University of Reading

Biocontrol as a key component to manage brown rot on cherry

PhD in Crop Science

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Declaration of original authorship

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Brown rot, caused by *Monilinia* spp., is one of the most important diseases in stone fruits worldwide. Brown rot can cause blossom wilts and fruit rots in the orchard as well as latent infections of fruit, leading to post-harvest fruit decaying. Current control methods rely on scheduled spraying of fungicides. However, the continuing pressure to reduce fungicide use has seen an increase in research into alternative management methods, such as biological control. NIAB EMR recently identified two microbes that significantly reduced sporulation of *Monilinia laxa* under laboratory conditions. These two isolates were a bacterial species *Bacillus subtilis* (B91) and yeast-like fungus *Aureobasidium pullulans* (Y126) and are currently being formulated into commercial products. We are investigating how to optimise the use of these two potential biocontrol products in practice, in terms of suppressing *Monilinia* sporulation on overwintered mummies and preventing infection of blossoms and fruits.

When applied to mummified fruits in winter Y126's population was stable through the winter but at a low concentration. The B91 survived a little longer with the population reaching that of the control group by week 4. Neither Biological control (BCA) treatments had an affected the population of *M. laxa* when compared to the control treatment of sterile distilled water. The interaction time between the BCAs and *M. laxa* showed the longer the interaction time the lower the spore count of *M. laxa*.

Another study was performed looking into the ability of our BCAs to colonise and survive on blossoms. B91 did not survive well on blossoms but could survive on fruits. However, its antagonistic compounds need to be in relatively high concentration to be effective against *M. laxa.* Therefore, it is best used as a fungicide, ensuring the antagonistic compounds are at a high concentration when applied in the field. Y126 can persist throughout the season and was marginally, though not statistically significantly, more effective at long term reduction in *M. laxa.* This could be because Y126 works through competition, therefore the interaction time with the pathogen could be important for efficacy and something worth investigating further. The difference between the BCAs highlights the need to understand each BCA's ecology to ensure maximum efficacy.

In a latent infection experiment, we inoculated trees with *M. laxa* and then treated them with the two biocontrol isolates two weeks before harvest. Post-harvest disease development was assessed after four days of storage in 2019 and two weeks in 2020. There was a significant reduction in rot incidence ($p < 0.001$) of 29% (Y126) and 27% (B91) in 2019 and 62 % (Y126) and 80 % (B91) in 2020 when the harvested fruit was stored at cold store levels.

With new products to be introduced into the environment, it's important to understand the effects they may have on the plant's microbiome. Using next-generation sequencing techniques, we looked at the impact B91 and Y126 has on the blossom and cherry microbiomes. There was a treatment effect in both the bacterial and fungal communities on the blossom and ripe cherry. But the biggest variability was between blocks (Geographical effect) and between the years in which we experimented ($p < 0.0001$). This research will assist in the development of management strategies, especially spray timings for brown rot on stone fruit, integrating BCAs with other management practices.

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Contribution of authors

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Biocontrol agents to manage brown rot disease on cherry

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Abstract This review focuses on biological control as a management strategy for brown rot disease on cherry. Specifically, the strain *Monilina laxa* as much of the research so far has been focused on *Monilina fructicola* and other stone fruit. Brown rot is one of the most economically important stone fruit diseases worldwide. Current control methods rely heavily on fungicide and costly physical controls. Biological control agents (BCAs) have been found which antagonise *M. laxa* and other *Monilinia* species. However, only a few have been developed into commercial products. This is primarily because they are still not as effective and consistent as the fungicides currently available to growers. Current biocontrol research focuses heavily on post-harvest applications but more could be done to look at the potential for BCAs to be used in the field to protect blossoms and fruits, reducing post-harvest losses. Future research needs to understand the ecology of these BCAs and their population dynamics in relation to external conditions, in order to optimise their use in integration with other management strategies. Disease management in the near future will rely more on the use of BCAs than conventional fungicides.

Keywords Biocontrol · *Monilinia* spp. · Brown rot · Stone fruit · Cherry · *P. avium*

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Introduction

Sweet cherry (*P. avium*) is the principal economic cherry species that are traded worldwide (Piaskowski et al., 2018). Market demand means that much of the fruit grown in the UK is sold straight from the field. In the UK, the demand for stone fruit exceeds production, with much of the demand, therefore, being met by import. This unmet demand has led to recent extensive plantings of cherry orchards, leading to an increase of 382% in UK cherry production from 2012 to 2017 (DEFRA 2019). Cherry's soft skin is prone to wounding from pests and natural fruit cracking. Combined with the long juvenile period of the trees this leaves the crop vulnerable to fungal infections. Sweet cherry has a lengthy juvenile stage, which means improving the crop and disease resistance through conventional breeding has a long time-scale: breeding programs can sometimes take 15 to 25 years to release a new variety. (Piaskowski et al., 2018).

The *Monilinia* genus includes some of the most important fruit pathogens worldwide (Xu & Fountain,

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2019). Thorough reviews of research into *Monilinia* species were conducted by Wormald (1954) and Byrde and Willetts (1977) and more recent research findings were reviewed by Rungjindamai et al. (2014), Oliveira Lino et al. (2016) and Xu and Fountain (2019). The three most common phytopathogenic *Monilinia* species are *M. fructicola, M. fructigena* and *M. laxa.* These pathogens are capable of causing a range of diseases including blossom blight, twig canker and brown rot. Their host range is mainly stone fruit though *M. laxa* and *M. fructigena* can also infect pome fruit such as apples and pears (Rungjindamai et al., 2014). Different *Monilinia spp.* can infect through different parts of plant tissue as well as having different symptoms depending on the host species (Holb, 2008). If conditions are favourable, brown rot disease can result in fruit losses of 50–75% within an orchard, with additional potential post-harvest fruit losses of 25–50% in transit and storage (Lahlali et al., 2020).

Biology and epidemiology of *Monilinia laxa* on cherry

The present review focuses on *M. laxa* as a cherry pathogen, because although *M. laxa* and *M. fructigena* both cause blossom wilt and fruit rot on cherry only *M. laxa* can infect both healthy and damaged fruit (Rungjindamai et al., 2014). *M. fructigena* can only infect through fruit wounds and *M. fructicola* is not present in the UK. *M. laxa* has received less attention than *M. fructicola* because *M. fructicola* is the main brown rot pathogen on stone fruit in Oceania and North and South America (De Cal et al., 2009). *M. fructicola* has also been reported on European cherries in Germany (2009), Spain (2006), Italy (2009), Romania (2010) and Slovenia (2009), France (2001), Hungary (2010), Poland (2010) (Steffen et al., 2015) and Bulgaria (Bobev et al., 2020). Where possible examples of research into *M. laxa* and cherry have been highlighted but other examples have been drawn upon for areas that we feel are relevant to highlight.

The teleomorph of *M. laxa* is rarely seen in nature. The majority of infections are by the asexual conidia that can be produced at temperatures as low as −4 °C (Tian & Bertolini, 1999). These are usually spread by wind and water splash but can be spread by vectors (Byrde and Willetts, 1977). The lifecycle of *M. laxa* in the field means that there is a continuous supply of conidia throughout the entire fruiting season from overwintered mummified fruit, newly infected and blighted flowers, and spurs infected the previous season. The greatest source of inoculum usually comes from mummified fruit left in the orchard over the winter (Villarino et al., 2010). The number of conidia per unit area of infected tissue is 10 times higher on a mummified fruit than on an overwintered fruit spur or newly infected flower, and *M. laxa* may sporulate for up to three years on the same mummified fruit (Stensvand et al., 2001). These mummies, therefore, serve as the dominant source of inoculum infecting flowers in the spring. The incidence of post-harvest brown rot is positively correlated with the number of mummified fruit in the orchard (Villarino et al., 2010), so their removal could greatly reduce disease development in subsequent years.

M. laxa can infect intact fruit directly as well as through wounds and natural openings although wounds are still the major infection site. Intact cherry fruit becomes more susceptible with age, young fruitlets are resistant to infection by *M. laxa* conidia until they reached the stage when they began colouring (Xu et al., 2007). This age-related susceptibility has also been observed for brown rot caused by *M. laxa* on peaches (Maria Villarino et al., 2011). The severity of *M. laxa* can be influenced by climate with high humidity and high precipitation favouring infection. High humidity during blossom facilitates the disease because other floral parts become available as infection sites (in addition to stamens and stigma) (Weaver, 1950). The key factors influencing the development of cherry brown rot in the UK are fruit age and availability of inoculum; the latter is likely to be positively related to the amount of rainfall during the early season (Xu et al., 2007).

Current Brown rot control methods on cherry

Chemical

Chemical control is still the main method of controlling brown rot within cherry orchards. Scheduled applications of fungicides on cherry and cherry blossom in orchards are used by most cherry growers in the UK to protect against brown rot (Xu et al., 2007). However, the overuse of fungicide can lead to phytotoxicity for blossoms and resistant strains emerging. In 1984 *M. laxa* isolates resistant to the main fungicide used at the time,

the benzimidazole benomyl, were reported in America. In 2006 more resistant strains were reported, this time to the related compound thiophanate-methyl (Egüen et al., 2016). In 2012, one-third of isolates recovered from stone fruits, including cherry, within Greece were resistant to the benzimidazoles benomyl and thiophanate-methyl (Malandrakis et al., 2012). It has also been reported that the population of thiophanate-methyl resistant strains of *M. laxa* has increased in Spanish peach orchards since 2007 (Egüen et al., 2016). This emergence of resistant strains, along with new legislation banning certain chemicals, has led to a reduction in the availability of chemical controls available to growers for use on cherry. Fenhexamid was the second most used fungicide in the UK on cherry, after copper oxychloride, in 2016 (DEFRA, 2016). It has been shown to be effective on *M. laxa* (Malandrakis et al., 2013) but careful observations are needed to detect early resistance and so help monitor the effectiveness of this product.

There is also pressure from consumers to reduce fungicide use due to concerns about the environmental impact of chemical use and residues in fruit. With a lack of an alternative to control postharvest disease chemical fungicide use could be managed by reducing the number of application times and the doses used, throughout the season. To do this requires effective integrated pest management systems (IPM), which would utilise disease prediction models to indicate when sprays are needed and the optimum dose (Usall et al., 2015).

Physical

Pre-harvest physical controls such as the removal of mummies are an effective way of controlling *M. laxa* in the field when incorporated into a pest management programme (Rungjindamai et al., 2014). However, in order to be effective, all diseased parts of the plant must be removed before harvest - including mummies, branches and rotten fruit - and disposed of properly, such as burial (Usall et al., 2015). Pre-harvest physical controls also include plastic covers, which are now widely used within the UK to reduce fruit splitting from rain, reducing wounds easily infected by brown rot. However, *M. laxa* is able to infect intact fruit at almost the same rate as wounded fruit (Xu et al., 2007). Moreover, the plastic covers can be expensive to install and the removal of diseased material is very labour intensive so these options are not always a viable option for growers. Fruit bagging is another potential physical control that can also protect against pest damage and improve fruit quality (Sharma et al., 2015) however this practice is also very expansive and time consuming and may not protect against early blossom infections.

There are also potential physical controls for the disease post-harvest. There are many successful studies looking at *M. fructicola* that have shown reduced post-harvest rot with surface sterilisation of fruit with hot water or boron dips. These methods can kill surface conidia to reduce new infections occurring post-harvest (Rungjindamai et al., 2014). Rapid cooling of the fruit after harvest also reduces post-harvest rots (Usall et al., 2015) by reducing respiration and fruit ripening. A recent study into post-harvest storage found that when stored at 20 °C and 100% relative humidity all fruit were infected with *M. fructicola,* but in fruit stored at 0–4 °C only 4% of those artificially inoculated with *M. fructicola* developed infections. It was concluded that in these cold store conditions there would be a low risk of infection due to the low disease development. However, this may not be the case for *M. laxa* as it is able to grow at lower temperatures, as mentioned earlier, compared with *M. fructicola*, which has a much higher optimal temperature of 22.5–27.5 °C. (Bernat et al., 2019).

Commercial biocontrol products

The use of biocontrol agents to control postharvest brown rots in stone fruit was first experimentally tested by Pusey (1984) using a soil bacterium (Aiello et al., 2019). Since then several antagonist microorganisms have been investigated and reported as effective against postharvest diseases on stone fruit. Despite these advances in research there is still no commercial product available that is specifically designed to treat brown rot in cherry (Janisiewicz et al., 2014). However, a few that have been taken forward for commercial use have been approved for the use on cherry to target brown rot. A strain of *B. subtilis* (QST713) has been approved by the European Union (Reg. (EC) No 839/2008) and is commercially available as Serenade (Serenade Max®, Bayer CropScience). It is currently the only *B. subtilis* product approved and available as a pre-harvest control for brown rot (Usall et al., 2015) though it is mainly used against *Botrytis cinerea* on outdoor grown lettuce and strawberries (Reiss & Jørgensen, 2017). Bio-ferm has produced two products 'BoniProtect' and 'Blossom Protect' to combat *Botrytis cinerea* and *Penicillium*

expansum, both post-harvest rots on apple. These products use a strain of *Aureobasidium pullulans* that uses competition as its mode of action. These *A. pullulans* biocontrol products are approved by the manufacturer as compatible for concurrent use with some fungicides, such as Fluopyram andTrifloxystrobin (Mounir et al., 2007). Recently *S. cerevisiae* and *B. amyloliquefaciens* have been approved for use in some European countries for control of, among other disease brown rot on stone fruit in the form of Julietta® and Amylo-X® respectively (Casals et al., 2021).

Research on biocontrol of brown rot diseases on cherry

Candidate biocontrol organisms

B. subtilis, a well-known biological control agent, is effective thanks to its ability to form endospores, tolerate a range of environments and produce antagonistic compounds. In fact, 45% of its genome is dedicated to the production of antagonistic compounds such as antibiotics. The species is also highly suited to being developed into formulated products as it grows rapidly in liquid culture (Calvo et al., 2017). *B. subtilis* endospores produce antibiotics at low concentrations. Rungjindamai et al. (2013) suggested that these disrupters of the pathogen cell membrane are *B. subtilis* mode of action. After treating *M. laxa* with cell-free culture filtrate and living cells, both showed similar levels of antagonism. However, other modes of action may contribute to *B. subtilis* antagonistic properties. Some strains of *B. subtilis* have been shown also to induce pathogen resistance pathways within the host plant, and stimulate mutualistic symbionts, or to produce plant hormones that can influence plant cell enlargement and division (Reiss & Jørgensen, 2017; Rungjindamai et al., 2013). *B. subtilis* strain CPA-8 has also proven effective as a post-harvest treatment for *M. laxa* on peach (Casals et al., 2010). Rungjindamai et al. (2013) characterised two candidate strains inhibiting *M. laxa* which were sourced from indigenous populations within the UK. One of these is a *B. subtilis* strain (B91) that was able to suppress conidia formation as well as survive at a wide range of temperatures under lab conditions.

In vitro studies of *B. amyloliquefaciens* (strain BUZ-14) showed growth inhibition of up to 73% in *M. laxa* and *M. fructicola* (Calvo et al., 2017). It was also able to survive on peach fruit surfaces at low temperatures, making it a potential candidate for post-harvest disease control. Two other strains (SF14 and SP10) also showed a significant decrease in disease severity of *M. laxa* on apple (80.3% and 100% respectively). However, they were still less effective than the fungicide in the trial, thiophanate-methyl, especially against *M. fructigena*. It was also observed with BUZ-14 and SF14 that a minimum concentration for efficacy was 10^7 CFU/ ml and 10^6 CFU/ ml. This concentration would need to be maintained in order for the biocontrol to be effective (Lahlali et al., 2020), which may be hard to achieve. An extensive survival rate study is therefore needed to assess the potential of the organism.

P. synxantha has been suggested as a potential BCA against *Monilinia spp.* Its antagonistic properties include competition and the production of toxic metabolites. *P. synxantha* was able to significantly reduce brown rot incidences postharvest on peach, caused by either *M. fructicola* or *M. fructigena,* at a range of temperatures (25 °C - 0 °C) (Aiello et al., 2019). Both *A. faecalis and P. agglomerans* are known biocontrol agents. *A. faecalis* a biocontrol agent used in the control of post-harvest diseases in citrus, and several strains of *P. agglomerans* have been found to control fungal or bacterial postharvest pathogens causing pear and apple blights (Smits et al., 2015). They both have now been recognised as bacterial antagonists of *Monilinia* sp. (Lahlali et al., 2020). However, control of *M. fructicola* or *M. fructigena* may not translate into control of *M. laxa*. Studies will need to be carried out it confirm if these microbes can control the *M. laxa*.

Aureobasidium pullulans is a biocontrol agent that occurs naturally on the surface of fruits and other plant surfaces. This yeast-like fungus is common in a range of environments. The production of large quantities of yeast-like propagules has enabled it to proliferate across the globe (Zalar et al., 2008). Strain L47 has proven to be effective at controlling a range of post-harvest pathogens on a range of fruits and vegetables including *M. laxa* on sweet cherries (Schena et al., 2003). The main antagonistic properties of *A. pullulans* arise from competition for nutrients and space (Schena et al., 2003). An increase in exogenous nutrients from the application of nutrient yeast dextrose broth reduced the antagonistic behaviour of *A. pullulans* (isolate LS-30) against *B. cinerea* and *P. expansum*, suggesting that competition from nutrients is a leading factor in its

efficacy as a biocontrol agent (Castoria et al., 2001). Some strains, such as isolate L47, also induce resistance through the production of hydrolytic enzymes by the host (Ippolito & Nigro, 2000; Schena et al., 1999). Castoria et al. (2001) detected extracellular exochitinase *N*-acetyl-β-D-glucosaminidase (Nagase) and β-1-3-glucanase on apple wounds treated with the biocontrol agent and suggested that these two enzymes could contribute to LS-30's antagonistic activity. However, it is not clear yet if these enzymes can be attributed to the antagonist or the host (Castoria et al., 2001).

Penicillium frequentans strain pf909 is a microbe found on peach twigs and flower. It is a known inhibitor of *M. laxa*, with competition as well as the production of antibiotics as its mode of biocontrol activity (De Cal et al., 1988). It shows promise to be formulated into a commercial biocontrol as it has genetic stability after 36 months of storage, can persist in orchards for up to two weeks and, when tested in combination with commonly used fungicides, was insensitive to field doses of 15 of the 21 fungicides tested, allowing it to be easily integrated into current pest management systems (Guijarro et al., 2019).

Factors affecting biological control

Environmental stresses such as direct UV irradiation, drought, varying nutrient availability and high and low temperatures and climate changes can all affect the efficacy of a BCA and so must be taken into consideration when planning application times. Factors that limit the growth of a BCA in vitro may not translate to efficacy in the field when also interacting with the host and pathogen. More field tests will help understand how these factors will affect BCA efficacy. Climate change must also be considered when researching BCAs as it will alter the exposure of the microbes to these environmental factors.

Rain, hail or mist have strong effects on BCAs and will heavily influence the application time of BCAs just as they do for chemical fungicides. For example, *Penicillium frequentans* (strain Pf909) was shown to be washed off fruit surfaces if applied within hours of rainfall (Guijarro et al., 2019). However, if given time to establish, the population loss of *B. amyloliquefaciens* from rainfall was reduced to insignificant levels (Vilanova et al., 2018). This shows the potential of BCAs to have an advantage over synthetic fungicides if given the opportunity to establish early, as they will be less affected by rainfall. Finding the optimum growing conditions for BCAs and giving them time to establish will go towards planning spray times to optimise their efficacy in the field.

A BCA must not only survive environmental stresses but must thrive by successfully colonising and persisting on the host plant. Knowing the growing environment and niche in which it can survive will inform growers of the best stage of the growing season to apply the biocontrol, be that on blossom or fruit. However, currently there has been little research into the fate of BCAs once they have been applied in field conditions (Ruano-Rosa et al., 2016). This has been in part because appropriate tools to study survival have not been available. The introduction of propidium monoazide (PMA) dyes, DNA-binding dyes that cannot penetrate living cell walls but react only with dead cells, combined with qPCR can tell us the quantities of living cells in a sample. This knowledge of the viable population will enable studies to look at the survival rate during different field conditions and at different times of the growing season (Ippolito & Nigro, 2000).

The populations of microbes on a plant, the microbiome, will vary depending on the geographic region, the species of plant, and the environment. When looking into the growth rate of two *A. pullulans* strains (L1 and L8) on Gala apples, Mari et al. (2012a) found both increased their population (8 and 2-fold respectively) during the first 48 h after treatment. However, in a previous study they had observed weak population growth of the same antagonists on peach (Mari et al., 2012b). This shows how population dynamics can vary, under similar environmental conditions depending on fruit species. Therefore, the species of crop must be researched when looking at BCA populations. (Mari et al., 2012a; Mari et al., 2012b). As well as surface microbes the disease pressure in the field also affects the efficacy of BCAs, increased disease pressure reduces BCA efficacy. Disease pressure in the field is also linked to environmental factors (Casals et al., 2021).

The concentration and dose at which a BCA is applied and the consequent relative and absolute density in the phylloplane microbiome is important for its efficacy. This will vary between BCAs, for example, *P. frequentans* Pf909 is only effective when the conidial concentrations are substantially higher than those of *M. fructicola* (Guijarro et al., 2017). Lahlali et al. (2020) when studying *B. amyloliquefaciens* on apple fruit, found it only effective when its concentration

was maintained at 10^7 CFU/ ml. Therefore, studies into the optimum concentration and dose, as well as timing, of a BCA application are important for formulation. As these will vary depending on the species of host and the species of *Monilinia* specific studies need to be carried out looking into *M. laxa* and Cherry.

Strategies for using biocontrol

Microbial antagonists are living organisms so have optimum conditions that enable them to survive and proliferate; these conditions must be met to use them effectively. Because fruit is stored in constant and controlled conditions post-harvest it is plausible that post-harvest application of BCAs would offer greater potential for success than application in field conditions. However, a pre-harvest application would allow beneficial microbes to colonise the fruit surface before harvest, since, for example, they cannot otherwise protect wounds were sustained during harvest. More generally, it is likely to be advantageous to the use of BCA microbes to allow them to establish and grow before infection, especially if their main mode of action is competition. As most *M. laxa* infections occur in the orchard (Bernat et al., 2017) pre-harvest applications of microbial antagonists could be an effective control for the post-harvest decay of fruit (Sharma et al., 2009). For example, Smilanick et al. (1993) found poor control of post-harvest brown rots on nectarine using *P. corrugata* and *P. cepacia* applied shortly after harvest. Concluding that early application in the field would improve control (Ippolito & Nigro, 2000).

