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SCIENCE SECTION

Introduction

In the final year of the project the DNA collected from the two previous years was used to look at the effect of the two BCAs on the microbiome of the blossom and fruit. 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 compared the two BCAs [*Bacillus subtilis* (B91) and *Aureobasidium pullulans* (Y126)] to a fungicide treatment and a control of sterile distilled water to see what impact they had on the blossom and cherry microbiomes.

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).

Materials and methods

Four treatments were looked at: two BCAs (B91 and 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.

After samples were collected they were washed in Maximum Recovery (Sigma-Aldrich) for one hour on a shaking incubator at 180 rpm. DNA was extracted from the washes using TRI Reagent (Sigma-Aldrich) following the manufacturer's instructions with an additional step of ethanol (70%) precipitation to remove any residual salts. DNA samples were sent to NOVOGENE (UK) LTD for Library prep and 16S and ITS Amplicon sequencing. The Data received was then clustered at 97% similarity into operational taxonomic unit (OTU) using reference databases. OTUs were analysid using R.

Results

The first questions we wanted to answer was; does the treatment of BCAs or tissue type affect the microbial community? To do this we conducted a Principal components analysis (PCA). The PCA showered that 37% of the variation in PC1 within the 2019 bacterial data was due to block effect. There was a slight treatment effect ($PC3\ P < 0.02$) and tissue type ($PC4\ P < 0.04$) effect with clear clustering for the control treatment, B91 and fungicide (Figure 1). A similar pattern as seen in the fungal communities though not as pronounced. The effect size of each factor; represented but the PCA percentage variance being: block 12.3%, treatment 7%, tissue type 10.7%.

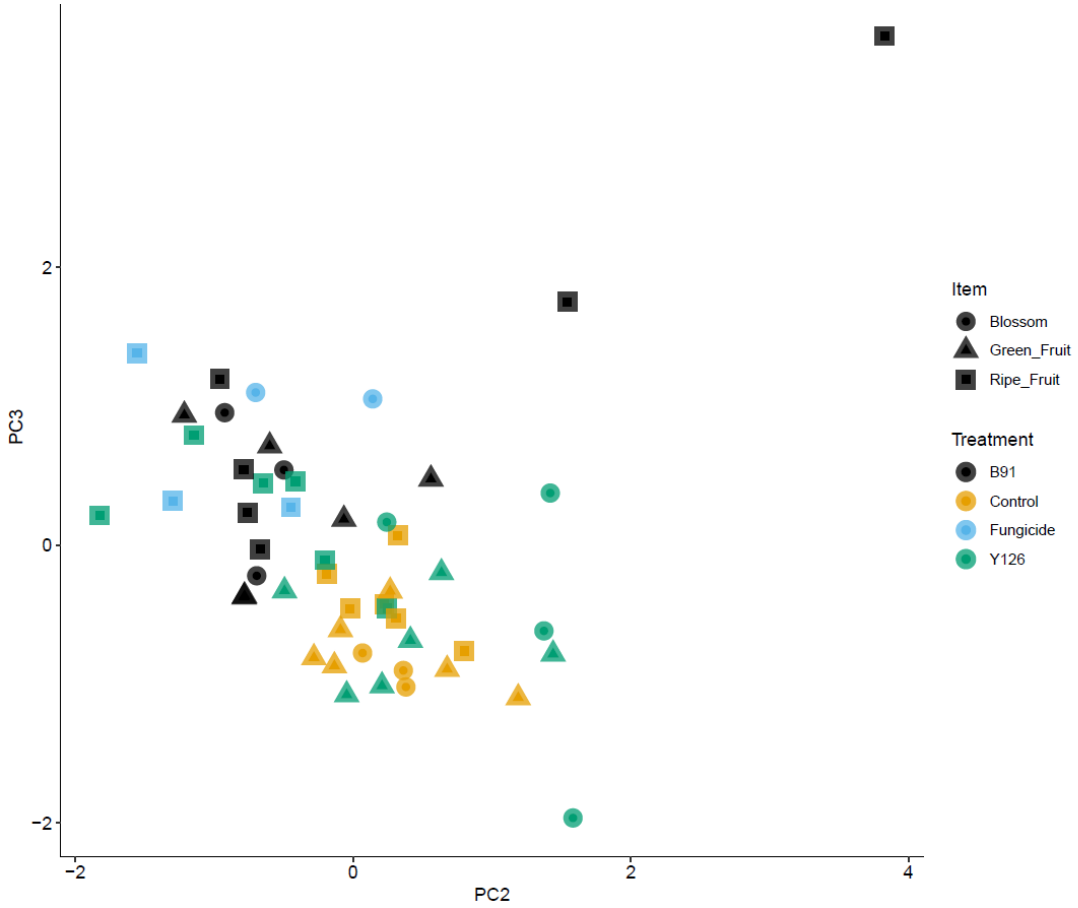

Figure 1: PCA plot of PC2 vs PC3 showing 2019 bacterial community. The graph shows a 2D plot of each sample based on the scores of PC2 and PC3. Tissue type (Item) by shape and Treatment by colour. Black = B91, Yellow = Control, Blue = Fungicide, Green = Y126.

