Final Report

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Title: The Epidemiology and Management of *Cladosporium* on Raspberry

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CONTENTS

1. Industry Summary

In recent years, UK growers have reported that fungal infections caused by the genus *Cladosporium* are becoming more prevalent on raspberries. Current management strategies are generalised from other soft fruit pathogens due to a lack of research on the epidemiology and management of *Cladosporium.* This work began to study the epidemiology of *Cladosporium* on raspberry to highlight potential management strategies for further investigation. Growers may be able to use these methods to alleviate future outbreaks of this disease.

The first experiments identified the predominant species of *Cladosporium* on UK raspberries and found *C. cladosporioides* was the most prevalent. As this species is a common airborne spore, air flow management may be important for primary inoculum control. Next, different stages of raspberry development were inoculated with three separate isolates of *C. cladosporioides* to determine at which stages fruit are susceptible to *Cladosporium* skin lesions and stigmata infections. Green to ripe fruit were susceptible to *Cladosporium* stigmata infections, however, only ripening and ripe fruit developed skin lesions. This highlights the need for growers and agronomists to monitor disease from the ripening stage of development and onwards for effective management (in the absence of aphid infestations).

Next, the presence of *Cladosporium* and other microorganisms on the raspberry fruit surface were investigated using microbiome experiments. These experiments investigated whether fruit age, location of fruit within a polytunnel, location of fruit across polytunnels on a farm and across two dates significantly impacted the diversity and abundance of organisms on the fruit surface. As fruit ripened, less *Cladosporium* was reported on the fruit surface. This suggested there may be potential antagonistic organisms present on fruit to investigate as future biocontrols. *Cladosporium* was also more prevalent on fruit at the outer edges of tunnels than the centre, indicating that air movement may facilitate *Cladosporium* dispersal. The number of *Cladosporium* airborne spores were investigated to determine whether polytunnel infrastructure increased the number of spores and changed the diurnal pattern. Spores followed a diurnal pattern inside the polytunnel and open field, however, more spores were trapped inside the polytunnel than in the open field, indicating that venting might be a useful strategy to reduce the airborne inoculum load.

Finally, we investigated the use of Biological Control Agents (BCAs) and resistant varieties as potential management strategies. We tested five commercially available BCA products (not all licensed on raspberry) in plate assays, and then took the three most effective products to field experiments. In field trials, the *Trichoderma* sp. was the most effective at reducing skin lesions, followed by *Bacillus subtilis* (strain QST 713) and *B. amyloliquefaciens* (strain FZB24), which had similar efficacy in field applications. Further testing of these products with applications closer to industry standard sprays and dosages are essential.

From this series of experiments, we have begun to understand the epidemiology of *Cladosporium* on raspberry. This will direct future researchers conducting more in-depth investigations of suitable management strategies. This research also provides growers and agronomists with a time frame of when to look for visible symptoms, highlights that managing airflow will be important in reducing the inoculum load, encourages consideration of the wider environmental landscape and how that impacts disease risk, and finally, provides some products that require further testing for future commercialisation.

2. Introduction

Cladosporium is a genus of fungi that has been increasingly reported by growers in the last 10 years on raspberries (Farwell *et al*., 2023; Swett *et al*., 2019). When present, *Cladosporium* can cause black and green patches on the fruit surface making fruit unmarketable and causing substantial losses. *Cladosporium* can be quite prevalent, with one farm in Kent having over 50% of fruit infected with *Cladosporium* post-harvest (O'Neill *et al*., 2012). Previous research from the USA has focused on how damage from *Drosophila suzukii* (also known as Spotted Winged Drosophila; SWD) caused wounding on the fruit surface, providing nutrients for *Cladosporium* to develop (Lewis *et al*., 2019; Swett *et al*., 2019). Research has also indicated that *Cladosporium* can develop close-to-harvest, instead of predominantly being a post-harvest disease, when conditions are conducive to its development (Swett *et al*., 2019). Therefore, more research is essential to understand the epidemiology of *Cladosporium* on raspberry to determine management strategies that may be effective.

Previous studies from the USA found multiple species of *Cladosporium* were infecting raspberries, including *C. cladosporioides* and *C. pseudocladosporiodes* (Swett *et al*. 2019). Determining which species were prevalent on UK fruit allowed future experiments to focus on the predominant species. Raspberries were collected from growers across the UK and the species determined by sequencing two DNA regions (actin and trans-elongation factor 1α). The NCBI database was searched to find similar sequences to determine the species identity. Phylogenetic trees were also built using maximum likelihood to see if the NCBI database returned the same results for species ID as the phylogenetic trees.

To target management strategies to the most susceptible stages of fruit development, more research was needed to determine when fruit are most susceptible. Previously, *Cladosporium* was considered to be a primarily post-harvest disease (Dennis, 1975), however recent research indicated *Cladosporium* skin lesions can develop on fruit pre-harvest (Swett *et al.*, 2019). However, this research did not include the green stage of fruit development within their studies. Raspberries were inoculated in the field to determine when fruit were susceptible to *Cladosporium* skin lesions and stigmata infections, with green, ripening and ripe fruit being inoculated. This will provide growers with a targeted window of when to look for visible symptoms of *Cladosporium* and when to apply effective management strategies.

The inoculum and ecology of *Cladosporium* on the fruit surface was investigated using next-generation sequencing to understand how specific species and genera changed during fruit ripening, between the centre and outer edge of polytunnels, across locations on a farm, and across

sampling dates. This provided insight into the factors impacting the amount of *Cladosporium* inoculum on the fruit surface, including whether there were any antagonists that could potentially be screened as biocontrols in the future.

The number and diurnal patterns of airborne *Cladosporium* spores were investigated to determine whether the inoculum load in the air was different inside a raspberry polytunnel compared to an open field. Currently, no published research has investigated how polythene infrastructure may impact the dispersal dynamics of any plant pathogen. Understanding how the primary inoculum load is impacted by polythene coverings could show whether growers should vent tunnels to reduce the incidence of *Cladosporium*.

Some previous research showed that there are some management techniques warranting further investigation. In previous studies, *Cladosporium* mycelial growth was reduced in plate assays when using biological control agents (El-Dawy *et al*., 2021; Rolim *et al*., 2019), however no research has attempted field applications of BCAs against *Cladosporium.* Commercially available Biological Control Agents (BCAs) were assessed in plate assays and the best were then taken for field inoculations across two years to determine whether the proportion of fruit infected with *Cladosporium* skin lesions decreased. Research performed in the 1980s found differences between susceptibility to *Cladosporium* across raspberry varieties, however, robust statistical testing could not be performed on the data (Knight, 1980). Modern raspberry varieties were inoculated in field conditions with a mixed *C. cladosporioides* inoculum to determine whether any were more or less susceptible to *Cladosporium* skin lesions and stigmata infections.