Extending the argument, if biocontrols were able to colonise and survive on cherry blossom there would be a potential to reduce blossom blight disease from *M. laxa* as well as protect fruit from latent infections. This was shown to be the case when *A. pullulans* and *E. purpurascens* were applied to blossom. They successfully reduced the incidence of post-harvest rot of brown rot in cherry (Sharma et al., 2009). There have been recent studies that suggest the BCAs not only have the potential to colonise blossom but also to be dispersed by pollinators. Joshi et al. (2020) successfully used Japanese orchard bees as a vector to deliver *B. subtilis* (Serenade®) to apple blossoms (Joshi et al., 2020). Further innovations have been made in the delivery system of BCAs, specifically Serenade®, using bees as a delivery system for *B. subtilis*. The success of this research has led to new commercial products from Biobest "Flying Doctors®" (Dedej et al., 2004; Biobest 2013; Joshi et al., 2020).

Another potential preharvest application timing would be early winter. If the BCA was able to colonise mummified fruits for extended periods it might reduce the overwintering inoculum on mummified fruit within the orchard. However, the survival rate and efficacy of these microbial antagonists are often limited by field environmental conditions (Gerbore et al., 2014). Therefore, more research into field application of BCAs is needed to assess their survival rate and ability to tolerate variable conditions including low nutrient availability, fluctuating temperatures and UV radiation, allowing early colonisation of fruit surfaces (Ippolito & Nigro, 2000).

Perspectives and conclusion

With the current emphasis on reducing the use of fungicides, there is a real need for alternative tools like the BCAs discussed above. The use of BCAs in disease management is not new, and several antagonistic microorganisms have been identified which could help combat brown rot on stone fruit. However, few have made it to the market. The main reason for this is that their potential effectiveness at reducing disease is less than fungicides, both within the field and post-harvest. On top of this, because they are living organisms, and more affected by environmental factors than fungicides as well as the disease pressure within the field meaning their efficacy can vary greatly through the season and year to year (Casals et al., 2021). This makes them unattractive to growers as they are perceived as too unreliable.

Research into the ecology of the biological control organism, including its mode of action and the interaction of its population with the pathogen and the fruit microbiome should improve reliability. As this will be dependent on the target pathogen (*M. laxa*) and the host (cherry), specific studies will need to carried out to ensure that BCAs is optimised. Few studies have been conducted in relation to the interactions of the microbiome with BCAs although these could severely impact BCA efficacy. Unlike BCAs, chemical controls will not be affected by population dynamics on plant surface but could disrupt the natural microbiome by killing off-target organisms; this imbalance could affect

disease pressures in the future or have already influenced which problems are currently serious.

Understanding the effect of the time BCAs have to interact with the *M. laxa* on the crop, along with the disease life cycle, could help improve the efficacy of BCAs by making application timings more effective. We would predict that – other things being equal - a longer interaction time between the BCA and *M. laxa* would lead to increased efficacy. A simple way to increase the interaction time between the *M. laxa* and BCA would be to apply the BCA in the field before harvest. This could reduce the amount of pathogen inoculum present at harvest. Knowing the optimum length of time a BCA must interact with a pathogen to be effective would enable agronomists and growers to utilise the BCA at the optimum time in the growing season, in response to the disease prediction models. It is important that interaction time and microbiome studies are conducted within commercial orchards to ensure that results can be replicated by growers.

Knowing the concentration at which the BCA is effective is relatively simple to discover. However, maintaining that concentration in the field as well as on the shelf is more complex. Formulation research can help improve both lifetimes, but it is therefore necessary to select for BCAs that are able to survive well in liquid culture as well as tolerating environmental stresses. Field experiments must be conducted throughout the growing season to learn the survival rate and state of each BCA once applied in the field. Many BCAs are used, similarly to chemical pesticides, with an inundative strategy, relying on high concentrations for rapid, but possibly short term, effects. In order for this to work it is imperative to know the survival and multiplication rates of the BCA in the field and to combine these with accurate disease forecasts. The multiplication and survival rates of BCAs in the field and cold store under different environmental pressures need to be studied to optimise time and method of use.

As *M. laxa* is able to infect multiple parts of the cherry plant the ability of BCAs to colonise, survive and multiply in a target niche is paramount to its effectiveness as they may be affected by the tissue type: flower, wood, fruit etc. Therefore, more research into the behaviour of BCAs when applied to cherry, the trophic networks of which they are part, and their overall ecology will be crucial in their effective use. For control of *M. laxa* on cherry, the key factors determining disease are overwintering on mummified fruit and the incidence of spring blossom infections. An effective BCA should have the potential to persist and survive on mummified fruit over winter to reduce the primary inoculum of the disease during spring and then further survive and colonise blossoms to protect against blossom blight and latent infections. Survival rate studies on different tissue types and at the key times mentioned above should lead to more efficient use of BCAs.

It is important that we continue to develop BCAs as a tool for disease management in more sustainable food production systems in the future. However, the efficacy and reliability of BCAs have been a stumbling block to commercialisation. The problems are real, but BCAs should not be seen as an alternative to synthetic pesticides, rather as part of integrated pest management systems. Being able to apply BCAs with fungicides or other BCAs simultaneously or sequentially could help improve the efficacy, but reduce the side-effects, of an IPM system. Research into discovering new antagonistic microbes is important but more needs to be done to understand their ecology once found. Looking into their compatibility with other management strategies especially current fungicide products, as seen with 'boniprotect', will be crucial for the success of a new biocontrol product. Optimising application times by understanding their interactions with their physical and biotic environments, as mentioned in this review, will also help make BCAs more commercially viable.

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Chapter 2

Biocontrol to reduce the overwintering inoculum of *Monilinia laxa* on cherries

Biocontrol to reduce the overwintering inoculum of *Monilinia laxa* on cherries

Abstract

Brown rot caused by *Monilinia laxa* is a major disease on stone fruit. The initial infections of blossoms in spring are from the overwintered inoculum that builds up on mummified fruits left in the field. To combat this the removal of all mummified fruits from the orchard is recommended. However, this can be very labour intensive so alternative methods are needed. We looked at the ability of two biocontrol agents (BCAs) to survive on mummified fruits over winter and their potential to reduce *M. laxa* inoculum. The first BCA, *Aureobasidium pullulans's,* population declined rapidly in the first two weeks after which the population stabled. The *Bacillus subtilis* survived a little longer with the population reaching that of the control group by week 4. Neither BCAs affected the population of *M. laxa.* We also looked into the effect of interaction time to see if longer exposure of *M. laxa* to the BCA would affect it. The longer the interaction time the lower the spore count of *M. laxa*.

Introduction

Monilinia sp. are the main causal agent of brown rot, a major disease in stone fruits worldwide (Rungjindamai et al. 2014). The main species prevalent in the UK is *Monilinia laxa* (Mari et al. 2012). It can infect blossoms, twigs, wounded and intact fruits causing blossom blights, cankers and fruit rots (Xu et al. 2007). Infected fruits that are left in the orchard over winter become mummified (Casals et al. 2015). Most *M. laxa* infections are by conidia, asexual spores, which are spread by wind, water splash as well as some vectors (Byrde and Willetts 1977). The optimum temperature for conidium production for *M. laxa* is 10 ^{o}C (Tamm and Fluckiger 1993). However, it can grow at even lower temperatures with Tian and Bertolini

(1999) observing mycelial growth at -2 °C and germination of *M. laxa* conidia as low as -4 °C. This enables the pathogen to build up inoculum over winter on infected fruits that have mummified in orchards. These mummies are the primary source of inoculum causing blossom blight infections the following spring and an increase in post-harvest brown rot (Villarino et al. 2010).

When looking at non-chemical control methods for brown rot the removal of mummified fruits from the orchard is effective (Casals et al. 2015). However, this is very labour intensive and expensive. BCAs could be another ecological alternative method to help reduce the source of inoculum from mummified fruits, greatly reducing the disease pressure in subsequent years (Holb 2008). To control the overwintered *M. laxa* inoculum the BCAs need to be able to survive on the fruit surface within the orchard and through the winter. Because BCAs are living organisms, they are affected by environmental conditions such as UV radiation, fluctuating nutrient levels and climatic change (Ippolito and Nigro 2000). To assess the viability of BCAs, field studies are needed to understand their ability to survive in the field. As well as the survival in the field, a good understanding of the effect of interaction between the BCA and the brown rot pathogen over time is needed. This knowledge will inform the best application time for improved efficacy of the BCA.

This study looked at two microbial biocontrols, new strains of the known biocontrol agents *Bacillus subtilis* (B91) and *Aureobasidium pullulans* (Y126). Isolated from the indigenous population of cherries within an orchard in the UK, these two microbes not only can suppress conidium formation of *M. laxa* but have shown to be able to survive a range of temperatures under lab conditions (Rungjindamai et al. 2013). The modes of action of these BCAs were

investigated by Rungjindamai et al. (2013). Y126 competes with the pathogen for nutrients and space and B91 produces antagonist compounds.

This paper aims to determine whether targeting BCAs against mummified fruit could be an effective means of reducing the primary inoculum on mummified fruits. To do this we aimed to evaluate the survival of *B. subtilis* (B91) and *A. pullulans* (Y126) on mummified fruit over winter and their effect on *M. laxa*. We would expect that an increased interaction time of the BCA with a pathogen would lead to increased biocontrol efficacy (Ruano-Rosa et al. 2016). Preliminary analysis by Rungjindamai et al. (2013) shows that the agents studied here may not survive below 0 °C (Y126) and 10 °C (B91) and therefore we hypothesise a rapid decline in the viable population size, potentially affecting biocontrol efficacy. Yeasts are generally more tolerant of changing environmental conditions, such as humidity, when compared with bacteria (Ippolito and Nigro 2000). Combining this with the lower survival temperature we expected Y126 to perform better in the field than B91.

Methods

Experiment design

To assess the survival of the two BCAs overwinter, mummified fruits were treated with four treatments; B91, Y126, Luna Sensation (a commercial fungicide) and sterile distilled water as a control. The fruits were separated into groups of 5 mummies and placed into green mesh bags so they were exposed to the external environment. For each treatment, there were 6 bags for each replication, one for each time point (Table 1). Four cherry trees were selected in a non-commercial orchard and acted as four blocks. On each tree were hung the mesh bags

on branches around the same height for each treatment and time point (96 sample bags, 460 fruits). The experiment was carried out in the winter of 2017 and 2018.

Table 1: The table shows the date of collection for each of the time points for each of the years and weeks after application.

Time Point	Weeks after application of treatments	Date of Collection	
		2017	**2018**
1	2	5.12.17	3.12.18
2	4	19.12.17	17.12.18
3	6	2.01.18	2.01.19
4	8	16.01.18	14.01.19
5	10	30.01.18	28.01.19
6	12	13.02.18	11.02.19

To estimate the effect of interaction time, the mummified fruits were prepared the same but instead of 6 time points, there were 3 interaction times. All bags were placed into the field at the same time. Bags allocated to the first interaction time were immediately treated; a week later bags allocated to the 2nd interaction time received the first treatment and the first interaction time received the second treatment. The 3rd interaction time treatments were applied to all three groups in the third week. This meant that the bags allocated to the 1st interaction time received three treatments (hence higher BCA inoculum) and the BCAs had the longest interaction with the pathogen on the fruit surface.

Mummified fruits

For 2017 mummified damsons from two unknown cultivars were supplied by Michael Shaw at the University of Reading. In 2018 mummies of cultivar Kordia was collected from a non-

commercial cherry orchard in East Malling Kent. Cherries were kept in a cold store 4 ^{o}C until needed. For the Interaction time study, plum fruits were mummified artificially. Plums of variety Victoria were purchased and surface sterilised before being inoculated with *M. laxa* mycelium via wounding. Inoculated plums were placed on trays in a glasshouse compartment to mummify for 6 weeks.

Inoculum production and application

Single colonies of the BCAs were grown in liquid media for 24 hours on a shaking incubator (180 rpm, 25^{0}C). B91 in LB broth (ThermoFisher) and Y126 in Potatoes dextrose broth. A spectrophotometer was used to measure the concentration which was adjusted to OD600 0.2 (B91) and 0.01 (Y126) for an estimated CFU of 1x10^{8} / ml. The fungicide, Luna® Sensation, was prepared according to the manufacturer's instructions. Mummified fruits were sprayed within polythene bags to avoid cross-contamination with treatments until runoff with a handheld sprayer. Ten Sprays were needed to reach runoff at ~0.2 ml per spray, estimating the application concentration of 4 x 10^{7} CFU per fruit.

PMA treatment and DNA extraction

The mummified fruits were washed in Maximum Recovery (Sigma-Aldrich) for one hour on a shaking incubator at 180 rpm. The wash was then spun down and the pellet and resuspended in 500 µl of sterile distilled water. The suspension was then treated with PMAxx™ (Propidium monoazide) of concentration 25 µM for B91, 50 µM for Y126 and 60 µm for *M. laxa* (Tut et al. 2021; Vilanova et al. 2017). The samples were then incubated at room temperature, in the dark for 15 minutes on a rocker at 35 rpm. This was followed by a light exposure stage of 20

minutes to bind PMAxx™ to the DNA of degraded cells. Samples were kept on ice on a rocker at 35 rpm to ensure full light exposure and reduce heating from the light source. DNA was then extracted from the samples using TRI Reagent (Sigma-Aldrich) following the manufacturer's instructions with an added ethanol precipitation step.

Colony forming unit (CFU) analysis

The CFU of B91, Y126 and *M. laxa* of each sample was quantified using qPCR (Bio-Rad CFX96) with SYBR green (Bioline). Each reaction contained 10 µl of extracted DNA, 8 µl of SensiFAST™ SYBR® no-rox kit and 1 µl of each forward and reverse primer (Table 2) with a total volume of 20 µl. Reactions were performed in triplicate under the following conditions: 94 °C for 3 min followed by 40 cycles of 15 s at 94 °C, 30 s at annealing temperature (Table 2) and 30 s at 72 °C. The CFU was calculated using the standard curve generated from 5 standards of ten-fold serial dilution of genomic DNA of a known concentration present on each plate.

Table 2: Primers for each microbe including sequence and annealing temperature.

Target	Name	Sequence (5'-3')	Annealing Temperature (°C)	Reference
M. laxa	Mlx368-F	CCAAGGGCTCCGTAGGTAA	55	(Guinet et al. 2016)
	Mlx368-R	TCCACACCGTCGAACAATAA	55	(Guinet et al. 2016)
B. subtilis	Bs_dnaK1154 f	ACACGACGATCCCAACAAGC	60	(Tut et al. 2021)
	Bs_dnaK1254 r	AGACATTGGGCGCTCACCT	60	(Tut et al. 2021)
A. pullulans	ELO2-F	CACTCTTGACCGTCCCTTCGG	62	(Zalar et al. 2008)
	ELO2-R	GCGGTGATGTACTTCTTCCACCAG	62	(Zalar et al. 2008)

For the interaction time experiment, the washes were not treated with PMA as the CFU of *M. laxa* was estimated with a haemocytometer. First, the mummified fruits were washed in 200 ml of Maximum Recovery (Sigma-Aldrich) for one hour on a shaking incubator at 180 rpm. The wash was then filtered through a sterile muslin. A serial dilution was then performed before a haemocytometer was used to count the *M. laxa* spores.

Data Analysis

To assess the effect of the four treatments on *M. laxa* CFU over time the data was log-transformed and a two-way repeated measures ANOVA was performed. For CFU per fruit of B91 and Y126, an ANOVA was used to assess the temporal effects decomposed into linear and quadratic. To assess the effect of interaction time the CFU of *M.laxa* from mummified fruits was log-transformed and a two-way repeated measures ANOVA was also used to evaluate the effect of the treatments over time.

Results

When applied in the field the population of Y126, from two weeks after the application, was around $1x10^5$ CFU/ ml in 2017 and $1x10^4$ CFU/ ml in 2018. The population stabilised and stayed around $1x10^4$ CFU/ ml by week 12. There was no significant effect of time on the population (2017 $P = 0.25$, 2018 $P = 0.36$). By the second time point at week 4 the populations had reduced closed to that of the control (Figure 1).

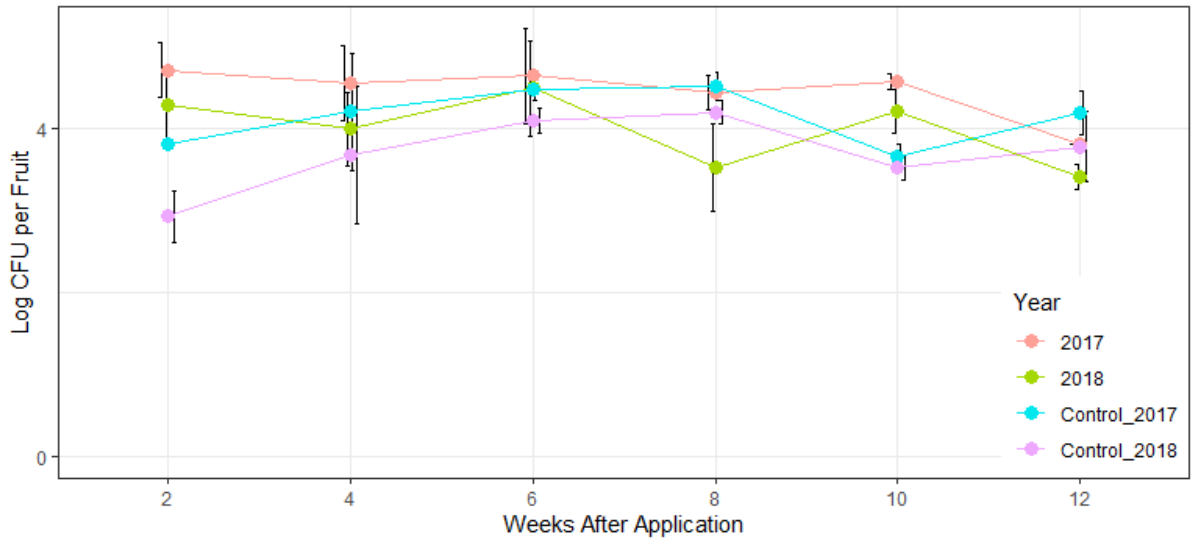

Figure 1: The log CFU of *A. pullulans* over 12 weeks after the initial application of the BCA for the two years (red = 2017, green = 2018) and in the control group that received no BCA inoculation (blue = control of sterile distilled water in 2017 and purple = control of sterile distilled water in 2018). The error bars represent standard error.

The population of B91 reduced slightly in the first two weeks from its initial concentration of $4x10^7$ CFU/ ml down to around $1x10^7$ CFU/ ml in 2017 and $1x10^6$ CFU/ ml in 2018. The population then fluctuated (Figure 2) but gradually reduced over time to around $1x10^5$ CFU/ ml by week 12. There was no significant effect of time on the population (2017 P = 0.46, 2018 P = 0.78). The results from the two years followed a similar growth pattern that was opposite to the background B91 population measured in the control (Figure 2). There is a difference in B91 populations between the three treatments measured: B91, fungicide and SDW control (2017 P < 0.0001, 2018 P = 0.0003).

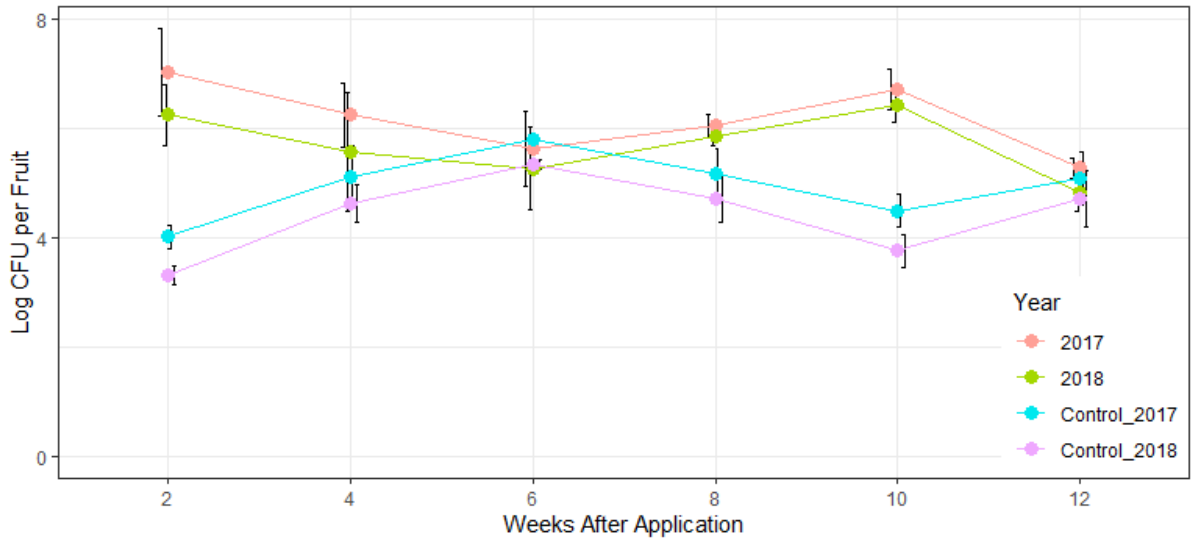

Figure 2: The log CFU of *B. subtilis* over 12 weeks after the initial application of the BCA for the two years (red = 2017, green = 2018) and the log CFU of *B. subtilis* in the control group that received no BCA inoculation (blue = control of sterile distilled water in 2017 and purple = control of sterile distilled water in 2018). The error bars represent standard error.

The BCAs did not affect the CFU of *M. laxa* on mummified fruits overwinter in either year (2017 P = 0.11, 2018 P = 0.13). As expected the CFU of *M. laxa* was effected by time (2017 P = 0.06, 2018 P = 0.01) . Figures 3 and 4 show the CFU for the 4 treatments. In each year, the CFU of *M. laxa* remained relatively constant from week 4 throughout the winter. The pairwise analysis showed the only significance was between treatments and time point 1 at two weeks (P = 0.017) in 2018.

