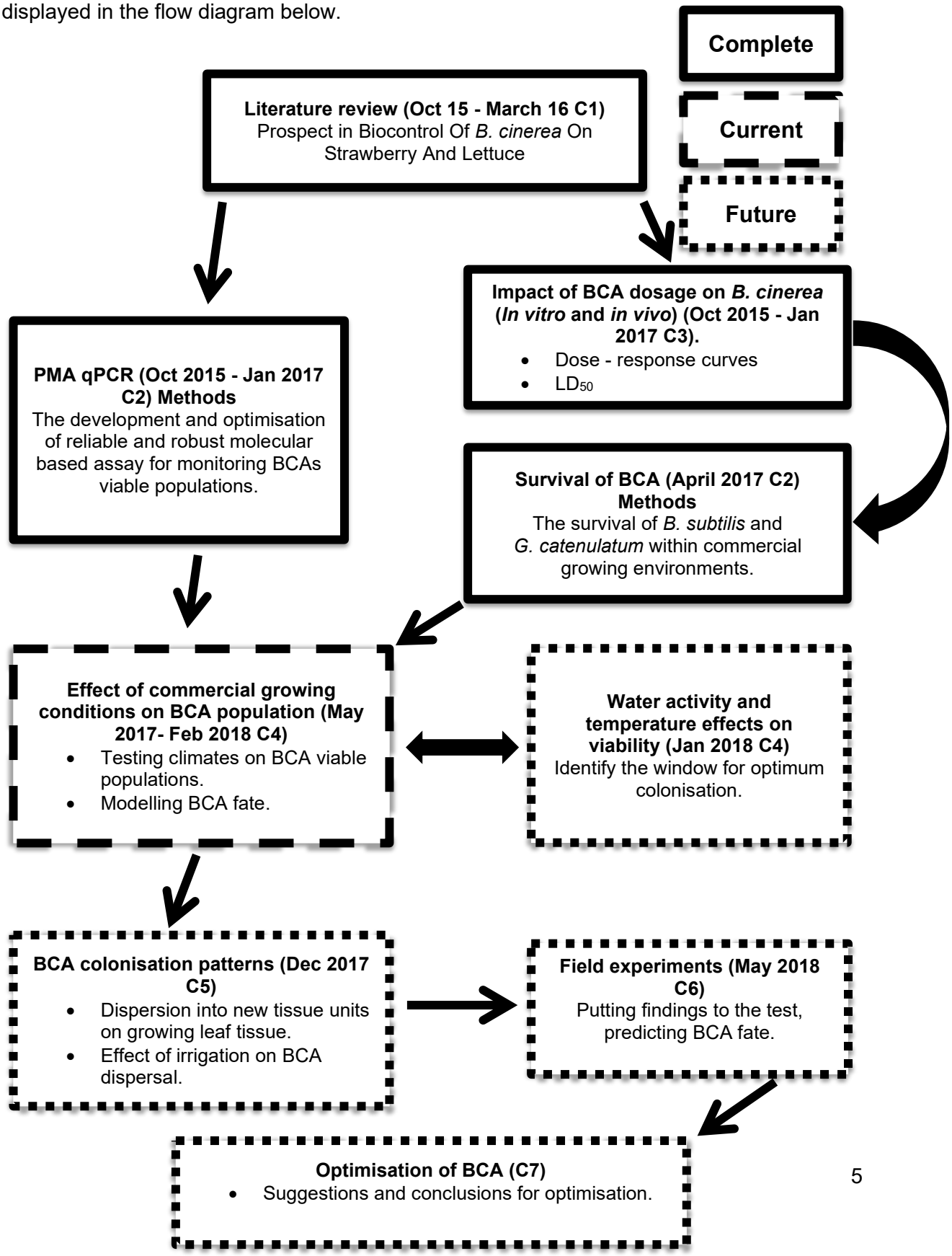
It is too early in the project to identify any financial benefits from the work.

Action points for growers

- No action points have been generated from the work so far.

1. Introduction

This is the 2nd year of the PhD project; the overall project plan and the work achieved are displayed in the flow diagram below.



This is the second reporting year of CP 140 (Optimising the use of biocontrol agents to improve the control of *B. cinerea* in key vegetable and fruit crops), and currently the research has progressed significantly from the last reported stage. The determined LD_{50} doses were used as a guide for developing the PMA-qPCR tool for enumerating the viable BCA populations. Subsequently the developed tool was used to monitor *B. subtilis* and *G. catenultaum* viable populations on lettuce in the winter period within a poly tunnel and glasshouse. This was important for evaluating the frequency of sampling in order to obtain data on the survival and reproduction of *B. subtilis* and *G. catenulatum* in the simulated different climatic treatments. The BCA propagules were thus monitored in eighteen climatic treatments which represented U.K commercial growing regimes.

Because components of the commercial formulation of Serenade interfere with the PMA-qPCR protocol, a pure isolate of *B. subtilis* was utilised for these experiments. For PreStop, where formulation components do not interfere with the PMA-qPCR analyses, the formulated product was used in these experiments.

Determination of the LD_{50} concentrations of Serenade and PreStop required for *in vivo* efficacy against *B. cinerea*

1. Background for research

The initial stage in the optimisation of BCA use against *B. cinerea* is via the development of a dose – response curve for relative efficacy. Several techniques exist for analysing and evaluating how BCA dose affects *B. cinerea* disease potential; including ranking by disease severity (Narwal *et al*., 2009), molecular, and imaging techniques (Fang and Ramasamy, 2015). The current research utilised a traditional leaf disc assay technique for analysing the impact of biocontrol doses on *B. cinerea* macro conidial infection potential on true lettuce leaves. This was done as the method is rapid and effective for gaining data that directly relates to macro-conidial infection rates by *B. cinerea* as well as being economical (Cohen, 1993; Sedegui *et al*., 1999). The median effective dose refers to the dosage which produces a response in half the population, and consequently from a different perspective can be defined as the mean tolerance (LD_{50}) (Finney and Tattersfield, 1952).

1.1 Objectives

(1) Develop dose - response curves of efficacy of Serenade and PreStop against *B. cinerea*

(2) Identification of the mean LD_{50} doses of Serenade and PreStop to *B. cinerea*

1.2 Materials and methods

Design of experiment and lettuce development

1.2.1 Experimental treatments

Experiments were conducted in the glasshouse on lettuce leaves. Initially the BCA products were applied at a range of doses and allowed to settle for six hours. Subsequently lettuce plants were sprayed with a high inoculum load of *B. cinerea* macro conidial suspension (10^6 spores/ml), with an infection period of two days. Five treatments per BCA product (10^5, 10^6, 10^7, 10^8 and 10^9 cfu/ml for Serenade, and 10^5, 10^6,10^7,10^8 and 10^9 spores/ml for PreStop) were tested. Each dose including a positive control (only *B. cinerea* suspension), and a negative control (water only) contained ten replicates of each treatment. The experiment was repeated twice.

1.2.2 Experimental description

The BCA product was sprayed with a hand sprayer onto lettuces and allowed to settle for six hours. A period of six hours was allowed for BCA establishment on lettuce leaves. Lettuce plants were assigned for dose confirmation, and the leaves were initially sampled immediately after this period to establish a base line of inoculum of the BCA treatments using PMA-qPCR. The lettuce plants were then sprayed with a high inoculum dose of *B. cinerea* excluding the negative controls. The *B. cinerea* suspension contained macroconidia, the primary inoculum of *B. cinerea* for lettuce infection. Positive controls were only sprayed with *B. cinerea* suspension. The pathogen was allowed to become an established infection for 48 hrs. After this period, two of the oldest true leaves were collected from each plant (blocking effect), surface sterilized, and traditional leaf disc assays were used to determine lesion development and/or infection. Leaf disc assays were designed to directly simulate the section in which each leaf disc was obtained (Figure 1).

1.2.2 Experimental environmental conditions and replication

The range in temperature and relative humidity in the glasshouse during the experimental period was 17-22°C, and 60-95% respectively. Each treatment group (including positive and negative controls) contained twenty replicates each broken down into two experiments comprised of ten replicates each. The experiment was designed as a random block design, a total of forty five blocks containing nine rows and five columns. Leaf age and size variability can arise among each leaf within the same plant; consequently the oldest two leaves per plant were used to reduce variability.

For avoiding contact between lettuce plants they were positioned 15cm apart. Plants were randomly selected for treatment. The treated plants were actively marked and placed back into their original

position. The application of the BCA products was done as recommended by the user instructions for foliar application.