When combined, this research begins to unravel the epidemiology of *Cladosporium* on raspberry and alludes to management strategies that growers may be able to use in the future, and highlights the need for further research on this understudied pathogen.

3. Materials and methods

For a detailed description of the materials and methods of the below experiments, please refer to the thesis. For the purpose of this report, a brief description of the methods are detailed.

3.1. Determining the Predominant *Cladosporium* Species on UK Fruit

Raspberries were collected from commercial farms across the UK and some farms from Spain and incubated in ambient room conditions to allow the development of *Cladosporium* skin lesions. *Cladosporium* isolates were then collected from these fruits and isolated using PDA media

(supplemented with 9.3 g NaCl per 100 mL to inhibit bacteria and other fast growing fungi). Once pure isolates were obtained, DNA was extracted using the Sigma extraction buffers (Extract-N-Amp™, 2022). Isolates were then subjected to PCR amplification of two regions, the trans-elongation factor 1α (TEF1α) and actin (ACT) regions (for primer sequences and PCR set-up, see thesis section 3.3.1.2). Samples were then sent to Eurofins Genomics for Sanger sequencing of the forward and reverse of the TEF1α and ACT regions. Only high quality sequences were taken forward, with a consensus sequence created for both regions. These consensus sequences were then run through the NCBI BLAST database to search for a species ID. Similar sequences from the database were used as reference sequences to build a phylogenetic tree using maximum likelihood and 1000 bootstrap replications for both regions to compare results from the NCBI database and the phylogenetic trees (for a full break down of the software and thresholds used, please refer to section 3.3.1.3 of thesis).

3.2. When are Raspberries Susceptible to *Cladosporium* Skin Lesions and Stigmata Infections?

Field inoculations were performed on raspberry plants across two years (2019 and 2020) to determine whether green, ripening and ripe fruit are susceptible to *Cladosporium* skin and stigmata infections. In 2019, four rows of three plants were inoculated with a mix of three isolates of *Cladosporium cladosporioides*, with no control treatment. A single branch was selected from each plot (a plot consisting of one plant, with 12 plots in total) across four inoculation dates. In 2020, two rows of 16 pots were divided into four randomised blocks, with each plant in the blocks designated to a treatment with either one of three *C. cladosporioides* isolates or a control, to determine whether there were differences between isolates in their pathogenicity. In each pot, two or three branches were selected for inoculation across three dates (for more information, refer to section 3.3.2 and 3.3.3 in thesis). Plants were inoculated in field conditions, left for 24 hours, then picked and taken into a lab to be incubated in ambient room conditions for four days until assessment. In 2019, presence or absence of *Cladosporium* skin lesions was recorded. In 2020, fruits were scored for the number of infected drupelets and the number of infected stigmata. All the data were analysed in R v. 4.0.3.; in 2019, data were analysed with a generalised linear mixed model, and in 2020 they were analysed with an ordinal logistic regression.

3.3. Investigating the Inoculum Load on the Surface of Raspberries

Raspberries were collected from a commercial raspberry grower in Kent, UK, and the fruit washed to remove surface epiphytes. The fruit washes were centrifuged to produce a pellet from which DNA was extracted using the TRI reagent protocol (Sigma-Aldritch TRI Reagent® Protocol, 2023). The DNA was then sent to Novogene UK for next generation sequencing. The following factors

were investigated for their effects on the epiphytic microbiome communities: fruit age (green, ripening and ripe fruit), location of fruit within a tunnel (the very centre vs. the outer edge), location of the polytunnel on the farm (one of four polytunnels spaced out over the farm) and date of sampling (samples taken on the 2nd and 9th of October 2021). In total, 48 samples were taken, with 40 fruits in each sample. For a breakdown of the bioinformatics and statistical methods used to analyse the data, refer to section 4.3.1.2 of the thesis. Statistical analyses identified how the before-mentioned factors affected the alpha diversity (how diverse the genera/species were), the beta diversity (the overall community structure), and how individual genera/species abundance differed.

3.4. Variations in Airborne *Cladosporium* Inoculum in a Polytunnel vs an Open Field

Two experiments were conducted to determine whether *Cladosporium* airborne spore numbers were higher inside a raspberry polytunnel versus 100 m away in an open field, conducted from the 10th to the 31st of October 2022. One experiment compared the day vs. night (08:00 to 20:00 vs. 20:00 to 08:00; samples taken from the 10th to the 31st of October), with the second experiment further breaking the day into three collection periods (morning 08:00 to 12:00, afternoon 12:00 to 16:00, evening 16:00 to 20:00 and night 20:00 to 08:00; samples taken from the 10th to the 21st of October). Airborne spores were collected using two Rotorod spore traps set 1.4 m above the ground: one attached to the centremost pole within a raspberry polytunnel, and one 100 m away in the field. Rotorod traps collect spores using arms that are coated with a thin film of Vaseline on the collecting edge, and are then spun at maximum speed using a 12 V battery for the allotted collection period. The rods were collected at the end of the allotted period and taken to a -80 °C freezer for storage. DNA extractions were performed using the MasterPure Yeast DNA Purification Kit (LGC Biosearch Technologies), with the same protocol used in Fraaije *et al.* (2021). To calculate estimates of the number of *Cladosporium* spores, a standard curve was created from DNA extracted from a known number of *Cladosporium* spores, diluted in four 10-fold dilutions. This standard curve was used in qPCR reactions to then calculate an estimate for the number of spores in each sample (refer to section 4.3.2 of thesis).

3.5. Assessing the Efficacy of Biological Control Agents Against *Cladosporium*

Commercially available biocontrol products were first screened in dual culture plate assays (Table 1) by applying 20 µl of *C. cladosporioides* inoculum to one side of a 9 mm Petri dish and 20 µl of the commercial product 30 mm away. The diameter of *Cladosporium* growth was recorded 9 days post biocontrol application (thesis section 5.3.1).

Table 1. Biological control agents tested in plate assays and field applications.