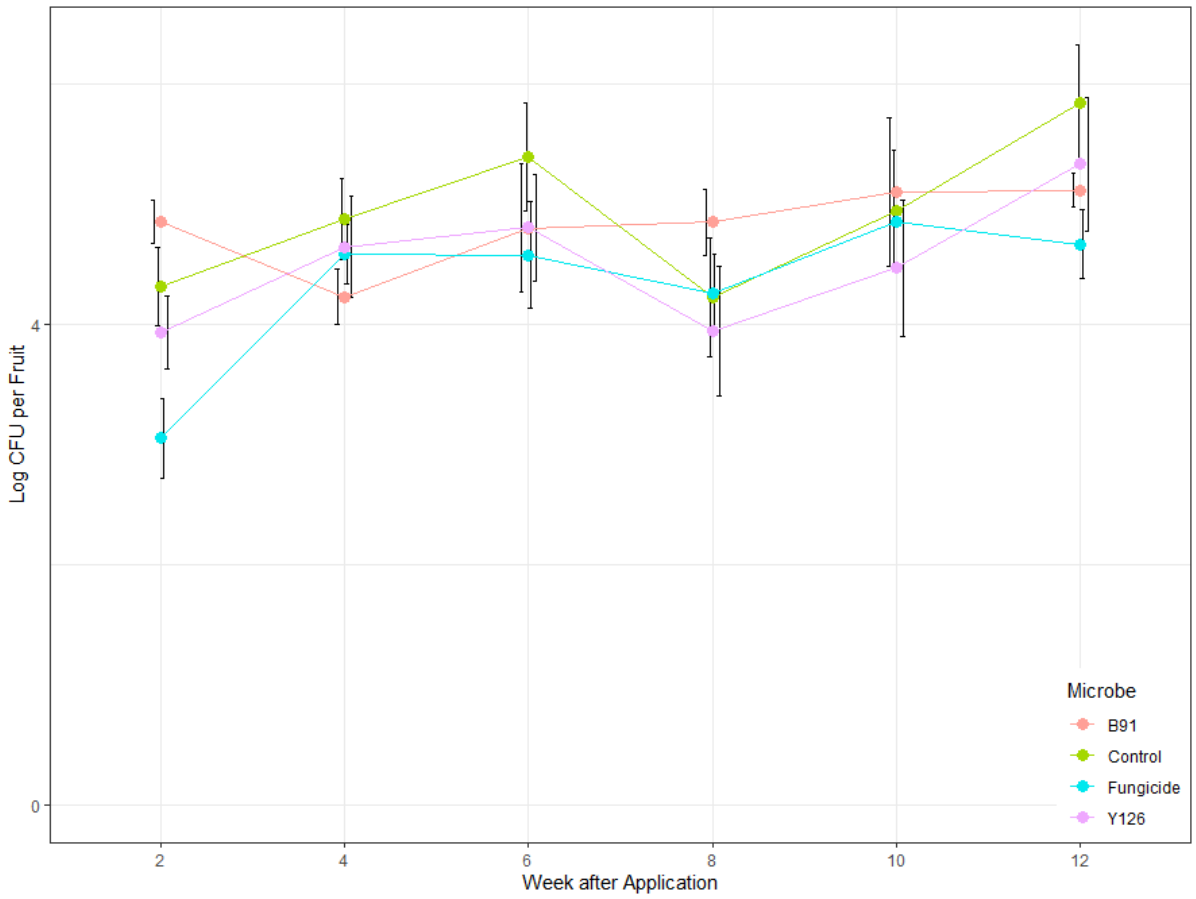

Figure 3: Log CFU of *Monilinia laxa* per mummified fruit across six time points in 2017 given in weeks after application of treatments. Each line represents the treatment that was applied to the mummified fruits on the 28th of November 2017.

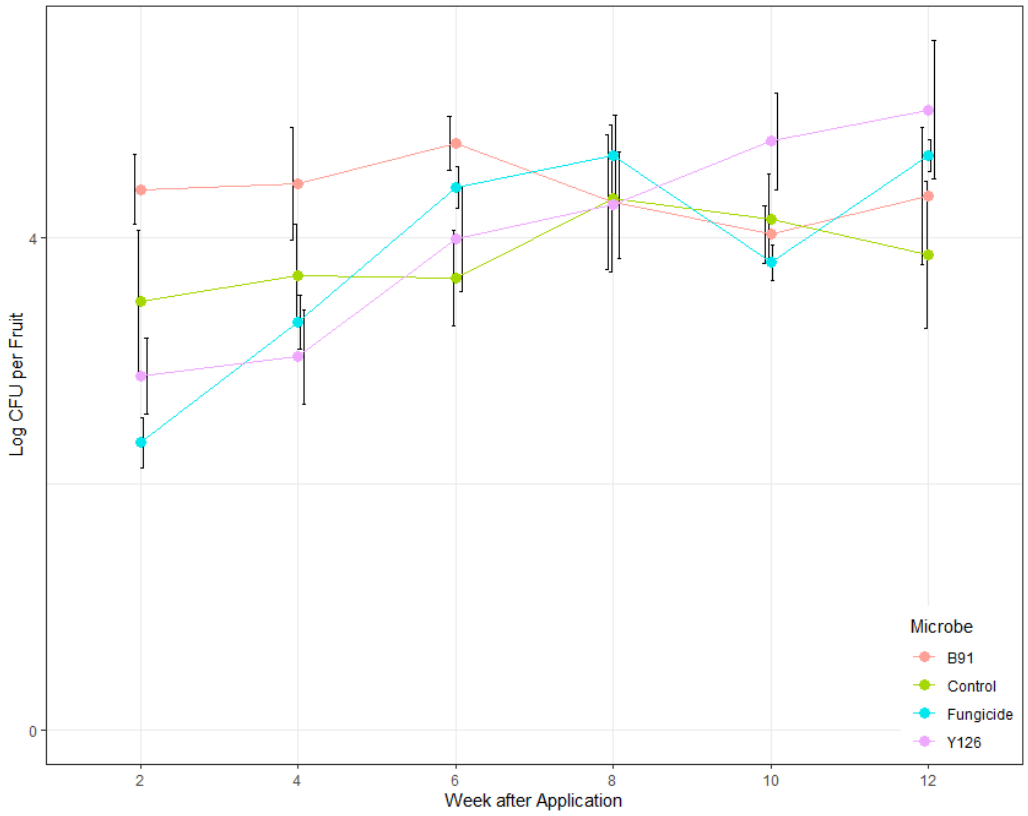

Figure 4: Log CFU of *Monilinia laxa* per mummified fruit across six time points in 2018 given in weeks after application of treatments. Each line represents a treatment.

There was a difference in the spore concentration among all four treatments ($P < 0.0005$). The longer the interaction for each of the treatments the lower the number of *M. laxa* spores (Figure 5). The number of *M. laxa* spores was significantly lower for both BCAs compared to the control (B91 $P = 0.03$, Y126 $P = 0.0003$). The number of spores present in the control also reduced with the longer interaction time (Figure 5).

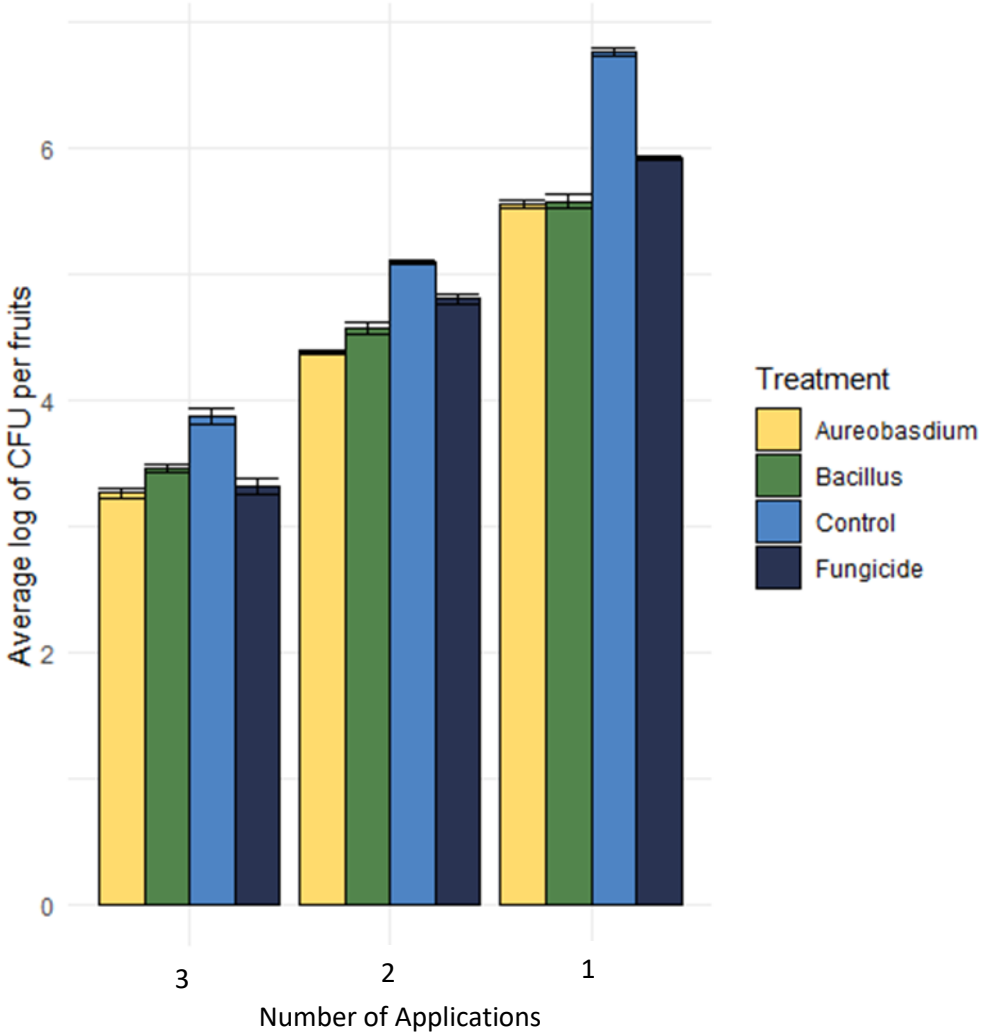

Figure 5: The (log transformed) number. of *Monilinia laxa* spores per fruit for the four treatments over three interaction times. The 1st interaction time having the longest exposure with 3 applications of the treatments and the 3rd the shortest exposure with one application of treatments.

Discussion

There was little difference between the applied treatments of Y126 and the background levels of the microbe measured in the control by week 4. This would suggest that the treatment would need to be reapplied regularly through the winter in order to have any effect on *M. laxa*. The first time point was taken two weeks after application and so a follow up experiment looking at how the BCA survives in the first two weeks in the field will be needed to assess when and how quickly the population decline happened. The two BCA treatments were not measured using the qPCR at the time of application. Any follow up experiments should include and accurate CFU at the time of application.

B91 performed a little better on the mummified fruits in the field than Y126. The population of *B. subtilis* decline was more gradual and reached the background levels measured in the control group by week 6. The population seemed to fluctuate in a cyclical pattern that was also seen in the control group, though in the inverse. Repeating this experiment, extending the time over which the measurements are taken may show if the pattern visible in figure 2 is repeatable. Interestingly the results from both years are very similar and follow the same pattern in both the control and the treated results. It should be noted that the primers used in the qPCR were not strain specific so the CFU for the BCA treatments also include the background levels seen in the control treatments.

Despite the survival rate of B91, it had little effect on the CFU of *M. laxa*. None of the treatments affected *M. laxa* apart from the first time point in 2018, at two weeks ($P < 0.02$). B91's main mode of action is through the inhibitory compounds. Even though B91 survived well in the first 4 weeks in the field, its lack of effect on *M. laxa* would suggest that these inhibitory compounds were not in a high enough concentration. Y126 did not survive well in

the field over winter and consequently did not affect *M. laxa*. The mode of action for Y126 is to compete with microbes for space and nutrients and so needs to sustain a larger population to be a success as a control agent. Mummified fruits already have a large established colony of *M. laxa* and so may have the advantage over Y126. Y126 could be more successful if it can colonise and establish the fruit surface before *M. laxa* therefore earlier inoculation would be worth testing.

The longer the interaction time the lower the spore count of *M. laxa*. However, this was also true for the control group. The control was an application of sterile distilled water, the spray may have washed off spores with each application or the BCAs may have been dispersed by rain. The BCAs did have a significant reduction on the number of *M. laxa* spores when compared to the control. The applications for the Interaction time were done weekly compared to the survival experiment that had one application and the first time point was measured after two weeks. As the survival experiment did not affect the CFU of *M. laxa* but the Interaction time did have a treatment effect, this gives us an indication that frequent applications may be necessary to control overwintering *M. laxa*.

Y126 did not survive well on mummified fruits in the field in winter but when the interaction time with *M. laxa* was increased by multiple applications it was able to reduce the spore count compared to the control group. B91 survived well for the first 4 weeks in the field but this did not influence the CFU of *M. laxa*. The is probably due to the inhibitory compounds being too low of a concentration to have an effect. A dose response experiment would shed light on what concentrations of these BCAs need to be sustained in the field to ensure efficient control of the pathogen.

The interaction time confirmed that the longer the BCA has to interact with the pathogen on the fruit surface the more effective it is. However, this is also true for the control of sterile distilled water, this could be due to the spray washing off spores. Future experiment should include a control of media used to suspend the BCAs. Combining the knowledge of interaction time with a dose response experiment and the known survival of these BCAs at different times in the growing season will help inform the best times to apply these products. The initial findings suggest that multiple treatments are necessary to ensure prolonged exposure to the BCAs. The BCAs are not well suited to winter conditions so applications in early spring may be a strategy to investigate going forward.

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Chapter 3

The use of biocontrol to reduce blossom wilt on cherry

The use of biocontrol to reduce blossom wilt on cherry

Abstract

Monilinia Laxa is the main causal agent of brown rot in the UK and one of the most destructive diseases within UK cherry orchards. Brown rot can cause blossom wilts in spring, these infected blossoms can lead to and fruit rots in the orchard as well as post-harvest. Biological control agents (BCAs) have been proposed as a potential method for brown rot control. One of the main hurdles of commercialisation of BCAs is their ability to survive field conditions. This study will look at two potential BCAs a bacterial species *Bacillus subtilis* (B91) and yeast-like fungus *Aureobasidium pullulans* (Y126) and their potential to colonise and survive on cherry blossom and fruits in the field and reduce the *M. laxa* population. Y126 showed promise as it was able to persist on blossoms and throughout the season. B91 was unsuccessful at surviving in high concentrations on blossom but the population recovered once fruit appeared. Neither BCAs had much effect on the concentration of *M. laxa* though Y126 was marginally more effective at long term reduction in *M. laxa.* The difference between the BCAs seen in this study highlights the need to understand each BCA's ecology to ensure maximum efficacy.

Introduction

Brown rot is one of the most devastating diseases affecting cherries worldwide. The first symptom of the disease is blossom blight seen in spring (Holb 2008). These early infections and blighted blossoms then lead to post-harvest rots causing crop losses in the field and in storage (Luo et al. 2005). Therefore, controlling infection at the blossom stage, and so

reducing blossom wilt, is an important disease management action for brown rot and brown rot blossom blight.

The main causal agent of the brown rot blossom blight within the UK is *Monilinia laxa*. Its life cycle means that it can infect and proliferate in the field throughout the season on various tissues. Infected fruits that are left on trees and in the orchard serve as a source of material for overwintering *M. laxa* allowing inoculum to build over winter (Casals et al. 2015). In early spring when blossoms appear the conidia of *M. laxa* colonise and infect blossoms. Blossoms are susceptible from bud breakthrough to petal fall and all parts of the flower are a potential infection site (Holb 2008). Blighted blossoms do not develop into fruits, reducing crop yield. Infected blossoms can often remain on the tree and become a source of inoculum that infects developing fruits. If the infection extends into the woody tissue, often via the peduncle after blossom infection, a necrotic lesion can form, called a canker (Ritchie 2000).

Blossom blight is mainly controlled with one to three applications of fungicides during the bloom period (Holb and Kunz 2013). However, legislative restrictions and an increase in environmental concerns have led to a demand for other control methods, such as biological controls (BCA). BCAs are seen as a potential tool in an integrated management system and have been studied extensively (Holb 2008). However, few have successfully made it to commercialisation (Janisiewicz et al. 2014). One of the barriers to commercialization is their survival rate in the field. BCAs are living organisms with optimal growing conditions so are affected by environmental conditions such as UV radiation, low nutrient levels and climatic change (Ippolito and Nigro 2000). Due to this, field studies are needed to understand BCAs tolerance and ability to survive in the field. Yeasts are generally considered to be more

tolerant of unfavourable environmental conditions when compared to bacterial species, especially in the presence of low humidity (Ippolito and Nigro 2000).

This study looked at two potential microbial biocontrols, bacterial species *B. subtilis* (B91) and yeast-like fungus *A. pullulans* (Y126). Discovered in the indigenous population of cherries within a UK orchard, these two microbes showed an ability to suppress conidia formation as well as survive a range of temperatures under lab conditions (Rungjindamai et al. 2013). Y126 and B91 have different antagonist properties and modes of action. Y126 competes with the pathogen for nutrients and space and B91 produces antagonist compounds (Rungjindamai et al. 2013). Regardless of this, we would expect that both BCAs will work better when applied early, so that they have time to establish on the flower *before M. laxa*.

The dose at which the microbe is applied and the concentration that it can maintain in the field has an effect on its efficacy as a BCA. The concentration needed will vary between BCAs; the bacterial antagonist *Pseudomonas frequentans* (Pf909) is only effective against *Monilinia* spp. when the concentration is significantly higher than the pathogen (Guijarro et al. 2017). Therefore, comparing optimum dose levels with survival rates in the field will be important for formulation as well as the timing of applications. Maintaining an optimum concentration level in the field is vital, as regular applications would become costly and time consuming for growers.

There have recently been studies of the effects of pollinators on BCAs. Bees, with hives equipped with BCA dispensers, successfully spread the biocontrol *B. subtilis* within blueberry orchards reducing the incidence of mummified fruits made by *Monilinia vaccinii-corymbosi*

(Dedej et al. 2004). Newly open blossoms in apple and pear orchards were also successfully inoculated with *B. subtilis* by bees living in hives with hive mounted dispensers (Joshi et al. 2020). This could be a useful approach for continued inoculation of blossom throughout the season.

For our BCAs to be successful we must assess their ability to survive environmental stresses and thrive by successfully colonising and persisting on the host plant, in this case, cherry blossoms. I looked at the ability of B91 and Y126 to colonise and survive on blossoms and reduce the *M. laxa* inoculum in the field; the application time of the BCA; the importance of dose; and the effect of pollinators on the BCAs.

Materials and methods

Experiment design:

To assess the survival rate of BCAs on blossom a field trial was conducted in the spring of 2019 through to summer/fruit harvest 2019. There were five treatments: Two BCAs, *Bacillus subtilis* (B91) and *Aureobasidium pullulans* (Y126); a fungicide (Luna Sensation); Serenade, a commercial biocontrol; and a sterile distilled water treatment as a control. For treatments B91, Y126 and control, samples were taken at six time points (1 = 1 week after application, blossom; 2 = 4 weeks after application, blossom; 3= 9 weeks after application, green fruit; 4= 10 weeks after application, green fruit; 5= 15 weeks after application, ripe fruit; 6= 18 weeks after application, ripe fruit) to assess biocontrol viability. For Serenade and Luna Sensation only the first and last time points were taken. There were three block repeats. Each branch was allocated to a time interval and each tree was allocated to a treatment. Four trees , of

culitvar Kordia, made up one block (Serenade and Luna Sensation were applied to one common tree). There were 3 replicates, so twelve trees in total.

In 2020, two small studies were conducted on potted trees (cultivars Kordia and Sweetheart) under cover to look at the application time of BCAs and the effect of pollinators on microbial dispersal, respectively. For the application time experiment, there were three spray treatments: two BCAs, B91 and Y126, and a sterile distilled water treatment as a control. For each spray treatment, there were two *M. laxa* inoculation times 'before', applied 4 days before BCA treatment, 'after' applied 1 day after treatment. For the control, there was also a branch that was not inoculated with *M. laxa* so that background levels on *M. laxa* could be estimated. There were 4 experimental blocks, each experiment block contained three potted trees, each tree a different treatment. Within a treatment tree, two branches were selected one each for the two *M. laxa* inoculations.

For the pollinator experiment, a single potted tree was treated as a block; there were a total 8 trees (i.e. blocks). For each tree, four branches of similar size were used. One was treated with B91, one with Y126 and two were untreated. The two untreated branches were either 'open' or 'bagged'. The 'open' branch was exposed and accessible to pollinators. 'Bagged' branches were bagged in green mesh to exclude pollinators but allow airborne microbes. Fifty blossoms from each branch were collected into sterile tubes for washing and DNA extraction.

<u>Inoculum production and application:</u>

Single colonies of B91 and Y126 were grown in liquid media (Liquid Broth and Potatoes dextrose broth respectively) for 24 hours on a shaking incubator (180 rpm, 25^0C). The

concentration was measured using a spectrophotometer and adjusted to OD600 0.2 (B91) and 0.01 (Y126) for a CFU of $1x10^8$ / ml.

The BCA suspensions and the control of sterile distilled water were transferred to handheld sprayers. Blossoms on each branch were sprayed with treatments until runoff with a handheld sprayer. Branches were prepared by removing old and new (un-opened) blossoms, so treated blooms were of similar age. *M. laxa* inoculum was grown on ripe plums. Spores were harvested from the surface of the fruit using a sterile scalpel, then suspended in sterile distilled water and adjusted to $1x10^4$ spores ml^{-1} with a haemocytometer.

PMA treatment and DNA extraction:

Samples were collected from the field at the six time intervals and the number of blossom or fruit recorded. Samples were then washed in Maximum Recovery (Sigma-Aldrich) for one hour on a shaking incubator at 180 rpm. The washes were collected, spun down and the pellet resuspended in 500 µl of sterile distilled water. Samples were then treated with PMAxx™ using the method from Tut et. al., 2021 (Tut et al. 2021). The PMA treatment was followed by DNA extraction using TRI Reagent (Sigma-Aldrich) following the manufacturer's instructions with the additional step of ethanol (70%) precipitation to remove any residual salts.

Quantitative Real-time PCR (qPCR):

To quantify B91, Y126 and *M. laxa* genomic DNA a qPCR (Bio-Rad CFX96) with SYBR green (Bioline) were used. Primers were selected from published literature (Guinet et al. 2016; Hertwig et al. 2015; Zalar et al. 2008). All reactions were performed in triplicate with the final volume of 20 µl containing 10 µl of extracted DNA, 8 µl of SensiFAST™ SYBR® no-rox kit and 1

µl of each forward and reverse primer. The cycle conditions for B91, Y126 and *M. laxa* were; 94 °C for 3 min followed by 40 cycles of 15 s at 94 °C, 30 s at annealing temperature and 30 s at 72 °C. Annealing temperatures for each microbe were B91: 60 °C, Y126: 62 °C, *M. Laxa*: 55 °C. Within each plate, there was negative control containing 10 µl of DNAse-free water instead of DNA sample and standards made from a ten-fold serial dilution of genomic DNA from an inital concentration of 10^8 CFU. These standards were used to generate a standard curve. The CFU was calculated using the standard curve and adjusted to CFU per blossom/fruit.

Dose Response:

South African cherries, cultivar Lapins, purchased from a supermarket, were used to study the dose response. They were surface sterilised and treated by pipetting five µl of biocontrol suspension into a shoulder wound by a sterile needle. There were 17 treatments: Four concentrations of each Biocontrol and Bayer's Serenade (active ingredient: Bacillus subtilis strain QST 713) (10^8, 10^7, 10^6, 10^5 ,10^4 CFU/ml), Luna Sensation (active ingredients: 250 g/L fluopyram and 250 g/L trifloxystrobin) and a control (sterile distilled water). Once treated, the cherries were placed in sterilised trays. There were three cherries per treatment per tray; eight trays were used, each as a block, and placed inside a 20^0C incubator. After 24 hours the cherries were inoculated with *M. laxa* (10^5 Spores/ml) by pipetting 5 µl into the wound. On day 4 of incubation, three days after *M. laxa* inoculation, cherries were assessed for visible rot symptoms.

