CRANFIELD UNIVERSITY

GURKAN TUT

OPTIMISING THE USE OF BIOCONTROL AGENTS TO IMPROVE THE CONTROL OF *BOTRYTIS CINEREA* IN KEY VEGETABLE AND FRUIT CROPS

Environment and Agrifood
PhD in Environment and Agrifood

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Supervisor: Prof. Naresh Magan
Supervisor: Prof. Xiangming Xu
Supervisor: Dr Angel Medina-Vaya

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This thesis is submitted in partial fulfilment of the requirements for the degree of PhD

ABSTRACT

For sustainable agriculture, efficient usage of biocontrol agents (BCAs) is crucial. As BCAs are living organisms effective biocontrol ability is governed by complex ecological processes. Because of this, biocontrol of plant diseases can become constrained. Research on the ecology, mechanisms of action and population ecology in the phyllopshere environment is critical for modelling the efficacy of BCAs for control of foliar plant pathogens, especially *Botrytis cinerea*. The aim of the research was to obtain ecological knowledge on *Bacillus subtilis* QST 713 and *Gliocladium catenulatum* J1446, and if feasible use this ecological information to apply these two BCAs against *B. cinerea*. Thus the objective initially was to (i) develop a molecular based assay to quantify viable population changes of the two BCAs, and use this novel assay for investigating: (ii) the dose response relationship of *B. cinerea* to the bacterial and fungal BCA, (iii) impact of relative humidity (RH) and temperature used in UK agronomic production systems on BCA populations, (iv) produce a simple model to predict BCA fate, (v) identify the colonisation and dispersion kinetics of the two BCAs on expanding foliage, and finally from the collected ecological knowledge (vi) suggest optimisations strategies for the two BCAs. This study successfully developed a PMAxx^{TM}-qPCR method for quantifying the kinetics of viable population changes for both the BCAs. The dose response relationship of *B. cinerea* to the BCAs' was deciphered and *G. catenulatum* median effective dose was 1×10^8 spores/ml, while for *B. subtilis* this was 3×10^8 CFUs/ml^{-1}. However, this changed with temperature, formulation, and leaf tissue type. Both temperature and humidity impacted on viable population dynamics of the two BCAs, and showed that viable populations were sustained, increased or reduced depending on abiotic factors (temperature, R.H.), with efficacy best at conditions close to the BCAs optimum

growth conditions. From the collected data, models were produced and tested for their ability to predict the fate of each BCA in commercial growing sites. The dispersion and colonisation kinetics of the two BCAs were analysed on growing lettuce and strawberry leaves. The two BCAs behaved in different ways, while their ability to disperse and colonise virgin leaf tissue was effective, the rate depended on the season (temperature and RH) and host. This study has developed significant new ecological knowledge on these two BCAs and their behaviour when applied to strawberry and lettuce leaf surfaces, and their establishment to control *B. cinerea* in these agronomic crop systems.

Keywords:

Abiotic factors; *Bacillus subtilis* QST 713; Biological Control; *Botrytis cinerea*; Climate; Colonization; Dew point; Dispersion; Dose of application; Dose response models; Dose; Ecology; Formulation; *Gliocladium catenulatum* J1446; Grey mould disease; LD_{50}; Lettuce; Optimization; PMA; Population density; Population dynamics; Predictive modelling; Pre-harvest; PreStop; qPCR; Quantitative method; Relative humidity; Sereande ASO; Strawberry; Temperature; Viable Cells; VPD.

ACKNOWLEDGEMENTS

A special thank you to Prof Naresh Magan my primary supervisor for supporting and advising me over the period of this PhD. I am also thankful to my supervisor Prof Xiangming Xu for supporting and teaching me. I am additionally thankful and grateful to Cranfield University and NIAB-EMR staff members for their discussions and advice as well as the AHDB for funding this research, and especially to Philip Brain for teaching me and helping with my statistical work.

I would like to thank my family for their absolute love and support during this process. Especially my son Serzad Tut for putting a smile on my face every day, and my other half Fatma Tut for keeping me motivated even on the rainy days. Finally, please take a moment of silence for all the lettuce and strawberry plants that have fallen during the making of this thesis.

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LIST OF EQUATIONS

1. $\boldsymbol{Total\ amount\ of\ dead\ cells} = \boldsymbol{Log10}\ (\boldsymbol{Total\ amount\ of\ cells}) -$

   $\boldsymbol{Log10}\ (\boldsymbol{Total\ amount\ of\ viable\ cells})$

2. ΔCt = Ct value obtained without PMAxx™(Negative control) −

   Ct value obtained with PMAxx™(Treatment)

3. Total Area $= \frac{\text{Total area of leafs in pixel}}{\text{Mean area of standard square in pixel}} =$ Number of standard squares in leaf area ×

   Area of a single sqaure

4. $\frac{\text{CFU}}{\text{mm2}} = \frac{\text{Total quantified viable population of BCA with PMAqPCR}}{\text{Total Area}}$

5. $(E, \%) = [(C - T) / C] \times 100$

6. $Log_{10}(N_n) - Log_{10}(N_0)$

LIST OF ABBREVIATIONS

Adj MS	Adjusted mean squares error
Adj SS	Adjusted sums of squares error
AHDB	Agriculture and Horticulture Development Board
AI	artificial intelligence
ANOVA	Analysis of Variance
ASPIRE	Agriculture Services Programme for Innovation, Resilience and Extension
ATP	Adenosine Triphosphate
BCA	Biocontrol agent
CFU	Colony forming units
CI	Confidence interval
Coefvar	Coefficient of variation
Cq	Quantification cycle
CSP	Cold shock protein
D0	Day zero
DEFRA	Department for Environment, Food and Rural Affairs
DEPC	Diethyl pyrocarbonate
DF	Degrees of freedom
DNA	Deoxyribonucleic acid
DRR	Dose response relationships
EMR	East malling research
EPA	Environmental Protection Agency
EXP	Experiment
FAO	Food and agriculture organisation
IQR	Interquartile range
IT	Information Technology
LET	Lettuce
LLV	Lamina and lateral veins
LPP	Leaf plant parts
MANOVA	Multivariate analysis of variance
MEA	Malt extract agar
MOA	Mechanism of action
NA	Nutrients agar
NC	Negative control

ND	Nano drop
NIAB	National Institute of Agricultural Botany
NTC	No template control
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PI	Prediction interval
PMA	Propidium monoazide
POULD	Proportion of uninfected leaf discs
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
REML	Restricted maximum likelihood
Resid	Residual
RH	Relative humidity
RT-qPCR	Reverse transcription qPCR
S.E	Standard error
SDW	Sterile distilled water
STD	Standard
STDEV	Standard deviation
STRW	Strawberry
TAE	Tris base, acetic acid and EDTA
TRI	Trizol
UV	Ultraviolet
UV-C	Ultraviolet C
UVR	Ultra violet radiation
VIF	Variance inflation factor
VPD	Vapour pressure deficit

GENERAL INTRODUCTION

The nature of phyllosphere ecology is complex and can hinder the development of effective BCAs, making biocontrol efficacy difficult to predict, especially in naturally fluctuating environments. To obtain reproducible biocontrol, reliable predictions of interactions between BCAs and pathogens are critical for control. There is a lack of published studies on BCA survival, reproduction and dispersal. For improving biocontrol efficacy attention must be directed to understanding the ecology of the specific BCA. There is thus a necessity to focus on understanding the inter-relations between BCAs and their abiotic external conditions, the foliar surfaces where they are applied and how these all affect viable population establishment and thus influence their disease control efficacy (Droby et al., 2009, Lacroix, 2011, Liu et al., 2013, Sharma et al., 2009, Spadaro and Droby, 2016).

A recent study focused on the dispersal of *Bacillus subtilis* on strawberry crops, and suggested limited dispersal of BCAs as the primary cause for variability in biocontrol efficacy under field conditions (Wei et al., 2016). Application of BCAs, especially in the phyllosphere, requires rapid germination and establishment to effectively outcompete the pathogen. Thus it is important to have significant BCA viable spores or cells present to prevent pathogen dominance at an early stage of infection (Hadas et al., 2007, Karabulut et al., 2001, Porat et al., 2002, Prusky et al., 2001, Qin et al., 2006).

Current modelling studies suggest that the outcomes of biocontrol are dependent on the rate of BCA colonisation of healthy and diseased host tissues and the rate of mortality of the BCA. Surprisingly, there are little knowledge on this subject, especially comparing a bacterial and fungal BCA and the influence of abiotic factors on colonisation of phyllosphere tissue, especially of strawberry and lettuce leaves,

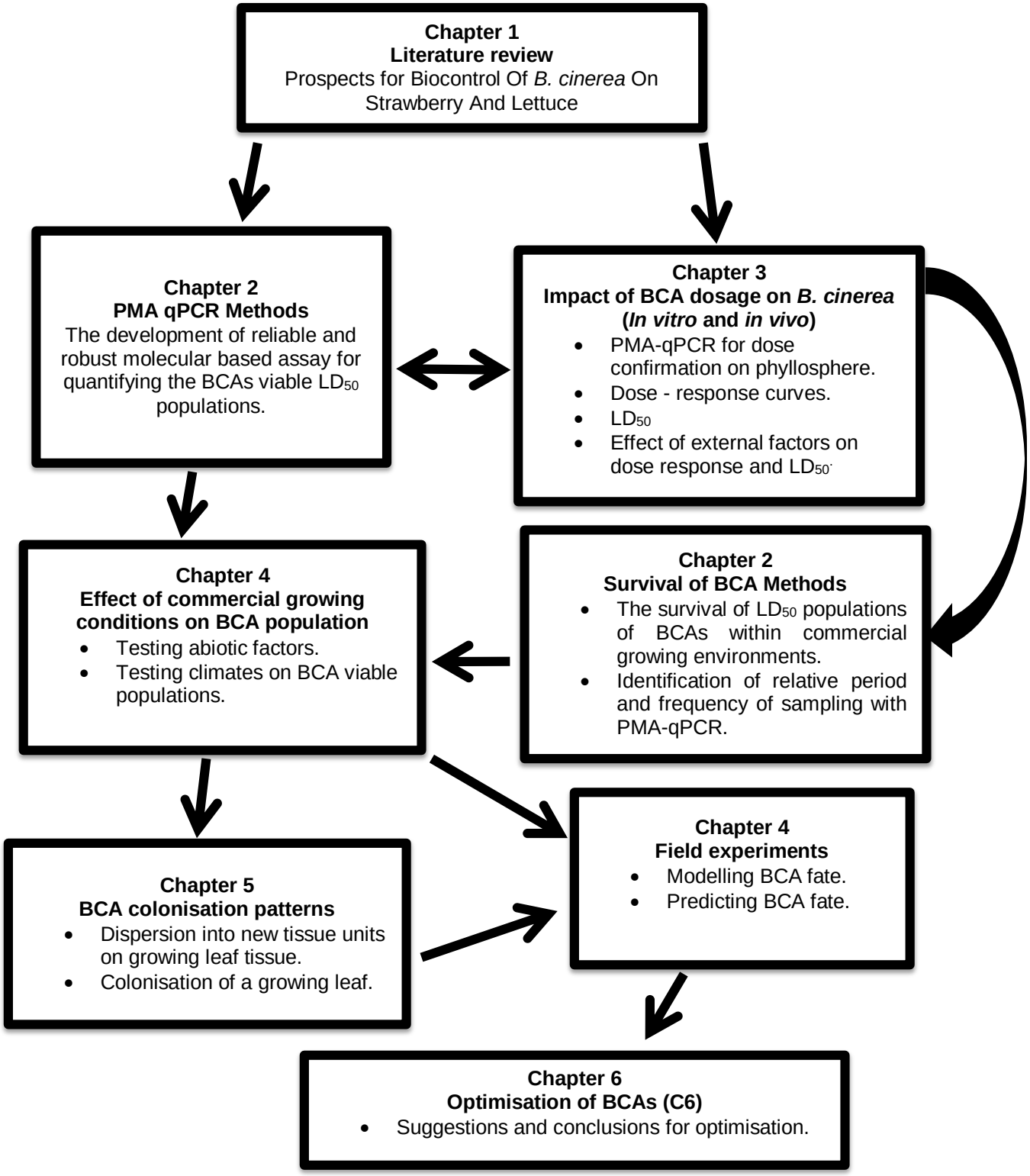
with concomitant quantification of the kinetics of viable BCA populations (Wei et al., 2016).

The survival, reproduction, dispersal and control efficacy of the BCA once applied to a crop is affected by a number of abiotic and biotic factors. Key abiotic factors in the phyllosphere include temperature, relative humidity and leaf wetness periods and their interactions. Temperature is one of the primary factors which influence BCA establishment and competition with the pathogen. For instance, low temperatures affect *B. subtilis* cell viability (Willimsky et al., 1992); and fluctuations in temperatures can affect antifungal metabolite production by this species (Kilani-Feki et al., 2016). This can significantly slow down the cell production rate for this type of BCA. Relative humidity can be key for colonizing crop foliar surfaces; this is especially the case for both bacterial and fungal BCAs. It is known that most bacteria require at least 98% RH (0.98 water activity, a_w) while most non-xerophilic fungi require at least >90-93% RH (>0.90-0.93 a_w) for effective germination and growth (Stevenson et al., 2015, Sperber, 1983). Thus, while BCAs are effective in post-harvest systems where both temperature and RH is controlled, this is more difficult in the phyllosphere where there are significant fluxes in both temperature and RH over a 24 hr period (Cota et al., 2008, Magan, 2001, Magan and Aldred, 2007, Medina and Magan, 2010, Medina-Martínez et al., 2015, Parts et al., 2013).

The main research question for the thesis was: what are the impacts of temperature and relative humidity on the population dynamics of two commercial BCAs, (a bacterium and a filamentous fungus) in the phyllosphere of lettuce and strawberry crops, and how can this knowledge be used to inform and improve biocontrol strategies. The overall approach and links between the components of the project are shown in the flow diagram.

Commercial involvement

The Agriculture and Horticulture Development Board provided funding for the research under the PhD studentship code CP140. The research was conducted in collaboration between NIAB-EMR and Cranfield University. There are no direct commercial involvement in the project. The industry representative for the project was Richard Pett, JEPCO (Marketing) Ltd.



The overall project plan and the work carried out are displayed in the flow diagram above.

CHAPTER 1

LITERATURE REVIEW

1 Prospects For Biocontrol Of *Botrytis cinerea* On Strawberry And Lettuce

1.1 Economic importance

Botrytis cinerea infects around 200 plant species worldwide (Dean et al., 2012). The estimation of the costs of *Botrytis* damage is difficult to quantify because of its broad host range and the lack of reliable figures (Dean et al., 2012). Yet the global cost for chemical control of *Botrytis* is estimated to be around €38.16/ha per crop cycle (Steiger, 2007). At the postharvest stage 15 – 50 % of fruit and vegetables are lost to pathogenic decay (all decay causing pathogens) (FAO, 2011), with maximum losses occurring in developing countries due to the lack of essential technologies (Sharma et al., 2009). Crops susceptible to *B. cinerea* include strawberry and lettuce (Droby and Lichter, 2007).

Strawberry belongs to the *Rosaceae* family, is an economically important berry fruit globally, and consumed for its satisfying flavour and nutritious value (Padmanabhan et al., 2016). The current world leading producers of strawberries include China, USA, Mexico, Turkey and Spain (Padmanabhan et al., 2016). Strawberry is the most valuable fruit crop in the UK in terms of revenue (DEFRA, 2015). Strawberries were worth £244 million in 2014; and their production was increased by 14% in 2013 reaching a new high of 104 thousand tonnes (DEFRA, 2015). Strawberry development is dependent on the achene quantity and distribution on the surface

area of the receptacle tissue. Receptacle tissue growth is regulated by auxins produced in the achenes. Malformed fruit can occur due to damage to achenes through diseases that prevent auxin synthesis such as grey mould (Padmanabhan et al., 2016).

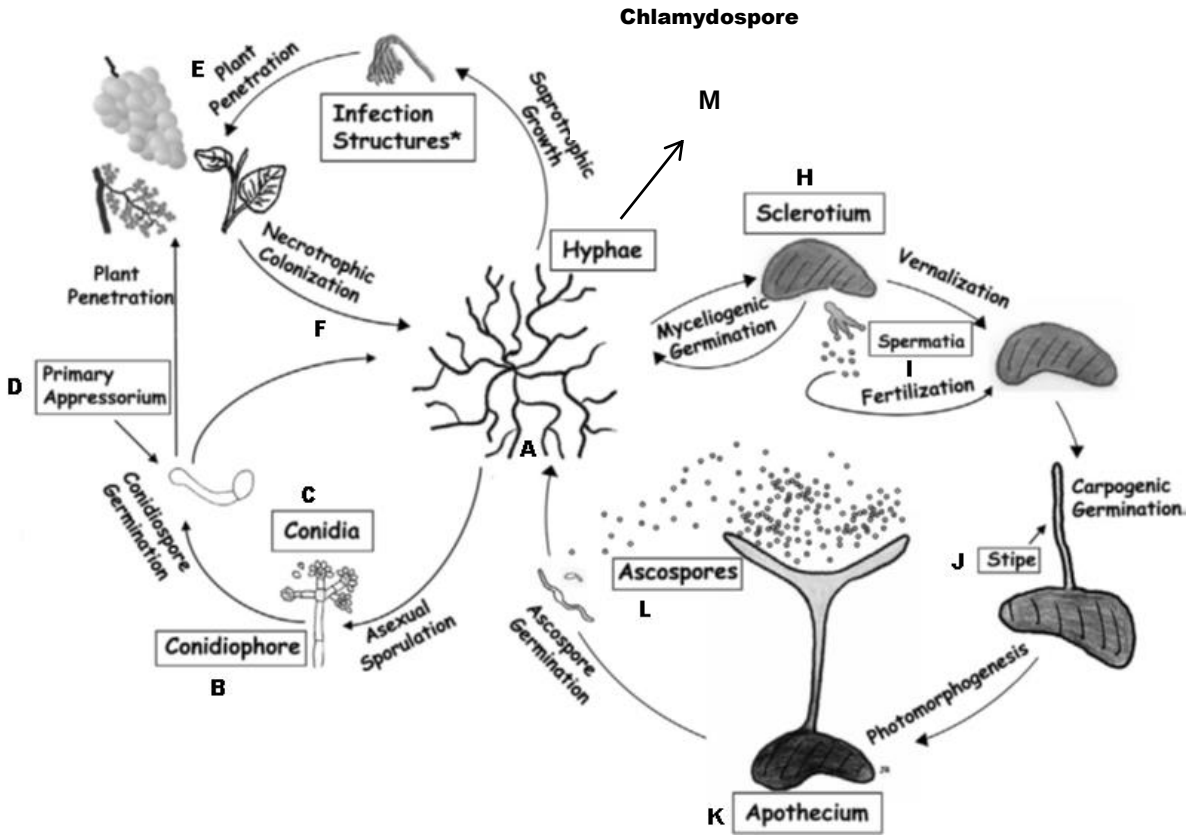
Lettuce is a globally grown crop: with one of the leading producers being the USA (Jackson et al., 1996). Since the popularity of bagged salads increased in the UK, lettuce production has become more economically important (DEFRA, 2015). Lettuce growth can become limited by pathogens such as *B. cinerea* (Snowdon, 1990). Lettuce production in the UK increased by 38% in 2014 (nearly 12 thousand tonnes) (DEFRA, 2015).

1.2 *Botrytis cinerea* life cycle

The genus *Botrytis* contains 22 species and one hybrid, and is entirely composed of plant pathogens. *B. cinerea* is an anamorph (asexual stage) of the teleomorph (sexual stage) *Botryotinia fuckeliana* (Staats et al., 2004). All species in the *Botrytis* genus are specialised pathogens on single plant species, except for *B. cinerea* (Staats et al., 2004). The basis for the previous classification is morphological characters as well as the host range and physiology. Recent classification of the genus *Botrytis* centres on DNA sequence of three nuclear protein coding genes (G3PDH, HSP60 and RPB2) (Amselem et al., 2011, Staats et al., 2004).

The hyphae of *B. cinerea* branch out in a septate manner (Fig 1.1 A), having a colour of hyaline-brown. Hyphae produce conidiophores (Fig 1.1 B) that are tall, slender and irregularly branched in the terminal portion, and have enlarged round pointed cells containing clusters of conidia moulded alongside short denticles (Fig 1.1 C). Conidia are ovoid shaped with a smooth surface; and have a mean length of 10 µm

and width of 5 µm. The colour of the conidia can be hyaline or grey (Barnett and Hunter, 1998). Conidiophore germination is the primary method via appresoria (Fig 1.1 D) for plant penetration (Fig 1.1 E). After the plant surface is penetrated the fungus further colonizes by necrotrophy (Fig 1.1 F). The fungus uses the nutrients available to form hyphae which follow a saprotrophic growth (Fig 1.1 G); in turn leaving infection structures (dead debris containing conidia) which can infect new plant tissue, and induce necrotrophic colonization to form hyphae. Sclerotia are survival structures formed by mycelial branches fusing together (Fig 1.1 H). Initially sclerotia are hyaline, but as melanic pigments become deposited into the outer rind, the sclerotia become brown or black (Williamson et al., 2007). Sclerotia are protected from UV radiation, desiccation and microbial attack with the build-up of a melanised coat and β-glucans which also cover the internal mycelia (Williamson et al., 2007). Within temperate mesophilic conditions, myceliogenic germination of sclerotia leads to the production of spermatia (Fig 1.1 I) and carpogenic germination forms the stipe (Fig 1.1 J) which leads to the elongation of the apothecium through photomorphogenesis (Fig 1.1 K), the apothecium releases ascospores (Fig 1.1 L) that germinate to form hyphae (Fig 1.1 A). In extreme conditions such as >30°C and <0°C (Romanazzi and Feliziani, 2014) chlamydospores are formed for short term survival. Chlamydospores are formed by transforming vegetative mycelium parts, released by hyphal disintegration (Fig 1.1 M) (Holz et al., 2007).



(Romanazzi and Feliziani, 2014).

Figure 1.1 Life cycle of *B. cinerea* including the different stages of sexual and asexual development

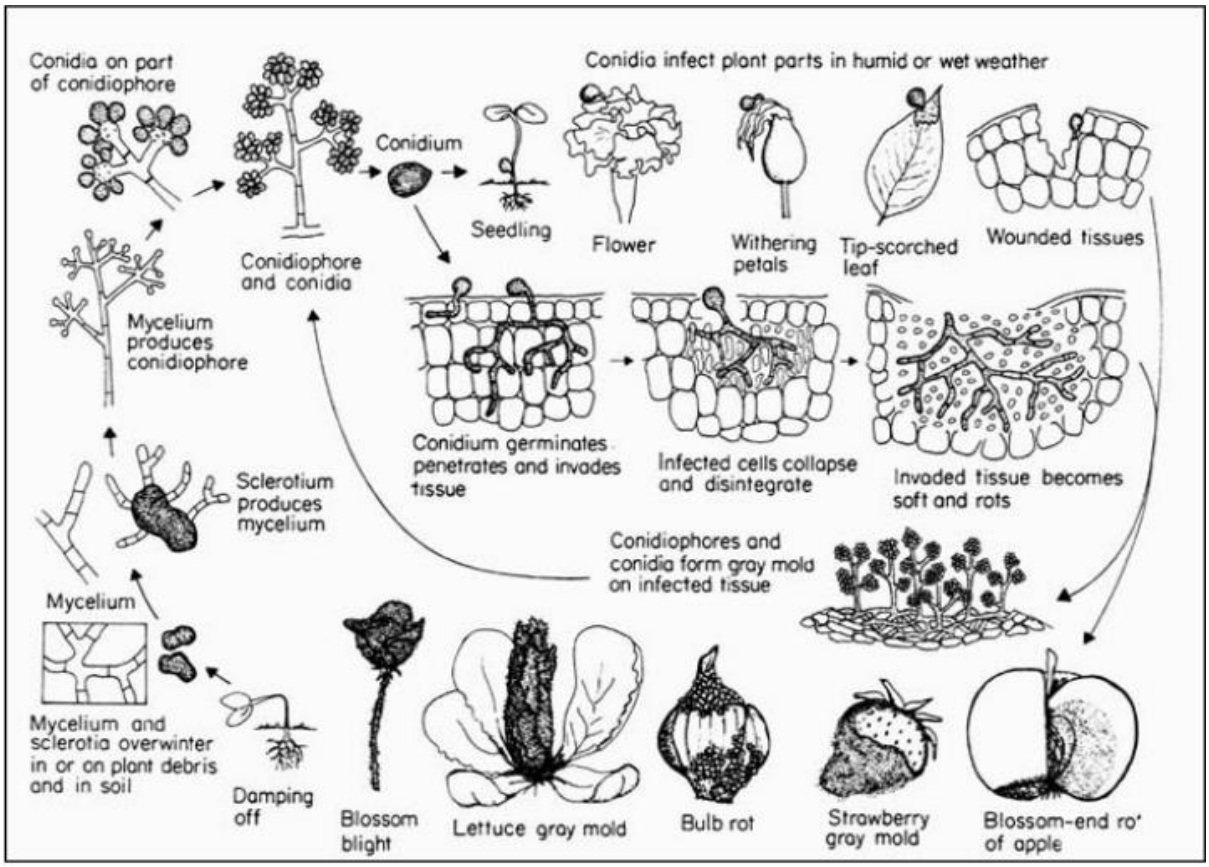
B. cinerea survives in several forms including: hyphae, mycelia, microconidia, macroconidia, sclerotia, apothecia, ascospores, and chlamydospores. Conidia which are large, complex and capable of plant penetration are termed macroconidia, while conidia that are smaller, predominantly involved in reproduction, and lack plant penetration ability are termed microconidia. Macroconidia and ascospores are the primary inoculum sources and disperse through environmental mechanisms such as wind and rain (Holz et al., 2007). The pathogen life cycle can consist of a somatic/vegetative/anamorph stage. Under unfavourable growth conditions

(comparatively in aging cultures or in the presence of other organisms) sclerotia are formed by myceliogenic germination. After sclerotial formation if conditions are favourable (mesothermal) fertilization occurs, and sclerotia can germinate to produce apothecia (teleomorph stage) instead of mycelia and conidia (Beever and Weeds, 2007). Asexual reproduction of hyphae forms conidia. Whereas sexual reproduction occurs after asexual reproduction, as crosses between large quantities of microconidia form sexual bodies (Fukumori et al., 2004). As isolates of *B. cinerea* are heterothallic, sexual reproduction and meiotic recombination are key sources of genetic variation in pathogenicity (Holz et al., 2007).

The telemorph or the sexual stage of *B. cinerea* is *Botryotinia fuckeliana*, and is characterized by the production of one to several apothecia that arise from sclerotia (Holz et al., 2007). Sclerotia are initially whitish then become black and lenticular, and are 2-7 mm in length and width, with a thickness of 1 – 3 mm (Holz et al., 2007). Apothecia appear flat and hollow and are shaped cupulate to plane, with a colour of pale brown having a 1.5 – 3 mm diameter with a stipe of 3 – 15 mm × 0.5 – 1 mm. Mature apothecium fruiting surface (hymenium) exposes cylindrical asci 100 – 150 µm × 6 – 10 µm containing unicellular and hyaline ascospores 6 – 10 µm × 4 – 6 µm (Holz et al., 2007).

Within temperate climates such as the UK the primary source of inoculum on crops are sclerotia, and macroconidia. Within winter and other unfavourable conditions sclerotia and mycelia survive among crop debris (infected dead host tissue). In crops, dead flowers, fruit, and leaves contain masses of mycelia and conidia (Williamson et al., 2007). *B. cinerea* growth requires a minimum temperature of 0 °C and, a maximum of 30 °C, and has an optimum of 20 °C, and grows well under humid conditions (Romanazzi and Feliziani, 2014). Such growth conditions allow this

pathogen to become a real problem in protected crops (Romanazzi and Feliziani, 2014). The development of grey mould disease in different crops can be viewed in Fig 1.2.



(Agrios, 2004)

Figure 1.2 Development of grey mould disease on different crops

1.3 Infection of strawberry crops by *B. cinerea*

1.3.1 Dispersal, infection and symptoms

In the UK (temperate regions) by early spring an abundance of inoculum is present in the environment. The predominant sources of inoculum for strawberries are over-wintering macroconidia on dead leaves (Braun and Sutton, 1987, Bristow et al., 1986, Sutton, 1990, Williamson, 1994). Dispersion primarily occurs by wind and rain

in fields and in protected crops (Aylor, 1990). On petals within temperate, humid and wet conditions, infections induce abundant sporulation. On developing fruitlets, inoculum loads of high as 100-fold occur on fruitlets with attached petals in comparison to fruitlets where petals had abscised (Elmer et al., 1995). Conidia in certain cases are regarded as “short lived propagules”: in direct sunlight (Rotem and Aust, 1991), extreme temperatures and dryness (Yunis and Elad, 1989), but on fruit surfaces long term survival of *B. cinerea* conidia is recognised (Spotts, 1985, Walter et al., 1998). *B. cinerea* conidia survived 16 weeks in the field on kiwifruits when applied early in the growing season (Walter et al., 1998).

The infection processes in strawberry plants can be summarised as: macroconidia of *B. cinerea* lands on strawberry flowers, leaves and setting fruit and initiates infection through necrotrophy where the pathogen co-opts programmed cell death pathways in the host (Sutton, 1990). Mummified fruits, dead leaves, straw mulch and neighbouring infected crops serve as the primary inoculum for infection. Diseased strawberry fruits are covered with grey tuft (Fig 1.3), which contains mycelium conidiophores and conidia of the pathogen (Bristow et al., 1986, Sutton, 1990, Williamson, 1994).

(Romanazzi et al., 2016)

Figure 1.3 Grey mould infection on a strawberry fruit

Strawberry plants can be infected on contact, with the primary infective unit being conidia of *B. cinerea* formed on host tissues, mycelium, and sclerotia residing on the surface of the soil (Sutton, 1990). In Canada during early spring even newly emerged and partially expanded green strawberry leaves are susceptible. The infection remains latent until the onset of leaf necrosis (Sutton, 1990). Assessments from a wide range of fruit growing regions ranked conidia of *B. cinerea* as a significant part of the aerial microflora (Bisiach et al., 1984). *B. cinerea* in the soil only represents a small proportion of the fungal biomass (Dorado et al., 2001).

Minor brown lesions under the calyx are indicators of infection. Resumption of infection activity within the lesion areas induces sporulation, and spore structures that form under the plant's calyx are small stalks containing clusters of spores at the tip, this process develops the lesion size rapidly (Sutton, 1990). On infected berries a velvety grey-brown coat of mycelium and then spores form. Rotted regions are initially soft and mushy and become dry and leathery in non-humid conditions. Millions of spores are formed on each infected berry. The rotting stage is commonly known as grey mould disease due to the development of "grey fuzz" (Bristow et al., 1986, Sutton, 1990).

1.3.2 Flower infection pathway

Initial studies in strawberries suggested senescent flower parts are important sources of pathogen inoculum for the subsequent spread of infection into ripening fruits (Boff et al., 2003, Powelson, 1960). Flowering is fundamental for *B. cinerea's* ecology and epidemiology; epidemiological studies have shown correlations between the colonisation of senescent floral tissue in the spring with the severity of grey mould disease at harvest (Elmer and Michailides, 2007). Flower infections can result in latent infections of immature fruit (Williamson, 1994). When strawberry plant

flowers senesce they become highly susceptible to infection by macroconidia (Jersch et al., 1989). Conidia initiate infection on styler tissues, which follows a slow systemic hyphal growth into the ovule, and then the pathogen enters a latent phase (Williamson, 1994). Conidial infection of stamens and or petals is followed by systemic hyphal growth through floral tissues to the receptacle and results in latent infections (Pezet and Pont, 1990). A sole botryticide application of fenhexamid at anthesis controlled botrytis fruit rot in strawberries (Mertely et al., 2002). Such studies indicate the importance of flowering infection. Yet multiple infection pathways take place, and conidial infections of ripening berries later within the growing season are as important as latent infections of green structural tissues earlier in the growing season (Wilcox, 2002).

1.3.3 Fruit infection pathway

Systemic infection and the growth of *Botrytis* through filaments of infected stamens into the receptacle to produce a symptomless infection are suggested to be the main fruit infection pathway (Bristow et al., 1986). When strawberries are ripening there is abundant inoculum for both canopy and fruit infections (Keller et al., 2003). Inoculum is dispersed between flowers and ripened fruits to pedicels, laterals and immature fruit surfaces (Holz et al., 2003). Initially fruit surfaces can also be contaminated by conidia through active sporulation on aborted fruitlets and calyptras. Conidia residing on the receptacle/cap scar zone showed a resting state, as opposed to a latent infection. Conidia trapped at the base of the fruit are at an ideal position to infect the fruit in favourable conditions (Sarig et al., 1996). Fruit pedicels are susceptible to infection at the early stage of berry development (Dashwood and Fox, 1988), but the fruit pedicel increases in resistance as the season progresses (Holz, 1999).

The “classic” preharvest polycyclic epidemic starts as host defences deteriorate and symptoms become prevalent, and ultimately a new cluster of conidia are dispersed to new infection sites (Keller et al., 2003). After flowering in dry conditions *B. cinerea* starts a resting over-summering stage in the canopy. During the growing season wet periods result in the production of abundant conidia, which disperses into the canopy contaminating the outer hairy surface of the fruit without inducing infection. At harvest the conidia covering the fruit surface re-disperses to the picking wound. Accumulation of conidia on the fruit surface occurs early in the growing season due to infections on floral tissues (Elmer et al., 1995). *B. cinerea* initiates a saprophytic phase on the fruit, and during favourable conditions large quantities of conidia are produced, resulting in the contamination of external surfaces of ripening fruits. The leakage of exudates from matured fruit provides stimuli for conidial fruit infection (Holz et al., 2003).

1.4 Infection of lettuce crops by *B. cinerea*

1.4.1 Dispersal, infection and symptoms

Conidia are dispersed by wind, air movement, agitation and water splash. Dry conditions inhibit the spread of the disease as non-humid conditions do not favour germination of conidia (Fiume and Fiume, 2005). Limited information is available on how *B. cinerea* infects lettuce plants; this is especially the situation for the infection pathways. *B. cinerea’s* conidial anamorphic state or asexual stage ensues on lettuce leaves, where firm and cylindrical conidiophores form (Fig 1.4). Extensive use of transplants assists in *B. cinerea* infection levels, and causes significant losses. The adaptability of *B. cinerea* is demonstrated with the conditions required for infection. Conidia germinate at a relative humidity of > 90 % and in temperatures between 1 to

30 °C. The optimal temperature for growth is between 18 to 20 °C. Fog, rain, high humidity periods, and moderate temperatures, favour disease development (Fiume and Fiume, 2005).

The first documented case of infection by *B. cinerea* on lettuce was on the roots (MacNeill, 1953). The leaves remained unaffected with no morphological changes, while there was an abundance of the pathogen on the roots. The natural etiology of lettuce infection is unclear. *B. cinerea* is regularly isolated from lettuce seeds both before and after surface sterilization (Sowley et al., 2010). Lettuce plants with no previous infection by *B. cinerea* were successfully inoculated, while lettuce plants with previous infections could not be further inoculated (Shafia, 2009), suggesting infections are caused by redistribution of spores on the surface of the plant. Both in *Asteraceae* and other families symptomless infections by *B. cinerea* are widespread in the roots and upper parts of wild species (Gange et al., 2007, Shafia, 2009). Seed-borne and airborne infection of lettuce seedlings by *B. cinerea* cause long-lived systemic endophyte-like infections in all tissues of the plant (Gange et al., 2007, Shafia, 2009, Sowley et al., 2010), similar to endophytic infections established in other *steracae* by other fungi (Gange et al., 2007), and the *B. cinerea* infections previously found in Primula × polyantha (Barnes and Shaw, 2002).

The effects are specifically evident in romaine cultivars as they are very susceptible to the disease. On lettuce plants grey mould disease symptoms can also appear on leaves (Jarvis, 1980, Maude, 1980). Initial symptoms of grey mould disease of lettuce plants embed water-soaked brownish grey rot, which ensues on the plants leaves and stems that mechanically become damaged through the general practice of handling and planting (Fiume and Fiume, 2005). Damaged wet plant tissues and leaves provide humid conditions, therefore when in contact with the soil they can be

infected by conidia on the soil surface. *B. cinerea* progresses from infected leaves and stems into the healthy crown of the lettuce plants, and decays the main stem, causing death overtime. A dense bushy grey growth of mycelia covers the diseased area (Fiume and Fiume, 2005).

1.4.2 Systemic infection

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 (Shafia, 2009, Sowley et al., 2010). Lettuce is the second documented host with systemic, symptomless progression of *B. cinerea*, alongside the commercial hybrid Primula (Barnes and Shaw, 2002). Systemic symptomless progressive infections are known in many fungi and pathogens including *Leptosphaeria maculans*, *Cephalosporium graminearum* and smuts (Kan and Al, 2006).

B. cinerea invades lettuce seedlings in the seed-bed or in the field after direct seeding. At or close to the soil line seedlings collapse from a soft, tan-to-brown water soaked rot of the stem. Dampening of seedlings by *B. cinerea* occurs mainly in humid cold frames, as well as in the field especially when seedlings are contaminated with sclerotia and mycelium, or if *B. cinerea* is present in the soil, living on marcescent lettuce tissues (Fiume and Fiume, 2005). Active mycelium in dead plant tissue and sclerotia transfer pathogenesis in the field season to season and in greenhouses to each cropping. Mycelia from scleroita can infect lettuce plants through senescence of lower leaves and root tissue close to the soil surface (Fiume and Fiume, 2005). Endophytic colonization of *B. cinerea* can be confirmed via the regular and patterned resumption of the fungus from excised healthy tissue. As the plant grows the infection spreads to newly formed tissues (Sowley et al., 2010).

Hyphae are present in the cortical tissue of roots, and throughout the stems. When the seed is contaminated with *B. cinerea*, seedling blight of lettuce occurs and progresses onto the germinating seedlings, ultimately advancing polycyclically in the developing crop (Burgess et al., 1997, Harold et al., 1997).

1.4.3 Necrotrophic infection

In greenhouses lettuce infected with *B. cinerea* is a significant problem. Once conidia have infected the lettuce plant (usually the leaf Fig 1.4), the disease develops upwards throughout the head until all inner leaves are altered into a slimy mass (Sherf and MacNab, 1986, Shim et al., 2014). The pathogen rots the stem and leaves at the centre before reaching the margin of the outer leaves, in turn collapsing the plant before any visual confirmation of rot from the outside. After thorough rotting of the lettuce head sclerotia form throughout the decayed mass. An ashen-grey layer of conidia spores form just above the surface of diseased area. The layers of conidia spores are distinct on the undersides of leaves, and other plant tissues which retain moisture. The lettuce plant dries and withers after collapsing (Sherf and MacNab, 1986, Shim et al., 2014).

(Shim et al., 2014)

Figure 1.4 Infection of lettuce by *B. cinerea* showing characteristic lesion formation

1.5 Factors predisposing host tissue to *B. cinerea* infection

Several factors predispose host tissues to *B. cinerea* infection, including cuticle integrity, contact of insects, invertebrates and vectors carrying *B. cinerea* inoculum, and plant nutrition. In terms of cuticle integrity the structure and thickness of the cuticle and the epidermal layers are regarded as main factors of resistance against *B. cinerea* (Serrano et al., 2015). In general the stomata number and or natural openings are independent of susceptibility to *B. cinerea* (Bernard and Dallas, 1981, Pucheu-Plante and Mercier, 1983). But, another study suggested the quantity of stomata within the berry epidermis was negatively correlated with resistance, while the quantity and thickness of epidermal and hypodermal cell layers, cuticle and wax contents were positively correlated with resistance (Gabler et al., 2003). In some cases insects, invertebrates and vectors can aid in *B. cinerea* infection by carrying inoculum and damaging fruit. For example, in the UK, strawberry berries can be damaged by, but not limited to, avians, small rodents and slugs (Eskelson et al., 2011), as these pests can cause large mechanical wounds on the fruit, which can be a direct entry site for conidia. The effects of specific nutritional ions on host susceptibility and progression of *B. cinerea* epidemics have been reported (Goodman et al., 1986, Jarvis, 1980). For strawberries variable results of calcium uptake are reported, but increasing the fruit calcium content reduced *B. cinerea* (Cheour et al., 1990).

1.6 Current control methods of *Botrytis cinerea* in strawberries and lettuce

In order to control a pathogen it is essential to understand its life cycle. The life cycle in plant pathology includes a disease-causing biotic agent which interacts with a susceptible host, and conditions which favour disease development (Fig 1.2).

Disease management strategies eliminate one of these factors. The fungal pathogen has pathogenicity factors (cell wall degrading enzymes) which colonize and consume plant tissues, while counteracting host resistance mechanisms e.g. phytoalexins, and the potential to survive, reproduce and disperse in unfavourable conditions (Staats et al., 2004).

1.6.1 Chemical control

Commonly used fungicides against *B. cinerea* include maneb, thiram, dichlofluanid, carbendazim, fludioxonil and pyrimethanil. From these pesticides maneb, thiram in formulations with benomyl and carbofuran, and carbendazim are listed as banned pesticides (UTZ, 2015). Chemical control can also be used to treat seeds, bulbs, heads and fruit. Development of resistance towards chemical control in *B. cinerea*, and the negative impact on health and environment (Bernhardt et al., 2017) as well as the negative perception of the public to pesticides have hindered this approach. Regulatory authorities especially in Europe have limited the use of current and new pesticides; applications of each chemical family are generally restricted to a single use per cropping season to avoid maximum residual level values (Elad et al., 2004). Fungicides provide excellent control of some diseases, but for others they can be ineffective, unavailable and may induce a gradual increase of fungicide resistance (Elad et al., 2004). Fungicide resistance can potentially arise rapidly to an extent where disease control is lost. In other cases fungicide resistance can be a gradual process, resulting in partial loss of control (Elad et al., 2004).

To effectively manage strawberry fruit rot after harvest there is a requirement of regular preharvest application of botryticides during peak flowering periods since flowers are highly susceptible to *B. cinerea* infection by macroconidia (Jersch et al., 1989). Aerial parts of the plants are sprayed with fungicides (Elad et al., 2004).

Independent research shows weekly applications of captan and thiram are effective in reducing the grey mould incidences both preharvest and postharvest (Blacharski et al., 2001). Additionally Fenhexamid (750 g/ha), applied as a programme of treatments from early flowering demonstrated adequate control of *B. cinerea* in the UK (Duben et al., 2001). Currently in the UK partial and or complete resistance of *B. cinerea* to fungicides is an ongoing problem, and has been reported in Benzimidazole, Dicarboximide, Anilo-pyrimidine and Phenylsulphamide (DEFRA, 2009). Detailed information on *B. cinerea* resistance to chemical methods can be found (Fillinger and Walker, 2016). Chemical control is the main method of managing *B. cinerea* on plants.

1.6.2 Cultural practices on strawberry crops to control grey mould

In order to minimize the infection probability, environmental conditions can be modified. Modification examples include selecting growing areas not suitable for grey mould development. Suitable cultivation sites are dry and warm, and should be absent of high humidity and stagnant air, especially in the fruit zone (Padmanabhan et al., 2016). Humidity in the canopy can be reduced by the removal of dead debris and canopy management (Mari et al., 2007, Mari et al., 2009, Padmanabhan et al., 2016). The following procedures are required to reduce *B. cinerea* infection on strawberry plants; planting in an area with good air circulation, appropriate heating and ventilation (in greenhouses), excellent sanitation procedures including but not limited to the removal of dead/dying debris from the soil surface, removing ripened and rain damaged fruit, avoiding berry soil contact, and picking fruit at the right maturity to avoid over ripening (Mari et al., 2007, Mari et al., 2009, Padmanabhan et al., 2016). Careful handling of fruit reduces fruit damage and therefore reduces the incidence of grey mould on strawberry fruit (Mari et al., 2009).

1.6.3 Cultural practices on lettuce crops to control *B. cinerea* infection

In regards to the management and control of grey mould in lettuce a number of management strategies can be employed to minimize losses. Accurate sanitation after each lettuce growing cycle through removing any plant debris, providing appropriate ventilation, spacing the plants, and reducing humidity. Avoiding excessive watering and sprinkler irrigation systems which wet the top of lettuce beds is crucial. Avoiding extensive nitrogen feeding encourages the rotting of these fungal propagules e.g. macroconidia, and deep plowing buries sclerotia which decreases their potential to germinate and cause infection. The removal and burning of infected plants from the lettuce field can reduce further infections, and the secondary inoculum potential. Weeding the fields and employing crop rotations with resistant crops such as corn can also limit *B. cinerea* infections (Fiume and Fiume, 2005, Peruzzi, 2000). *B. cinerea* requires > 90 % relative humidity to germinate and infect lettuce plant tissues, and thus not allowing the humidity to reach that level is key to stopping germination (Fiume and Fiume, 2005, Peruzzi, 2000).

1.6.4 Alternative technologies for controlling *B. cinerea* infection

B. cinerea infection and grey mould development can also be reduced by alternative management methods including but not limited to applying, organic and inorganic salts, natural antifungals, disinfectant agents and physical methods. In organic culture using inorganic and organic salts pre-harvest has gained popularity. The use of calcium chloride at preharvest stages can be effective against grey mould development, but can modify the maturity of the berry, and leave a distinct residual, in turn harming its marketability (Romanazzi et al., 2016). For instance in southern Italy it is a successful treatment preharvest to control postharvest decay on table grapes (Romanazzi et al., 2012). Disinfectant agents (acetic acid, ethanol,

electrolyzed oxidizing water) can be added into the water used for crop surface sterilization, especially when the process of washing is included in packaging. Disinfectant agents have successfully controlled decay on table grapes, as well as the use of electrolyzed water in packing houses in decreasing conidia contamination of *B. cinerea* (Romanazzi et al., 2016). Example includes the use of acetic acid (Sholberg et al., 1996) and ethanol (Gabler et al., 2005) to control post-harvest decay on table grapes. Physical methods (UV-C irradiation, ozone, conservation agriculture, hypobaric and hyperbaric treatments) can control grey mould development; and the benefits embed the lack of direct contact with the fruit and vegetable (Romanazzi et al., 2016). The control by physical methods remains as long as the procedure is repeated constantly. Physical means have been demonstrated to control grey mould on table grapes (Romanazzi et al., 2012, Sanzani et al., 2009).

1.7 Biocontrol and current products

“Biological control is the use of parasitoid, predator, pathogen, antagonist, competitor populations to suppress a pest population making it less abundant and thus less damaging than it would otherwise be” (Van Driesche and Bellows, 1996). Biocontrol agents (bacteria, yeast and fungi) are gaining popularity to control diseases on crops due to their eco-friendly nature (Korsten, 2006, Mari et al., 2007).

Organisms that naturally survive in the plants phyllosphere and/or endosphere can be potentially used for the development of effective BCAs. The first documented BCA that controlled *B. cinerea* on strawberries was a *Trichoderma* species (Tronsmo and Dennis, 1977). A classical study showed *Bacillus subtilis* can control the brown rot pathogens of stone fruits (Pusey and Wilson, 1984). *B. subtilis* was shown to

control *B. cinerea* infection and grey mould development on strawberries (Zhao et al., 2007).

Agrochemical companies are interested in developing antagonists into commercial bio-products. Current global products against *B. cinerea*, that have been patented, and are being used commercially include ASPIRE (*Candida oleophila*), YieldPlus (*Pichia anomala* J121), and BIOSAVE-110 (*Pseudomonas syringae*). The current prominent bio-products in the UK are Serenade (*Bacillus subtilis* QST 713) and Prestop (*Gliocladium catenulatum* J1446).

1.7.1 *Bacillus subtilis* strain QST 713 (Serenade)

1.7.1.1 General information

Bacillus subtilis is a mesophilic, gram positive, catalase positive bacterium found in but not limited to soils, hay and grass. The cells are round and smooth, and the endospores are rod shaped and have a length of 4 µm – 10 µm and a diameter of 0.25 µm – 1.0 µm. *B. subtilis* produces endospores to survive extreme conditions. The organism is a facultative anaerobe. The cell and the endospore are extensively flagellated which allows improved mobility in liquids. *B. subtilis* cell wall is rigid, and composed of peptidoglycan (Schaechter et al., 2006).

The life cycle of *B. subtilis* is formed of three different physiological phases: vegetative growth, sporulation, and germination. The transition in the mode of development is regulated by nutrient availability, detected by the bacterium (Moir, 2006, Rosenberg et al., 2012). Many signalling pathways convey information on growth rate and nutrition directly to the cell cycle machinery, which allows cells to constantly sample their environment and adjust the cell cycle process accordingly (Wang and Levin, 2009).

Vegetative growth is symmetric and characterized through binary fission cell growth, which occurs when nutrients are abundant. Chromosomal replication is directly associated with the vegetative cell division cycle (Wang and Levin, 2009). Under certain conditions the whole separation of sister cells do not occur, and thus cells remain linked together due to multiple rounds of binary fission, resulting in the formation of long chains (Chai et al., 2010). During vegetative growth transcriptional variability among different bacterial cells is strictly associated with nutrient availability (Rosenberg et al., 2012).

Environmental cues such as nutrient deprivation, high mineral compositions, pH, temperature, and high cell density induces differentiation of vegetative cells into endospores. The increase in cellular mass is associated with the accumulation of secreted peptides, which are sensed via cell surface receptors that stimulate the sequential activation of the master regulator SpoA. This phosphorelay activation involves the transfer of phosphate groups from ATP by the use of histidine kinases and two intermediate proteins Spo0F and Spo0B, to the transcription factor Spo0A. Spo0A-P controls expression of a multitude of genes, initiating a chain of events that take several hours to complete and finalises in the release of mature spores from its mother cell compartment (Molle et al., 2003, Veening et al., 2009). The choice to sporulate is stochastic (Chastanet et al., 2010).

Spores remain dormant for a prolonged time, and retain outstanding resistance to environmental damage such as heat, radiation, toxic chemicals and extreme pH. Under favourable (temperate and mesophilic) environments spores break dormancy and restart growth through germination and outgrowth. Outgrowth occurs when a germinated spore transforms into a growing cell (Setlow, 2003).

1.7.1.2 Current research

B. subtilis can control *Fusarium* wilt of chickpea (Hervas et al., 1998, Abed et al., 2016), *Pythium torulosum* damping-off of tomato (Jacobsen et al., 2004), Cercospora leaf spot of sugarbeet (Arzanlou et al., 2016, Jacobsen et al., 2004), and *Ralstonia* wilt disease in several plants (Aliye et al., 2008, Ji et al., 2008, Lemessa and Zeller, 2007). *Bacillus* based biocontrol was superior on more resistant cultivars. *B. subtilis* synthesizes a variety of biologically active compounds with a broad range of activity against phytopathogens (Shafi et al., 2017), and are capable of inducing host systemic resistance (Aliye et al., 2008, Bais et al., 2004, Butcher et al., 2007), and have the potential of forming multicellular structures and biofilms (Bais et al., 2004, Branda et al., 2001). These traits increase the value of *B. subtilis* as a BCA, and the strain QST 713 has been formulated into a commercial product labelled Serenade.

Competition for iron and nutrients through siderophore synthesis is recognised as a significant antagonistic trait in *B. subtilis* (de Boer et al., 2003, Saha et al., 2016). Antifungal lipo-peptides of *B. subtilis* are strong inhibitors of pathogenic fungi (Knox et al., 2000) and antifungal lipo-peptides of *B. subtilis* can suppress fungi (Priest, 1993). Lytic enzymes (chitinase, protease, β-1,3-glucanase) have antifungal properties due to their ability to degrade fungal cell walls (Boller et al., 1983). The production of antifungal compounds is suggested to be the primary mode of action of *B. subtilis* (Toral et al., 2018). *B. subtilis* synthesized lipo-peptides function through their implication on plant tissue colonization, direct antagonism of phytopathogens, and inducing plant resistance (Ongena et al., 2007, Toral et al., 2018). Fengycin and surfactin type lipo-peptides are suggested to act as inducers of plant defences, as they are suggested to interact with plant cells as bacterial determinants to activate the immune response via the phenomenon known as stimulation of the induced

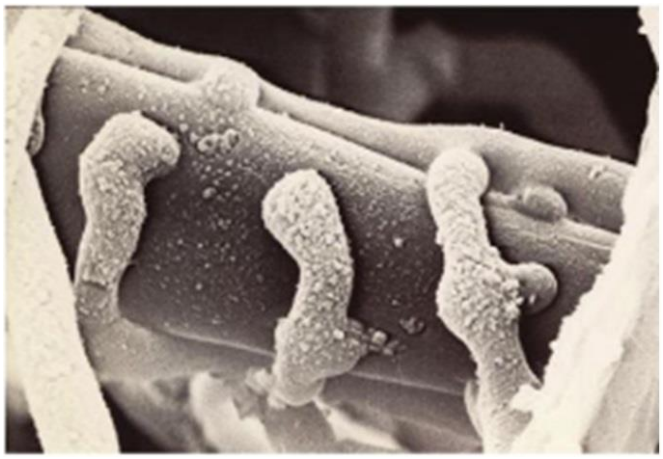
systemic resistance (Ongena et al., 2007). In bean and tomato plants the role of surfactins and fengycins in plant defence induction was demonstrated by the similar protective activities of the purified compounds, in comparison to the activity directly from *B. subtilis* strains synthesizing the compounds (Ongena et al., 2007). Iturin and fengycin peptides produced by *B. subtilis* are involved in antibiosis of several pathogens in different plant species. Iturin A synthesized by *B. subtilis* RB14 can control damping-off of tomato caused by *Rhizoctonia solania* (Asaka and Shoda, 1996). Moreover to control phyllosphere diseases input of both iturins and fengycins are required in antagonising *Podosphaera fusca* infecting melon leaves (Romero et al., 2007).

The constant release of various low molecular weight compounds and macromolecules from plant roots produce a nutritionally and physico-chemically specific environment for microbiota growing in the rhizosphere (Bais et al., 2006). Certain released molecules act as chemical signals for motile bacteria such as *B. subtilis* to move onto the root surface through chemotaxis. In the rhizosphere the BCA can utilize the root oozed products for persistence and growth (de Weert et al., 2002). Surfactins are involved in pellicle formation at the air liquid interface of roots (Hofemeister et al., 2004, Kinsinger et al., 2003). When *B. subtilis* cells establish at the phytosphere, they can release their antibiotic arsenal. The production of surfactins is required for root colonization (Fan et al., 2017), but also for reducing the infections caused by bacterium pathogens such as *Pseudomonas syringae* on *Arabidopsis* plants (Bais et al., 2004).

1.7.2 *Gliocladium catenulatum* strain J1446 (Prestop)

1.7.2.1 General information

Gliocladium species are commonly found as saprophytes in many environments, and are pathogenic against numerous fungi including *B. cinerea* (Helyer et al., 2014). *Gliocladium* has been isolated from plant debris and organic matter around the world. Species that are potent BCAs and registered as biofungicides include *G. catenulatum* strain J1446 and *G. virens* strain GL-21. Conidia can be moved in soil, organic matter, and by soil dwelling insects and mites, but is dispersed by wind and water (Helyer et al., 2014). Fine hair like hyphae are produced from germinated spores, which can contact a plant pathogen (Helyer et al., 2014). Commercial formulations can contain up to 2×10^8 cfus/g (colony forming units per gram). *G. catenulatum* hyphae eliminate host fungi by enzymatic activity without the need of penetration and parasitism (Helyer et al., 2014). The BCAs hyphae coil around the pathogen hyphae. This *G. catenulatum* strain also develops a close relationship with healthy roots, ultimately colonizing and protecting them against pathogen attack (Fig 1.5).