			A1			
	A5				A8	
			A2			
A6						A9
			A3			
	A7				A10	
			A4			

Figure 1. Leaf disc procurement position on lettuce leaves (not to scale). An example of a lettuce leaf collected can be viewed on the left side of Figure 1. The right side of Figure 1 displays the position of leaf disc procurement. Leaf discs were obtained from three different sections the apex (A1), midrib (A2, A3, and A4) and lamina/lateral veins (A5, A6, A7, A8, A9, and A10) for representing the whole leaf.

1.2.3 Lettuce cultivars used in the study

Lettuce cultivar Carter was provided via Premier plants and collected from Darnicle Hill Nursery. The plants were at their two true leaf stage. They were grown in plastic plant pots (9cm x 9cm x 10cm) filled with compost and compressed until a flat surface was achieved; subsequently the lettuce peat blocks were replanted into these pots for carrying out the experiment in the Glasshouse until early head development.

Preparation of the pathogen and BCAs, and their applications

1.2.4 B. cinerea cultivation and inoculum production

For procurement of viable inoculum, *B. cinerea* isolates were obtained from lettuce in commercial growing environments including Laurence J Betts Ltd, Anglia salads Ltd, JEPCO and Scotland's Rural College. In experiment one the study utilised a single isolate obtained from the Carter cultivar. In the second experiment all available *B. cinerea* isolates of lettuce (a total of eight isolates) were mixed and utilised together as a mixed spore suspension.

Each collected *B. cinerea* isolate per plant was grown independently on a PDA plate for 10 days at 20°C. Subsequently 20mls of maximum recovery diluent (MRD) was pipetted onto the plate and the condia dislodged with a sterile scraper. The suspension was decanted through a funnel containing four layers of muslin cloth, and the suspension collected in a 25ml vial. The collected conidial suspensions were poured into a beaker and shaken for 1 minute. Consecutively 10µl of the mixed conidia suspension was pipetted onto a sterile haemocytometer (IMPROVED NEUBAUER depth 0.1mm 1/400mm^2) and the cover slip placed firmly and held for 2 minutes. Macro conidial counts were made under a light microscope, and the spore concentration diluted with sterile water to obtain a final spore suspension containing 10^6 spores/ml.

1.2.5 Preparation of the Serenade and PreStop biocontrol organisms

For Serenade (Bayer) the formulation was found to contain 5×10^{10} cfu/ml. This was serially diluted to obtain 10^5, 10^6, 10^7, 10^8, and 10^9 cfu/ml. For each dosage a 250ml suspension was produced via the serial dilution technique. For instance 25ml of Serenade into 225ml of water equated to 10^{-1} dilution and had a concentration of 5×10^9 cfu/ml; transferring 25ml from the 10^{-1} dilution into 225ml of water equated to 10^{-2} dilution which contained 5×10^8 cfu/ml and so on.

PreStop was available in a dehydrated dry powder formulation. Independent haemocytometer readings indicated that 5g of PreStop in 1000ml of water contained 4.47×10^8 spores/ml (100g/20L). A PreStop suspension of 500ml containing 8.94×10^9 spores/ml was prepared by adding water to 50g of the powder to produce the final volume. The suspension was mixed thoroughly for 5 minutes by hand to ensure rehydration. The serial dilution technique was employed to produce each dose as described previously. PreStop suspensions were prepared freshly on the day of spraying.

1.2.6 Spraying of plants with biocontrol agents and the pathogen

Lettuce plants were positioned 15cm apart on the glasshouse stage/platform, and allowed to settle for two days. The freshly prepared BCA product dosages were transferred into a hand sprayer, labelled and the cap was tightened to produce fine droplets. Subsequently the plant to be treated was selected with a random number generator. The randomly selected plant was placed into an isolated location and sprayed with the correct dose treatment until the visible surface was close to the point of wash off.

After a waiting period of six hours for the BCA propagules to settle, the *B. cinerea* conidia suspension of 10^6 spores/ml was sprayed onto each plant in an identical design and manner. Subsequently treatments allocated for dose confirmation via the PMA-qPCR technique were procured randomly. For each plant two of the eldest leaves were sampled, placed into plastic containers and labelled.

Positive controls were subject to a sole application of *B. cinerea* macroconidia suspension. An infection period of two days was ensued; succeeded by random sampling of each plant.

Disease assessment

1.2.7 Leaf collection, surface sterilization and leaf disc assays

Eldest two leaves were excised with scissors from each lettuce plant, and the leaf pair was attached via a peg, labelled and placed into a plastic container. Scissors were sterilized for 10 seconds in 70% ethanol per use. Initial washing for each leaf commenced under a slow running tap of water for 5 minutes, ensued by a hand agitated wash in Tween 80 suspension (1 drop in 200 ml of sterile distilled water) for one minute. In succession rinsing of each leaf transpired in SDW for 1 minute thrice; and subsequently placed in 70% ethanol suspension for one minute, and rinsed in SDW for 1 minute twice.

Succeeding leaf surface sterilization, leaves were placed on three layers of blue roll to dry under a class 1 cabinet for two hours; subsequently the leaves were placed onto a tile, firmly positioned and a cork borer size 4 was used to collect a leaf disc with an area of 3.14 cm^2. From each leaf 10 discs were collected; Figure 1 indicates the procurement location. The distance between collections of each leaf disc was dependant on leaf size and width. Leaf discs were transferred with a tweezer onto PDA agar and placed firmly 0.75cm apart, and positioned identical to Figure 1. The plate was covered, sealed with parafilm and incubated at 20°C for seven days. Post the incubation period each leaf disc was inspected for confirming or rejecting lesion formation, and hyphae growth.

Refer to annual report of 2016 of CP140 for technical methods i.e. PDA preparation, PMA treatment, DNA extraction, and qPCR materials and methods. The mean LD_{50} values were determined for Serenade and PreStop by the statistical software GenStat through the probit analysis.

1.3 Results

The mean LD_{50} values were determined for Serenade and PreStop by the statistical software GenStat through the probit analysis. Initially data on the biocontrol potential was compared with the negative control percentage (uninfected leaf discs) for Serenade (Figure 2) and for PreStop (Figure 3). The relative biocontrol obtained has been broken down into different leaf sections and presented as a bar chart for Serenade (Figure 4) and for PreStop (Figure 5). The log of dose vs response data was also plotted for the PreStop treatments (Figure 6) and for Serenade (Figure 7).

In this case the logit equation was used to transform and produce the dose - response curve. The mean LD_{50} doses are shown in Table 3. In all cases the analyses for goodness of fit was done for each leaf disc. Subsequently, a probit analysis was done. This was used instead of logit transformation because of the better fit to the data sets for each leaf disc (Figure 8).

The dose response curves for Serenade showed that 5×10^9 cfu/ml was the most effective dose, while 5×10^5 cfu/ml was the least effective dose, and that the threshold dose was 5×10^8 cfu/ml against an initial inoculum of the pathogen of 10^6 spores/ml (Figure 2). The 5×10^9 cfu/ml application of the biocontrol agent produced a higher control percentage in contrast to the untreated leaf discs.