Data Analysis:

For the data collected from the survival of BCAs on blossom, the CFU per flower/fruit was calculated from the qPCR. The data was log transformed before a two-way repeated measures ANOVA was performed to evaluate the effect of the treatments overtime on the CFU per blossom of M. laxa. For CFU per Blossom of B91 and Y126, an ANOVA was used to assess the temporal effects decomposed into linear and quadratic. The pollinator CFU data were also log transformed and orthogonal contrasts (Table 1) were used to look at the treated trees compared to the untreated trees and then untreated, bagged trees and the unbagged, untreated trees. Each treatment of biocontrol was assessed separately. Dose response data were logit transformed and an ANOVA was performed.

Table 1: The two contrasts used for the pollinator data.

	Is there a difference in CFU on treated trees compared to untreated trees?	On the untreated trees, is there a difference in CFU between the trees exposed to pollinators and those closed to pollinators?
Bagged (untreated)	0	1
Open (untreated)	-1	-1
Treated (open)	1	0

Results

Dose Response:

When looking at the dose of BCAs, as expected there was a difference seen between treatments ($P < 8 \times 10^{-12}$) and concentrations ($P < 7 \times 10^{-5}$). The fungicide treatment was the most effective with no rots recorded (Table 2). The three BCA treatments (B91, Y126 and Serenade) did not perform significantly better than the control ($P > 0.8$, $P > 0.9$, $P > 0.3$,).

When looking at the individual concentrations, only B91 at its highest concentration of 1 x 10^8 had a significant effect on rot reduction compared to the control ($P < 0.05$).

Table 2: The percentage of rotted fruit for each treatment and concentration. Significance groupings (Tukey HSD, p=0.05) are presented.

Treatment	Concentration (CFU)	Percentage of Rot	Significance groupings
P < 0.00001	P < 0.0001		
B91	1 x 10^8	37.50	b
	1 x 10^7	50.00	bc
	1 x 10^6	50.00	bc
	1 x 10^5	62.50	c
	1 x 10^4	87.50	d
Y126	1 x 108	83.33	d
	1 x 10^7	85.71	d
	1 x 10^6	50.00	bc
	1 x 10^5	62.50	c
	1 x 10^4	50.00	bc
Serenade	1 x 10^8	62.50	c
	1 x 10^7	60.00	c
	1 x 10^6	50.00	bc
	1 x 10^5	28.57	b
	1 x 10^4	28.57	b
Control		50.00	bc
Fungicide (Luna Sensation)		0.00	a

Field Survival:

Looking at the CFU per blossom of *M. laxa* it was not affected by either of the BCAs ($P = 0.6$). There was no significant interaction between treatment and time ($P = 0.6$). When looking at all treatments including the serenade treatment and fungicide there was also no effect seen between treatment ($P > 0.05$) and interaction between treatment and time ($P > 0.05$). Though no statistical difference was seen between treatments the best performing treatment by time point 6 was Y126 with the fungicide treatment having the highest average CFU of *M. laxa* by timepoint 6, 13 weeks after application.

The Average CFU per blossom for B91 on day 1 was around 1 x 10^4. This is a dramatic reduction from the initial inoculation concentration of 1 x 10^8 per ml. There was then an increase at time point 3 (5 weeks after application) once green fruit had developed (Figure 1) ($P < 4 \times 10^{-12}$). This pattern matched that of *M. laxa* CFU per blossom. The CFU per blossom of Y126 sustained its population for longer (Figure 2) with the CFU on blossom staying near the application concentration of 1 x 10^8 per ml for the first two time points, 1 day and 9 days after application (Figure 2) ($P = 0.02$). The level of *M. laxa* also remained low following a similar pattern as the Y126 after the first week from inoculation (Figure 2).

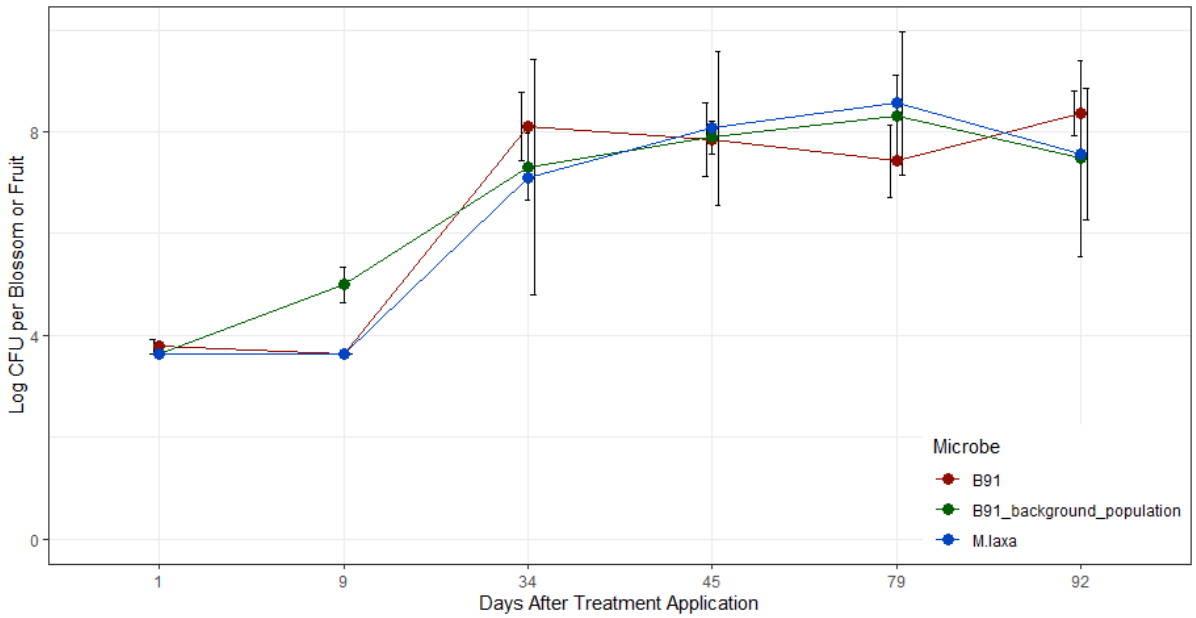

Figure 1: The log transformed CFU of B91 (red), B91 background levels taken from the control treatment (green) and *M. laxa* (Blue) over time. Time is given in days after the application of treatment. Time points: 1 = 18/04/19 Blossom, 2 = 25/04/19 Blossom, 3= 21/05/19 Green fruit, 4= 31/05/19 green fruit ,5= 04/07/19 Ripe fruit, 6= 17/07/19 ripe fruit. Correlation between B91 and *M. laxa* is -0.05 ($P > 0.5$). Error bars are standard error.

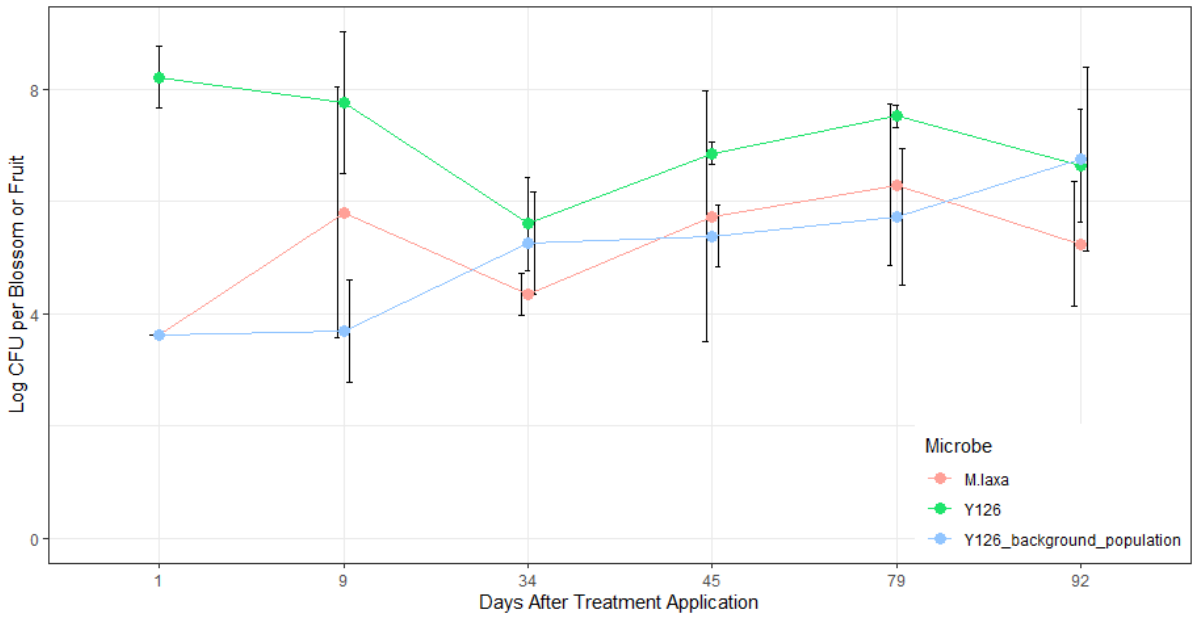

Figure 2: The log transformed CFU of Y126 (Blue) and *M. laxa* (Red) over time. Time is given in days after the application of treatment. Time points: 1 = 18/04/19 Blossom, 2 = 25/04/19 Blossom, 3= 21/05/19 Green fruit, 4= 31/05/19 green fruit, 5= 04/07/19 Ripe fruit, 6= 17/07/19 ripe fruit. Correlation between Y126 and *M. laxa* is -0.11 ($P > 0.5$). Error bars are standard error.

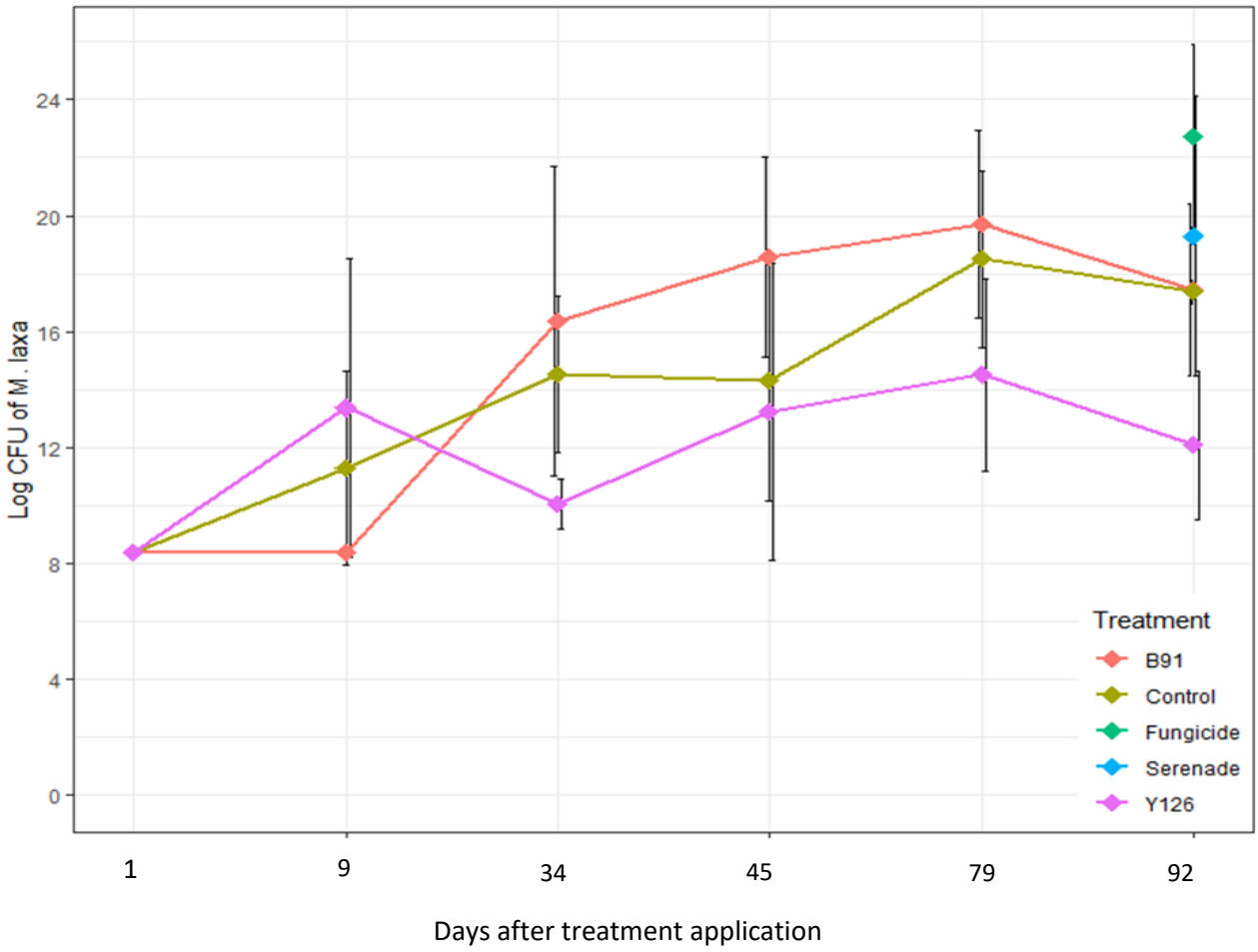

Figure 3: The log transformed CFU of *M. laxa* for the 5 treatments over time. Time is given in days after the application of treatment. Time points: 1 = 18/04/19 blossom, 2 = 25/04/19 blossom, 3= 21/05/19 green fruit, 4= 31/05/19 green fruit, 5= 04/07/19 ripe fruit, 6= 17/07/19 ripe fruit. No points are shown for Serenade and fungicide for time point 1 as the *M. laxa* was below the detectable level. Only two time points (1 and 6) were taken for Serenade and fungicide treatments. Error bars are standard error.

Application Time:

The application time of the treatments led to little difference in CFU per blossom in the Y126 and B91 treatments ($P < 0.1$, $P > 0.5$). However, both BCAs reduced the CFU per blossom of

M. laxa down to background levels (no inoculation) (Figure 4) compared to the control treatment ($P < 0.0005$).

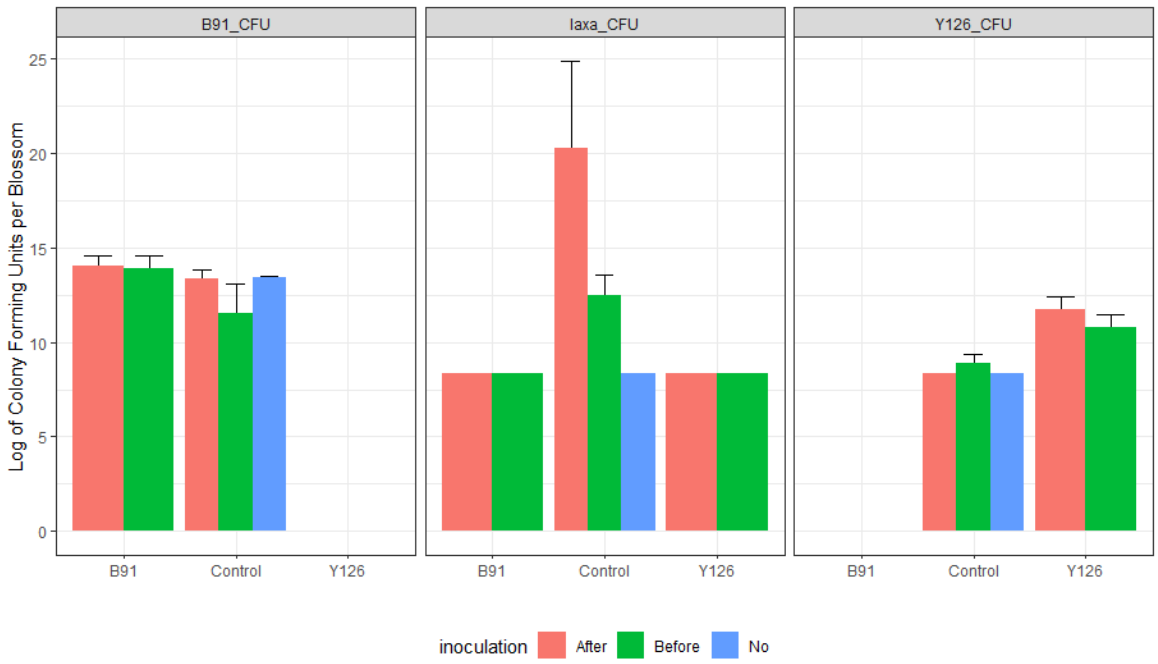

Figure 4: The Log transformed CFU per blossom for; B91 (B91_CFU), *M. laxa* (laxa_CFU) and Y126 (Y126_CFU) for each treatment; B91, Control (SDW) and Y126 and each inoculation time; *Monilinia laxa* applied After the treatment and *Monilinia laxa* applied Before the treatment. There was also a 'No inoculation' of *M. laxa* for the control treatment to assess background levels of the pathogen. No results show for the BCA CFU treated with the opposite BCA as it was not tested for. Error bars show standard error of mean. Where no error bars are visible (laxa_CFU) the threshold of detection of CFU was recorded as the result.

<u>Pollinator dispersal:</u>

There was no difference in CFU per blossom between the treated trees and those exposed to pollinators (unbagged untreated trees) for B91 but there was also no difference between the

untreated pollinator excluded trees (bagged) and unbagged untreated trees (P > 0.5). The first contrast (Table 1) (Is there a difference in CFU on treated trees compared to untreated trees?) for Y126 showed that there was a difference in CFU per blossom between the treated and the untreated trees that were exposed to the pollinators (P < 0.05). Y126 was more abundant on the treated trees than the untreated trees (Figure 5). There was no clear significant difference seen when comparing the trees that pollinators had access to (untreated unbagged trees) and those that pollinators could not access (untreated bagged trees) (P = 0.06).

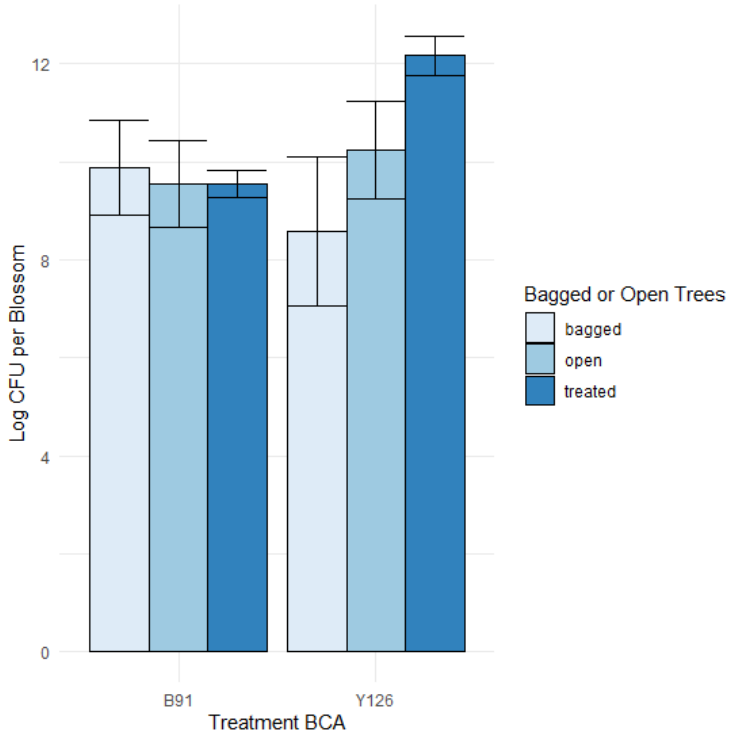

Figure 5: The Log transformed number of colony forming units per blossom for each BCA treatment Y126 and B91. For each treatment type there were levels; Treated, where trees were treated with the BCA, Open, where trees were untreated but left open for pollinators to access them and bagged, where trees were untreated but bagged to exclude pollinators. Error bars show the standard error of the mean.

Discussion

The field survival rate experiment showed that the population of B91 rapidly dropped within the first day of application onto blossom with Y126 able to sustain the population for the first few weeks. The population of B91 recovered once fruit formed indicating either that the BCA is better adapted at growing on fruit than on blossom or that the surface area is greater for fruit compared to blossom. The CFU results were recorded per blossom and per fruit so the increase CFU of B91 could be a result in the increase in surface area available on fruit compared to blossom. The population of *M. laxa* also increased greatly, in line with the BCA, so the BCA population size may not be at a level to affect the pathogen. B91 produces antagonistic compounds against *M. laxa.* The population pattern of *M. laxa* in the B91 treated trees suggests that the population level was not able to produce sufficient compounds to affect *M. laxa.* B91 may only be useful as an alternative fungicide where high concentrations of its antagonistic compounds can be produced in media and sprays can be timed when the disease pressure is high.

Y126 population remained at a high level in the first week after application on blossom. After the first time point (1 day after application) the *M. laxa* population follows closely below the population of Y126 (Figure 2). The Y126 mode of action is through competition, so the CFU must be maintained at a high level to be effective against *M. laxa.* Keeping the population of Y126 at a high level through formulation and regular application could be a method integrated into a disease management system, though further work would be needed to verify this theory. It should also be noted that the BCAs were applied to the field as pure cultures in a growth medium, with proper formulation we would expect to see better survival rates. The

growth medium may have also affected the growth of *M. laxa* by providing nutrients on the blossom surface.

When comparing the dose response with the survival rate, neither B91 nor Y126 was sustained at a level needed to reduce rot in fruit. The Cherries in the dose response experiment differed from the field experiment because they were wounded and the BCAs were only applied 24 hours before the *M. laxa* inoculation. The dose level needed for detectable protection might be different if the BCAs - especially Y126 - were able to establish on the fruit surface for longer before *M. laxa* infection. There might also be different outcomes of fruit rot in the dose experiment if the fruit is intact. Timepoint 6 suggests that Y126 treatment could cause lower levels of *M. laxa* A further dose experiment with different interaction times and conditions that mimic field conditions could confirm this.