(Helyer et al., 2014)

Figure 1.5 *G. catenulatum* coiling around a plant root in a beneficial manner to protect against pathogen attack

Colonization of the root zone appears to be an important characteristic in *G. catenulatum* ecology. But true symbiotic relationships with its associated organisms have not been studied yet. *G. catenulatum* activity occurs around 5 °C – 34 °C with optimal activity at 15 °C – 25 °C, temperatures >42 °C being detrimental to the fungal mycelium and spores (Helyer et al., 2014). Optimal pH is 5 – 6, but the fungus can cope and grow between pH 3 - 8.2 (Tahvonen et al., 1999). The commercial strain of *G. catenulatum* J1146 is used as a biofungicide to control diseases on amenity turf, herbs, ornamentals, tree and shrub seedlings, and vegetables. The main targets of *G. catenulatum* are plant pathogens involved in damping-off diseases and foliar stem diseases (Helyer et al., 2014).

Colonies plated on malt extract agar spread broadly and grow in ten days to a size of 2.5 cm – 3.5 cm in diameter at 20 °C (Tahvonen et al., 1999). The colonies are woolly, whitish, and the conidial areas in the centre turn green (pale olive - grey green) when the culture is 7 – 10 days old, whereas in older cultures the conidial areas are dark green. The reverse of the cultures can be colourless, pinkish and yellowish, due to the pigments in the mycelium. The BCA produces transparent, slimy septate hyphae. Conidiophores are upright and branch repeatedly at their apices; the terminal branches give rise to phialides (Helyer et al., 2014). The *G. catenulatum* primary conidiophores are verticillium-like, and the secondary conidiophores are penicillium-like. Conidiophores are usually irregularly branched. The size of phialides of penicillate conidiophores in the basal area is 12 µm – 17 µm long and approximately 2.5 µm wide. Primary conidiophores appear warted when observed directly, but are smooth in liquid mounts (Tahvonen et al., 1999, EPA, 2002). Conidia of *G. catenulatum* are green, ellipsoid, bilaterally symmetrical and contain an oblique scar of attachment, and have a size of 5.0 µm – 6.5 µm × 2.5 µm

– 3.0 µm. In secondary conidiophores, conidia can remain attached to each other and form a long chain. Chlamydospores are intercalate at the terminal, and have a diameter of 7-10 µm (Tahvonen et al., 1999, EPA, 2002).

1.7.2.2 Current research

G. catenulatum has a broad spectrum of activity against plant pathogens (Lahdenpera and Korteniemi, 2005, McQuilken et al., 2001, Parikka et al., 2016, Mahmoud, 2016). A study on biological control strategies in *Fusarium* rot and stem rot of cucumbers showed under semi-commercial growth conditions *G. catenulatum* reduced *Fusarium* root and stem rot, and *Pythium* root rot of cucumbers when applied before pathogen inoculation (Punja and Yip, 2003, Rose et al., 2003). *G. catenulatum* lived on cucumber roots for a minimum of 50 days when applied to rockwool blocks at seeding at concentrations above 1×10^{10} cfu/g root fresh weight (Chatterton et al., 2008).

After *G. catenulatum* application within 7 days hyphae extensively colonize cucumber roots and produce a dense network over the root surface and internally in root epidermal cells. The application of *G. catenulatum* onto rockwool blocks before inoculation with *Fusarium oxysporum* resulted in a significant decrease in pathogen levels on the roots and crown, in comparison to plants inoculated only with *Fusarium oxysporum* (Punja and Yip, 2003). Additionally on ornamental beddings *G. catenulatum* applications controlled *Pythium ultimum* and *Rhizoctonia solani* caused diseases (McQuilken et al., 2001) as well as damping-off on ginseng seedlings (Rahman and Punja, 2007). *G. catenulatum* has also demonstrated its efficacy in decreasing anthracnose development on blueberry blossoms and developing fruit, caused by *Colletotrichum acutatum* (Verma et al., 2006) as well as suppressing sporulation of *Botrytis spp.* on deceased onion leaves (Köhl et al., 1995). Recently

Gliocladium species have been found with antagonistic effects against grey mould disease of tomato (Khonglah and Kayang, 2018). Overall, studies suggests *G. catenulatum* has activity towards both rhizosphere and phyllosphere-infecting pathogens (Singh et al., 2018). The BCA also secretes cell wall degrading enzymes which can degrade *F. oxysporum* hyphae, for instance β-1,3-glucanase activity was identified on colonized cucumber roots (Chatterton and Punja, 2009). The root colonisation ability coupled with mycoparasitism appears to be important for *G. catenulatum* efficacy.

G. catenulatum mechanisms of actions involved in controlling pathogens are uncertain, but progress has been made (Nicot et al., 2016). The BCA is rhizosphere competent and can endophytically colonize roots and stems of cucumber plants (Chatterton et al., 2008). The parasitism of *G. catenulatum* has been observed against *R. solani*, *P. ultimum*, *B. cinerea* an *S. sclerotiorum* (Huang, 1978, McQuilken et al., 2001). Microscopic analysis demonstrated the BCA coils loosely around hyphae of *P. ultimum* and *R. solani*, causing partial destruction, and eliminates the hyphal cell (Huang, 1978, McQuilken et al., 2001).

The dissolution of pathogen cell walls by *G. catenulatum* probably involves enzymatic hydrolysis (Lahdenpera and Korteniemi, 2005). The Per3 gene which encodes a perilipin-like protein is upregulated in the presence of scleroita (Sun et al., 2015), and the production of β-1,3-glucanase is involved in fungi cell wall degradation (Chatterton and Punja, 2009). Another isolate of *G. catenulatum* demonstrated the ability of coiling around and penetrating the hyphae of *R. solani* which granulated and disintegrated its cell (Turhan, 1990). *G. catenulatum* coils around *B. cinerea* hyphae (Simay, 1988), and suppresses the pathogen on dead lily leaves (Köhl et al., 1995). Crchi1 an endochitinase gene was successfully cloned

from *Gliocladium roseum*, and its expression was induced by the presence of *R. solani's* cell wall, and repressed with glucose (Gan et al., 2007). The ability to synthesize chitinases and β-1,3-glucanases are a recognised characteristic of rhizosphere-competent biocontrol fungi, and crucial for mycoparasitic procedure (Viterbo et al., 2002).

1.8 Biocontrol function, screening, and development

1.8.1 How does a BCA function: mechanisms of action

Studies have focused on using antagonists against pathogens, but lacked identification of how antagonist exclude the pathogens (Janisiewicz et al., 2000). Understanding the mechanism of action will contribute to improving biocontrol efficacy; through identifying more efficient antagonists and strains, as well as finding paths to manipulating the organisms control characteristics (Janisiewicz et al., 2000). The following mechanisms of action (MOA) are proposed for the control of pathogens: competition of space and nutrients between the antagonist and pathogen, production of antibiotics (antibiosis), direct parasitism, and induced resistance (Janisiewicz et al., 2000).

The BCA must adapt to environmental conditions and nutritional challenges to efficiently compete for space and nutrients eventually supressing the pathogen on the phyllosphere (Di Francesco et al., 2017). The BCA must survive in conditions which are unfavourable to the pathogen. The primary characteristic of competition of space and nutrients is the rapid colonization of the BCA on to the phyllosphere. This MOA enhances as the applied biocontrol concentration is increased and the pathogen concentration decreased (Guijarro et al., 2017). Research supported this quantitative affiliation, as mutant *Pichia 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 (Droby et al., 1991). The mutant strain could not replicate, and the initially applied concentration remained constant, whereas the wild type reproduced by 20 fold in 24 hours. These findings suggested that rapid colonization is crucial for overpowering the pathogen. A similar study demonstrated the importance of the initially applied BCA concentration and its potential to colonize the plant surface (McLaughlin et al., 1990). The lack of data for interactions between antagonists and pathogens cause problems for identifying antagonistic strains with an enhanced rate of colonization, there is a requirement to understand the features of competition on the phyllosphere, and the characterization of the genes involved in colonization. Research showed that for nutrient competition direct attachment by the BCA to the pathogen's hyphae is critical, since direct attachment prevents spore germination (Arras et al., 1998). In certain cases direct attachment is not necessary, comparatively *B. cinerea* was suppressed by *Aureobasidium pullulans* through multiple MOAs functioning together for a better efficacy, which also included antibiosis (Castoria et al., 2001). Antibiotic production is the key MOA in several BCAs; similarly *B. subtilis* produces iturin to suppress pathogen establishment (Gueldner et al., 1988). Most recently *B. subtilis* iturinic lipopeptides have been documented for control of plant pathogens (Dunlap et al., 2019).

Direct parasitism was first acknowledged with *P. guilliermondii* cells attaching to *B. cinerea* hyphae; once dislodged the hyphal surface became concave and partial degradation of the cell wall was apparent at the attachment site (Wisniewski et al., 1991). A more recent study found that *Candida saitoana* attached firmly to the hyphae of *B. cinerea* and caused swelling (El-Ghaouth et al., 1998). Parasitic antagonists produce lytic enzymes (gluconase, chitanase, and proteinases) that

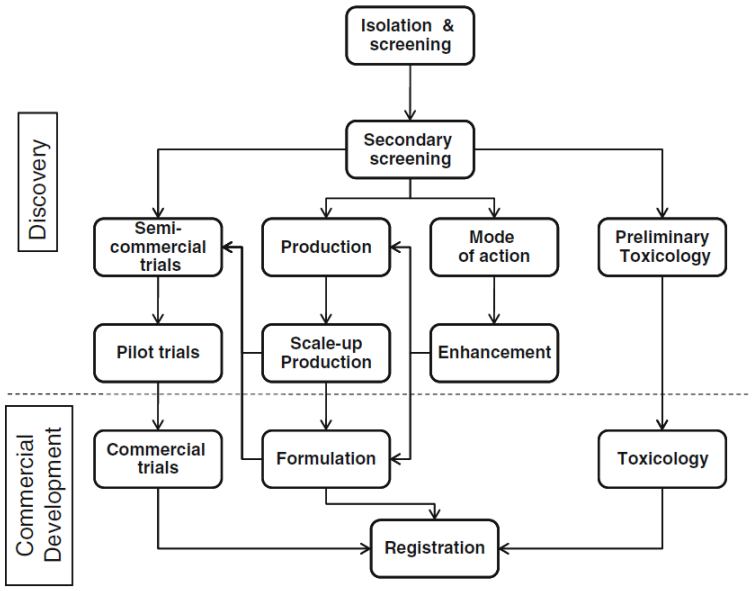
degrade the cell wall of pathogenic fungi (Chernin and Chet, 2002). A more recent study with the BCA *Trichoderma gamsii* identified parasitism as the primary MOA against the root rot pathogens (Chen et al., 2016). The application of parasitic BCAs to a wound site enhances their activity and suppresses the pathogens access to nutrients (El Ghaouth et al., 2004).

Antagonists 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). A recent study showed *B. subtilis* was capable of inducing systemic resistance in peanut plants against fungal plant pathogens (Figueredo et al., 2017). 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 BCAs have a role in disease resistance. On fruit surfaces BCAs 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). Recently research on the yeast BCA *Hanseniaspora opuntiae* was found to release compounds which protected the host against *Corynespora cassiicola* and *B. cinerea* (Saab et al., 2018).

1.8.2 Screening

BCA identification, development and commercialization are expensive and extensive processes. BCAs are applied to field and protected crops and require distinct consideration in biosafety, ease of use, activity range, growth requirements, shelf-life

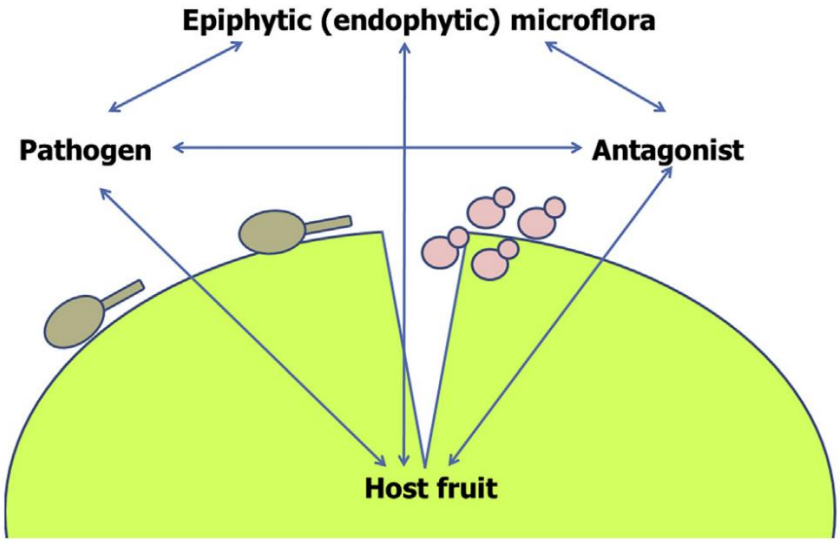
and patent potential (Fig 1.6). The dotted line in Fig 1.6 represents the information needed in key research areas before proceeding to the stage of commercial development.



(Nunes, 2012)

Figure 1.6 Factors involved in the development of a biocontrol product

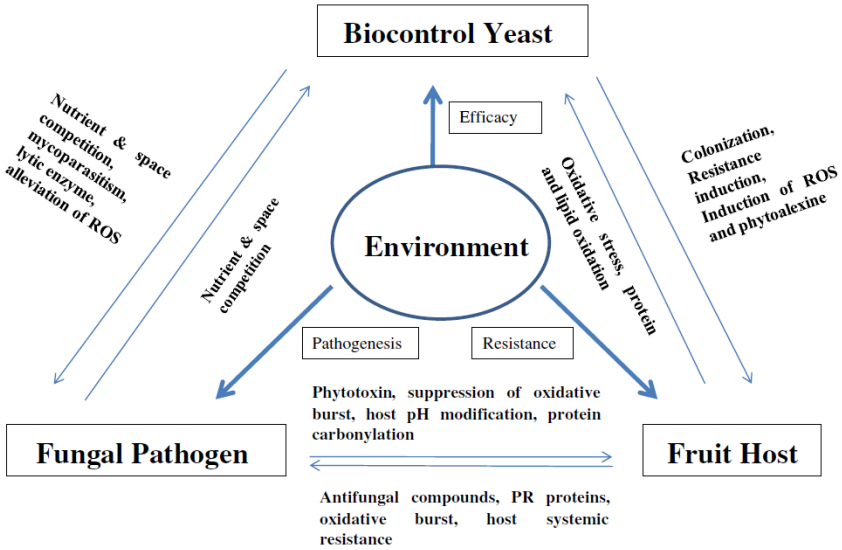
Detailed studies in relation to the interactions between the host, pathogen, BCA, and epiphytic microflora are required to unravel and optimise BCA research (Fig 1.7).



(Spadaro and Droby, 2016)

Figure 1.7 Diagram of quadritrophic system between host, pathogen, antagonist, and epiphytic microflora

The complex nature of interactions between the BCA, host and pathogen confines the information obtainable on the MOA of BCAs (Fig 1.8).



(Liu et al., 2013)

Figure 1.8 Diagram of possible interactions between host, pathogen and antagonist and their environment

Biocontrol systems are complex and influenced by a number of factors. BCAs are applied to plants both pre- and post-harvest by spraying, dipping and drenching. BCA survival, reproduction, dispersion and efficacy can be affected by environmental factors including temperature, humidity, pH, oxidative stress, water activity and wind speed (Liu et al., 2013). Oxidative stress in BCAs cause responses that increase the amounts of a range of antioxidants which regulate transcriptional changes, which results in negative effects on cells by damaging their DNA, protein and lipids (Sui et al., 2015).

1.8.3 Problems and obstacles of BCA development and commercialization

The main problem with biocontrol products is that they do not consistently and efficiently control the target diseases on a commercial level, which limits the product's reliability and in turn its reputation for recurrent use. In the final stage of BCA development, the formulated product is tested on target diseases at different locations with a range of application methods, and such largescale tests are costly and consequently employed with companies desiring to commercialise the bio-product.

Prior to any effort of commercialisation, the development of the BCA must be cost-effective and the formulated product has to retain its original characteristics (pure, genetically stable, viable, and preserve its MOA) after mass production. The BCA must use industrial by-products for nutrients, and fermentation should be completed in 30 hours (Hofstein et al., 1994). Downstream processing involves drying, addition of inert ingredient's, emulsifiers and adjuvants which may affect the BCA characteristics adversely (Hofstein et al., 1994).

BCAs are prepared as dry or liquid formulations. Advantages of dry formulations are prolonged shelf life, protection from contamination, and ease of, shipping, distribution and storage (Melin et al., 2006, Melin et al., 2007). Whereas liquid formulations are cost efficient, and the lack of dehydration and rehydration preserves initial concentrations (Abadias et al., 2003). Both types of formulations contain an outer layer which shelters the BCA from environmental stress. In depth research covers the details of dry and liquid formulation and their effects on the BCA (Abadias et al., 2003, Abadias et al., 2000, Abadias et al., 2001). Results from trials of formulated products: in both commercial and semi-commercial conditions indicate that inconsistency and variability in the level of disease control is the primary barrier to the widespread use of biocontrol products (Droby et al., 2009). For improving the control consistency and variability several means are integrated including: addition of salts and organic acids (Karabulut et al., 2001), food additives (Qin et al., 2006), glucose analogs (El-Ghaouth et al., 2000) and physical treatments (Porat et al., 2002). Additives may show synergistic effects, but there is a lack of knowledge on the interactions of antagonists, complementary treatments, pathogens and commodities. Most recent advances in formulations have been focused on *Trichoderma* (Cumagun, 2014).

Biological control of diseases is viewed with scepticism, since control efficiencies are required to be 95 – 98 %; currently these requirements can solely be achieved with the input of fungicides and synthetic chemicals, suggesting that for improved efficacy some form of optimisation is needed for the currently available BCAs.

1.9 Overcoming BCA problems through optimisation

BCAs are applied by spraying, similar to that for synthetic chemicals. Serenade is formulated with *B. subtilis* strain QST 713; and PreStop with *G. catenulatum* strain J1446. They are applied in the same manner as a contact pesticide. Serenade is optimally applied with a hydraulic sprayer and for a good crop coverage requires 10 litres of Serenade into 400 litres of water per hectare, but the users dilute or concentrate depending on how much area needs to be covered. PreStop is applied as 100 g of dry product in 20 litres of water. Spraying a biocontrol for high coverage uses the competition of space and nutrients MOA. Each BCA is independent in how it protects the host and controls the target pathogen. Therefore gaining ecological information on impacts of abiotic and biotic factors specific to the BCA may help to enhance the BCAs survival, reproduction, dispersion and colonisation. This may help to optimise BCAs effectiveness in protecting the host and controlling the target pathogen (Droby et al., 2009, Liu et al., 2013, Magan, 2001, Sharma et al., 2009, Spadaro and Droby, 2016, Sui et al., 2015). Thus, abiotic factors and the timing of application of BCAs under their most favourable conditions may be critical for successful biocontrol (Ment et al., 2018, Sui et al., 2015).

Research on biocontrol has evolved more into a production systems approach, with attention towards industrial problems. To integrate BCAs into all stages of crop protection and growth the following need investigation: the effect of using chemical and biological approaches and mixed applications of BCAs on disease control (Droby et al., 2009, Lima et al., 2006). Biocontrol products are costly to produce and purchase; and using these products in a manner that requires a high volume of product will affect the choice of farmers. Genetic modification of BCAs may be the answer in the future, but currently there are more pressing questions: can the

population density applied affect the colonization of the BCA? Can different environmental factors affect survival, reproduction, dispersion and the efficacy?

The initially applied concentration (CFU/ml^{-1}) could influence the MOA; for instance quorum sensing regulates biofilm formation and antibiotic production, and a specific concentration could aid in biofilm formations, superior antibiotic production, and rapid phyllosphere colonisation. The efficient colonization on the crop surface requires the BCA to use specific features including adherence, colonization and reproduction on the plants surface; these characteristics are associated with biofilm formation (Annous et al., 2009). Most recently biofilm production of the BCA *A. pullulans* has been demonstrated to improve biocontrol of the causal agent of sour rot in citrus *Geotrichum citri-aurantii*. The research focused on stimulating biofilm production of the BCA with ammonium sulfate, which lead to better antagonistic activity towards the disease as well as superior survival of the yeast BCA in wound sites. The biofilm was capable of deforming the pathogen hyphae (Klein and Kupper, 2018). Such findings encouraged work towards identifying the key parameters of the environment which will promote biofilm formation for survival, reproduction and dispersal of *B. subtilis* (Pandin et al., 2017). Additionally *B. subtilis* produces lipo-peptides which are involved in inhibiting the establishment of pathogens due to their antibiotic nature. During the fermentation process, *B. subtilis* produces a mixture of secondary metabolites that function as antifungal inhibitors including but not limited to surfactin, bacylisin, fengycin, bacyloomicin, subtilin and iturin (Athukorala et al., 2009, Mora et al., 2011, Ramachandran et al., 2014). Identifying the environmental conditions that increase survival, reproduction and lipo-peptide production can be crucial to improving control against pathogens.

In large scale crop production it can become difficult to keep up excellent sanitation procedures, in turn there is always rotting material functioning as secondary inoculum especially in the field. BCAs are advised to be applied pre-infection, this ideology is convincing, but can a BCA be useful post infection? Since parasitic BCAs consume mycelia and spores there is potential in identifying the most economically viable concentration to reduce the secondary inoculum potential of infected plant material. Direct parasitism can control *B. cinerea* mycelium and spores (El-Ghaouth et al., 1998, Wisniewski et al., 1991). Identifying the climatological factors which induce survival, reproduction, hyper parasitism and colonization of *G. catenulatum* could improve biocontrol efficacy.

Combination approaches are useful within cropping systems. To improve control strategies on a systems approach in the context of predictive models there is a need for early detection techniques, as well as cultural and biological methods tailored towards each crop. A success story of similar management strategies comes from New Zealand for the management of grey mould on kiwi (Michailides and Elmer, 2000). The study demonstrated adopting summer cropping and using an open canopy with pre-harvest predicative and post-harvest curing models efficiently reduced *B. cinerea* infection and losses to grey mould disease.

It is speculated that BCA's control of target diseases is unsatisfactory due to their lack of survival, reproduction and dispersion on the crop surface. An application may become meaningless without prolonged survival, reproduction and dispersion, as climatological factors could eliminate the BCA population within days. For each BCA the environmental cues that induce survival and reproduction have to be identified and manipulated to observe if this improves control of their target diseases consistently.

1.10 The effect of climate on BCA survival, reproduction, dispersion and efficacy

Our understanding of how climatic variables affect BCA survival, reproduction, dispersion and efficacy is lacking. Such an understanding is critical to identifying the right conditions to apply biocontrol agents. Once applied to a crop, survival, reproduction, dispersal and efficacy are affected by climatic conditions including precipitation, light, temperature, humidity, and wind. The period and intensity of a rainfall event impacts the colonisation of the BCA by washing it off and diluting the initially applied concentration on the crop surface. A study found that after 8 days in the field 50% of *B. subtilis* was absent, and suggested the bacteria was washed-off by rainfall (Wei et al., 2016). The loss of biocontrol activity was confined to the duration of the rain event, and suggested rain reduced the biocontrol efficacy through physically removing the organism from the target zone (Behle et al., 1997). Microbial antagonists are prone to degradation when in direct sunlight. Solar UV radiation (UVR 290 nm to 400 nm) causes cellular damage on nucleic acids, proteins and lipids that induce mutations, cell inactivation and death; as microbial antagonists are too small for developing protection against UVR (Coekmues et al., 2000). 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 antifungal metabolite production (Kilani-Feki et al., 2016). The amount of water vapour present in the air and available to the BCA can be critical for colonising the crop phyllosphere: as an increase in humidity increases the amount of water vapour that can be readily utilised by the BCA, and therefore is a critical bottleneck for microbial growth (Cota et al., 2008, Magan, 2001, Parts et al., 2013). Wind

disperses organisms, and thus can also play an important role for dispersing BCAs from one zone into another, as well as dispersing BCAs from colonised tissue to virgin expanding tissue (Bock et al., 2012, Smith et al., 2013).

Aims and Objectives

The overall aim of the study was to obtain ecological knowledge on *B. subtilis* QST 713 and *G. catenulatum* J1446, and if possible utilize this knowledge to produce strategies for applying the two BCAs, ultimately improving consistency and efficacy against *B. cinerea* on lettuce and strawberry crops. The main research question for the thesis thus was: what are the impacts of temperature and relative humidity on the population dynamics, of the two commercial BCAs on lettuce and strawberry crops, and how can knowledge of this be used to inform and improve biocontrol strategies.

BCA's are living organisms and as with every organism, reproduction is critical to its survival. Identifying which conditions induce survival, reproduction, dispersal and efficacy is crucial to expanding their use. Studies on modelling the BCA population dynamics in relation to climatic conditions is fundamental for their use to control and manage foliar pathogens (Medina-Martínez et al., 2015), because abiotic factors significantly affect BCA's populations in the phyllosphere of these two crops (Magan, 2001, Scherwinski et al., 2008, Andrews, 1992).

The project objectives

i. Develop a molecular-based assay to quantify viable populations of *B. subtilis* QST 713, and *G. catenulatum* J1446 (Chapter 2).
ii. Determine the dose response relationship of *B. cinerea* with *B. subtilis* QST 713 and *G. catenulatum* J1446, and observe whether temperature, formulation and leaf tissue type impact upon the viability (Chapter 3).

iii. Use of the developed assay to quantify temporal viable populations of *B. subtilis* QST 713, and *G. catenulatum* J1446 at different temperature and relative humidity combinations in lettuce and strawberry phyllosphere (Chapter 4).

iv. Produce a simple model to predict the viable population fate of *B. subtilis* QST 713 and *G. catenulatum* J1446 and test the predication ability (Chapter 4).

v. Identify *B. subtilis* QST 713 and *G. catenulatum* J1446 dispersion and colonisation pattern on expanding lettuce and strawberry leaves (Chapter 5).

vi. Optimisation strategies for *B. subtilis* QST 713 and *G. catenulatum* J1446 as BCAs (Chapter 6).

Chapters 2 to 5 are presented as draft paper manuscripts with the final Chapters (Chapter 6, 7 and 8) integrating the overall Discussions, Conclusions and Future Work suggestions.

CHAPTER 2

2 Development of a molecular assay to monitor change in viable populations of *Bacillus subtilis* QST 713 and *Gliocladium catenulatum* J1446 in commercial formulations and the phyllosphere

2.1 Abstract

Optimising the use of biocontrol agents (BCAs) requires tracking and monitoring of the microorganisms after application in the crop phyllosphere to understand their survival, establishment and reproduction. There are no methods for the sensitive tracking and monitoring of *B. subtilis* and *G. catenulatum* viable populations. The objective of this Chapter was to develop a method that can monitor and quantify temporal viable populations of these two formulated BCAs in the crop phyllosphere. A molecular tool based on propidium monoazide (PMA) (PMAxx™-qPCR) capable of tracking, monitoring and quantifying viable populations of these two BCAs was developed. This involved treating the samples with PMAxx™ (12.5 µM - 100 µM), followed by 15 min incubation and then exposure to an 800W halogen light for 30 min. Then the DNA extraction and quantification followed the usual qPCR process. The purpose of the chapter was to provide a platform system for using the PMAxx™-qPCR technique for these two BCAs to enable the blocking of DNA from dead BCA cells, and result in quantifying DNA from only live BCA cells. Validation studies showed that this method can block the DNA from dead cells, which resulted in the quantification of DNA from only the viable cells, and therefore provided analyses of

viable population changes of these two BCAs in commercial formulations, and on aerial plant surfaces.

2.2 Introduction

The efficacy of a BCA is usually evaluated by the dose response relationships against their target pathogens (Smith et al., 1997). This requires accurate quantification of the viable populations in the applied formulations in relation to climatic conditions and relative control of the target pathogen. Traditionally, this is done by culturing the viable populations or microscopic counts. However, such methods are labour intensive (Turano and Pirali, 1988). In addition, some BCAs may remain viable under environmental stress conditions but enter into a hibernation phase, which makes them unculturable, resulting in an underestimation of viable populations present (Papadimitriou et al., 2016, Liu et al., 2016).

BCA detection/quantification has been modernized with nucleic acid targeting techniques such as the polymerase chain reaction (PCR) and quantitative-PCR (qPCR) (Sanzani et al., 2014). PCR lacks specificity for quantitative research as the amplification curves are absent, and results are dependent on the end product (Silva and Domingues, 2015). While qPCR both detects and quantifies the population and has been developed for *Pantoea agglomerans* (Braun-Kiewnick et al., 2012), *Pseudomonas fluorescens* (Pujol et al., 2006) and *Cryptosporiopsis kienholzii* (Spotts et al., 2009). However, the conventional qPCR technique cannot discriminate viable (live) and non-viable (dead) cells, because DNA from both are normally quantified. This limits the understanding of the population dynamics of formulated BCAs (Fittipaldi et al., 2012).

Propidium monoazide (PMA) a membrane impermeable nucleic acid intercalating dye when used as a pre-treatment before qPCR enables the blocking of DNA from dead cells, which results in the sole quantification of DNA from live cells (Nocker et al., 2006, Fittipaldi et al., 2012). PMA enters cells with ruptured membranes and intercalates with the cell DNA. The PMA-DNA complex once photo-activated becomes a permanent structure as the PMA dye releases an azido group resulting in a covalent cross link between the PMA and the DNA complex. This irreversible change in DNA structure obstructs access to the modified DNA and therefore stops amplification by PCR techniques (Nocker et al., 2006, Nocker et al., 2007, Nocker and Camper, 2009). The success of PMA-qPCR in quantifying viable populations has been reported for *Listeria monocytogenes*, Salmonella and *E.coli* species within simple matrices (Elizaquível et al., 2012a), identifying viable fungi such as *Alternaria* spp. (Crespo-Sempere et al., 2013), and the bacterial BCA, *P. agglomerans* (Soto-Muñoz et al., 2014). This method was used effectively to monitor viable populations of *P. agglomerans* in the phyllosphere of citrus fruit (Soto-Muñoz et al., 2015).

The objective of Chapter 2 was to develop a method with sufficient competence in quantifying viable populations of *B. subtilis* QST 713 and *G. catenulatum* J1446 in commercial formulations. For developing a new and improved method based on the PMA approach, studies examined whether PMAxx™ can be applied to inhibit DNA amplification of dead cells in formulated products, and then included experiments to assess the capability of PMA-based qPCR quantification of viable BCA populations in the phyllosphere.

2.3 Materials and Methods

2.3.1 B. subtilis QST 713 and G. catenulatum J1446

Serenade ASO, a biocontrol product containing *B. subtilis* strain QST 713, was purchased as a liquid formulation from Fargro, UK. Serenade ASO was stored at room temperature. PreStop, a fungal biocontrol product containing *G. catenulatum* strain J1446, was also purchased from Fargro as a wettable powder formulation. PreStop was stored in a cool dry place at < 8 °C, and once opened was frozen at –20 °C. The batch of both the biological products was less than six-months old when used in experiments.

2.3.2 Cell suspensions and viable population reduction treatments

For both biocontrol products, viable populations were determined by quantifying CFUs with the viable plate count technique, and total populations were estimated with a Neubauer haemocytometer under a microscope. Cell viability was confirmed by culturing *B. subtilis* on nutrient agar and *G. catenulatum* on malt extract agar at 25 °C for 48 h. Thus, the non-viable population was estimated as the difference between the total and viable populations. The two products were serially diluted with maximum recovery diluent (Sigma). After dilution 500 µL of the BCA suspension was transferred into a 1.5 ml Eppendorf. The total cell concentration in the 500 µL volume used in the study for *B. subtilis* contained 7.96 $\log_{10}$ units of cells, and of the 7.96 $\log_{10}$ units in 500 µL 1 $\log_{10}$ unit was previously dead in formulation, while 6.96 $\log_{10}$ units were alive. The total cell concentration in the 500 µL volume used in the study for *G. catenulatum* contained 8.64 $\log_{10}$ units, and of the 8.64 $\log_{10}$ units 2.94 $\log_{10}$ units were previously dead in formulation, while 5.7 $\log_{10}$ units were alive.

To increase the total amount of dead cells, the cell suspensions of 500 µL containing the BCAs were treated with heat on a dri-block heater. The first heat treatment was

95 °C for 5 mins, and this increased the total amount of dead cell concentration from 1 $\log_{10}$ units into 2.75 $\log_{10}$ units of dead cells for *B. subtilis*. The same heat treatment for *G. catenulatum* increased the total amount of dead cells from 2.94 $\log_{10}$ units into 5.75 $\log_{10}$ units of dead cells. The second heat treatment was at 95 °C for 10 mins, and this increased the total amount of dead cells from 1 $\log_{10}$ units into 3.44 $\log_{10}$ units of dead cells for *B. subtilis*. The second heat treatment for *G. catenulatum* increased the total amount of dead cells from 2.94 $\log_{10}$ into 6.22 $\log_{10}$ units of dead cells.

Before heat treatments, the formulated versions of the BCAs contained a large portion of dead cells. Because of this a separate experiment was set up in which the serial dilution method was used to dilute the amount of dead cells present in the BCA suspensions, and for *B. subtilis* this was carried out with the pure isolate, while for *G. catenulatum* this was carried out with the formulated version. The total cell concentration and the total amount of dead cells are stated as follows for *B. subtilis* 1 $\log_{10}$ units of cells were previously dead, and for *G. catenulatum* 3.21 $\log_{10}$ units of cells were previously dead. These BCA suspensions were serially diluted and from each serial dilution this changed the total amount of dead cells by a factor of 10 i.e. for *B. subtilis* this changed the total amount of dead cells into 0.1 $\log_{10}$ units, while for *G. catenulatum* this changed the total amount of dead cells into 2.21 $\log_{10}$ units. The serial dilution method was followed up to four serial dilutions, which diluted the total amount of dead cells up to a factor of 10000. Refer to Table 2.1 for the exact mean total amounts of dead cells.

The following equation was used to calculate mean total amount of dead cells:

$$\boldsymbol{Total\ amount\ of\ dead\ cells} = \boldsymbol{Log10}\ (\boldsymbol{Total\ amount\ of\ cells}) - \boldsymbol{Log10}\ (\boldsymbol{Total\ amount\ of\ viable\ cells})$$

Table 2.1 List of treatments to produce different BCA cell concentrations

Treatment	Organism	Formulated	Total volume	Mean total cell concentration (log_{10}) confirmed with Haemocytometer	Mean total amount of live cells (log_{10}) confirmed with plate counts	Mean total amount of dead cells (log_{10})
Room temperature	*B. subtilis*	Yes	500 µL	7.96	6.96	1.00
Room temperature	*G.catenulatum*	Yes	500 µL	8.64	5.7	2.94
95 °C for 5 mins	*B. subtilis*	Yes	500 µL	7.96	5.21	2.75
95 °C for 5 mins	*G.catenulatum*	Yes	500 µL	8.64	2.89	5.75
95 °C for 10 mins	*B. subtilis*	Yes	500 µL	7.96	4.52	3.44
95 °C for 10 mins	*G.catenulatum*	Yes	500 µL	8.64	2.42	6.22
Serial dilution 1	*B. subtilis*	No	500 µL	9.4	9.3	0.1
Serial dilution 2	*B. subtilis*	No	500 µL	8.4	8.39	0.01
Serial dilution 2	*G.catenulatum*	Yes	500 µL	6.6	5.39	1.21
Serial dilution 3	*B. subtilis*	No	500 µL	7.4	7.399	0.001
Serial dilution 3	*G.catenulatum*	Yes	500 µL	5.6	5.39	0.21
Serial dilution 4	*B. subtilis*	No	500 µL	6.4	6.3999	0.0001
Serial dilution 4	*G.catenulatum*	Yes	500 µL	4.6	4.579	0.021

*Serial dilution series 1 for *G. catenulatum* was not included because the PMA treated counterpart was unidentifiable.

2.3.3 PMAxx™ treatment

PMAxx™ (Biotum, Hayward, USA) was diluted into DEPC water to produce a 2.5 mM concentration and stored at -20 °C. At room temperature under dimmed lighting conditions, PMAxx™ solution (0 µL – 20 µL) was transferred to each BCA suspension to produce a final concentration of 0 µM, 12.5 µM, 25 µM, 50 µM, and 100 µM. This was followed by an incubation stage in which BCA suspensions were encased in aluminium foil and placed into a lightproof sealable container, which was shaken on a rocker at 35 revs/min for 15 minutes. In a cold room (4 °C – 8 °C) a photo-activation system was assembled with a rocker fitted with an icebox that contained ca. 2 L ice, with the interior covered by two layers of aluminium foil (reflective side), and two light sources with a light output of 800 W fitted on the top with an angle of ca. 45°. After incubation, cell suspensions were transferred into the system at a distance of 20 cm from the light source to initiate photo-activation.

Photo-activation consisted of 1 min light treatment followed by 2 mins cooling for 30 cycles with constant agitation at 35 revs/min (Papadimitriou et al., 2016). After photo-induced cross linking of PMAxx™ and exposed DNA in dead cells, BCA suspensions were centrifuged at 5000 × g for 10 mins at 4 °C, and the supernatant was discarded.

2.3.4 DNA extraction and qPCR

DNA from cell pellets was extracted with TRI Reagent® (SIGMA-ALDRICH) following the manufacturer's protocol. Extracted DNA was filtered with Millex-VV syringe filter unit 0.1 µm (PVDF, 33 mm and gamma sterilized) and purity as well as concentration was determined on a NanoDrop spectrometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE). The integrity of the DNA was determined by electrophoresis on a 1.5% agarose gel run at 60 V for 90 mins within TAE buffer solution and stained with GelRed (Biotum, Hayward, USA). The DNA was quantified with the ABI-7500 qPCR detection system (Applied Biosystem Division, Perkin-Elmer Co., Foster City, CA, USA). Reactions were prepared in a clear 96 well qPCR plate, and sealed with an adhesive cover. The final volume in each reaction for *B. subtilis* was 12 µL, and for *G. catenulatum* was 15 µL. The final volume contained 5 µL SensiFAST™ SYBR® No-ROX Kit (Bioline meridian bioscience, U.K), and 35 ng of DNA extracted with and without PMAxx™. *B. subtilis* reactions contained 434 nM of Bs_dnaK1154 forward primer (5′-ACACGACGATCCCAACAAGC-3′), and 434 nM of Bs_dnaK1254 reverse primer (5′-AGACATTGGGCGCTCACCT-3′) (Hertwig et al., 2015). *G. catenulatum* reactions contained 344 nM of Gc1-1 forward primer (5′-CCGTCTCTTATCGAGCCAAGAT-3′), and 344 nM of Gc3-2a reverse primer 5′-GCCCATTCAAAGCGAGGCATTA-3′) (Paavanen-Huhtala et al., 2000). The PCR conditions used for both BCAs were 94 °C for 3 mins followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. There were two replicates for each treatment

combination. Reactions were run in triplicate, and in each qPCR run controls with no template (NTC) which contained 3 – 5 µL of DEPC water instead of DNA were used, and the negative controls which contained only DEPC water were included. Software-defined storage (SDS) software version 1.5.1 (Applied Biosystems) was used to analyse and calculate Ct values automatically. All samples that reached fluorescence values above the threshold were treated as a positive reaction, which was determined by the software.

2.3.5 Effect of PMAxx™ concentration on BCA CFUs

The toxicity effect of PMAxx™ concentrations was tested with $\log_{10}$ viable populations of cells at 4, 5, 6, 7, 8 and 9 CFUs/ml^{-1} for *B. subtilis*, and 2, 3, 4, 5, 6, and 7 CFUs/ml^{-1} for *G. catenulatum.* Each tested concentration contained one independent biological replicate, and this was plated a total of four times to produce four technical replicates in each of the six cell concentrations for each of the four PMAxx™ concentration treatments (12.5 µM, 25 µM, 50 µM and 100 µM). Negative controls of 0 µM PMAxx™ treatments were included for each cell concentration.

2.3.6 Testing viable BCA quantification with PMAxx™-qPCR from the phyllosphere of lettuce

For production of *G. catenulatum* J1446 inoculum, 5 g of PreStop powder was topped up to 1 L with tap water and hand shaken vigorously. *B. subtilis* QST 713 colonies were cultured on nutrient agar, divided into four equal parts and transferred to a 1 L vacuum filter flask containing pre-autoclaved tryptone soya broth (Sigma) and grown on a rotary shaker (110 rpm) at 20 – 25 °C for 10 days. The formulated product Serenade ASO of *B. subtilis* QST 713 was not directly used because the formulation components have qPCR inhibiting materials and additives which cause complications in the produced standard curve for estimating the population. Before

plant spraying total populations were estimated with a Neubauer haemocytometer under a microscope, and the total viable cells for both BCAs were determined by plate counts. Nutrient agar was used for *B. subtilis*, and malt extract agar for *G. catenulatum.*

A randomised block design containing thirty five blocks with seven rows and five columns was used for each experiment. Each BCA contained a total of two experiments (polytunnel and glasshouse) and six independent biological replicates per experiment. Each replicate was formed with the oldest leave of five independent lettuce plants (the single oldest leaf from each plant). All experiments followed seven common steps: (1) plant propagation and selection; plants were sown, grown and selected for being pest-disease free and healthy with a minimum of six leaves. (2) BCA cultivation and concentration calibration; plate counts were used to determine the concentration of the cultivated BCAs, and were adjusted as necessary to obtain 8 $\log_{10}$ CFU/ml^{-1} for *B. subtilis*, and 8 $\log_{10}$ Spores/ml^{-1} for *G. catenulatum*. (3) Plant treatment; plants were sprayed with the appropriate BCA at a fine droplet setting until just before run-off. (4) Plant drying; after treatment plants were allowed to dry for 1 h in the glasshouse, and then placed into their designated glasshouse and/or polytunnel. (5) Sampling was done on days 0, 2, 4, 6, 8 and 10 after spraying. In total for each BCA there were two experiments which contained a total of twelve biological replicates. On each sampling day the oldest leaf was collected from five pre-determined plants (one leaf per plant), and immediately pooled and placed into a falcon tube containing maximum recovery diluent (Sigma). (7) Surface washing, filtration and cell pellet collection; the leaves were soaked in maximum recovery diluent until full, sealed and shaken on a rotary shaker at 100 rpm for 30 mins at 10 °C. The contents were filtered with a wet muslin cloth (four layers), and the cells

were pelleted by centrifugation at 2000 × g for 15 minutes at 4 °C. The supernatant was decanted and the cell pellet supplemented up to 500 µl with maximum recovery diluent solution, and then transferred into a 1.5 ml Eppendorf for storage at 4 °C. (8) PMAxx™ treatment (25 µM for *B. subtilis* and 50 µM for *G. catenulatum*) refer to subsection 2.3.3. (9) DNA extraction and qPCR refer to subsection 2.3.4. Data loggers (EasyLog EL-USB-2 standalone USB temperature and RH %, dew point data logger) were used for monitoring the temperature, relative humidity and dew point in the glasshouse and polytunnel.

2.3.6.1 Standard curves of cycle threshold to copy number of DNA and viable population

PMAxx™ treated standard curves were produced for *B. subtilis* and *G. catenulatum*. For *B. subtilis* the DNA from the pure isolate, and for *G. catenulatum* the DNA from the formulated version of PreStop was used to produce standards of Ct to copy number generated (Fig 2.1), and Ct to $\log_{10}$ total viable population (Fig 2.2). The BCA cell concentrations for standards were PMAxx™ treated. For *B. subtilis* 25 µM, while for *G. catenulatum* 50 µM PMAxx™ concentration was used to treat standard cell suspensions. For both BCAs in each standard point haemocytometer counts determined the total cell concentration, and viable plate count technique determined total viable cell concentration.

The standard curve was produced with a minimum of four standard points having three replicates each. In the standard the copy number of the DNA marker ranged from $\log_{10}$ 11 to 8 units (Fig 2.1). For obtaining the viable population of a sample; the Ct value allows the estimation of DNA marker copy number through the use of a standard curve, which can also be used to estimate the total viable population with

efficient primers when there is a linear relationship between the copy number of target DNA sequence to population (Fig 2.2).

Monitoring viable population change was also possible without the use of a standard curve since the following equation can be applied:

$$\Delta \mathrm{Ct} = \mathrm{Ct\ value\ obtained\ without\ PMAxx™(Negative\ control)} - \mathrm{Ct\ value\ obtained\ with\ PMAxx™(Treatment)}$$

The equation above calculates the viable population change from the initial application, and in result the use of this equation can quantify and evaluate if viable cell numbers have increased, decreased or remained stable. But the exact population size cannot be estimated, as well as this the initially introduced inoculum must contain minimal amounts of pre-dead cells. Because of this a PMAxx™ treated standard curve was more reliable to allow the estimation of the viable BCA population treated with PMAxx™.

For producing a standard curve of Ct to viable population another type of calibration was necessary. For identifying the direct relationship between Ct and viable populations the following steps were achieved: (1) BCA cell concentrations with known viable populations were serially diluted using 10 fold dilutions, (2) following this these standards were PMAxx™ treated, and the (3) viable cell concentration were re-determined with the viable plate count technique for each dilution series of the standard points, (4) subsequently DNA extraction from each dilution series containing a specific BCA standard cell concentration was achieved, and this produced a linear relationship between Ct value and the number of viable BCA cells. Therefore the standard curve was labelled in relation to concentration of viable BCA cells extracted instead of DNA copy number produced. The use of this standard

curve allowed direct total viable population estimation. However this was only restricted to the section of the standard curve in which the copy number of DNA was directly proportional to the number of viable cells present in a linear a form.

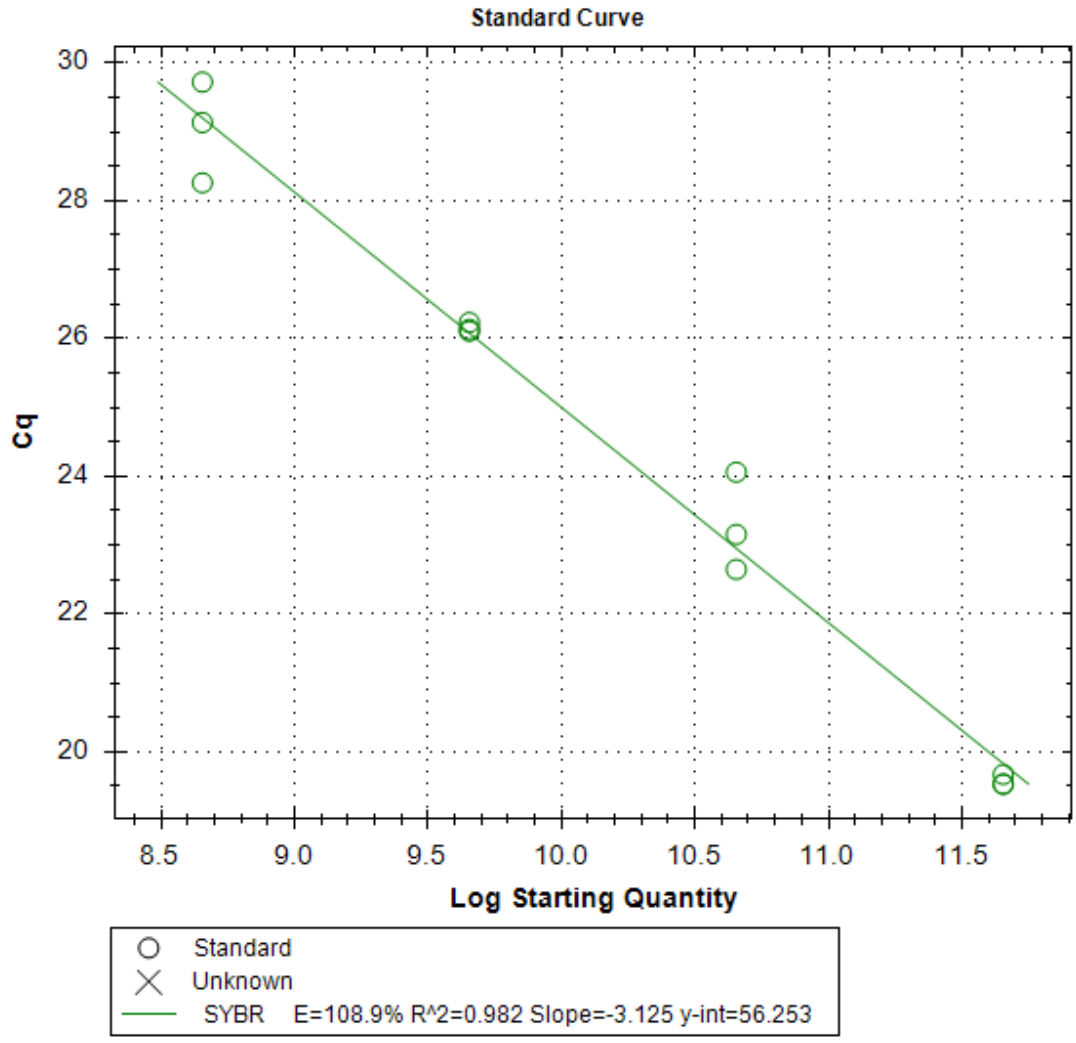


Figure 2.1 The PMA treated standard curve for *B. subtilis* QST 713 of Ct to copy number generated from genomic DNA.

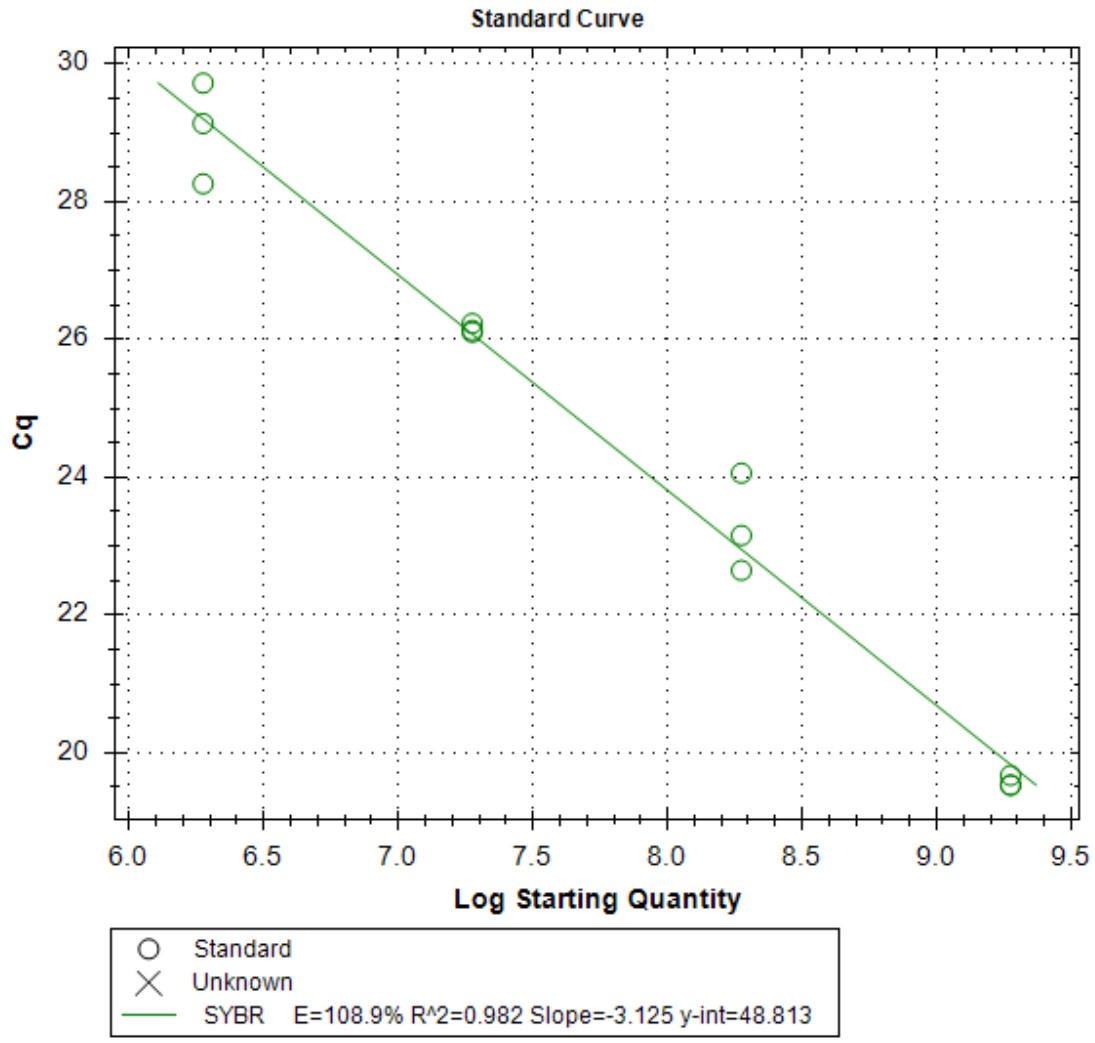


Figure 2.2 The PMA treated standard curve for *B. subtilis* QST 713 of Ct to log_{10} total amount of viable population.

In Fig 2.1 and Fig 2.2 the DNA was extracted from pure culture mixed with maximum recovery diluent. The Ct values were obtained with SYBR Green qPCR and plotted against the copy number generated of genomic DNA (Fig 2.1), and the total amount of viable population (Fig 2.2). The linear relationship between the copy numbers of DNAk (a single copy of genomic DNA fragment from *B. subtilis* with a 101 base pair length) was the marker for the PMAxx™-qPCR analyses that was used to measure the bacterial viable population. The standard curve showing the relationship between Ct and log_{10} viable population (Fig 2.2) was deduced from number of copies of DNAk marker in *B. subtilis* from four different total viable cell concentrations of log_{10} 9.2, 8.2, 7.2, and 6.2. These standard curves illustrate the linear regression, efficiency, and sensitivity of PMAxx™-qPCR for quantification of *B. subtilis* viable population employing the DNAk marker to genomic DNA. The standard curve examples shown in Fig 2.1 and Fig 2.2 were run on BIO-RAD CFX96TM real time PCR detection system (BIO-RAD), and the CFX Manager™ Software version 3.1 (BIO-RAD) was used to analyse and calculate the

standard curves automatically by adhering to the manufactures guidelines. The standards for each copy number and $\log_{10}$ viable cell concentration were run in triplicates on the qPCR plate.

The same standard curve production procedure of Ct to copy number and $\log_{10}$ viable population was carried out for *G. catenulatum* J1446 and generated similar results.

2.3.6.2 Conversion from total viable BCA population to viable population in leaf area

During the sampling period of BCA's from leafs an image was acquired next to standard square paper with a known area. Subsequently the pixel area of a single square at three different locations on the standard was measured with image J, following this the total area of leafs were measured with image J. In succession the following formula was utilised to calculate a given total area.

$$\text{Total Area} = \frac{\text{Total area of leafs in pixel}}{\text{Mean area of standard square in pixel}}$$

$$= \text{Number of standard squares in leaf area} \times \text{Area of a single sqaure}$$

After calculating the total area the following formula was used to calculate CFU/mm^2

$$\text{CFU/mm2} = \frac{\text{Total quantified viable population of BCA with PMAqPCR}}{\text{Total Area}}$$

2.3.7 Statistical analysis

For testing the capability of blocking DNA quantification of dead cells with PMAxx™ two experiments were completed for each BCA. Each experiment was set-up as a randomised block design containing fifteen blocks with three rows and five columns. Each experiment contained a single independent biological replicate for each treatment combination: type of treatment (room, heat treatment of 95 °C for 5 mins,

and 95 °C for 10 mins) and PMAxx™ concentration (0 µM, 12.5 µM, 25 µM, 50 µM and 100 µM). We used ΔCt values to evaluate microbial quantification with the PMAxx™-qPCR method in relation to heat treatment and PMAxx™ doses. The ΔCt is the difference in Ct-value obtained without PMAxx™ treatment and the Ct-value obtained with PMAxx™ treatment (Barbau-Piednoir et al., 2014). The effect of PMAxx™ in suppressing DNA amplification of dead cells in relation to heat treatment, and the sensitivity of this quantification method were assessed through a restricted maximum likelihood (REML) analysis. In the REML analysis the fixed factor was the PMAxx™ concentrations, and the treatments were room temperature, heat treatment of 95 °C for 5 mins and 95 °C for 10 mins. The Ct data was used for the REML analysis and therefore was not transformed. The serial dilution treatments were pooled from two standard curves for the PMAxx™ and non-PMAxx™ treated. These treatments were not included in statistical analysis, and were solely used for graphical purposes as a representative of ΔCt (signal reduction) for BCA suspensions with concentrations that contained a lower amount of dead cells than the room temperature treatments in Section 2.4.4.