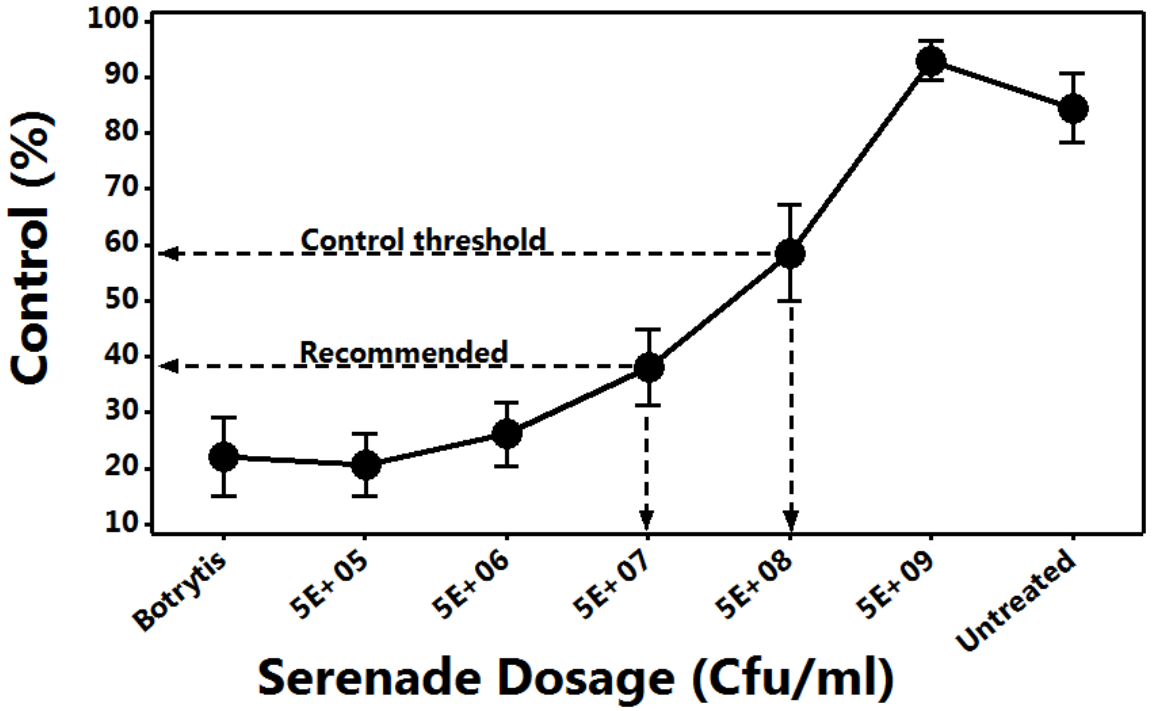


Figure 2. Preliminary dose – response curve of *B. cinerea* to Serenade: for Figure 2 and 3 leaf disc assays were incubated at 20°C for 7 days, and the preliminary dose – response curve of *B. cinerea* to Serenade and PreStop was produced by counting the total number of uninfected leaf discs across both experiments out of a sample population of two hundred per treatment. The data was converted to percentages, and the mean percentage along with the error bars for each treatment was plotted and joined.

The dose response transpired for PreStop control showed that there was an increase in disease control as the inoculum of the BCA was increased (Figure 3). The inoculum of 8.94×10^9 spores/ml of the BCA was the most effective dosage, while 8.94×10^5 spores/ml was the least effective dose. The threshold dose was 8.94×10^8 spores/ml of *G. catenulatum* to control a high inoculum load of *B. cinerea* (10^6 spores/ml).

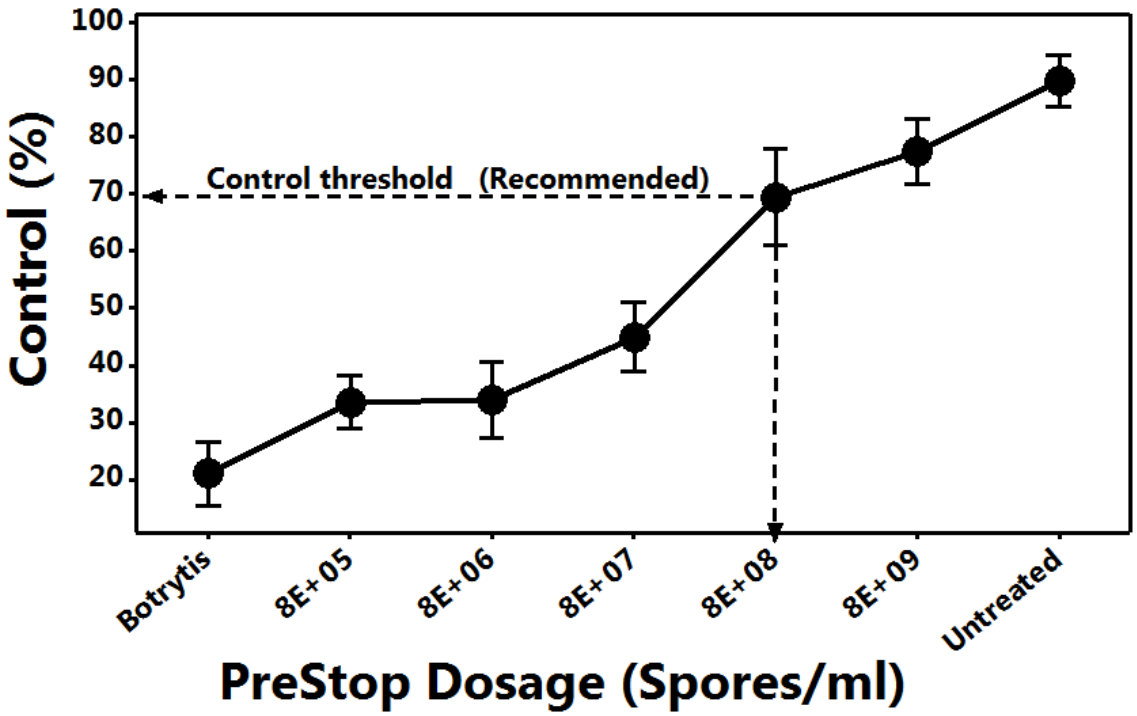


Figure 3. Preliminary dose – response curve of *B. cinerea* to PreStop

Figure 4 suggests the apex and midrib sections are difficult for controlling *B. cinerea* infection when using Serenade at low dosages, in contrast, for the lamina and lateral veins, the threshold dose was 5×10^8 cfu/ml. A higher concentration of the BCA at 5×10^9 cfu/ml produced better pathogen control in the apex and midrib compared to the untreated leaf discs.

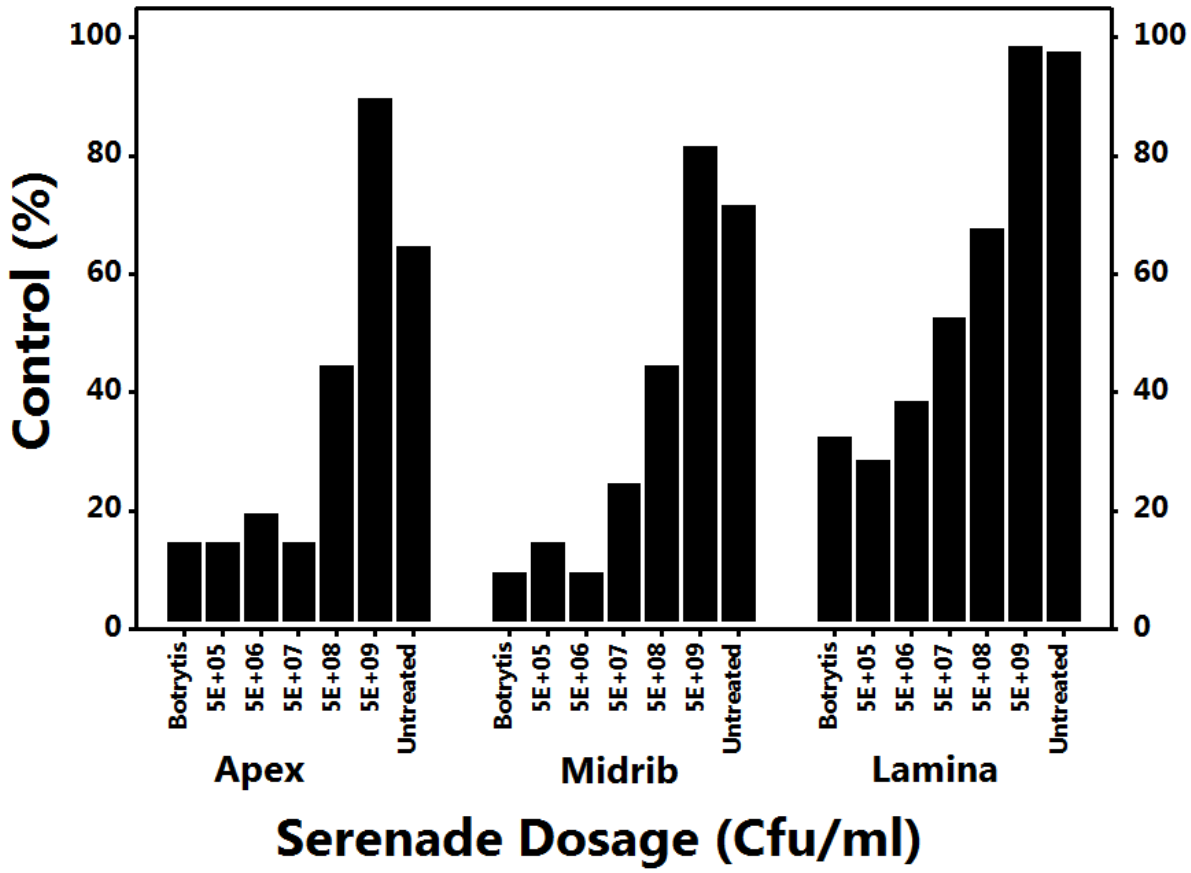


Figure 4. Serenade bar chart of incidence of control on lettuce leaf sections: for Figures 4 and 5 leaf disc assays were incubated at 20°C for 7 days; the bar chart of incidence for the apex section (A1) was produced by counting the total number of uninfected leaf discs across both experiments out of a sample population of 20 leaf discs per treatment. The bar chart for the midrib section (A2, A3 and A4) was produced by counting the total number of uninfected leaf discs across both experiments out of a sample population of 60 per treatment. The bar chart for the lamina and lateral veins section (A5, A6, A7, A8, A9, and A10) was produce by counting total number of uninfected leaf discs across both experiments from a sample population 120 per treatment. The data was converted to percentages and the mean uninfected leaf disc percentage was plotted for each section.