The application time of the BCAs relative to inoculation did not affect their survival or ability to reduce *M. laxa*. This is useful as it will mean that protection will not be affected too much if the disease model is slightly out and the timings of the sprays are timed just before or after high disease pressure. Both BCAs reduced the inoculum of *M. laxa* when compared with the control of SDW. B91 survival rate was low on the blossom for both application times as the results were similar to the background levels recorded in the control treatment. This result is similar to that of the field survival rate experiment, so was to be expected. However, B91 was still effective at reducing the *M. laxa* population down to background levels. This supports previous studies that *B. subtilis* mode of action is through antagonistic compounds (Rungjindamai et al. 2013). These compounds may have concentrated within the growth medium before application. In which case it would seem the BCA may not need to survive for

the antagonists to be effective. Y126 had a better survival rate compared to B91 and was also effective in reducing *M. laxa* inoculum concentration to that of the control treatment. As Y126 relies on competition as its antagonism mode of action a high concentration must be maintained for it to be effective (Ippolito et al. 2005).

The pollinators did not affect the BCAs once applied to the trees. Although there as a slight increase in Y126 on the open but untreated bags, this was not significant, so Bees were not a vector. B91, as seen in the other experiments did not survive well on the blossoms and therefore may not have been in high enough concentration to be spread by pollinators. In previous literature, pollinators passed through the BCA in a liquid or powder form when exiting the hive (Dedej et al. 2004; Joshi et al. 2020). Therefore, BCAs must be in high concentration and either suspended in a liquid or powdered form, similar to the literature formulations, to be spread effectively by pollinators. Human mediated spray will still be the most effective way to apply these BCAs.

To get a more accurate dose response curve a follow up experiment would be needed that more closely mimicked the field conditions. For Y126 a longer establishment time and interaction time with the pathogen will better explore how it might be used in the field. We expect that good formulation of these BCAs will also improve their survival rate and efficacy within the field, so for any further experiments, using a formulated product would be preferred.

These experiments have highlighted that the different modes of action of these BCAs may greatly affect the way that they are best utilised within the field. B91 does not seem to survive

well on blossom but can survive on fruits. However, its antagonistic compounds need to be in relatively high concentration to be effective against *M. laxa*. Y126 can persist at low levels within the field though not at a level to significantly reduce *M. laxa*. An interaction time study between Y126 and *M. laxa* would be helpful to know the optimum establishment time for Y126 before increased disease pressure from *M laxa*. A follow-up study with multiple field applications and post-harvest rot assessments would inform effective spray regimes. The application time experiment showed that both BCAs reduced *M. laxa* inoculum on Blossom initially but along with the field survival experiment that they have a low survival rate overall and multiple applications would be needed throughout the season.

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Chapter 4

Biocontrols *Bacillus subtilis* and *Aureobasidium pullulans* to reduce *Monilinia laxa* post-harvest rot on cherry

Biocontrols *Bacillus subtilis* and *Aureobasidium pullulans* to reduce *Monilinia laxa* post-harvest rot on cherry

Abstract:

Brown rot caused by *Monilinia* spp. is one of the most important diseases in stone fruits worldwide. Latent infections of fruit by the pathogen often manifest once the fruit is ripe, presenting as post-harvest rots. Two microbial strains (*Bacillus subtilis* -B91 and *Aureobasidium pullulans* - Y126) have shown antagonistic properties towards the pathogen *M. laxa* in previous studies. This study assessed the potential of these two biocontrols under field conditions to reduce latent infection of cherry by *M. laxa* in the form of post-harvest rots. The study was carried out for two consecutive years in cherry orchards in Kent. When applied pre-harvest, both biological controls (B91 and Y126) successfully reduced the incidence of post-harvest rots between ~ 30% - 60%. This promising result helps towards the commercialisation of these biological controls, reducing the current reliance on fungicides in commercial cherry production.

Introduction

Brown rot caused by *Monilinia* spp. is one of the most important diseases in stone fruits worldwide (Holb, 2019). In addition to causing blossom wilts and fruit rots in the orchard, this pathogen can also cause latent infections of fruit that lead to post-harvest rot (Larena et al. 2005). Latent infections are characterised by *M. laxa* penetrating young fruits but remaining symptomless (latent) until fruit ripening near or after harvest. Growth of latent infection resumes as the fruit matures, often manifesting as post-harvest rots. In the first stage of fruit formation, the green fruitlet is photosynthetically active and is susceptible to infection. This

susceptibility is thought to be due to the active stomata forming an easy entry point for the fungi. The following stage, pit hardening, is the least susceptible due to the increase in production of secondary compounds including phenolics such as catechin and epicatechin that have antifungal properties. Once the pericarp forms and hardens, the concentration of phenolic compounds declines and the production of sugars increases, leading to increased susceptibility to *M. laxa*. Physical changes such as the thinning or fracturing of the cuticle associated with fruit maturity can also increase fruit susceptibility (Gatto et al. 2011; Oliveira Lino et al. 2016).

Latent infections that occurred in the field can quickly develop into visual rots after harvest and easily spread via contact within cold storage (Fourie and Holz 2003). The ability of *M. laxa* to develop rapidly at 5-10 °C can lead to widespread rot post-harvest. With the restriction of fungicide application post-harvest, the spread of rot can lead to significant post-harvest crop loss (Martini and Mari 2014). Current control methods rely on scheduled spraying of fungicides. However, there is continuing pressure to reduce fungicide. This has led to an increase in research into alternative management methods, such as biological control (Usall et al. 2015). In 2013, two microbial strains (*Aureobasidium pullulans* - Y126, a yeast-like fungus; *Bacillus subtilis* - B91, a bacterium) were isolated from cherry within the UK (Rungjindamai et al. 2013). These two strains showed promise in suppressing *M. laxa* sporulation on mummified fruit as well as being able to survive over a range of temperatures under lab conditions.

Though many previous studies have focused on the post-harvest application of biocontrols, pre-harvest applications of microbial antagonists could be an effective control measure to

reduce post-harvest decay. Pre-harvest application of BCAs would not only reduce pre-harvest infection and disease development but also allow beneficial microbes to colonise the fruit surface before harvest hence reducing secondary contact spread in cold storage (Ippolito and Nigro 2000). The present study aimed to test the hypothesis that B91 and Y126 applied close to harvest would reduce post-harvest cherry fruit rot caused by *M. laxa*.

Methods

In 2019, there were three treatments: fruit treated with each of the two biocontrol microbes in addition to fruit that did not receive biocontrol microbes but were treated with sterile distilled water as a control. All products were applied 24 h before artificial inoculation of fruit with *M. laxa*. In 2020, there were nine treatments: [1-8] four products (fungicide, sterile distilled water and the two biocontrol microbes (B91 and Y126)) applied 24 hours before or after *M. laxa* inoculation, and [9] fruits that were not subjected to artificial inoculation with *M. laxa* but were harvested to assess the background level of latent infection.

Single colonies of B91 and Y126 were grown in liquid media (liquid broth and potato dextrose broth, respectively) for 24 h on a shaking incubator (180 rpm, 25°C). Propagule concentration was estimated with a spectrophotometer and adjusted to OD600 0.2 (B91) and 0.01 (Y126) to achieve a propagule concentration of ~ $1x10^8$ CFU ml^{-1}. *M. laxa* inoculum was grown on ripe plums. Spores were harvested from the surface of the fruit using a sterile scalpel, then suspended in sterile distilled water and adjusted to $1x10^5$ spores ml^{-1} with a haemocytometer. The biocontrol suspensions and sterile distilled water were transferred to handheld sprayers. Fungicide Lunar sensation (250 g/L fluopyram and 250 g/L trifloxystrobin, Bayer,

www.cropscience.bayer.co.uk) was prepared at 3 ml/ 10 L according to manufacturers instructions.

In 2019, the experiment was carried out on three trees of cultivar Kordia in an open-air orchard at NIAB EMR. Three similar branches, at the same height from each tree were selected, one for each treatment. Two weeks before harvest, fruits on each branch were sprayed with an appropriate biocontrol strain until runoff. Twenty-four hours later, all fruits, including the control treatment, were sprayed with *M. laxa* spore suspension. In 2020, 10 trees of cultivar Kordia at NIAB EMR in the same open-field orchard as 2019 were selected, each with nine branches selected and randomly assinged a treatment. There were two treatment timings relative to the pathogen inoculation – 'before' and 'after'. For the 'before' timing, the treatments were applied 24 h before *M. laxa* was applied; for the 'after' timing, the treatments were applied 24 h after *M. laxa* was applied to the fruit. Two weeks before harvest, fruits on each branch were sprayed with the 'before' treatments (two biological controls, fungicide and water as a control) until runoff. Twenty-four hours later, all fruits, excluding the control with no pathogen inoculum (no), were sprayed with *M. laxa* spore suspension. Twenty four hours after *M. laxa* inoculation, the treatments were applied to the fruit on those branches allocated to the 'after' timing.

Two weeks after the 'before' timing treatments, all visibly healthy ripe fruits were harvested and stored on sterile trays: in 2019 at 20°C and in 2020 at 4°C. Throughout the incubation period, fruit with visible rot was recorded on days 1, 2 and 4 and removed to prevent secondary contact spread. The assessment was carried out for 4 days in 2019 when the

control treatment reached 100% rot, and until day 15 in 2020, generally considered the maximum shelf life (Habib et al. 2017).

For each branch, data consisted of a total number of healthy fruit and rotted fruit at a given assessment time. Individual trees functioned as blocks. The data were logit transformed and then ANOVA was conducted to assess the treatment effects on the incidence of post-harvest fruit rotting. Individual contrasts (Appendix a) were used to answer specific questions detailed in the results.

Results

Treatment with BCAs reduced the incidence of post-harvest fruit rotting (2019: $P < 0.001$; 2020: $P < 0.001$, Figure 1). In 2019, the two biocontrol treatments (B91 and Y126), when applied 24 h before pathogen inoculation, had a lower incidence of fruit rotting than the SDW control on all assessment dates ($P < 0.001$). There was little difference between the two biocontrol treatments.

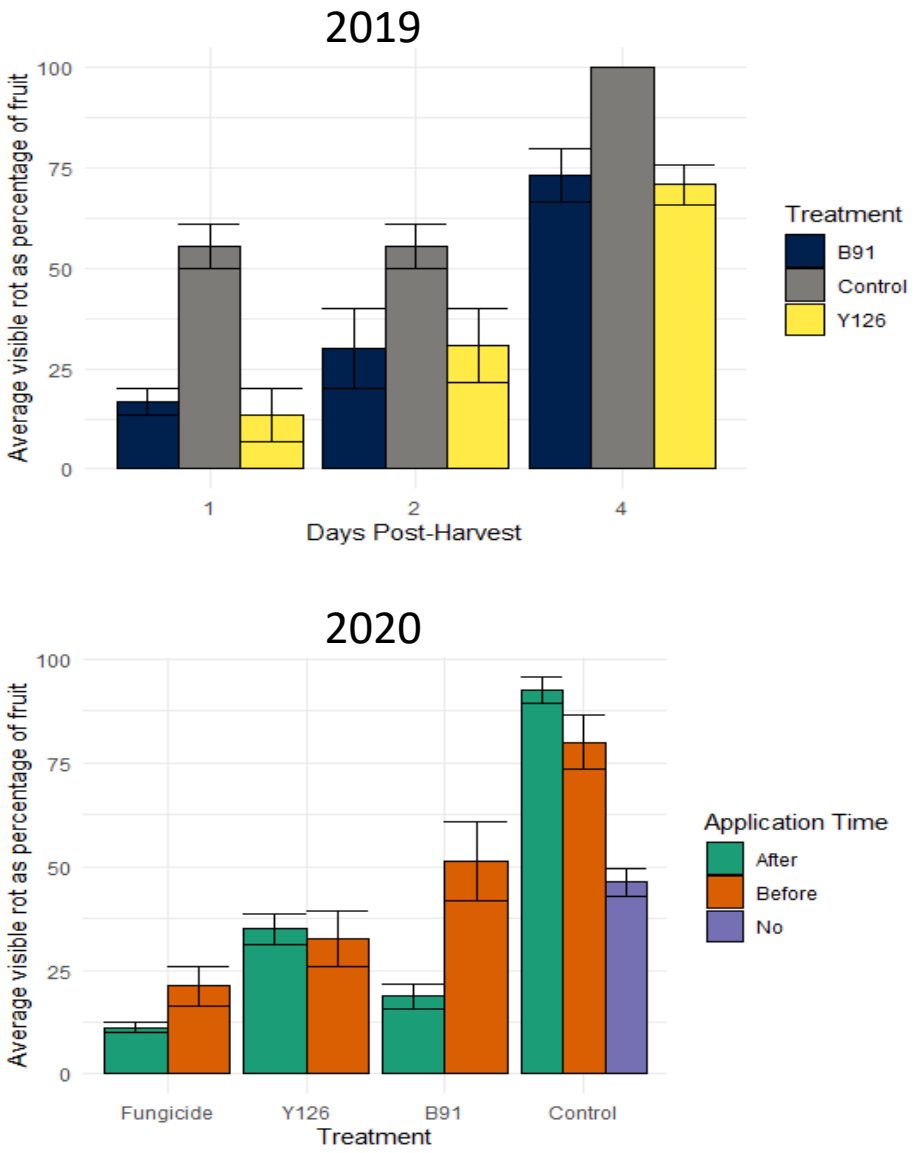

Figure 1: The mean percentage of cherry fruits with visible rot in 2019 and 2020. Error bars showing the standard error of the mean. 2019 shows percentage rot assessed on days 1,2 and 4 post harvest for two BCA treatments and the control of SDW. 2020; the mean percentage of cherry fruits with visible rot by day 15 of post-harvest storage at 4°C in 2020. Four products: B91, Y126, fungicide (Lunar sensation) and sterile distilled water (control) were applied 24 h before or after inoculation of fruit with *M. laxa* inoculation; the 'No' control received only sterile distilled water. The M. laxa inoculation was applied two weeks before harvest and the treatments 24hrs before or after this inoculation.

In 2020, a similar pattern was observed with both biocontrol microbes reducing the incidence of post-harvest rot incidence compared to the SDW control with the same inoculation time ($P < 0.0001$) (Figure 1). There was no significant difference between inoculation time within the control treatment ($P > 0.05$). There was also no significant difference between the inoculation time of the fungicide treatment ($P > 0.001$) though figure 1 shows a pattern of a slight reduction when applied ater the *M. laxa* inoculation. The fungicide also performed better than the two BCAs ($P > 0.001$). Averaged over spray timings, there was little difference in the reduction of post-harvest rots between the BCAs ($P > 0.1$). However, for B91 the application time had a strong effect with the BCA being more effective when applied after the *M. laxa* inoculation ($P < 0.001$). Unlike the fungicide and B91 treatments, there was little difference in the incidences of post-harvest rots between the application times of Y126 ($P > 0.1$).

Discussion

This study assessed the potential of two specific biocontrol strains (B91 and Y126) to reduce post-harvest rot on cherry caused by *M. laxa* inoculated two weeks before harvest. The 2019 experiment showed that the application of either biocontrol agent two weeks before harvest, the day before inoculation, reduced the incidence of post-harvest fruit rot by 70-75% on day 1 and by 25-30% by day 4 when incubated under ambient conditions. The 2020 experiment confirmed the effects of the two biocontrol strains. Application time (before or after *M. laxa* inoculation) was also studied in 2020. Both Y126 and B91 reduced the disease incidences post-harvest compared to the inoculated control by 59% and 36%, respectively for the 'before' application time, and 62 % and 80 % for the 'after' application. These results are similar to, if not better than, the 2019 results obtained at 20°C post-harvest storage, so these microbes

appear to remain effective at 4°C. B91 applied after inoculation had control efficacy almost as good as the fungicide control. This is a promising step forward for biological control of cherry brown rot to reduce the reliance on fungicides for combating these diseases.

The fungicide, as expected, reduced *M. laxa* infections and hence post-harvest rots. It was slightly more effective when it was applied after the *M. laxa* inoculation. This is probably due to its mode of action. The active ingredient fluopyram prevents spore germination *M. laxa* so needs to be present on the fruit surface to be effective. The application before pathogen inoculation may also have resulted in a lower concentration of fungicide present on the fruit surface to interact with *M. laxa* compared to the 'after' application; a side-target effect on resident microbiomes may have led to the pathogen experiencing less competition from the resident microbiome (Busby et al. 2016). B91 had a similar pattern to the fungicide, being slightly more effective when applied after the *M. laxa* inoculation. One important mode of action for B91 is through the production of toxins (Rungjindamai et al. 2013). Thus, we had hypothesised that the B91 would work better when applied before *M. laxa* as it would enable the biocontrol to establish on the fruit surface and produce these toxins. The opposite was observed, refuting our hypothesis. Because the organism was grown in liquid media for 24 hours before application, it is possible that the toxins were present in high concentrations in the application solution. This should be considered when formulating the biocontrol microbe into a product. Indeed, ensuring high concentrations of these toxins within their product this is the formulation strategy used for Serenade, a commercially formulated product of a specific *B. subtilis* strain (Yánez-Mendizábal et al. 2012). There was little difference between the treatments Fungicide (applied before inoculum) and B91 (applied after inoculum) showing the importance of the application time and the effect it can have on the biocontrol's efficacy.

If this is the case then B91 if commercialised will be considered a fungicide and would need to be handled with the same precautions and safety measures.

There was no significant difference between the incidence of post-harvest fruit rots at the two inoculation times of Y126, indicating that it is effective even if it has not time to establish before *M. laxa* innoculations. Y126 works primarily through competition with the pathogen (Rungjindamai et al. 2013) so this observation demonstrates that it can compete successfully with *M. laxa* even when applied 24 h after *M. laxa* inoculation.

These experiments showed that the BCA treatments reduced post-harvest rots, but it is not clear whether there was a latent infection present in the unaffected fruit (i.e., visibly healthy) since we terminated the experiment when the control had reached nearly 100% fruit rotting. Therefore, we are unable to establish if the latent infection was being successfully suppressed or prevented from being established by the BCAs. Using molecular techniques to analyse the fruits with no visible rots to assess if a latent infection is present will shed some light on these questions.

There is a need to investigate how the BCAs interact with other management strategies used to combat brown rot and how biocontrol could be successfully integrated with other pest and disease management practices within orchards. The poor survival of BCAs in the field has often been cited as a barrier to the effective deployment of BCAs in commercial agriculture and horticulture but applications closer to harvest have shown more promise (Sharma et al. 2009). This may also be the case for this experiment as the BCAs showed a good level of protection. Knowledge of the survival rate of BCAs in the field could assist in timing BCA

applications more effectively. The difference in the incidence of post-harvest rot due to application time relative to pathogen inoculation shows the importance of understanding the ecology of the biocontrol microbes and their mode of action to optimise their efficacy.

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Chapter 5

The effect of biological control agents on the microbiota of cherry blossom and fruit

The Effect of Biological Control Agents on Microbiota of Cherry Blossom and Fruit.

Abstract

With the advances in next-generation sequencing techniques, we are starting to piece together the microbiome of the plant's phyllosphere. However, it is still not clear how and to what extent these microbes affect the introduced biocontrol agents (BCAs) and what impact the BCAs have on the plant microbiome. In this study, we looked at our two BCAs [*Bacillus subtilis* (B91) and *Aureobasidium pullulans* (Y126)] to see what impact they had on the blossom and cherry microbiomes compared to a fungicide treatment and a control of sterile distilled water. We found that the biggest effect was the difference between blocks (Geographical effect) and between years ($P < 3 \times 10^{-16}$). We also observed a treatment effect of B91, Y126 and the fungicide for both years and both the fungal and bacterial communities. This effect was greater than the significant difference of tissue type between the microbiome of blossom and ripe cherry fruit. Because BCAs can impact the plant's microbiome the effects should be researched before new BCAs are brought to market to ensure that there are no off-target effects to the new product.

Introduction

Past research into microbial biocontrol agents (BCAs) has often been binary, focusing on the relationship between BCA and the pathogen or host plant. This research is important to help understand these primary relationships and assess the commercial viability of new BCAs. However, the plant phyllosphere is not a sterile surface but hosts a community of different bacterial and fungal species (Massart et al. 2015). The microbial community of the phyllosphere referred to as the microbiota, has been estimated at 10^6–10^7 cells/cm^2 (Lindow

and Brandl 2003). And this community has a role to play in plants' overall health. It can aid the host plant with nutrient acquisition, interact with pathogens and insects, induce host resistance against pathogens and help plants to tolerate other abiotic stresses (Massart et al. 2015).

A strong driver to study plant-associated microbiomes is that they are home to the majority of biocontrol agents and are the first place researchers can look to find novel BCAs (Massart et al. 2015). Two BCAs used in this study were discovered this way by screening microbes from the indigenous populations on cherries and plums within the UK (Rungjindamai et al. 2013). Once novel strains have been identified further research is needed to assess their survival and efficacy on the host plant under field conditions (Massart et al. 2015). A key part of this research is to understand the relationship between the introduced BCA and the microbial community of the host plant (Ippolito and Nigro 2000). It's important to know any off-target effects of a new BCA product as well as the effect of the microbiome on the BCA's efficiency.

Many factors affect the phyllosphere microbiome. One factor influencing the phyllosphere microbiome is the host genotype (Vorholt 2012). Just a single mutation in the host plant genome could modify the microbiome (Bodenhausen et al. 2014). The cherry phyllosphere is ever-changing with short-lived environments. In this study, we focus on the anthosphere (flower) and the carposphere (fruit). As well as host species, the microbiome is heavily influenced by environmental factors such as humidity, UV exposure, competition within the community, and pesticides (Vorholt 2012).

Using plate assays, two fungicides (metiram and captan) were tested to see their effect on the microbiome on 10 year old apple tree leaves (Royal Gala) in New Zealand. The fungicides initially reduce the growth of microorganisms. Some populations recovered quickly within just two to six days. However, there was a decline in species richness (Walter et al. 2007). The use of BCAs may also pose the same risk as these fungicides by affecting off-target microbes. These concerns were raised in a 2003 study that looked at four other BCAs (*Pythium oligandrum*, *Talaromyces flavus*, *Coniothyrium minitans* and *Ampelomyces quisqualis (Brimner and Boland 2003).* These BCAs use a range of modes of action from parasitism to competition, and it was suggested they could adversely affect non-target organisms in the soil microbiome (Brimner and Boland 2003). However, more recent studies of the effect of BCAs on plant microbiomes have shown minimal or short term effects (Sylla et al. 2013). It is suggested that the effect of host plant and seasonal changes have more of an influence on microbial communities, so any off-target effects of the BCA are confined to one growing season (Chen et al. 2013). But the effect of BCAs on these communities should be investigated before a product is released.

In 1999 the effect of BCA *Bacillus thuringiensis* on the microbial community of the phyllosphere of Brussel sprout leaves was analysed using plating techniques. No effect was found on the culturable microbes (Russell et al. 1999). However, since then techniques that no longer rely on culture to study microbial communities have improved our understanding (Abdelfattah et al. 2018). Looking at the same BCA on the phyllosphere of pepper plants these culture-independent techniques (phospholipid fatty acid analysis and 16S rRNA gene-directed PCR–denaturing gradient gel electrophoresis) showed that *B. thuringiensis* did have a

significant effect on the structure of the phyllosphere microbial community (Zhang et al. 2008).