For testing the effect of PMAxx™ on cell viability a separate single experiment was completed on each BCA. Each experiment was set-up as a randomised block design containing thirty blocks with six columns and five rows. Each experiment contained a single independent biological replicate for each treatment combination of: PMAxx™ dose (0 µM, 12.5 µM, 25 µM, 50 µM and 100 µM) to a $\log_{10}$ BCA concentration (4, 5, 6, 7, 8, and 9 CFU/ml^{-1} for *B. subtilis*, and 2, 3, 4, 5, 6, and 7 CFU/ml^{-1}). Each biological replicate was plated four times to produce four technical replicates in each treatment combination. The effect of PMAxx™ on cell viability was tested with Analysis of Variance (ANOVA). The data on BCA viability were $\log_{10}$ transformed

prior to the ANOVA. All statistical significances were judged at a P value of <= 0.05. GenStat (version.18) statistical package was used.

Time series plots were generated with the $\log_{10}$ transformed viable population data for visualising the developed PMAxx™-qPCR method in quantifying viable populations of *B. subtilis* QST 713 (pure isolate) and *G. catenulatum* J1446 (formulated) from the phyllosphere of lettuce.

2.4 Results

2.4.1 Effect of PMAxx™ on blocking DNA amplification of dead BCA cells

This experiment was set-up separately for testing the effect of PMAxx™ on blocking DNA amplification from dead BCA cells. Cell suspensions of *B. subtilis* and *G. catenulatum* of three different total amounts of dead cells were subjected to PMAxx™ dosages of 12.5 µM, 25 µM, 50 µM, and 100 µM, a control with no PMA treatment was also included. For *B. subtilis* the amounts of dead cells were a total of 1, 2.75 and 3.44 $\log_{10}$ units, while for *G. catenulatum* this was a total of 2.94, 5.7 and 6.22 $\log_{10}$ units of dead cells, refer to Table 2.1. Heat treatment method was used to increase the amount of dead cells. PMAxx™ blocked amplification of DNA from the dead BCA cells ($P < 0.01$) (Fig. 2.3), as well as this blocked amplification of increasing amounts of dead cells in each tested PMAxx™ concentration ($P < 0.05$) (Fig. 2.4 and Fig 2.5). For both BCAs, the mean Ct from the PMAxx™ treatment varied with the amount of dead cells present ($P < 0.05$).

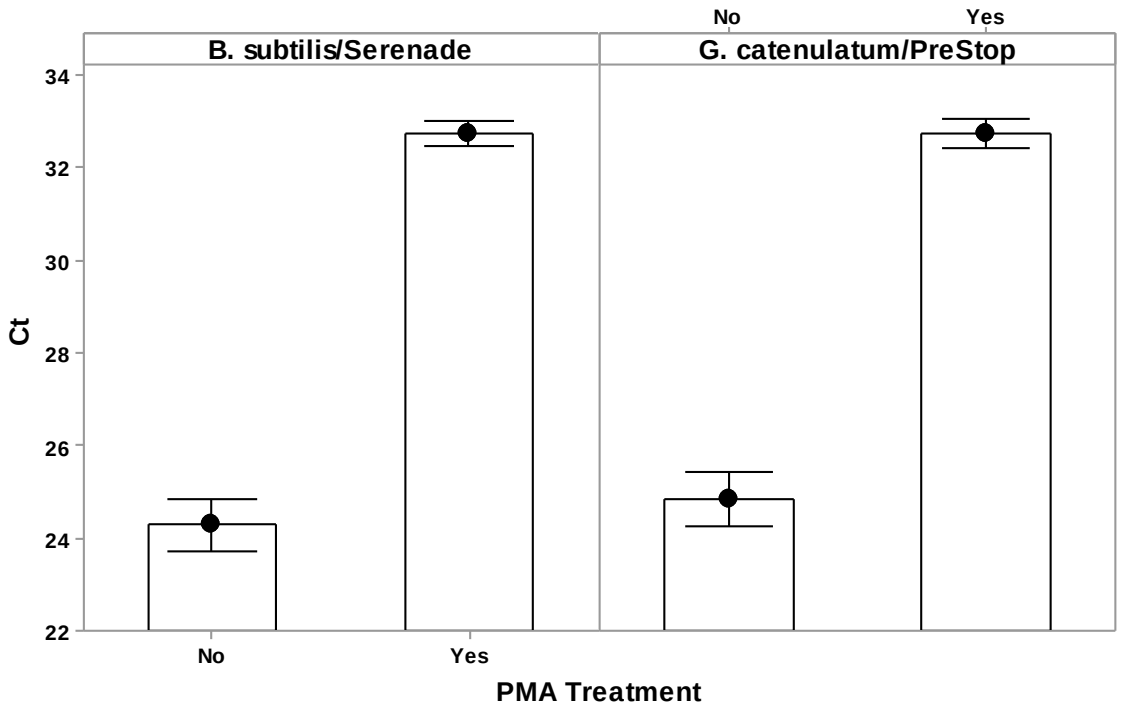


Figure 2.3 Effect of PMAxx™ on blocking DNA amplification from dead BCA cells

On the X axis No defines the overall non-PMA treated mean Ct value for BCAs: for *B. subtilis* the amounts of total dead cells tested are and pooled together from negative PMAxx™ treatments of 0 µM for 1, 2.75, and 3.44 log_{10} units, while for *G. catenulatum* the amounts of total dead cells tested are and pooled together from negative PMAxx™ treatments of 0 µM for 2.94, 5.75 and 6.22 log_{10} units. On the X axis Yes defines the overall PMAxx™ treated mean Ct value for BCAs: for *B. subtilis* the amounts of total dead cells tested are and pooled together from positive PMAxx™ treatments of 12.5 µM, 25 µM, 50 µM, and 100 µM for 1, 2.75 and 3.44 log_{10} units, while for *G. catenulatum* the amounts of total dead cells tested are and pooled together from positive PMAxx™ treatments of 12.5 µM, 25 µM, 50 µM, and 100 µM for 2.94, 5.75 and 6.22 log_{10} units. All cell suspensions subjected to PMAxx™ or DEPC water (for No PMA) followed incubation for 15 mins and photo-activation (800 W) for 30 mins. The black circular points show the mean of six replicates for the non PMA treatment, and the mean of 24 replicates for the PMA treatment, and each replicate was run in triplicate on the qPCR plate, and the error bars represent the standard error.

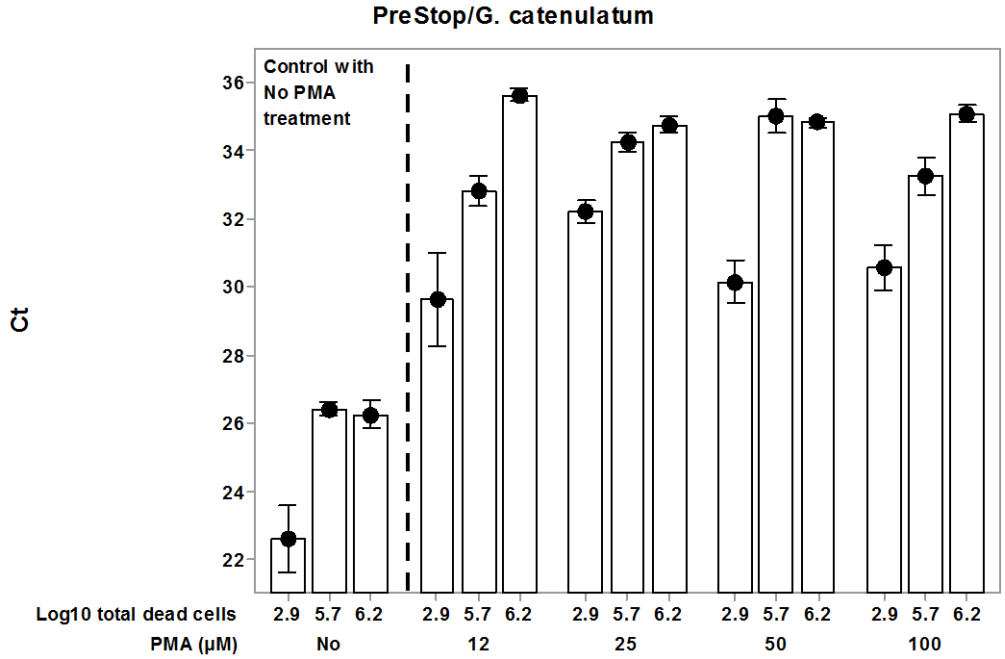


Figure 2.4 Impact of PMAxx™ concentrations on blocking DNA amplification of increasing log_{10} total dead cells from *G. catenulatum*

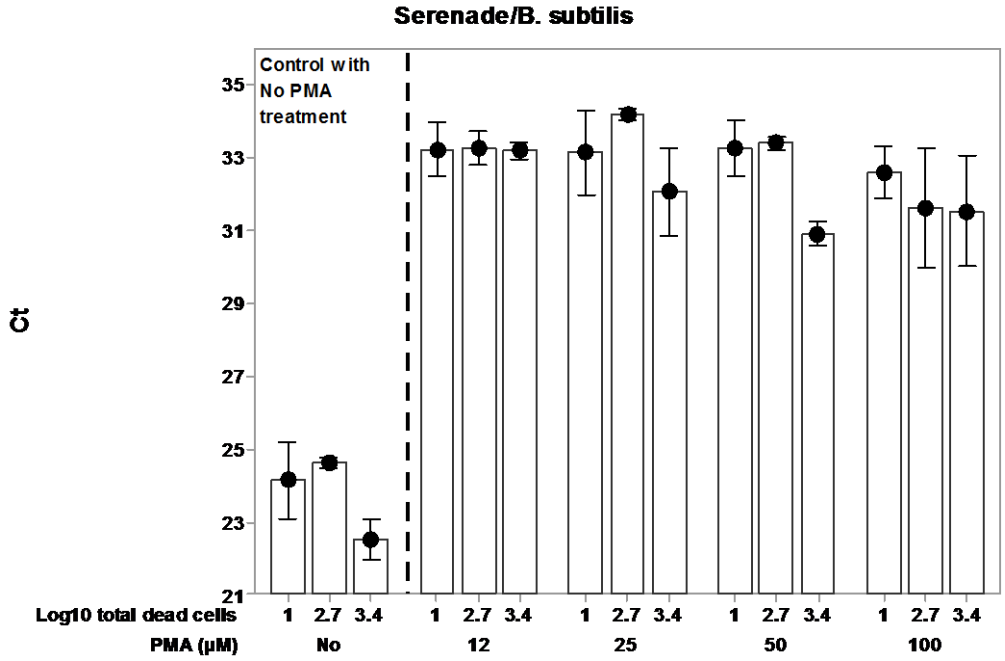


Figure 2.5 Impact of PMAxx™ concentrations on blocking DNA amplification of increasing log_{10} total dead cells from *B. subtilis*

For Fig 2.4 and 2.5 on the X axis No defines the non PMA treated mean Ct value for *B. subtilis* and *G. catenulatum*. The total dead cell amounts tested are displayed on the X axis and for *B. subtilis* this was a total of 1, 2.75 and 3.44 $\log_{10}$ units, while for *G. catenulatum* this was a total of 2.94, 5.75 and 6.22 $\log_{10}$ units of dead cells, and the values of 12, 25, 50 and 100 on the X axis defines the amount of PMA used in (µM) for the treatment. All cell suspensions subjected to PMAxx™ (12 µM, 25 µM, 50 µM and 100 µM) or DEPC water (for No PMA) followed incubation for 15 mins and photo-activation (800 W) for 30 mins. Each treatment combination contained two independent biological replicates, and each biological replicate was run in triplicate on the qPCR plate, and the mean of this is represented with the black circular points, and the error bars represent the standard error.

2.4.2 PMAxx™ concentration response at different amounts of total $\log_{10}$ dead cells

The data collected for investigating the effect of PMAxx™ on blocking DNA amplification from dead cells was arranged in relation to the amount of logarithmic dead cells and increasing PMAxx™ concentration. This arrangement enabled investigation into the impact of increasing PMAxx™ concentration on Ct value of the same amount of $\log_{10}$ total dead cells. For *B. subtilis* the total dead cells tested were 1, 2.75 and 3.44 $\log_{10}$ units, while for *G. catenulatum* this was a total of 2.94, 5.75 and 6.22 $\log_{10}$ units of dead cells. Each cell suspension was subjected to PMAxx™ concentrations of 12.5 µM, 25 µM, 50 µM, and 100 µM. Increasing PMAxx™ concentration had no effect on Ct of the same treatment of total dead cell amount. Fig 2.6 and Fig 2.7 plots the mean Ct values against PMAxx™ concentrations in different total dead cell concentrations. However, there were no significant ($P > 0.05$) differences in Ct values when PMAxx™ concentrations were increased in each of the tested total dead cell amounts. There was also no statistically significant ($P > 0.05$) relationship between PMAxx™ concentration used and the total dead cell amount tested.

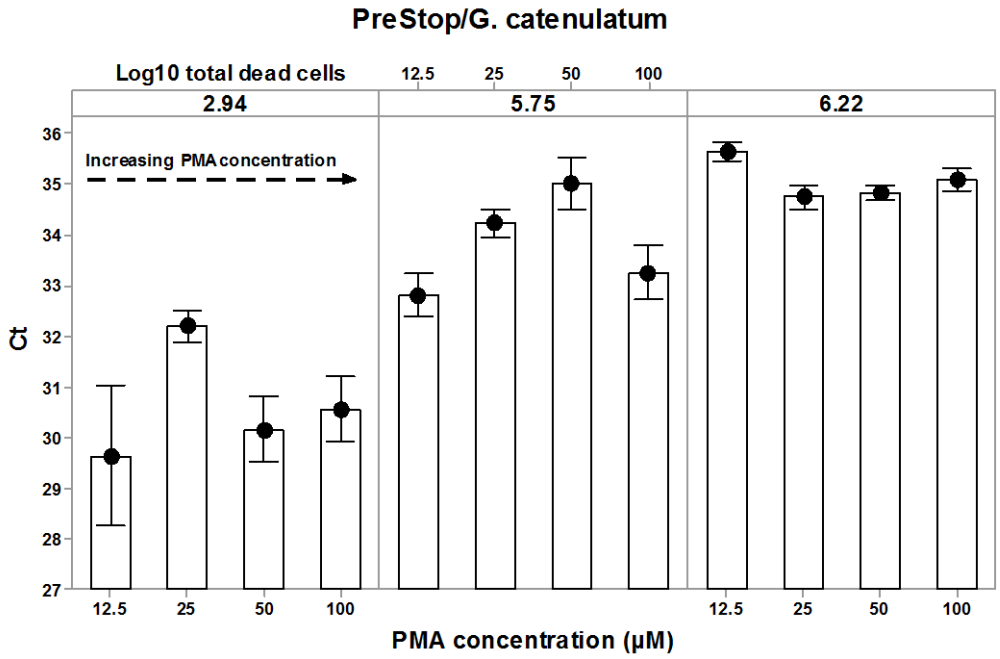


Figure 2.6 Dose response of cycle threshold (Ct) to increasing PMAxx™ concentrations on the same total amounts of *G. catenulatum* dead cells

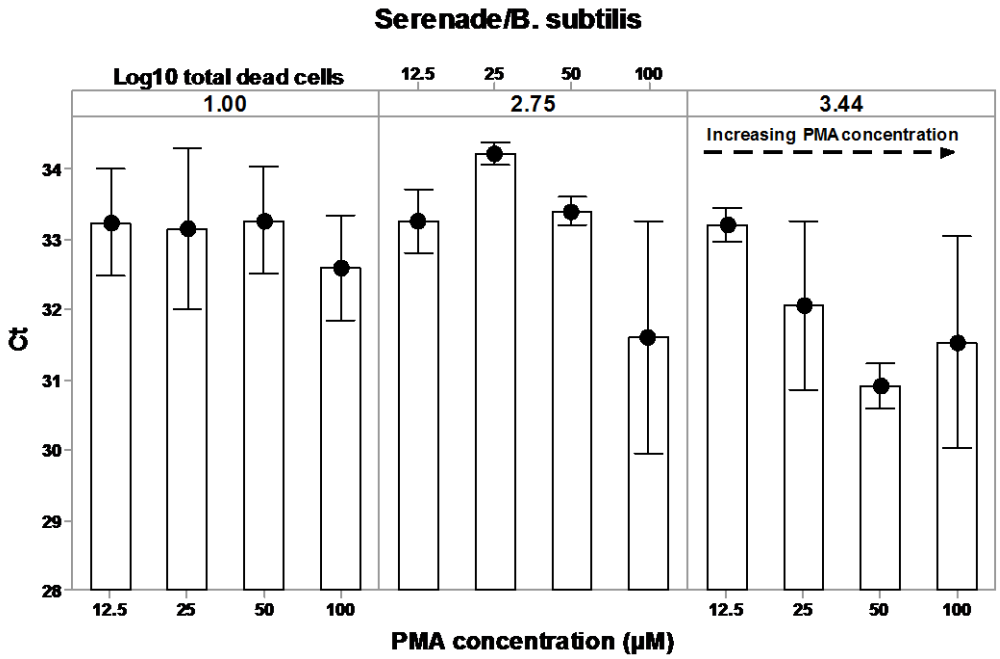


Figure 2.7 Dose response of cycle threshold (Ct) to increasing PMAxx™ concentrations on the same total amounts of *B. subtilis* dead cells

For Fig 2.6 and Fig 2.7 Ct values were obtained in relation to treatment combinations of cell suspensions containing increasing PMA concentrations on the same total dead cell amounts. The amounts of dead cells were increased by heat treatment. The increasing PMA concentrations and the tested total dead cell amounts are displayed on the X axis and for *B. subtilis* this was a total of 1, 2.74 and 3.44 log_{10} units, while for *G. catenulatum* this was a total of 2.94, 5.75 and 6.22 log_{10} units of dead cells, and the values of 12, 25, 50 and 100 on the X axis defines the amount of PMA used in (µM) for the treatment. The mean Ct of each treatment combination between PMAxx™ concentration and total amount of dead cell are represented with a black circle. The points show the mean of two independent biological replicates and each replicate was run in triplicate on the qPCR plate, and the error bars represent the standard error. Detection limit of Ct < 35.

2.4.3 Testing PMAxx™ concentration on CFUs to determine toxicity potential

This experiment was set-up separately for testing the effect of PMAxx™ concentration on viable CFU counts on agar plates. Log_{10} CFU/ml^{-1} of 4, 5, 6, 7, 8, and 9 for *B. subtilis* and 2, 3, 4, 5, 6 and 7 for *G. catenulatum* were treated with 0 µM (negative control), 12.5 µM, 25 µM, 50 µM, and 100 µM of PMAxx™ and then plated. For both BCAs in all tested BCA concentrations PMAxx™ doses had no impact on the CFU/ml^{-1} ($P > 0.05$) (Fig 2.8). This data was also used to determine the relationship of the tested PMAxx™ concentration on a range of CFU/ml^{-1}. Each tested PMAxx™ concentration and the negative control was plotted as a linear standard curve to show the PMAxx™ impact if any on producing standard curves with PMAxx™ treated samples. The relationship between CFU/ml^{-1} before and after treatment with PMAxx™ can be observed on Fig 2.8. All fitted linear standard curves had R^2 values of > 99.6 % (Fig 2.9).

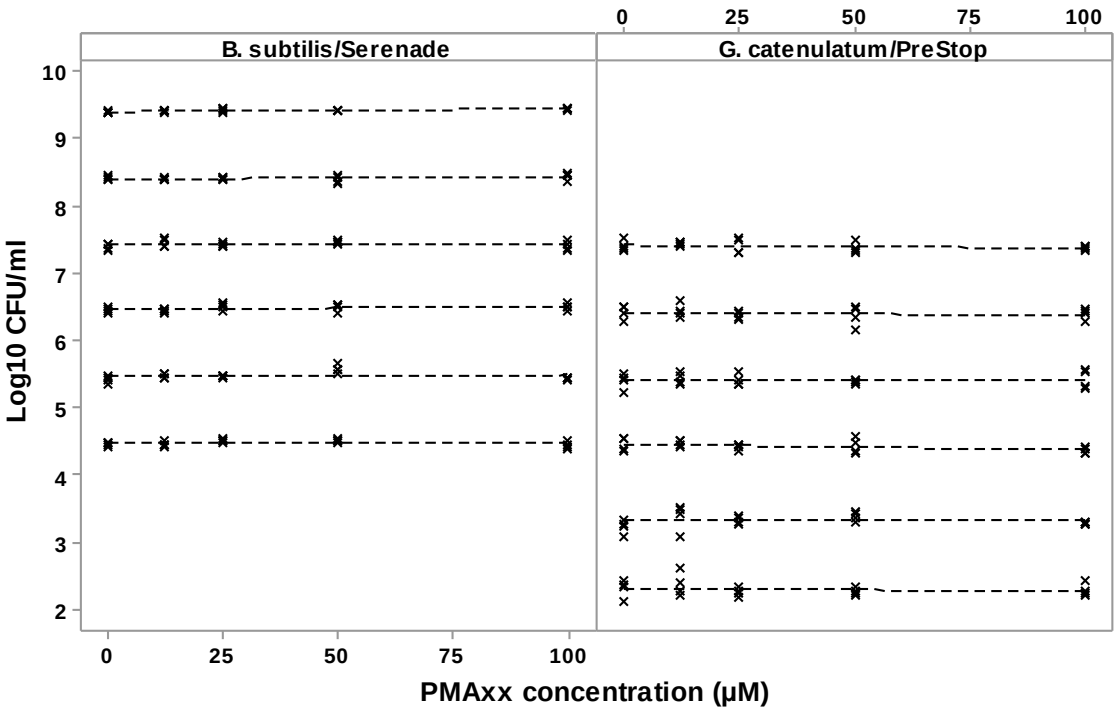


Figure 2.8 Effect of increasing PMAxx™ dose on viable plate counts.

In Fig 2.8 viable plate count technique determined viable CFU/ml^{-1} counts before (negative control 0 µM) and after PMAxx™ treatment (12.5 µM, 25 µM, 50 µM, and 100 µM). The 0 µM treatment of PMAxx™ was used in each cell concentration as a negative control. Cell concentrations were treated with 0 µM, 12.5 µM, 25 µM, 50 µM, and 100 µM of PMAxx™ followed by incubation for 15 mins and photo-activation (800 W) for 30 mins. Analysis of variance compared the means of CFU/ml^{-1} for each PMAxx™ concentration in each tested cell concentration, and suggested no negative difference in CFU/ml^{-1} ($P > 0.05$). The PMAxx™ concentration response with R^2 values for *B. subtilis* are between 0 % – 36 %, while for *G. catenulatum* ranged between 0 % – 16 %. Each biological replicate was plated four times to confirm CFU/ml^{-1}, and therefore each treatment combination contains four technical replicates. The standard error for each treatment was < 1.25.

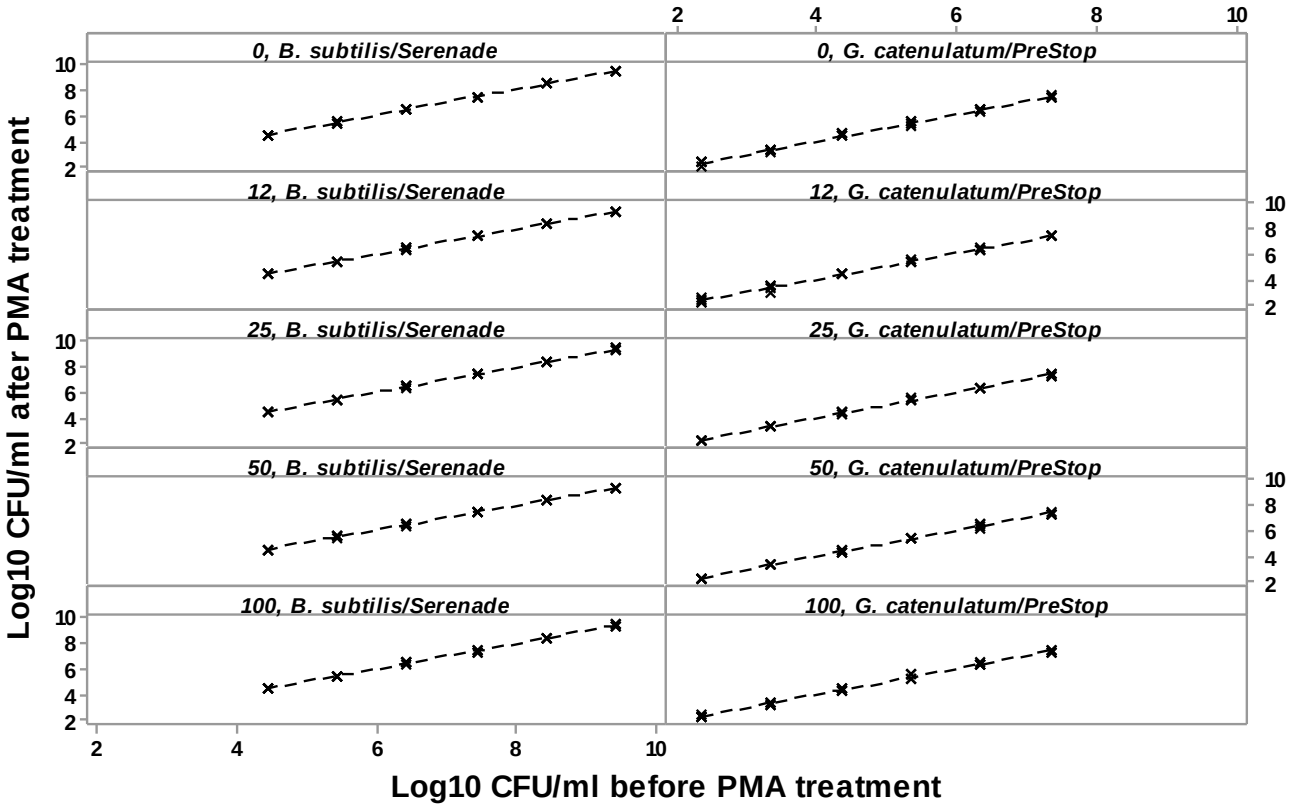


Figure 2.9 Linear standard curves of the relationship between CFU/ml^{-1} before and after PMAxx™ treatment.

In Fig 2.9 BCA cell concentrations were treated with 0 µM (negative control), 12.5 µM, 25 µM, 50 µM, and 100 µM PMAxx™ followed by incubation for 15 mins and photo-activation (800 W) for 30 mins. Viable plate count technique determined the viable CFU/ml^{-1} for all treatments. The graph has been categorized by organism/product and PMAxx™ dose, and a linear regression was fitted for each PMAxx™ dose. For all treatments the R^2 values for both BCAs are > 99.6 % suggesting the tested PMAxx™ concentrations have no negative effects on CFU/ml^{-1}. Each data point on the standard curve contains four replicates obtained from a single biological replicate. The standard error and deviations for the mean of each treatment group were < 1.25.

2.4.4 Sensitivity range of PMAxx™-qPCR on blocking DNA from dead cells

The data collected for investigating the effect of PMAxx™ on blocking DNA amplification from dead cells was pooled and arranged in relation to $\log_{10}$ total

amount of dead cells. To investigate assay sensitivity in blocking DNA from dead cells the ΔCt was calculated according to the formula below (Barbau-Piednoir et al., 2014).

$$\Delta\mathrm{Ct} = \mathrm{Ct\ value\ obtained\ without\ PMAxx™(Negative\ control)} - \mathrm{Ct\ value\ obtained\ with\ PMAxx™(Treatment)}$$

The formula above obtains a ΔCt value which translates into the signal reduction for the amount in change of cycles in qPCR. Therefore when ΔCt (signal reduction) was plotted against known amounts of dead cells the graph demonstrated the sensitivity range of the developed PMAxx™-qPCR molecular tool (Fig 2.10 and Fig 2.11).

The research pooled together the ΔCt from each PMAxx™ treatment concentration in all amounts of the tested total dead cells because (1) all concentrations with PMAxx™ blocked DNA amplification from dead cells at the qPCR stage significantly within all tested amounts of total dead cells ($P < 0.05$) (Section 2.4.1), (2) increasing the concentration of PMAxx™ from 12.5 µM was insignificant in changing the ΔCt at the same total dead cell concentration ($P > 0.05$) (Section 2.4.2), and (3) the tested PMAxx™ concentrations did not reduce the amount of CFU/ml^{-1} ($P > 0.05$) (Section 2.4.3). For *B. subtilis* the total amount of dead cells plotted in $\log_{10}$ units were 0.0001, 0.001, 0.01, 0.1 (serial dilution treatments), 1 (room temperature), 2.75 and 3.44 (heat treatments), while for *G. catenulatum* the total amount of dead cells in $\log_{10}$ units plotted were 0.021, 0.21, 1.21 (serial dilution treatments), 2.94 (room temperature), 5.75 and 6.22 (heat treatments). For both BCAs the increase in the amount of $\log_{10}$ total dead cells lead to the increase of ΔCt (signal reduction; $P < 0.05$) (Fig 2.10 and Fig 2.11). The overall mean ΔCt (signal reduction) to $\log_{10}$

mean of total dead cells was plotted in Fig 2.10 for *G. catenulatum* and Fig 2.11 for *B. subtilis*. Also the viable population reduction method of heat treatment has an independent effect of decreasing Ct in *B. subtilis* and increasing Ct of *G. catenulatum,* and in result impacts the ΔCt ($P < 0.05$).

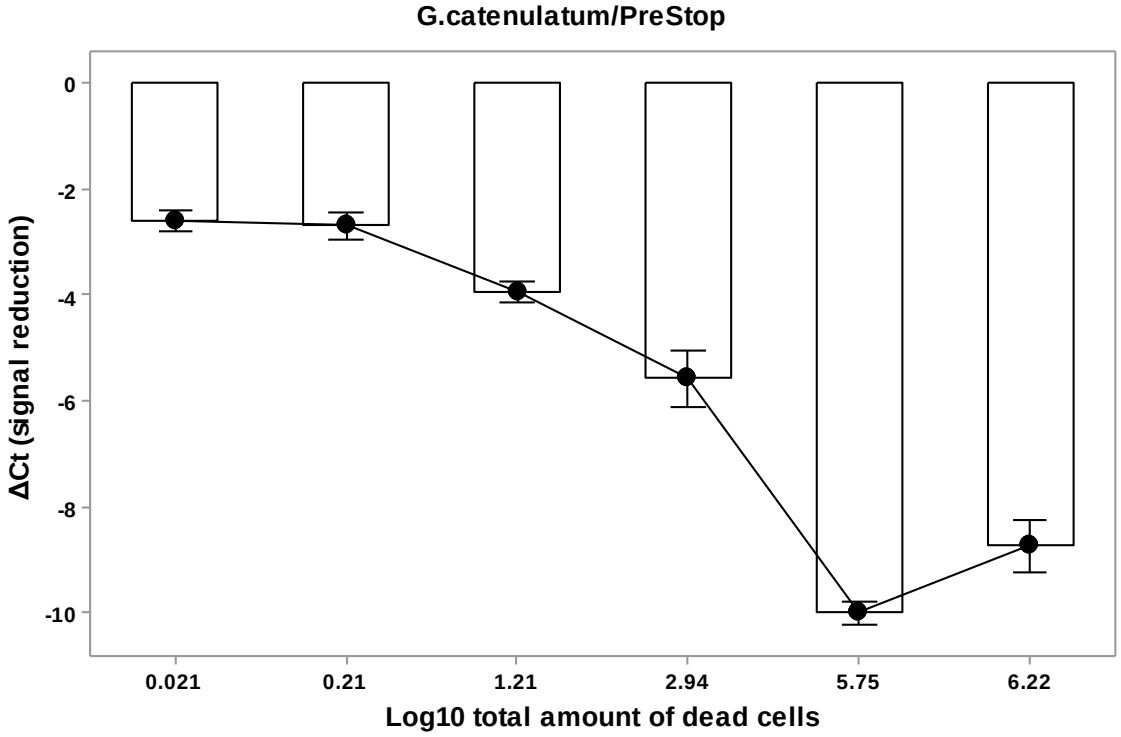


Figure 2.10 Interval plot of overall mean ΔCt (signal reduction) to overall mean log_{10} total dead cell amount of *G. catenulatum.*

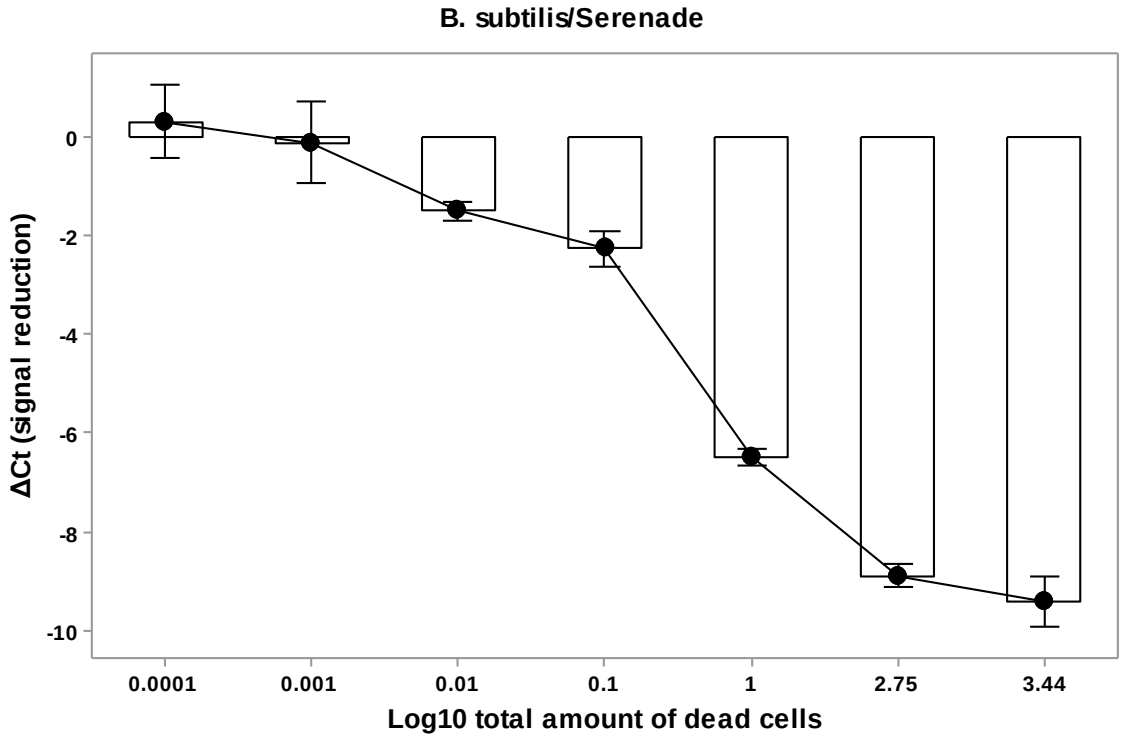


Figure 2.11 Interval plot of overall mean ΔCt (signal reduction) to overall mean log_{10} total dead cell amount of *B. subtilis.*

In Fig 2.10 and Fig 2.11 the overall mean ΔCt (signal reduction) of both BCAs were plotted against their overall mean log_{10} total amount of dead cells. For *B. subtilis* the total amount of dead cells plotted in log_{10} units were 0.0001, 0.001, 0.01, 0.1 (serial dilution treatments), 1 (room temperature), 2.75 and 3.44 (heat treatments), while for *G. catenulatum* the total amount of dead cells plotted in log_{10} units were 0.021, 0.21, 1.21 (serial dilution treatments), 2.94 (room temperature), 5.75 and 6.22 (heat treatments). The ΔCt (signal reduction) determined by PMAxx™-qPCR was represented by the mean of two biological replicates, and each replicate was repeated thrice on a qPCR plate for the following log_{10} mean total amounts of dead cells: for *B. subtilis* at 0.0001, 0.001, 0.01, 0.1 (serial dilution treatments), and for *G. catenulatum* at 0.021, 0.21, 1.21 (serial dilution treatment). The ΔCt (signal reduction) determined by PMAxx™-qPCR was represented by the mean of eight biological replicates, and each replicate was repeated thrice on a qPCR plate for log_{10} mean amounts of total dead cells for *B. subtilis* at 1 (room temperature), 2.75 and 3.44 (heat treatments), and for *G. catenulatum* at 2.94 (room

temperature), 5.75 and 6.22 (heat treatments). Log_{10} mean amounts of total dead cells for all treatments were determined from the difference between total cell counts on a haemocytometer and viable plate counts. The error bars represent the standard error.

In Fig 2.10 and Fig 2.11 the relationship of ΔCt (signal reduction) with the mean log_{10} amount of total dead cells for *B. subtilis* shows a blocking ability for the PMAxx™-qPCR tool of at least up to 3.44 log_{10} amount of total dead cells, while for *G. catenulatum* shows the blocking ability for the PMAxx™-qPCR was up to 5.75 log_{10} amount of total dead cells.

2.4.5 Phyllosphere quantification of BCA with the PMAxx™-qPCR technique

The method showed promise in blocking DNA quantification from dead BCA cells, and in result this experiment was initiated and set up independently. The PMAxx™-qPCR technique quantified the two BCA's from the phyllosphere of lettuce in two separate semi-commercial growing environments (polytunnel and glasshouse). Viable population of *B. subtilis* declined in both tested environments, yet the pattern of decline was rapid in the polytunnel and gradual in the glasshouse, and both contained episodes of viable population increase at day ten (Fig 2.12). Viable population change of *G. catenulatum* ensued in smaller numbers overtime regardless of viable population increase or decrease, and similar population patterns were observed in both environments (Fig 2.13). The use of Serenade ASO proved difficult to produce standard curves with the serial dilution method, and therefore the pure isolate *B. subtilis* QST 713 was utilised.

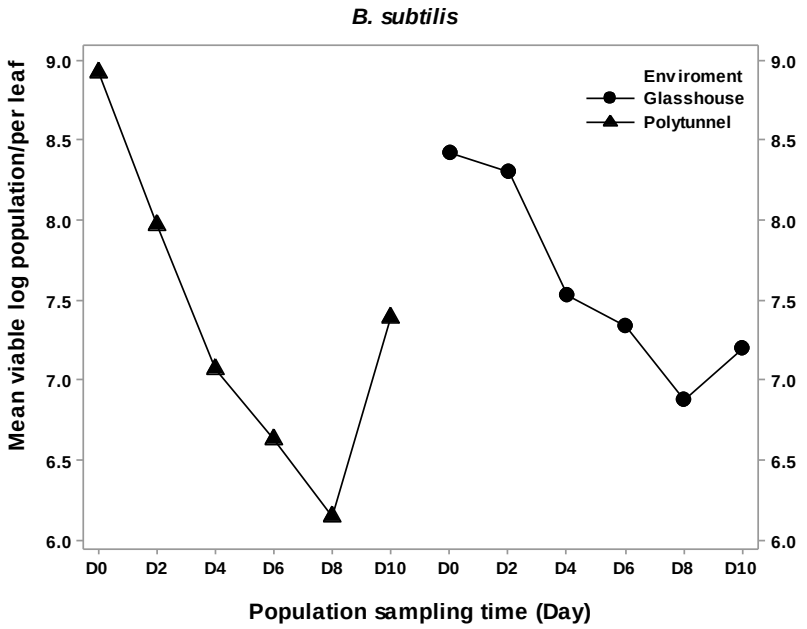


Figure 2.12 Temporal viable population of *B. subtilis* in a glasshouse and polytunnel

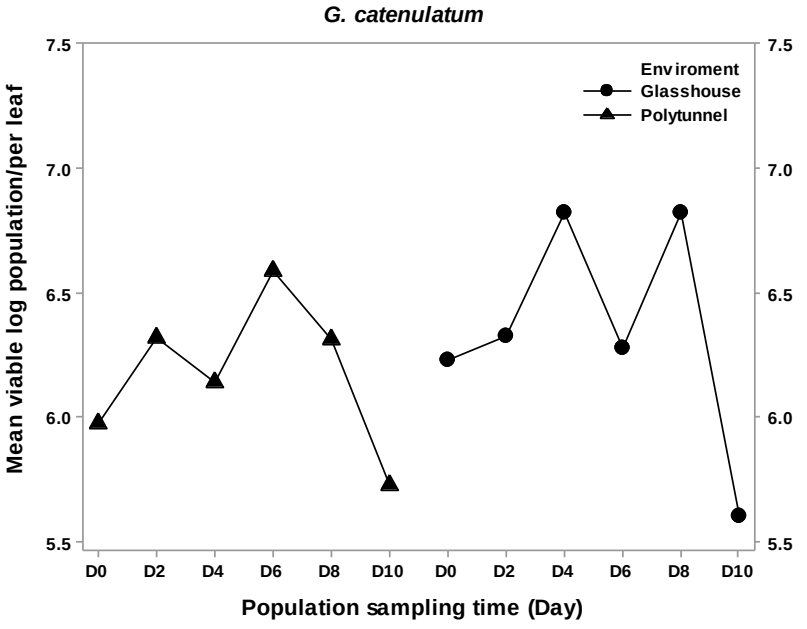


Figure 2.13 Temporal viable population of *G. catenulatum* in a glasshouse and polytunnel

In Fig 2.12 and Fig 2.13 for each sampling day the oldest single leaf from five lettuce plants were collected, suspended and agitated with maximum recovery diluent (Sigma), and then filtered with four layers of muslin cloth. Following this cell suspensions were PMAxx™ treated (incubation for 15 mins and photo-activation 800 W for 30 mins), after *G. catenulatum* cells were re-suspended into maximum recovery diluent and then ground. Post DNA extraction samples were filtered with Millex-VV syringe filter unit 0.1 µm and then quantified with qPCR to obtain the viable population. The standard errors were 1.25.

2.5 Discussion

PMAxx™-qPCR tools allows accurate monitoring of viable BCA populations because of its efficient blocking ability of DNA from dead cells, and the quantification of viable but non-culturable cells. This study focussed on selection of PMAxx™ dose to minimise inhibitory effects of PMAxx™ on DNA amplification of BCAs in their commercial formulation, and tested the limits of this method in blocking DNA from dead cells, in addition to determining the ability of PMAxx™-qPCR in blocking the quantification of DNA from dead BCA cells in the phyllosphere of lettuce. This method will allow research on the fate of two BCAs following their application in relation to climatic conditions, host development stages and other agronomic practices. Results from these studies may allow designing better formulations for improved BCA survival, and development of BCA application strategies to improve disease control efficacy.

Effective control of plant diseases requires better strategies for using BCAs because of limited availability of fungicides. To optimise the use of BCAs, we first need to quantify viable BCA populations in their natural environments. Thus, we developed the PMAxx™-qPCR methods for the two commercial BCAs, widely used to control diseases on vegetable and fruit crops. Traditional

methods (culture-dependent) and independent techniques (Trias et al., 2008, Turano and Pirali, 1988), quantitative PCR for bacteria (Braun-Kiewnick et al., 2012) , yeasts (Spotts et al., 2009) and moulds (Larena and Melgarejo, 2009) have been developed to monitor BCA survival and fate. The modern technique PMA-qPCR monitors viable population of BCAs *P. agglomerans* CPA-2 (Soto-Muñoz et al., 2014) and *Lactobacillus plantarum* PM411 (Daranas et al., 2018) on aerial plant surfaces. PMAxx™-qPCR was recently used for distinguishing dead cells from live within microbial ecosystems (Emerson et al., 2017). PMAxx™-qPCR sensitivity and specificity are among the best in the current available techniques.

One of key parameters in this method is the selection of PMAxx™ dose. Optimal PMAxx™ dose was selected based on several criteria, including elimination of amplification of dead cells, preservation of live cells, and reliable signal reduction. The tested dosages of 12.5 µM, 25 µM, 50 µM, and 100 µM had no toxic effects on population densities of *B. subtilis* (log_{10} 4 to 9 CFUs/ml^{-1}) and *G. catenulatum* (log_{10} 3 to 7 CFUs/ml^{-1}). A non-significant toxicity effect was found at 50 µM for *P. agglomerans* CPA-2 (Soto-Muñoz et al., 2014), whereas cytotoxic effect for *L. monocytogenes* was significant at 50 µM (Pan and Breidt, 2007). A study on *Legionella pneumophila* suggested minimal toxicity occurred at 100 µM whereas a 200 µM treatment was toxic and reduced viable cells by 0.4 log_{10} units (Yáñez et al., 2011). In the present research the lowest dose of PMAxx™ caused a plateau for both BCAs in all three different cell suspensions with different amounts of total dead cells; suggesting that exploration of low

PMAxx™ concentrations can be critical to identify the dose response relationship of dead cell densities to PMAxx™. Yet studies on *Legionella* biofilms identified no significant PMA (30 µM - 100 µM) effect of blocking DNA from dead cells (Taylor et al., 2014).

A critical parameter to consider is the matrix of the cells when dealing with formulated BCAs, due to the impact of the matrixes on DNA amplification efficiency and PMA uptake (Nkuipou-Kenfack et al., 2013). Formulated BCAs were amplifiable when filtration was used after DNA extraction, but obtaining appropriate standard Ct curves to quantify true population instead of relative values was unfeasible as the concentration of formulation additives (dyes and adjuvants) and their effect on the qPCR process were unknown. Also, there are no methods available for separating the BCA cells from formulation additives because the additives dyed the DNA of the organism, in addition to this companies were not willing to cooperate due to industrial proprietary of formulations. There was close correlation in estimated viable population sizes between PMAxx™-qPCR and viable plate count technique when tested in laboratory environments, this section of the research did not require further tests into correlation of PMAxx™-qPCR and viable plate count technique in an *in vivo* setting because the DNA markers used for each BCA were from published sources which had already established their ability for monitoring populations in different environments (Hertwig et al., 2015, Paavanen-Huhtala et al., 2000). Similar results were found on *E. coli, Staphylococcus aureus* and *L. monocytogenes* provided that similar population densities of dead

concentrations were used, and that storage as well as pre-conditioning of cells were minimised and if possible avoided as this impacted the quantified viable population (Martinon et al., 2012).

Some differences observed between PMAxx™-qPCR and viable plate count technique can be accounted for by the presence of viable but not culturable cells with compromised membranes that contain entry points in the cell membrane. In the present study, initial signal reductions from non-heat treated cell suspensions was confirmed by microscopic counts for both BCAs, as reported in previous studies (Seinige et al., 2014, Rudi et al., 2005). Signal reduction with presumed viable cell suspensions of both BCAs are possibly due to membrane compromised cells, particularly for *G. catenulatum* J1446 as freeze-drying process can cause distresses to microorganisms, possibly modify permeability of cytoplasmic membrane and damages the cell wall (Sinskey and Silverman, 1970, Abadias et al., 2000, Abadias et al., 2001). Liquid formulations also involve a form of cell drying and can contain dyes, additivities, and adjuvants; the effects of these additional factors on cell mortality, cytoplasmic membrane integrity and cell wall properties are unexplored and could explain *B. subtilis* QST 713 initial signal reduction (Abadias et al., 2003). PMAxx™ may bind DNA from damaged cells, while injured cells may repair their membrane and persist as viable but not culturable (Knight, 2000). Even though cell drying might impact permeability of the cell membrane, permeability differences alone was unlikely to lead to PMAxx™ infiltration, because PMAxx™ is cell membrane impermeable. Work on non-formulated strains of these two BCAs was more

promising for *B. subtilis* QST 713 and therefore ultimately selected for further research in the confirmation study of PMAxx™-qPCR on blocking DNA from dead *B. subtilis* cells on the lettuce phyllosphere. On the contrary the use of the non-formulated strain of *G. catenulatum* J1446 proved difficult to utilise for quantitative DNA extraction, however this might be the case because the pure isolate was cultivated from agar plates instead of being propagated in liquid suspension, and therefore the formulated version of *G. catenulatum* was used in the confirmation study of PMAxx™-qPCR on blocking DNA from dead *G. catenulatum* cells on the lettuce phyllosphere. In *G. catenulatum*'s case independent preliminary findings (data not shown) suggested the selected formulation type (dry) and possibly the choice of dye (white) were beneficial to the PMAxx™-qPCR process in comparison to using the pure isolate. The heat treatment method employed to increase the amount of dead cells may have also changed the amount of DNA readily extractable and quantifiable and therefore the Ct (Dashti et al., 2009). Because of this the amounts of dead cells tested could produce a lower signal reduction at the largest relative values of dead cells, since they were subjected to a longer period of thermal heat treatment. Therefore there is rational in using UV light treatment in reducing viable populations in future studies. Furthermore the excess amount of pre-present dead cells in formulated versions can be overcome with addition of excess viable cells from an independently cultivated source.

For the two BCAs, the developed tool can estimate viable population sizes by blocking the accessibility of DNA from dead cells, and in result eliminating there

amplification in the qPCR stage. Mean viable population mortality to mean signal reduction (non-PMAxx™ - PMAxx™ treated) increased on the log_{10} scale at least up to the value of 3.44 units for *B. subtilis,* and up to the 5.75 units for *G. catenulatum*. Mean of signal reduction was connected between the amounts of dead cells in the interval plots for representing the limit of PMAxx™ blocking ability being dependent on amount of dead cells present. The limit of dead cell quantification inhibition has also been reported for probiotic bacteria (Desfossés-Foucault et al., 2012) *L. plantarum* PM411 (Daranas et al., 2018) and *P. agglomerans* CPA-2 (Soto-Muñoz et al., 2014) but with varying ranges. In the present study, a mean difference of 10 cycles representing 3.44 log_{10} reductions for *B. subtilis* and 5.75 log_{10} reductions for *G. catenulatum* were found after heat treatment followed by a PMAxx™ treatment, and the experiments carried out on the phyllosphere of lettuce justified these findings since the quantified difference from mean maximum viable population and mean minimum viable population was around ~2.9 log_{10} units plus 1 log_{10} units of pre-present dead cells for *B. subtilis*, equalling a total of 3.9 log_{10} units. While for *G. catenulatum* (formulated) the quantified difference from mean maximum viable population and mean minimum viable population was around ~1.5 log_{10} units plus 2.94 log_{10} units of pre-present dead cells, equalling a total of 4.4 log_{10} units. For *G. catenulatum* the range up to 5.75 log_{10} units of total dead cells was unexplored because the conditions in the polytunnel and glasshouse were incapable of reducing the fungi's viable population completely. In comparison research on *P. agglomerans* CPA-2 had a maximum of 16 cycle difference

representing 10^5 CFU/ml^{-1} (Soto-Muñoz et al., 2014). Research on *S. aureus* identified that in population densities with excess dead cells the inhibition limit of DNA amplification from dead cells was a factor of 1000 (Schmidlin et al., 2010). The present developed PMAxx™-qPCR tools has a sensitivity range of 3.44 log_{10} reductions for *B. subtilis* and 5.75 log_{10} reductions for *G. catenulatum*; however, the true viable population may differ in formulations because they may contain heavy presence of inhibitors and may prevent correct estimation of the true viable population. But, for most ecological studies, we are really interested in the population changes over time or under different conditions. The inability of estimating the true viable population will not impact on such ecological studies. In agreement with similar studies (Elizaquível et al., 2012a, Martin et al., 2013), the present result suggested that 25 µM for *B. subtilis* and 50 µM for *G. catenulatum* are the optimal PMAxx™ concentration that can satisfy the criteria of eliminating amplification of dead cells, preserving live cells, reliable signal reduction, and overcoming possible formulation matrix effects.

2.6 Conclusions

This study developed the molecular tool PMAxx™-qPCR for quantifying viable population in commercial formulations, and in environmental samples for *B. subtilis* QST 713 and *G. catenulatum* J1446. The method is a valuable tool for studying ecological fate of BCAs under natural environment to optimise their use for disease management.

CHAPTER 3

3 Dynamics of the two biocontrol agents for efficacy against *B. cinerea in vitro* and *in vivo* in relation to different abiotic factors using dose response models

3.1 Abstract

The study evaluated the dose response relationship of the biocontrol agent's *B. subtilis* and *G. catenulatum* against a high inoculum dose of *B. cinerea* and developed dose response models (probit and polynomial) to identify the LD_{50} efficacy both *in vitro* and *in vivo.* Inoculum doses, formulation, temperature and foliar leaf part affected the control achieved by the two BCAs based on the efficacy dose models. *In vitro* competition assays on modified PDA plates tested a range of doses (log_{10} 3 – 10 CFUs or spores/droplet) at 4 °C, 10 °C and 20 °C on the development of *B. cinerea* colonization. This suggested that the dose response relationship was influenced by BCA formulation and temperature. *In vivo* studies in semi-commercial greenhouses on lettuce plants examined the BCA dose (log_{10} 5 – 9 CFUs or spores/ml^{-1}) for controlling a high inoculum pressure of *B. cinerea* (log_{10} 6 spores/ml^{-1}). Using leaf disc assays, this showed that the dose response relationship changed with leaf parts analyzed.

3.2 Introduction

B. subtilis, a gram positive *Rhizobacterium* produces endospores for survival and lipo-peptides (iturins, fengycins, surfactins) for plant colonization, induction

of plant defense responses and control of plant pathogens (Ongena et al., 2007). The ability of *B. subtilis* to compete for space and nutrients is important for survival and colonization of plants (Hibbing et al., 2010). Serenade ASO a globally used broad spectrum bio-fungicide contains *B. subtilis* strain QST 713 and is registered in Europe to target *B. cinerea* in lettuce and strawberry crops (Fischer et al., 2013). *Gliocladium catenulatum* a saprophytic filamentous fungi that survives globally in organic matter and as a endophyte in roots and stems, is rhizosphere-competent and reportedly pathogenic against a range of fungal plant pathogens (Helyer et al., 2014). *G. catenulatum* has been suggested to be parasitic to fungal pathogens (Huang, 1978), destroying hyphal cells (McQuilken et al., 2001), produces enzymes (chitinase and β-1,3-glucanases) for hydrolysis of fungal cell walls (Lahdenpera and Korteniemi, 2005), and is effective at competing for space and nutrients on plants (Chatterton et al., 2008). PreStop a broad spectrum bio-fungicide contains conidia and mycelium of *G. catenulatum* strain J1446 and is registered in Europe for targeting *B. cinerea* in lettuce and strawberry crops.

Probit models characterise dose response relationships based on lognormal probability distribution, and assume efficacy of a given organism is smallest at low densities, maximum at intermediate densities, and plateaus or decreases at high densities. This can be used to develop a probabilistic model for biocontrol based on the theory that the pathogen population decreases in response to the inoculum density of the BCA. Key parameters which need to be taken into account are the probability of host infection and pathogen activity in the

presence of the BCAs (Montesinos and Bonaterra, 1996). Probit models for defining dose response relationships for these two BCAs have not been previously developed. They would be very valuable to obtain information on the effective dose for reduction of the pathogen by 50 % (LD_{50}) and the relationship between efficacy parameters in the context of the BCA inoculum density. This would be beneficial in also evaluating the impact of external factors (i.e. formulation, temperature, relative humidity and leaf parts) on the dose response relationships and inoculum dose factors. This Chapter addresses these key questions by attempting to unravel the dynamics of inoculum dose of these two BCAs at a high pathogen pressure in the context of fluxes in abiotic and biotic factors.

