Project title:	Biocontrol as a key component to manage brown rot disease on cherry
Project number:	SF/TF 170: CTP_FCR_2017_3
Project leader:	Xiangming Xu, NIAB EMR and Michael Shaw, University of Reading
Report:	Annual report, October 2020
Previous report:	2019
Key staff:	Sophia Bellamy
Location of project:	NIAB EMR
Industry Representative:	NA
Date project commenced:	October 2017

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

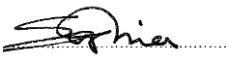
AUTHENTICATION

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

Sophia Bellamy

PhD Student

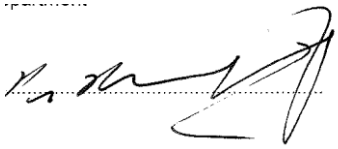
NIAB EMR

Signature ....  ..... Date .....21/10/2020.....

Prof Xiangming Xu

Head of Department

NIAB EMR

Signature .....  ..... Date .....21/10/2020.....

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CONTENTS

GROWER SUMMARY

Headline

Two microbial biocontrol agents (BCAs) (*Aureobasidium pullulans* and *Bacillus subtilis*) have shown biocontrol promise against brown rot disease of stone fruits.

Background

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

Summary

Y126 and B91 are being studied for their efficacy against *M. laxa* in terms of reducing sporulation on mummified fruits, blossom wilt and latent fruit infections in cherry. In year 2 (Y2), we did a preliminary investigation into the use of these two microbes for reducing latent infection of cherry fruit, hence reducing post-harvest rot development. The results of this were promising showing a 30% reduction in post-harvest rot. In year 3 (Y3), this experiment was repeated on a larger scale including a fungicide treatment and different inoculation times. Both biological controls were able to significantly reduce the post-harvest disease incidences and in the case of B91 as effective as the fungicide control.

Financial Benefits

Further research is needed to fully assess the direct effects of these two biocontrol microbes on commercial fruit production. However promising results in a latent infection trial showed the two biocontrol agents significantly reduce the disease incidence post-harvest when applied two weeks before harvest. This is supported by Y3 results that the two organisms reduced incidences of post-harvest rotting by 30 % - 75 %.

Action Points

There are no grower action points at this stage of the project.

SCIENCE SECTION

Introduction

Latent infections are characterised by the penetration of *Monilinia laxa* of young fruits but remaining symptomless (latent) until fruit riping near harvest. The latent infection resumes as the fruit matures, often manifesting as post-harvest rots. It is understood that infections of *M. laxa* via wounds result in visible symptoms in the orchard pre-harvest, and latent infections occur on intact fruits but usually manifest as visible fruit rot post harvest. A study looking at *M. fructicola,* another dominate brown rot pathogen, on prune showed a positive correlation between blossom blight incidence, latent infections on immature fruits and post-harvest rots (Luo *et al.*, 2005).

Infection of *M. laxa* on stone fruit is closely related to environmental factors. The optimum temperature for *M.laxa* sporulation is around 10°C. It can produce conidia at even lower temperatures. Germination of *M. laxa* conidia may occur at temperatures as low as -4°C (Tamm and Fluckiger, 1993; Tian and Bertolini, 1999). However, infection rarely occurs at temperatures lower than 10°C (Casals *et al.*, 2010).

Infection of fruit by *M. laxa* is affected by fruit maturity: fruit tends to be increasingly susceptible as it matures (Gell *et al.*, 2008). As the fruit matures, there are changes in its biochemical and physiological composition that result in changes in its susceptibility to infection (Biggs, 1988; Wade and Cruickshank, 1992). 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 such as catechin, epicatechin, and phenolic compounds. Once the pericarp forms and hardens, the concentration of chlorogenic andneochlorogenic acid reduces. This leads to increased susceptibility of fruit to *M. laxa* as chlorogenic andneochlorogenic acid can affect melanin production in *M. laxa* that is essential for penetration (Oliveira Lino *et al.*, 2016).

Many of the current studies on latent infection are carried out on different *Prunus* species, *Monilina* species with a range of techniques. These variations may account for inconsistencies in latent infection at different stages of fruit maturity (Oliveira Lino *et al.*, 2016). It is, however, generally understood that the susceptibility of stone fruits to the pathogen increases with fruit maturity (Oliveira Lino *et al.*, 2016). Latent infections can quickly develop into visual rots and easily spread via contact within cold storage (Fourie and Holz, 2003). The ability of *M. laxa* being able to develop rapidly at 5-10°C can lead to the rapid spread of 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).

Pre-harvest applications of microbial antagonists could be an effective control measure for reducing post-harvest decay of fruits. Theoretically, a pre-harvest application would allow beneficial microbes to colonise the fruit surface before harvest. Therefore, if wounds were sustained during harvest, these would be colonised by the antagonist suppressing the spread of the pathogen (Sharma *et al.*, 2009).

In 2013, two microbial strains of *M. laxa* were identified from indigenous populations within the UK (Rungjindamai, Xu and Jeffries, 2013): *Aureobasidium pullulans* (Y126), a yeast-like fungus, and *Bacillus subtilis* (B91), a bacterium. These two strains showed promise in suppressing pathogen sporulation as well as being able to survive over a range of temperatures under lab conditions.