Our understanding of these microbial communities has improved even more in recent years thanks to advances in next-generation sequencing techniques (NGS). Since sequencing has become more accessible, these tools have been used to answer many questions around BCAs such as their effect on species richness and diversity of the resident microbiome. They can also be harnessed to answer questions around BCA efficacy and whether it can be influenced by the resident microbiome (Massart et al. 2015). In this study, we will look at the changes of the cherry microbiome through the growing season from blossom to ripe fruit and the potential effect of the two BCA microbes when applied as pure isolates [*Bacillus subtilis* (B91) and *Aureobasidium pullulans* (Y126)] and a fungicide (Luna Sensation). We would expect to see that the changes in plant niche during fruit development (blossom, green fruit and ripe fruit) will have the largest effect on their surface microbiome. We would also expect, but to a lesser extent, there to be an effect of the introduced BCA (treatment). This could be short-lived and might only last as long as the survival of the BCA seen in Chapter 3.

Materials and methods

Experiment design:

To assess the effect of BCAs on the microbiota of the anthosphere and carposphere, samples were collected in 2019 at the same time as those in Chapter 3. There were four treatments: two BCAs (*B.subtilis* (B91) and *A. pullulans* (Y126)), a fungicide treatment (Luna Sensation), and a sterile distilled water treatment as a control. For treatments B91, Y126 and control, samples were taken at six time points to assess BCA viability (1 = 1 week after application,

blossom; 2 = 4 weeks after application, blossom; 3= 9 weeks after application, green fruit; 4= 10 weeks after application, green fruit; 5= 15 weeks after application, ripe fruit; 6= 18 weeks after application, ripe fruit). Only the first and last time points for Luna Sensation were taken. There was one treatment per tree with four trees adjacent to each other making one block. One branch per tree was used per time interval. Branches of a similar height were chosen and numbered and randomly assigned to each time point. There were three blocks with twelve trees in total. In 2020 the experiment was repeated on a smaller scale with four treatments: B91, Y126, a fungicide treatment and sterile distilled water as a control, and samples were taken only at two time points (at blossom and ripe fruit). There were six blocks, one tree serving as a single block containing all four treatments.

Inoculum production and application:

The BCAs were grown in liquid media from single colonies for 24 hours on a shaking incubator (180 rpm, 250C): B91 in LB broth (ThermoFisher) and Y126 in Potatoes dextrose broth. The concentration was adjusted to $1x10^8$ CFU / ml (OD600 0.2 for B91 and 0.01 for Y126) using a spectrophotometer. A handheld sprayer was used to apply individual treatments to blossom in the field. To ensure that blossoms were of a similar age, old and new (un-opened) blossoms were removed from the branch before treatment.

DNA extraction:

Samples were collected from the field at six time intervals in 2019 and two time points in 2020; the number of blossoms (~50) or fruit (~10) was recorded at each sampling time. Samples were then washed in Maximum Recovery (Sigma-Aldrich) for one hour on a shaking incubator at 180 rpm. The washes were then collected, spun down and the pellet

resuspended in 500 µl of sterile distilled water. DNA was extracted using TRI Reagent (Sigma-Aldrich) following the manufacturer's instructions with an additional step of ethanol (70%) precipitation to remove any residual salts. The quality and concentration of extracted DNA were assessed using a spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific, Cambridge, UK) and a fluorometer (Qubit 2.0, Life Technologies, Carlsbad, USA).

Sequence Processing and Operational Taxonomic Unit (OTUs) Generation:

DNA samples were sent to NOVOGENE (UK) LTD for Library prep and 16S and ITS Amplicon sequencing. Once the quality of the DNA sample was assured, PCR amplification was performed using 16S and ITS primers (Table 1). The PCR product was purified before library preparation. Sequencing was performed using sequencing by synthesis technology with the Illumina NovaSeq 6000 platform. Raw data were received from NOVOGENE (UK) LTD. Sequences were then clustered at 97% similarity into OTUs and taxonomy was predicted for generated OTUs using the reference databases Unite V7 (fungi) and RDP trainset 15 (bacteria), using the SINTAX algorithm (Cole et al. 2014; Nilsson et al. 2019).

Table 1: Primers applied in Amplicon Metagenomic Sequencing. Primers sourced by NOVOGENE (UK) LTD.

Types	**Region**	**Fragment Length**	**Primer**	**Primer sequences（5'- 3'）**
Bacterial 16S	V3-V4	466bp	341F	CCTAYGGGRBGCASCAG
			806R	GGACTACNNGGGTATCTAAT
Fungal ITS	ITS1-1F	321bp	ITS1-1F-F	CTTGGTCATTTAGAGGAAGTAA
			ITS1-1F-R	GCTGCGTTCTTCATCGATGC

Data Analysis:

The OTU table was normalized by rarefaction (Vegan package in R (Dixon 2003)). The rarefied OTU table was then used to calculate alpha diversity indices; Chao1, Simpson and Shannon metrics (Phyloseq package in R (McMurdie and Holmes 2013)). Then a permutation ANOVA of rank of diversity metrics was used to assess the effect of treatment, block and the difference between tissue type (flower & fruit). Chao1 index estimates the number of species present in a given sample and is a useful tool when assessing samples that may contain groups that may be of low abundance. The Simpson diversity index looks at both the abundance and the number of taxa so will give more weight to the dominant species in a sample. This means that species that are of low- abundance will not have much effect on the diversity. The Shannon and Simpson indexes summarize the diversity in the population taking into account the evenness within the community.

The beta (Bray-Curtis (Bray and Curtis 1957)) diversity was calculated to quantify the differences in species populations between samples. Non-metric multidimensional scaling (NMDS) plots were used to visualise sample locations in two dimensions based on the calculated beta-diversity indices. ADONIS, a permutation MANOVA using F-tests based on sequential sums of squares (Anderson 2001), was calculated for the beta diversity matrix to answer our primary research question: to what extent is the observed difference in microbiota due to tissue types and treatments. This research question was further answered through a Principal components analysis (PCA). PCA decomposes variation within the OTU data into orthogonal vectors in decreasing order of variation accounted for. An ANOVA was performed on the first 4 PCs to assess the relative contribution of experimental factors (block, treatment and tissue type). However, it was not possible to include the full model for 2019

data due to the confounding effects between time points and tissue type. Each year was analysed separately, the 2019 data was then subsetted to omit time points two to five, making it balanced with 2020 data. The two years were then pooled and analysed together. Differential abundance analysis was then used to look at each BCAs effect on the relative abundance of individual OTUs compared to the fungicide and the control (DeSeq2 package in R (Love et al. 2014)). P values were adjusted using Benjamini-Hochberg Procedure and significance was taken at 0.05 (Benjamini and Hochberg 1995).

Results

The total bacterial reads were higher than fungal counts with 10,690,696 and 8,897,216 reads respectively but with fewer OTUs: 881 for bacteria and 3,471 for fungi. Rarefaction curves of transformed (log_{10}) OTU counts showed that sequencing depth was adequate in both years (appendix b & c).

Alpha diversity:

The data from both years were pooled and Chao1, Simpson and Shannon metrics were used to show alpha diversity. The alpha diversity metrics did not reveal significant differences between treatments within the fungal population, thus indicating a similar level of microbial diversity. However, there was a difference between blossom and ripe fruit (Table 2). Figure 2 shows that the biggest variation was seen in the samples collected from the control treatment and ripe fruits. The bacterial communities differed between treatments and tissue type (Table 2). For both the Shannon and the Simpson indices, the samples from the ripe fruit are more diverse (Figure 1). There is no significant difference in the Chao1 index between treatments or between blossom and ripe fruit, indicating similar community richness. The

most significant effect on the alpha diversity is between the two years for both the bacterial and fungal communities.

Table 2: Alpha diversity of the bacterial and fungal communities for factors: year, treatment, tissue type and block effect, by means of Chao1, Shannon, and Simpson indices. Bold values indicate P value < 0.05.

Indices	**Year (P values)**	**Treatment (P values)**	**Tissue Type (P values)**	**Block (P values)**	**Residuals**
Bacteria					
Chao1	**$2 * 10^{-16}$**	0.41	0.30	0.60	253.20
Shannon	**$2 * 10^{-16}$**	**0.026**	**0.005**	0.35	224.90
Simpson	**0.025**	**0.04**	**0.0008**	0.27	245.10
Fungi					
Chao1	**$2 * 10^{-16}$**	0.37	0.19	0.10	134.70
Shannon	**$2 * 10^{-16}$**	0.17	**$2 * 10^{-16}$**	0.59	140.78
Simpson	**0.001**	0.10	**0.0008**	0.41	160.27

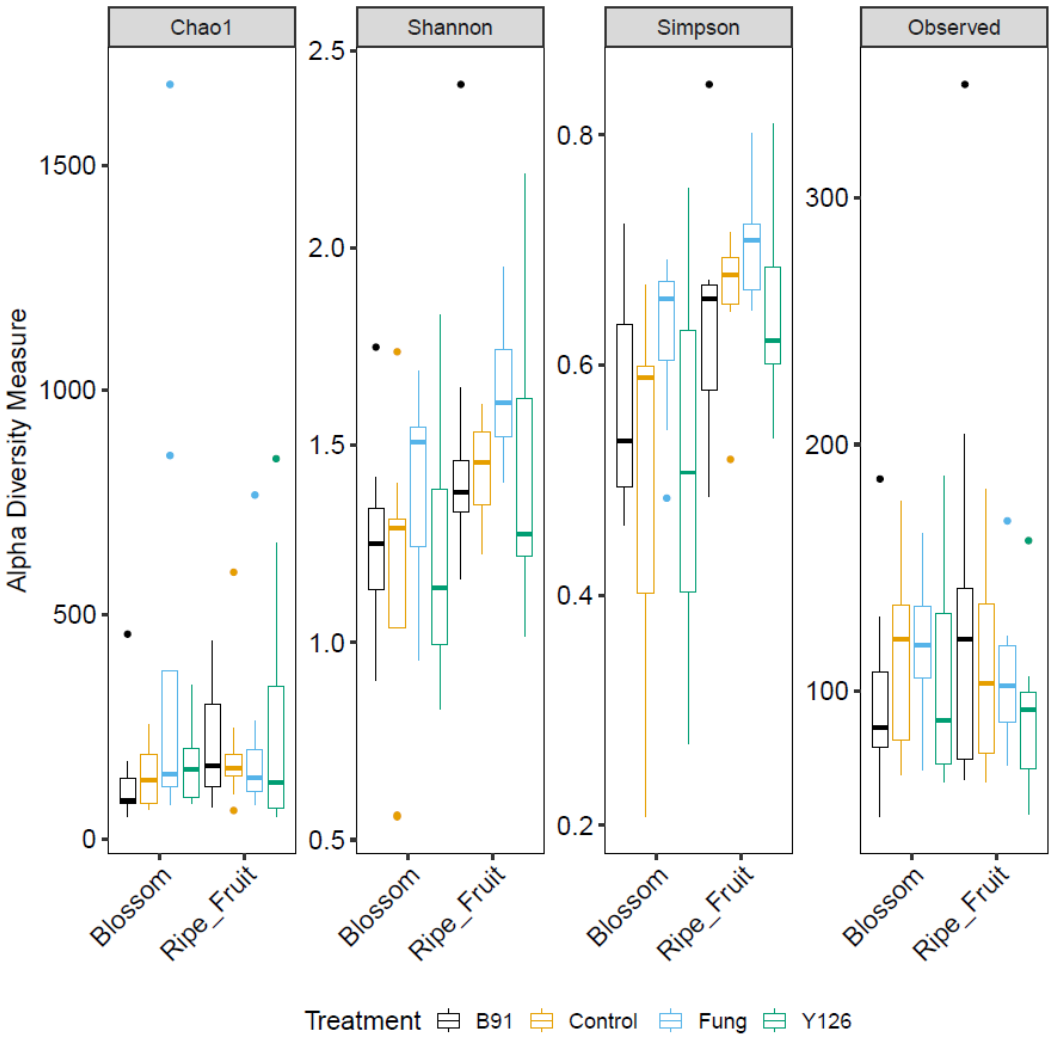

Figure 1: Alpha diversity measures of observed Operational Taxonomic Units (OTUs), Chao1, Shannon and Simpson, for bacterial OTUs. Data is for both years with 6 reps for 2020 data and 3 for 2019. The Axes show the tissue type blossom or Ripe fruit - and the colours indicate each treatment; B91, Y126, control and Fungicide (Fung). Shannon and Simpson show that fungicide treatment (Blue) increases bacterial diversity.

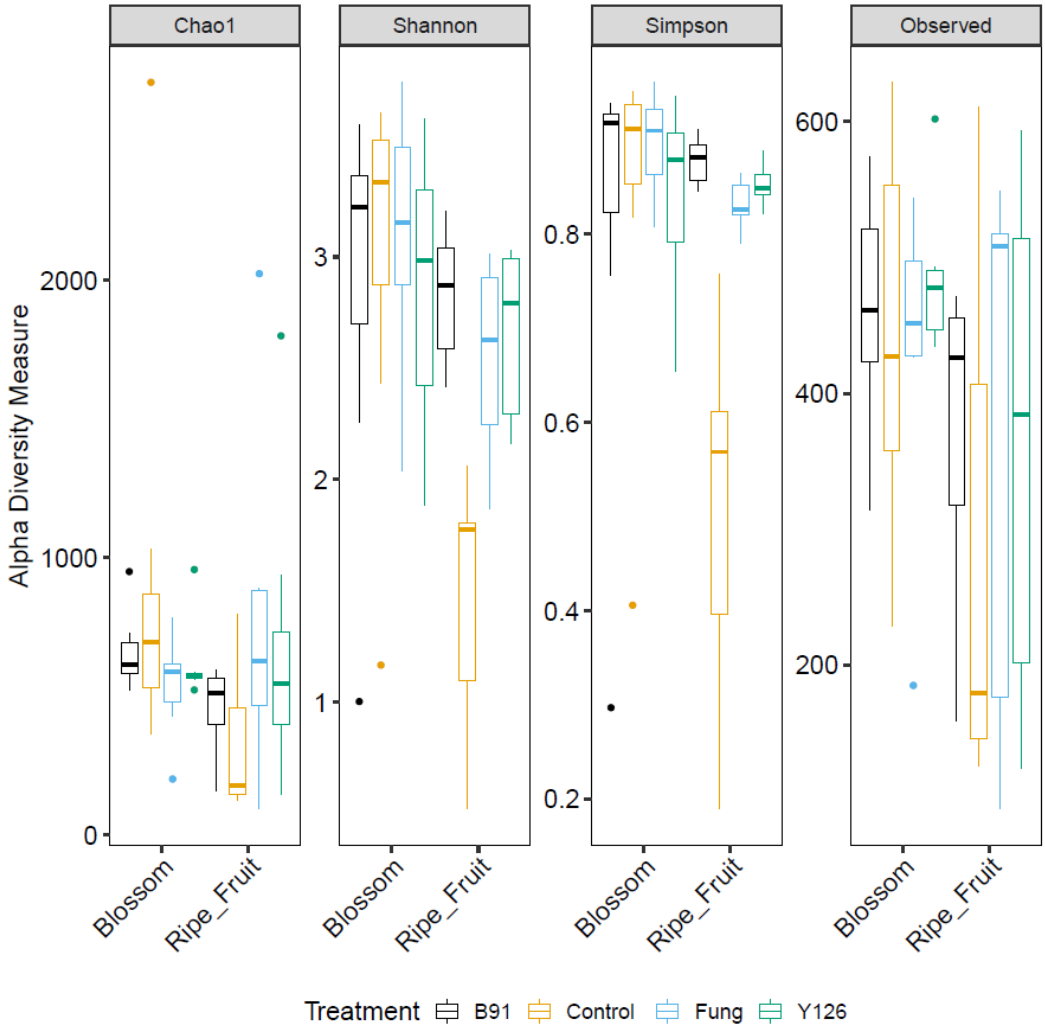

Figure 2: Alpha diversity measures of observed Operational Taxonomic Units (OTUs), Chao1, Shannon and Simpson, for fungal OTUs. Data is for both years with 6 reps for 2020 data and 3 for 2019. The Axis show the tissue type, blossom and Ripe fruit and the colours indicate each treatment; B91, Y126, control and Fungicide (Fung).

When looking more closely at the frequency of different taxonomic groups in each treatment and tissue type it became clear that *Bacillus* species are present in all treatments on ripe fruit at similarly high levels with little on blossom (Figure3). In 2019 the presence of *Bacillus* was less prevalent seen in Appendix f as taxonomic group *Bacillales.* It is still present in low but

similar concentrations across all treatments on ripe fruit (B91, control and Fungicide) and green fruit (Y126) (Appendix f).

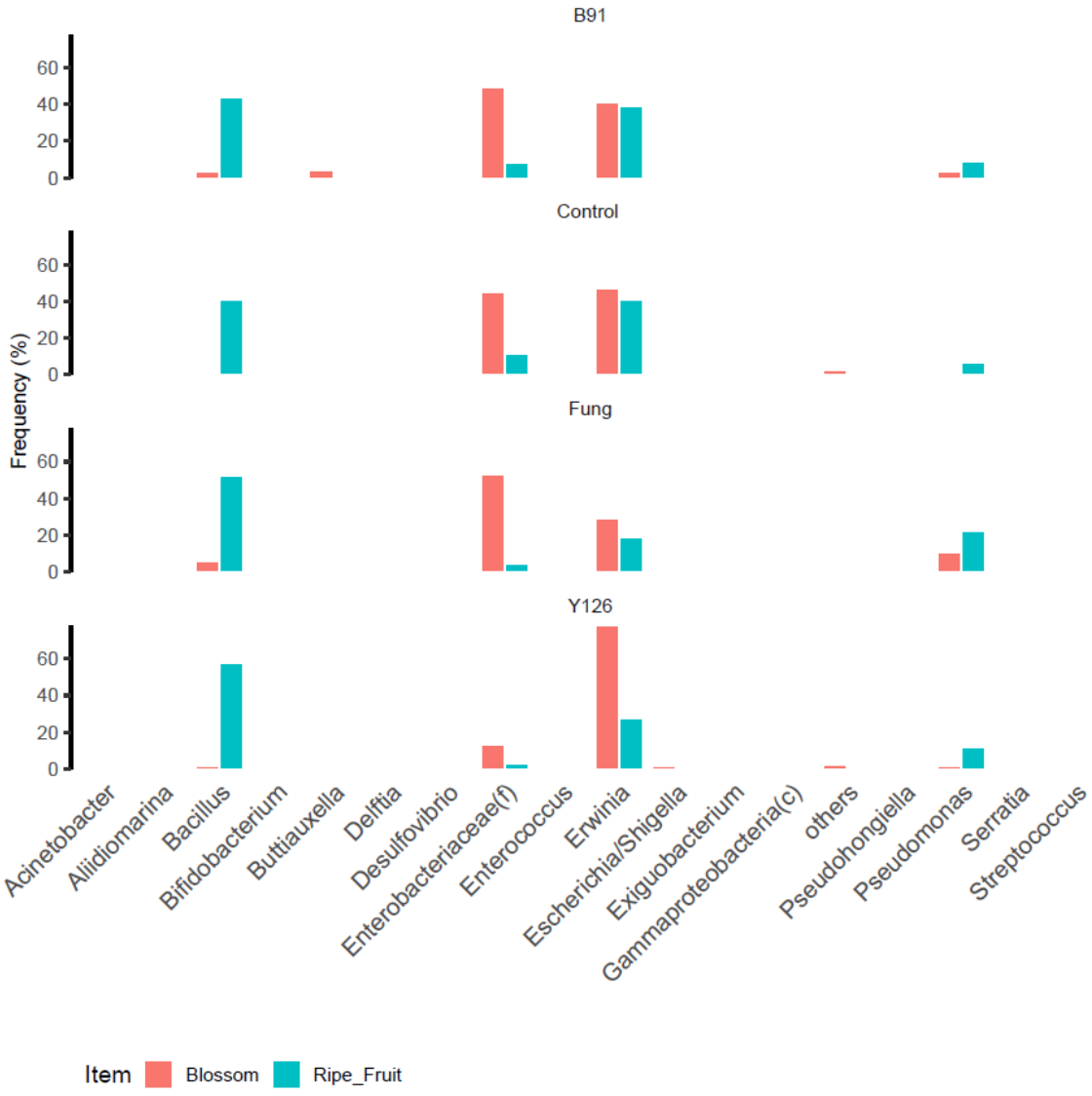

Figure 3: Taxonomy graph of bacteria from 2020 data only. Plots of percentage frequency of reads assigned to different taxonomic groups for each treatment (B91, Control, Fungicide and Y126). Colour denotes tissue type (item).

Does the treatment or tissue type affect the microbial community?

In 2019 bacterial OTUs the PCA showed there was a very strong block effect with Block 1 (orchard edge) distinctly different from blocks 2 and 3 (Appendix d); PC1 explains 37% of the variation in PC1 within the 2019 data, the ANOVA showed that the only significant effect in PC1 was block ($P < 0.000001$). There was a slight treatment (PC3 $P < 0.02$) and tissue type (PC4 $P < 0.04$) effect with clear clustering for the control treatment, B91 and fungicide (Figure 4). This was also similar in the NMDS plot with axis 1 separated by block and axes 2 vs 3 plot showing a similar clustering pattern of treatments (Appendix e). PCA analysis showed that block explains nearly 40% of the variation in the beta diversity indices with treatment and tissue type accounting for 5.7 % and 5.4% respectively.