The objective of the research was to evaluate the dose response relationships and inoculum dose features of these two BCAs against high inoculum pressure of *B. cinerea* under different abiotic (temperature and formulation) and biotic (leaf part) factors. The intention was to establish if *B. subtilis* QST 713 and *G. catenulatum* J1446 biocontrol dynamics are influenced by abiotic and biotic factors.

3.3 Material and Methods

3.3.1 Isolation and culture conditions of *B. subtilis* QST 713, *G. catenulatum* J1446 and *B. cinerea*

Serenade ASO (Serenade) and PreStop were purchased from Fargro Ltd. Serenade was an aqueous formulation and contained *B. subtilis* strain QST 713, and PreStop was a dry formulation and contained *G. catenulatum* strain

J1446. For both bio-fungicides culturing conditions, growth, and formulation components were unknown due to industrial proprietary. Serenade was stored at room temperature, while PreStop was stored in a cool dry location below 8 °C, and once opened, frozen at - 20 °C. The batches used were less than 6 months old. Each BCA was isolated from their formulations and grown *in vitro* on culture media. Serenade was serially diluted thrice into maximum recovery diluent (Sigma) and 10 µL was spread-plated onto nutrient agar, and incubated at 30 °C for three days. Concentrates were produced by collecting the bacterial colonies on the media into maximum recovery diluent solution and transferring the supernatant (*B. subtilis* suspended into maximum recovery diluent) onto the next plate for repeating the process. The concentrate was stored at 20 °C. One gram of PreStop was mixed with 200 ml of maximum recovery diluent, shaken vigorously for 15 secs; serially diluted twice and 10 µL plated, and then spread-plated on malt extract agar, and incubated at 22 °C for 10 days. Concentrates were produced by collecting the surface fungal growth into maximum recovery diluent solution and transferring the supernatant (*G. catenulatum* suspended into maximum recovery diluent) onto the next plate and repeating the process. The hyphae and mycelium were separated from the macroconidia in the suspension by filtration (Whatman 25 µ).

For *in vitro* dual culture assays three single-spored *B. cinerea* isolates were isolated from separate strawberry fruits (cultivar Elsanta) infested with grey mould at the commercial site of Driscoll's on the same day, and plated on potato dextrose agar, followed by incubation at 20 °C for 10 days in the dark.

The fungal growth from each plate was placed into the same beaker, and mixed with maximum recovery diluent solution, and stored at 4 °C. From the mixed isolate suspension, 200 µL was plated onto PDA then spread-plated, followed by incubation for 10 days at 20 °C in the dark. For *in vivo* plant experiments four isolates of *B. cinerea* were collected from lettuce (Cos Romia, little gem, Lollo Verdi, and Apolo) infested with grey mould from the field of Laurence J Betts in West Malling, Kent, and three *B. cinerea* isolates were collected from lettuce (Carter) infested with grey mould in a semi-commercial glasshouse at NIAB-EMR. Collected isolates were isolated on potato dextrose agar and incubated at 20 °C for 10 days of 8 h light / 16 h dark cycles. For acquiring conidia from *B. cinerea* cultures 10 mls of sterile distilled water was added to the surface and the *Botrytis* culture was agitated for conidial release. Supernatants were collected, filtered (Whatman 25 µ), serially diluted, and confirmed with microscopic counts to contain macroconidia concentrations of about $\log_{10}$ 6 spores/ml^{-1}. The first experiment used all three Carter isolates, while the second experiment used all the lettuce isolates, using a mixture of spores combined as a mixed single inoculum.

3.3.2 Dual culture co-inoculations

The viable plate count technique was used to confirm BCA dosage (Serenade/ *B. subtilis,* PreStop/*G. catenulatum*). Serenade and *B. subtilis* tests were performed on mixed media of 50 % potato dextrose agar and 50 % nutrient agar, while PreStop and *G. catenulatum* tests were performed on 50 % potato dextrose agar and 50% malt extract agar. Testing was carried out in a single

vent 90 mm × 16.2 mm Petri dish, and the inoculum doses tested were between $\log_{10}$ 3 to 10 CFUs for Serenade and spores for PreStop per droplet in the formulation. For non-formulated strains the concentrations used were between $\log_{10}$ 3 to 9 CFUs for *B. subtilis* and spores for *G. catenulatum* per droplet. Each dosage contained the same population density. A modified dual culture technique (Landa et al., 1997) was used, in which an agar plug of 34 mm in diameter was removed with a surface sterilised cork borer, and then re-plugged with a mycelial plug of the same size containing both the agar and the *Botrytis* fungal layer. In succession the BCA droplet was applied. Both the plug site of the pathogen, and the application site of the BCA droplet in the Petri plate were opposite to each other, in addition to being 1 cm away from the edge of the plate (Fig 3.1). The plates were sealed with parafilm and incubated at 4 °C, 10 °C or 20 °C for 7 days in the dark. The positive control contained the *B. cinerea* mycelial plug only, and the negative control contained no microorganisms. After 7 days, images of dual cultures and positive controls were obtained and analysed with image J to calculate the *B. cinerea* mycelial area colonised.

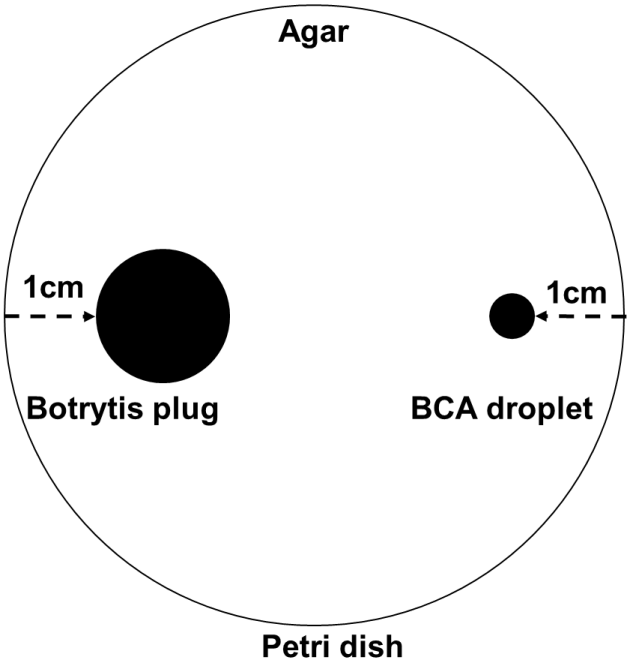


Figure 3.1 Plate design of *in vitro* dual culture co-inoculations

Fig 3.1 is not to scale, and this modified dual culture assay design was used in all tested dosages for both BCAs. The Botrytis plug size was 34 mm in diameter and constant across treatments. The BCA droplet size changed in relation to the BCA dosage treatment.

The BCA efficacy was calculated by the inhibited area of *Botrytis* mycelial colonisation using the formula below. The formula includes: efficacy (E, %), *Botrytis* mycelial colonisation of positive control (C) and *Botrytis* mycelial colonisation with the BCA treatment (T).

$$(E, \%) = [(C - T) / C] \times 100$$

Probit analysis was completed by transforming the efficacy data to probit values using the probit table to obtain the LD_{50} (Finney and Tattersfield, 1952). The dosage of BCA for obtaining 50 % inhibition (LD_{50}) of *B. cinerea* mycelial colonisation was estimated for each temperature. Standard errors of the LD_{50}

were calculated, and they were separated with a Two-Sample T-Test at a significance of P=0.05. The randomised block design contained ten experiments, up to ten replicates per treatment, and three pseudo repeats per replicate.

3.3.3 Preventive effect of BCAs against *B. cinerea* on older lettuce leaves

Lettuce cultivar Carter was obtained from Premier plants, propagated into standard compost in independent pots (9cm x 9cm x 10cm), and placed in a semi-commercial pest and disease free glasshouse until early head development. During experimentation lettuce plants were hand watered daily, and the glasshouse temperature and relative humidity ranged between 17°C – 22°C and 60 % - 95 % respectively. Refer to Section 3.3.1 for *B. cinerea* isolates used.

3.3.3.1 Inoculation and disease evaluation

The viable plate count technique was used to confirm the BCA dosage, and the previously developed PMAxx™-qPCR technique (see Chapter 2) was used to confirm differences between dosages on older leaves. At the early head development of the lettuce plants they were spray-inoculated with the BCA (treatment) and sterile distilled water for negative and positive controls. Dosages tested were $\log_{10}$ 5, 6, 7, 8 and 9 CFU/ml^{-1} for Serenade and spores/ml^{-1} for PreStop. After a BCA settling period of 4 h, plants were spray inoculated with *B. cinerea* (macro conidial dosage of $\log_{10}$ 6 spores/ml^{-1}), apart from the negative control which was sprayed with sterile distilled water once

more. After 48 h, older leaves of similar size and shape were collected, and surface sterilized. Sterilization started with a wash under a slow running tap (cold), followed by a 1 min wash in tween 80 solution (1 drop in 200mls sterile distilled water), re-washed with sterile distilled water twice, then surface sterilized with a wash in 70% ethanol for 1 min, and residual alcohol was removed by washing twice in sterile distilled water, followed by leaf drying under a fume hood for 2 h. From each older lettuce leaf a total of ten leaf discs (10 mm diameter) were obtained and leaf parts included the apex (1 disc), midrib (3 discs) and lamina with lateral veins (6 discs), and disc location selection depending on the leaf size. The discs were placed on potato dextrose agar at an equal distance from each other (0.75 cms), the plate was sealed with parafilm and incubated at 20 °C for 10 days in the dark. After the incubation period the leaf discs were assessed for incidence of disease and or symptoms (i.e. lesions, hyphae and necrosis). The BCA efficacy was calculated by the incidence of diseased leaf discs and using the following formula: the efficacy (E, %), mean diseased of positive control (C), diseased of treated (T).

$$(E, \%) = [(C - T) / C] \times 100$$

Probit analysis was completed by transforming the proportion of uninfected leaf discs data (non-diseased leaf discs) to probit values using the probit table to obtain the LD_{50} inoculum levels (Finney and Tattersfield, 1952). The dosage of the BCA for obtaining 50 % inhibition (LD_{50}) of *B. cinerea* conidial infection was estimated for the whole leaf and for each leaf disc. Standard errors of the LD_{50} were calculated, and the LD_{50} were separated according to the independent leaf

disc and leaf part with Paired Sample T-Test at a P=0.05 significance level. The randomised block design contained two experiments, a total of ten replicates per treatment, and two pseudo repeats per replicate.

3.3.4 Data analyses

Genstat (version 18) and MiniTab (version 17) were used for data analyses. A polynomial regression model was fitted to the data to estimate the efficacy relationship between dosage of BCA (Serenade/*B. subtilis*/ PreStop/*G. catenulatum*) and *B. cinerea* mycelial colonisation inhibition on an agar plate at 4 °C, 10 °C and 20 °C. As a preliminary test accumulated analysis of deviance was used to determine effect of experiment × dose × leaf position on proportion of uninfected leaf discs and significance evaluated at P=0.05 level. Both experiments were combined for the whole leaf analysis in defining the dose response relationship and LD_{50}, but due to experimental differences ($P < 0.05$) were not used for comparison of dose response relationship and LD_{50} between leaf disc positions and parts. For all statistical analysis dosages were log-transformed, efficacy was probit transformed, and analysis was performed at a P=0.95 confidence level.

3.4 Results

3.4.1 Effect of BCA inoculum dose, formulation and temperature on *in vitro B. cinerea* control

3.4.1.1 (a) *In vitro* efficacy of the BCAs on *B. cinerea* control

Findings (Figure 3.2, Figure 3.3 and Table 3.1) suggested that inhibition of *B. cinerea* mycelial colonisation occurred in all tested temperatures with each

BCA, and was positively correlated with dosage. However the control efficacy of each BCA and its control parameters (mean, maximum, minimum, range) directly depended on inoculum dose, temperature and formulation type (formulated or not). Formulated strains mainly retained greater efficacy at each temperature and inoculum dose (see Table 3.1). The efficacy range was lowest (0 – 20 %) in $\log_{10}$ inoculum dosages of < 6 CFU or spores/droplet, and intermediate (30 - 50 %) in inoculum dosages of 7 – 9 CFU or spores/droplet. For the highest inoculum dosages tested with Serenade and PreStop, 10 CFU or spores/droplet resulted in the highest efficacy of 96% for Serenade and 91% for PreStop (see Table 3.1).

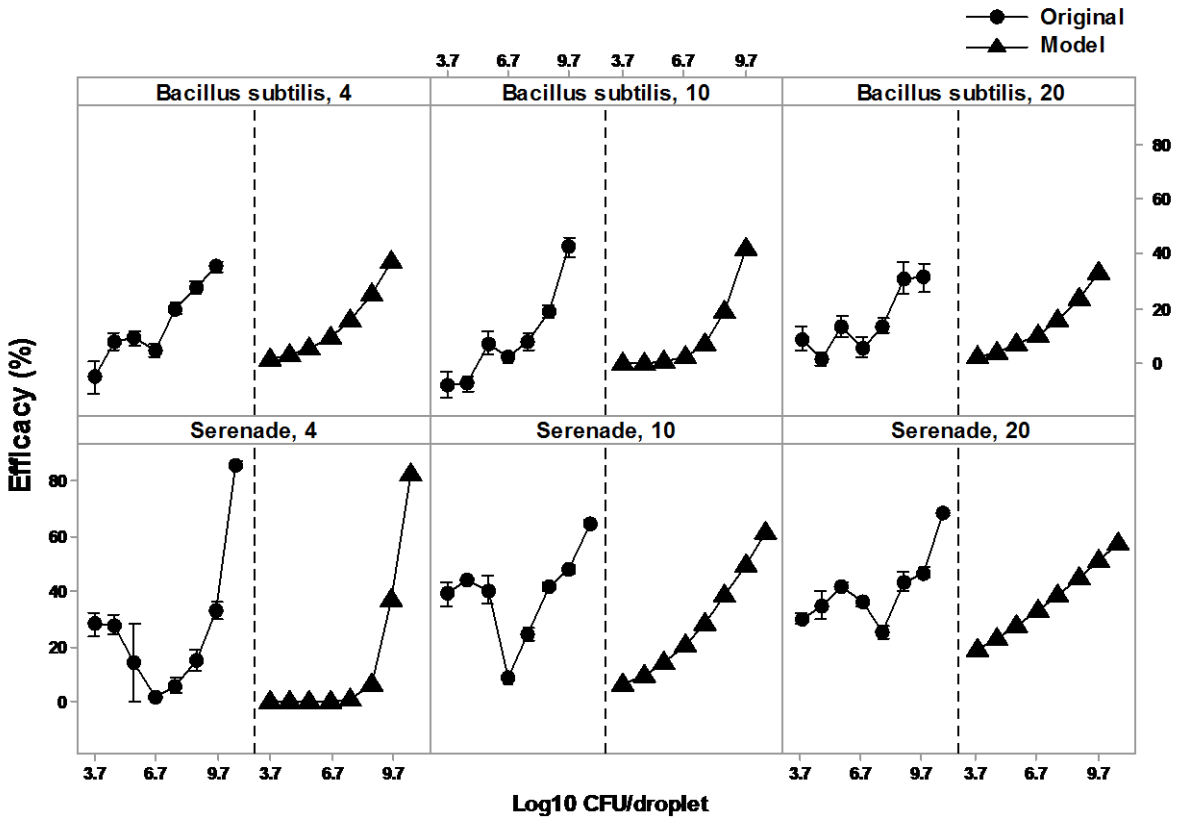


Figure 3.2 *In vitro* competition assays of original and fitted model efficacy curves of *B. subtilis* and Serenade inoculum doses inhibiting a large *B. cinerea* mycelial plug at 4°C, 10°C and 20°C.

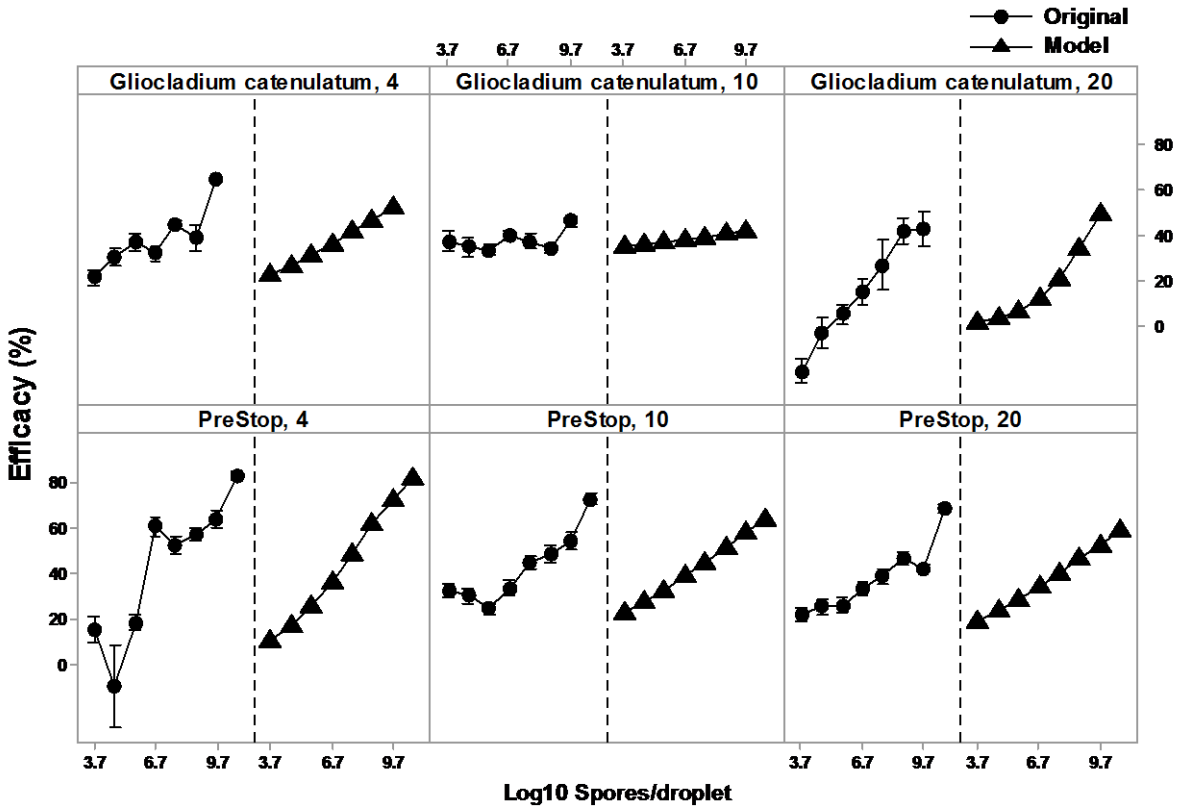


Figure 3.3 *In vitro* competition assays of original and fitted model efficacy curves of *G. catenulatum* and PreStop inoculum doses inhibiting a large *B. cinerea* mycelial plug at 4°C, 10°C and 20°C.

In Fig 3.2 and Fig 3.3 the tested BCA doses are presented as log-transformed values. Tested BCA inoculum doses ranged from $\log_{10}$ 3.7 – 10.7 CFU or spores/droplet for the formulated strains, and 3.7 – 9.7 CFU or Spore/droplet for non-formulated strains. All experiments were carried out for 7 days. The quantitative summaries for original and fitted model efficacies were obtained from up to ten replicates per BCA inoculum dose. Black circles represent the mean for original efficacy data, while black triangles represent the mean for the model data, and error bars represent standard errors of the mean. The competition assays were carried out on a 50 % potato dextrose agar and 50 % nutrient agar for *B. subtilis* and Serenade, and on a 50 % potato dextrose agar and 50 % malt extract agar for *G. catenulatum* and PreStop at 4 °C, 10 °C and 20 °C for 7 days.

Table 3.1 Efficacy description of BCA doses for *in vitro* competition assays

BCA	Temp(°C)	Log_{10} CFUs or Spores / droplet	Mean Efficacy (%)	SE mean	Maximum Efficacy (%)
QST 713	4	3.6	-5	5.89	32
QST 713	10	3.6	-8	4.72	12
QST 713	20	3.6	9	4.43	30
J1446	4	3.6	22	3.24	35
J1446	10	3.6	38	4.00	49
J1446	20	3.6	-20	5.37	-9
PreStop	4	3.6	16	5.56	32
PreStop	10	3.6	33	2.80	46
PreStop	20	3.6	22	2.68	34
Serenade	4	3.6	28	4.17	35
Serenade	10	3.6	40	4.36	46
Serenade	20	3.6	31	2.10	34
QST 713	4	4.6	8	2.98	30
QST 713	10	4.6	-7	2.69	7
QST 713	20	4.6	1	2.39	20
J1446	4	4.6	31	4.09	45
J1446	10	4.6	36	4.35	48
J1446	20	4.6	-3	6.66	18
PreStop	4	4.6	-9	18.44	26
PreStop	10	4.6	30	3.50	47
PreStop	20	4.6	26	3.46	48
Serenade	4	4.6	28	3.44	34
Serenade	10	4.6	44	0.79	45
Serenade	20	4.6	35	4.81	42
QST 713	4	5.6	9	2.96	26
QST 713	10	5.6	7	4.33	28
QST 713	20	5.6	14	3.91	35
J1446	4	5.6	38	3.96	49
J1446	10	5.6	34	2.40	44
J1446	20	5.6	6	4.34	26
PreStop	4	5.6	19	3.07	30
PreStop	10	5.6	25	2.35	33
PreStop	20	5.6	26	3.20	40
Serenade	4	5.6	14	14.34	43
Serenade	10	5.6	41	5.16	49
Serenade	20	5.6	42	1.61	45
QST 713	4	6.6	5	2.32	28
QST 713	10	6.6	2	1.74	16
QST 713	20	6.6	6	3.69	63
J1446	4	6.6	32	2.92	43
J1446	10	6.6	41	1.96	54
J1446	20	6.6	15	5.65	47
PreStop	4	6.6	61	4.45	79
PreStop	10	6.6	34	3.12	47
PreStop	20	6.6	34	2.77	49
Serenade	4	6.6	2	0.71	7
Serenade	10	6.6	9	2.03	24
Serenade	20	6.6	37	1.80	53
QST 713	4	7.6	20	2.01	30

QST 713	10	7.6	8	3.19	24
QST 713	20	7.6	14	2.92	24
J1446	4	7.6	46	1.45	51
J1446	10	7.6	38	3.17	48
J1446	20	7.6	27	10.60	48
PreStop	4	7.6	53	3.93	69
PreStop	10	7.6	45	2.98	58
PreStop	20	7.6	39	3.29	49
Serenade	4	7.6	6	2.77	26
Serenade	10	7.6	25	2.07	35
Serenade	20	7.6	25	2.46	49
QST 713	4	8.6	28	2.12	42
QST 713	10	8.6	19	2.50	28
QST 713	20	8.6	31	5.86	56
J1446	4	8.6	40	5.71	62
J1446	10	8.6	35	1.93	38
J1446	20	8.6	43	5.81	53
PreStop	4	8.6	58	2.93	69
PreStop	10	8.6	49	3.64	68
PreStop	20	8.6	47	2.73	58
Serenade	4	8.6	15	3.95	38
Serenade	10	8.6	42	1.67	52
Serenade	20	8.6	44	3.56	59
QST 713	4	9.6	35	1.78	46
QST 713	10	9.6	43	3.61	55
QST 713	20	9.6	31	5.06	55
J1446	4	9.6	65	0.60	66
J1446	10	9.6	47	2.19	54
J1446	20	9.6	43	8.15	73
PreStop	4	9.6	64	4.01	81
PreStop	10	9.6	55	3.77	68
PreStop	20	9.6	43	1.36	50
Serenade	4	9.6	33	3.05	51
Serenade	10	9.6	49	1.53	60
Serenade	20	9.6	47	2.01	64
PreStop	4	10.6	83	1.83	91
PreStop	10	10.6	73	2.60	89
PreStop	20	10.6	69	2.12	78
Serenade	4	10.6	87	1.25	96
Serenade	10	10.6	65	0.93	72
Serenade	20	10.6	69	0.87	75

QST 713 refers to the BCA *B. subtilis* and J1446 refers to the BCA *G. catenulatum* in pure isolate form.

3.4.1.2 (b) Effect of formulation and temperature on *in vitro* LD_{50} values

Table 3.2 shows the effect of interactions between the BCA treatments and abiotic factors of temperature and formulation on the LD_{50} values. Temperature was shown to influence the LD_{50} of *B. subtilis* in pure isolate ($P < 0.05$) and formulated *G. catenulatum* ($P < 0.05$) treatments. Formulation influenced the

LD_{50} of *B. subtilis* ($P < 0.05$). The combination and interaction effect of both formulation and temperature on LD_{50} was found for both BCAs ($P < 0.05$). Comparison between LD_{50} values was achieved with the Two-Sample T-Test at a significance level of P=0.05.

Table 3.2 Determined LD_{50} values of BCAs at 4 °C, 10 °C and 20 °C

Compound	Temp(°C)	Log_{10} LD_{50} CFU/droplet	Standard Error	Compound	Temp(°C)	Log_{10} LD_{50} Spores/droplet	Standard Error
QST 713	4	10.4	0.602	**J1446**	4	9.09	1.429
Serenade	4	9.9	0.125	**PreStop**	4	7.6	0.358
QST 713	10	9.8	0.186	**J1446**	10	7.7	0.408
Serenade	10	9.6	0.187	**PreStop**	10	8.3	0.305
QST 713	20	11	0.456	**J1446**	20	9.6	0.282
Serenade	20	9.4	0.363	**PreStop**	20	9.1	0.38

3.4.2 PMAxx™-qPCR

The PMAxx™-qPCR technique was applied to establish the inoculum dose differences in each treatment after application onto the phyllopshere. For PreStop the mean Ct for the applied log_{10} inoculum doses were 30.71 for 5, 30.11 for 6, 29.12 for 7, 28.50 for 8, and 24.75 for 9 spores/ml^{-1}, and the STD error for each mean was < 0.5 while the STD deviation of each mean was < 1.1 excluding the log_{10} 9 spores/ml^{-1} inoculum which produced 1.63. The relationship of Ct to *G. catenulatum* inoculum dose was linear up to log_{10} 8 spores/ml^{-1}, and 10 fold more cells produced one extra qPCR cycle. For Serenade as the concentration was serially diluted to produce the desired inoculum dose the Ct decreased; this possibly occurred because the formulation may have contained qPCR inhibiting dyes, additive and/or

adjuvants, which were also diluted with the serial dilution method, but to pinpoint the true cause the formulation components must be known. Therefore the PMAxx™-qPCR method could not be used to compare formulations with different inoculum doses using the serial dilution method for Serenade.

3.4.3 *In vivo* dose response relationship and inoculum dynamics of the BCAs on *Lactuca sativa* leaves under high *B. cinerea* inoculum pressure

The effect of inoculum dose and interaction with leaf part on relative proportion of uninfected leaf discs were investigated with an Accumulated analysis of deviance; and independently inoculum dose ($P < 0.01$), and leaf disc part ($P < 0.01$) affected the relative proportion of uninfected leaf discs, but lacked a combination effect with leaf disc position and part (P 0.215). Considering this, data from both experiments were united to define the overall dose response relationship and inoculum dose features for the whole lettuce leaf (Fig 3.4, Fig 3.5, Fig 3.6 and Table 3.3).

Figure 3.4 shows the effect of Serenade and PreStop application on lettuce leaf discs inoculated with *B. cinerea*. As inoculum dose of BCA was increased the control level achieved improved. On occasion complete control (100%) of *B. cinerea* was achievable with the highest inoculum dose of $\log_{10}$ 9.7 CFUs or spores/ml^{-1}. The efficacy was consistent in both experiments against a high inoculum of the pathogen (6 spores/ml^{-1}). For both BCAs the inoculum doses used gave significant control (P=0.01).

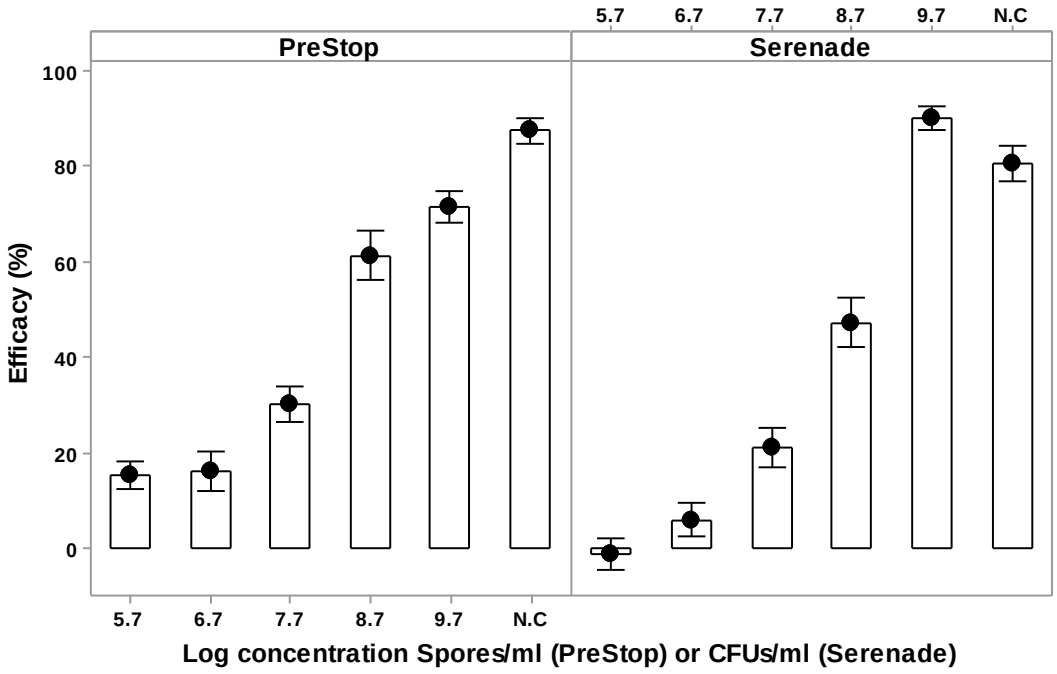


Figure 3.4 Interval plots for preliminary dose response *of B. cinerea* to BCAs on *L. sativa* leaf discs obtained from older lettuce leaves in the glasshouse at 17 °C - 22 °C with a RH range of 60 % - 100 %

For Fig 3.4 leaf disc assays were incubated on potato dextrose agar at 20 °C for 10 days and the infected leaf discs were counted out of a sample population of two hundred per dosage for both experiments. Efficacy was calculated in relation to the equation in subsection 3.3.3.1$(E, \%) = [(C - T) / C] \times 100$. The mean efficacies per dosage are represented as black circles, the error bars represent standard errors around each mean. The N.C on the X axis stands for the negative control, and the BCA dosages applied are shown as $\log_{10}$-transformed values.

Table 3.3 provides the estimated LD_{50} inoculum doses for *B. cinerea* control. Highest dosage of $\log_{10}$ 9.7 CFU or spores/ml^{-1} led to a higher mean amount of proportion of uninfected leaf discs > 90% compared to the negative control for Serenade (Fig 3.5), while for PreStop reached the highest mean amount of

proportion of uninfected leaf discs 80 % (Fig 3.6). For both BCAs a minimum inoculum dose of log_{10} 8.7 CFU or spores/ml^{-1} was needed to reach and/or surpass the LD_{50}, and a clear sigmoidal dose response relationship was displayed.

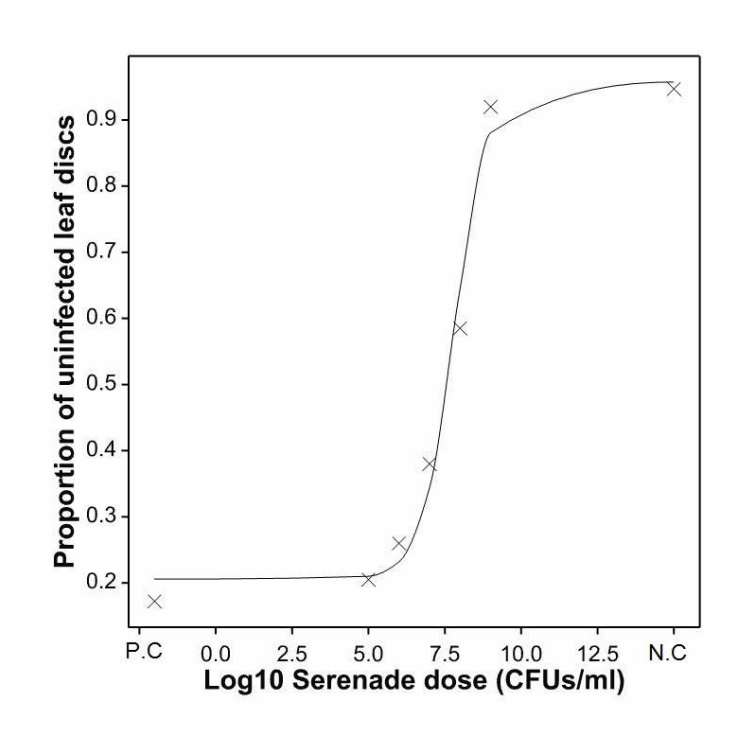


Figure 3.5 Probit modelled dose response curve of *B. cinerea* infection to Serenade inoculum concentration

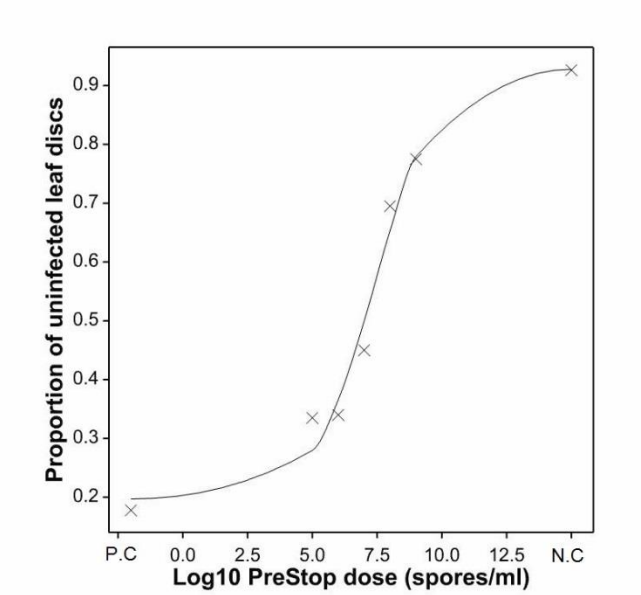


Figure 3.6 Probit modelled dose response curve of *B. cinerea* infection to PreStop inoculum concentration

In Fig 3.5 and Fig 3.6 the Probit analyses in Genstat were performed on the proportion of uninfected leaf discs. The X axis labelling contain: log_{10}-transformed applied dosages of 5.7, 6.7, 7.7, 8.7 and 9.7 CFU or spores/ml^{-1}, the positive control response (P.C) containing only Botrytis treatment, and the high dose immunity negative control response (N.C) containing only sterile distilled water illustrated the proportion of population with no response which are not protected against *B. cinerea*. This data was combined with the slope of the logit curve. The model assumes at a BCA log_{10} dosage of 15 (negative control) labelled as N.C high dose immunity levels are obtained. A non-linear model with binominal distribution using a logit link was fitted. For Fig 3.5 the disarray in the dose response curve complicated interpretation and was due to highest dosage of log_{10} 9 CFU/ml^{-1} containing higher proportion of uninfected leaf discs in contrast to the negative control.

Table 3.3 The determined LD_{50} of Serenade and PreStop against a high inoculum dose *of B. cinerea on L. sativa* leaf discs obtained from older lettuce leaves in the glasshouse at 17 °C - 22 °C with a RH range of 60 % - 100 %

BCA	Log_{10} LD_{50} Estimate CFU or Spores/ml^{-1}	S.E.	lower 95%	upper 95%
Serenade	8.31	0.4822	7.09	9.01
PreStop	7.91	0.5368	6.39	8.97

3.4.4 Effect of spatial leaf disc parts on dose response relationship of BCAs for *B. cinerea* control

Figure 3.8 and Table 3.4 show the comparison of different leaf disc types and effect of BCA treatments and the relative LD_{50} control concentrations. Leaf disc acquisition locations are illustrated in Fig 3.7. Inoculum dose affected the proportion of uninfected leaf discs in each leaf disc type ($P < 0.01$), and there was an interaction between leaf disc part × LD_{50} BCA dose. For either BCA in each leaf disc position and part the fitted probit model formed a sigmoid dose response curve but this relationship was challenged in the midrib leaf disc and/or parts (A2, A3 and A4) especially for PreStop (Fig 3.8). An experiment effect was found on proportion of uninfected leaf discs ($P < 0.01$), but carried no effect on the interaction of biocontrol × leaf disc positions and part × experiment (P 0.343); together with an independent leaf disc position and part effect ($P < 0.01$). Due to this data from both experiments were separated for defining dose response relationship (Fig 3.8) and dose features for each leaf disc position and part.

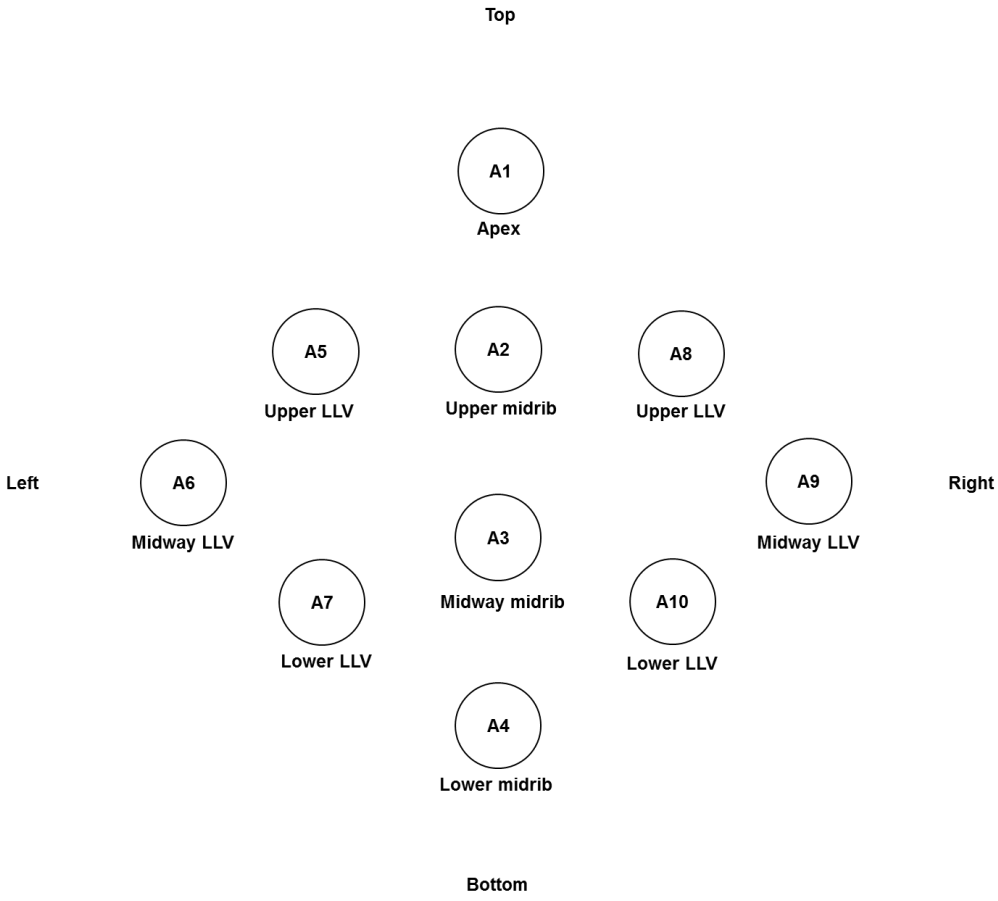


Figure 3.7 Leaf disc acquisition locations obtained from older *L. sativa* leaves

The illustration in Fig 3.7 is not to scale. In figure 3.7 LLV stands for lamina and lateral veins.

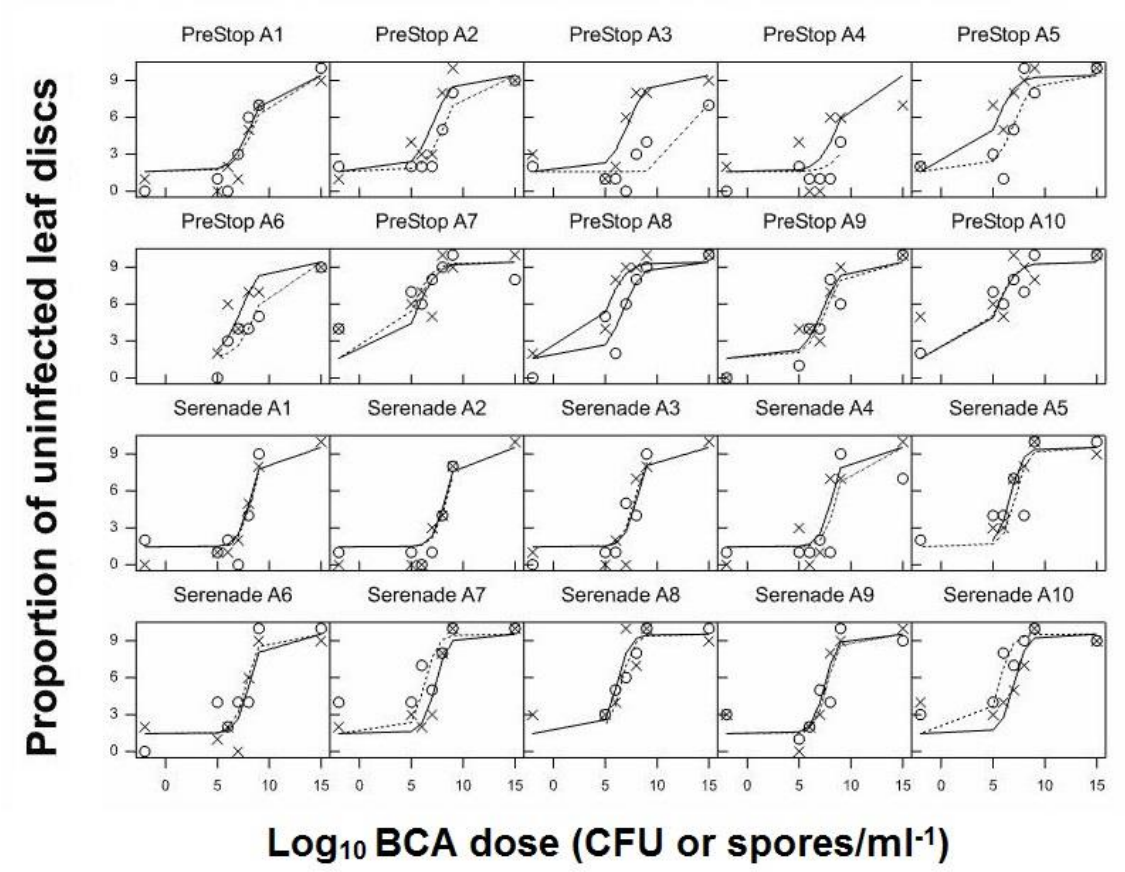


Figure 3.8 Proportion of uninfected *L. sativa* leaf discs against inoculum dose in each leaf part obtained from older lettuce leaves in the glasshouse at 17°C - 22 °C with a RH range of 60 % - 100 %

In Fig 3.8 Y axis represents proportion of uninfected leaf discs out of ten, and the X axis represents the log_{10} transformed dosage. On the probit model -2.5 identifies the positive control (inoculated with *B. cinerea*), log_{10} transformed dosages are observable in the centre (5, 6, 7, 8 and 9 CFU or spores/ml^{-1}), and negative controls (untreated) are pinpointed by the value of 15 located at top of the curve. Dose response curve development was produced by joining the means of each inoculum dose, and fitting the probit equation. Dose response curve of experiment one is identified by the black lines, and experiment two by the dashed lines. The means for experiment one are represented as X and for experiment two as O. Fig 3.8 displays the goodness of fit between the data and the probit dose response curve. Data labels are as follows: A1 apex, A2 upper midrib, A3 midway midrib, A4 lower midrib, A5 upper lamina and

lateral veins, A6 midway laminar and lateral veins, A7 lower lamina and lateral veins, A8 upper lamina and lateral veins, A9 midway lamina and lateral veins, and A10 lower lamina and laterals veins.

A sigmoidal dose response relationship was found for both BCAs in the apex (A1), midrib (A2-A4) and lamina lateral veins (A5-A10). However, for the midrib leaf parts the sigmoidal dose response relationship deteriorated further down the midrib for PreStop to an extent where the curve pattern was absent. For Serenade, the dose response curve shifted to the right and needed a higher inoculum dose for the initial increase in the proportion of uninfected leaf discs. Ineffective inoculum doses on proportion of uninfected leaf discs in the apex and lamina and lateral veins were $\log_{10}$ dosages < 6 CFU or spores/ml^{-1} for both BCAs, but in the midrib were dosages < 8 spores/ml^{-1} for PreStop and < 7 CFU/ml^{-1} for Serenade. The inoculum dose needed for reaching the LD_{50} for each leaf part differed (Table 3.4).

Table 3.4 Determined LD_{50s} of Serenade and PreStop on leaf disc parts

Leaf disc part	Log_{10} LD_{50} Serenade CFU/ml^{-1}	SE	Log_{10} LD_{50} PreStop Spores/ml^{-1}	SE
A1	8.97	0.52	9.17	0.75
A2	9.08	0.51	8.35	0.71
A3	8.70	0.50	11.39	0.93
A4	9.09	0.53	10.30	1.07
A5	7.60	0.48	6.84	0.71
A6	8.64	0.51	8.73	0.73
A7	7.51	0.48	5.99	0.72
A8	7.03	0.49	6.61	0.72
A9	8.31	0.50	8.12	0.67
A10	7.00	0.48	5.96	0.73

For comparing LD_{50} of leaf disc position and part Paired Sample T-Test used LD_{50}'s from each experiment in each leaf disc position instead of the mean of proportion of uninfected leaf discs. For Serenade differences in LD_{50} primarily occurred between different leaf discs of different leaf parts ($P < 0.05$). For PreStop differences in LD_{50} occurred in the same and different leaf disc parts ($P < 0.05$). The collective LD_{50} for Serenade on the apex, midrib and lamina lateral veins were significantly different from each other ($P < 0.05$). The collective LD_{50} for PreStop on the apex was the leaf part which was different from the midrib and lamina and lateral veins. The

LD_{50}'s were calculated for each experiment on each leaf disc position and part and only averaged out for viewing in Table 3.4 because the inoculum dose response relationship and LD_{50} were very close in both experiments.

3.5 Discussion

This study has examined the dose response relationship for these two BCAs and control of a high inoculum of *B. cinerea* both *in vitro* and *in vivo* for the first time. This has allowed the development of dose response relationships, and produced models for *B. subtilis* and *G. catenulatum* inoculum doses which will control *B. cinerea* on different phyllosphere leaf regions. The research investigated the acquisition of the applied formulated BCA's on the phyllosphere with the PMAxx™-qPCR tool in combination with the serial dilution method, and found that this was possible for comparing formulated *G. catenulatum* inoculum doses. The advantage of PMAxx™-qPCR involves the ability to distinguish the specific BCA accurately while avoiding the requirement of difficult interpretations of the organism and their amount on agar plates from environmental samples.

In vitro studies with both BCAs reduced mycelial growth of a *B. cinerea* inoculum. The formulated BCAs performed better, had higher efficacy rates, and thus resulted in lower LD_{50} inoculum concentrations. Temperature impacted the two BCAs dose response relationship and LD_{50}. Temperature influences reproduction in both BCAs, while temperature also impacts synthesis of lipo-peptides in *B. subtilis* and hydrolytic enzymes in *G. catenulatum*. *B. subtilis* growth becomes constrained around 11 °C, and cold shock proteins, fatty acids and SigB proteins are produced for cellular survival (Price, 2000), which reduce

sensitivity to environmental stress (Bernhardt et al., 1997). Lipo-peptides produced by *B. subtilis* are involved in antifungal activity, biofilm formation and colonization. Examples include iturin over synthesis in solid-state fermentation at lower temperatures (Ohno et al., 1995), surfactin overproduction at 37 °C (Akpa et al., 2001), and increase in mycosubtilin production when temperature changes from 37°C to 25°C (Fickers et al., 2008). *G. catenulatum* can grow between 5 °C – 34 °C, and survive up to of 42 °C, but the optimum growth ranges are 15 °C – 25 °C (Helyer et al., 2014). Information on growth rates are limited yet temperature affects the biomass of *Gliocladium* species (Harman and Kubicek, 1998). This fungi possibly copes with environmental stress by the production of heat shock and cold shock proteins (Tiwari et al., 2015). Enzymes produced by *G. catenulatum* are involved in antifungal activity. A study confirmed the production of β-1,3-glucanase and chitinase (McQuilken et al., 2001) and the stability of chitinase up to 40°C for controlling *B. cinerea* (Ma et al., 2012), in addition to this another study identified the production of a perilipin protein encoded by the Per3 gene involved in enhanced mycoparasitic activity at 28°C against sclerotia (Sun et al., 2015). Research on fungal metabolites identified production limitations occurred at low temperatures < 15 °C and at high temperatures > 45 °C (Diğrak and Özçelik, 2001). The production of cold shock proteins in both BCAs, in addition to the synthesis of hydrolytic enzymes and mycoparasitic proteins by *G. catenulatum*, as well as the production of SigB proteins and lipo-peptides by *B. subtilis* may have been vital in adaptation, growth and potential to control *B. cinerea* mycelial growth. The impact of

temperature on the characteristics of both BCAs resulted in a direct effect on dose response relationship and LD_{50} against *B. cinerea*.

Findings suggest the two BCAs prevented the *B. cinerea* macroconidia activity at high inoculums on older lettuce leaves. The maximum mean efficacy of the BCAs on older leaves were obtained at the highest dose; for Serenade this achieved 92 % and was greater compared to grey mould disease of apples with *B. subtilis* GA1 (80 %) (Toure et al., 2004), and *Botrytis* blight of geranium with *B. subtilis* QST 713 (Serenade max) (Elmhirst et al., 2011); while PreStop achieved an efficacy of 77 % and was greater than reported for *Botrytis* blight of geranium with PreStop (Elmhirst et al., 2011), and was similar to *B. cinerea* stem infection in cucumber and tomato with *G. catenulatum* (> 75 %) (Dik et al., 1999). The LD_{50} for Serenade was $\log_{10}$ 8.3 CFU/ml^{-1} and for PreStop was 7.9 spores/ml^{-1} for preventing a high inoculum of *B. cinerea* ($\log_{10}$ 6 spores/ml^{-1}) on older lettuce leaves. There is currently no literature available on the LD_{50} of both BCAs against *B. cinerea* for comparison.

Findings showed both BCAs LD_{50} inoculum dose for preventing *B. cinerea* in lamina and lateral veins were at a tenfold greater dose of the pathogen, contrasted with the midrib and apex where a hundred to thousand fold greater inoculum dose was needed. Explanations for this include: leaf disc part and/or leaf parts affected the BCAs and *B. cinerea* differently as well as having different physical and chemical properties. The presence of a pre-symptomless systemic *B. cinerea* infection of lettuce starting from the roots and traveling into the stem, petiole and leaves point out the vascular tissue in the midrib as a

location to reside for *B. cinerea*, therefore justifying the requirement of a higher LD_{50} and the positive infections from negative controls in the midrib (Sowley et al., 2010). Also leaf disc parts and/or leaf parts may have conditions favoring *B. cinerea*, but a lack of research on lettuce leaf surface features and their nutrient contents (Mirik and Öksel, 2016), cuticle features (Serrano et al., 2015), wound and stomata sites (Dugan and Blake, 1989, Faulkner and Robatzek, 2012) and cell wall degrading enzymes (Kubicek et al., 2014) on lettuce with *B. cinerea* infection potential cause a barrier for completely understanding the differences observed, but can be suggested as the cause of decline in efficacy, shift of the dose response curve and a higher LD_{50} for both BCAs in the apex and midrib in contrast to lamina and lateral veins. The mechanisms of action of competition for space and nutrients (Hibbing et al., 2010), lipo-peptide synthesis (Ongena et al., 2007), and induced host resistance (Gond et al., 2015) for *B. subtilis*, and competition for space and nutrients (Chatterton et al., 2008), antifungal enzyme production (Lahdenpera and Korteniemi, 2005), and hyper parasitism (Sun et al., 2015, Huang, 1978) for *G. catenulatum* may be responsible for the increase in proportion of uninfected leaf discs and the prevention of *B. cinerea* macroconidia activity on older lettuce leaves.

Our findings draw attention to the importance of abiotic (temperature and formulation) and biotic (leaf parts) factors role in defining dose response relationships, dose response models, and LD_{50}. Above all the significance of multiple factors coupling together to cause a substantial impact on biocontrol were noted. Applying the identified LD_{50} doses are a starting point for optimizing

biocontrol success with discretion to disease pressure. On a final note for the first time *B. subtilis* QST 713 and *G. catenulatum* J1446 dose response relationships, dose response models and inoculum dose features have been investigated simultaneously with abiotic and biotic factors, shedding light onto the ability of abiotic and biotic factors directly impacting biocontrol, and in result bringing attention to these factors for application strategies.

3.6 Conclusions

This Chapter has shown that both BCAs inhibit the colonization of *B. cinerea* inoculum *in vitro* on agar plates in a range of temperatures, with formulated versions having an advantage in biocontrol. In addition studies with the older lettuce leaf and its leaf parts show the two formulated BCAs inoculums were able to effectively control a high inoculum of *B. cinerea*. Above all the dose response relationships and LD_{50} were changed by temperature, formulation, and leaf tissue type.

CHAPTER 4

4 Effect of abiotic factors on viable populations of BCAs in the phyllosphere of lettuce and strawberry leaves

4.1 Abstract

There is little information on the effect of temperature, relative humidity (RH) and vapour pressure deficit (VPD) under UK conditions on the viable populations of these two commercial BCAs. The PMAxx™-qPCR molecular assay was used to quantify the viable BCA populations in the phyllosphere of lettuce and strawberry crops under UK agronomy climatic conditions. This showed that temperature, RH and VPD all significantly affected the viable populations of the two BCAs. For both BCAs on both crops, increasing temperature improved the population survival and/or reproduction up to the optimal growth temperature under theoretical healthy plant transpiration rates. Increasing the RH improved the population survival and/or reproduction within sub-optimal and optimal growth temperatures. Of the tested climatic conditions both BCAs were compatible with some parts of UK agronomy climates for production of these two crops. The viable population responses obtained for the different UK agronomy climates were modelled to predict BCA fate, and the potential of the models were tested in real time within commercial growing environments.

4.2 Introduction

The research in Chapter 3 on the dose-response relationships determined the median effective population density needed to control a high inoculum of *B. cinerea* and how biotic and abiotic factors impacted on this. Identifying the best population density required for biocontrol is an important step in optimization of BCA formulations to control their target pathogens, especially in the phyllosphere (Gotor-Vila et al., 2017a). Findings in Chapter 3 showed that the dose response relationship may be influenced by both biotic and abiotic factors. The key bottleneck in foliar applications of BCAs is the establishment of the right doses of the BCA for control of the target fungal pathogen under fluctuating abiotic factors that affects establishment in the phyllosphere. Indeed, studies have suggested that abiotic factors are the primary cause of the loss of introduced inoculum populations of BCA and the loss of efficacy for pathogen control (Magan, 2001, Sui et al., 2015, Liu et al., 2013). Thus, it was important to examine the integration of optimal *B. subtilis* and *G. catenulatum* inocula into UK commercial production systems.