Species	Product Name	Plate Screen Media	Maximum Dose (g/L)	Tested in the field
***Bacillus amyloliquefaciens* strain FZB24**	Taegro™	PDA	0.37	Yes
***Bacillus subtilis* strain QST 713**	Serenade™	PDA	8.00*	Yes
***Bacillus amyloliquefaciens* subsp. *plantarum* D747**	Amylo-X™	PDA	2.50	No
***Aureobasidium pullulans* strain DSM 14940 and DSM 14941**	Botector™	3% Raspberry	1.00	No
***Trichoderma* sp.**	NA	3% Raspberry	2.00	Yes

*Product supplied in liquid form, therefore units were mL/L.

For field trials, preventative and curative applications of *B. subtilis* (strain QST 713), *B. amyloliquefaciens* (strain FZB24) and a *Trichoderma* sp. were performed across two years (2022 and 2023) compared to a *Cladosporium* treated control (positive) and an untreated control (negative). Biocontrol agents were applied preventatively (24 hours before *C. cladosporioides* inoculum application) or curatively (24 hours after *C. cladosporioides* inoculum application) to ripening fruit at the maximum dose recommended on the label (table 1), using a pressurised hand held sprayer. After 24 hours, ripe fruit were picked and incubated in ambient room conditions for four days, and then assessed for the presence or absence of *Cladosporium* skin lesions (thesis section 5.3.2).

3.6. Screening Raspberry Varieties for Resistance to *Cladosporium* Skin and Stigmata Lesions

Five varieties of raspberries (a popular UK proprietor variety, Cascade Gem, Paragon, Malling Bella and Malling Charm) were screened for their susceptibility to both skin and stigmata infections. Plants were arranged into an incomplete randomised block design (due to plant losses) and either inoculated with a mixed *C. cladosporioides* inoculum during ripening or were left untreated as a control (to determine if inoculation helped to increase the incidence of disease). Nine rounds of inoculations were performed in 2021 and three rounds in 2022. After 24 hours, ripe fruit were picked and washed twice in 0.1% Tween-20 for one minute, then in sterile water for one

minute, then dried in a flow hood for approximately 20 minutes. Fruit were finally incubated in ambient room conditions for four days. Fruit were then assessed for the absence or presence of skin lesions, and 20 stigmata were assessed for presence of *Cladosporium* (see section 6.3 from thesis).

4. Results

4.1. Determining the Predominant *Cladosporium* Species on UK Fruit

A total of 41 *Cladosporium* isolates were collected from UK fruit, with the predominant species being *C. cladosporioides*, making up 41.5% of isolates. Other species present included *C. sphaerospermum* (14.6%), *C. europeaum* (14.6%), *C. fusiforme* (12.2%), *C. limoniforme* (9.8%), and *C. ramotellum* (7.3%).

Phylogenetic trees corroborated with the nrBLAST database (Fig. 1; see section 3.4.1. of thesis).

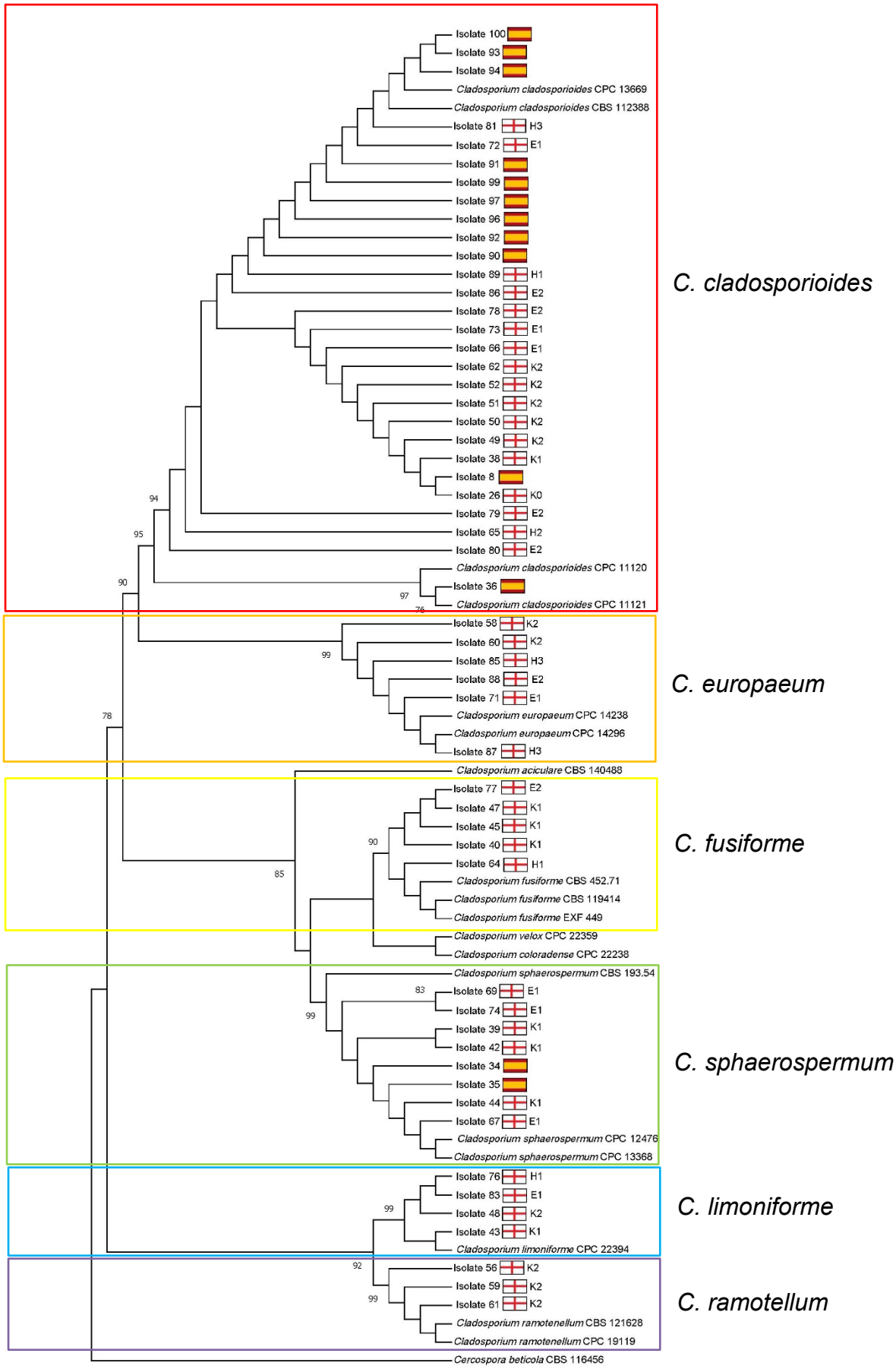
Isolate 100
Isolate 93
Isolate 94
Cladosporium cladosporioides CPC 13669
Cladosporium cladosporioides CBS 112388
Isolate 81 H3
Isolate 72 E1
Isolate 91
Isolate 99
Isolate 97
Isolate 96
Isolate 92
Isolate 90
Isolate 89 H1
Isolate 86 E2
Isolate 78 E2
Isolate 73 E1
Isolate 66 E1
Isolate 62 K2
Isolate 52 K2
Isolate 51 K2
Isolate 50 K2
Isolate 49 K2
Isolate 38 K1
Isolate 8
Isolate 26 K0
Isolate 79 E2
Isolate 65 H2
Isolate 80 E2
Cladosporium cladosporioides CPC 11120
Isolate 36
Cladosporium cladosporioides CPC 11121
Isolate 58 K2
Isolate 60 K2
Isolate 85 H3
Isolate 88 E2
Isolate 71 E1
Cladosporium europaeum CPC 14238
Cladosporium europaeum CPC 14296
Isolate 87 H3
Cladosporium aciculare CBS 140488
Isolate 77 E2
Isolate 47 K1
Isolate 45 K1
Isolate 40 K1
Isolate 64 H1
Cladosporium fusiforme CBS 452.71
Cladosporium fusiforme CBS 119414
Cladosporium fusiforme EXF 449
Cladosporium velox CPC 22359
Cladosporium coloradense CPC 22238
Cladosporium sphaerospermum CBS 193.54
Isolate 69 E1
Isolate 74 E1
Isolate 39 K1
Isolate 42 K1
Isolate 34
Isolate 35
Isolate 44 K1
Isolate 67 E1
Cladosporium sphaerospermum CPC 12476
Cladosporium sphaerospermum CPC 13368
Isolate 76 H1
Isolate 83 E1
Isolate 48 K2
Isolate 43 K1
Cladosporium limoniforme CPC 22394
Isolate 56 K2
Isolate 59 K2
Isolate 61 K2
Cladosporium ramotenellum CBS 121628
Cladosporium ramotenellum CPC 19119
Cercospora beticola CBS 116456
94
95
90
97
76
99
78
90
85
83
99
99
92
99
C. cladosporioides
C. europaeum
C. fusiforme
C. sphaerospermum
C. limoniforme
C. ramotellum