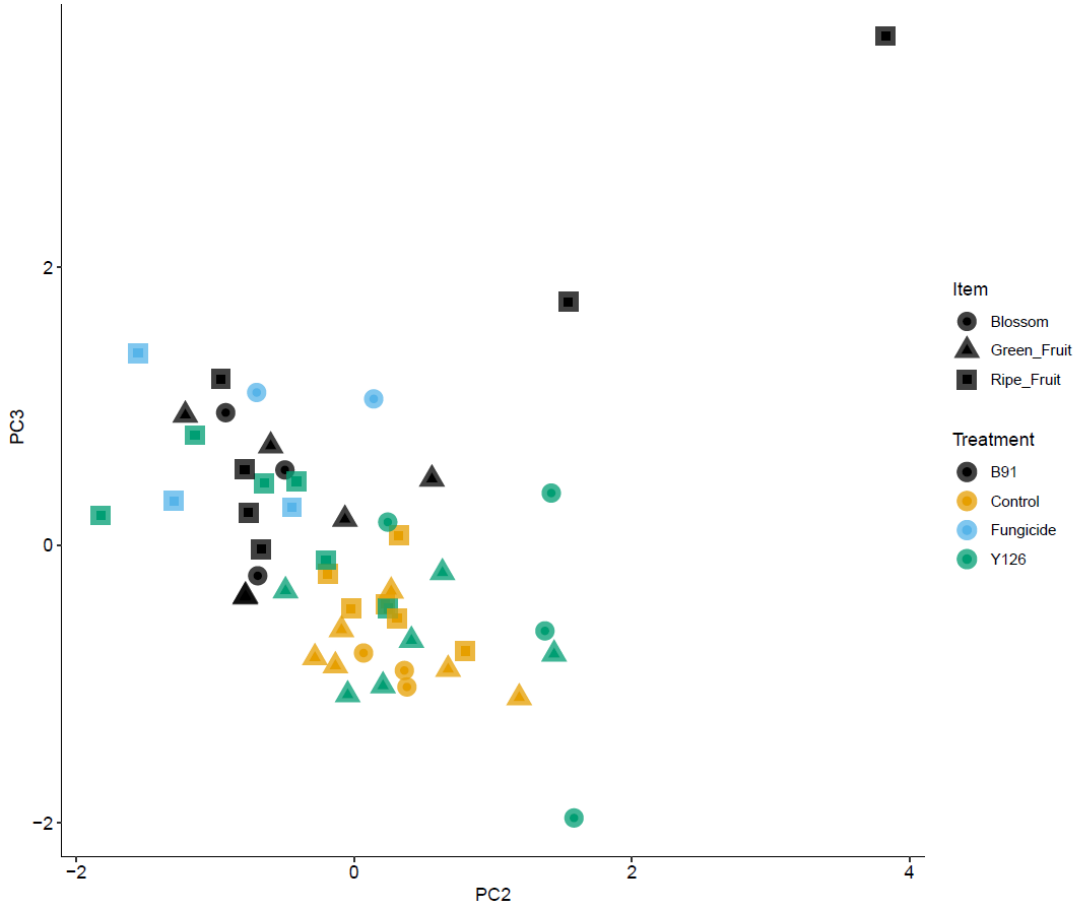

Figure 4: PCA plot of PC2 vs PC3 showing 2019 bacterial community. The graph shows a 2D plot of each sample based on the scores of PC2 and PC3. Tissue type (Item) by shape and Treatment by colour. Black = B91, Yellow = Control, Blue = Fungicide, Green = Y126.

In 2019 fungal OTUs the PCA showed some blocking effect (PC1 $P < 0.0003$) though the block effect is not as clear as seen in the bacterial community. There is some treatment (PC1 $P < 0.004$) and tissue type effect (PC2 $P < 0.02$) (Figure 5). The effect size of each factor; represented but the PCA percentage variance is: block 12.3%, treatment 7%, tissue type 10.7%.

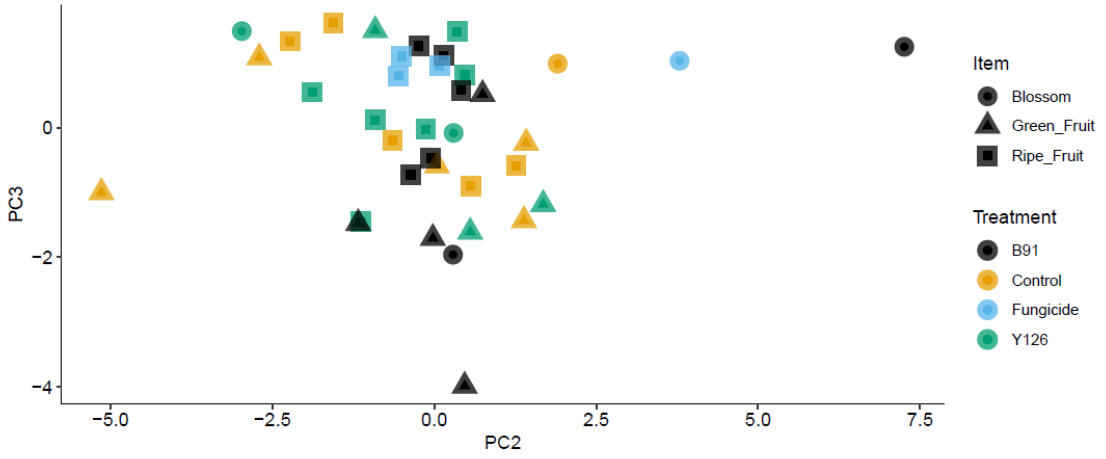

Figure 5: PCA plot of PC2 vs PC3 showing 2019 fungal community. The graph shows a 2D plot of each sample based on the scores of PC2 and PC3. Tissue type (Item) by shape and Treatment by colour. Black = B91, Yellow = Control, Blue = Fungicide, Green = Y126.

The 2020 data had just two tissue types, blossom and ripe fruit. The PCA of the bacterial OTUs showed a tissue type effect (PC1 $P < 0.05$) and a treatment effect (PC2 $P < 0.02$) (Figure 6) with the percentage variance for each factor being treatment 12.5% and tissue type 6.0%. ADONIS analysis highlighted that beta diversity (Bray-Curtis) values were significantly influenced by both treatment ($P < 0.01$) and tissue type ($P < 0.001$). There were three samples (D6, D3, D7 shown in Figure 6 A.) with much lower PC1 scores; these three samples were from trees in the edge of the orchard row, two samples from the control treatment and one from Y126 treatment. The edge of the orchard consisted of a large open area used as access for tractors and other farm vehicles. Figure 6B shows a reanalysis of the data omitting the three samples D3, D6 and D7.

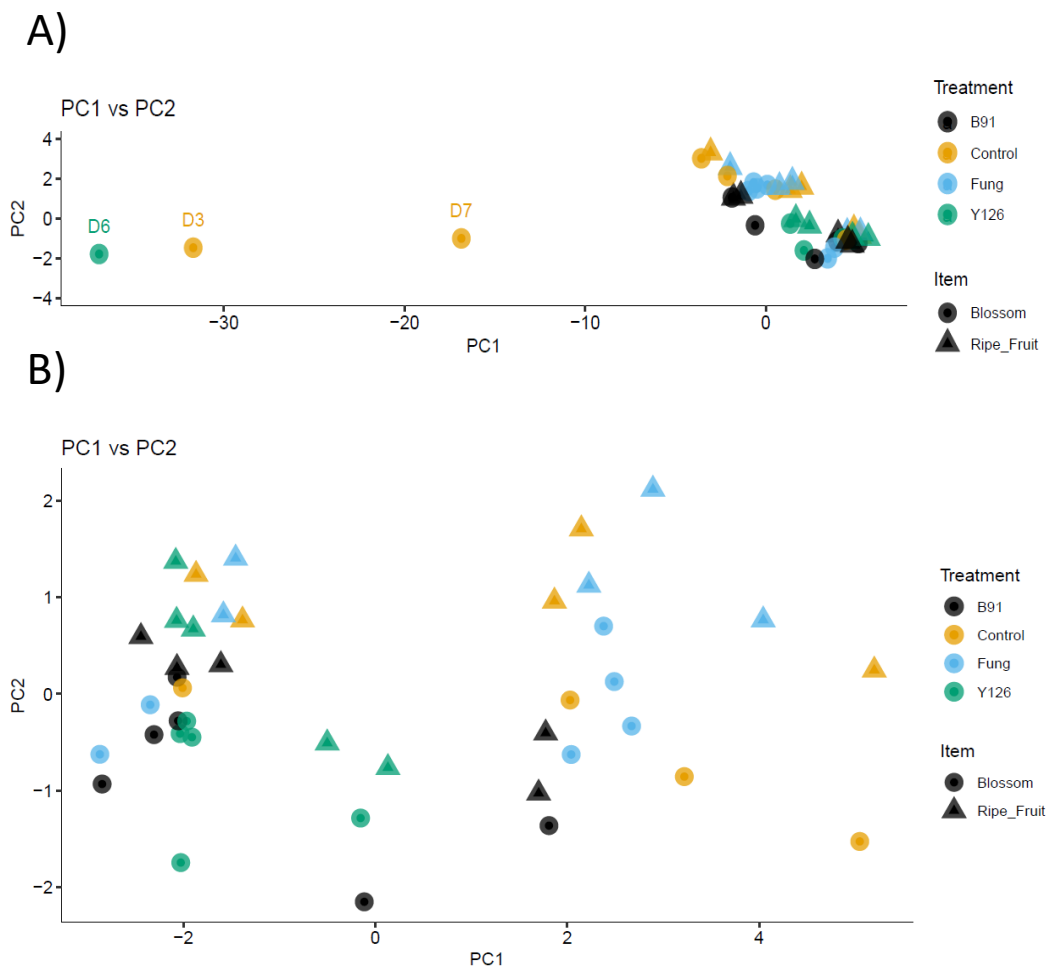

Figure 6: 2020 bacterial community shown in a PCA plot of PC1 vs PC2 showing tissue type (Item) by shape and Treatment by colour. Black = B91, Yellow = Control, Blue = Fungicide, Green = Y126. A) shows all samples B) Graph excludes outlier samples; D3 (tree 1, Control), D6 (tree 1, Y126), D7 (tree 1, Control).

In the fungal population there was a clear tree ($P < 0.002$) and tissue type ($P < 0.009$) effect on PC1. PC3 showed a treatment effect ($P < 1 \times 10^{-4}$) (Figure 7). The percentage variance from the PCA model for each factor was: block 16 %, treatment 11 %, tissue type 4.5 %. There was

a similar pattern in the beta diversity (Bray-Curtis) indices; the Adonis analysis showed a significant difference in both treatment ($P < 0.001$) and tissue type ($P < 0.001$).

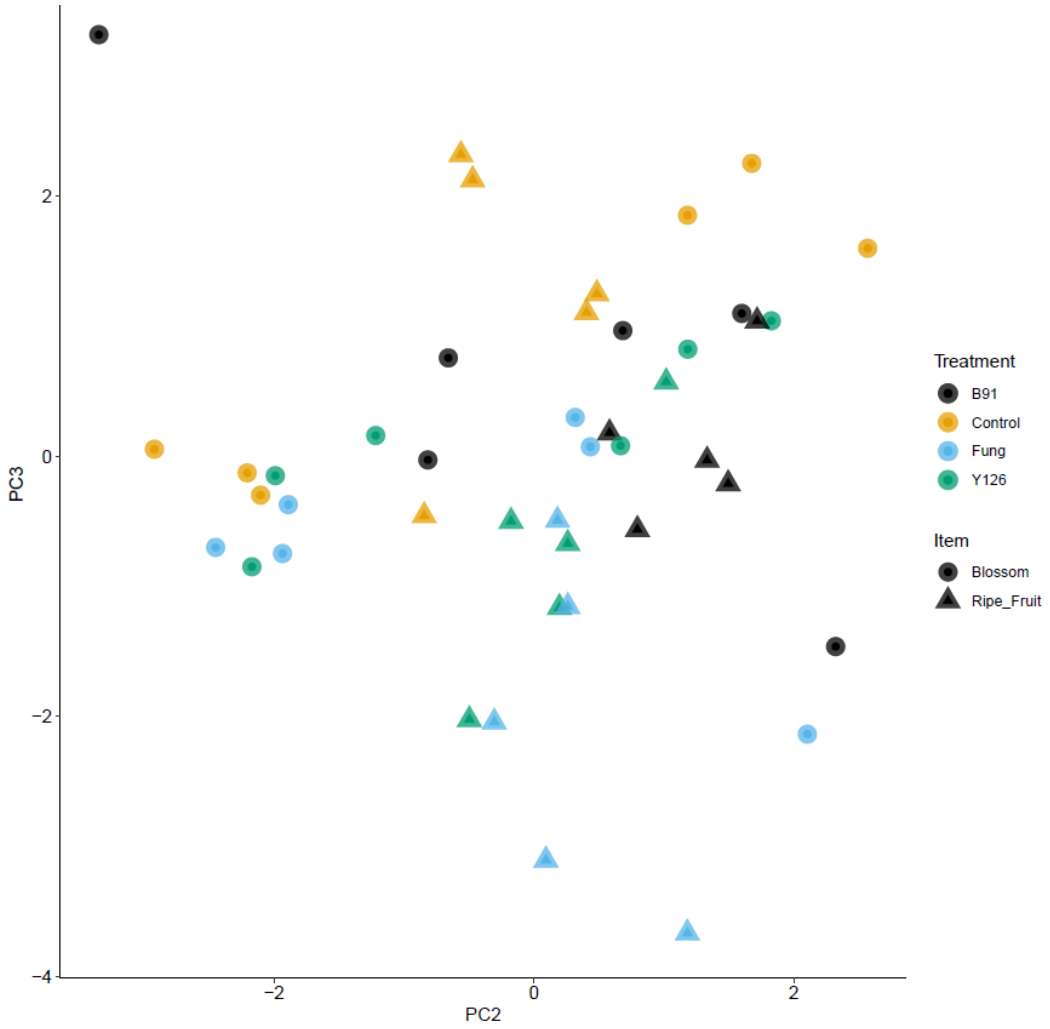

Figure 7: Fungal community from 2020 experiment in a PCA plot of PC2 vs PC3 showing tissue type (Item) by shape and Treatment by colour. Black = B91, Yellow = Control, Blue = Fungicide, Green = Y126.

Combining the data from both years the PCA shows an effect of tissue type (PC3 $P < 0.05$), treatment (PC1 $P < 0.02$) and block (PC1 $P < 7 \times 10^{-12}$), similar to the separate analysis. However, the largest effect is due to year-to-year differences (PC1 $P < 3 \times 10^{-16}$). From the ADONIS the percentage effect of each factor emphasizes the effect size of year compared to

the other factors in both fungal and bacterial community. For the bacterial community: Year = 15.90%, treatment = 5.25%, tissue type = 17.0% and for the fungal community: Year = 26.31%, treatment = 8.87%, tissue type = 8.13%.

How does BCA affect the microbiome?

We looked at the OTUs that had increased in abundance relative to the control in the samples treated with B91 or Y126. *Bacillus* and *Aureobasidium* did not increase in the BCA treated samples meaning that they did not increase their relative abundance. Differential analysis was performed on the combined data from two years to see how each BCA affected the relative abundance of individual OTUs in comparison to fungicide and the control. Table 3 shows the number of OTUs for which the abundance was significantly different between the BCAs and the two other treatments (control and fungicide).

There were 253 fungal OTUs, out of 1693, that were significantly different (Adjusted P-value < 0.05) between B91 and the control. 215 of these OTUs increased and 38 decreased, with $\log_2$ fold changes ranging from 23 to – 8. The results were similar in the comparison of B91 with Fungicide treated samples (333 OTUs with an adjusted P-value < 0.05) with the main difference being the greater number (221) of OTUs that decreased in abundance. The number of bacterial OTUs for the B91 vs Control and B91 vs the Fungicide analysis were similar. 46 OTUs (vs control) and 31 OTUs (vs fungicide) out of a total of 600 had an adjusted P-value of < 0.05 with the majority of these being an increase in abundance, only 8 OTUs had a negative log fold change in both comparisons.

In the comparison of Y126 against fungicide and the control, there was little difference between the number of OTUs for bacteria and fungi (Table 3). There were more OTUs that differed in the fungal community in the control comparison (364 out of 1693) compared to the fungicide treatment (317 out of 1693) this was the opposite for the bacterial OTUs with the control comparison having 68 OTUs out of 600 with an adjusted P-value of <0.05 and the fungicide treatment of 92 OTUs. The number of OTUs that increase in abundance was relatively similar to the number that decreased (table 3).

Table 3: Differential analysis of each BCA (Treatment 1) vs the control and Fungicide (Treatment 2). The number of OTUs that differed between treatments (adjusted $P < 0.05$). The number of OTUs that had a $\log_2$ fold change (LFC) both increased (Increase) and decreased (Decrease) in abundance.

Treatment 1	**Treatment 2**	**Fungi**			**Bacteria**		
		No. OTUs	Increase	Decrease	No. OTUs	Increase	Decrease
B91	**Control**	253	215	38	46	37	8
B91	**Fungicide**	333	221	112	31	23	8
Y126	**Control**	364	170	194	68	30	38
Y126	**Fungicide**	317	148	169	92	59	42
Total OTUs		1693			600		

Discussion

The 2019 data was unbalanced due to the fungicide treatment having only two time points while the other treatments had six time points. Furthermore, the time points were confounded with tissue types in 2019. To pool the data from the two years, the 2019 data was subsetted to include only time points 1 and 6 (Blossom and ripe fruit) to be in line with the 2020 data also with these two time points. Year had the greatest effect on the alpha diversity with a significance seen in all three metrics (Chao1, Simpson and Shannon) for both

fungi and bacteria. Treatment did not seem to have an effect on the fungal alpha diversity but tissue type did. This was mainly due to the low diversity seen in the control treatment for ripe fruit (Figure 2). Within the bacterial community, there was a significant effect of treatment ($P < 0.03$) with lower diversity in samples treated with Y126. Y126 mode of action is through competition. This could explain the lower diversity seen within these treatment samples. There is a greater effect of tissue type on alpha diversity. Blossom may have lower bacterial diversity since they are a new emerging niche when compared to ripe fruit that has longer atmospheric exposure. A study into the microbial ecology of different organs of tomato (*Solanum lycopersicum*) found lower diversity in new leaves and flowers compared to roots, stems and fruit (Ottesen et al. 2013).

The largest effect in both the PCA and Bray-Curtis analyses of differences between samples was between years (when the data were combined across both years). Another major factor affecting the microbial community of both blossom and fruit was the block effect. This was most pronounced in the bacterial community in samples taken in 2019 where block effects explained 37% of the variation in the population (Appendix d). There was a strong distinction between block one, located at the edge of the orchard, and blocks two and three that were further up the row within the orchard. In the bacterial community assessed from the 2020 blossom samples, there were three samples with much lower OTU levels. These samples came from two treatments (two control treatments and one Y126 treatment) and all samples were from Tree 1 that was located at the edge of the orchard. The edge of the orchard is bordered by an open space that is an access way for farm vehicles to access the orchard. This could lead to the edge of the orchard being exposed to more wind and turbulence that could decrease

the humidity at the tissue surface. There is both a strong geographical effect on the microbiome even within the orchard and the populations change from year to year.

We hypothesised that the tissue type would affect the population. This was the case for both years. In 2019 there was a significant difference between tissue types in the microbial community, assessed either by the Euclidean metric (PCA) or Bray-Curtis similarity. Both blossom and fruit are ephemeral organs of the plant, so form unique environments for microbes to colonise. As well as being different habitats for microbes to colonise they also exist at different times of the fruiting season, with different abiotic factors. Blossoms appear for a short period in spring when temperatures may be cooler and rainfall heavier than in summer months when ripe fruit appears.

We also hypothesised that if there was any effect on the microbial population due to treatment, this would be more pronounced in the fungicide treatment than the BCA treatments. There was a treatment effect in both years in both the bacterial and fungal communities. In the bacterial population, the control treatment forms a clear cluster; the B91 treatment and Fungicide treatment are clustered close together; Y126 is most scattered with no obvious clustering (Figure 4). Y126 works through competition so it may be more affected by the microbiome than the B91 and Fungicide that use chemical inhibitors. However, these patterns aren't as clear in the 2020 results that show two clusters with no obvious link. This could be due to a less consistent treatment effect, though there is a clear cluster of Y126 on the left (Figure 6b). The fungal communities were also affected by treatment for both years (2019, PC1 $P < 0.004$ and 2020 PC3 $P < 10^{-4}$) (Figure 5 and 7). Contrary to our initial hypothesis,

the treatments had a greater effect than tissue type when looking at the percentage variance for both the bacteria and the fungal community in 2020 and the bacterial community in 2019.

A differential analysis showed how each BCA affects the microbiome compared to the fungicide treatment and the control. The biggest difference in the bacteria population is between the Y126 treatment and Fungicide with a $\log_2$ fold increase of 8.3% and a decrease of 7%. Similar species can be seen to increase or decrease in different treatments. Even though the BCAs themselves may not be prevalent in high numbers they seem to affect the microbiome making the environment more or less attractive to some species. For example, Y126 seemed to increase *Erwinia rhapontici* populations compared to the control and fungicide treatments. *Erwinia rhapontici* is an opportunistic plant pathogen (Huang et al. 2003). Though it does not infect cherry the increase of unintended plant pathogens in the field is something that needs to be considered when looking into potential BCAs.

In chapter three we saw B91 did not survive well in blossom and the Y126 population declined after a week in the field. A look at the OTUs that increased in abundance for the BCA treatments did not include *Bacillus* and *Aureobasidium.* This was expected as it is similar to what was observed in chapter three. In Chapter 3 we also observed that the population of B91 in all treatments was very low on blossom but in the fruit, there were relatively high levels of B91. We also observed this in the current study when we looked at the percentage frequency of different taxonomic groups. *Bacillus* was present at similarly high levels on ripe fruit but not on the blossom for each treatment in 2019 (Figure 3). This suggests B91 persists in high concentrations on cherry fruit naturally.

The biggest effect on the microbial community was the difference between blocks and between years ($P < 3 \times 10^{-16}$). This punctuates the difficulties when it comes to phyllosphere microbiome studies as these microbial populations can vary greatly within the field and between seasons. Unlike the rhizosphere, the phyllosphere is considered a short-lived environment this can lead to large changes in the microbial community from season to season (Vorholt 2012). Microbial epiphytes are exposed to greater fluctuations in the environment compared to endophytes that reside in the endosphere. This can have a large effect on the microbial community within a season and within an orchard. We saw a large difference in population from the edge of the orchard compared to blocks positioned further in. The edge of the orchard could be exposed to more UV as well as temperature and humidity fluctuations and environmental pollutants from farm vehicles.

The treatments B91, Y126 and fungicide also affected the bacterial and fungal communities on blossom and fruit of cherry, and in most cases more so than tissue type. The effect of any new plant protection product that can affect the microbiome of the plant should be fully explored to ensure that there are no off-target effects to the new product. We still do not fully understand the role that the microbiome has on plant health and how it can potentially improve or worsen the efficacy of BCA products. With the accessibility of NGS technologies improved microbiome studies should start to be considered as part of the research needed when looking at any new products. Improving our understanding of how these microbes interact with the plant, the pathogen and BCAs will improve pest management strategies in the future.

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Chapter 6

Discussion and Conclusions

General Discussion

The Two BCAs that I looked at in this research have been shown to be effective antagonists against *M. laxa* sporulation. Their ability to survive at a range of temperatures have given promise of their potential as BCAs for stone fruit (Rungjindamai et al. 2013). To assess their survival in the field, I targeted two key times in the pathogen's life cycle: blossom and mummified fruits. I also assessed their effect on latent infections and the interaction time between BCAs and the pathogen to help assess their viability for commercialisation.