For a BCA to be reliable, consistent and effective, establishment in the phyllosphere is essential, and can only be successful if the inoculum has the right resilience under the climatic regimes where the pathogen represents a significant problem. Previously, the inconsistent efficacy of BCAs was attributed to the lack of establishment of the chosen BCA under the environmental conditions conducive for pathogen infection (Andrews, 1992, Andrews and Hirano, 2012, Cray et al., 2013, Elad, 1996, Liu et al., 2013, Magan, 2001,

Spadaro and Droby, 2016, Sui et al., 2015). Thus, resilience under changing abiotic factors is important to produce ecologically competent BCAs that can become established at the right threshold level to have an impact on the pathogen, in this case *B. cinerea*.

Information on the effect of temperature, RH and VPD changes on *B. subtilis* and *G. catenulatum* viable population in the plants' phyllosphere are lacking, although many studies have suggested that abiotic factors are the cause of the loss of biocontrol efficacy. The optimum inoculum potential range of the two BCAs for control of *B. cinerea* was identified in Chapter 3. There is however a need to understand the changes in viable BCA populations under relevant abiotic conditions of temperature, RH and VPD which occur under UK agronomic conditions for lettuce and strawberry. Thus, the objective of this study was to examine the impact of temperature, RH and VPD on viable populations of *B. subtilis* and *G. catenulatum* in the phyllosphere of lettuce and strawberry crops, and to use the data to produce and test the use of a simple model for predicting BCA fate in these climatic conditions.

4.3 Materials and Methods

4.3.1 BCA preparation

Refer to Chapter 3 Materials and Methods Subsection 3.3.1 for BCA isolation and culturing methods. The following modifications were made: (1) for production of *G. catenulatum* J1446 inoculum, 5 g of PreStop powder was placed in 1 L of tap water and shaken vigorously; (2) The *B. subtilis* QST 713

colonies were cultured on nutrient agar, divided into four equal parts and transferred to a 1 L vacuum filter flask containing pre-autoclaved tryptone soya broth (Sigma) and grown on a rotary shaker (110 rpm) at 20 – 25 °C for 10 days. The concentration of both BCAs before each spraying event was determined by plate counts on three replicates of nutrient agar for *B. subtilis*, and malt extract agar for *G. catenulatum.* The BCA preparation procedure was the same in commercial trials for predicting BCA fate.

4.3.2 Plant propagation

Berry Plants provided strawberry plug plants (Malling Centenary), and Premier Plants provided lettuce seedling plants (Carter) in peat blocks. Plants were grown in pots (9 cm x 9 cm x 10 cm) using standard compost (All Purpose Enriched Compost Miracle-Gro). Plants were grown in a semi-commercial pest and disease free glasshouse. This was achieved by adhering to strict cultural practices detailed in Section 1.6.2 and 1.6.3, and when pest and/or diseases arose the plants were discarded, the glasshouse was cleaned out, and thorough sterilization processes were instigated. Only when the glasshouse was free from pest and disease were the plants sown and grown again. Strawberry plants were at their early flowering stage, while lettuce plants were in early head development before experimentation. Plant were watered on a daily basis.

For commercial field trials, lettuce plants were sown in the field of Laurence J Betts at West Malling, and received supplementary overhead irrigation. While strawberry plants were sown in groups of ten, and each group contained

independent soil bags. Strawberry plants were watered by drip irrigation and belonged to the AHDB mildew incidence trials.

4.3.3 Environmental treatments and experimental design

A randomized block design was used to investigate the effect of climatic conditions on the viable population of *B. subtilis* and *G. catenulatum*. Selected climatic treatments (see Table 4.1) represented UK climatic conditions for strawberry and lettuce production. The rationale in selecting these temperature and RH combinations was to (1) cover a wide range of UK growing climates, (2) have several sets of common vapour pressure deficits (VPD in kPa) which allowed the study of the effects of temperature under the same evaporative demand, (3) have an increasing RH range for each temperature. This design allowed testing the effect of changing temperature and RH independently, and changing temperature and moisture together. Each climatic treatment was repeated at least twice, and contained a total of ten replicates (five replicates per repeat experiment) and each replicate contained approx. 6 leaves obtained from plants placed on a grid inside a controlled environment cabinet. The time period of each treatment was 10 days with samples taken every 48 h (Day (D)0, D2, D4, D6, D8 and D10). Refer to Section 2.3.6 Testing viable BCA quantification with PMAxx™-qPCR from the phyllosphere of lettuce, and Section 2.4.5 Phyllosphere quantification of BCA with the PMAxx™-qPCR technique for details on the definition of the frequency and period of quantifying BCAs with the PMAxx™-qPCR technique. The experiments also contained

negative controls that were untreated lettuce and strawberry plants in each climatic treatment.

Table 4.1 Experimental conditions and levels of temperature and RH considered for representing commercial growing conditions in the U.K.

Temp°C	RH% 1	RH% 2	RH% 3	RH% 4
10°C	65%	75%	85%	95%
A	(0.43 kPa)	(0.31 kPa)	(0.18 kPa)	(0.06 kPa)
16°C	60%	68%	76%	90%
B	(0.72 kPa)	(0.58 kPa)	(0.43 kPa)	(0.18 kPa)
22°C	55%	64%	73%	84%
C	(1.19 kPa)	(0.95 kPa)	(0.72 kPa)	(0.43 kPa)
28°C	50%	59%	68%	81%
D	(1.89 kPa)	(1.54 kPa)	(1.19 kPa)	(0.72 kPa)
34°C	45%	55%	64%	78%
E	(2.93 kPa)	(2.41 kPa)	(1.89 kPa)	(1.19 kPa)

4.3.3.1 Treatments and experimental design for commercial trials for predicting BCA fate

In commercial trials, the natural field conditions determined the climatic treatments on lettuce leaves, and the natural polytunnel climatic conditions determined the climatic treatments on strawberry flowers. There were three climatic treatments in each commercial trial, and each treatment contained five replicates of real time quantifications (D2, D4, D6, D8 and D10 viable BCA population quantification with PMAxx™-qPCR). In the lettuce commercial trial for each replicate four older leaves were collected from two independent lettuce plants, while in the strawberry flower commercial trial for each replicate ten flowers were collected from ten independent strawberry plants. In both

commercial trials, a randomised block design was used for sampling. In each commercial trial, the viable population was quantified with the PMAxx™-qPCR, while simultaneously using the general linear model developed to predict the BCA viable population, and these two values were then tabulated.

4.3.4 General methodology description

All experiments followed seven common steps: (1) plant propagation and selection; plants were sown, grown and selected for being pest-disease free and healthy with a minimum of six leaves. (2) BCA cultivation and cell density calibration; plate counts were used to determine the concentration of the cultivated BCAs, and were adjusted as necessary to obtain a median effective dose (see Chapter 3). (3) Plant treatment; plants were sprayed with the BCA at a fine droplet setting just before run off. (4) Plant drying: after treatment, plants were allowed to dry for 1 h in the glasshouse and then placed into a climatic chamber, alongside sampling and image acquisition of D0 subjects. (5) Exposure to climatic regimes: all treatments had the same light dark cycles of 14 h light and 10 h dark (3 h at 30 % light, 3 hrs at 50 % light, 4 hrs at 70 % light, 2 h at 100% light, followed by 2 h at 10 % light, and 10 h of dark). (6) Sampling was done on days 0, 2, 4, 6, 8 and 10. The older leaves were collected from three pre-determined plants (two leaves per plant), imaged alongside a standard (Panasonic DMC-SZ3) and immediately placed into a falcon tube containing 10 mls of maximum recovery diluent (Sigma). (7) Surface washing, filtration and cell pellet collection; the leaves were soaked in the maximum recovery diluent solution until full, sealed and shaken on a rotary

shaker at 100 rpm for 30 mins at 10 °C. The contents were filtered with a wet muslin cloth (four layers) and cells pelleted by centrifugation at 2000 × g for 15 minutes at 4 °C. The supernatant was decanted and the cell pellet supplemented with maximum recovery diluent solution and transferred into a 1.5 ml Eppendorf, and stored at 4 °C.

Constant temperature and RH conditions (step 5) were achieved with two climatic chambers (Panasonic model MLR-352, and Sanyo format 650). Prior to experimentation climate chambers were calibrated using external data loggers (EasyLog EL-USB-2 standalone USB temperature and RH %, dew point data logger). The same data loggers were used for monitoring the temperature, relative humidity and dew point in each chamber throughout the experiment. The total surface area of the leaves in each replicate was calculated with image J.

For commercial trials, the general methodology description was identical except for the following differences: step 5 was excluded, for step 6, healthy opened strawberry flowers were sampled instead of leaves, and strawberry plants were treated with a range of agricultural products two weeks before experimentation. In step 7, an additional soil removal step was initiated before muslin cloth filtration that involved samples being spun at 100 × g for 1 minute at 4°C.

4.3.5 PMAxx™ treatment

Refer to Chapter 2, Material and Methods Section 2.3.3 PMAxx™ treatment.

4.3.6 Grinding of *G. catenulatum* J1446 cells

Briefly, after PMA treatment, cells were pelleted by centrifugation at 5,000 × g for 10 minutes at 4 °C. The supernatant was decanted and conidia suspended in maximum recovery diluent solution with a final volume of 1 ml. After slow pipetting for homogenisation, five stainless steel beads (6 mm) were transferred into each sample, and *G. catenulatum* cells were ground with the use of the genome grinder 2000 set at 1750 rpm for 20 minutes. After samples were ground the steel beads were removed with a magnet and sterilized with 5 % bleach and 70 % ethanol.

4.3.7 DNA extraction

Refer to Chapter 2, Material and Methods Section 2.3.4 DNA extraction and qPCR.

4.3.8 Minimisation and suppression of qPCR inhibitors

For improving the dissolution of the extracted DNA into 8 mM sodium hydroxide, five steel beads (6 mm) were transferred into each sample which contained a total volume of 1 ml and the DNA pellets were ground using the genome grinder 2000, set at 500 rpm for 1 minute followed by a cool down period of 2 minutes, the cycle was repeated fifteen times. After the grinding phase the DNA was pelleted at 1500 × g for 5 minutes at 4 °C and the supernatant (clear DNA suspended in 8 mM sodium hydroxide) was diluted into 8 mM sodium hydroxide. The dilution depended on the clarity of the DNA sample. Filtration of the diluted DNA was completed with a Millex-VV Syringe Filter Unit 0.1 µm (PVDF, 33 mm and gamma sterilized), and the DNA purity and concentration

was measured on a Nanodrop spectrometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE).

4.3.9 qPCR

Refer to Section 2.3.4 (Chapter 2) for DNA extractions and qPCR methods. The following amendments were made: (1) The BIO-RAD CFX96TM real time PCR detection system (BIO-RAD) was used to quantify DNA, and (2) CFX Manager™ Software version 3.1 (BIO-RAD) was used to analyse and calculate the Ct values automatically by adhering to the manufactures guidelines. (3) Reactions were prepared in a white/green semi-skirted 96 well qPCR plate (BIO-RAD). (4) The final volume in each reaction for both BCAs was 44 µL which contained 10 µL SensiFAST™ SYBR® No-ROX Kit (Bioline Meridian Bioscience, U.K). (5) Each reaction well contained 400 ng/µL of bovine serum albumin. (6) The cycling conditions used for both BCAs were 95 °C for 3 mins followed by 40 cycles of 45 s at 95 °C, 60 s at 61 °C and 60 s at 72 °C.

4.3.10 Viable BCA population quantification with PMAxx^{TM}-qPCR from the phyllopshere of plants

Refer to Chapter 2, Section 2.3.6.1 Standard curves of cycle threshold to copy number of DNA and viable populations, and Section 2.3.6.2 Conversion from total viable BCA population to viable populations in leaf area.

4.3.11 Conversion of Ct to CFU/mm^2

Refer to Chapter 2, Section 2.3.6.1 Standard curves of cycle threshold to copy number of DNA and viable population, and then Section 2.3.6.2 Conversion from total viable BCA population to viable population in leaf area.

4.3.12 Conversion of CFUs/mm^2 to population change for observing population biology with $Log_{10}(N_n) - Log_{10}(N_0)$

The equation $Log_{10}(N_n) - Log_{10}(N_0)$ defines the impact of a climatic treatment in relation to the produced population change, and breaks interpretation into three parts: (1) producing a value of zero (0). The value of zero indicates no viable population change, which translates into the climatic treatment being sustainable to the introduced viable population, and in lay terms, the description of survival can be employed. (2) Producing a positive value, i.e., a positive value indicates viable population change, which translates into the climatic treatment being beneficial to the introduced viable population to an extent in which the viable population has surpassed the original size, and in lay terms, the description of growth and/or reproduction is used. (3) Producing a negative value, i.e., a negative value indicated a viable population change, which translates into the climatic treatment being harmful to the introduced viable population to an extent in which the viable population size has decreased, and in lay terms, the description of mortality is used. However, the descriptions are used only to describe the overall outcome for the viable population, and by no means suggests that in a positive relationship, mortality is not occurring, and

vice versa. Table 4.2 demonstrates how the $Log_{10}(N_n)$ - $Log_{10}(N_0)$ equation was utilized.

Table 4.2 Demonstration of how the equation $Log_{10}(N_n)$ - $Log_{10}(N_0)$ was put to use for observing viable population change in climatic treatments

Organism	Plant	Day (N_n)	Log_{10} (N_n) CFUs/mm²	Day (N_0)	$Log_{10}(N_0)$ CFUs/mm²	$Log_{10}(N_n)$-Log_{10} (N_0)	Replicate	Temp°C	RH%
B. subtilis	Strawberry	2	7.71	0	8.48	-0.76	1	11	66
B. subtilis	Strawberry	4	8.07	0	8.48	-0.40	2	11	66
B. subtilis	Strawberry	6	6.73	0	8.48	-1.74	3	11	66
B. subtilis	Strawberry	8	6.70	0	8.48	-1.77	4	11	66
B. subtilis	Strawberry	10	5.39	0	8.48	-3.08	5	11	66

The population change equation of $Log_{10}(N_n)$ - $Log_{10}(N_0)$ was employed instead of CFUs/mm^2 because the interest of the research and funding bodies was to categorize climatic treatments as sustaining, harmful or beneficial in an explicit and simple manner. The use of CFUs/mm^2 directly would have overcomplicated any climatic treatment impacts on population biology and introduced other uncontrollable variables onto the observations. Equations similar to this have been employed by several studies for expressing population biology (Calvo-Garrido et al., 2014a, Calvo-Garrido et al., 2014b).

4.3.13 Statistical analyses

Before statistical analysis, the data sets of CFUs/mm^2 were transformed with the log_{10} function. One-way ANOVA was used to compare between all climatic treatments for each BCA on each crop, separated by time. When analysis proved statistically significant that climatic treatment and not time alone was affecting log_{10} CFUs/mm^2 an ANOVA was performed to test if differences were

due to temperature, RH or VPD. For testing if differences were due to temperature, ANOVAs were performed on data sets with the same vapour pressure deficit at different temperatures. For testing if differences were due to RH, ANOVAs were performed on data sets with the same temperature but different RH regimes. For testing if differences were due to VPD ANOVAs were performed for each BCA on each crop containing all VPDs. The relationship of the BCA with each climatic regime on each crop was defined using a general linear model. The general linear models were used to predict BCA populations alongside real time quantification to assess model reliability (commercial trials). All data analysis was performed with the software package MiniTab (V. 17) at P=0.95 confidence level.

4.4 Results

One-way ANOVA was performed on the $\log_{10}$ CFUs/mm^2 data set for *B. subtilis* and *G. catenulatum* climatic treatments (see Table 4.1) on lettuce and strawberry plants. The statistical test showed that there were significant differences in viable populations of *B. subtilis* on lettuce and strawberry plants, and of *G. catenulatum* on lettuce and strawberry plants because of climatic treatments ($P < 0.05$). Two-way ANOVA indicated a significant effect of temperature, RH and VPD on viable populations of *B. subtilis* and *G. catenulatum* on both crops ($P < 0.05$).

Consideration of all the statistical analysis as a whole suggests that the viable populations of both BCAs in both crops was strongly influenced by temperature,

RH and VPD. However, the relationship between viable population change and the three factors in the phyllosphere was complex. The BCAs appeared to prefer specific climatic regimes (temperature and RH combinations) ($P < 0.05$). For both BCAs in both crops, as temperature was increased, the viable population density increased ($P < 0.05$) but this was restricted to theoretical sub-optimal and optimal plant transpiration rates; the optimum plant transpiration rates for lettuce and strawberry plants are not known. However, in general research suggested approx. 0.8 - 1 kPa as an ideal VPD for plants (Shamshiri, 2014). A clear pattern was found for the effect of RH for both BCAs with viable population density highest at temperature closest to their *in vitro* optimum for growth ($P < 0.05$). Viable population densities increased on leaves of both crops for *B. subtilis* at VPD of 0.6, 1.4, and 2.4 kPa ($P < 0.05$), and for *G. catenulatum* at VPD of 0.3 kPa on lettuce ($P < 0.05$).

4.4.1 Temporal viable population of the two BCAs in UK agronomic climates

Temporal BCA survival of the introduced viable populations depended on the climatic treatment. Survival became limited in the lowest RHs on strawberry plants. Instances in which the introduced BCA populations surpassed their original amount were few and included: 28 °C at 68 % (D3) on day eight in strawberry, and 35 °C at 57 % (E2) on day six in lettuce for *B. subtilis*, and for *G. catenulatum* in both crops at the climate of 16 °C at 89 % on day four. The changes in temporal viable populations of the BCAs were constant and gradual,

and consequently lead to differences in viable populations with time. In all cases, the co-variate of time impacted on viable population density ($P < 0.05$).

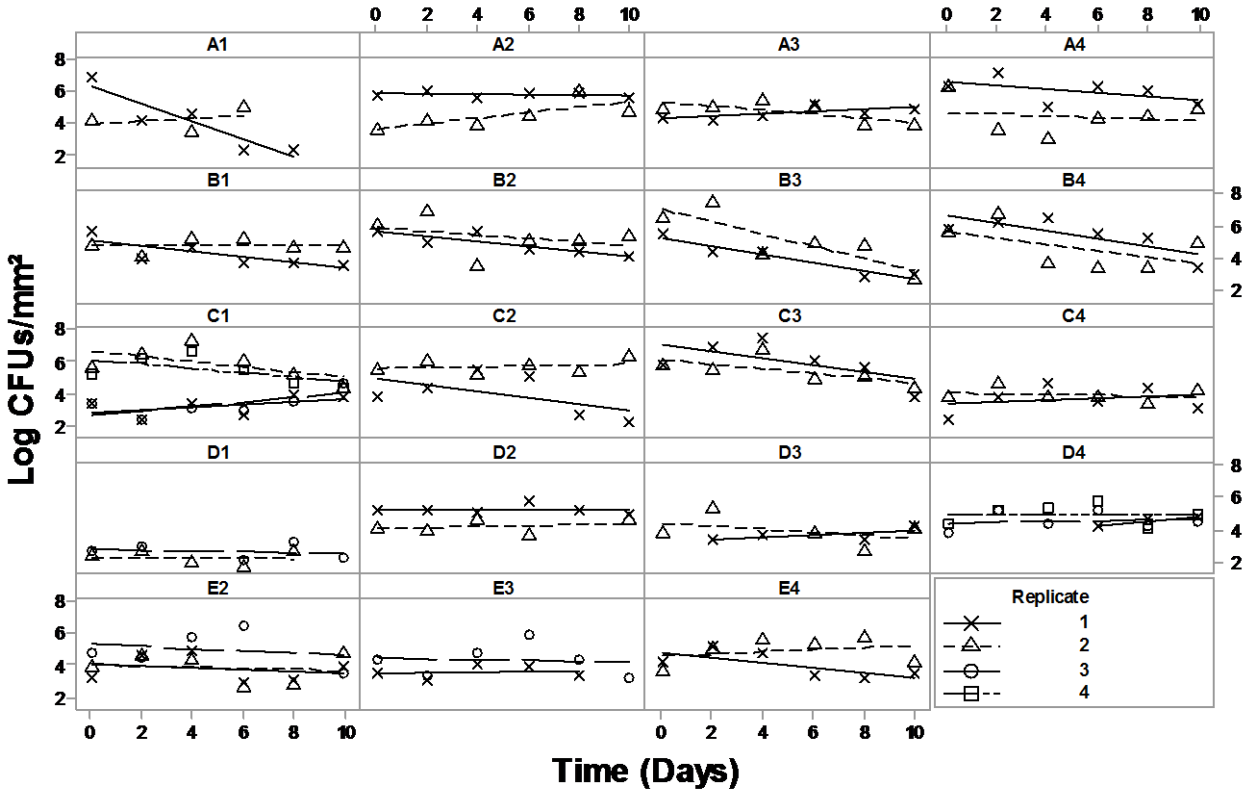


Figure 4.1 Temporal viable populations of *B. subtilis* QST 713 on lettuce plants exposed to a range of temperature and RH% regimes

For Figures 4.1-4.4 Plants were treated with the BCA (median effective dose), then allowed to dry for 1 h before being exposed to climatic regimes of temperature and RH. Log_{10} CFUs/mm^2 on the Y axis is the viable population of BCAs in a given area of leaf surface. Values shown are the mean CFUs on six leaves divided by the total leaf area. Negative controls (untreated) did not produce any positive reaction > Ct 35 and therefore their population was non-quantifiable. The X axis represents the length of the experiment and the labels represent the sampling frequency. The x, △, O, and ☐ represent the replicates. Each climatic treatment, temperature, RH and VPD can be found in Table 4.1 and are listed as following: 11°C at 66% (A1), 11°C 76%

at (A2), 11°C at 82% (A3), 11°C at 92% (A4), 16°C at 61% (B1), 16°C at 69% (B2), 16°C at 74% (B3), 16°C at 84% (B4), 22°C at 52% (C1), 22°C at 65% (C2), 22°C at 68% (C3), 22°C at 78% (C4), 28°C at 57% (D1), 28°C at 62% (D2), 28°C at 68% (D3), 28°C at 85% (D4), 34°C at 56% (E2), 34°C at 72% (E3), and 34°C at 88% (E4).

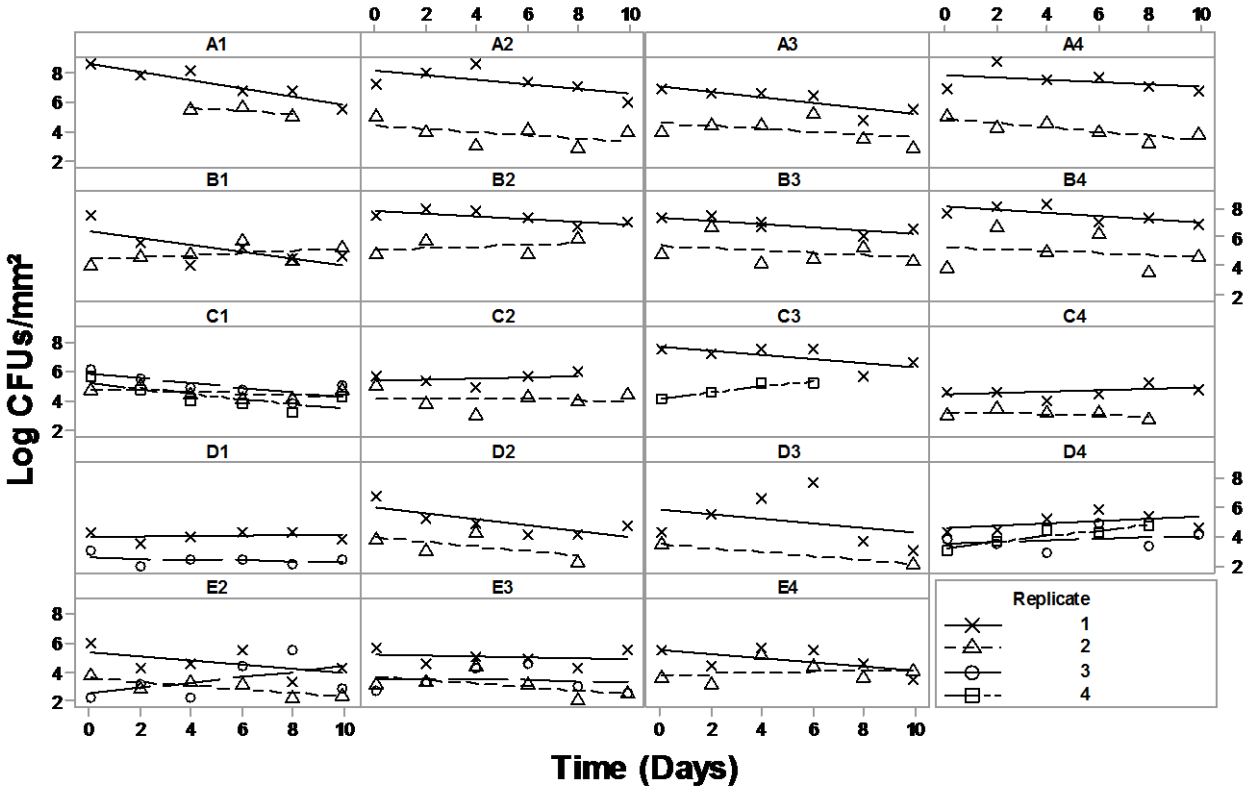


Figure 4.2 Temporal viable populations of *B. subtilis* QST 713 on strawberry plants exposed to a range of temperature and RH% regimes

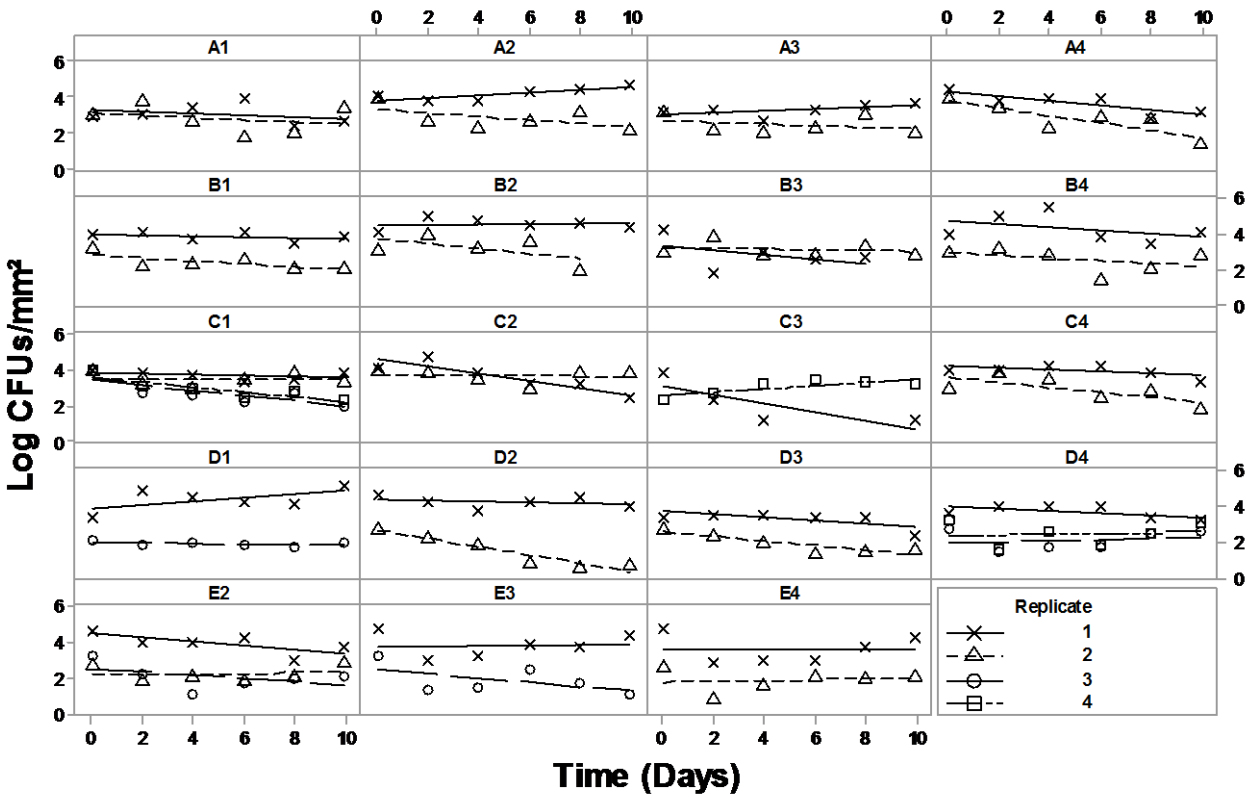


Figure 4.3 Temporal viable populations of *G. catenulatum* J1446 on lettuce plants exposed to a range of temperature and RH% regimes

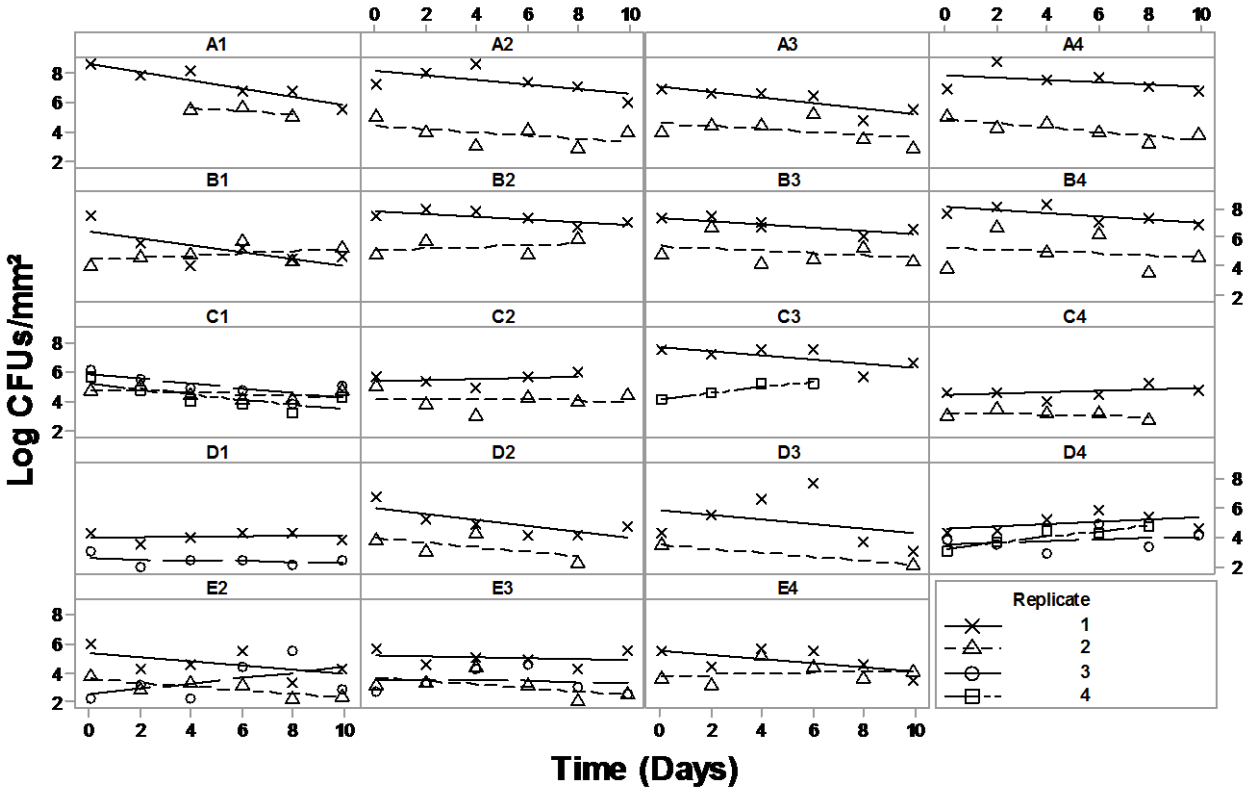


Figure 4.4 Temporal viable populations of *G. catenulatum* J1446 on strawberry plants exposed to a range of temperature and RH% regimes

Chapter 4 focuses on the impact of abiotic factors (temperature, RH and VPD) on viable population. Time, in days, was an unavoidable co-variate since in general growers rely on an application for around 10 days. Therefore, this section of the research was relevant to investigate the longevity of the two BCAs on both crops in UK agronomy climates. Therefore for observing viable population changes a transformation of the data was required in which the impact of a climatic treatment can be categorised into sustainable, beneficial or harmful (see Section 4.3.12). In the results the following equation was applied $Log_{10}(N_n) - Log_{10}(N_0)$ and the replicates of population change were averaged.

This equation permits observation of viable population change in an explicit manner, and enabled direct interpretation of the nature of UK agronomy climates on the BCAs population biology. This equation was applied for graphical representation of the data in Sections 4.4.2, 4.4.3 and 4.4.4. However, for statistical analysis the data sets of $\log_{10}$ CFUs/mm^2 was employed and time (in days) was provided as a co-variate for ease of interpretation.

4.4.2 Effect of temperature on the viable population change of the two BCAs

Figures 4.5, 4.6, 4.7 and 4.8 show a small portion of the results illustrating the effect of temperature on the introduced BCA populations of the bacterial and fungal BCAs, respectively. Statistical analysis showed that for the bacterial BCA temperature significantly affected the introduced BCA populations on lettuce (4 out of 10 tests) and on strawberry (7 out of 10 tests). For the fungal BCA, on lettuce (6 out of 10 tests) and on strawberry (5 out of 10 tests) ($P < 0.05$). The temperature effect either led to an increase in viable populations (Figures 4.5, 4.6, 4.7 and 4.8) relative survival or a decrease of the population (Figures 4.5, 4.6, 4.7 and 4.8). This was directly influenced by VPD. An increase and/or survival of the introduced viable populations of the BCAs in relation to temperature was found close to or at healthy plant transpiration rates (i.e., VPD of 0.6 and 0.8 kPa) (Shamshiri, 2014) which is close to, or at, the BCAs *in vitro* optimal temperatures, i.e., for *B. subtilis* at 22 °C – 28 °C (Figs 4.5 and 4.6), and for *G. catenulatum* at 16 °C - 22 °C in both crops (Fig 4.7 and 4.8). The effect of increasing or decreasing temperature can be beneficial on the BCA populations.

However, the same temperature can also have a different effect if tested with a different VPD ($P < 0.05$). Overall, in both crops an increase in temperature favoured the increase and or survival of the bacterial BCA while a decrease in temperature favoured the increase and or survival of the fungal BCA. This is of course partially because of the different ecology of the two BCAs. The optimal temperature for growth of *B. cinerea* is considered to be between 18 to 20 °C (Fiume and Fiume, 2005).

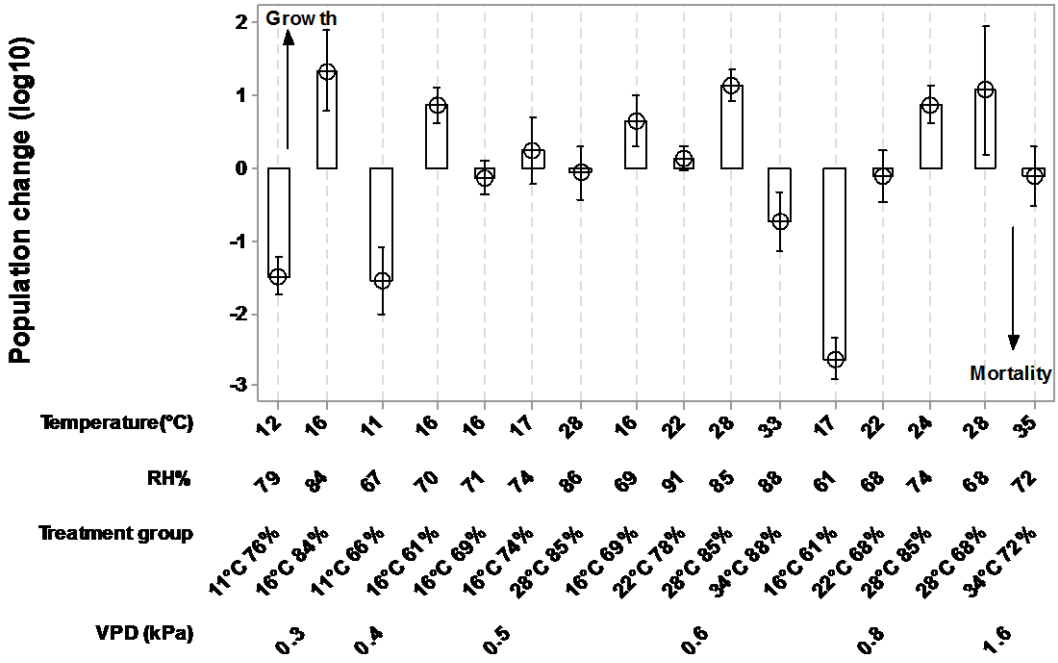


Figure 4.5 Viable population changes of *B. subtilis* QST 713 on strawberry plants in different temperatures at the same VPD

In Figure 4.5 – 4.8 the Y axis represents the mean BCA population change from the introduced viable population, the equation in Section 4.3.12 was applied: population change = $Log_{10}(N_n)$ - $Log_{10}(N_0)$. On the X axis the displayed VPD, RH% and temperature are the true mean values obtained with a data logger, while allocated treatment groups are categorical in which two of the

most similar climatic treatments were pooled together and their mean climate was displayed as a single treatment, since it was physically impossible to obtain identical climates. Each mean in the figure contains up to ten replicates. The mean of the data sets are displayed with a black circle, and the standard errors of each mean are represented by interval bars. Negative controls lacked a positive qPCR reaction (> 35 Ct) and therefore the BCA population was assumed zero when Ct > 35. The arrow pointing up on the graph indicates that positive values represent growth to an extent in which the introduced viable population was exceeded, while the arrow pointing down on the graph indicates that negative values represent mortality in which the population decreased below the introduced amount. The treatment groups on the X axis label relates to the targeted climatic treatments in Table 4.1.

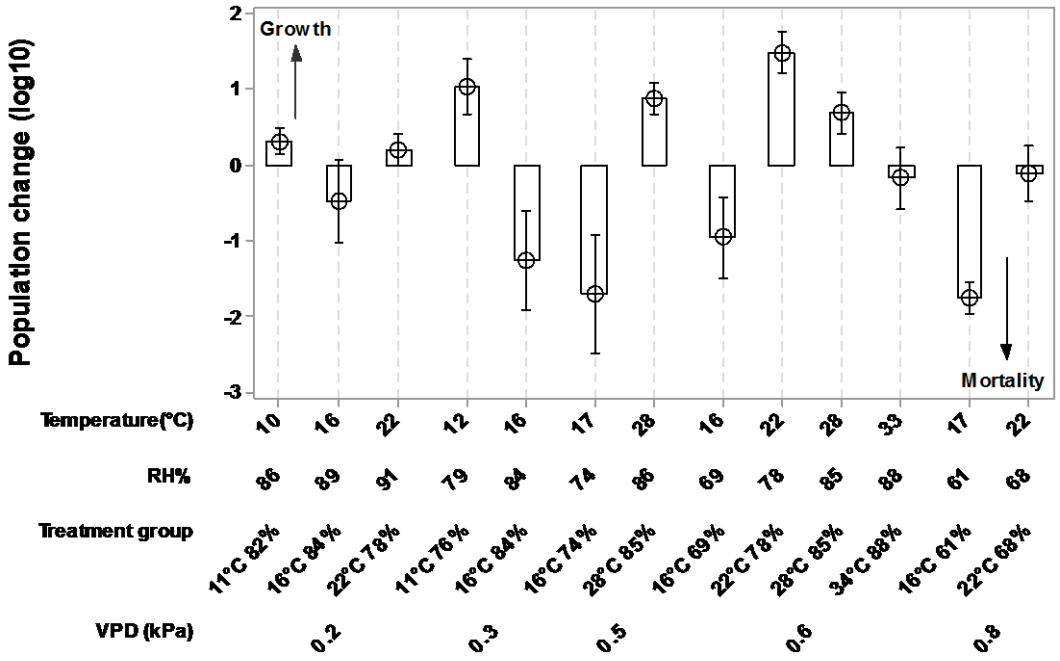


Figure 4.6 Viable population change of *B. subtilis* QST 713 on lettuce plants in different temperatures at the same VPD

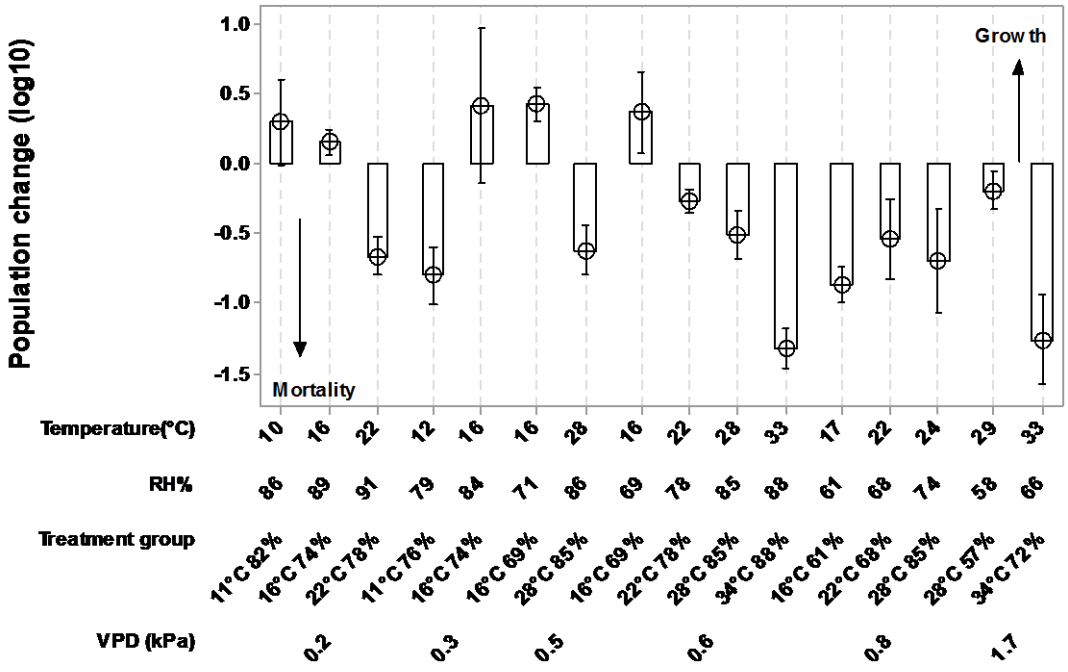


Figure 4.7 Viable population change of *G. catenulatum* J1446 on strawberry plants in different temperatures at the same VPD

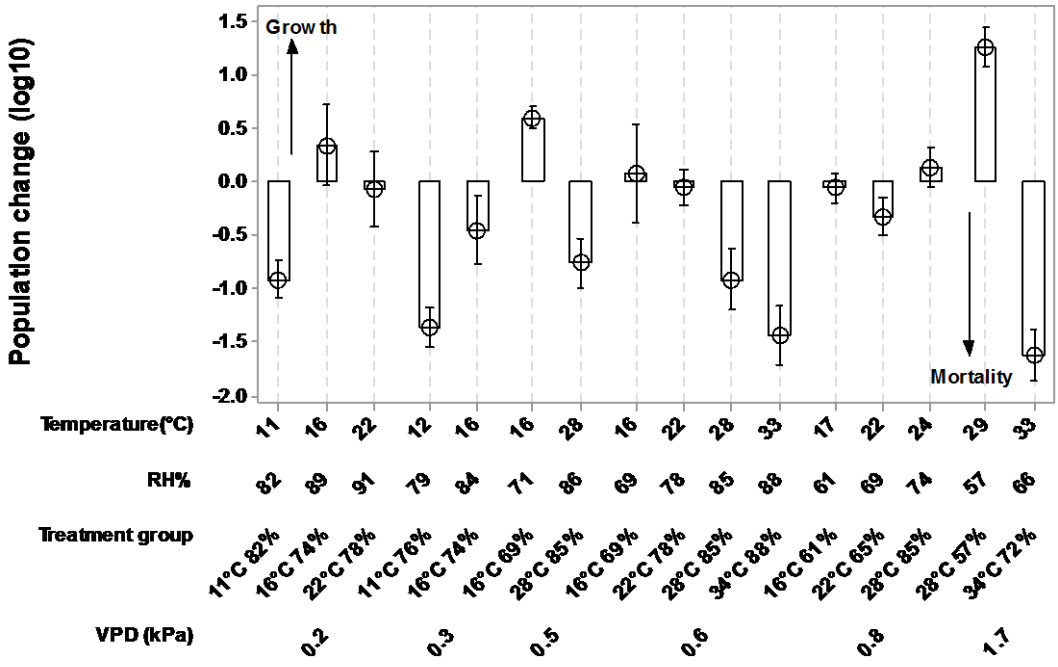


Figure 4.8 Viable population change of *G. catenulatum* J1446 on lettuce plants in different temperatures at the same VPD

Overall, the results suggest that temperature affects introduced viable populations of both BCAs significantly under the same VPD ($P < 0.05$), and the effect on viable populations can be positive, conserved or negative and this directly depends on the evaporative demand of the environment and the host. Temperature increase was beneficial when VPD is close to healthy plant transpiration rates (Figs 4.5, 4.6, 4.7 and 4.8 at VPD of 0.6 and/or 0.8 kPa). This suggests a strong interaction between the BCA population change × temperature × host × transpiration rates.

4.4.3 Effect of RH on the viable population change of the two BCAs

RH significantly affected the introduced viable populations of the two BCAs. In the five temperature groups, statistically significant differences due to RH were found for the bacterial BCA on lettuce (2 out of 5 tests), and on strawberry (3 out of 5 tests). For the fungal BCA, on lettuce (1 out of 5 tests), and on strawberry (2 out of 5 tests). Differences generally occurred at sub-optimal and optimal temperatures for growth of the BCAs ($P < 0.05$). The optimal growth temperature for *B. subtilis* can range from approx. 22 °C – 35 °C (Cook, 1996, Schaechter et al., 2006), while for *G. catenulatum* optimal growth temperature ranges from 15 °C – 25 °C (Helyer et al., 2014).

Figures 4.9 and 4.10 show examples of the effect of RH on the bacterial and fungal BCA in terms of survival, growth and mortality. For both BCAs on strawberry, in general, low RH across all the temperature treatments led to a greater decrease in the population densities. Preferences in terms of RH for both BCAs appeared to be both temperature and host specific, and this resulted in either an increase, or better survival of the introduced population inocula. For the bacterial BCA there was a significant benefit when RH was increased. This was observed in strawberry crops at 11 °C 66 %,11 °C 76 %,11 °C 82 %, and 11 °C 92 % (A1-4), 16 °C 61%, 16 °C 69 %, 16 °C 74 %, and 16 °C 84 % (B1-4), 22 °C 52 %, 22 °C 65 %, 22 °C 68 %, and 22 °C 78 % (C1-4) as shown in Figure 4.9, and for the fungal BCA in both crops at 22 °C 52 %, 22 °C 65 %, 22 °C 68 %, and 22 °C 78 % (C1-4) in Figure 4.10.

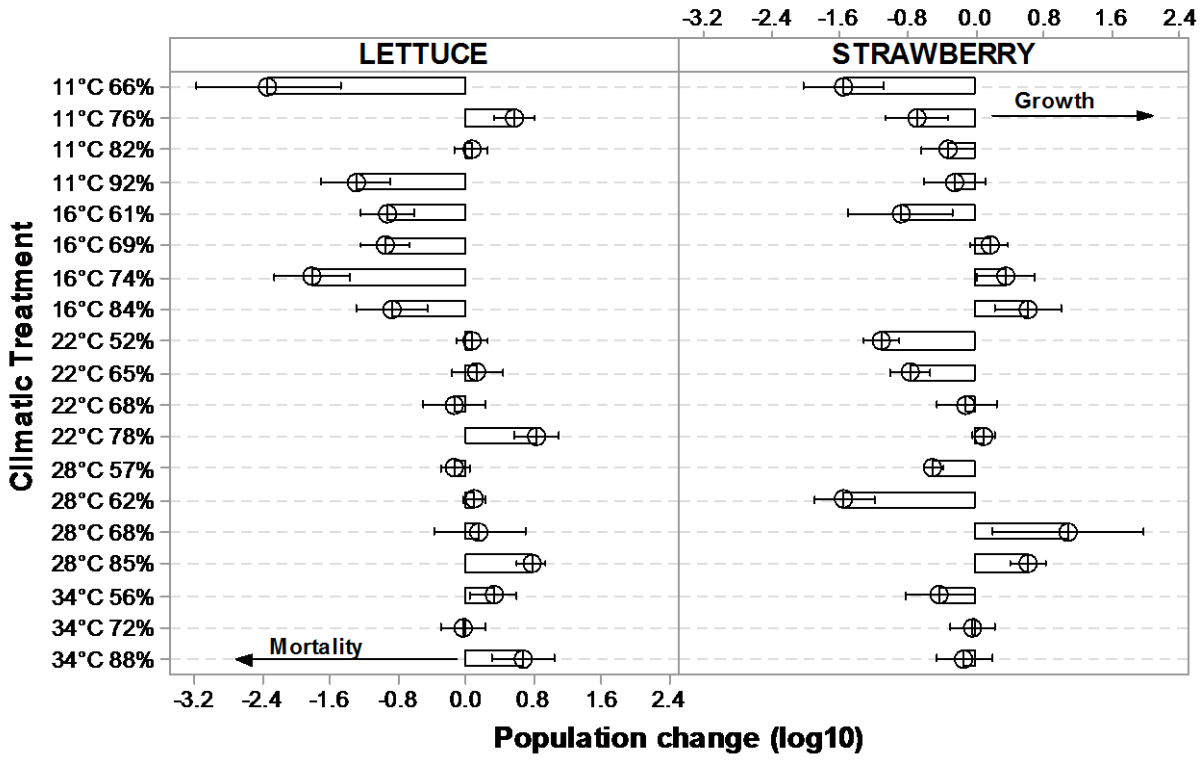


Figure 4.9 Viable population change of *B. subtilis* QST 713 on lettuce and strawberry plants with increasing RH% at the same temperature

In Figure 4.9 and 4.10 the X axis represents the mean BCA population change from the introduced viable population, the equation in section 4.3.12 was applied: population change = $Log_{10}(N_n)$ - $Log_{10}(N_0)$. The climatic treatments displayed on the Y axis are from two of the closest climates being pooled together and their mean being represented as a climatic treatment group. This strategy was applied because obtaining identical climates was physically impossible. Each mean in the figure contains up to ten replicates. The mean of the data sets are displayed with a black circle, and the standard errors of each mean are represented by interval bars. Negative controls lacked a positive qPCR reaction (> 35 Ct) and therefore the BCA population was assumed zero when Ct > 35. The arrow pointing right on the figure indicates that positive values represent growth in which the introduced viable population was exceeded, while the arrow pointing left on the figure indicates that negative values represent mortality in which the population decreased below the introduced viable population. The climatic treatments on the Y axis relates to the targeted climatic treatments in Table 4.1.

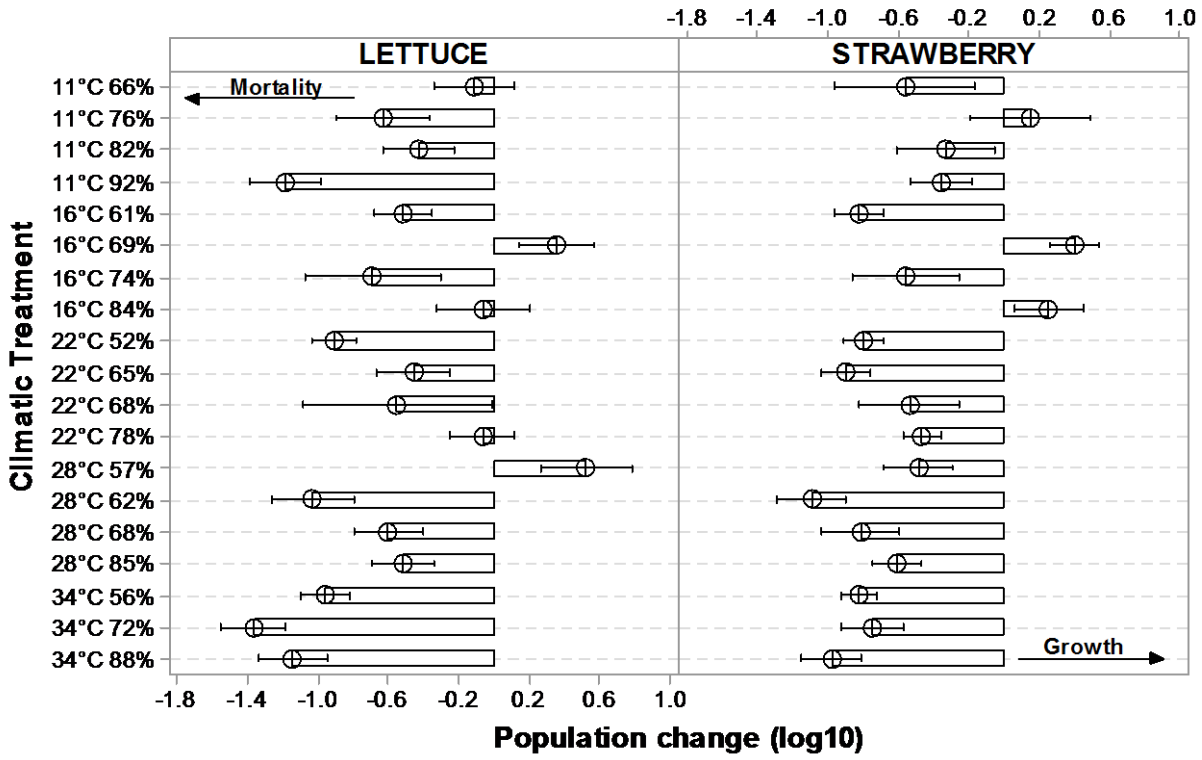


Figure 4.10 Viable population change of *G. catenulatum* J1446 on lettuce and strawberry plants with increasing RH% at the same temperature

Increasing RH led to better survival of the introduced BCAs, but this was constrained to sub optimal and optimal growth temperatures of the BCAs. As a whole for both BCAs in both crops the favoured RH increased with temperature, but exceptions occurred at lethal temperatures for growth of the BCAs (i.e., 11 °C for *B. subtilis* and 34 °C for *G. catenulatum* on both crops). This indicated that viable populations of these two BCAs differed depending on temperature, RH, and host, but there was no clear trend in the differences.

4.4.4 Effect of VPD on the viable population change of the two BCAs

A total of fifteen VPD treatments were obtainable to use from all the available data. Increasing VPD significantly affected the introduced viable population of the two BCAs. Out of the fifteen VPD treatments significances ($P < 0.05$) were found. A single statistical test containing the fifteen VPDs was performed for each BCA and crop combination. Thus, significances were found for *B. subtilis* on lettuce (7 out of 15 VPDs) and, on strawberry (10 out of 15 VPDs). For *G. catenulatum* on lettuce (6 out of 15 VPDs) and on strawberry (3 out of 15 VPDs). Figures 4.11 and 4.12 demonstrate the effects for all of the data when VPD is increased for the bacterial and fungal BCAs, respectively. For the two BCAs on both crops a pattern to describe the overall relationship of VPD to viable population change was absent. This being said for the bacterial BCA an increase between the VPD ranges of 0.4 kPa to 1.4 kPa on lettuce resulted in viable population increase (Fig 4.11), yet the opposite effect was observed on strawberry plants between the VPD ranges of 0.5 kPa to 1.1 kPa (Fig 4.11). For the fungal BCA a decrease between the VPD ranges of 1.1 kPa to 0.5 kPa resulted in viable population increase on both crops (Fig 4.12).