Figure 1. A phylogenetic tree of the ACT gene for 72 isolates of *Cladosporium*. The tree was created using maximum likelihood with a Kimura 2-parameter evolutionary model and 1000 bootstrap replications. The numbers above branches represent bootstrap values. The flags represent the country of origin, the codes represent areas within the country, and numbers denote different farms. Area codes are as follows: E = Essex, K = Kent, H = Herefordshire.

4.2. When are Raspberries Susceptible to *Cladosporium* Skin Lesions and Stigmata Infections?

In 2019, skin lesions were only present on ripening and ripe fruit, with no significant difference between these stages; there was a 13.2% (s.e. ±8.3%) incidence on ripening fruit and 26.5% (SEM ±11.5%) on ripe fruit. In 2020, again only ripening and ripe fruit had skin lesions with an incidence of 17.4% (SEM ± 9.0%) on ripening fruit and 50.2% (SEM ± 12.1%) on ripe fruit, with ripe fruit significantly more susceptible to skin lesions than ripening fruit ($p < 0.001$). Inoculations significantly increased the number of infected fruit ($p < 0.05$). Out of the three *C. cladosporioides* isolates tested, isolate three also caused significantly higher skin lesion severity scores than isolate two ($p < 0.05$), but not isolate one. For more detailed results, see section 3.4.2. of the thesis.

There was no difference in susceptibility to stigmata infections across the ripening stages (green, ripening and ripe fruit; $p = 0.166$), with all stages being susceptible to *Cladosporium* stigmata infections. Once again, isolate three had significantly higher infection scores than isolates one and two ($p < 0.001$). For more detailed results, see section 3.4.3. of the thesis.

4.3. Investigating the Inoculum Load on the Surface of Raspberries

In the fungal microbiome, *Cladosporium* was the most ubiquitous genus, making up 43.9% of the total number of fungal sequences. For bacteria, *Erwinia* was the most abundant genus, making up 23.2% of bacterial sequences.

4.3.1. Differences in Fungal and Bacterial Diversity

The fungal alpha diversity (the number of unique sequences) was significantly higher on ripening and ripe fruit than green fruit (Shannon and Simpson $p < 0.01$; Fig. 2). In contrast, bacterial diversity showed the opposite trend, with lower diversity on ripening and ripe fruit than green fruit (Shannon and Simpson, $p < 0.05$; Fig. 3). Sampling date also affected fungal diversity, with higher diversity taken on the 2nd than the 9th of October 2021 (Shannon and Simpson, $p < 0.01$; Fig. 2). Date did not affect bacterial diversity indices. Finally, location of fruit within the tunnel affected

fungal diversity, with more unique fungal sequences found on raspberries collected from the outer edge (Chao1, $p < 0.05$; Fig. 2). Bacterial microbiomes did show an interaction between the within-tunnel location and fruit age, with outer green fruit having higher diversity than inner green fruit, but ripening and ripe fruit having higher diversity when collected from the centre rather than the outer edge (Shannon, $p < 0.05$; Fig. 3). For more information, see section 4.4.1.2. of the thesis.