For PreStop (Figure 5) the apex and midrib sections were more difficult to control *B. cinerea* infection; in contrast to the lamina and lateral veins. The threshold dose in all leaf sections was 8.94 × 10^8 spores/ml.

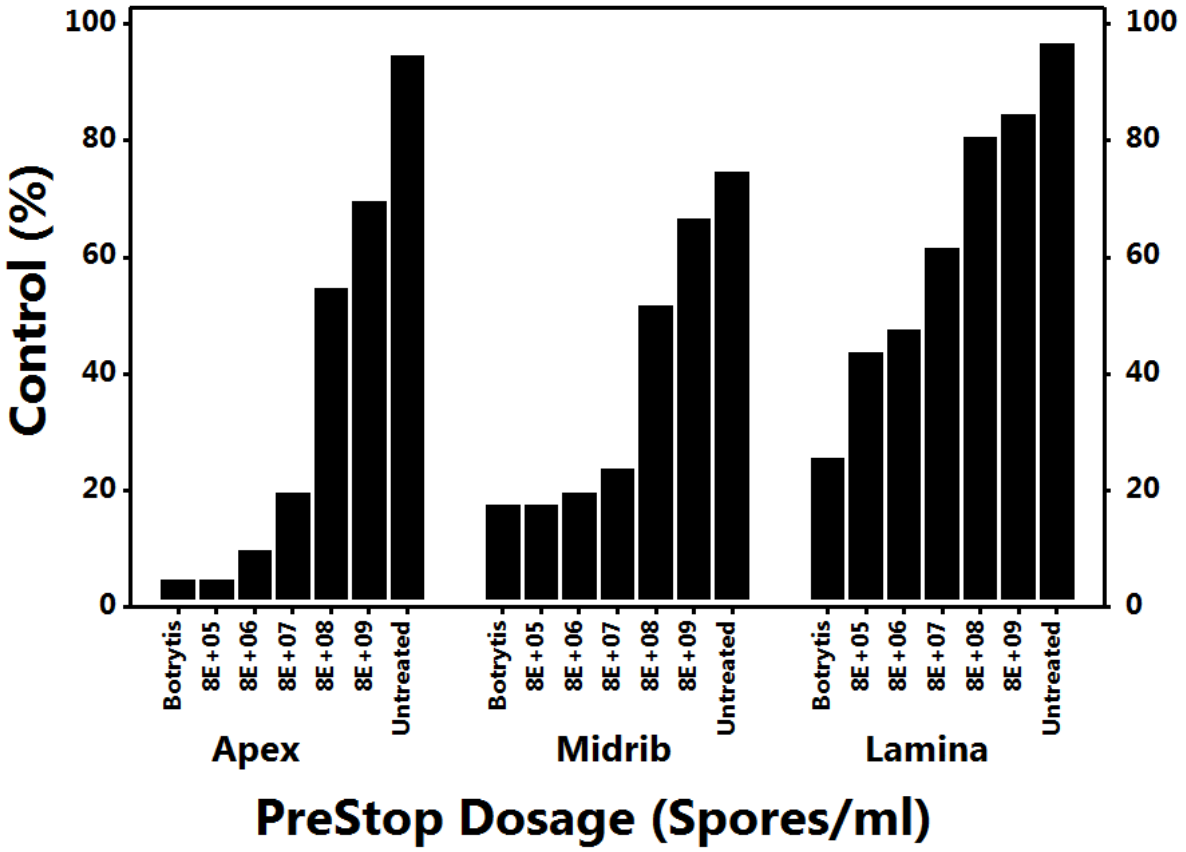


Figure 5 PreStop bar chart of incidence of control on lettuce leaf sections

Significance tests were not necessary to determine the dose which produced significant differences between the BCAs and the control. Since significance directly depends on the number of replicates, consequently twice as many replicates can change the significant dose. Thus the present work was focused on determining the dose which produced 50% reduction in comparison with the control. In the positive control there were infections of about 20%. Consequently the total response range was 70%. Doses which gave a 50% reduction within this range were considered as the effective LD_{50} concentration. The LD_{50} concentrations of the BCAs and their 95% confidence intervals are shown in Table 3.

Table 3. Mean LD_{50} doses for PreStop and Serenade: the proportion of uninfected leaf discs was transformed with the logit equation to produce a linear scaled dose – response curve, and from the transformed dose- response curve the dosage which produced 50% response from the total response was acquired as the LD_{50}.

Nonlinear regression analysis				
Summary of analysis				
Source	d.f.	deviance	Mean deviance	Deviance ratio
Regression	7	810.14	115.734	27.73
Residual	6	25.04	4.174	
Total	13	835.18	64.245	
Dispersion parameter is estimated to be 4.17 from the residual deviance.				
Effective doses				

Log10 scale					
Group	LD	estimate	s.e.	lower 95%	upper 95%
PreStop	50.00	7.456	0.838	5.765	8.573
Serenade	50.00	7.758	1.213	6.254	8.749
Natural scale					
Group	LD	estimate		lower 95%	upper 95%
PreStop	50.00	2.56E+08		5.21E+06	3.34E+09
Serenade	50.00	2.86E+08		8.97E+06	2.80E+09

Figure 6 shows the relationship between the proportion of non-infected leaf discs relative to the concentration of the applied BCA for PreStop. At a low dose, 35% of the population responds, while at a high dose 20% do not respond.

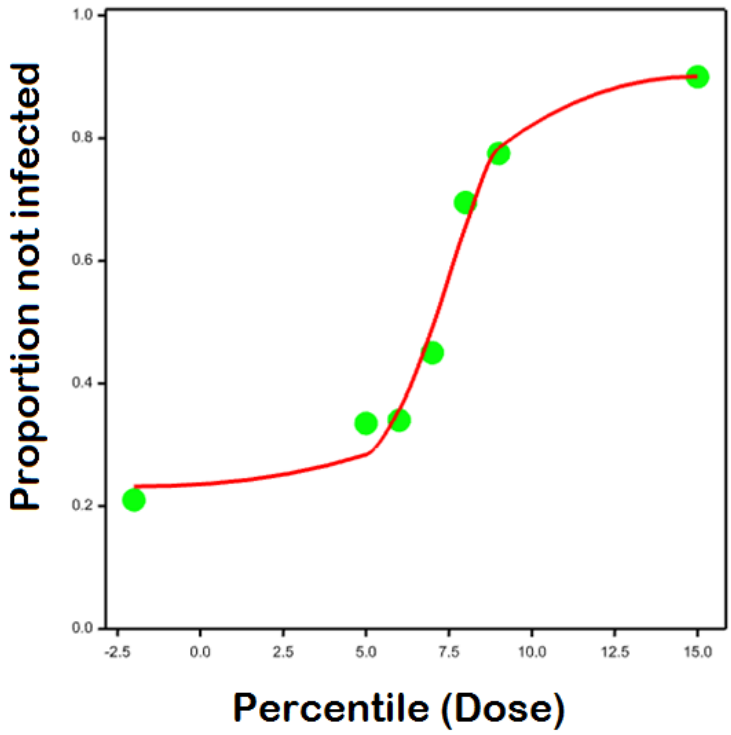


Figure 6 Dose – response curve of proportion of leaf discs not diseased for PreStop treatments. For figure 6 and 7 the proportion of lesion/infection free was analysed via PROBITANALYSIS in Genstat. Figure 6 and 7 dose – response curves contain the estimates (dosages), the control of response (positive control), and the high dose immunity which equals to infected at high dose (proportion of population with no response, and are not immune) together with the slope of the logit curve and the LD_{50} (dose which produces 50% response of the potential responders). The model estimates at the LD_{50} value of 15 the high dose immunity level was acquired. Models for Figure 6 and 7 were fitted utilising a non-linear model with binominal distribution utilising a logit link (observed and fitted percentage of responses).

Figure 7 shows the similar dose response curve for Serenade. In this case the interpretation was more complex due to the highest dosage containing least infected leaf disc proportion in contrast to the negative control (untreated). This outcome causes disarray in dose – response curve production.