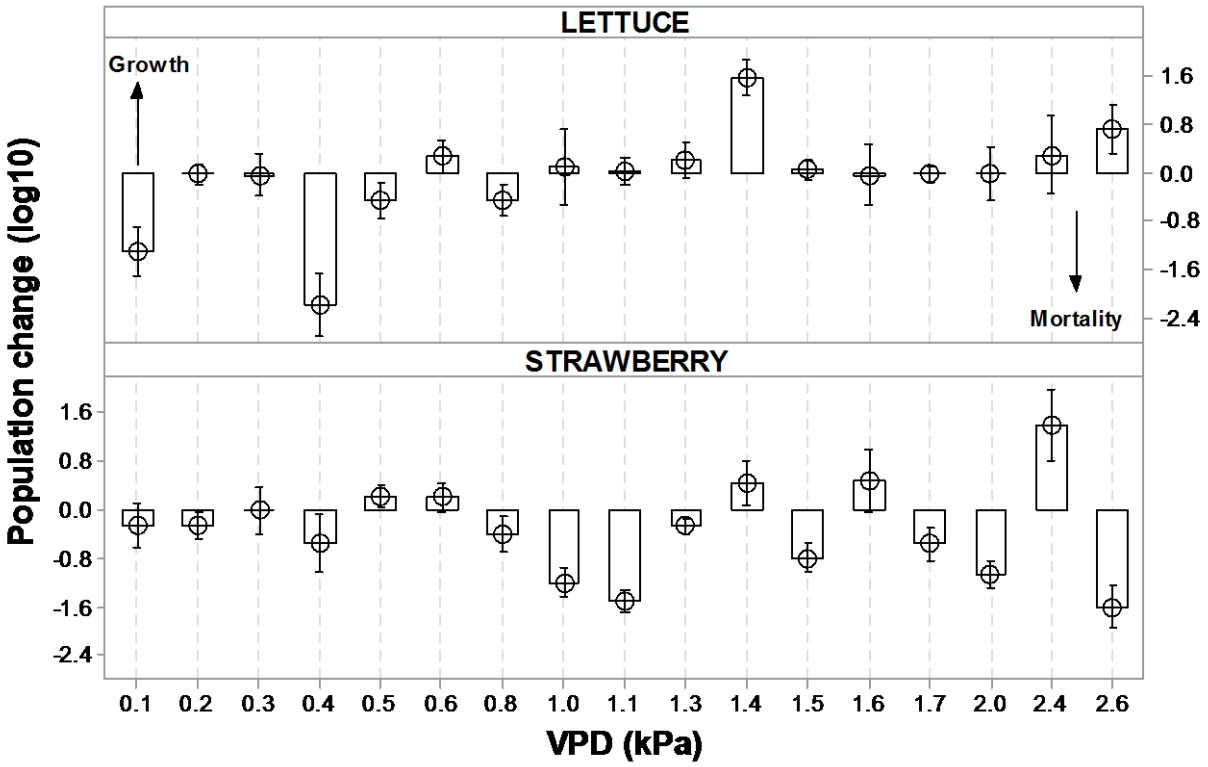


Figure 4.11 Viable population change of *B. subtilis* QST 713 on lettuce and strawberry plants with increasing VPD

In Figure 4.11 and 4.12 the Y axis represents the mean BCA viable population change from the introduced viable population, the equation in section 4.3.12 was applied: population change = $Log_{10}(N_n)$ - $Log_{10}(N_0)$. On the X axis the VPD (kPa) are displayed in which two of the closest VPDs were pooled together and their mean was displayed as a single VPD treatment. This strategy was applied because changes in VPD demonstrates the difference in the evaporative demand of the environment, as well as the inability of producing identical climates. Each mean in the figure contains up to twenty replicates. The mean of the data sets are displayed with a black circle, and the standard errors of each mean are represented by interval bars. Negative controls lacked a positive qPCR reaction (> 35 Ct) and therefore the BCA population was assumed zero when Ct > 35. The arrow pointing up on the figure indicates that positive values represents growth of the population in which the introduced viable population was exceeded, while the arrow pointing down on the figure indicates that negative values represent mortality in which the population decreased below the introduced viable population. The VPDs on the X axis relates to the targeted VPDs in Table 4.1.

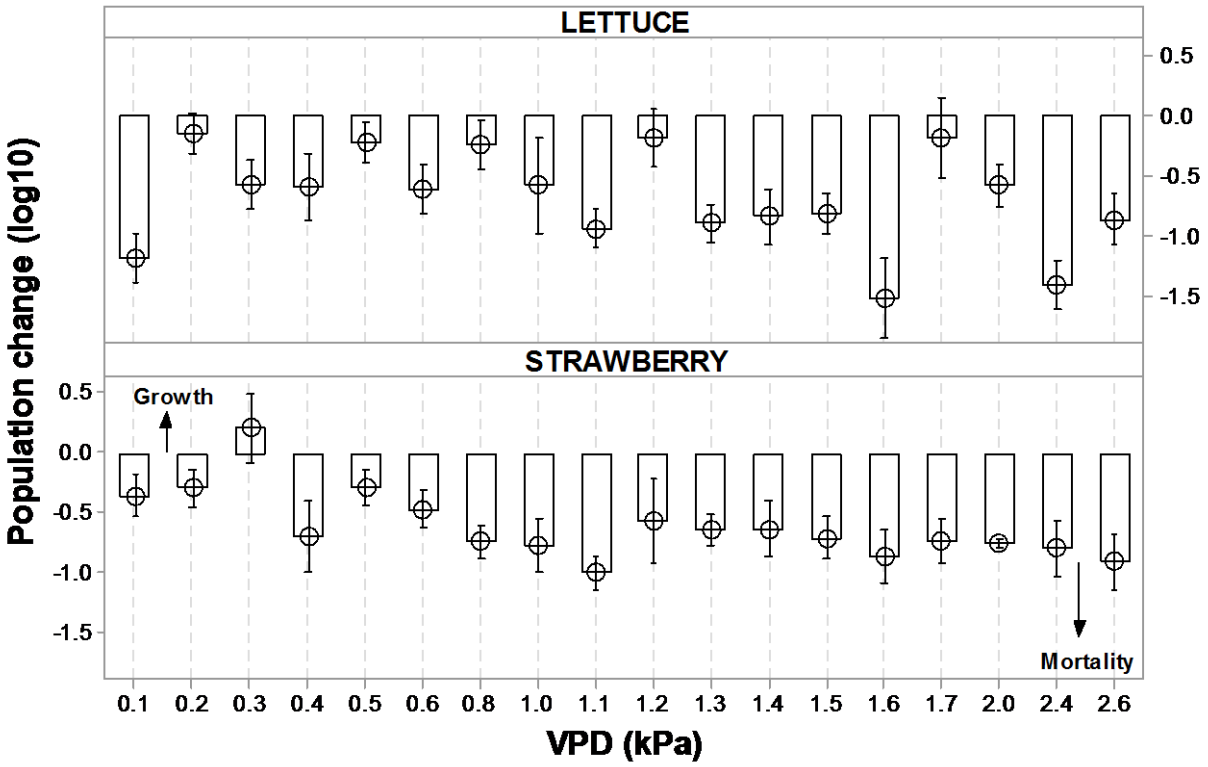


Figure 4.12 Viable population change of *G. catenulatum* J1446 on lettuce and strawberry plants with increasing VPD

4.4.5 UK commercial growing climates

Table 4.3 presents the average population changes for *B. subtilis* and *G. catenulatum* on both crops. Also to understand the relationship and compatibility between climates and the BCAs on their target hosts an ANOVA general linear model was fitted (Table 4.4), with the data models coding as -1, 0, +1.

Table 4.3 Summary of viable population change log_{10} (N_n) - log_{10} (N_0) in the introduced viable populations of the two BCAs in UK commercial growing climates

		B. subtilis QST 713 on lettuce					*B. subtilis* QST 713 on strawberry				
		Time (days)					Time (days)				
≈ Temp°C	≈ RH%	2	4	6	8	10	2	4	6	8	10
11	66	-2.67	-1.53	-1.83	-4.59	*	-0.77	-0.41	-1.75	-1.78	-3.09
11	76	0.39	0.15	0.51	1.29	0.52	-0.20	-0.37	-0.45	-1.21	-1.20
11	82	0.06	0.34	0.49	-0.32	-0.20	0.16	0.10	0.47	-1.23	-1.19
11	92	-0.86	-2.29	-1.02	-1.02	-1.29	0.49	0.05	-0.18	-0.88	-0.75
16	61	-1.21	-0.33	-0.82	-1.06	-1.19	-0.59	-1.29	-0.29	-1.40	-0.86
16	69	0.03	-1.31	-1.14	-1.18	-1.16	0.70	0.29	-0.05	0.11	-0.50
16	74	-0.08	-1.77	-1.62	-2.26	-3.24	2.24	-0.07	-0.28	0.05	-0.25
16	84	0.74	-0.71	-1.32	-1.46	-1.57	1.67	0.89	0.85	-0.30	-0.03
22	52	-0.06	0.71	-0.10	-0.07	-0.08	-0.41	-1.06	-1.33	-1.83	-0.90
22	65	0.49	0.63	0.69	-0.64	-0.44	-0.83	-1.45	-0.49	-0.40	-0.62
22	68	0.44	1.31	-0.24	-0.45	-1.67	0.00	0.51	0.49	-1.91	-0.96
22	78	1.14	1.15	0.58	0.78	0.58	0.25	-0.19	0.01	0.19	0.22
28	57	0.24	-0.43	-0.70	0.40	-0.39	-0.93	-0.46	-0.24	-0.44	-0.47
28	62	-0.06	0.27	0.04	0.11	0.14	-1.14	-0.73	-2.57	-2.05	-2.05
28	68	1.56	*	-0.02	-1.11	0.27	1.32	2.39	3.49	-0.57	-1.25
28	85	1.11	0.74	1.38	0.03	0.65	0.16	0.46	1.30	0.73	0.38
34	56	0.64	1.07	0.11	-0.58	0.12	-0.64	-0.67	0.38	-0.32	-0.84
34	72	-0.73	0.42	0.91	-0.13	-1.17	-0.14	0.74	0.35	-0.80	-0.33
34	88	1.21	1.28	0.46	0.62	-0.08	-0.76	0.90	0.44	-0.46	-0.80
		G. catenulatum J1446 on lettuce					*G. catenulatum* J1446 on strawberry				
		Time (days)					Time (days)				
≈ Temp°C	≈ RH%	2	4	6	8	10	2	4	6	8	10
11	66	0.39	0.01	-0.16	-0.77	0.00	-0.67	0.35	-0.73	-1.46	-0.29
11	76	-0.83	-0.96	-0.50	-0.24	-0.61	0.55	0.51	-0.12	0.03	-0.23
11	82	-0.53	-0.86	-0.40	0.03	-0.36	0.21	-0.17	-0.40	-0.20	-1.09
11	92	-0.61	-1.13	-0.84	-1.45	-1.92	0.21	0.13	-0.28	-1.06	-0.78
16	61	-0.41	-0.56	-0.19	-0.81	-0.60	-0.53	-1.04	-0.84	-0.99	-0.71
16	69	0.89	0.41	0.49	-0.34	0.38	0.94	-0.12	0.34	0.43	0.39
16	74	-0.81	-0.72	-0.94	-0.60	-0.06	-0.06	-0.37	-0.42	-1.21	-0.92
16	84	0.60	0.69	-0.87	-0.70	-0.02	0.78	0.14	-0.06	0.48	-0.12
22	52	-0.74	-0.91	-1.06	-0.75	-1.09	-0.33	-0.56	-0.99	-0.72	-1.37
22	65	0.30	-0.32	-0.92	-0.48	-0.86	-0.73	-0.76	-1.38	-0.83	-0.79
22	68	-0.76	-1.09	0.80	0.72	-1.10	0.03	-0.72	-0.68	-0.66	-0.19
22	78	0.47	0.37	-0.16	-0.13	-0.86	-0.30	-0.25	-0.45	-0.60	-0.71
28	57	0.64	0.52	0.40	0.22	0.86	-0.38	0.12	-0.42	-1.02	-0.74
28	62	-0.53	-0.90	-1.22	-1.14	-1.36	-0.49	-0.74	-1.28	-1.76	-1.19
28	68	-0.14	-0.34	-0.73	-0.65	-1.13	0.10	-0.45	-1.19	-1.13	-1.41
28	85	-0.83	-0.40	-0.70	-0.43	-0.22	-0.58	-0.34	-1.00	-0.67	-0.45
34	56	-0.85	-1.14	-0.92	-1.19	-0.68	-0.49	-1.37	-0.60	-0.99	-0.67
34	72	-1.87	-1.61	-0.83	-1.28	-1.24	-0.77	-1.50	-0.43	-0.28	-1.03
34	88	-1.86	-1.40	-1.12	-0.82	-0.52	-1.42	-1.26	-0.68	-0.96	-0.57

The symbol (*) indicates population was not quantifiable.

For the general linear models a regression approach fitted a model for each BCA × crop × climatic treatment (Table 4.4). Within the model, climatic treatments were coded as indicator variables, for viable population changes, and this allowed the analysis and calculation of coefficients for each BCA × crop × climatic treatment (predicted viable population change for a given climatic

treatment every 2 days), in addition to this as time (days) was provided as a covariate this also produced independent coefficients for BCA × crop. This was necessary because time also has an independent effect depending on the BCA and crop combination. For *B. subtilis* this was a viable population reduction of log_{10} -0.0687 on lettuce, and -0.0660 on strawberry. For *G. catenulatum* this was also a log_{10} viable population reduction of -0.0554 on lettuce, and -0.0760 on strawberry. This implies viable population changes occur due to the climatic treatment the BCA was subjected to, in addition to the time the BCA occupied the target crop.

Table 4.4 Predictive coefficients of viable population change of the two BCAs under fixed UK commercial growing climates in the phyllosphere of lettuce and strawberry plants every two days.

			Log_{10} coefficient of population change every two days			
Label code	**≈Temp°C**	**≈RH%**	***B. subtilis* lettuce**	**SE of Coefficient**	***B. subtilis* strawberry**	**SE of Coefficient**
A1	11	66	-0.533	0.343	1.613	0.398
A2	11	76	0.571	0.282	0.558	0.347
A3	11	82	0.099	0.282	0.059	0.347
A4	11	92	0.632	0.282	0.741	0.347
B1	16	61	0.020	0.282	0.051	0.347
B2	16	69	0.627	0.282	1.529	0.378
B3	16	74	0.108	0.294	0.924	0.347
B4	16	84	0.545	0.282	1.277	0.347
C1	22	52	-0.005	0.205	-0.283	0.287
C2	22	65	0.363	0.282	-0.197	0.361
C3	22	68	1.218	0.282	1.169	0.378
C4	22	78	-0.694	0.282	-1.024	0.361
D1	28	57	-2.070	0.307	-1.725	0.347
D2	28	62	0.293	0.294	-0.598	0.378
D3	28	68	-0.639	0.323	-0.380	0.421
D4	28	85	0.268	0.254	-0.665	0.295
E2	34	56	-0.301	0.240	-1.332	0.287
E3	34	72	-0.521	0.294	-1.160	0.287
E4	34	88	*	*	*	*
			Log_{10} coefficient of population change every two days			
Label code	**≈Temp°C**	**≈RH%**	***G. catenulatum* lettuce**	**SE of Coefficient**	***G. catenulatum* strawberry**	**SE of Coefficient**
A1	11	66	-0.163	0.261	-0.289	0.224
A2	11	76	0.403	0.261	0.340	0.224
A3	11	82	-0.221	0.261	-0.150	0.224

A4	11	92	0.136	0.261	-0.015	0.224
B1	16	61	0.072	0.261	-0.067	0.224
B2	16	69	0.834	0.272	0.710	0.234
B3	16	74	-0.095	0.272	-0.060	0.234
B4	16	84	0.371	0.261	0.518	0.245
C1	22	52	0.128	0.189	-0.188	0.166
C2	22	65	0.572	0.261	0.287	0.224
C3	22	68	-0.408	0.285	-0.165	0.245
C4	22	78	0.352	0.261	0.335	0.224
D1	28	57	0.028	0.261	0.214	0.224
D2	28	62	-0.218	0.261	-0.218	0.224
D3	28	68	-0.502	0.261	-0.213	0.224
D4	28	85	-0.341	0.216	-0.057	0.186
E2	34	56	-0.347	0.216	-0.233	0.186
E3	34	72	-0.246	0.261	-0.419	0.191
E4	34	88	*	*	*	*

The climatic treatment of 34 °C at 88 % RH could not be predicted as the variance inflation factor was not producible (meaning the variance between the replicates were too large for prediction).

4.4.6 Predicting BCA fate in real time at commercial growing environments

The generation of general linear models with the ability of predicting viable population changes of BCAs in relation to the climate enabled progress into evaluating the potential of produced models for prediction, and to compare the predicted viable BCA populations to the viable populations quantified using the PMAxx™-qPCR method in real time. Thus, the commercial trials provided a platform for determining the effectiveness and revealed the reliability of these models, alongside suggesting the necessity of including other abiotic variables such as rainfall, wind and UV for refining the prediction of BCA fate (Table 4.5).

Table 4.5 Quantified and predicted viable populations for the two BCAs in commercial growing environments

Crop	Experiment	Day	Rainfall amount (mm)	*B. subtilis* QST 713 Predicted log_{10} CFUs/mm²	*B. subtilis* QST 713 Quantified log_{10} CFUs/mm²	*G. catenulatum* J1446 Predicted log_{10} CFUs/cm²	*G. catenulatum* J1446 Quantified log_{10} CFUs/cm²
Lettuce	1	2	0	*	*	4.3	2.6
Lettuce	1	4	0	*	4.4	2.3	2.4
Lettuce	1	6	0.5-1	*	*	0	2.7
Lettuce	1	8	7-8	*	*	*	*
Lettuce	1	10	5-6	*	*	*	*
Lettuce	2	2	0	6.1	5.9	4.2	1.7
Lettuce	2	4	0.5-1	6.2	4.3	*	*
Lettuce	2	6	7-8	4.9	3.3	*	*
Lettuce	2	8	5-6	3.8	3.8	*	*
Lettuce	2	10	0	*	*	*	*
Lettuce	3	2	0.5-1	4.5	4.0	*	*
Lettuce	3	4	7-8	3.4	4.6	*	*
Lettuce	3	6	5-6	2.5	4.1	*	*
Lettuce	3	8	0	1.4	2.6	*	*
Lettuce	3	10	0	*	*	*	*
Strawberry	1	2	0	*	*	4.4	3.9
Strawberry	1	4	0	*	4.2	6.6	7.5
Strawberry	1	6	0	4.5	*	*	*
Strawberry	1	8	0	4.0	5.8	*	*
Strawberry	1	10	0	3.3	3.4	*	*
Strawberry	2	2	0	4.7	4.1	10.1	3.2
Strawberry	2	4	0	5.3	4.1	7.6	3.9
Strawberry	2	6	0	*	*	5.3	3.5
Strawberry	2	8	0	4.4	4.7	2.8	2.4
Strawberry	2	10	0	*	*	0	2.6
Strawberry	3	2	0	6.0	5.8	3.9	4
Strawberry	3	4	0	5.9	3.9	1.7	3.3
Strawberry	3	6	0	*	*	0	2.4
Strawberry	3	10	0	6.5	3.9	0	2.4

The (*) symbol in the prediction section means prediction of the BCA fate was not attempted because of the absence of a quantified sample for comparison, or unquantified D0. The (*) symbol in quantified section indicates a missing value because of the following reasons: (1) Ct was above 35, (2) absence of melt curve, and (3) lack of quantification.

The generated general linear models were examined for their ability in predicting BCA fate. The predicted and quantified viable populations varied in some instances. The capacity for prediction was not possible for either the BCA or specific crop type.

4.5 Discussion

This is the first study to investigate the impact of individual and interacting abiotic factors on viable populations of these two BCAs in the phyllosphere of lettuce and strawberry crops under such a wide range of temperature and RH combinations. The development of the PMAxx™-qPCR allowed the sensitive tracking and quantification of the true viable populations of these two BCAs in the phyllosphere of leaves of these two crops. These abiotic factors all significantly affected BCA viable populations. Subjecting the BCAs to sub-optimal and optimal growth temperatures led to viable population increases. Under these conditions, increasing RH led to an increase in the viable populations for both BCAs. For the two BCAs, an explicit pattern for viable population response in relation to VPD was lacking.

In the phyllosphere the bacterial and fungal BCA viable population biology are complex and depends on the interaction between temperature, microclimate RH and host. In the majority of UK commercial growing climates represented by temperature and RH combinations, differences in the introduced viable populations of both BCAs in lettuce and strawberry phyllospheres were found. The viable population sizes varied with specific climatic conditions, indicating that these factors influenced BCA mortality and/or reproduction. Of the abiotic factors examined, temperature has usually been suggested to be the most important factor influencing the development of an organism. *B. subtilis* cell growth becomes limited at <11 °C (Price, 2000) and is optimal over the range 25-37°C (Cook, 1996, Schaechter et al., 2006). The fungal BCA,

G. catenulatum is active at 5-34 °C, but optimal temperatures for growth is between 15-25 °C (Helyer et al., 2014). Unfavourable temperatures may cause mortality, while optimal temperatures lead to population growth in the crop phyllosphere. In addition, the viable population densities were highest at optimal temperatures for each BCA, but were affected by RH. Overall, temperature appeared to be a major abiotic factor for the establishment and development of the BCA population in strawberry and lettuce phyllospheres. Previous studies on *Beauveria brongniartii,* an entomogenous fungal BCA (Kessler et al., 2003), *Beauveria bassiana* an ascomycetal fungal BCA (Studdert and Kaya, 1990), *Staphylococcus aureus* a gram-positive bacterium (Valero et al., 2009), *B. subtilis* IB-15, *B. polymyxa*, Bacillus sp. 739 (Melent'ev et al., 2000), *Bacillus amyloliquefaciens* CPA-8 a bacterial BCA (Gotor-Vila et al., 2017b), and yeast BCAs (Artes et al., 1995) have all concluded that temperature significantly influenced the development and survival of their BCA, and was probably the key factor. However, these studies did not examine the interaction of temperature with RH on the phyllosphere of their target crops. The study revealed when BCAs are subjected to non-optimal temperatures, a higher rate of mortality occurs, reducing BCA viable population sizes and hence likely affect biocontrol. Temperatures < 22 °C caused a reduction in the viable population of *B. subtilis*, while temperatures > 22 °C led to a reduction in the viable population of *G. catenulatum*. Extreme low and high temperatures were lethal for the BCAs in the phyllosphere of lettuce and strawberry in the present study. Extended periods in unfavourable temperatures, especially on foliar surfaces, will thus

impact on BCA performance. This has also been found with some other BCAs (Kessler et al., 2003, Melent'ev et al., 2000).

The other important factor that influenced the development of BCA populations in the present study was RH. This is a critical bottleneck as often RH ranges of 95 – 100 % are required for population establishment, especially in the phyllosphere of crops (Magan, 2001, Hallsworth and Magan, 1999). The present study suggests the RH preferences exist for both BCAs, depending on air temperature as well as the host plant. An increase in the viable BCA population size occurred with increasing RH. This pattern was most evident in the optimum growth temperature ranges for these two BCAs. A study with the BCA *Candida oleophila* found that excess water was required for rapid reproduction in apple wounds to achieve biocontrol (Mercier and Wilson, 1995). The present study is one of the few studies that have demonstrated the impact of RH alone and when interacting with other factors on the viable population in a bacterial and fungal BCA. We clearly showed that for effective phyllosphere establishment, RH is a critical factor together with temperature. Perhaps formulation of the BCA then becomes critical in influencing the potential for effective establishment in the phyllosphere of different target crops. In the phyllosphere RH plays an important role and the formulation medium may prevent desiccation and improve BCA survival (Magan, 2001, Barbosa-Cánovas et al., 2008). Absence of a relationship between viable BCA population size and RH in non-optimal temperatures on both crops may also suggest that the host can adequately provide water requirements in non-optimal temperatures for

BCA survival. The presence of a relationship between viable BCA population size and RH in optimal temperatures on both crops suggests that this is a key factor for BCA growth under suitable temperatures. Overall, the preference in RH is dependent on both temperature and the phyllosphere of the host.

Grounds for investigating VPD came from the effect of beneficial temperatures being constrained to theoretical healthy plant transpiration rates which was estimated from the influence of VPD (Vadez et al., 2014, Yang et al., 2012, Shamshiri, 2014), and the effect of beneficial RH being constrained to sub-optimal and optimal BCA growth temperatures. VPD represents both the heat energy and the amount of water vapour available in the air, in addition to demonstrating the evaporative demand of a given climate, and therefore could be used to observe the combined effect of temperature and RH climatic treatments. Both temperature and RH impacted on the viable populations of the BCA, and were further influenced by host factors, which resulted in complex interactions between the factors affecting BCA viable populations. Thus, consideration needs to include interactions between these factors, including temperature, RH, potential source of water in the environment, and plant surface characteristics (Ruinen, 1961, Beysens, 1995, Andrews and Hirano, 2012). Because VPD was the only known factor capable of representing some of these factors it was considered, and the tested range was between 0.1-2.6 kPa. Overall, VPD affected the viable populations of both BCAs in the phyllosphere of lettuce and strawberry. However, viable population responses to VPD appeared to be sub-grouped (low, medium and high VPDs). In general,

for the bacterial and fungal BCA in lettuce and strawberry plants an overall viable population response pattern was absent, and the impact of VPD was independent of the crop and sub-group of VPD. Such findings may be due to the existence of native microbiota. A decrease in VPD raises available water potential, which controls the competitive activity between the BCA and the native phyllosphere microbiota. Thus, better adapted organisms to a specific climatic regime may outcompete the other (Edel-Hermann et al., 2009, Toyota et al., 1996).

The generated models prediction ability of BCA fate in each host in the context of commercial growing environments was tested. Inconsistent prediction of BCA fate and lack of quantification from lettuce plants may have resulted from the presence of overhead irrigation and rainfall, as the model was incapable of embedding this climatic factor. Field sampling contained separate challenges including the presence of excess soil on older leaves, and invertebrates. Time constrains limited the optimisation of the molecular assay for field sampling with excess soil. Moreover the cultivar employed was a winter type and the trials were initiated in early autumn season which was inappropriate for a physiologically satisfied winter variant crop. This resulted in an excess amount of tannin production, as well as this humic acid may have been present in the soil leading to further complications for quantification with qPCR and difficulties on the molecular assay as a whole (Opel et al., 2010).

Inconsistent prediction of BCA fate in strawberry plants could have been due to the sampling of flowers instead of leaves, as this removed the dilution effect

from leaf growth. Furthermore if residues of products remained from the previous AHDB trials their effects on the BCAs were unknown. In other words the model was incapable of consistently predicting BCA fate because of numerous unknown variables, which also included other abiotic and biotic factors (Sui et al., 2015, Liu et al., 2013, Magan, 2001). However, this can be partially derived from the mathematical approach, as population growth curves are complex with several different stages. Research on *C. oleophila* showed that utilising in-depth modelling has potential for consistently predicting BCA fate (Lahlali et al., 2008). Also the population ecology of the BCA could be different in fixed and fluctuating environments. Time constrains limited our ability to further optimise the models.

The factor of surface wetness emerged within commercial trials, but was absent in data collection and therefore not incorporated into the model predictions. For this reason, surface wetness requires discussion for *Botrytis* disease risk. In lettuce field trials, overhead irrigation, rainfall, fog and/or dew events, and in strawberry trials, dew and/or fog events were likely to lead to surface wetness (Rowlandson et al., 2015). Leaf wetness periods of 32 h in optimal temperatures of 20°C lead to a *Botrytis* infection probability of 60 %, while on flowers after wetness periods of 24 h at 20 °C botrytis infection reached 100 % (Bulger et al., 1987). Such findings are also supported by recent *Botrytis* risk forecasting models (Rasiukevičiūtė et al., 2013). This being the case, another study suggested low daytime VPD and high night time temperature as key factors for increasing strawberry flower infections instead of surface wetness

periods (Xu et al., 2000). In the lettuce field trials, a rainfall event for up to 96 h ensued, which lead to continuous leaf wetness throughout this period. In comparison, flower wetness periods were intermittent and emerged only a few hours a day. In the two commercial trials, optimum temperature for *Botrytis* was not achieved consistently. Therefore, the factor of surface wetness possibly requires further investigation, especially in combination with BCA × target crop × *Botrytis* infection. Furthermore, the surface wetness factor possibly contributed to the found differences between the models predicted and PMAxx™-qPCR quantified viable populations.

The present findings highlight the importance of an ecological understanding of BCAs in order to improve their biocontrol performance. The same temperatures at different RH and the same RH at different temperatures do not produce the same response. For the reproductive success of *B. subtilis* temperatures > 16 °C and < 34 °C at high RH values, and for *G. catenulatum* temperatures > 10 °C and < 22 °C at intermediate RH values were best for establishment. These findings show that these two BCAs are capable of surviving in the phyllosphere in climates of 20 °C at high RH levels that favour *B. cinerea* infection. On addition, this was the first time viable population studies were achieved with a technologically advanced molecular tool for these two BCAs under UK commercial growing climates. The findings from this study can improve the development of strategies for the timing of applications of BCAs with regard to climatic regimes and the relevant host for optimising the potential for their establishment in the phyllosphere to maximise disease control.

4.6 Conclusions

Abiotic factors of temperature, RH and VPD affected the viable populations of *B. subtilis* and *G. catenulatum* in lettuce and strawberry. Survival and reproduction of the BCAs in relation to temperature depended on both the evaporative demand of a given environment and the theoretical healthy plant transpiration rates. Optimum RH conditions encouraged survival and reproduction in sub-optimal and optimal growth temperatures of the BCA. For the two BCAs, viable population responses to increasing VPDs were specific to the crop and either low, medium or high VPDs.

CHAPTER 5

5 Spatial dispersal and colonization kinetics of biocontrol agents on developing leaves

5.1 Abstract

After biocontrol agents (BCA) are sprayed onto the phyllosphere, the foliar parts of plants, especially the leaves continue to grow leading to expansion of the surface area over which the BCAs need to colonise. This chapter examines the dispersion and colonization kinetics of the two BCAs (*B. subtilis, G. catenulatum)* in a semi-commercial glasshouse in the winter and spring seasons on lettuce and strawberry leaves naturally colonized by other phyllosphere microbiota. The PMAxx™-qPCR technique and leaf sectioning were used to differentiate and compare the viable population sizes residing on the leaf tissues of different ages. Differences in dispersion of *B. subtilis* were found between older and newly formed leaf tissue on lettuce in the winter period. Leaf expansion led to population changes of the two BCAs. However, neither negative nor positive correlation with viable populations for *B. subtilis* was found in relation to leaf expansion. For *G. catenulatum* there was a negative change in populations in relation to leaf expansion. These findings were also subject to other temporal effects, since leaf expansion and tissue age were inseparable from abiotic and biotic factors. The hurdles that this implies in effective establishment of BCAs, especially where the main mechanism is

competition for nutrients and space in the phyllosphere as foliar surfaces expand over time are discussed.

5.2 Introduction

The objective of applying BCAs to foliar surfaces for disease control is to obtain effective establishment in order to gain advantage over the pathogen. Research has focused on the persistence, survival and reproduction of BCAs in the phyllosphere (Alexandrova et al., 2001, Boff et al., 2001), together with dose response relationships (de Catalunya, 1996, Yu and Sutton, 1998), and mechanism of actions (Jacobsen, 2006, Narayanasamy, 2013). Chapters 3 and 4 have provided information on the impact that abiotic factors have on the establishment of these two BCAs. The main areas where BCAs have not been effectively studied is related to rates of colonisation of foliar surfaces, especially where this is changing. For example, leaves expand over time resulting in new uncolonised tissue becoming available for colonisation which are rich in nutrients for both fungal pathogens, the BCAs and other phyllosphere microbiota. Where a BCA relies on competition for nutrients and space this may be critical in determining successful control. Few studies have examined this aspect of the establishment of BCAs. For bacteria such as *B. subtilis*, formulation may be important, but also the availability of water in the phyllosphere as they require films of water to grow and colonise new tissue.

Previous research on dispersion and colonisation characteristics of BCAs have not examined this aspect and its role in determining the level of disease control

achieved (Lu et al., 2004, Prieto et al., 2009). Research on the dispersion and colonisation of the phyllosphere by BCAs is thus important in optimising the level of control achievable (Lindow and Brandl, 2003, Monier and Lindow, 2004). Few studies have focused on the dispersion and colonisation of foliar surfaces by BCAs (Collins et al., 2003, Crane and Bergstrom, 2014, Faize et al., 2006, Romero et al., 2004). Often the lack of an effective technique to achieve this has limited the ability to obtain such information.

Both *B. subtilis* and *G. catenulatum* are BCAs recommended for control of *B. cinerea* on foliar surfaces. However, the relative establishment after application as the phyllosphere expands due to new tissue formation has not been previously quantified. Without effective establishment and dispersion of the BCAs, relative control of *B. cinerea* may well be affected. New nutritionally rich tissue will thus be unprotected and prone to pathogen colonisation affecting the ability for competitive exclusion by the BCA (Collins et al., 2003, Crane and Bergstrom, 2014, Faize et al., 2006, Romero et al., 2004, Wei et al., 2016). Therefore, there is a need for quantification of the BCAs as the foliar tissues evolve.

The objectives of this Chapter were to examine the impact of (1) leaf expansion, and (2) leaf tissue age on viable populations of the two BCAs. The viable population sizes on leaf tissues of different ages (aged, developing, and new tissue) were quantified to understand the dispersion and colonisation kinetics for these two BCAs. These studies were carried out on both lettuce and strawberry leaves to provide fundamental ecological information to improve the

understanding of the establishment of these two BCAs in the evolving foliar phyllosphere environment.

5.2 Materials and Methods

5.2.1 BCA preparation

Refer to Chapter 4, Materials and Methods, Sub-section 4.3.1 for BCA isolation and culturing methods.

5.2.2 Plant propagation

Refer to Chapter 4, Materials and Methods, Sub-section 4.3.2 for plant propagation.

5.2.3 Treatments and experimental design

A randomized block design was used to investigate the effect of leaf tissue age and leaf expansion on the viable populations of *B. subtilis* and *G. catenulatum.* Experiments were conducted in a semi-commercial glasshouse located at NIAB-EMR East Malling. The rationale in selecting the semi-commercial glasshouse was to (1) use a natural growing environment (2) carry out an experiment during winter (gradual leaf expansion), (3) as well as during the spring (rapid leaf expansion). This design allowed testing the effect of leaf tissue age and leaf expansion on BCA viable populations. A single experiment was repeated in winter and spring, and each experiment contained nine replicates per treatment, with each replicate containing approx. 18 leaves obtained from plants placed on a grid inside the glasshouse. The time period of each treatment was 15 days. In the winter studies, samples were taken on D0,

D6, D10 and D15, and in the spring they were taken on D0, D4, D8 and D15. The experiments also contained negative controls that consisted of untreated lettuce and strawberry plants in each experiment.

5.2.4 General methodology description

All experiments contained eight common steps. (1) Plant propagation and selection: plants were sown, grown and selected for being pest-disease free and healthy with a minimum of six leaves. (2) BCA cultivation and concentration calibration: plate counts were used to determine the concentration of the cultivated *B. subtilis* and *G. catenulatum* (PreStop), and were adjusted as necessary to obtain the median effective dose (see Chapter 3). (3) Plant treatment: plants were sprayed with the BCA as a fine droplet misting just before run off. (4) Plant drying: after treatment plants were allowed to dry for 1 h in the glasshouse, after this period sampling and image acquisition of D0 leaves was made. (5) Sampling times are as described previously. The older leaves were collected from nine pre-determined plants (two leaves per plant), and immediately placed into a sterile sealable plastic box. (6) Leaf sectioning: each leaf was separated into three sections, the outer region (newly developing tissue units), the developing regions (developing tissue units in-between the outer region and centre of the leaf), and the aged regions (aged tissue units located around the midrib) see Figure 5.1. (7) Image acquisition: leaf sections were imaged alongside a standard (Panasonic DMC-SZ3) and immediately placed into a falcon tube containing maximum recovery diluent (Sigma). (8) Surface washing, filtration and cell pellet collection: the excised sections were

soaked in the maximum recovery diluent until full, sealed and shaken on a rotary shaker at 100 rpm for 30 mins at 10 °C. The contents were filtered with a wet muslin cloth (four layers) and cells pelleted by centrifugation at 2000 × g for 15 minutes at 4 °C. The supernatant was decanted and the cell pellet supplemented with maximum recovery diluent solution and transferred into a 1.5 ml Eppendorf, and stored at 4 °C.

The temperature and relative humidity depended on the climate within the semi-commercial glasshouse. Three data loggers were used to monitor the climate in the glasshouse throughout each experiment (EasyLog EL-USB-2 standalone USB temperature and %RH, dew point data logger). The same data loggers were used for monitoring the climate in the winter and spring. The total surface area of the leaf sections in each replicate was calculated with image J.

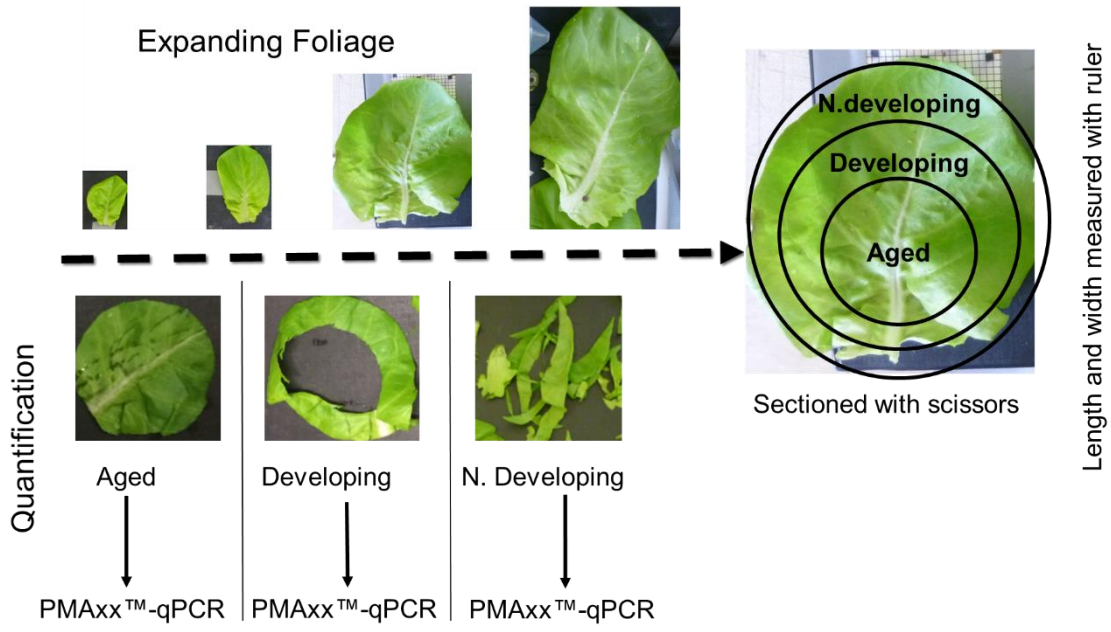


Figure 5.1 Diagram of leaf sectioning procedure on lettuce foliage

5.2.5 PMAxx™ treatment

Refer to Chapter 2, Material and Methods, Section 2.3.3 PMAxx™ treatment.

5.2.6 Grinding of *G. catenulatum* J1446 cells

Refer to Chapter 4, Material and Methods, Section 4.3.6.

5.2.7 DNA extraction

Refer to Chapter 2, Material and Methods, Section 2.3.4 DNA extraction and qPCR.

5.2.8 Minimizing and suppressing of qPCR inhibitors

Refer to Chapter 4, Material and Methods, Section 4.3.8.

5.2.9 qPCR

Refer to Chapter 4, Material and Methods, Section 4.3.9.

5.2.10 Viable BCA population quantification with PMAxx^{TM}-qPCR from the phyllopshere of plants

Refer to Chapter 2 Section 2.3.6 Testing viable BCA quantification with PMAxx^{TM}-qPCR from the phyllosphere of lettuce, and Section 2.3.6.1 Standard curves of cycle threshold to copy number of DNA and viable population.

5.2.11 Conversion of Ct to CFU/cm^{-2}

Refer to Chapter 2 Section 2.3.6.1 Standard curves of cycle threshold to copy number of DNA and viable population, and then Section 2.3.6.2 Conversion from total viable BCA population to viable population in leaf area.

5.2.12 Conversion of CFUs/cm^{-2} to population change for observing population biology with $Log_{10}(N_n) - Log_{10}(N_0)$

The equation $Log_{10}(N_n) - Log_{10}(N_0)$ defines the impact of temporal leaf expansion and leaf tissue type in relation to the produced population change, and divides interpretation into three parts: (1) producing a value of zero (0). The value of zero indicates no viable population change, which translates into the temporal leaf expansion and leaf tissue type being sustaining to the introduced BCA viable population (CFUs/cm^{-2}), and in lay terms, the description of survival is employed. (2) Producing a positive value, i.e., a positive value indicates viable population change, which translates into the temporal leaf expansion and leaf tissue type being beneficial to the introduced viable population to an extent that the viable population has surpassed the original size (CFUs/cm^{-2}), and in lay terms, the description of growth and/or reproduction is used. (3) Producing a negative value, i.e., a negative value indicates viable population change, which translates into the temporal leaf expansion and leaf tissue type being harmful to the introduced viable population (CFUs/cm^{-2}) to an extent in which the viable population size has decreased, and in lay terms, the description of mortality is used. These descriptions are only used to describe the overall outcome to the viable population (CFUs/cm^{-2}), and by no means suggests that in a positive relationship, mortality is not occurring, and vice versa. Table 5.1 demonstrates how the $Log_{10}(N_n) - Log_{10}(N_0)$ equation was utilized.

Table 5.1 Demonstration of how the equation Log_{10} (N_n) - Log_{10} (N_0) was put to use for observing viable population change with temporal leaf expansion and leaf tissue type

Organism/ Product	Plant	Climate Temp (°C) RH(%)	Leaf tissue type	EXP	Replicate of experiment	Day (N_n)	Log_{10} (N_n) CFUs/cm²	Day (N_0)	Log_{10}(N_0) CFUs/cm²	Log_{10}(N_n)-Log_{10}(N_0)	Replicate for temporal leaf expansion	Replicate for leaf tissue type
B. subtilis QST 713	Lettuce	16°C 69%	Newly Developing	1	1	6	4.34	0	5.65	-1.31	1	1
B. subtilis QST 713	Lettuce	16°C 69%	Newly Developing	1	1	10	4.41	0	5.65	-1.24	1	2
B. subtilis QST 713	Lettuce	16°C 69%	Newly Developing	1	1	15	4.38	0	5.65	-1.27	1	3
B. subtilis QST 713	Lettuce	16°C 69%	Newly Developing	1	2	6	4.44	0	6.66	-2.22	2	4
B. subtilis QST 713	Lettuce	16°C 69%	Newly Developing	1	2	10	5.28	0	6.66	-1.38	2	5
B. subtilis QST 713	Lettuce	16°C 69%	Newly Developing	1	2	15	4.64	0	6.66	-2.01	2	6
B. subtilis QST 713	Lettuce	16°C 69%	Newly Developing	1	3	6	4.26	0	6.21	-1.94	3	7
B. subtilis QST 713	Lettuce	16°C 69%	Newly Developing	1	3	10	5.19	0	6.21	-1.02	3	8
B. subtilis QST 713	Lettuce	16°C 69%	Newly Developing	1	3	15	5.44	0	6.21	-0.77	3	9

In Table 5.1 the column EXP stands for experiment. Since the experiment design contained the potential of investigating the association between: viable population (CFUs/cm^{-2}) and temporal leaf expansion, in addition to viable population (CFUs/cm^{-2}) and leaf tissue type, this was explored by pooling the data accordingly (see column replicate for temporal leaf expansion and replicate for leaf tissue type). The mean leaf size for lettuce at day 6 was 79 cm^{-2}, at day 10 was 89 cm^{-2}, and at day 15 was 123 cm^{-2}. For obtaining a total of 9 replicates for each day and leaf size samples from developing and aged tissues were also pooled into the data set (data not shown in Table 5.1).

The population change equation of Log_{10} (N_n) - Log_{10} (N_0) was employed instead of CFUs/cm^{-2} in graphical representations because the interest of the research and funding bodies was to categorize temporal leaf expansion and leaf tissue types as sustaining, harmful or beneficial in an explicit and simple manner, as this was critical for timing of application in strategy development. The use of CFUs/cm^{-2} directly would have overcomplicated interpreting the influence of temporal leaf expansion and leaf tissue type on population biology and introduced other uncontrollable complicated variables onto the graphical observation. Equations similar to this have been employed by several studies

for expressing population biology (Calvo-Garrido et al., 2014a, Calvo-Garrido et al., 2014b).

5.2.13 Statistical analyses

Before statistical analysis, the data sets of CFUs/cm^{-2} were transformed with the $\log_{10}$ function. One-way ANOVA was used to compare the CFUs/cm^{-2} between leaf tissue types for each BCA on each crop, separated by temporal leaf size. Statistical analysis was completed separately for the winter and spring experiment. When analysis proved statistically significant that leaf tissue type and not temporal leaf size contributed to differences in $\log_{10}$ CFUs/cm^{-2} an ANOVA was performed to identify which tissue types contained different CFUs/cm^{-2}. When analysis proved statistically significant that temporal leaf size alone and not leaf tissue types contributed to differences in $\log_{10}$ CFUs/cm^{-2} an ANOVA was performed to identify which temporal leaf sizes contained different CFUs/cm^{-2}. Finally when analysis proved statistically significant that both the leaf tissue types and temporal leaf size were contributing to differences in $\log_{10}$ CFUs/cm^{-2} ANOVA was performed to identify which tissue types and temporal leaf sizes contained differences in CFUs/cm^{-2}. All data analysis was performed with the software package MiniTab (V. 17) at P=0.95 confidence level.

5.3 Results

5.3.1 *B. subtilis* viable populations colonising leaves of lettuce and strawberries

Figure 5.2 shows the changes in viable populations on growing lettuce leaves in the winter and spring seasons. Significant changes in the viable bacterial

population sizes on leaf area (CFUs/cm^{-2}) occurred in association with temporal leaf expansion in the spring lettuce experiment ($P < 0.05$). In the spring experiment as the lettuce leaf grew over time, the viable populations on leaf area (CFUs/cm^{-2}) declined in a linear way up to 71 cm^{-2} where the maximum loss of viable populations developed (Fig 5.2).

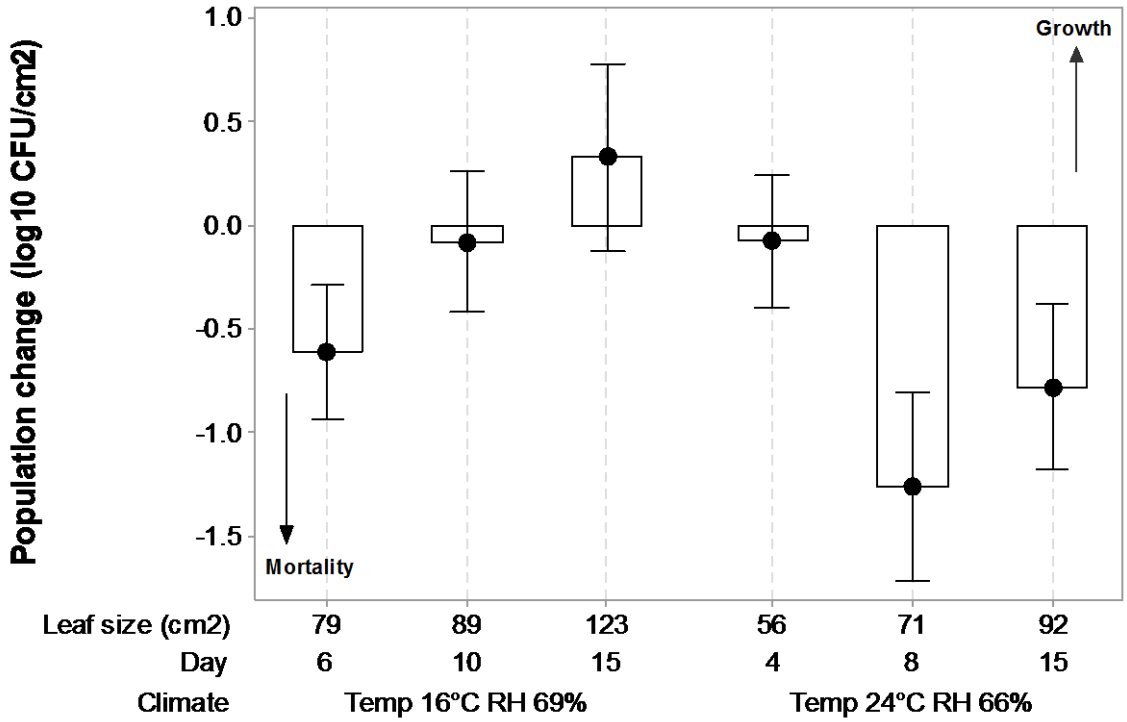


Figure 5.2 Viable population change in leaf area (CFU/cm^{-2}) of *B. subtilis* QST 713 on lettuce leaf as the leaves develop in the winter (left) and the spring (right) seasons

In Figure 5.2 and 5.3 the Y axis represents the mean BCA population change from the introduced viable population in each temporal leaf size, the equation in Section 5.3.12 was applied: population change = Log_{10} (N_n) - Log_{10} (N_0). On the X axis the displayed climate, day and leaf size (cm^{-2}) are the true mean values. The climates on the X axis label relates to the experiments carried out in the winter (16 °C RH 69 %) and spring (24°C RH 66 %). The X axis

label of leaf size (cm^{-2}) refers directly to the whole leaf size. The mean of the data sets are displayed with a black circle and represent the viable population change ($CFUs/cm^{-2}$) in log_{10} as the leaf grows, and is coupled with the co-variate time labelled as day in the X axis. Each mean in the figure contains nine replicates from a single experiment. The standard errors of each mean are represented by interval bars. Negative controls lacked a positive qPCR reaction (>35 Ct) and therefore the BCA population was assumed zero when Ct >35. The arrow pointing up on the graph indicates positive values represent growth for temporal leaf sizes in which the introduced viable population was exceeded on the leaf area ($CFUs/cm^2$), while the arrow pointing down on the graph indicates negative values represent mortality for temporal leaf sizes in which the population decreased below the introduced amount on the leaf area ($CFUs/cm^2$).

Figure 5.3 shows the changes in viable populations on growing strawberry leaves in the winter and spring seasons. Significant changes in the bacterial viable population sizes on the leaf area ($CFUs/cm^{-2}$) occurred in association with temporal leaf expansion in the strawberry winter experiment ($P < 0.05$). In the winter experiment as the strawberry leaf grew over time the viable population size on the leaf area ($CFUs/cm^{-2}$) increased in a linear form up to 32 cm^2 where the maximum gain of viable populations developed up to this surface area (Fig 5.3).

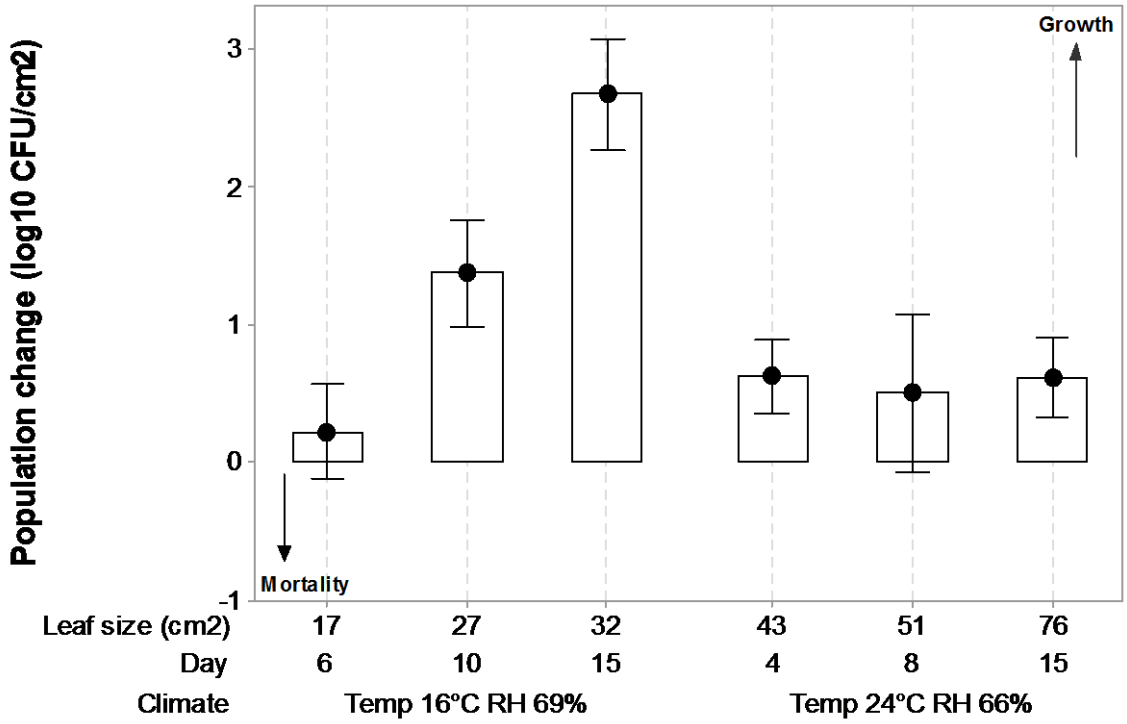


Figure 5.3 Viable population change in leaf area (CFU/cm^{-2}) of *B. subtilis* QST 713 on strawberry leaf as the leaves develop in the winter (left) and the spring (right) seasons

5.3.2 *B. subtilis* viable population dispersion on the leaf surfaces of lettuce and strawberry

From the four experiments conducted the viable population sizes (CFUs/cm^{-2}) were different on leaf tissue types in the winter lettuce leaf experiment ($P < 0.05$). The aged leaf tissues had greater populations (CFUs/cm^{-2}), which were higher than the original applied inoculum level when compared to the developing and newly developing tissues (Fig 5.4). Interestingly, the opposite trend was found in the spring experiment with lettuce where the youngest

(newly developed) leaf tissues had the highest viable population size (see Fig 5.4).