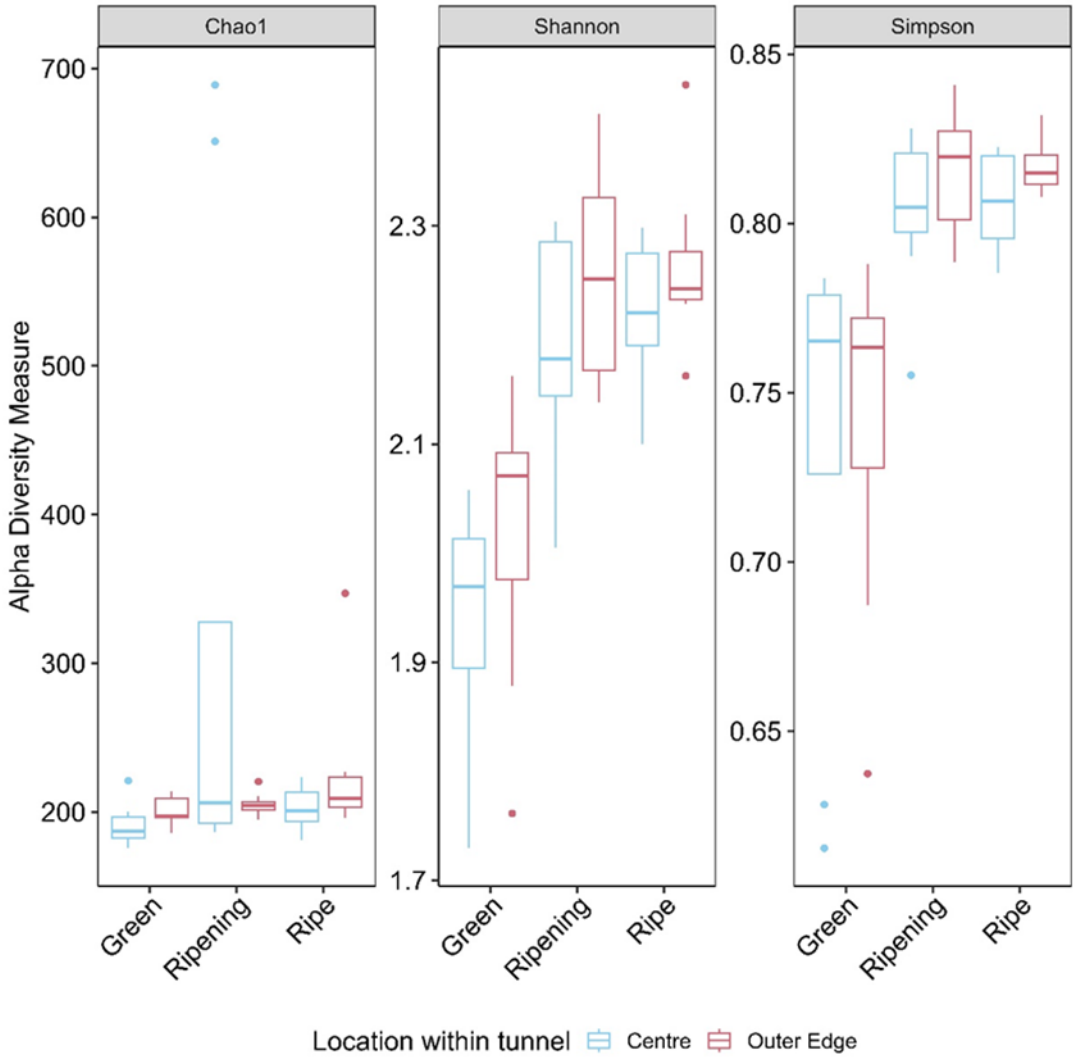

Figure 2. The alpha diversity measures for fungal Amplicon Sequence Variants (ASVs) of Chao1, Shannon and Simpson diversity indices. Four polytunnels were sampled across two repeats in time (2nd and 9th of October 2021). The x-axis denotes fruit ripeness, and colour denotes location within the polytunnel.

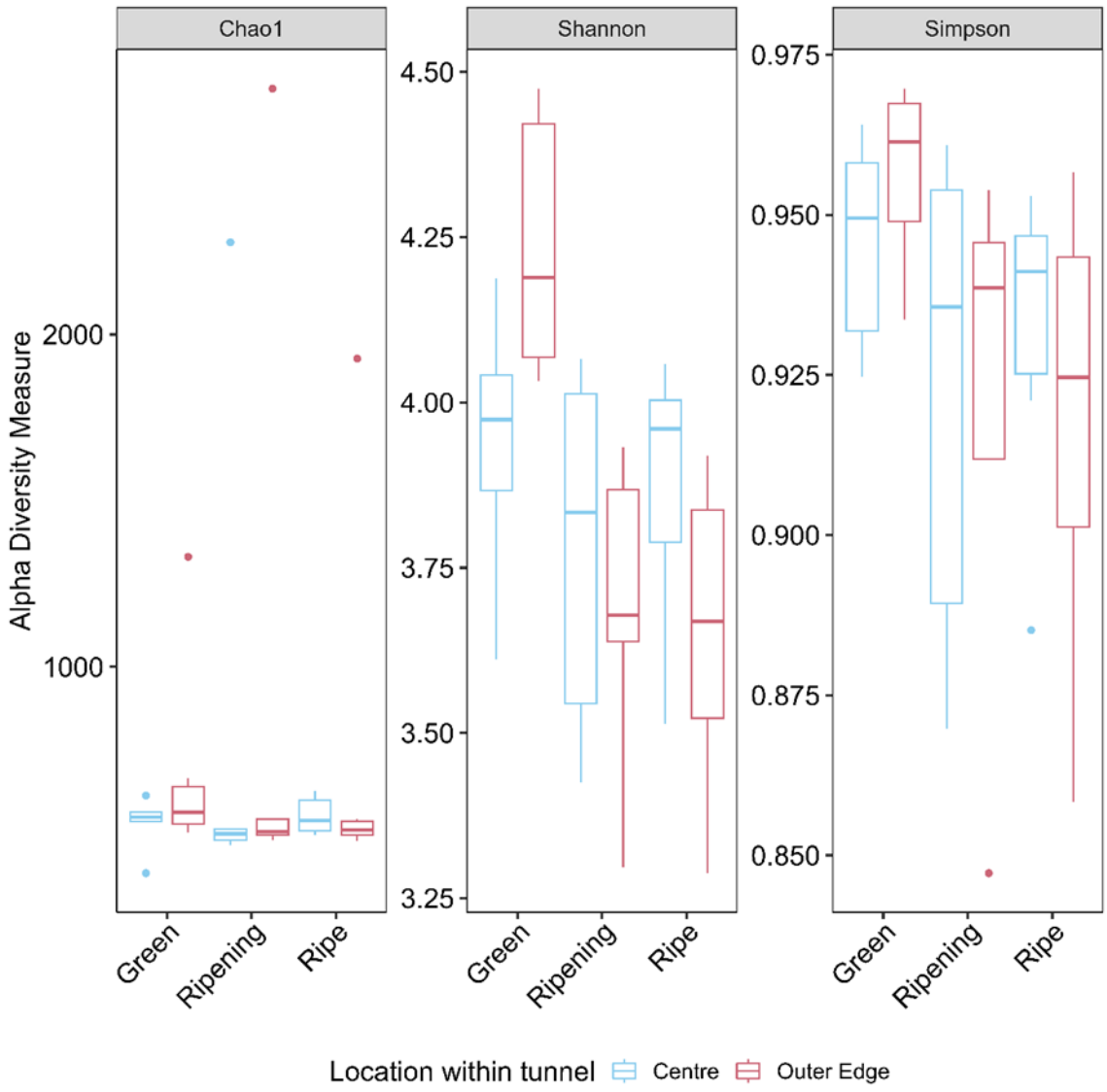

Figure 3. The alpha diversity measures for bacterial Amplicon Sequence Variants (ASVs) of Chao1, Shannon and Simpson diversity indices. Four polytunnels were sampled across two repeats in time (2nd and 9th of October 2021). The x-axis denotes fruit ripeness, and the colour denotes location within the polytunnel.