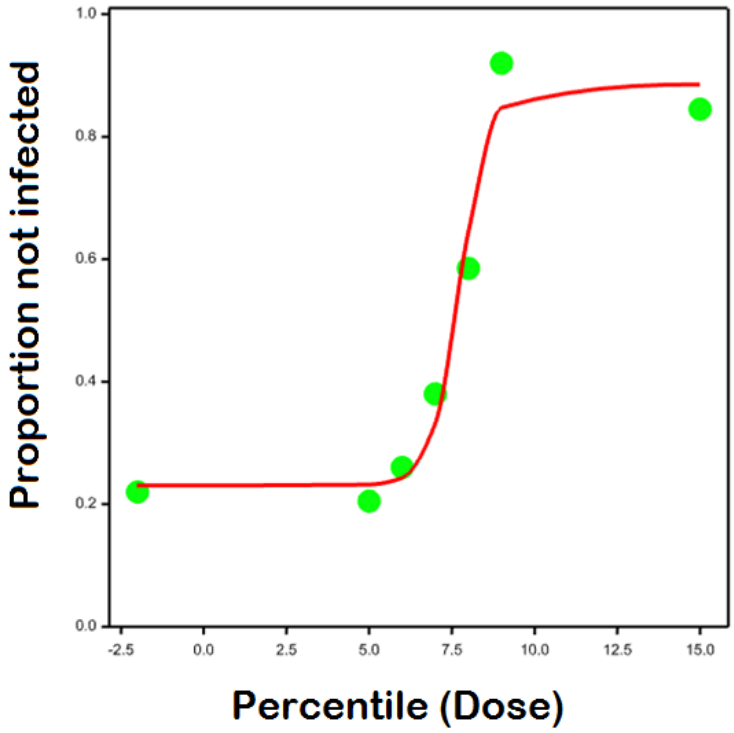


Figure 7 Dose – response curve proportion of leaf discs not diseased for Serenade treatments

A plot of percentage uninfected (out of ten) leaf discs vs treatment (Figure 8) was produced via probit analyses in GenStat. Each sub-Figure in Figure 8 provides information on the BCA used and the position. Dose - response curves were fitted to each graph independently for determining the dose which controlled total infection by 50%. This allows comparison between leaf positions.

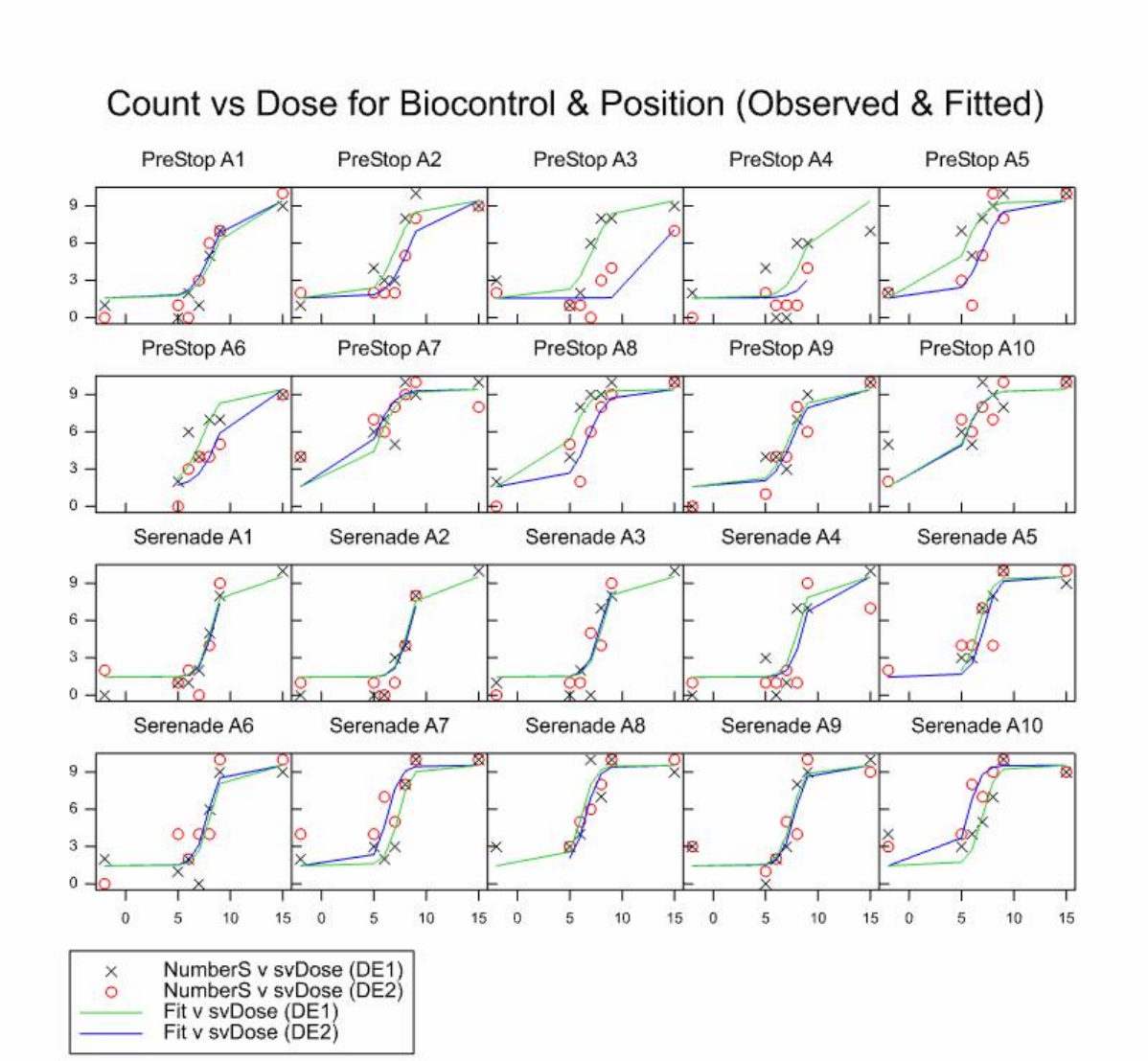


Figure 8 Count of uninfected leaf discs vs dose for position: The Y axis in Figure 8 represents the number of leaf discs not infected out of ten, and the X axis indicates the treatment. In Figure 8 zero pinpoints the positive control (solely *B. cinerea* infected), the dosage treatments are approximately within the centre, and the negative controls (untreated/non-infected) are pinpointed by the value 15 at the top. Consequently higher response (presented as control percentage) directly correlates to the number of non-infected leaf discs. Figure 8 suggests highest response occurs with the most concentrated dosages, positive control contains the highest and the negative control contains the least number of infections. Each point within each graph in Figure 8 represents the mean of the data and the dose – response curves are produced through joining of each treatments mean. The results in Figure 8 are additionally the findings of the Probit analyses since each curve was fitted with the probit equation. The green dose – response curves fitted with the probit equation are for experiment one and the blue dose – response curves are for experiment two. The red circles and X symbols represent

the mean data on each graph. Figure 8 demonstrated both the goodness of fit between the data and the probit curve.

1.4 Discussion

The dose response curves for Serenade and for PreStop were different and suggest different thresholds for achieving 50% control of *B. cinerea* leaf infection.

In summary:

Serenade: thresholds were 5×10^8 cfu/ml, and the transformed dose – response curve indicates a mean LD_{50} dose of 3×10^8 cfu/ml. Control of *B. cinerea* conidia (10^6 spores/ml) occurred through elimination of macroconidia and or suppression of macroconidia germination. Maximum control and or response of *B. cinerea* macroconidia were gained with 5×10^9 cfu/ml; while 5×10^5 cfu/ml procured the minimum control and or response. Increasing Serenade dosage increased the control percentage and proportion of uninfected leaf discs. Greater dosages may contain higher quantity of lipopeptides that directly eliminate and or suppress the macroconidia of *B. cinerea* from germinating. In addition a greater dose contains a higher number of bacterial cells which compete for nutrients and space on the lettuce leaves thereby limiting the colonisation potential of *B. cinerea* macroconidia (Ongena *et al*., 2007). The Serenade dose of 5×10^9 cfu/ml procured superior control in contrast to the negative control (untreated). Findings were anticipated since *B. subtilis* are endophytic thus mobility among plant cells walls to actively encounter and eliminate and or suppress systemic infections was feasible in a high dose treatment; even in company with a *B. cinerea* treatment (Gao *et al*., 2015).

PreStop: threshold dose was 8.94×10^8 spores/ml, while the transformed dose – response curve produced a LD_{50} dose of 3×10^8 spores/ml. Control of *B. cinerea* macroconidia (10^6 spores/ml) occurred through hyperparasitism and induced resistance of the host. Maximum control and/or response of *B. cinerea* were with 8.94×10^9 spores/ml of PreStop; while 8.94×10^5 spores/ml resulted in the minimum level of control. Increasing PreStop dose reduced the *B. cinerea* macroconidia infection potential. Greater dosages may contain a higher content of lytic enzymes (β-1,3-glucanase) which aid in the breakdown of fungal cell walls. In addition a greater dose contains a larger quantity of *G. catenulatum* conidia and or hyphae/mycelium capable of hyper parasitizing the pathogens macroconidia, as well as gaining an edge for competition for nutrients and space (Lahdenpera and Korteniemi, 2005; Chatterton and Punja, 2009).