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 2) with the percentage variance for each factor being treatment 12.5% and tissue type 6.0%. 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. 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 ($P < 1 \times 10^{-4}$). The percentage variance from the PCA model for each factor was: block 16 %, treatment 11 %, tissue type 4.5 %.

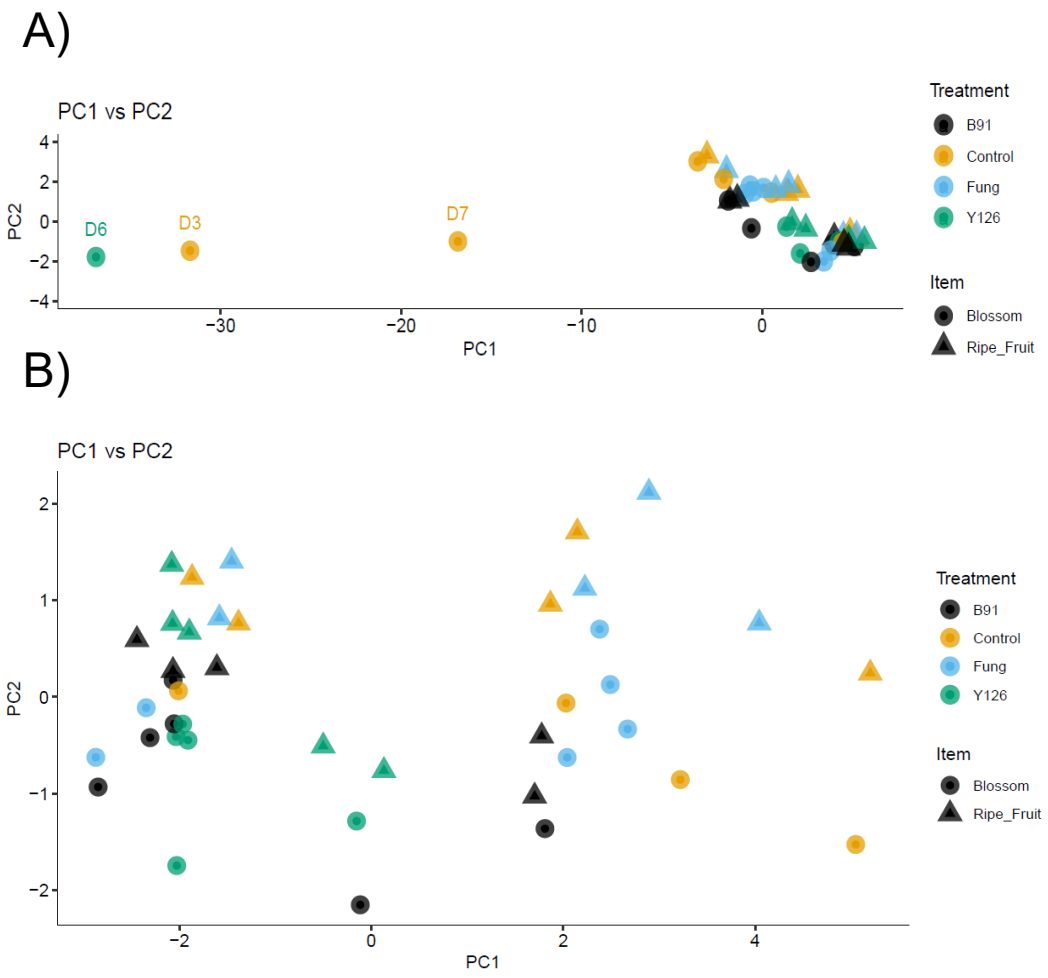

Figure 6: 2020 bacterial community shown in a PCA plot of PC1 vs PC2 showing tissue type (Item) by shape and Treatment by colour. Black = B91, Yellow = Control, Blue = Fungicide, Green = Y126. A) shows all samples B) Graph excludes outlier samples; D3 (tree 1, Control), D6 (tree 1, Y126), D7 (tree 1,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

Next, we looked at how the BCAs 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 1 shows the number of OTUs for which the abundance was significantly different between the BCAs and the two other treatments (control and fungicide).

Table 1: 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

When introducing a new product into the environment it's important to understand any 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 beneficially to see how they affect the plant, the pathogen (*M. laxa*) 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.

Conclusions

The full project looked at multiple aspects of the use of our two BCAs. Previous reports discussed the results of our survival studies of BCAs on blossom and mummified fruits and the effect of pre-harvest fruit applications on post-harvest rots.

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 could be used early in the 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). Currently 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 arson that can be used in tandem with other techniques to combat this pathogen.

Knowledge and Technology Transfer

- Berry Gardens Research & Agronomy Conference 2021 - Presentation
- 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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