This study is to assess the efficacy of B91 and Y126 in terms of reducing latent infection of *M. laxa* on cherry fruit. We expect that applying these two strains close to harvest would lead to reduced latent infection, hence post-harvest rot on cherry.

Materials and Methods

Treatments

There were nine treatments: [1-8], fungicide, sterile distilled water and the two biocontrol microbes (B91 and Y126) applied 24 hours before or after *M. laxa* inoculation (without wounding), and [9] fruits that received no *M. laxa* were harvested to assess the background level of latent infection.

Inoculum production

Single colonies of B91 and Y126 were grown in liquid media (liquid broth and potato dextrose broth, respectively) for 24 hours 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}. The biocontrol suspensions and the control of sterile distilled water were transferred to handheld sprayers.

M. laxa inoculum was grown on ripe plums, spores were harvested and suspended in sterile distilled water and adjusted to $1x10^5$ spores ml^{-1} with a haemocytometer. Fungicide, Lunar sensation was prepared according to manufactures instructions.

Application

Ten trees of cultivar Kordia in an open-field orchard at NIAB EMR were selected, each with nine branches selected at random, one for each treatment. There where two inoculation times ‘before’ and ‘after’. For the before timing the treatments were applied before *M. laxa*

was applied and for after timing the treatments were applied 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 that the 'after' timing treatments were applied.

Disease assessment

Two weeks after treatment, visibly healthy ripe fruits were harvested and stored at 4°C on sterile trays. Visible rot was assessed on days 4, 6, 9 and 15; on each assessment, diseased fruits removed to prevent secondary contact spread.

Data analysis

Data was collected as a binomial representation of presence/absence of rot. The presence and absence of brown rot was assessed to see if there was a relationship between the presence of rot and the treatment or the time of application. A chi-squared test was performed, followed by an ANOVA.

Results

Figure 1 shows the mean percentages of visible rot for all combinations of products and application time. After two weeks of incubation at 4°C both the biocontrol organisms and fungicide treated fruit had a significant reduction in rot incidences compared to the control treatment that had been inoculated with *M. laxa* and received sterile distilled water. The incidences of rot in the fungicide treatments were lower than the biological control treatments. However, this difference is not significant when the fungicide was applied before *M. laxa* inoculation.