The primary characteristic of competition of nutrients and space is the rapid colonization of the BCA on to the crops surface. This mechanism of action enhances as the applied biocontrol concentration is increased and the pathogen concentration decreased. Droby *et al.* (1991) supported this quantitative affiliation, as mutant *P. guilliermondii* lacking the function to reproduce lost its potency against *B. cinerea* on grape fruit, even with applications as high as 10^{10} cfu/ml.

Lettuce plants not treated with *B. cinerea* or biocontrol product (negative control) displayed *B. cinerea* infection and or lesion development. Bar chart of incidence of control for Serenade, and

PreStop; probit fixed dose response curves; suggest leaf disc positions A1 (apex), A2, A3, and A4 (midrib) require a higher dose of treatment to control *B. cinerea*, or are immune to treatment. Lettuce plants can harbour *B. cinerea* in their roots, stems and leaves without any visual symptoms suggesting the occurrence of systemic infections. As the plant grows the infection in the lettuce seed or seedling spreads to fresh tissue (Sowley, Dewey, and Shaw, 2010; Fiume and Fiume, 2005). Post infection *B. cinerea* colonises leaf tissue systemically in a symptomless manner. Systemic infection observation occurred after the leaf disc assay stage due to placement onto PDA inducing favourable conditions for necrotrophic infection thereby causing lesion formation and *B. cinerea* hyphae growth. Findings indicate the influence of pre-infection and determine the necessity of high dose treatment and or immunity to treatment with PreStop in the midrib (A2, A3 and A4).

In contrast endophytes such as *B. subtilis* are capable of endophytic colonisation of lettuce tissue; thereby containing capacity for suppressing and or eliminating systemic infections of *B. cinerea* at a high dose treatment (5×10^9 cfu/ml). Research in lettuce infection by *B. cinerea* justifies the differences due to systemic infections; hence pre-systemic symptomless infections influenced the dose – response curve and ultimately the LD_{50} dose value. Due to *B. subtilis* possessing endophytic mechanisms the impact was not as emblematic. In comparison *G. catenulatum* lacks endophytic mechanisms for foliar applications. Lettuce infections in the research are prominently residing on the midrib (A2, A3, and A4) suggesting systemic symptomless colonisation transpired from the petiole.

Justification of experimental differences include; utilization of different *B. cinerea* isolates for each experiment, lettuce plants were fifteen-twenty days older in experiment two, and climate in the glasshouse may have been different as two to three weeks were in-between each experiment. Experiment one contained preliminary *B. cinerea* isolate acquired from Cater; while experiment two contained a mixture of *B. cinerea* isolates collected from the field on a range of lettuce cultivars and the initial Carter isolate. Studies by Choquer *et al*. (2007); Movahedi and Heale, (1990) suggests that isolate differences can impact virulence factors.

Utilization of the LD_{50} dose carries significance as mean tolerance in a population are considered; an application of Serenade fundamentally functions through the available lipo-peptides in the formulation, and the continuously produced lipo-peptides by the *B. subtilis* cells post application (Ongena and Jacques, 2008); since lipo-peptides are bio-degradable, and their biodegradability directly depends on the protein structure (Liu *et al*., 2015; Lin and Grossfield, 2014; Biniarz *et al*., 2017). *B. subtilis* cells are impacted by quorum sensing that effect growth rate and lipo-peptide synthesis; consequently consistency and efficacy (Eldar *et al*., 2017; Lazazzera, 2000). Applying the mean LD_{50} dose for Serenade potentially provides superior consistency and greater efficacy. Temperature can play a key role in BCA reproduction, as temperature can directly affect the growth rate (Ratkowsky *et al*., 1982). Low temperatures can affect the cell viability of *B. subtilis* (Willimsky *et al*., 1992); and fluctuations in temperature can affect the lipo-peptide production (Kilani-Feki *et al*., 2016). Hence a critical approach suggests temperature dependency for long term control with the mean LD_{50} dose. Temperature affects both *B. cinerea* and biocontrol product, consequently a lack of

evidence indicates the LD_{50} dose potentially functions in a range of climates unless justified ineffective via testing in a range of climates.

Presence of antagonists such as *G. catenulatum* and their compounds can induce defence responses in plants (Ippolito and Nigro, 2000): *C. saitona* when applied to plants caused papillae in host cell walls by inducing chitinase activity (El-Ghaouth *et al*., 1998). Similarly *A. pullulans* provoked an ephemeral increase of β-1,3-glucanase, peroxidase, and chitinase enzymes on apple wounds, which stimulated wound healing (Ippolito and Nigro, 2000). Information on the relationship of host defence development and bio protection by antagonists are limited, but the build-up of antifungal compounds in protected tissues suggests BCA have a role in disease resistance. On fruit surfaces BCA form extra cellular mucilage along the host cells walls; which the BCA uses to adhere to the fruit surface, and possibly release active chemical elicitors which provide signals for recognition and subsequent responses (Castoria *et al*.,1997; El-Ghaouth *et al*., 1998). Consequently the LD_{50} dose of *G. catenulatum* upholds reliability for controlling a high inoculum load of *B. cinerea* consistently with a high efficacy.

Section 2: The survival of *B. subtilis* and *G. catenulatum* within commercial growing environments

2.1 Background for research

Currently there is a lack of knowledge on BCA survival on crops within commercial growing environments. Additionally there are no concurrent findings on the survival of *B. subtilis* and *G. catenulatum* propagules within a polytunnel and glasshouse during the winter. The selected commercial settings potentially display a range of climatic conditions which would allow testing of the two BCAs, *B. subtilis* and *G. catenulatum* propagules, on their survival capabilities over time. Determining the survival competence defines the importance in testing a range of climatic treatments on biocontrol agent viable populations. This will be beneficial in understanding the effectiveness and length of biocontrol achieved and the necessary sampling which may be required to assess efficacy under different environmental conditions.

Thus the objective was to determine the temporal dynamics of BCA survival, in order to identify the relative period and frequency of sampling for assessing efficacy in subsequent experiments.

2.2 Materials and methods

2.2.1 Experimental treatments

For confirming the period and frequency of sampling BCA propagules in commercial growing environments experiments were conducted on lettuce within a poly tunnel, and a glasshouse during the winter. *B. subtilis* and *G. catenulatum* propagule sampling occurred at six time points (Day 0, 2, 4, 6, 8, 10). Consequently sampling time equals to treatment, thus six treatments per environment, totalling twelve treatments per BCA. Per treatment five replicates were sampled.

Experiment description

As new leaves emerge after ten days that specific time frame was selected as the period of sampling. The frequency of sampling occurred every two days due to restraints in funding; specifically due to the labour requirements from sample collection to DNA requiring twenty hours of lab work (minimum). Sampling from healthy tissue was critical as the research focuses on BCA viable population on healthy lettuce plants, subsequently for increasing the probability of direct sampling of BCA propagules from healthy leaf tissue for the total period of ten days thirty five plants were placed into each environment. A total of five true leaf samples were acquired every two days for each treatment (D0, D2, D4, D6, D8, and D10) per BCA (*B. subtilis* or *G. catenulatum*) and environment (poly tunnel or glasshouse). Post sample acquisition PMA treatment ensued, followed by DNA extraction, and DNA samples were stored at -20°C for qPCR analysis for determining the viable population.

Experimental design

Refer to Table 4. for details on the climatic conditions during BCA survival experiments.

Table 4. Descriptive Statistics of climatic variables in the poly tunnel and glasshouse during survival of BCA experimentation. Data was collected with data loggers set to sample the temperature, humidity and dew point every thirty minutes. The data set was analysed with Minitab.