4.3.2. Differences in Fungal and Bacterial Community Compositions

The beta diversity refers to the similarity between samples. On the NMDS plot (Fig. 4), samples with a more similar community structure are closer together, and samples more dissimilar are further apart.

For fungal samples, fruit age explained 21.8% of variation in samples ($p < 0.001$; Fig. 4A), and sampling date explained 14.2% ($p < 0.001$; Fig. 4A). Other factors contributed to variations in sample fungal microbiomes to a lesser extent, including polytunnel location on the farm explaining 7.4% ($p < 0.01$; Fig 4A) and the within-tunnel location a further 2.4% ($p < 0.01$; Fig. 4A).

For bacterial samples, fruit age explained 14.4% ($p < 0.001$; Fig. 4B) of the variation in sample microbiomes. The polytunnel location also greatly affected the microbiome, explaining 14.5% ($p < 0.05$; Fig. 4B) of the variance. Sampling date explained a further 5.3% ($p < 0.01$; Fig. 4B) of the variance, and there was no significant difference between the locations of fruit within a tunnel (see section 4.4.1.3. of thesis).

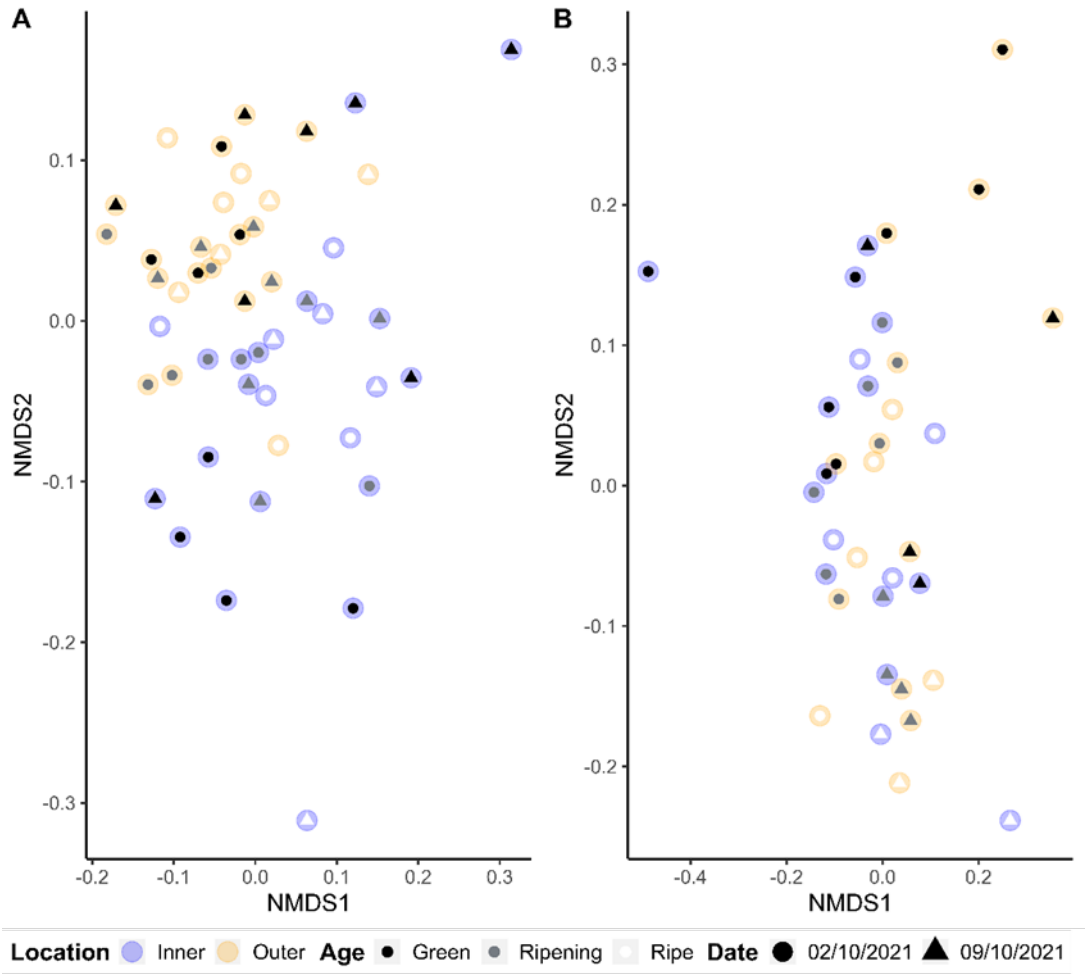

Figure 4. The first two dimensions of the NMDS analysis based on Bray-Curtis indices for A) fungi and B) bacteria from the surface of raspberries collected on two dates (2nd and 9th of October 2021), from two locations within polytunnels (inner vs. outer) and across three ripening stages (green, ripening and ripe).

4.3.3. Differences in Specific Fungal and Bacterial Genera Between Factors

In total, 58 fungal sequences significantly differed across fruit ripening stages. *Cladosporium* had a significantly higher abundance on green fruit than on ripening and ripe fruit. The opposite was found for *Podosphaera* (powdery mildew) and *Epicoccum nigrum* (see table 4-2 in thesis). A total of 75 fungal sequences differed between fruit collected from the centre of polytunnels than the

outer edge. This included sequences of *Cladosporium, Botrytis* and *Alternaria*, which were all more abundant at the outer edge (see table 4-3 in thesis).