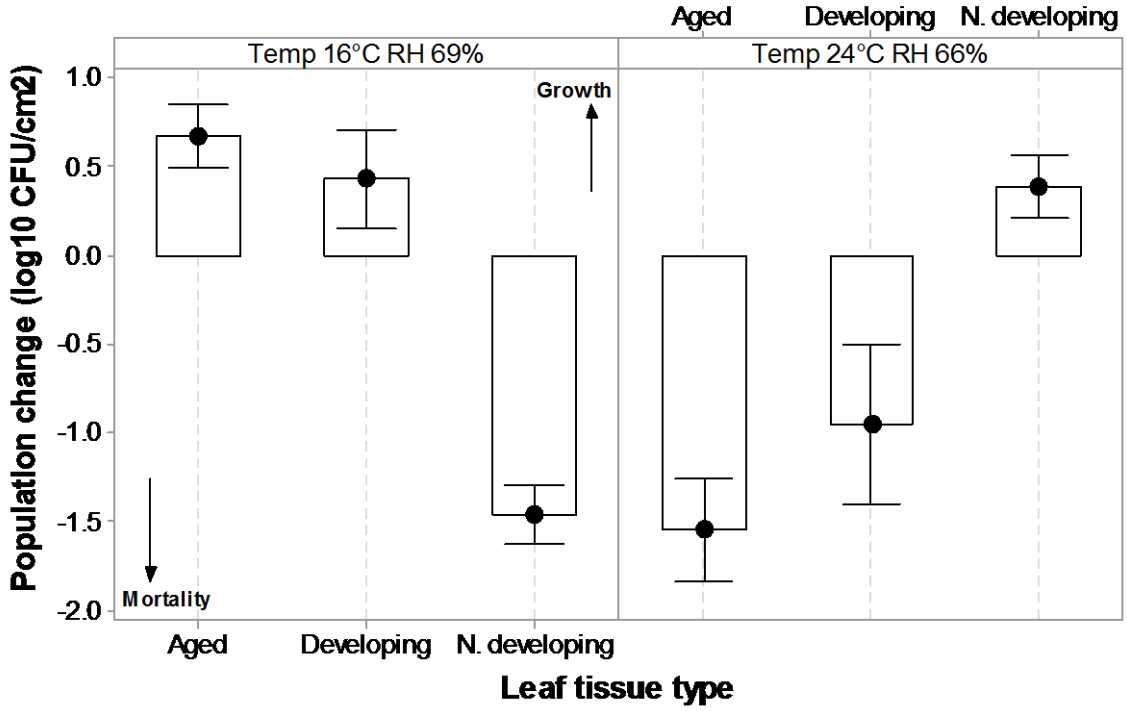


Figure 5.4 Viable population change in leaf area (CFUs/cm^{-2}) of *B. subtilis* QST 713 on lettuce leaf tissues of different ages in the winter (left) and the spring (right) seasons

In Figure 5.4 and 5.5 the Y axis represents the mean BCA population change from the introduced viable population in each leaf tissue type, the equation in Section 5.3.12 was applied: population change = Log_{10} (N_n) - Log_{10} (N_0). On the X axis the leaf tissue ages are displayed. The climate variable has been panelled and relates to the experiments carried out in the winter (16 °C RH 69 %) and spring (24 °C RH 66 %). The mean of the data sets are displayed with a black circle and represent the viable population change (CFUs/cm^{-2}) in log_{10} for each tissue type. Each mean in the figure contains nine replicates from a single experiment. The standard errors of each mean are represented by interval bars. Negative controls lacked a positive qPCR reaction (>35 Ct) and therefore the BCA population was assumed zero when Ct >35. The arrow pointing up on the graph indicates positive values represent growth in which the

introduced viable population was exceeded on the leaf area (CFUs/cm^{-2}) for that leaf tissue type, while the arrow pointing down on the graph indicates negative values represent mortality in which the population decreased below the introduced amount on the leaf area (CFUs/cm^{-2}) for that leaf tissue type.

Figure 5.5 shows that there was no significant difference in the viable population sizes (CFUs/cm^{-2}) between the leaf tissue types on different ages of strawberry leaves in both the winter and spring seasons. However, in all tissue types the viable population size (CFUs/cm^{-2}) exceeded the original application, except in the newly developing tissues in the spring.

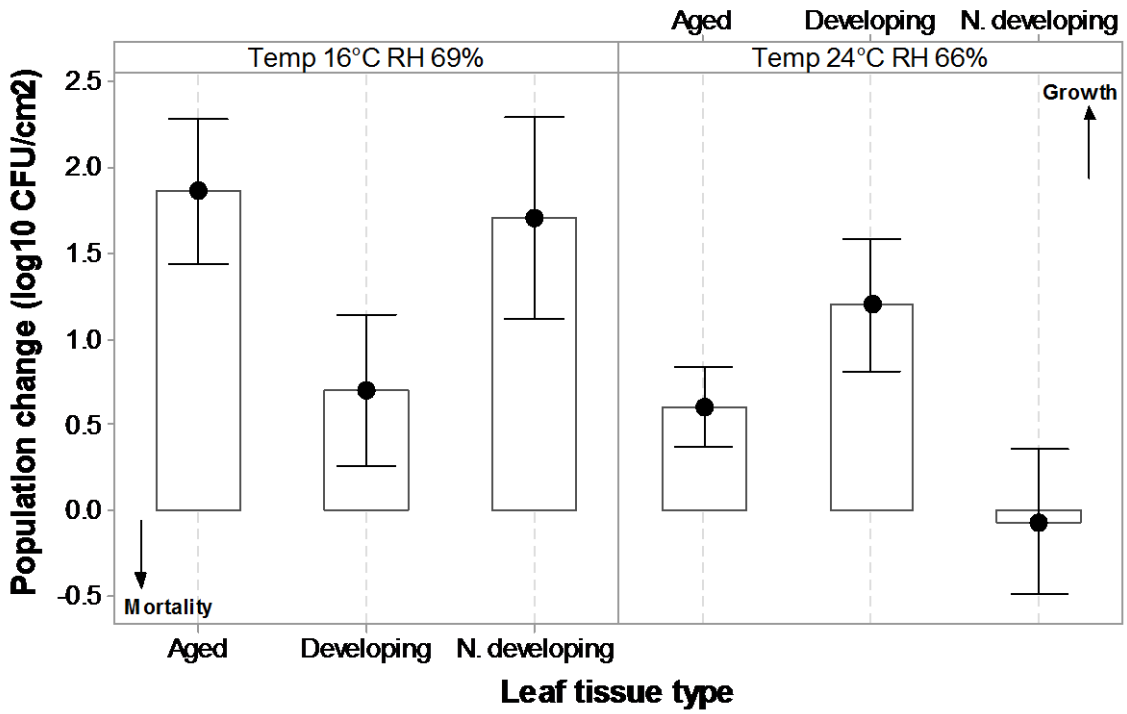


Figure 5.5 Viable population change in leaf area (CFUs/cm^{-2}) of *B. subtilis* QST 713 on strawberry leaf tissues of different ages in the winter (left) and the spring (right) seasons

5.3.3 *G. catenulatum* viable populations colonising leaves of lettuce and strawberries

Figure 5.6 presents the changes in viable populations (CFUs/cm^{-2}) on growing lettuce leaves in the winter and spring seasons. Significant changes in fungal viable population sizes in leaf area (CFUs/cm^{-2}) occurred in association with lettuce leaf expansion over time in the winter and spring experiments ($P < 0.05$). In both seasons as the leaves grew over time the viable population declined but at different rates, and then a recovery stage ensued. Despite this, the introduced viable population was not gained.

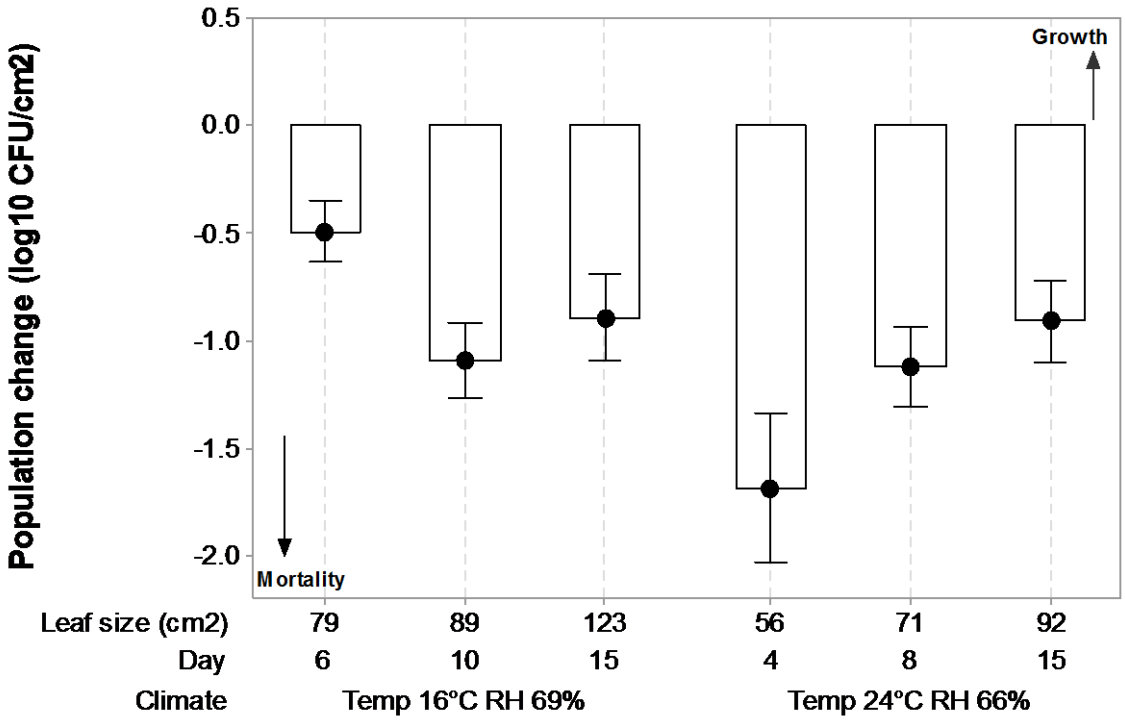


Figure 5.6 Viable population change in leaf area (CFU/cm^{-2}) of *G. catenulatum* J1446 on lettuce leaves as the leaves develop in the winter (left) and the spring (right)

In Figure 5.6 and 5.7 the Y axis represents the mean BCA population change from the introduced viable population in each temporal leaf size, the equation in section 5.3.12 was applied: population change = Log_{10} (N_n) - Log_{10} (N_0). On the X axis the displayed climate, day and leaf size (cm^2) are the true mean values. The climates on the X axis label relates to the experiments carried out in the winter (16 °C RH 69 %) and the spring (24 °C RH 66 %). The X axis label of leaf size (cm^{-2}) refers directly to the whole leaf size. The mean of the data sets are displayed with a black circle and represent the viable population change ($CFUs/cm^{-2}$) in log_{10} as the leaf grows, and is coupled with the co-variate time labelled as day in the X axis. Each mean in the figure contains nine replicates from a single experiment. The standard errors of each mean are represented by interval bars. Negative controls lacked a positive qPCR reaction (>35 Ct) and therefore the BCA population was assumed zero when Ct >35. The arrow pointing up on the graph indicates positive values represent growth for temporal leaf sizes in which the introduced viable population was exceeded on the leaf area ($CFUs/cm^{-2}$), while the arrow pointing down on the graph indicates negative values represent mortality for temporal leaf sizes in which the population decreased below the introduced amount on the leaf area ($CFUs/cm^{-2}$).

Figure 5.7 shows the changes in viable populations ($CFUs/cm^{-2}$) on growing strawberry leaves in the winter and spring seasons. Significant changes in fungal viable population sizes in leaf area ($CFUs/cm^{-2}$) occurred in association with temporal leaf expansion in the winter and spring experiments ($P < 0.05$). In the winter seasons as the leaf expanded over time a significant decrease in the viable populations emerged, when compared to the spring season. In the winter season, the initial sharp decline in viable populations were influenced by the recovery episodes in which the viable populations stabilised.

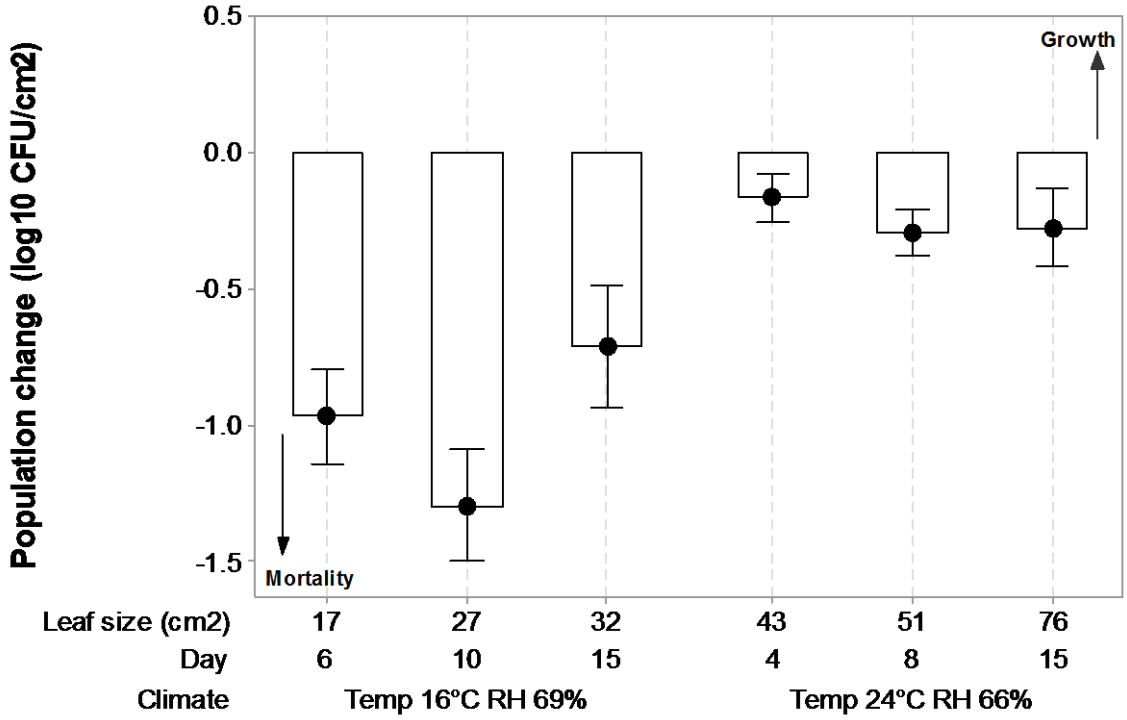


Figure 5.7 Viable population change in leaf area (CFUs/cm^{-2}) of *G. catenulatum* J1446 on strawberry leaf as the leaves develop in the winter (left) and the spring (right)

Although the fungal viable populations (CFUs/cm^{-2}) declined with leaf expansion, there was a recovery stage in which the viable population started exceeding the previous size. On the whole, smaller leaves retained higher fungal viable populations (CFUs/cm^{-2}) with a few exceptions (lettuce, spring experiment), but in no circumstance was the original inoculum level exceeded in the expanding leaf area.

5.3.4 *G. catenulatum* viable population dispersion on leaf surfaces of lettuce and strawberry

Figure 5.8 shows the changes in the viable populations (CFUs/cm^{-2}) of this BCA between different tissue types of lettuce leaves. There were no significant differences in viable populations (CFUs/cm^{-2}) between tissue types. Indeed there was no increase in the populations on lettuce leaves in the winter and spring seasons when compared to the initial application. Yet, new lettuce leaf tissue contained the highest viable population sizes.

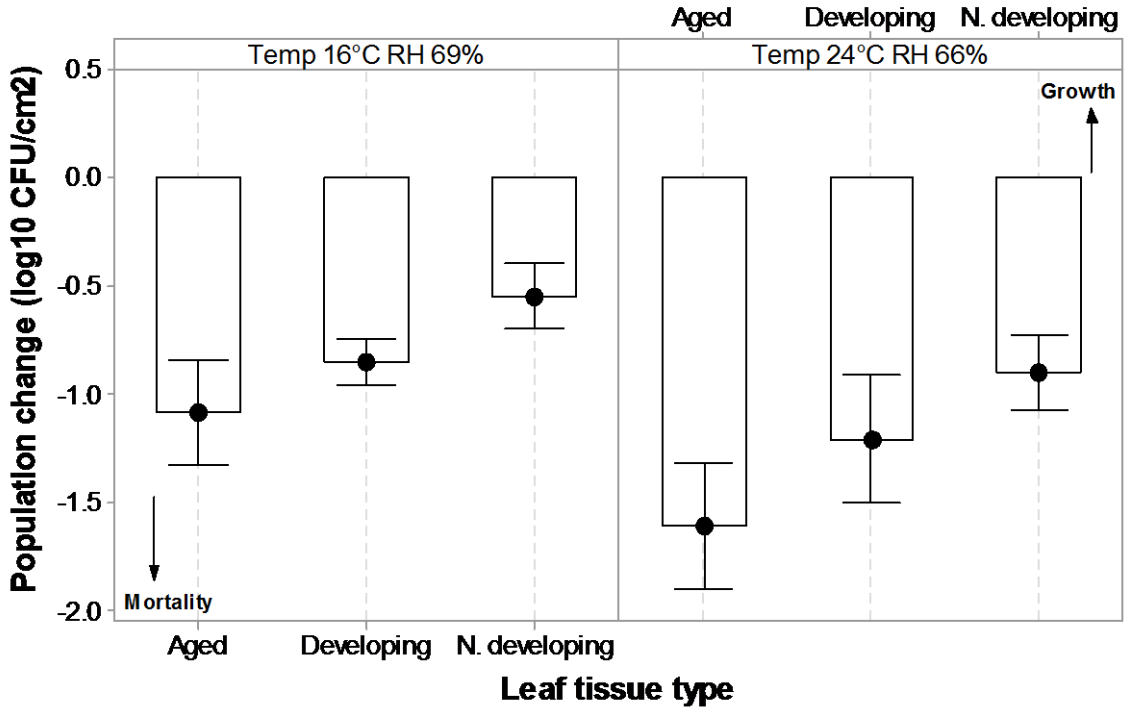


Figure 5.8 Viable population change in leaf area (CFUs/cm^{-2}) of *G. catenulatum* J1446 on lettuce leaf tissues of different ages in the winter (left) and the spring (right) seasons.

In Figure 5.8 and 5.9 the Y axis represents the mean BCA population change from the introduced viable population in each leaf tissue type, the equation in Section 5.3.12 was applied: population change = Log_{10} (N_n) - Log_{10} (N_0). On the X axis the leaf tissue ages are displayed. The climate variable has been panelled and relates to the experiments carried out in the winter (16 °C RH 69 %) and spring (24 °C RH 66 %). The mean of the data sets are displayed with a black circle and represent the viable population change (CFUs/cm^{-2}) in log_{10} for each tissue type. Each mean in the figure contains nine replicates from a single experiment. The standard errors of each mean are represented by interval bars. Negative controls lacked a positive qPCR reaction (>35 Ct) and therefore the BCA population was assumed zero when Ct >35. The arrow pointing up on the graph indicates positive values represent growth in which the introduced viable population was exceeded on the leaf area (CFUs/cm^{-2}) for that leaf tissue type, while the arrow pointing down on the graph indicates negative values represent mortality in which the population decreased below the introduced amount on the leaf area (CFUs/cm^{-2}) for that leaf tissue type.

Figure 5.9 shows the results for strawberry leaves. A pattern existed in the spring strawberry leaves where the aged leaf tissue had the highest populations (CFUs/cm^{-2}) followed by the developing and then the newly developing tissues, although differences were small. In the winter season there was very little pattern in viable populations (CFUs/cm^{-2}), even so the developing tissues had the highest populations. There were no significant differences in viable populations between tissue types on strawberry leaves in the winter and spring season.

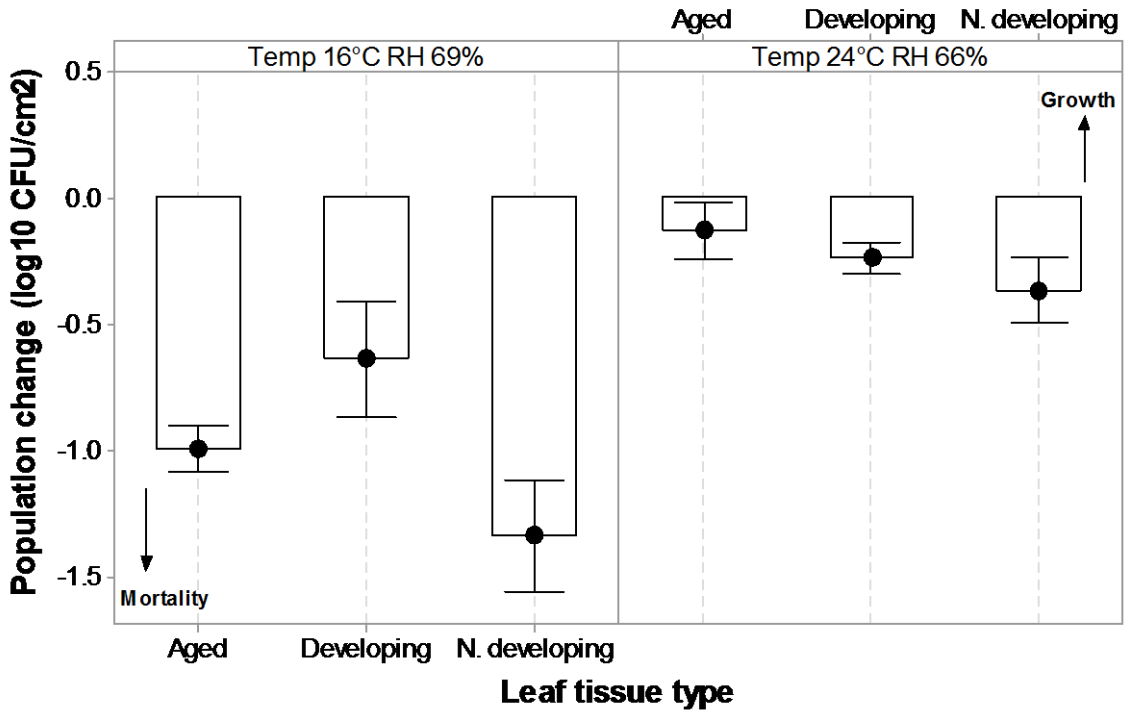


Figure 5.9 Viable population change in leaf area (CFUs/cm^{-2}) of *G. catenulatum* J1446 on strawberry leaf tissues of different ages in the winter (left) and the spring (right) seasons

For both BCAs the dispersion and colonization characteristics differed in relation to the host plant (lettuce and strawberry) and season (winter or spring). Simple relationships for colonization and dispersion characteristics were absent for the two BCAs, yet a quantifiable viable population existed for at least 15 day in both hosts on all tissue types.

5.4 Discussion

This study has shown the usefulness of using the PMAxx™-qPCR technique to obtain data for the first time on the kinetics of changes in viable populations of two different BCAs on developing leaves of both lettuce and strawberries. The establishment and occupation of the leaf tissue as the leaf size expands over time will thus have a significant impact on the potential control of the target pathogen, in this case *B. cinerea.* However, this association was subject to other temporal effects such as abiotic and biotic factors, which cause change in biocontrol populations (Andrews and Hirano, 2012, Lindow, 2006, Ruinen, 1961). The results suggest that *B. subtilis* colonisation kinetics varies markedly, while that of the fungal BCA *G. catenulatum* was more consistent in terms of temporal colonisation of the different tissue ages in lettuce and strawberries. This could partially be due to the bacteria requiring water films for effective reproduction and colonisation of new tissue. For the fungal BCA, once established, mycelial colonisation can occur more easily without water films allowing more rapid establishment on new virgin leaf tissue. This discussion focuses on temporal leaf expansion and leaf tissue age influence on viable BCA populations, to obtain information on dispersion and colonisation kinetics for the two BCAs. Considering the temporal leaf expansion and leaf tissue age factors are inseparable from other temporal abiotic and biotic factors (Andrews and Hirano, 2012, Lindow, 2006, Ruinen, 1961), numerous abiotic and biotic factors were probably also influencing BCA viable populations, in terms of dispersion and colonisation kinetics.

Simply put dispersion can be categorized as the locomotion ability, while colonization embeds reproduction potential after movement onto a new site. The present study suggests that dispersion and colonisation kinetics are independent factors subject to other temporal abiotic and biotic factors. For this reason a BCA may lack satisfactory movement yet reproduce at adequate rates on preferred sites, in which the analysis of the whole leaf indicates sufficient coverage, as shown with *B. subtilis* in the lettuce winter experiment: and vice versa. In contrast, the fungal BCA can disperse evenly across the host, but lacks rapid reproduction ability to continuously colonise an expanding leaf to retain the original viable population size on the leaf area, as commonly observed with *G. catenulatum.*

The tissue the BCA resides in possibly influences the viable population by leading to reproduction or mortality. The random settling on specific tissue types is variable especially for *B. subtilis*, except that this bacterial BCA was probably dependant on the environment for establishment. For the fungal BCA, it may be able to escape this necessity due to its ability to produce mycelia and establish effectively. The establishment is further influenced by presence of water, ultra violet radiation, nutrients, indigenous and epiphytic microbiota, microclimate, transpiration sites, topography, and wind speeds (Andrews and Hirano, 2012, Lindow, 2006, Ruinen, 1961).

At present, direct comparisons of our results with other studies are difficult because of methodological differences, experimental design and the utilised BCAs. Related research on *B. subtilis* (isolate) on sugar beet leaves reinforces

our findings as the organism displayed a preference for particular leaf sections leading to uneven coverage, except when applied with a glucose analogue, which allowed the isolate to disperse evenly across the leaf (Collins et al., 2003). This suggested that nutrient availability influences the dispersion kinetics and the lack of a nutrient supply causes inadequate dispersion. Moreover, studies on a non-pathogenic mutant of *Erwinia amylovora* revealed that the organism controls the pathogenicity on the leaf of apple seedlings through dispersing onto and colonising the midrib. This matches our findings as the midrib is located on mature older leaf tissue (Faize et al., 2006).

Studies of another *B. subtilis* (strain UMAF6614) on melon leaves using electron microscopy showed that the BCA dispersed into and colonised the junction lines of epidermal cells as cell aggregates (Romero et al., 2004). Applying this information clarifies our findings; since environmental conditions stimulate plant cell expansion and/or replication (Gonzalez et al., 2012, Volkenburgh, 1999), consequently the ratio of expanding to replicating cells possibly modifies the sustainable viable population size. Also tissue age may have a role in influencing the ratio between the choice of expanding on/in existing tissues and/or colonising new tissue where there is less competition from other microbiota. Recent research on *Bacillus amyloliquefaciens* with green fluorescent tagging suggests this BCA also colonises in a aggregated form while residing on certain sections of the wheat leaf and glume (Crane and Bergstrom, 2014). In theory, increase of plant cell replication may improve BCA reproduction and result in their colonisation potential of newly developed tissue

units. These are invaluable resources for nutrients, while plant cell expansion may improve a BCAs dispersion since expanding plant cells may physically distribute the BCA (Crane and Bergstrom, 2014, Gonzalez et al., 2012, Romero et al., 2004, Volkenburgh, 1999).

Differences in viable population sizes of the two BCAs on leaf tissue of different ages, focuses on the dispersion kinetics which probably varies because of numerous factors such as water, ultra violet radiation, nutrients, indigenous and epiphytic microbiota, microclimate, transpiration sites, topography, and wind speeds (Andrews and Hirano, 2012, Lindow, 2006, Ruinen, 1961). These abiotic and biotic factors present on leaves require synchronised investigation with viable population dispersion and colonisation, and indicates the complexity of the factors and mechanisms involved for a BCA to disperse on a leaf.

For *B. subtilis* leaf expansion induced the colonisation kinetics of the bacterium positively or negatively, and the outcome depending on host and climate. In comparison, for *G. catenulatum* leaf expansion induced the colonisation kinetics of this strain negatively. However, the rate of population size reduction in leaf area was influenced by host and climate. This suggests an interaction between climate × host × leaf expansion rates on the two BCA's colonisation kinetics. Previous studies with the BCA, *Ulocladium atrum,* in relation to survival on strawberry leaves suggested population density exponentially declined over time, although this study did not focus on leaf expansion. However, they sampled leaves for 70 days in a growing environment, suggesting that leaf expansion must have occurred (Boff et al., 2001). This reinforces the present

findings that leaf expansion may reduce viable population sizes as leaf area expands.

Our findings suggest that *B. subtilis* may become constrained by lack of dispersion and colonisation, whereas *G. catenulatum* biocontrol may become limited by the lack of colonisation. On the whole this study underlines that dispersion of bacterial and fungal BCAs and colonisation abilities are affected by both abiotic and biotic factors. The ecological information obtained has importance for BCA application strategies, especially for timing of applications, as well as knowledge on how the two BCAs colonise developing lettuce and strawberry leaves.

5.5 Conclusions

This study has shown that it is important to consider the fluctuations in BCA populations which might occur when leaves are expanding and new tissues are being produced. The present study suggests that the kinetics are very important and perhaps formulation of BCAs does not take account of the establishment of the BCA under such plant physiological changes, and the relationship between the BCA and the target pathogen. New tissue may be rapidly colonised by other phyllosphere microflora including *B. cinerea* and this can result in more difficulty in excluding the pathogen and competing effectively.

CHAPTER 6

6 General Discussion On Optimising BCAs

6.1 Tools for enhancing knowledge on BCA ecology and compatibility

The phyllosphere environment of plants is colonised by a range of microorganisms which changes in time and space in response to abiotic fluxes which they are exposed to. Thus, applied BCAs must be able to effectively compete with the resident populations and compete for the limited nutrients available on such foliage surfaces (Cray et al., 2013, Lievens et al., 2015). It is essential to thus be able to quantify the viable populations of a BCA on the phyllosphere surfaces under different climatic regimes. This is important in understanding where the BCA can compete effectively in this environment to be able to control resident fungal pathogens such as *Botrytis cinerea*.

Previous studies suggest that the competence of BCA to outcompete the native microbiota and plant pathogens in the phyllosphere may be significantly affected by formulation of the BCA and efficiency of application and subsequent establishment (Cray et al., 2013). However, knowledge of the fluctuations in formulated BCAs in the phyllosphere, especially using molecular techniques have not previously been extensively studied despite this being critical for successful establishment in the phyllosphere for effective disease control. This

project has developed, for the first time, a PMAxx™-qPCR method for the quantification of viable BCA viable in the phyllosphere (see Chapter 2).

The developed molecular assay PMAxx™-qPCR for quantifying viable population changes in the BCAs in formulations was tested by monitoring of the viable populations of *B. subtilis* QST 713 and *G. catenulatum* J1446 in the phyllosphere of lettuce and strawberry crops.

The developed PMAxx™-qPCR system had the advantage of distinguishing between live and dead BCA cells, and allowed the assessment of growth kinetics of viable populations (Nocker et al., 2006, Fittipaldi et al., 2012). In theory, this approach may also be advantageous as a tool for the rapid screening and selection of the best formulations and storage conditions required for commercializing BCAs. The developed PMAxx™-qPCR tool provides ecological benefits by: (1) including viable but not culturable cells (Papadimitriou et al., 2016, Liu et al., 2016), (2) improving tracking, monitoring and quantification of live populations with a high level of specificity and sensitivity (Silva and Domingues, 2015), (3) being time efficient, and accurate for avoiding misinterpretations from environmental samples (Elizaquível et al., 2012b).

There were certain constraints on the project due to funding which resulted in the prioritisation of the development of a functional PMAxx™-qPCR tool. This of course had some implications: (1) the unavailability of a PMA-Lite™ LED photolysis device, resulted in the development of an independent PMA

activation system in relation to manufacturers recommendations. This approach has also been adopted in other studies (Crespo-Sempere et al., 2013, Elizaquível et al., 2012a, Miotto et al., 2020, Soto-Muñoz et al., 2014). (2) The highest possible light intensity (800 W) and exposure time (30 mins) was applied to ensure PMA activation as an alternative to identifying the optimal lighting conditions. This was used previously (Miotto et al., 2020, Soto-Muñoz et al., 2014). (3) DNA from dead BCA cells were unblocked for *B. subtilis* QST 713 resulted in a background level of 3.44 $\log_{10}$ dead cells, and for *G. catenulatum* J1446 a total of 5.75 $\log_{10}$ dead cells. This limitation has also been noted in other studies (Crespo-Sempere et al., 2013, Elizaquível et al., 2012a, Miotto et al., 2020, Soto-Muñoz et al., 2014). (4) In the initial development, the findings from the assay were compared with culture-based and microscopy methods to validate findings. However, in the second phase for phyllosphere-based testing this was achieved for confirming applications rates, but not used throughout the whole thesis. The justification for introducing this limitation in phyllosphere testing was because: (a) it was not physically possible to carry out both techniques for a prolonged period due to the labour requirements; (b) the impact of PMAxx™ method was not observable via viable plate count techniques, as these methods are not capable of demonstrating if PMAxx™ has blocked the DNA from dead cells; (c) other research findings highlighted that a consensus between the two methods was not probable in the phyllosphere (Knight, 2000, Liu et al., 2016, Papadimitriou et al., 2016). Consequently for phyllosphere studies, the PMAxx™-qPCR was important as the majority of the

BCA populations were viable but not culturable in this environment (Pinto et al., 2015).

The PMAxx™-qPCR tool was used to examine the impact of abiotic factors on the viable populations of both the bacterial and fungal BCA under UK climatic conditions. The results are beneficial in identifying the best timing of applications for potentially establishing such BCAs in the target crops. The present study has shown that fluctuations in temperature and RH can impact the BCA populations on both lettuce and strawberry leaves. Previously, the viability of BCAs has been influenced by the use of freeze drying and cell drying of formulations (Abadias et al., 2001, Abadias et al., 2003). It is thus important to maximise the formulations such that they can establish in the phyllosphere where they are exposed to extremes in temperatures, RH, and UV light which can all significantly decrease viability of formulations (Sui et al., 2015).

In summary, the developed PMAxx™-qPCR molecular assay enabled the monitoring of viable populations of *B. subtilis* and G*. catenulatum* in the phyllosphere of both lettuce and strawberry crops. More information on these BCAs, including effects of temperature, formulation and foliar leaf establishment was also needed in relation to relative control ability.

6.2 Optimising BCAs with population density

For reliable biocontrol, the BCA must be capable of controlling *B. cinerea* even under high disease pressure conditions. Chapter 3 evaluated and modelled the dose response relationships of *B. subtilis* QST 713 and *G. catenulatum* J1446

under high disease pressure of *B. cinerea* while simultaneously testing if abiotic and biotic factors affected biocontrol. This approach allowed the identification of a reliable and consistent inoculum dose.

The present study discovered a direct interaction between the BCA inoculum and *B. cinerea* colonisation and macroconidial infection. It also demonstrated the importance of choosing a suitable inoculum dose for controlling *B. cinerea* even at high densities under different conditions, i.e., temperature, formulation and leaf tissue type, providing new information on our understanding of these processes not available previously (Montesinos and Bonaterra, 1996, Smith et al., 1997, Larkin and Fravel, 1999, Gerlagh et al., 2003). Thus, Chapter 3 was the first study that investigated the relationship between abiotic and biotic factors and biocontrol of *B. cinerea* with *B. subtilis* QST 713 and *G. catenulatum* J1446. This showed that the population density of the BCA required for effective control and the economic costs of application of the inoculum dose and the level of control achieved was important (Larkin and Fravel, 1999, Fravel, 2005).

Factors listed in reviews for improving BCAs (Droby et al., 2009, Fravel, 2005, Liu et al., 2013, Sharma et al., 2009, Spadaro and Gullino, 2005, Andrews, 1992) have been more focused on the function of the BCA in the environment and climate or the traits/characteristics of the BCA. Consideration of the population density or number of introduced BCA propagules have been lacking. Some previous studies have focused on BCA inoculum dose of these two BCAs, have been carried out with very low inoculum doses of BCA and pathogen than that recommended. This has been shown for *B. subtilis* QST 713

(Reiss and Jørgensen, 2017, Peng et al., 2011) and for *G. catenulatum* J1446 (Dik et al., 1999, Peng et al., 2011).

The question which was not addressed was how these two BCAs behave post-application, in terms of viable populations and at least conserving their LD_{50} population densities until the next treatments under UK climatic conditions for the control of *B. cinerea,* even under high disease pressure. This would be beneficial economically to the grower. Therefore, the findings in Chapter 3 show that for BCAs to perform effectively, a population density threshold must be met, because less inoculum than this level affects the biocontrol function, as shown by the polynomial regression and probit models developed in the present study. This was not available previously. Furthermore, the present study has shown that the median effective population density of the BCA was directly related to abiotic and biotic factors. This goes beyond previous dose response models (Adams and Fravel, 1990, Bull et al., 1991, Hadar, 1979, Mandeel and Baker, 1991).

The developed *in vitro* polynomial regression model for the efficacy of each BCA dose against *B. cinerea* provides a foundation for understanding dose response relationships, and the importance of choosing the correct dose and environment for the desired efficacy and the expected minimum and maximum outcome. Despite limited commercial trials being carried out, both models clearly demonstrate BCA function when applied at the correct dose at a given temperature. Therefore applying such a strategy may possibly provide better cost effectiveness with better reliability of BCA efficacy and consistency in

controlled environments such as greenhouses where climate can be controlled. Overall, these findings provide the beginning of an epidemiological matrix for dependable, efficient and consistent biocontrol of *B. cinerea* using the BCAs *B. subtilis* QST 713 and *G. catenulatum* J1446.

Many studies suggested environment, climate, and host as primary causes of loss in biocontrol efficacy and consistency. However, the majority of the reviews of available information (Magan, 2001, Sui et al., 2015, Liu et al., 2013, Droby et al., 2009) support the critical importance of abiotic factors affecting the introduced BCA viable population densities, their survival and reproduction and therefore their ability to control microbial pathogens. For further optimising the two BCAs, research into how abiotic factors impact on their viable populations requires further studies.

6.3 Optimising BCAs with climate

Successful dominance over phyllosphere pathogens with *B. subtilis* QST 713 and *G. catenulatum* J1446 depends on effective establishment and resilience in the phyllopshere tissues. This also depends on a strong association between host tissue establishment and the mechanisms of actions (Köhl et al., 2019, Spadaro and Droby, 2016, Cray et al., 2013). The research in Chapter 4 examined the impact of temperature, RH and VPD within UK growing climates on the establishment ability of the BCAs on the phyllosphere. As well as identifying promising conditions for BCA establishment, to also provide

information on the best timing of applications for integration of such BCAs into UK horticultural practice as part of an IPM strategy.

Developing an understanding of viable populations in relation to UK growing climates with all significant climatic variables can facilitate the development of prediction models of BCA fate. The general linear models produced in Chapter 4 for each climatic regime can be applied to cropping systems that lack overhead irrigation and rainfall, and are foliage specific. Thus, the developed models are unable to consistently predict BCA fate, and therefore require further optimisation including inclusion of rainfall and more refined expert modelling.

Chapter 4 showed that viable populations of the BCAs are affected by temperature, RH and VPD. The definitions of these findings are simple in nature and pin-point that abiotic factors directly influence establishment of these BCAs. Thus, timing becomes critical when incorporating into plant protection practice.

Indeed the positive effect of temperature (Kessler et al., 2003), and RH (Lahlali et al., 2006) was found for other BCAs. However, a more unique study which brought together both bio-efficacy and abiotic factors was that with *Pichia guilliermondii* strain Z1, where interactions between this BCA and *Penicillium italicum* was examined over a range of temperatures and RHs to determine the influence of biocontrol efficacy (Lahlali et al., 2011). However, in the present study (Chapter 4) has made significant advances in examining a range of abiotic factors relevant to UK growing climates. However, in addition to those

abiotic factors examined, it has been shown that surface wetness may also be critical and is an important factor influencing *B. cinerea* disease risk as well as establishment of the BCAs (Bulger et al., 1987, Rasiukevičiūtė et al., 2013, Rowlandson et al., 2015).

This study has however identified the relationship between these viable populations of these two BCAs × BCA efficacy × UK agronomy climates × host. These studies have identified reliable climatic treatments for both viable population growth and plant protection. Viable populations of BCAs were quantified from whole leafs without introducing methods such as leaf sectioning and/or green fluorescent protein tagging to monitor dispersion. Since both these BCAs use competition for nutrients and space this possibly limits the findings as it introduces the prospect that the BCAs can be present in a sufficient amount yet lack coverage and therefore influence the protection ability (Collins et al., 2003, Crane and Bergstrom, 2014, Faize et al., 2006, Lu et al., 2004, Prieto et al., 2009, Romero et al., 2004, Wei et al., 2016). Two of the crucial abiotic factors were investigated (temperature and RH). Further studies are required on rainfall, UV light and leaf wetness interactions with these two BCAS.

Practically the findings in this study show the relationship of viable populations of BCAs with abiotic factors on the phyllosphere are a matrix where each factor can be a primary or a secondary influence, and this depends on the prevailing current environmental conditions. They also highlight the significance of applying BCAs at the correct inoculum dose combined with the suitable climatic regime.

6.4 Optimising BCAs with colonisation and dispersion

For BCAs to physically protect plant tissue units from *B. cinerea* they must be able to disperse and colonise existing and new tissue units. Chapter 5 examined the ability of *B. subtilis* and *G. catenulatum* to become established and colonise such foliage, in relation to both season and host.

Developing an understanding the viable populations in relation to leaf expansion and tissue age can lead to improved prediction of the dispersion and colonisation kinetics of BCAs for a given environment. Chapter 5 quantified the colonisation and dispersion of the two BCAs on such foliar tissues for the first time. In addition, this study provided evidence for changes in dispersion and colonisation depending on both the season and host.

Overall, the climate and host (strawberry or lettuce) influence the viable populations of both BCAs. Under some conditions the establishment and colonisation was improved, while under other conditions there were significant differences between the bacterial and fungal BCA. This type of information will be valuable for timing of applications of these types of BCAs.

This study has quantified both dispersion and colonisation kinetics simultaneously in two different seasons with two different hosts in a semi-commercial glasshouses. This showed that there was a link between type of BCA used and their dispersion and colonisation kinetics, which were dependent on both climate and host. It appears that the bacterial BCA requires water films to grow and colonise new foliar tissue. In contrast, the fungal BCA, was able to

colonise new tissue via hyphal growth facilitating better resilience. No previous studies have investigated these aspects (Collins et al., 2003, Crane and Bergstrom, 2014, Faize et al., 2006, Lu et al., 2004, Prieto et al., 2009, Romero et al., 2004, Wei et al., 2016).

Practically the findings suggest the dispersion and colonisation patterns of BCAs on the phyllopshere may be a matrix in which abiotic and biotic factors can become important for enhanced dispersion and colonisation of the BCA, and this requires integration with UK agronomic climates to maximise efficacy of such BCAs.

7 Conclusions

The present research initially developed the molecular tool PMAxx™-qPCR for quantifying viable population of *B. subtilis* QST 713 and *G. catenulatum* J1446 in formulation and on aerial plant surfaces. Subsequently dose response models were developed for *B. subtilis* QST 713 and *G. catenulatum* J1446 against *B. cinerea* in relation to temperature and formulation and leaf tissue type. These studies showed that the LD_{50} population density changed with these factors. Subsequently, the effects of abiotic factors (temperature and RH) allowed the production of simple predictive models on the viable populations of *B. subtilis* QST 713 and *G. catenulatum* J1446 in lettuce and strawberry. BCA population size was highest when temperature was close to the optimum for their growth and development. High RH encouraged survival and/or reproduction in sub-optimal and optimal growth temperatures of the BCA. In succession the colonisation and dispersal capabilities of *B. subtilis* QST 713

and *G. catenulatum* J1446 were examine for the first time. The populations of the BCAs were quantified on continuously growing leaves representing existing and new foliar issue which showed that colonisation and dispersion kinetics are impacted by season and host.

8 Future work

8.1 Effect of water availability and temperature on the viable population growth of the BCAs

More detailed information is required on the ecological abiotic boundaries for growth of populations of these BCAs. Thus media can be modified with glycerol to achieve different $\mathbf{a_w}$ levels (0.995-0.92 $\mathbf{a_w}$) x different temperatures to better understand the effect on growth of *B. subtilis* and conidial germination of *G. catenulatum.* The lag times prior to growth, relative growth rates, and sporulation potential would help identify the ecological windows for successful establishment on foliar surfaces. These studies could be extended to examine efficacy on detached leaves and subsequently on seedlings.

The ecological information collected can form a profile for understanding the environmental range of interacting a_w x temperature conditions for *B. subtilis* and *G. catenulatum* for colonisation and reproduction, and additionally define the relationship of a_w and temperature on BCA viable populations overtime in a stringently controlled environment.

8.2 Identify the effect of rainfall events on *B. subtilis* and *G. catenulatum* viable populations and their dispersion onto newly developing leaves

Simulation of precipitation events including rainfall amount, rainfall intensity, and wind speed and effects on BCA functioning are needed. The effect on viable populations of *B. subtilis* and *G. catenulatum* needs to be quantified in relation to rainfall events and whether they can disperse by rainfall onto newly developing leafs. In addition, surface wetness regimes and wetness time periods need to be included to identify temporal conditions which will allow growth and establishment on foliar surfaces of strawberry flowers and leaves, and on lettuce leaves in the presence and absence of *Botrytis*.

8.3 Testing biocontrol efficacy genes under different climatic conditions

In recent years, a number of studies have identified several genes involved in mechanism of actions of specific biocontrol organisms. The currently commercialised BCA *B. subtilis* QST 713 controls target pathogens through antibiosis. Research has identified several biosynthetic genes including bacciliomycin, iturin, surfactin, mycosubtilin and fengycin produced by *Bacillus spp* (Athukorala et al., 2009), but a recent study indicated that Bacyllomicin and fengycin are the primary antifungal lipopeptides (Mora et al., 2011) along with Iturin A (Arrebola et al., 2010) and itu C (Ramachandran et al., 2014). RT-qPCR can be utilized to monitor the expression of antibiotic production. *G. catenulatum* is a fungal parasite which antagonizes fungal plant pathogens, recently the *per3* gene which is upregulated 6.6 fold under the induction of

sclerotia was identified (Sun et al., 2015); the *per3 gene* can be monitored in different climatological environments to identify the optimum conditions in which *G. catenulatum* J1446 will be most effective.

8.4 Selective breeding BCAs for engineering abiotic stress tolerant strains

Selective breeding to produce better crops and livestock are commonplace in horticulture and agriculture. This process can be applied to BCAs for creating abiotic stress tolerant strains capable of thriving in unfavourable environments. By using rigorous climatic treatments with strict breeding protocols it may be possible to develop resilient BCAs that are adapted to surviving in stressed climates and/or environments, while simultaneously being able to reproduce, disperse and colonise their target crops (Agler et al., 2016, Birch and Walker, 2000, Gabriel and Northup, 2013, Marian and Shimizu, 2019, Williams and Lenton, 2007).

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APPENDICES

Additional detailed information on data analyses have been deposited with Cranfield University and NIAB-EMR and with the supervisors.

Appendix A Chapter 1

Appendix B Chapter 2

B.1 Statistics effect of PMAxx™ dosages on DNA amplification

B. subtilis/Serenade

Table B-1 REML variance components analysis of PMAxx™ dosages on DNA amplification for *B. subtilis*/Serenade

REML variance components analysis					
Tests for fixed effects					
Sequentially adding terms to fixed model					
Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	P value
PMA_concentration_M	164.93	4	41.23	37.0	<0.001
Condition_treatment	7.97	2	3.98	37.0	0.027
PMA_concentration_M.Condition_treatment	6.62	8	0.83	37.0	0.584

G. catenulatum/PreStop

Table B-2 REML variance components analysis of PMAxx™ dosages on DNA amplification for *G. catenulatum*/PreStop

REML variance components analysis					
Tests for fixed effects					
Sequentially adding terms to fixed model					
Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	P value
PMA_concentration_M	320.43	4	80.11	75.0	<0.001
Condition_treatment	98.08	2	49.04	75.0	<0.001
PMA_concentration_M.Condition_treatment	10.27	8	1.28	75.0	0.265

B.2 Statistics Increasing PMAxx™ dose and assay sensitivity

B. subtilis / Serenade

Table B-3 REML variance components analysis of increasing PMAxx™ dose and assay sensitivity for *B. subtilis/Serenade*

REML variance components analysis

Tests for fixed effects

Sequentially adding terms to fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	P value
PMA_concentration_M	3.95	3	1.32	33.0	0.286
Condition_treatment	24.17	2	12.08	33.0	<0.001
PMA_concentration_M.Condition_treatment	4.12	6	0.69	33.0	0.662

PreStop / G. catenulatum

Table B-4 REML variance components analysis of increasing PMAxx™ dose and assay sensitivity for *G. catenulatum*/PreStop

REML variance components analysis

Tests for fixed effects

Sequentially adding terms to fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	P value
PMA_concentration_M	6.37	3	2.12	58.0	0.107
Condition_treatment	6.69	2	3.35	58.0	0.042
PMA_concentration_M.Condition_treatment	9.08	6	1.51	58.0	0.190

B.3 Statistics effect of PMAxx™ concentration on BCA CFUs

B. subtilis/Serenade

Table B-5 REML variance components analysis in effect of PMAxx™ concentration on BCA CFUs for *B. subtilis*/Serenade

Analysis of variance

Variate: log_Ratio

Source of variation	d.f.	s.s.	m.s.	v.r.	P value
log_Conc	5	0.105631	0.021126	14.11	<.001
PMAxx_tm_Conc_M	4	0.035140	0.008785	5.87	<.001
log_Conc.PMAxx_tm_Conc_M					

	20	0.072983	0.003649	2.44	0.002
Residual	90	0.134723	0.001497		
Total	119	0.348477			

PreStop/*G. catenulatum*

Table B-6 REML variance components analysis in effect of PMAxx™ concentration on BCA CFUs for *G. catenulatum*/PreStop

Analysis of variance					
Variate: log_Ratio					
Source of variation	d.f.	s.s.	m.s.	v.r.	P value
log_Conc	5	0.271705	0.054341	5.55	<.001
PMAxx_tm_Conc_M	4	0.064872	0.016218	1.66	0.167
log_Conc.PMAxx_tm_Conc_M	20	0.094571	0.004729	0.48	0.967
Residual	90	0.881428	0.009794		
Total	119	1.312577			

Appendix C Chapter 3

C.1 Appx Effect of BCA inoculum dose, formulation and temperature on *in vitro B. cinerea* control

Table C-1 Determined LD_{50s} of *B. subtilis* QST 713 and its formulated product Serenade ASO separated with Two-sample-T tests

Determined LD50s of *B. subtilis* QST 713 and its formulated product Serenade ASO separated with Two-sample-T tests									
Using Residual Mean Square from Curve Fit									
Comp	Temp(°C)	Log_{10} LD50 (CFUs)	STD Error	QST 713 4	Serenade 4	QST 713 10	Serenade 10	QST 713 20	Serenade 20
QST 713	4	10.464	< 0.7		0.402	0.351	0.208	0.468	0.145
Serenade	4	9.944	< 0.7	0.402		0.762	0.223	0.024	0.190
QST 713	10	9.876	< 0.7	0.351	0.762		0.447	0.021	0.285
Serenade	10	9.670	< 0.7	0.208	0.223	0.447		0.007	0.573
QST 713	20	11.012	< 0.7	0.468	0.024	0.021	0.007		0.014
Serenade	20	9.439	< 0.7	0.145	0.190	0.285	0.573	0.014	

Table C-2 Determined LD50s of *G. catenulatum* J1446 and its formulated product PreStop separated with Two-sample-T tests

Determined LD50s of *G. catenulatum* J1446 and its formulated product PreStop separated with Two-sample-T tests									
Using Residual Mean Square from Curve Fit									
				J1446	PreStop	J1446	PreStop	J1446	PreStop
Comp	Temp (°C)	Log_{10} LD_{50} (Spores)	Standard Error	4	4	10	10	20	20
J1446	4	9.09	1.42		0.315	0.401	0.599	0.715	0.984
PreStop	4	7.641	<0.4	0.315		0.299	0.149	**0.001**	**0.005**
J1446	10	7.725	<0.4	0.401	0.299		0.341	0.433	0.396
PreStop	10	8.32	<0.4	0.599	0.149	0.341		**0.002**	0.102
J1446	20	9.621	<0.4	0.715	**0.001**	0.433	**0.002**		0.262
PreStop	20	9.118	<0.4	0.984	**0.005**	0.396	0.102	0.262	

C.2 Appx *In vivo* dose response relationship and inoculum dynamics of the BCAs on *Lactuca sativa* leaves under high *B. cinerea* inoculum pressure

Table C-3 Accumulated analysis of deviance for biocontrol × leaf disc position × BCA dose × experiment

Accumulated analysis of deviance					
Change	d.f.	Deviance	Mean deviance	Deviance ratio	P value
+ Biocontrol	1	4.8085	4.8085	4.81	0.028
+ Position	9	224.0953	24.8995	24.90	<.001
+ Dose	6	886.5111	147.7519	147.75	<.001
+ Expt	1	14.8166	14.8166	14.82	<.001
+ Biocontrol.Position	9	22.6342	2.5149	2.51	0.007
+ Biocontrol.Dose	6	35.1658	5.8610	5.86	<.001
+ Position.Dose	54	61.8969	1.1462	1.15	0.215
+ Biocontrol.Expt	1	10.9351	10.9351	10.94	<.001
+ Position.Expt	9	24.1628	2.6848	2.68	0.004
+ Dose.Expt	6	21.4162	3.5694	3.57	0.002
+ Biocontrol.Position.Dose	54	89.6700	1.6606	1.66	0.002
+ Biocontrol.Position.Expt	9	10.0898	1.1211	1.12	0.343
+ Biocontrol.Dose.Expt	6	16.2570	2.7095	2.71	0.012
+ Position.Dose.Expt	54	74.2201	1.3744	1.37	0.035
+ Biocontrol.Position.Dose.Expt	54	83.4913	1.5461	1.55	0.006
Residual	2520	2300.6306	0.9129		
Total	2799	3880.8013	1.3865		

Analysis of Serenade whole leaf LD_{50}

Table C-4 Summary of analysis in calculating the LD_{50} of Serenade on the whole lettuce leaf

Nonlinear regression analysis

Summary of analysis

Source	d.f.	deviance	Mean deviance	Deviance ratio
Regression	3	544.38	181.460	53.82
Residual	3	10.12	3.372	
Total	6	554.50	92.416	

Dispersion parameter is estimated to be 3.37 from the residual deviance.

Estimates of parameters

Parameter	estimate	s.e.
PrMortality	0.2057	0.0360
PrImmunity	0.0425	0.0268
* Linear		
Constant	-14.33	3.68
DoseNew[2]	1.834	0.460

Effective doses

Log_{10} scale

LD	estimate	s.e.	lower 95%	upper 95%
50.00	8.314	0.4822	7.095	9.014

Analysis of PreStop whole leaf LD_{50}

Table C-5 Summary of analysis in calculating the LD50 of PreStop on the whole lettuce leaf

Nonlinear regression analysis

Summary of analysis

Source	d.f.	deviance	Mean deviance	Deviance ratio
Regression	3	372.109	124.036	49.64
Residual	3	7.496	2.499	
Total	6	379.605	63.267	

Dispersion parameter is estimated to be 2.50 from the residual deviance.

Estimates of parameters

Parameter	estimate	s.e.
PrMortality	0.1968	0.0451
PrImmunity	0.0711	0.0299
* Linear		
Constant	-6.33	1.40
DoseNew[1]	0.855	0.180

Effective doses

Log_{10} scale

LD	estimate	s.e.	lower 95%	upper 95%
50.00	7.911	0.5368	6.398	8.975

C.3 Appx Effect of spatial leaf disc parts on dose response relationship of BCAs for *B. cinerea* control

Analysis of Serenade: separated leaf disc LD_{50}

Table C-6 Summary of analysis in calculating the LD_{50} of Serenade within each experiment for each leaf disc position

Nonlinear regression analysis

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio
Regression	22	645.9	29.359	18.19
Residual	112	180.7	1.614	
Total	134	826.6	6.169	

Dispersion parameter is estimated to be 1.61 from the residual deviance.