The overwintering of *M. laxa* on mummified fruits left in the field is a major issue increasing rates of blossom blight in the spring and subsequent post-harvest rots (Villarino et al. 2010). When assessing the survival rate of BCAs B91 and Y126 on mummified fruits in the orchard, the population of Y126 declined rapidly in the first two weeks after which the population stabilised for the rest of the winter. B91 survived a little longer with the population reaching that of the control group by week 4. However, neither BCA affected the population of *M. laxa* on the mummified fruits, indicating that the concentration needs to be higher than the current survival rate of the BCAs in the orchard over winter and suggesting that the pathogen maybe more adapted to these winter conditions. I also looked into the effect of interaction time to see if longer exposure of *M. laxa* to the BCA would reduce sporulation. The hypothesis was confirmed, that the longer the interaction time between the BCA and the pathogen, the lower the spore count of *M. laxa*. However this was also true for the control treatment which indicated that there my also be a physical effect from spray application.

Looking at the BCAs ability to protect blossom and fruit I determined that Y126 was able to survive well on blossoms and marginally though not significantly reduce the levels of *M. laxa*. B91 did not survive well on blossom but the population bounced back once ripe fruit developed in all treatments groups, indicating generally high levels occurring naturally in the environment. The application time experiment showed that both BCAs reduced *M. laxa* inoculum on blossom initially. If Y126 populations could be maintained at higher levels during blossom season by regular application or better formulation, they may be more effective in reducing *M. laxa*.

I also assessed the potential of our two specific biocontrol strains to reduce post-harvest rot on cherry caused by *M. laxa* by applying them two weeks before harvest. The experiment was replicated over two years. In both years, BCA treatments significantly reduced post-harvest rots compared to the control. B91 when applied after inoculation of *M. laxa* had disease control efficacy almost as good as the fungicide. The use of BCAs in the field has always been secondary to post-harvest uses (Ippolito and Nigro 2000) but this study shows there is potential for pre-harvest disease control that could reduce our reliance on fungicides. It would be useful to combine the latent infection analysis with the blossom survival rate to see if there is a key link between blossom application and a reduction in latent infection. Its has been established that early blossom infections increase latent infections (Luo et al. 2005). It could be that early and regular application on blossom could translate to a reduction in post-harvest loss.

When introducing a new product into the environment it's important to understand and non-target effects it can have on the microbiome. When looking at the effect of the two BCAs on

the blossom and cherry microbiome there was a significant effect on the bacterial and fungal communities. This is an area that could do with more research to assess if these changes are long term and whether there are any environmental implications to the use of these BCAs. We saw that certain species increased after BCA treatment. A further look into these organisms would be beneficial to see how they affect the plant, the pathogen and the efficacy of the BCA. The largest effect seen on the microbiome was that of year, between the two experiments conducted one year apart and block, between the orchard edge and the blocks further up the row. This emphasises the difficulties faced when studying the microbiome of the phyllosphere because it is sensitive to the changing environment. Future studies will need to consider this when planning experiments, looking closely at experimental blocking and to conduct studies over more years.

The Survival rate studies showed a very early decline in population for both BCAs. However, a more accurate CFU count at application of the BCAs would give a clearer indication of the exact decline at is occurring once BCAs are placed in the field. When replicating this study, I would recommend that more time points be taken within the first two weeks of application to accurately assess the rate and pattern of decline. The study would also benefit from a look at multiple applications to see if regular sprays at key times in the season or when disease pressure is high can help control *M. laxa* inoculum. It should be noted that the two survival rate experiments and microbiome study (Chapter 2, 3 and 5) where conducted in open air orchards. This is contrary to the industry standard of growing cherry under protective covers. These covers will change the humidity, UV and exposure to rain so could have an effect on both the pathogen and the BCAs. Future work would benefit from the use of more commercial orchards that closely resemble the industry standard.

There is still more work to be done to fully assess the potential of the BCAs and ensure that growers can maximise their potential. A look into whether the BCAs can be used in combination with each other or leading fungicides would help agronomists know how these products best fit within an integrated pest management system. *B. subtilis* and *A. pullulans* are well known BCAs that have been used commercially in the form of Serenade® and Bio-ferm's 'BoniProtect' and 'Blossom Protect' (Bellamy et al. 2021). These products are not marketed for cherry, but this research could translate to these products. In this study, I looked at two new strains that would benefit from further research especially the antagonistic compounds produced by them to ensure that there are no adverse environmental or health effects.

From this research, we have seen that these BCAs are more effective when used in certain environmental niches, and I would advise that they are best when targeting blossoms and fruit over mummified fruits. Though the BCA can survive at lower concentrations over the winter there does not seem to be an effect on the *M. laxa* sporulation, perhaps because the pathogen is better adapted to the colder temperatures. Y126 has a good survival rate on blossom and fruit so should be investigated for its potential use in early spring to help protect against blossom blight. B91 seems to be a better adapter to fruit surface over blossom and sprays may be better focused towards the end of the growing season.

Pre-harvest applications of BCAs on fruit has been shown to be effective at reducing post-harvest rots. BCAs successfully reduced post-harvest rot in Kordia cherry when they were applied two weeks before harvest. Integrating these BCAs into a spray regime close to harvest

could help growers reduce fungicide residues on fruit. Reducing the amount of fungicide used in our agricultural systems has been recommended in many countries and legally imposed in some (Oliveira Lino et al. 2016). Reduced fungicide use using BCAs is also something that would be appealing to some consumers.

The modes of action of the two BCAs will influence the best way to utilise them in the field. Y126 uses competition with the pathogen, which means that the populations should be maintained on blossom and fruit. This could be achieved by regular spraying, but more research is needed to ascertain the most efficient and cost-effective way to maintain the optimum populations in the field. B91, even when the naturally occurring population was high late in the season, it did not have as strong an effect on the pathogen as when it was applied with its growth medium. I believe this is because it uses antagonistic compounds that would have built up during the inoculum preparation stage (Rungjindamai et al. 2013). It will be a big but important task to optimise this BCA formulation to ensure that these antagonistic compounds are concentrated enough to affect the pathogen yet not damage the plant's natural microbiome. B91 could then be used in a similar way to a fungicide.

The greatest stumbling block for BCAs so far has been their low and often inconsistent efficacy (Massart et al. 2015). At the moment BCAs will not be able to compete with the efficiency of fungicide spraying and should not be seen as a replacement to these chemical sprays. When framing them as an alternative their efficacy will be compared to that of a chemical fungicide and will not look as appealing to growers. Instead, BCAs should be seen as an added tool in the grower's arsenal that can be used in tandem with other techniques to combat this pathogen.

Conclusions

- M. laxa seems to be better adapted to winter field conditions then the BCAs; B91 and Y126.
- A further look at the interaction time between the BCAs and the pathogen could help inform optimum number of sprays needed in a season.
- Y126 was able to colonise and survive on blossom but had little effect on M. laxa
- B91 did not survive well on blossoms but was able to thrive on fruit. However, it could not produce inhibitory compounds in high enough concentrations to effect M. laxa.
- When applied in the field two weeks before harvest both BCAs significantly reduced the incidences of post-harvest rots on cherry.

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Appendices

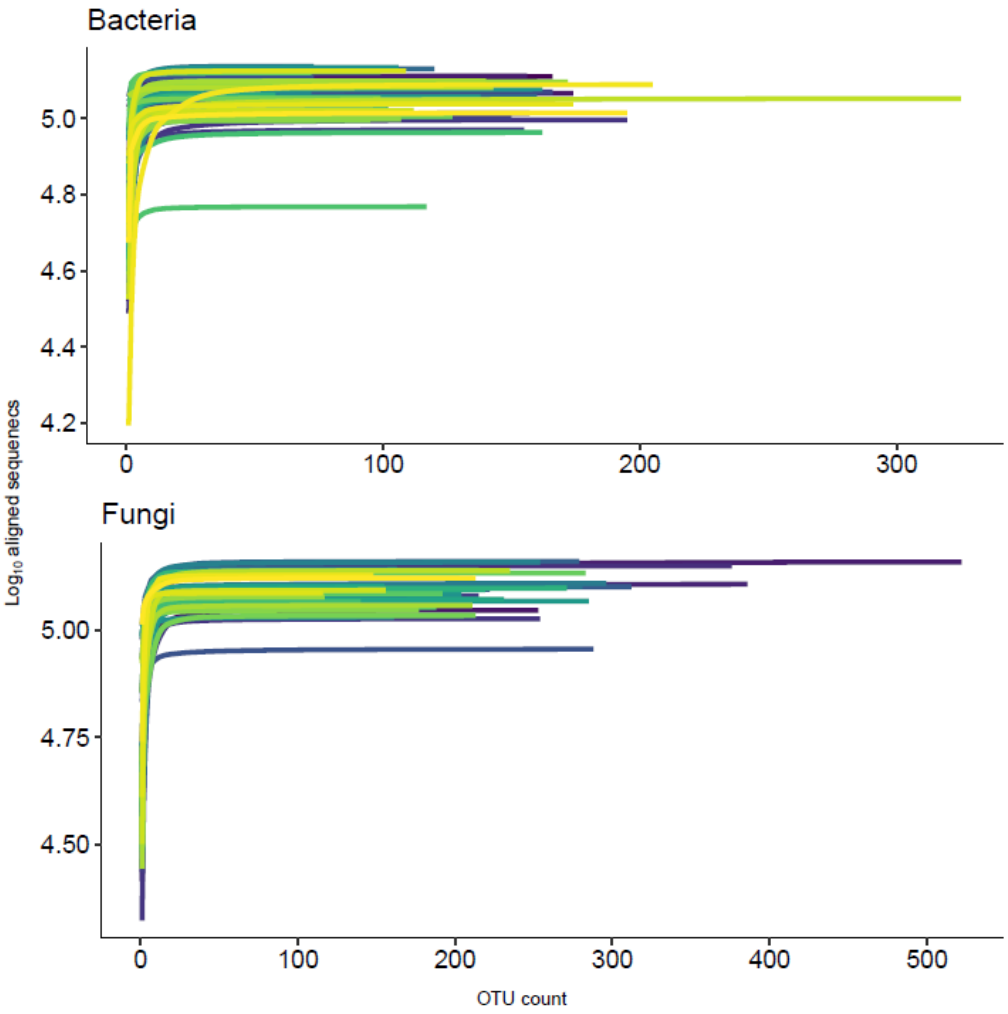
Appendices

All raw data is stored in NIAB EMR EMQA system within the Pest and Pathogen Ecology files. The Project code is 300039.

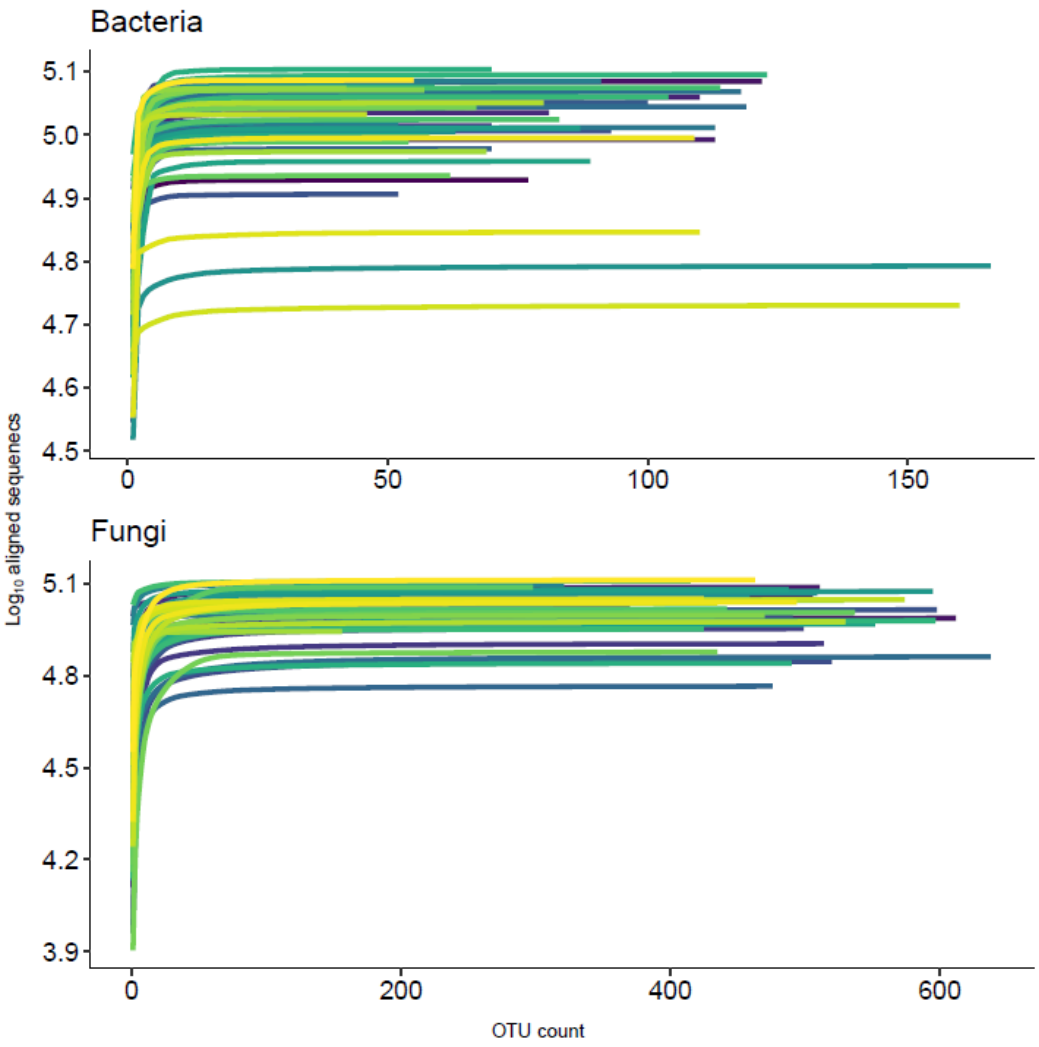
Data is filed in: P:\PPESCM\Xu\Brown rot CTP 300039
→Science
→WP (No.) WP1 = Chapter 2, WP2 = Chapter 3, WP3 = Chapter 4, WP4 = Chapter 5
→Data

Appendix a: Shows the individual contrasts used in the data analysis and the questions that they address.

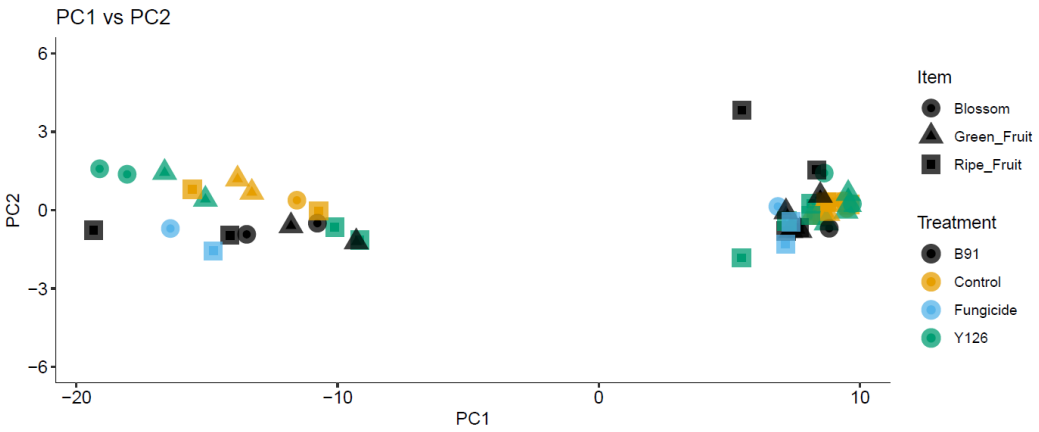
Treatment	time	Is there any control present?	Trend over time, in the control?	Is fungicide better or worse than BCAs?	Does timing affect fungicide?	Do BCAs differ?	Is B91 better applied early?	Is y126 better applied early?
B91	After	1	0	1	0	1	1	0
	Before	1	0	1	0	1	-1	0
Y126	After	1	0	1	0	-1	0	-1
	Before	1	0	1	0	-1	0	1
Fung	After	1	0	-2	1	0	0	0
	Before	1	0	-2	-1	0	0	0
SDW	After	-3	1	0	0	0	0	0
	Before	-3	-1	0	0	0	0	0



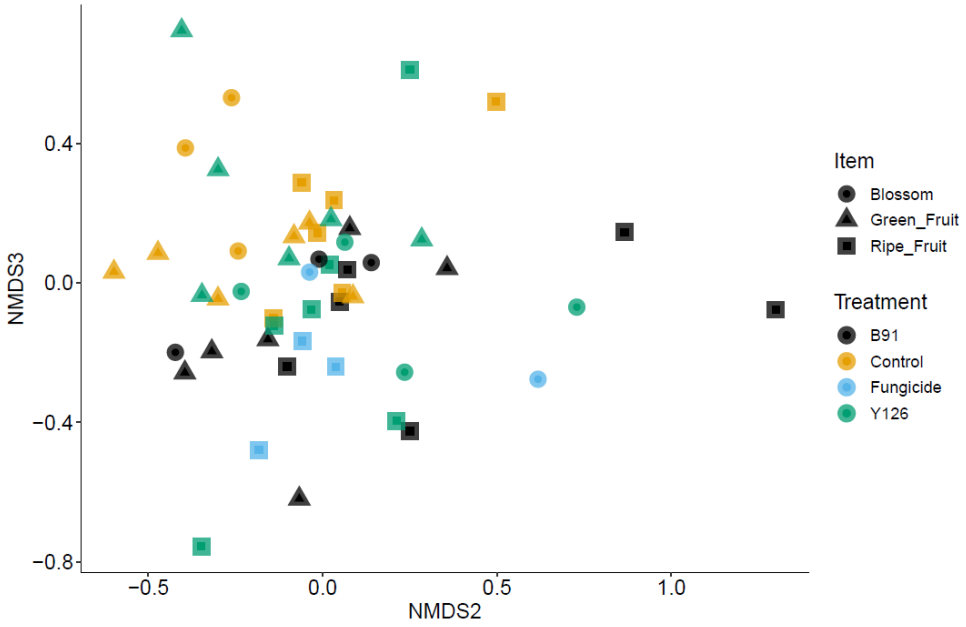
Appendix b: Rarefaction Curve Rarefaction curves of normalised Bacterial OTU counts (top) and Fungal OTU counts (bottom) from 2019 data. The plateau of the plot suggests sequencing depth is adequate.



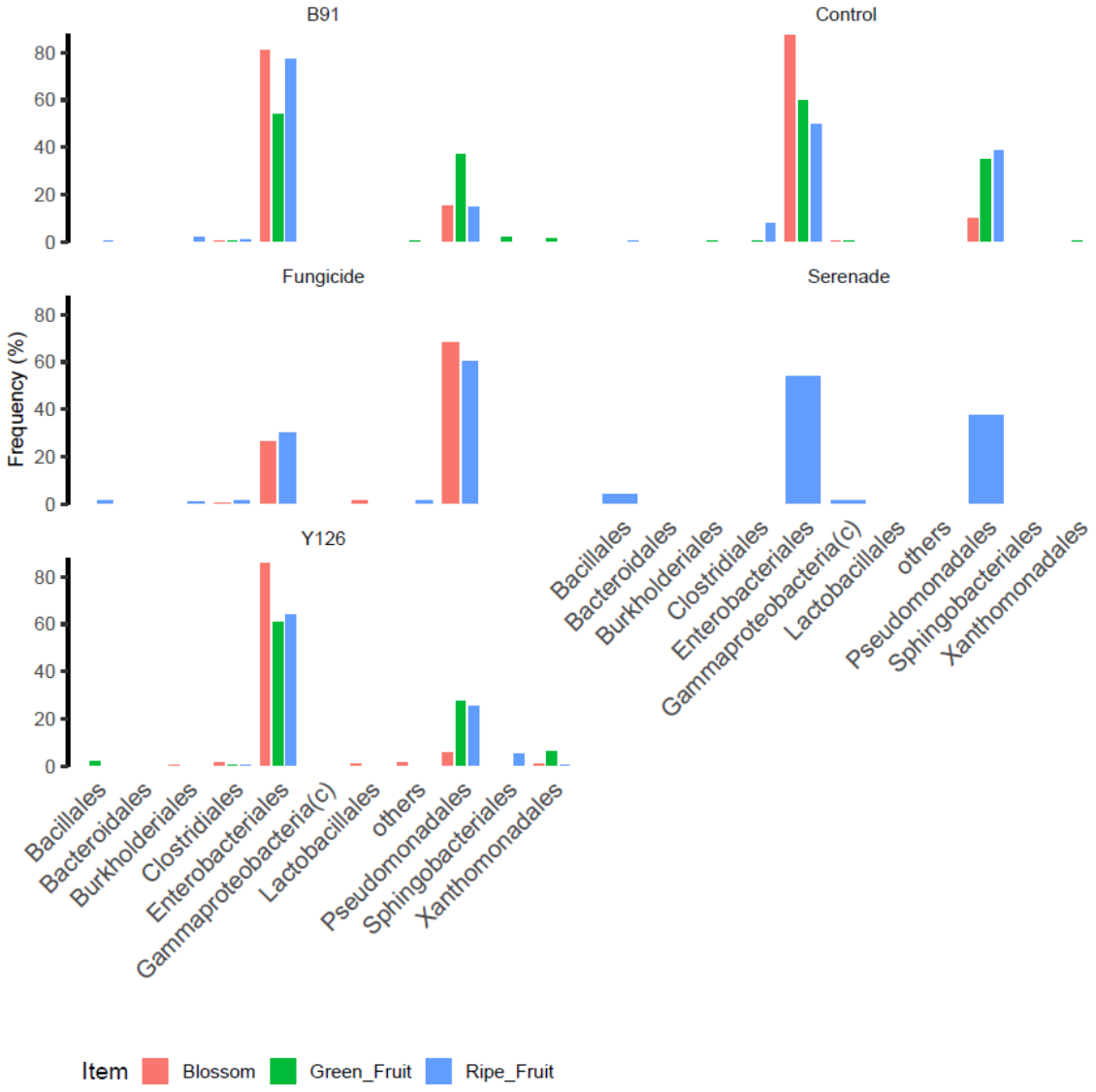
Appendix c: Rarefaction Curve Rarefaction curves of normalised Bacterial OTU counts (top) and Fungal OTU counts (bottom) from 2020 data. The plateau of the plot suggests sequencing depth is adequate.



Appendix d: PCA plot of the percentage of variance of PC1 vs PC2 showing Tissue type (Item) by shape and Treatment by colour. Bacterial OTUs from 2019 samples. The cluster on the left is Block 1 and the cluster on the right is Block 2 and 3.



Appendix e: NMDS ordination plot of axes 2 vs 3 of 2019 samples. Tissue type (Item) by shape and Treatment by colour.



Appendix f: Taxonomy graph of bacteria from 2019 data only. Plots of percentage frequency of reads assigned to different taxonomic groups for each treatment (B91, Fungicide, Y126, Control and Serenade). Colour denotes tissue type (item). Serenade and Fungicide samples were only taken at two time points so there was no green fruit present.