Across the ripening stages, 287 bacterial sequences significantly differed in abundance across fruit ripening stages. Of these, 37 were *Pseudomonas* sequences, with their prevalence varying across the ripening stages. The genus *Rouxiella* was also in higher abundance on ripening fruit, and the genus *Bacillus* was more abundant on green fruit than on ripening fruit (see table 4-4 in thesis). Fourty bacterial genera significantly varied in abundance between the centre of polytunnels vs. the outer edge. One *Bacillus* sequence was found to be more abundant at the centre of tunnels, and two *Erwinia* sequences were higher at the outer edge of tunnels. Four *Pseudomonas* sequences were higher at the outer edge, while another sequence was higher in the centre (see table 4-5 in thesis.

4.4. Variations in Airborne *Cladosporium* Inoculum in a Polytunnel vs. an Open Field

4.4.1. Daytime vs. Nighttime Variations in *Cladosporium* Spores

Cladosporium airborne spores were significantly affected by the time of sampling ($\chi^2(1) = 67.12$, $p < 0.001$) and location (polytunnel or open field; $\chi^2(1) = 68.70$, $p < 0.001$). More spores were trapped in the day than the night, and more inside the tunnel than the open field (Fig. 5A). There was also a significant interaction between time and location, with more *Cladosporium* spores being trapped inside the polytunnel than in the field in both the day and night ($\chi^2(1) = 5.17$, $p < 0.05$; Fig. 5B).

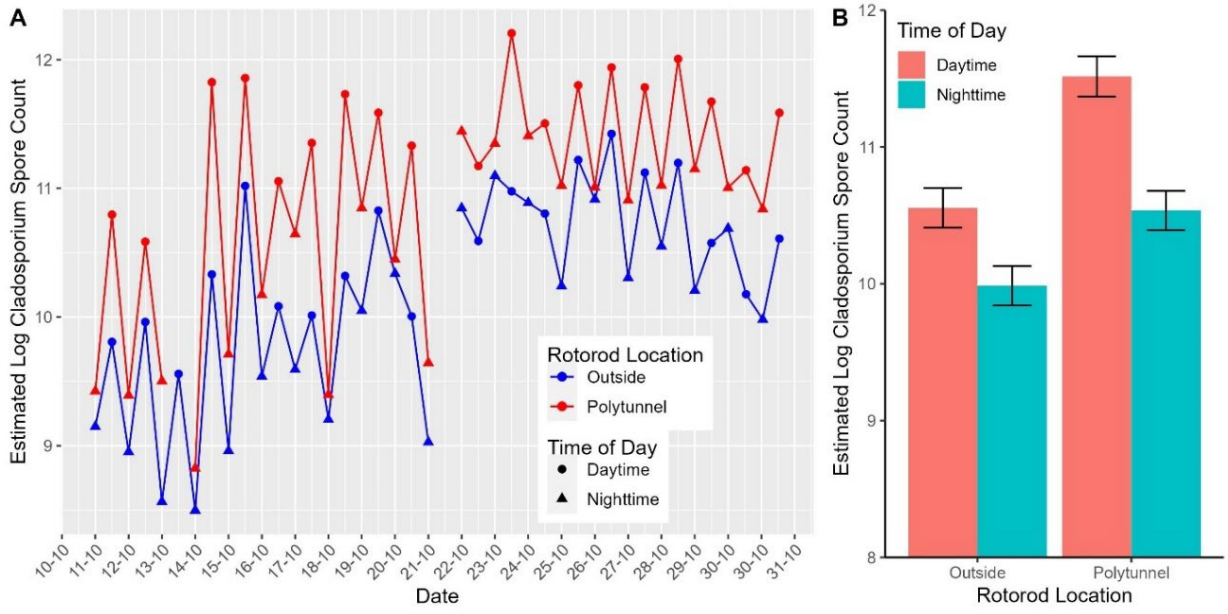

Figure 5. A) and B) the estimated log10 *Cladosporium* spore count averaged over location and time of day from spores collected using a Rotorod sampler from the centre of a polytunnel and in an open field over the day (08:00-20:00) and night (20:00-08:00) across 21 days in October 2022.

4.4.1. Diurnal Variations in *Cladosporium* Spores

Estimated *Cladosporium* spore counts varied between some of the time periods ($\chi^2(3) = 83.72$, $p < 0.001$) and between the polytunnel and open field ($\chi^2(1) = 77.70$, $p < 0.001$), but no significant interaction was found between these factors ($p = 0.14$). As before, more *Cladosporium* spores were found inside the tunnel than the open field. The number of spores was higher in the morning, afternoon and evening than in the night (morning vs. nighttime, $z = 7.26$, $p < 0.001$; afternoon vs. nighttime, $z = 8.34$, $p < 0.001$; evening vs. nighttime, $z = 5.56$, $p < 0.001$), and higher in the afternoon than the evening ($z = 2.73$, $p < 0.001$; Fig. 6).

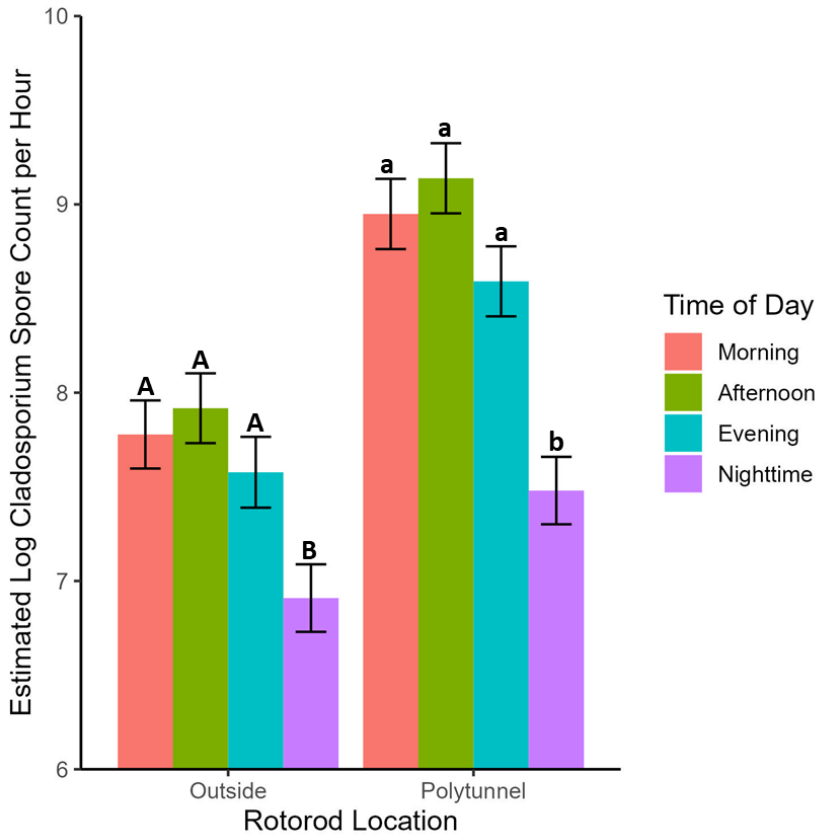

Figure 6. The estimated *Cladosporium* spore counts for samples collected from Rotorod samplers inside a polytunnel and in an open field across the day (morning 08:00-12:00, afternoon 12:00-16:00, evening 16:00- 20:00) and the night (20:00-08:00) for ten days. Letters indicate significant differences between factor levels ($p < 0.05$).