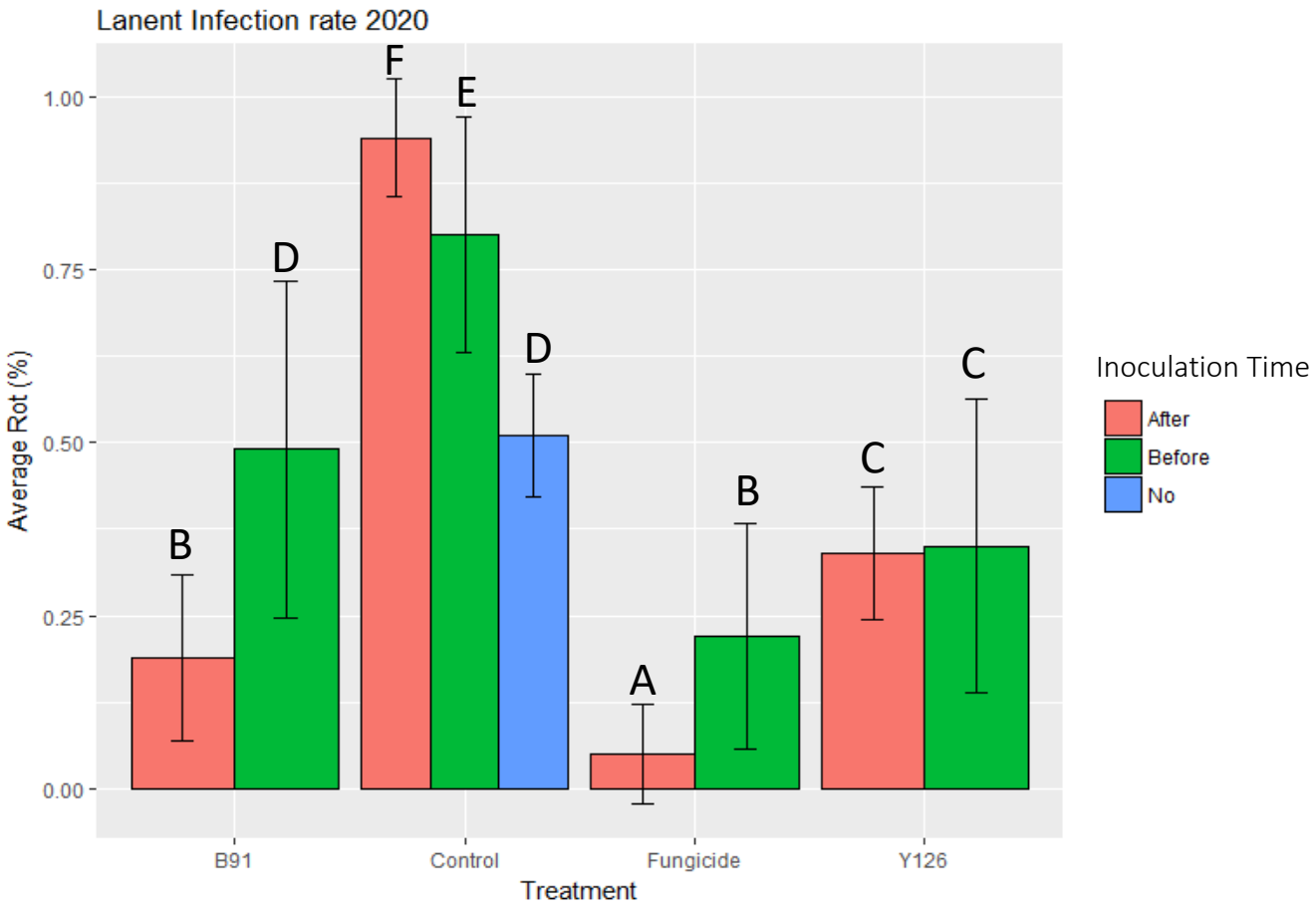

Figure 1: shows the mean percentages of presence for each treatment and application time, before or after *M. laxa* inoculation (the average across trees) with the standard error bars showing variation in trees. Significance groupings (Tukey HSD, p=0.05) are presented above each bar showing similarities between treatments/application times.

B91 treatment, significantly reduced rot incidences when compared with the *M. laxa* inoculated control for both applciaiton times ($p < 0.0001$). The fungicide performed significantly better ($p < 0.01$) compared to when B91 was applied before *M. laxa* inoculation. There was no significant difference between the two biocontrol microbes although there appeared to be a small reduction in post-harvest rot in the B91 when applied after *M. laxa* inoculation (Fig. 1).

Y126 also had significantly reduced post-harvest rot incidences when compared with the inoculated control treatment ($p < 0.0001$). The fungicide was able to significantly reduce rot incidences compared to Y126 when it was applied before the *M. laxa* inoculation.

The only significant difference between inoculation times within treatments was in B91 where the percentage of rot incidences was significantly lower ($p < 0.01$) when it was applied after the *M. laxa* inoculation.

Discussion and Conclusions

The results of the initial experiment in Y2 showed that the application of the two biocontrol agents can significantly reduce the incidence of post-harvest fruit rotting by nearly 30% when applied in the field two weeks before harvest. In Y3, we repeated the experiment with a larger sample size and more treatments including a fungicide treatment to fully explore the possibility of pre-harvest application of biological controls to reduce latent infections and subsequent post-harvest rotting. This years experiment confirmed the results from year two that the two biological control agents can significantly reduce the post-harvest rot incidence
The fungicide, as expected, reduced *M. laxa* infections and post-harvest rots. It was more effective, though not significantly when it was applied after the *M. laxa* inoculation. This is probably due to its mode of action. The application before may have resulted in a lower concentration of fungicide present on the fruit surface to interact with *M. laxa* compared to the 'after' application.

Surprisingly, B91 had a similar pattern to the fungicide being slightly more effective when applied after the *M. laxa* inoculation. One important B91 mode of action is through the production of toxins though it was 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. Because the organism was gown 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 into a product. This is the formulation strategy used for Serenade, a commercially formulated product of a specific *B. subtlis* strain. B91 was able to significantly reduce post-harvest rot when applied before the *M. laxa* inoculation so it also has the potential to reduce latent infections.

There was little difference between the inoculation times of Y126, indicating that is was able to prevent new infections on ripe fruit as well as suppress latent infections. Y126 works primarily through competition with the pathogen proving that it can compete successfully with *M. laxa* even when applied after *M. laxa* inoculation has occurred. Both biological controls Y126 and B91 were able to reduce the disease incidences post-harvest compared to the inoculated control by 45% and 30%, respectively for the 'before' application time, and 60% and 75% for the 'after' application. These results are similar to, if not better than, the

previous years results obtained at 20°C post-harvest storage. This suggests that these microbes are probably effective when fruits are stored at 4°C.

Over the two years of studies, B91 and Y126 have shown that when applied to cherry fruit two weeks before harvest they can significantly reduce post-harvest rot incidences under the conditions of both cold storage and ambient temperatures. A few specific biocontrol treatmetns achieved the control efficacy 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 this diseases.

Knowledge and Technology Transfer

- Industry Placement at the KTN July 2020- October 2020
- BSPP PhD Conference – Presentation at the BSPP PhD conference 2019
- AHDB Tree fruit day – Presentation at the AHDB tree fruit day 2019
- Biotechnology YES competition 2018
- BSPP Grand Challenges in Plant Pathology 2018
- XV Meeting of the IOBC-WPRS - Poster presentation at the International organisation for biological and integrated control conference in Lleida, Spain. Secured 500 euro travel grant.
- NIAB Poster day 2018 - Poster presentation at NIAB poster day 2018 in Cambridge, with the CTP.

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