Celsius(°C), Humidity(%rh), Dew Point(°C) in the poly tunnel

Variable	N	N×	Mean	SE Mean	TrMean	StDev	Variance	CoefVar	Minimum
Celsius(°C) P.T	6341	0	6.1322	0.0717	5.7419	5.7088	32.5908	93.10	-6.0000
Humidity(%rh) P.T	6341	0	80.903	0.140	82.360	11.163	124.606	13.80	39.000
Dew Point(°C) P.T	6341	0	2.8717	0.0517	2.9232	4.1200	16.9742	143.47	-8.3000

Variable	Q1	Median	Q3	Maximum	Range	IQR	Mode	N for Mode
Celsius(°C) P.T	2.5000	5.5000	9.0000	26.5000	32.5000	6.5000	7	292
Humidity(%rh) P.T	80.500	83.000	87.500	93.500	54.500	7.000	82.5	437
Dew Point(°C) P.T	0.0000	2.9000	6.0000	13.8000	22.1000	6.0000	5.9, 6.3	82

Descriptive Statistics: Celsius(°C), Humidity(%rh), Dew Point(°C) in the glasshouse

Variable	N	N×	Mean	SE Mean	TrMean	StDev	Variance	CoefVar	Minimum
Celsius(°C) G.H	3169	0	18.079	0.0394	17.881	2.218	4.919	12.27	14.000
Humidity(%rh) G.H	3169	0	61.858	0.227	61.713	12.783	163.409	20.67	24.500
Dew Point(°C) G.H	3169	0	10.335	0.0538	10.357	3.029	9.175	29.31	2.300

Variable	Q1	Median	Q3	Maximum	Range	IQR	Mode	N for Mode
Celsius(°C) G.H	16.500	17.500	19.500	31.500	17.500	3.000	16.5	619
Humidity(%rh) G.H	53.000	63.500	70.500	97.000	72.500	17.500	68.5	69
Dew Point(°C) G.H	8.200	10.500	12.600	19.000	16.700	4.400	9.9	61

Spraying of each plant occurred independently, analogous to the previously described model. The experiment was designed as a random block design, a total of thirty five blocks containing seven rows and five columns. For controlling leaf age and size variation on the same plant and among plants, and to induce the blocking effect solely the oldest leave was procured per replicate.

2.2.2 Lettuce collection and development

Refer to page 8.

2.2.3 Growth of *B. subtilis* QST 713

Within a flow cabinet 10µl of Serenade (neat product) was transferred with a pipette onto the centre of the nutrient agar. Subsequently the droplet was spread homogenously across the agar with a spreader following a circular motion. In succession the plate was sealed with parafilm, and the procedure repeated thrice. Plates were incubated at room temperature 18-22°C for two days. Post the incubation period; the agar containing the developed colonies were sliced with a scalpel into four equal sections, and transferred with a tweezer into a 1L vacuum filter flask containing 700mls of autoclaved tryptic soya broth. *B. subtilis* culture growth occurred in a shaker set at 110-130rpm in a temperature range of 25-37°C for seven to ten days. Determination of concentration post the growth period transpired via the viable plate count technique.

2.2.4 BCA dosage preparation, sample collection and processing

The grown *B. subtilis* suspension was serially diluted with water to contain approximately 5×10^8 cfu/ml. For producing a dosage of 4.4×10^8 spores/ml 5g of PreStop was mixed into 1L of water. Refer to page 8 plant spraying general description paragraph one for BCA spraying protocol. Post spraying stage samples assigned for D0 were procured, and the single oldest leaf was incised with scissors and attentively transferred with a tweezer into a 50ml falcon tube, sealed and labelled for; treatment, BCA and environment. Replicate selection order was randomised but placed into the same tube. Subsequently collected leaves were submerged in maximum recovery diluent (MRD), until the 50ml marker and sealed. The sample was placed onto a shaker set at 10°C with a shaking setting of 150rpm for 30 minutes.

After the surface biota fully suspended into the MRD solution, the contents were filtered through four layers of muslin cloth placed into a funnel, and passed into a new falcon tube. Conceding that the second falcon tube had a volume less than 50mls; the original sample was re-filled and shaken for 3

minutes and re-filtered. Successively centrifugation commenced at 3500 × g for 15 minutes at 4°C. Post centrifugation the supernatant was poured until 0.5ml of suspension remained. The supernatant and cell pellet were homogenised thoroughly with a pipette tip and transferred into a 1.5ml clear autoclaved micro centrifuge-eppendorf. Subsequently PMA treatment, DNA extraction and qPCR was accomplished (refer to annual report 1 for methodology).

2.2.5 Grinding of *G. catenulatum* propagules

Post PMA treatment PreStop centrifugation commenced at 5,000 × g for 10 minutes at 4°C. The supernatant poured, and 1ml of MRD pipetted into the cell suspension, ensued by slow pipetting for homogenization. A content of five stainless steel pellets were transferred into each sample with a tweezer. Samples were placed into the genome grinder 2000 set at 1750rpm for 15 minutes. Post grinding stage the steel beads were removed with a magnet and sterilized with 70% ethanol for 1 minute.

2.2.6 Statistics

The use of statistics on colony forming units per leaf was unacceptable since variations between surface areas of each leaf are not accounted, consequently the data can only be utilised for drawing assumptions.

2.3 Results

Figure 9 and 10 shows the survival of B. *subtilis* and *G. catenulatum*. Viable populations of *B. subtilis* declined in both environments until day ten. In contrast, PreStop viable populations remained constant up to day eight in both environments. The viable populations of both BCAs increased at specific time points, although this was clearer for PreStop.

Viable populations of *B. subtilis* for controlling a high pathogen inoculum load of *B. cinerea* macroconidia was not detected by day four in the poly tunnel and day six in the glasshouse (Figure 9). Viable populations of *B. subtilis* decreased; rapidly in the poly tunnel, and gradually in the glasshouse. The initial increase in viable population of *B. subtilis* transpires on day ten in both environments (Figure 9). The viable population of *B. subtilis* increased within the poly tunnel approximately by 1.5 log on day ten compared to the previous sampling point, in comparison the viable population increase of *B. subtilis* was subtle on day ten in the glasshouse.

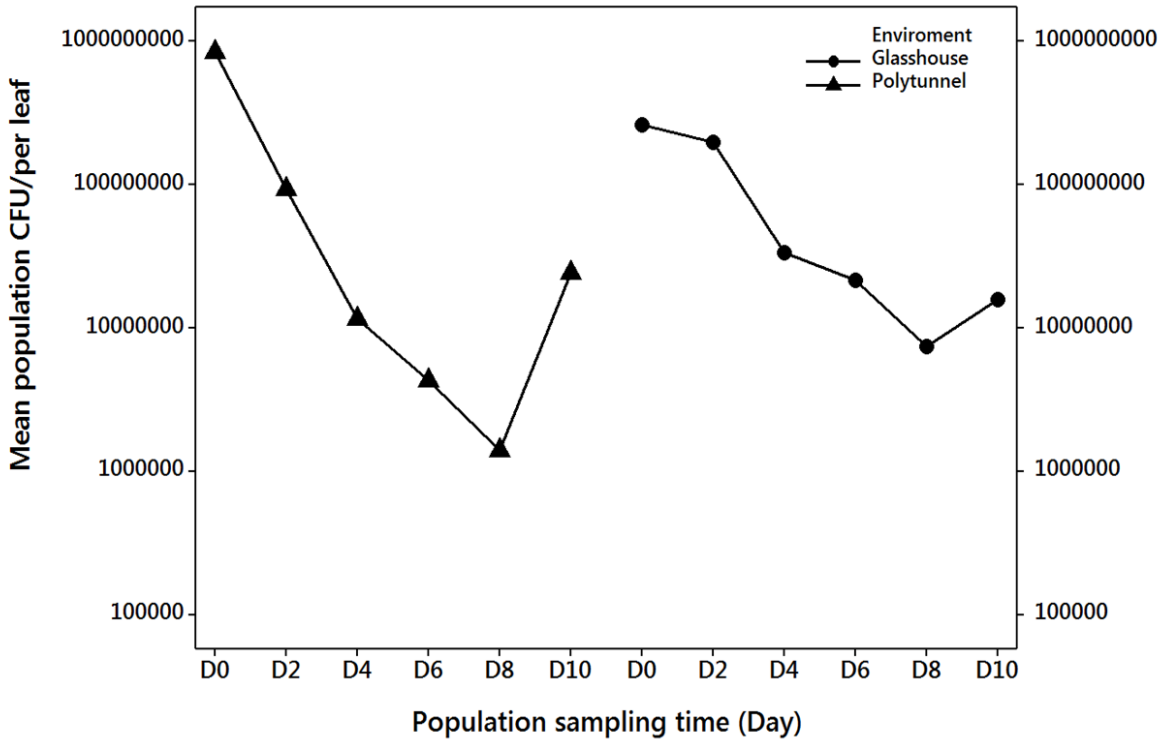


Figure 9. Time series plot of survival for *B. subtilis* in commercial growing environments. For Figure 9 and 10 each time point represented on the time series plot were acquired from a total of five leaves collected from five lettuce plants, the single oldest leaf was obtained from each plant. The leaf samples were washed with MRD and filtered. Post filtration samples were PMA treated and qPCR technique was used to enumerate the mean viable population on a single leaf.