Effective doses Log10 scale

Group	LD_{50} mean	Lower 95%	Upper 95%	S.E.
Serenade A1 DE1	8.90897	7.95297	9.84997	0.5052
Serenade A1 DE2	9.03697	8.01897	10.04297	0.5392
Serenade A2 DE1	9.00397	8.04297	9.95597	0.5098
Serenade A2 DE2	9.14997	8.16097	10.13397	0.5254
Serenade A3 DE1	8.77997	7.78597	9.73697	0.5195
Serenade A3 DE2	8.61097	7.67697	9.52897	0.4932
Serenade A4 DE1	8.86797	7.90897	9.79797	0.5032
Serenade A4 DE2	9.30697	8.22997	10.39097	0.5757
Serenade A5 DE1	7.35297	6.41997	8.25197	0.4879
Serenade A5 DE2	7.84397	6.93897	8.71397	0.4725
Serenade A6 DE1	8.77897	7.77197	9.75597	0.5284
Serenade A6 DE2	8.50797	7.55297	9.45497	0.5065
Serenade A7 DE1	8.04797	7.08597	8.98997	0.5071
Serenade A7 DE2	6.97197	6.06897	7.83297	0.4698
Serenade A8 DE1	6.81997	5.83897	7.78197	0.5175
Serenade A8 DE2	7.23797	6.32697	8.11397	0.4758
Serenade A9 DE1	8.20197	7.24497	9.12997	0.502
Serenade A9 DE2	8.42297	7.48297	9.35397	0.4984
Serenade A10 DE1	7.71597	6.80197	8.59897	0.4787
Serenade A10 DE2	6.28597	5.32197	7.19897	0.4996

Analysis of PreStop: separated leaf disc LD_{50}

Table C-7 Summary of analysis in calculating the LD_{50} of PreStop within each experiment for each leaf disc position

Nonlinear regression analysis

Summary of analysis

Source	d.f.	mean deviance	deviance	deviance ratio
Regression	22	556.0	25.272	16.07
Residual	117	184.0	1.572	
Total 139	739.9	5.323		

Dispersion parameter is estimated to be 1.57 from the residual deviance.

Effective doses Log10 scale

Group	LD50 mean	Lower 95%	Upper 95%	S.E
PreStop A1 DE1	9.37397	7.96497	10.85997	0.7825
PreStop A1 DE2	9.05597	7.71497	10.43497	0.7355
PreStop A2 DE1	7.86097	6.57297	9.14697	0.696
PreStop A2 DE2	9.01097	7.66397	10.39897	0.7396
PreStop A3 DE1	7.96697	6.68597	9.21197	0.6828
PreStop A3 DE2	14.97197	12.68097	17.07097	1.1861
PreStop A4 DE1	9.55297	8.08697	11.10797	0.8166
PreStop A4 DE2	16.53297	14.15997	19.10497	1.336
PreStop A5 DE1	6.06097	4.66497	7.32597	0.7188
PreStop A5 DE2	7.79197	6.45197	9.10897	0.7184
PreStop A6 DE1	8.08597	6.84697	9.29697	0.6626
PreStop A6 DE2	9.61997	8.18097	11.17297	0.8085
PreStop A7 DE1	6.33997	5.00197	7.57697	0.6956
PreStop A7 DE2	5.82597	4.35897	7.13397	0.749
PreStop A8 DE1	5.85597	4.36497	7.20597	0.7674
PreStop A8 DE2	7.54097	6.27297	8.76997	0.6751
PreStop A9 DE1	8.05497	6.79297	9.30397	0.6791
PreStop A9 DE2	8.37997	7.11597	9.62697	0.6789
PreStop A10 DE1	6.00497	4.56597	7.33297	0.7479
PreStop A10 DE2	6.08397	4.68997	7.34197	0.7162

Paired Sample T-Test for LD_{50} comparisons of different leaf disc positions

Table C-8 Summary of analysis in comparing the LD_{50} of Serenade for each leaf disc position

Serenade											
			P values								
Log_{10} LD_{50}	Standard error	Leaf disc position	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
8.97	0.52	A1		0.06	0.31	0.60	0.08	0.35	0.25	0.05	0.05	0.24
9.08	0.52	A2	0.06		0.25	0.95	0.07	0.29	0.24	0.04	0.03	0.23
8.70	0.51	A3	0.31	0.25		0.42	0.19	0.49	0.23	0.11	0.30	0.23
9.09	0.54	A4	0.60	0.95	0.42		0.01	0.43	0.29	0.00	0.09	0.27
7.60	0.48	A5	0.08	0.07	0.19	0.01		0.22	0.93	0.04	0.12	0.65
8.64	0.52	A6	0.35	0.29	0.49	0.43	0.22		0.22	0.13	0.41	0.22
7.51	0.49	A7	0.25	0.24	0.23	0.29	0.93	0.22		0.64	0.43	0.21
7.03	0.50	A8	0.05	0.04	0.11	0.00	0.04	0.13	0.64		0.05	0.98
8.31	0.50	A9	0.05	0.03	0.30	0.09	0.12	0.41	0.43	0.05		0.36
7.00	0.49	A10	0.24	0.23	0.23	0.27	0.65	0.22	0.21	0.98	0.36	

Table C-9 Summary of analysis in comparing the LD_{50} of PreStop for each leaf disc position

PreStop											
			P values								
Log_{10} LD_{50}	Standard error	Leaf disc position	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
9.17	0.76	A1		0.48	0.65	0.49	0.27	0.76	0.02	0.24	0.20	0.04
8.36	0.72	A2	0.48		0.49	0.36	0.12	0.28	0.22	0.10	0.69	0.14
11.39	0.93	A3	0.65	0.49		0.01	0.34	0.51	0.39	0.32	0.51	0.36
10.31	1.08	A4	0.49	0.36	0.01		0.26	0.37	0.31	0.25	0.38	0.29
6.85	0.72	A5	0.27	0.12	0.34	0.26		0.03	0.59	0.06	0.32	0.48
8.74	0.74	A6	0.76	0.28	0.51	0.37	0.03		0.23	0.02	0.48	0.16
5.99	0.72	A7	0.02	0.22	0.39	0.31	0.59	0.23		0.68	0.12	0.92
6.61	0.72	A8	0.24	0.10	0.32	0.25	0.06	0.02	0.68		0.27	0.57
8.12	0.68	A9	0.20	0.69	0.51	0.38	0.32	0.48	0.12	0.27		0.04
5.96	0.73	A10	0.04	0.14	0.36	0.29	0.48	0.16	0.92	0.57	0.04	

Summary of Paired T-Test's between median effective doses of different leaf tissue types

Table C-10 Paired T-Test's between median effective doses of different leaf tissue types

Paired T-Test's
Paired T-Test and CI: PreStop Midrib, PreStop lamina/lateral left
95% CI for mean difference: (-0.41, 7.80)
T-Test of mean difference = 0 (vs ≠ 0): T-Value = 2.32 P-Value = 0.068
Paired T-Test and CI: PreStop Midrib, PreStop lamina/lateral right
95% CI for mean difference: (-0.08, 8.08)
T-Test of mean difference = 0 (vs ≠ 0): T-Value = 2.52 P-Value = 0.053
Paired T-Test and CI: Serenade Midrib, Serenade lamina/lateral Left
95% CI for mean difference: (0.083, 1.989)
T-Test of mean difference = 0 (vs ≠ 0): T-Value = 2.79 P-Value = 0.038
Paired T-Test and CI: SerenadeMidrib, Serenade lamina/lateral Right
95% CI for mean difference: (0.391, 2.621)
T-Test of mean difference = 0 (vs ≠ 0): T-Value = 3.47 P-Value = 0.018
Paired T-Test and CI: PreStop lamina/lateral left, PreStop lamina/lateral right
95% CI for mean difference: (-0.230, 0.832)
T-Test of mean difference = 0 (vs ≠ 0): T-Value = 1.46 P-Value = 0.205
Paired T-Test and CI: Serenade lamina/lateral Left, Serenade lamina/lateral Right
95% CI for mean difference: (0.2362, 0.7035)
T-Test of mean difference = 0 (vs ≠ 0): T-Value = 5.17 P-Value = 0.004

Appendix D Chapter 4

D.1 Climatic regimes

Table D-1 Climatic treatments and mean levels of their temperature and relative humidity tested for representing UK agronomy climates

Climatic Treatment	Rep	Temp °C	RH (%)	Dew Point°C	VPD (kPa)	Climatic Treatment	Rep	Temp °C	RH (%)	Dew Point°C	VPD (kPa)
A1	1	11	67	5	0.4	C3	1	22	68	16	0.8
A1	2	11	66	5	0.4	C3	4	22	68	16	0.8
A2	1	11	76	7	0.3	C4	1	22	78	18	0.6
A2	2	12	79	9	0.3	C4	2	22	91	20	0.2
A3	1	10	86	8	0.2	D1	1	29	57	19	0.17
A3	2	11	82	8	0.2	D1	3	29	58	20	0.17
A4	1	14	93	13	0.1	D2	1	29	62	21	0.15
A4	2	11	92	9	0.1	D2	2	28	59	20	0.16
B1	1	17	61	9	0.8	D3	1	28	68	22	0.12
B1	2	16	70	10	0.5	D3	2	28	70	22	0.11
B2	1	16	71	11	0.5	D4	1	24	74	19	0.8
B2	2	16	69	10	0.6	D4	3	28	86	25	0.5
B3	1	16	80	13	0.4	D4	4	28	85	25	0.6
B3	2	17	74	12	0.5	E2	1	36	56	25	0.26
B4	1	16	89	14	0.2	E2	2	33	61	24	0.20
B4	2	16	84	13	0.3	E2	3	35	57	25	0.24
C1	1	22	57	13	0.11	E3	1	34	72	29	0.15
C1	2	22	52	13	0.13	E3	2	35	72	29	0.16
C1	3	22	57	13	0.11	E3	3	33	66	26	0.17
C1	4	22	52	12	0.13	E4	1	33	88	31	0.6
C2	1	23	65	15	0.10	E4	2	34	74	29	0.14
C2	2	22	69	16	0.8						

D.2 One-way ANOVA for BCAs on crops in different climatic regimes

Table D-2 One-way ANOVA's and MANOVA's for identifying if UK agronomy climates impact on viable population

One-way ANOVA's and MANOVA's

One-way ANOVA: B.sub Log10 CFUs mm² on LET versus Climatic Treatment

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Climatic Treatment	18	95.42	5.301	5.00	0.000

MANOVA B. sub Log10 CFUs on Lettuce versus Climatic Treatment with day as Covariate.

MANOVA for DAY

s = 1 m = -0.5 n = 107.0

Criterion	Test Statistic	F	Num DF	Denom DF	P
Wilks'	0.94424	12.754	1	216	0.000
Lawley-Hotelling	0.05905	12.754	1	216	0.000
Pillai's	0.05576	12.754	1	216	0.000

MANOVA for Climatic Treatment

s = 1 m = 8.0 n = 107.0

Criterion	Test Statistic	F	Num DF	Denom DF	P
Wilks'	0.69050	5.379	18	216	0.000
Lawley-Hotelling	0.44823	5.379	18	216	0.000
Pillai's	0.30950	5.379	18	216	0.000

One-way ANOVA: B. sub Log10 CFUs mm² on STRW versus Climatic Treatment

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Climatic Treatment	18	219.4	12.191	7.77	0.000

MANOVA B. sub Log10 CFUs on Strawberry versus Climatic Treatment with day as Covariate

MANOVA for DAY

s = 1 m = -0.5 n = 107.0

Criterion	Test Statistic	F	Num DF	Denom DF	P
Wilks'	0.96648	7.492	1	216	0.007
Lawley-Hotelling	0.03469	7.492	1	216	0.007
Pillai's	0.03352	7.492	1	216	0.007

MANOVA for Climatic Treatment

s = 1 m = 8.0 n = 107.0

Criterion	Test Statistic	F	Num DF	Denom DF	P
Wilks'	0.60019	7.994	18	216	0.000
Lawley-Hotelling	0.66614	7.994	18	216	0.000
Pillai's	0.39981	7.994	18	216	0.000

One-way ANOVA: Log10 CFUs PreStop mm² on LET versus Climatic Treatment

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Climatic Treatment	18	30.33	1.6848	1.88	0.019

MANOVA PreStop Log10 CFUs on Lettuce versus Climatic Treatment with day as Covariate.

MANOVA for DAY

s = 1 m = -0.5 n = 113.0

Criterion	Test Statistic	F	Num DF	Denom DF	P
Wilks'	0.95717	10.203	1	228	0.002
Lawley-Hotelling	0.04475	10.203	1	228	0.002
Pillai's	0.04283	10.203	1	228	0.002

MANOVA for Climatic Treatment

s = 1 m = 8.0 n = 113.0

Criterion	Test Statistic	F	Num DF	Denom DF	P
Wilks'	0.86755	1.934	18	228	0.014
Lawley-Hotelling	0.15267	1.934	18	228	0.014
Pillai's	0.13245	1.934	18	228	0.014

One-way ANOVA: Log10 PreStop CFUs mm² on STRW versus Climatic Treatment

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Climatic Treatment	18	21.58	1.1989	1.69	0.042

MANOVA PreStop Log10 CFUs on Strawberry versus Climatic Treatment with day as Covariate.

MANOVA for DAY

s = 1 m = -0.5 n = 114.0

Criterion	Test Statistic	F	Num DF	Denom DF	P
Wilks'	0.89678	26.473	1	230	0.000
Lawley-Hotelling	0.11510	26.473	1	230	0.000
Pillai's	0.10322	26.473	1	230	0.000

MANOVA for Climatic Treatment

s = 1 m = 8.0 n = 114.0

Criterion	Test Statistic	F	DF Num	DF Denom	P
Wilks'	0.87396	1.843	18	230	0.022
Lawley-Hotelling	0.14421	1.843	18	230	0.022
Pillai's	0.12604	1.843	18	230	0.022

Temperature: *B. subtilis* on lettuce

Table D-3 ANOVAs for comparing the impact of change in temperature on viable BCA population of *B. subtilis* on lettuce

TWO-WAY ANOVA's

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.8790	0.8790	1.75	0.207
Climatic Treatment	2	6.9110	3.4555	6.87	0.008

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	1.456	1.4556	1.56	0.243
Climatic Treatment	1	3.411	3.4112	3.66	0.088

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	14.8429	14.8429	23.14	0.002
Climatic Treatment	1	0.2650	0.2650	0.41	0.541

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.03417	0.03417	0.13	0.724
Climatic Treatment	1	0.14539	0.14539	0.56	0.472

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.7658	0.7658	1.02	0.325
Climatic Treatment	3	10.6853	3.5618	4.74	0.012

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	1.546	1.5458	3.52	0.090
Climatic Treatment	2	11.539	5.7695	13.13	0.002

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.00054	0.00054	0.00	0.979
Climatic Treatment	1	1.19668	1.19668	1.72	0.227

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.06467	0.06467	0.33	0.583
Climatic Treatment	1	0.96031	0.96031	4.91	0.062

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.03417	0.03417	0.13	0.724
Climatic Treatment	1	0.14539	0.14539	0.56	0.472

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.7568	0.7568	1.98	0.193
Climatic Treatment	1	6.7809	6.7809	17.72	0.002

Temperature: *B. subtilis* on strawberry

Table D-4 ANOVAs for comparing the impact of change in temperature on viable BCA population of *B. subtilis* on strawberry

TWO-WAY ANOVA's

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	2.900	2.8999	11.70	0.008
Climatic Treatment	1	6.608	6.6079	26.67	0.001

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	1.661	1.661	1.47	0.256
Climatic Treatment	1	16.940	16.940	14.98	0.004

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	5.4243	5.4243	22.44	0.001
Climatic Treatment	1	0.1456	0.1456	0.60	0.460

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.4828	0.4828	1.08	0.327
Climatic Treatment	1	2.7746	2.7746	6.18	0.035

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.0209	0.02095	0.05	0.832
Climatic Treatment	3	13.0362	4.34540	9.65	0.001

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	1.972	1.9717	2.71	0.122
Climatic Treatment	2	15.910	7.9552	10.95	0.001

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.5626	0.5626	1.10	0.330
Climatic Treatment	1	6.2520	6.2520	12.17	0.010

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	2.727	2.727	1.41	0.265
Climatic Treatment	1	13.598	13.598	7.03	0.026

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.00263	0.00263	0.01	0.941
Climatic Treatment	1	1.34402	1.34402	2.93	0.121

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.7205	0.7205	0.91	0.356
Climatic Treatment	2	3.8713	1.9357	2.45	0.123

Temperature: *G. catenulatum* on lettuce

Table D-5 ANOVAs for comparing the impact of change in temperature on viable BCA population of *G. catenulatum* on lettuce

TWO-WAY ANOVA's

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.7627	0.7627	1.79	0.197
Climatic Treatment	2	9.4491	4.7245	11.06	0.001

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.0002	0.00015	0.00	0.983
Climatic Treatment	1	7.4174	7.41744	23.42	0.001

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.5824	0.5824	1.11	0.323
Climatic Treatment	1	0.1986	0.1986	0.38	0.556

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.0468	0.0468	0.22	0.652
Climatic Treatment	1	18.4346	18.4346	85.47	0.000

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.09707	0.09707	0.37	0.559
Climatic Treatment	1	0.31812	0.31812	1.21	0.300

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.2342	0.2342	0.50	0.487
Climatic Treatment	3	7.6001	2.5334	5.45	0.008

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.2086	0.20864	1.88	0.191
Climatic Treatment	2	0.1694	0.08470	0.76	0.484

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	2.645	2.6448	19.58	0.002
Climatic Treatment	1	1.923	1.9232	14.24	0.004

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.00208	0.002079	0.01	0.937
Climatic Treatment	1	0.57583	0.575830	1.83	0.209

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.0205	0.0205	0.03	0.856
Climatic Treatment	1	18.7917	18.7917	31.85	0.000

Temperature: *G. catenulatum* on strawberry

Table D-6 ANOVAs for comparing the impact of change in temperature on viable population of *G. catenulatum* on strawberry

TWO-WAY ANOVA's

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	1.262	1.2622	3.69	0.069
Climatic Treatment	2	4.619	2.3097	6.75	0.006
Total	23	12.723			

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.7307	0.7307	0.35	0.563
Climatic Treatment	1	0.4872	0.4872	0.23	0.636
Total	15	28.0470			

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	1.460	1.4599	2.16	0.164
Climatic Treatment	1	2.187	2.1875	3.23	0.094

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.45988	0.45988	1.36	0.273
Climatic Treatment	1	0.03327	0.03327	0.10	0.761

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.1495	0.1495	0.70	0.413
Climatic Treatment	3	2.4888	0.8296	3.89	0.026

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	4.1545	4.15449	27.28	0.000
Climatic Treatment	1	2.3278	2.32779	15.29	0.002

Total	16	8.8600			
Analysis of Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	2.0835	2.08354	2.08	0.165
Climatic Treatment	1	0.0340	0.03397	0.03	0.856
Total	22	22.1863			
Analysis of Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.2775	0.2775	0.70	0.423
Climatic Treatment	1	0.6832	0.6832	1.73	0.221
Analysis of Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.07667	0.07667	0.53	0.486
Climatic Treatment	1	8.53516	8.53516	58.82	0.000
Analysis of Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	1.592	1.5920	5.48	0.035
Climatic Treatment	2	4.423	2.2114	7.61	0.006

RH%: *B. subtilis* lettuce

Table D-7 ANOVAs for comparing the impact of change in RH % on viable population of *B. subtilis* on lettuce

TWO-WAY ANOVA's			
Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	A1, A2, A3, A
Analysis of Variance			
Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	1.249	1.2486	1.14	0.292
Climatic Treatment	3	7.763	2.5876	2.36	0.086
Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	B1, B2, B3, B4
Analysis of Variance			
Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	19.742	19.7421	22.98	0.000
Climatic Treatment	3	3.361	1.1203	1.30	0.286
Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	C1, C2, C3, C4
Analysis of Variance			
Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	2.643	2.6429	1.91	0.174
Climatic Treatment	3	22.440	7.4800	5.40	0.003
Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	D1, D2, D3, D4
Analysis of Variance			
Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.0031	0.0031	0.01	0.927
Climatic Treatment	3	38.9593	12.9864	35.47	0.000
Factor	Type	Levels	Values
Climatic Treatment	Fixed	3	E2, E3, E4
Analysis of Variance			
Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.6596	0.6596	0.72	0.403
Climatic Treatment	2	1.6292	0.8146	0.89	0.421

RH%: *B. subtilis* Strawberry

Table D-8 ANOVAs for comparing the impact of change in RH % on viable population of *B. subtilis* on strawberry

TWO-WAY ANOVA's

Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	A1, A2, A3, A4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	12.245	12.2451	4.68	0.037
Climatic Treatment	3	13.177	4.3924	1.68	0.187
Total	44	129.245			

Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	B1, B2, B3, B4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	2.512	2.5124	1.59	0.214
Climatic Treatment	3	14.275	4.7585	3.02	0.041

Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	C1, C2, C3, C4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.6729	0.6729	0.74	0.394
Climatic Treatment	3	26.4333	8.8111	9.68	0.000

Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	D1, D2, D3, D4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.4277	0.4277	0.31	0.582
Climatic Treatment	3	12.3429	4.1143	2.96	0.043

Factor	Type	Levels	Values
Climatic Treatment	Fixed	3	E2, E3, E4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	1.186	1.1860	0.95	0.335
Climatic Treatment	2	4.541	2.2705	1.82	0.174

RH%: *G. catenulatum* lettuce

Table D-9 ANOVAs for comparing the impact of change in RH % on viable population of *G. catenulatum* on lettuce

TWO-WAY ANOVA's

Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	A1, A2, A3, A4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	1.823	1.8227	3.42	0.071
Climatic Treatment	3	3.003	1.0009	1.88	0.148

Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	B1, B2, B3, B4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	1.335	1.3351	1.63	0.208
Climatic Treatment	3	5.544	1.8478	2.26	0.096

Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	C1, C2, C3, C4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	4.395	4.3946	10.09	0.002
Climatic Treatment	3	5.817	1.9391	4.45	0.007

Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	D1, D2, D3, D4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	1.235	1.2347	0.88	0.352
Climatic Treatment	3	1.829	0.6097	0.44	0.728

Factor	Type	Levels	Values
Climatic Treatment	Fixed	3	E2, E3, E4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.6849	0.68486	0.54	0.467
Climatic Treatment	2	0.0938	0.04690	0.04	0.964

RH%: *G. catenulatum* Strawberry

Table D-10 ANOVAs for comparing the impact of change in RH % on viable population of *G. catenulatum* on strawberry

TWO-WAY ANOVA's

Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	A1, A2, A3, A4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	3.862	3.8620	3.44	0.070
Climatic Treatment	3	2.625	0.8749	0.78	0.512
Total	47	54.724			

Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	B1, B2, B3, B4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	1.443	1.4429	2.47	0.124
Climatic Treatment	3	5.297	1.7656	3.03	0.041

Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	C1, C2, C3, C4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	5.718	5.7182	17.56	0.000
Climatic Treatment	3	3.416	1.1386	3.50	0.022

Factor	Type	Levels	Values
Climatic Treatment	Fixed	4	D1, D2, D3, D4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	6.811	6.8114	10.77	0.002
Climatic Treatment	3	1.480	0.4932	0.78	0.511

Factor	Type	Levels	Values
Climatic Treatment	Fixed	3	E2, E3, E4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	0.8467	0.8467	1.39	0.245
Climatic Treatment	2	0.3102	0.1551	0.25	0.777

D.3 Dealing with the effect of VPD on viable populations of BCAs

VPD *B. subtilis* lettuce

Table D-11 ANOVAs for comparing the impact of change in VPD (kPa) on viable population of *B. subtilis* on lettuce

General Linear Model
TWO-WAY ANOVA's

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	12.97	12.9703	12.90	0.000
VPD (kPa)	17	96.49	5.6761	5.65	0.000
Total	235	325.49			

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	4.730	0.123	38.45	0.000	
DAY	-0.0691	0.0192	-3.59	0.000	1.01
VPD (kPa)					
0.1	0.706	0.284	2.49	0.014	1.43
0.2	0.221	0.208	1.06	0.290	1.23
0.3	0.501	0.236	2.12	0.035	1.30
0.4	-0.447	0.274	-1.63	0.104	1.40
0.5	0.425	0.208	2.04	0.042	1.23
0.6	0.086	0.208	0.41	0.681	1.23
0.8	0.866	0.198	4.37	0.000	1.22
1.0	-0.446	0.394	-1.13	0.259	1.85
1.1	-0.922	0.242	-3.81	0.000	1.31
1.2	-0.675	0.479	-1.41	0.161	2.28
1.3	1.248	0.284	4.40	0.000	1.43
1.4	0.542	0.394	1.38	0.170	1.85
1.5	-0.557	0.309	-1.80	0.073	1.52
1.6	-0.079	0.394	-0.20	0.841	1.85
1.7	-0.923	0.249	-3.71	0.000	1.33
2.0	-0.533	0.394	-1.35	0.178	1.85
2.4	0.584	0.430	1.36	0.176	2.02

VPD *B. subtilis* Strawberry

Table D-12 ANOVAs for comparing the impact of change in VPD (kPa) on viable population of *B. subtilis* on strawberry

General Linear Model
TWO-WAY ANOVA's

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	14.67	14.6682	8.62	0.004
VPD (kPa)	16	177.25	11.0779	6.51	0.000

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	4.960	0.164	30.28	0.000	
DAY	-0.0741	0.0252	-2.94	0.004	1.01
VPD (kPa)					
0.1	1.121	0.370	3.03	0.003	1.43
0.2	1.280	0.308	4.15	0.000	1.30
0.3	0.742	0.308	2.41	0.017	1.30
0.4	2.073	0.345	6.01	0.000	1.38
0.5	0.648	0.272	2.38	0.018	1.25
0.6	-0.185	0.289	-0.64	0.523	1.28
0.8	0.580	0.236	2.46	0.015	1.20
1.1	0.139	0.370	0.38	0.707	1.43
1.2	-1.742	0.873	-2.00	0.047	3.55
1.3	0.420	0.385	1.09	0.276	1.47
1.4	-0.621	0.512	-1.21	0.226	1.85

1.5	-0.258	0.402	-0.64	0.522	1.51
1.6	-0.484	0.370	-1.31	0.192	1.43
1.7	-0.856	0.272	-3.15	0.002	1.25
2.0	-1.706	0.512	-3.33	0.001	1.85
2.4	-1.186	0.512	-2.32	0.021	1.85

VPD *G. catenulatum* Lettuce

Table D-13 ANOVAs for comparing the impact of change in VPD (kPa) on viable population of *G. catenulatum* on lettuce

General Linear Model
TWO-WAY ANOVA's

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	8.913	8.9126	12.35	0.001
VPD (kPa)	17	61.692	3.6290	5.03	0.000
Total	247	236.060			

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	3.233	0.101	32.01	0.000	
DAY	-0.0557	0.0158	-3.51	0.001	1.00
VPD (kPa)					
0.1	0.254	0.240	1.06	0.290	1.42
0.2	0.272	0.175	1.55	0.123	1.22
0.3	0.221	0.199	1.11	0.269	1.29
0.4	-0.082	0.204	-0.40	0.687	1.30
0.5	0.086	0.175	0.49	0.626	1.22
0.6	0.333	0.179	1.86	0.064	1.23
0.8	0.411	0.164	2.51	0.013	1.20
1.0	0.642	0.333	1.93	0.055	1.84
1.1	-0.185	0.199	-0.93	0.354	1.29
1.2	0.282	0.333	0.85	0.398	1.84
1.3	0.246	0.240	1.02	0.307	1.42
1.4	-1.105	0.333	-3.32	0.001	1.84
1.5	1.067	0.240	4.45	0.000	1.42
1.6	-1.485	0.333	-4.46	0.000	1.84
1.7	-0.270	0.199	-1.36	0.176	1.29
2.0	-0.721	0.333	-2.16	0.032	1.84
2.4	-0.924	0.333	-2.78	0.006	1.84

VPD *G. catenulatum* Strawberry

Table D-14 ANOVAs for comparing the impact of change in VPD (kPa) on viable population of *G. catenulatum* on strawberry

General Linear Model
TWO-WAY ANOVA's

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	17.32	17.3231	30.24	0.000
VPD (kPa)	17	35.78	2.1048	3.67	0.000

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	3.2732	0.0888	36.86	0.000	
DAY	-0.0769	0.0140	-5.50	0.000	1.00
VPD (kPa)					

0.1	0.015	0.213	0.07	0.944	1.42
0.2	0.206	0.156	1.32	0.188	1.22
0.3	0.264	0.187	1.41	0.159	1.32
0.4	-0.022	0.182	-0.12	0.905	1.30
0.5	-0.035	0.156	-0.22	0.823	1.22
0.6	0.103	0.159	0.65	0.518	1.23
0.8	0.056	0.146	0.38	0.702	1.19
1.0	1.166	0.297	3.93	0.000	1.84
1.1	-0.230	0.182	-1.26	0.208	1.30
1.2	0.376	0.297	1.27	0.206	1.84
1.3	-0.349	0.213	-1.64	0.103	1.42
1.4	-0.572	0.297	-1.93	0.055	1.84
1.5	0.689	0.222	3.10	0.002	1.46
1.6	-0.923	0.213	-4.33	0.000	1.42
1.7	-0.136	0.177	-0.77	0.443	1.28
2.0	-0.516	0.297	-1.74	0.083	1.84
2.4	-0.508	0.297	-1.71	0.088	1.84

D.4 UK climatic regimes GLMs of BCA temporal population dynamics

General Linear Model: *B. subtilis* Lettuce CFUs mm² versus DAY, Climatic Treatment

Table D-15 General linear model of *B. subtilis* on lettuce in UK agronomy climates

General Linear Model

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	12.83	12.8277	12.75	0.000
Climatic Treatment	18	97.37	5.4097	5.38	0.000

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	4.802	0.116	41.39	0.000	
DAY	-0.0687	0.0193	-3.57	0.000	1.01
Climatic Treatment					
A1	-0.533	0.343	-1.56	0.121	2.33
A2	0.571	0.282	2.02	0.044	1.90
A3	0.099	0.282	0.35	0.727	1.90
A4	0.632	0.282	2.24	0.026	1.90
B1	0.020	0.282	0.07	0.944	1.90
B2	0.627	0.282	2.22	0.027	1.90
B3	0.108	0.294	0.37	0.714	1.97
B4	0.545	0.282	1.93	0.055	1.90
C1	-0.005	0.205	-0.02	0.981	1.48
C2	0.363	0.282	1.29	0.200	1.90
C3	1.218	0.282	4.32	0.000	1.90
C4	-0.694	0.282	-2.46	0.015	1.90
D1	-2.070	0.307	-6.73	0.000	2.07
D2	0.293	0.294	1.00	0.320	1.97
D3	-0.639	0.323	-1.98	0.049	2.18
D4	0.268	0.254	1.06	0.292	1.73
E2	-0.301	0.240	-1.26	0.210	1.65
E3	-0.521	0.294	-1.77	0.078	1.97

General Linear Model: *B. subtilis* strawberry CFUs/mm² versus DAY, Climatic Treatment

Table D-16 General linear model of *B. subtilis* on strawberry in UK agronomy climates

General Linear Model

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	11.69	11.6919	7.68	0.006
Climatic Treatment	18	218.96	12.1646	7.99	0.000

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	5.299	0.142	37.33	0.000	
DAY	-0.0660	0.0238	-2.77	0.006	1.01
Climatic Treatment					
A1	1.613	0.398	4.05	0.000	2.18
A2	0.558	0.347	1.61	0.109	1.90
A3	0.059	0.347	0.17	0.866	1.90
A4	0.741	0.347	2.14	0.034	1.90
B1	0.051	0.347	0.15	0.883	1.90
B2	1.529	0.378	4.04	0.000	2.07
B3	0.924	0.347	2.66	0.008	1.90
B4	1.277	0.347	3.68	0.000	1.90
C1	-0.283	0.287	-0.99	0.325	1.62
C2	-0.197	0.361	-0.55	0.586	1.97
C3	1.169	0.378	3.09	0.002	2.07
C4	-1.024	0.361	-2.83	0.005	1.97
D1	-1.725	0.347	-4.97	0.000	1.90
D2	-0.598	0.378	-1.58	0.116	2.07
D3	-0.380	0.421	-0.90	0.367	2.32
D4	-0.665	0.295	-2.25	0.025	1.65
E2	-1.332	0.287	-4.64	0.000	1.62
E3	-1.160	0.287	-4.04	0.000	1.62

General Linear Model: CFUs PreStop Lettuce CFUs/mm² versus DAY, Climatic Treatment

Table D-17 General linear model of *G. catenulatum* on lettuce in UK agronomy climates

General Linear Model

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	8.812	8.8121	10.20	0.002
Climatic Treatment	18	30.064	1.6702	1.93	0.014

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	3.350	0.105	32.03	0.000	
DAY	-0.0554	0.0173	-3.19	0.002	1.00
Climatic Treatment					
A1	-0.163	0.261	-0.63	0.532	1.89
A2	0.403	0.261	1.54	0.124	1.89
A3	-0.221	0.261	-0.85	0.397	1.89
A4	0.136	0.261	0.52	0.603	1.89
B1	0.072	0.261	0.28	0.782	1.89
B2	0.834	0.272	3.07	0.002	1.97

B3	-0.095	0.272	-0.35	0.726	1.97
B4	0.371	0.261	1.42	0.156	1.89
C1	0.128	0.189	0.68	0.499	1.47
C2	0.572	0.261	2.19	0.029	1.89
C3	-0.408	0.285	-1.43	0.153	2.06
C4	0.352	0.261	1.35	0.179	1.89
D1	0.028	0.261	0.11	0.916	1.89
D2	-0.218	0.261	-0.84	0.404	1.89
D3	-0.502	0.261	-1.92	0.056	1.89
D4	-0.341	0.216	-1.58	0.116	1.61
E2	-0.347	0.216	-1.61	0.110	1.61
E3	-0.246	0.261	-0.94	0.347	1.89

General Linear Model: PreStop Strawberry CFUs/mm² versus DAY, Climatic Treatment

Table D-18 General linear model of *G. catenulatum* on strawberry in UK agronomy climates

General Linear Model					
Analysis of Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
DAY	1	16.91	16.9111	26.47	0.000
Climatic Treatment	18	21.19	1.1771	1.84	0.022
Coefficients					
Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	3.2986	0.0894	36.89	0.000	
DAY	-0.0760	0.0148	-5.15	0.000	1.00
Climatic Treatment					
A1	-0.289	0.224	-1.29	0.199	1.89
A2	0.340	0.224	1.52	0.131	1.89
A3	-0.150	0.224	-0.67	0.503	1.89
A4	-0.015	0.224	-0.07	0.948	1.89
B1	-0.067	0.224	-0.30	0.767	1.89
B2	0.710	0.234	3.04	0.003	1.97
B3	-0.060	0.234	-0.26	0.797	1.97
B4	0.518	0.245	2.12	0.035	2.06
C1	-0.188	0.166	-1.13	0.259	1.49
C2	0.287	0.224	1.28	0.202	1.89
C3	-0.165	0.245	-0.67	0.502	2.06
C4	0.335	0.224	1.49	0.136	1.89
D1	0.214	0.224	0.95	0.341	1.89
D2	-0.218	0.224	-0.97	0.331	1.89
D3	-0.213	0.224	-0.95	0.343	1.89
D4	-0.057	0.186	-0.31	0.759	1.61
E2	-0.233	0.186	-1.25	0.211	1.61
E3	-0.419	0.191	-2.20	0.029	1.64

D.5 Predicting BCA fate

B. subtilis QST 713 prediction of viable population density

Table D-19 Prediction of viable population change with the general linear model of *B. subtilis* on lettuce

General Linear Model Predictions

$Log_{10}(N_n) - Log_{10}(N_0)$

General Linear Model Information

Terms

DAY Climatic Treatment

Variable	Setting
DAY	2
Climatic Treatment	22°C 78%

Fit	SE Fit	95% CI	95% PI
1.26688	0.320785	(0.633616, 1.90014)	(-0.738704, 3.27246)

Variable	Setting
DAY	4
Climatic Treatment	22°C 68%

Fit	SE Fit	95% CI	95% PI
0.0883292	0.308900	(-0.521471, 0.698129)	(-1.90997, 2.08663)

Variable	Setting
DAY	6
Climatic Treatment	11°C 92%

Fit	SE Fit	95% CI	95% PI
-1.29541	0.304836	(-1.89718, -0.693631)	(-3.29127, 0.700458)

Variable	Setting
DAY	8
Climatic Treatment	16°C 84%

Fit	SE Fit	95% CI	95% PI
-1.07318	0.308900	(-1.68298, -0.463381)	(-3.07148, 0.925118)

$Log_{10}(N_n) - Log_{10}(N_0)$

General Linear Model Information

Terms

DAY Climatic Treatment

Variable	Setting
DAY	2
Climatic Treatment	16°C 74%

Fit	SE Fit	95% CI	95% PI
-1.39334	0.336494	(-2.05761, -0.729065)	(-3.40893, 0.622251)

Variable	Setting
DAY	4
Climatic Treatment	11°C 92%

Fit	SE Fit	95% CI	95% PI
-1.08605	0.308900	(-1.69585, -0.476254)	(-3.08435, 0.912244)

Variable	Setting
DAY	6
Climatic Treatment	16°C 84%

Fit	SE Fit	95% CI	95% PI
-0.863829	0.304836	(-1.46560, -0.262053)	(-2.85969, 1.13204)

Variable	Setting
DAY	8
Climatic Treatment	16°C 84%

Fit	SE Fit	95% CI	95% PI
-1.07318	0.308900	(-1.68298, -0.463381)	(-3.07148, 0.925118)

Table D-20 Prediction of viable population change with the general linear model of *B. subtilis* on strawberry

General Linear Model Predictions

$Log_{10}(N_n) - Log_{10}(N_0)$

General Linear Model Information

Terms

DAY Climatic Treatment

Variable	Setting
DAY	2
Climatic Treatment	16°C 74%

Fit	SE Fit	95% CI	95% PI
0.741589	0.355299	(0.0402519, 1.44293)	(-1.48899, 2.97217)

Variable	Setting
DAY	4

Climatic Treatment 11°C 76%

Fit	SE Fit	95% CI	95% PI
-0.483020	0.343749	(-1.16156, 0.195518)	(-2.70653, 1.74049)

Variable Setting
DAY 6
Climatic Treatment 16°C 74%

Fit	SE Fit	95% CI	95% PI
0.337784	0.339265	(-0.331903, 1.00747)	(-1.88304, 2.55861)

Variable Setting
DAY 8
Climatic Treatment 11°C 82%

Fit	SE Fit	95% CI	95% PI
-0.537508	0.343749	(-1.21605, 0.141030)	(-2.76102, 1.68601)

Variable Setting
DAY 10
Climatic Treatment 11°C 82%

Fit	SE Fit	95% CI	95% PI
-0.739410	0.356993	(-1.44409, -0.0347302)	(-2.97104, 1.49222)

$Log_{10}(N_n) - Log_{10}(N_0)$
General Linear Model Information
Terms
DAY Climatic Treatment
Variable Setting
DAY 2
Climatic Treatment 11°C 76%

Fit	SE Fit	95% CI	95% PI
-0.281117	0.356993	(-0.985798, 0.423563)	(-2.51275, 1.95051)

Variable Setting
DAY 4
Climatic Treatment 16°C 74%

Fit	SE Fit	95% CI	95% PI
0.539686	0.342893	(-0.137162, 1.21653)	(-1.68331, 2.76269)

Variable Setting
DAY 6
Climatic Treatment 11°C 82%

Fit	SE Fit	95% CI	95% PI
-0.335606	0.339220	(-1.00520, 0.333991)	(-2.55641, 1.88520)

Variable Setting
DAY 8
Climatic Treatment 11°C 82%

Fit	SE Fit	95% CI	95% PI
-0.537508	0.343749	(-1.21605, 0.141030)	(-2.76102, 1.68601)

$Log_{10}(N_n) - Log_{10}(N_0)$
General Linear Model Information
Terms
DAY Climatic Treatment
Variable Setting
DAY 2
Climatic Treatment 11°C 82%

Fit	SE Fit	95% CI	95% PI
0.0681987	0.356993	(-0.636481, 0.772879)	(-2.16343, 2.29983)

Variable Setting
DAY 4
Climatic Treatment 11°C 82%

Fit	SE Fit	95% CI	95% PI
-0.133704	0.343749	(-0.812241, 0.544834)	(-2.35722, 2.08981)

Variable Setting
DAY 6
Climatic Treatment 16°C 74%

Fit	SE Fit	95% CI	95% PI
0.337784	0.339265	(-0.331903, 1.00747)	(-1.88304, 2.55861)

Variable Setting
DAY 10
Climatic Treatment 16°C 84%

Fit	SE Fit	95% CI	95% PI
0.215955	0.356993	(-0.488726, 0.920635)	(-2.01568, 2.44759)

G. catenulatum J1446 prediction of viable population density

Table D-21 Prediction of viable population change with the general linear model of *G. catenulatum* on lettuce

General Linear Model Predictions

$Log_{10}(N_n) - Log_{10}(N_0)$
General Linear Model Information
Terms
DAY Climatic Treatment
Variable Setting
DAY 2
Climatic Treatment 22°C 78%

Fit	SE Fit	95% CI	95% PI
0.0293107	0.246301	(-0.456591, 0.515212)	(-1.51576, 1.57438)
0.0165592	0.237949	(-0.485984, 0.452866)	(-1.55652, 1.52341)

Variable Setting
DAY 6
Climatic Treatment 16°C 74%

Fit	SE Fit	95% CI	95% PI
-0.699636	0.247950	(-1.18879, -0.210480)	(-2.24573, 0.846458)

$Log_{10}(N_n) - Log_{10}(N_0)$
General Linear Model Information
Terms
DAY Climatic Treatment
Variable Setting
DAY 2
Climatic Treatment 22°C 78%

Fit	SE Fit	95% CI	95% PI
0.0293107	0.246301	(-0.456591, 0.515212)	(-1.51576, 1.57438)

Table D-22 Prediction of viable population change with the general linear model of *G. catenulatum* on lettuce

General Linear Model Predictions

$Log_{10}(N_n) - Log_{10}(N_0)$
General Linear Model Information
Terms
DAY Climatic Treatment
Variable Setting
DAY 2
Climatic Treatment 16°C 74%

Fit	SE Fit	95% CI	95% PI
-0.346491	0.222782	(-0.785980, 0.0929979)	(-1.69550, 1.00252)

Variable Setting
DAY 4
Climatic Treatment 11°C 76%

Fit	SE Fit	95% CI	95% PI
0.269703	0.206900	(-0.138456, 0.677862)	(-1.06943, 1.60883)

$Log_{10}(N_n) - Log_{10}(N_0)$
General Linear Model Information
Terms
DAY Climatic Treatment
Variable Setting
DAY 2
Climatic Treatment 11°C 76%

Fit	SE Fit	95% CI	95% PI
0.390265	0.214090	(-0.0320764, 0.812607)	(-0.953255, 1.73378)

Variable Setting
DAY 4
Climatic Treatment 16°C 74%

```
      Fit    SE Fit           95% CI                 95% PI
-0.467053  0.216918  (-0.894974, -0.0391315)  (-1.81234, 0.878231)
Variable             Setting
DAY                        6
Climatic Treatment  11°C 82%
      Fit    SE Fit          95% CI                 95% PI
-0.331130  0.204448  (-0.734450, 0.0721907)  (-1.66879, 1.00653)
Variable             Setting
DAY                        8
Climatic Treatment  11°C 82%
      Fit    SE Fit           95% CI                 95% PI
-0.451692  0.206900  (-0.859850, -0.0435328)  (-1.79082, 0.887437)
Variable             Setting
DAY                       10
Climatic Treatment  16°C 74%
      Fit    SE Fit          95% CI                95% PI
-0.828738  0.226772  (-1.27610, -0.381379)  (-2.18033, 0.522854)
```

$Log_{10}(N_n) - Log_{10}(N_0)$

```
General Linear Model Information
Terms
DAY Climatic Treatment
Variable             Setting
DAY                        2
Climatic Treatment  11°C 82%
       Fit    SE Fit          95% CI                 95% PI
-0.0900059  0.214090  (-0.512347, 0.332336)  (-1.43353, 1.25351)
Variable             Setting
DAY                        4
Climatic Treatment  11°C 82%
      Fit    SE Fit          95% CI                95% PI
-0.210568  0.206900  (-0.618726, 0.197591)  (-1.54970, 1.12856)
Variable             Setting
DAY                        6
Climatic Treatment  16°C 74%
      Fit    SE Fit          95% CI                95% PI
-0.587615  0.215622  (-1.01298, -0.162250)  (-1.93209, 0.756859)
Variable             Setting
DAY                       10
Climatic Treatment  16°C 84%
       Fit    SE Fit          95% CI                 95% PI
-0.0036090  0.238337  (-0.473783, 0.466565)  (-1.36292, 1.35571)
```

Appendix E Chapter 5

E.1 Statistics

B. subtilis on lettuce

Table E-1 The impact of leaf size (time) and leaf tissue type on viable population of *B. subtilis* in the winter and spring on lettuce

MANOVA's and TWO-WAY ANOVA's

***B. subtilis* Lettuce winter ONE WAY ANOVA with covariate leaf size testing leaf tissue type**

```
MANOVA for LEAF AREA SIZE CM2
s = 1    m = -0.5    n = 15.0
                        Test              DF
Criterion          Statistic      F  Num  Denom      P
Wilks'               0.95990  1.337    1     32  0.256
```

Lawley-Hotelling	0.04178	1.337	1	32	0.256
Pillai's	0.04010	1.337	1	32	0.256
Roy's	0.04178				

MANOVA for Leaf tissue type

s = 1 m = 0.0 n = 15.0

	Test		DF		
Criterion	Statistic	F	Num	Denom	P
Wilks'	0.79346	4.165	2	32	0.025
Lawley-Hotelling	0.26031	4.165	2	32	0.025
Pillai's	0.20654	4.165	2	32	0.025
Roy's	0.26031				

***B. subtilis* Lettuce winter TWO-WAY ANOVA testing leaf tissue type with covariate leaf size**

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
LEAF AREA SIZE CM2	1	0.7338	0.7338	1.34	0.256
Leaf tissue type	2	4.5721	2.2861	4.16	0.025

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	4.269	0.398	10.73	0.000	
LEAF AREA SIZE CM2	0.00520	0.00450	1.16	0.256	1.00
Leaf tissue type					
Aged	-0.480	0.175	-2.75	0.010	1.33
Developing	0.106	0.175	0.61	0.547	1.33
Newly Developing	0.373	0.175	2.14	0.040	*

***B. subtilis* Lettuce spring ONE WAY ANOVA with covariate leaf size testing leaf tissue type**

MANOVA for LEAF SIZE CM2

s = 1 m = -0.5 n = 15.0

	Test		DF		
Criterion	Statistic	F	Num	Denom	P
Wilks'	0.79065	8.473	1	32	0.007
Lawley-Hotelling	0.26478	8.473	1	32	0.007
Pillai's	0.20935	8.473	1	32	0.007
Roy's	0.26478				

MANOVA for Leaf tissue type

s = 1 m = 0.0 n = 15.0

	Test		DF		
Criterion	Statistic	F	Num	Denom	P
Wilks'	0.85327	2.751	2	32	0.079
Lawley-Hotelling	0.17197	2.751	2	32	0.079
Pillai's	0.14673	2.751	2	32	0.079
Roy's	0.17197				

***B. subtilis* Lettuce spring TWO WAY ANOVA with testing leaf size**

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
LEAF SIZE CM2	3	9.749	3.2496	5.90	0.003

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	5.139	0.124	41.53	0.000	
LEAF SIZE CM2					
32.0561	0.530	0.214	2.47	0.019	1.50
56.4426	0.453	0.214	2.11	0.042	1.50
71.1890	-0.731	0.214	-3.41	0.002	1.50
91.6968	-0.252	0.214	-1.17	0.249	*

B. subtilis on strawberry

Table E-2 The impact of leaf size (time) and leaf tissue type on viable population of *B. subtilis* in the winter and spring on strawberry

MANOVA's and TWO-WAY ANOVA's

***B. subtilis* Strawberry winter ONE WAY ANOVA with covariate leaf size testing leaf tissue type**

MANOVA for LEAF SIZE CM2

s = 1 m = -0.5 n = 15.0

	Test		DF		

Criterion	Statistic	F	Num	Denom	P
Wilks'	0.44523	39.874	1	32	0.000
Lawley-Hotelling	1.24605	39.874	1	32	0.000
Pillai's	0.55477	39.874	1	32	0.000
Roy's	1.24605				

MANOVA for Leaf tissue type
s = 1 m = 0.0 n = 15.0

	Test		DF		
Criterion	Statistic	F	Num	Denom	P
Wilks'	0.83978	3.053	2	32	0.061
Lawley-Hotelling	0.19079	3.053	2	32	0.061
Pillai's	0.16022	3.053	2	32	0.061
Roy's	0.19079				

***B. subtilis* Straw winter TWO WAY ANOVA with testing leaf size**

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
LEAF SIZE CM2	3	40.95	13.650	13.57	0.000

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	6.433	0.167	38.49	0.000	
LEAF SIZE CM2					
11.6525	-1.066	0.290	-3.68	0.001	1.50
17.0998	-0.852	0.290	-2.94	0.006	1.50
27.1751	0.307	0.290	1.06	0.297	1.50
31.5260	1.610	0.290	5.56	0.000	*

***B. subtilis* Strawberry Spring ONE WAY ANOVA with covariate leaf size testing leaf tissue type**

MANOVA for LEAF SIZE CM2
s = 1 m = -0.5 n = 15.0

	Test		DF		
Criterion	Statistic	F	Num	Denom	P
Wilks'	0.97645	0.772	1	32	0.386
Lawley-Hotelling	0.02412	0.772	1	32	0.386
Pillai's	0.02355	0.772	1	32	0.386
Roy's	0.02412				

MANOVA for Leaf tissue type
s = 1 m = 0.0 n = 15.0

	Test		DF		
Criterion	Statistic	F	Num	Denom	P
Wilks'	0.95361	0.778	2	32	0.468
Lawley-Hotelling	0.04865	0.778	2	32	0.468
Pillai's	0.04639	0.778	2	32	0.468
Roy's	0.04865				

G. catenulatum on lettuce

Table E-3 The impact of leaf size (time) and leaf tissue type on viable population of *G. catenulatum* in the winter and spring on lettuce

MANOVA's and TWO-WAY ANOVA's

PreStop Lettuce Winter ONE WAY ANOVA with covariate leaf size testing leaf tissue type

MANOVA for LEAF SIZE CM2
s = 1 m = -0.5 n = 15.0

	Test		DF		
Criterion	Statistic	F	Num	Denom	P
Wilks'	0.59693	21.608	1	32	0.000
Lawley-Hotelling	0.67524	21.608	1	32	0.000
Pillai's	0.40307	21.608	1	32	0.000
Roy's	0.67524				

MANOVA for Leaf tissue type
s = 1 m = 0.0 n = 15.0

	Test		DF		
Criterion	Statistic	F	Num	Denom	P
Wilks'	0.98116	0.307	2	32	0.738
Lawley-Hotelling	0.01921	0.307	2	32	0.738

```
Pillai's            0.01884  0.307    2     32  0.738
Roy's               0.01921
```

PreStop Lettuce Winter TWO WAY ANOVA with testing leaf size

Analysis of Variance

```
Source           DF  Adj SS  Adj MS  F-Value  P-Value
  LEAF SIZE CM2   3   6.323  2.1076    15.83    0.000
Coefficients
Term             Coef  SE Coef  T-Value  P-Value   VIF
Constant       2.7210   0.0608    44.75    0.000
LEAF SIZE CM2
  45.729        0.622    0.105     5.90    0.000  1.50
  78.508        0.127    0.105     1.21    0.237  1.50
  89.241       -0.474    0.105    -4.50    0.000  1.50
  122.594      -0.275    0.105    -2.61    0.014     *
```

PreStop Lettuce Spring ONE WAY ANOVA with covariate leaf size testing leaf tissue type

MANOVA for LEAF SIZE CM2

```
s = 1    m = -0.5    n = 15.0
                        Test              DF
Criterion          Statistic      F  Num  Denom      P
Wilks'               0.85707  5.336    1     32  0.027
Lawley-Hotelling     0.16676  5.336    1     32  0.027
Pillai's             0.14293  5.336    1     32  0.027
Roy's                0.16676
```

MANOVA for Leaf tissue type

```
s = 1    m = 0.0    n = 15.0
                        Test              DF
Criterion          Statistic      F  Num  Denom      P
Wilks'               0.96667  0.552    2     32  0.581
Lawley-Hotelling     0.03448  0.552    2     32  0.581
Pillai's             0.03333  0.552    2     32  0.581
Roy's                0.03448
```

PreStop Lettuce Spring TWO WAY ANOVA with testing leaf size

Analysis of Variance

```
Source           DF  Adj SS  Adj MS  F-Value  P-Value
  LEAF SIZE CM2   3  13.306  4.4352    17.85    0.000
Coefficients
Term             Coef  SE Coef  T-Value  P-Value   VIF
Constant       2.3416   0.0831    28.19    0.000
LEAF SIZE CM2
  32.0561       0.931    0.144     6.47    0.000  1.50
  56.4426      -0.757    0.144    -5.26    0.000  1.50
  71.1890      -0.192    0.144    -1.34    0.191  1.50
  91.6968       0.018    0.144     0.13    0.899     *
```

G. catenulatum on strawberry

Table E-4 The impact of leaf size (time) and leaf tissue type on viable population of *G. catenulatum* in the winter and spring on strawberry

MANOVA's and TWO-WAY ANOVA's

PreStop Strawberry Winter ONE WAY ANOVA with covariate leaf size testing leaf tissue type

MANOVA for LEAF SIZE CM2

```
s = 1    m = -0.5    n = 15.0
                        Test              DF
Criterion          Statistic       F  Num  Denom      P
Wilks'               0.74729  10.821    1     32  0.002
Lawley-Hotelling     0.33817  10.821    1     32  0.002
Pillai's             0.25271  10.821    1     32  0.002
Roy's                0.33817
MANOVA for Leaf tissue type
s = 1    m = 0.0    n = 15.0
                        Test              DF
Criterion          Statistic      F  Num  Denom      P
```

Wilks'	0.93652	1.085	2	32	0.350
Lawley-Hotelling	0.06778	1.085	2	32	0.350
Pillai's	0.06348	1.085	2	32	0.350
Roy's	0.06778				

PreStop Strawberry Winter TWO WAY ANOVA with testing leaf size

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
LEAF SIZE CM2	3	8.222	2.7407	19.68	0.000

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	2.5471	0.0622	40.95	0.000	
LEAF SIZE CM2					
11.6525	0.746	0.108	6.92	0.000	1.50
17.0998	-0.224	0.108	-2.08	0.045	1.50
27.1751	-0.553	0.108	-5.14	0.000	1.50
31.5260	0.032	0.108	0.30	0.770	*

PreStop Strawberry Spring ONE WAY ANOVA with covariate leaf size testing leaf tissue type

MANOVA for LEAF SIZE CM2

s = 1 m = -0.5 n = 15.0

	Test		DF		
Criterion	Statistic	F	Num	Denom	P
Wilks'	0.87738	4.472	1	32	0.042
Lawley-Hotelling	0.13976	4.472	1	32	0.042
Pillai's	0.12262	4.472	1	32	0.042
Roy's	0.13976				

MANOVA for Leaf tissue type

s = 1 m = 0.0 n = 15.0

	Test		DF		
Criterion	Statistic	F	Num	Denom	P
Wilks'	0.84584	2.916	2	32	0.069
Lawley-Hotelling	0.18226	2.916	2	32	0.069
Pillai's	0.15416	2.916	2	32	0.069
Roy's	0.18226				

PreStop Strawberry Spring TWO WAY ANOVA with testing leaf size

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
LEAF SIZE CM2	3	0.4975	0.16584	1.87	0.154

Coefficients

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	2.5059	0.0496	50.52	0.000	
LEAF SIZE CM2					
25.3584	0.1852	0.0859	2.16	0.039	1.50
43.0844	0.0172	0.0859	0.20	0.842	1.50
51.4464	-0.1105	0.0859	-1.29	0.207	1.50
76.4011	-0.0919	0.0859	-1.07	0.293	*