Viable population of *G. catenulatum* for controlling a high inoculum load of *B. cinerea* was not detected by day eight in both environments (Figure 10). Viable population of *G. catenulatum* in both environments remains stationary with subtle changes on each sampling day. A viable population increase transpires on day two and day six in the poly tunnel, while viable population increase of *G. catenulatum* occurs on day four and day eight in the glasshouse (Figure 10).

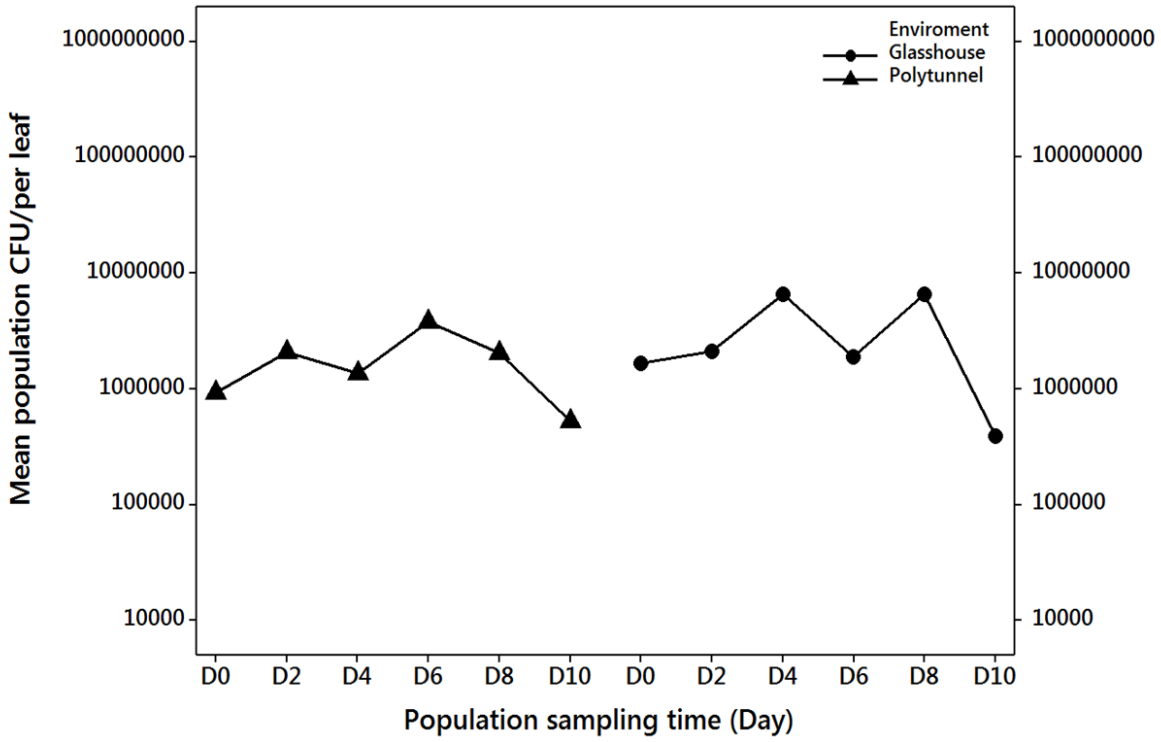


Figure 10. Time series plot of *G. catenulatum* survival in commercial growing environments

2.4 Discussion

B. subtilis and *G. catenulatum* viable populations are enumerable from lettuce plants up to ten days. The period directly represents a treatment reliabilities length; and proportionately new leaves develop post this period. Consequently future research of biocontrol agent propagule enumeration can ensue up to ten days.

B. subtilis and *G. catenulatum* sampling frequency of every two days was successful for monitoring viable population changes. Per contra higher the frequency of sampling; greater the monitoring capacity of population change. Frequency of sampling transpires every two days due to; funding limitations and labour demands (20 hours laboratory work from sample collection to DNA sample). The research determined the primary objective of identifying period and frequency of sampling for enumerating *B. subtilis* and *G. catenulatum* viable populations.

B. subtilis viable populations reduced continuously until day ten in both environments, subsequently on day ten viable populations increased from the previous sampling event. The justification for viable population increase possibly includes; *B. subtilis* cells entering the vegetative life cycle; proportionately the period may represent the lag time for the sporulation or binary fission to induce in the tested climatic environments for *B. subtilis*.

Absence of the viable population of *B. subtilis* required for controlling a high inoculum load of *B. cinerea* occurred on day four in the poly tunnel and day six in the glasshouse. The findings were anticipated since both environments contained climatic conditions below 20°C (Warth, 1978).

B. subtilis are mesophilic and consequently effected by low temperature, Nedwell, (1999) suggested low temperature limits growth in mesophiles through the loss of membrane function, and the inhibition in the uptake of key substrates through the impact of the low temperature on the cell membrane. Consequently the lower temperature in the poly tunnel reduced the reproduction capacity of *B. subtilis* in contrast to the glasshouse, thus clarifying the prompt absence.

Growth in *B. subtilis* viable population occurred on day ten in both environments; justification for the unexpected findings are due to cold shock proteins (CSP); *B. subtilis* possess a family of homologues small acidic proteins (CspB, CspC, and CspD) that are strongly induced in response to cold shock. CSPs are proposed to function in the process of adaptation to low temperatures (Graumann *et al*., 1997; Budde *et al*., 2006). Consequently *B. subtilis* survival/reproduction ensues, although not in a prolific manner to reserve and increase the original viable population. *B. subtilis* reproduction transpires in a range of temperatures from 11°C (Nichols *et al*., 1995) up to 52°C (Holtmann and Bremer, 2004). Yet adaptation responses of *B. subtilis* are initiated in the presence of rapid increase or decrease within the temperature range, or developing for extended periods bordering the upper or lower temperature limits (Brigulla *et al*., 2003; Holtmann *et al*., 2004). Hence the selection pressure for CSPs was greater in the poly tunnel, and post adaptation a profound reproduction phase was entered in contrast to the glasshouse. Findings suggest temperature may be critical for *B. subtilis* survival/reproduction.

Enumeration of the viable population from *G. catenulatum* required for controlling a high inoculum load of *B. cinerea* occurred up to eight days in both environments; viable population of *G. catenulatum* remained stationary with subtle changes on sampling days. Findings were feasible as PreStop contains *G. catenulatum* macroconidia. Macroconidia of *G. catenulatum* are survival structures, and survive in unfavourable environments for weeks; via their sturdy structure and the thick cell wall formed of beta glucans (Willetts, 1997). Macroconida of *G. catenulatum* function as a tool for colonising harsh environments including; habitats drier than the original, sub-zero temperatures and long periods with the absence of hosts; proportionately macroconida are resistant to water soaking. Macroconida grants an edge to the anamorph *G. catenulatum* through protecting and nurturing the fragile internal hyphae; initiation of favourable conditions induce germination, subsequently the abundantly stored hyphae are accessible to form mycelia and spores which antagonise *B. cinerea* (Willetts, 1997).

Hence survival of the control viable population of *G. catenulatum* occurred up to eight days, and increase in viable population of *G. catenulatum* in day two and day six in the poly tunnel, day four and day eight in the glasshouse suggests germination; as *G. catenulatum* activity ensues around 5 °C - 34 °C with optimal activity at 15 °C - 25 °C (Helyer, Cattlin, and Brown, 2014); optimal pH ranges are 5 - 6, but the *G. catenulatum* can cope and grow between pH 3 - 8.2 (Tahvonen *et al*., 1999). Thus far the experimental data of the climate fits into *G. catenulatum* activity range. Since viable population changes were indifferent in both environments, findings suggest humidity may be critical for *G. catenulatum* reproduction.

Conclusions

The LD_{50} dose was identified for Serenade and PreStop. To produce a response in 50% of the *B. cinerea* population (10^6 spores/ml) with Serenade an application of 3×10^8 cfu/ml, and with PreStop an application of 3×10^8 spores/ml are recommended. The viable population required for controlling a high inoculum load of *B. cinerea* represented as colony forming units per leaf for *B. subtilis* are reliable for a maximum of six days, and for *G. catenultaum* was reliable for a maximum of eight days. Findings also suggested temperature for *B. subtilis* and humidity for *G. catenulatum* are potentially critical for survival and reproduction.

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 *B. cinerea*.
- 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 *B. cinerea*.
- 2016 EMR Association/AHDB Soft Fruit Day poster presentation.
- 2017 AHDB Crops PhD Studentship Conference poster presentation.
- 2017 AHDB Crops PhD Studentship Conference research presentation.
- 2017 NIAB and NIAB EMR PhD outreach event presentation.
- 2017 NIAB and NIAB EMR PhD outreach poster presentation.

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Appendices

N/A

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