4.5. Assessing the Efficacy of Biocontrol Agents Against *Cladosporium*

4.5.1. Dual Culture Plate Assays

In plate assays, all of the tested BCAs significantly reduced the growth of *Cladosporium* compared to their respective controls (Fig. 7, 8 and 9). On the raspberry media, these were *A. pullulans* ($t(18) = 25.6$, $p < 0.001$) and the *Trichoderma* sp. ($t(18) = 68$, $p < 0.001$), and on PDA media, these were *B. amyloliquefaciens* strain D747 ($t(23) = 32.9$, $p < 0.001$) and strain FZB24 ($t(23) = 30.7$, $p < 0.001$) and *B. subtilis* ($t(23) = 30.7$, $p < 0.001$). For more information, see section 5.4.1 of the thesis.

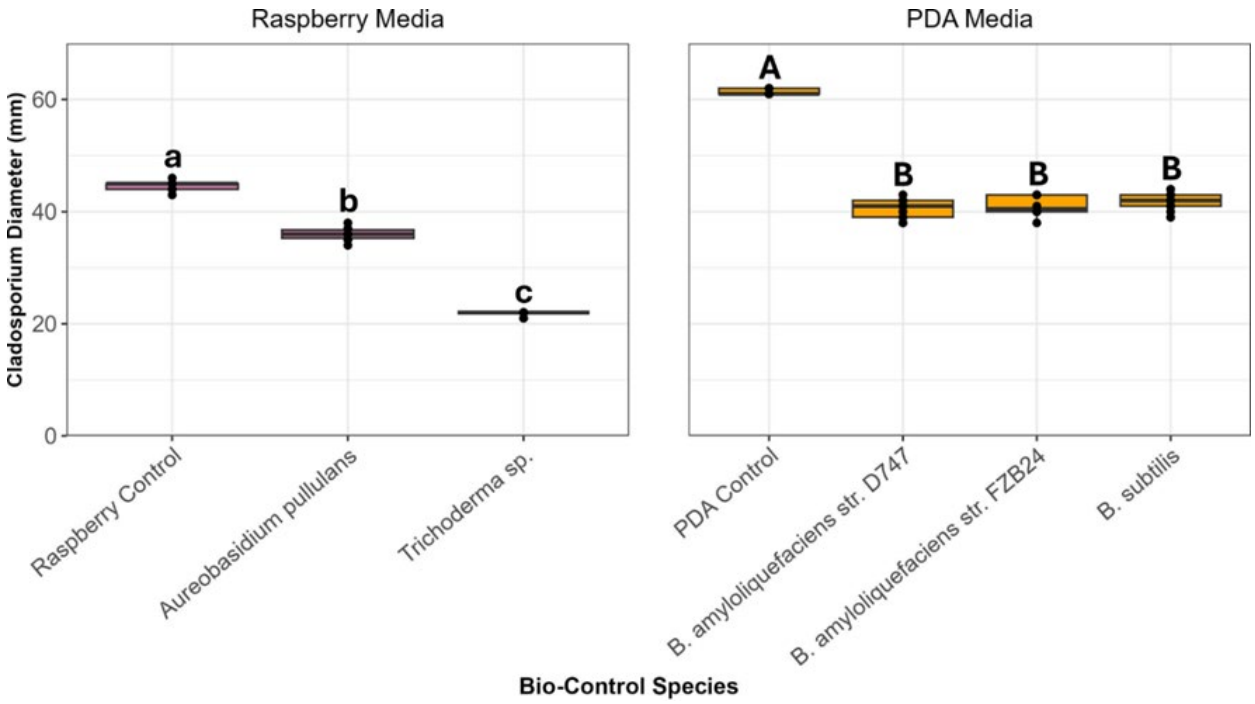

Figure 7. Mycelial diameter growth (mm) of *Cladosporium* colonies 9 days post-inoculation on control plates and plates treated with a BCA. Bacterial BCAs were tested on PDA and fungal BCAs on raspberry media. Letters denote significant differences ($p < 0.05$) between treatments in Tukey corrected post-hoc *t* tests.

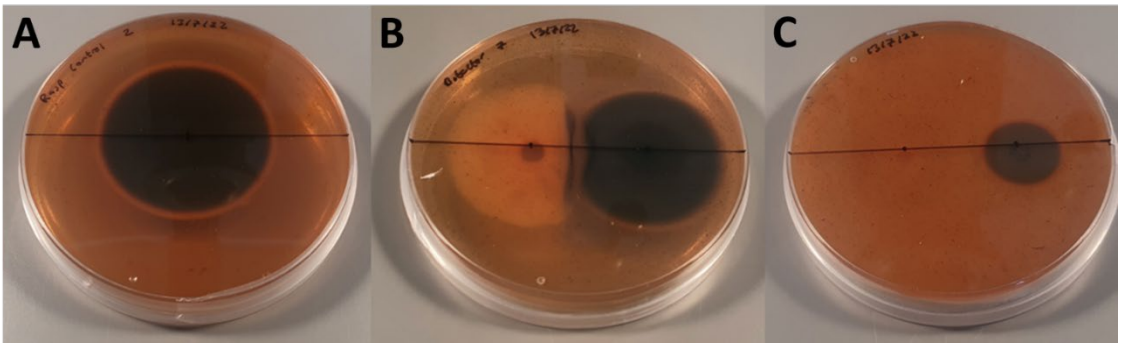

Figure 8. Dual plate screening images of BCAs against *Cladosporium* on 2% raspberry media. A) *Cladosporium* control, B) *Aureobasidium pullulans,* and C) *Trichoderma* sp.

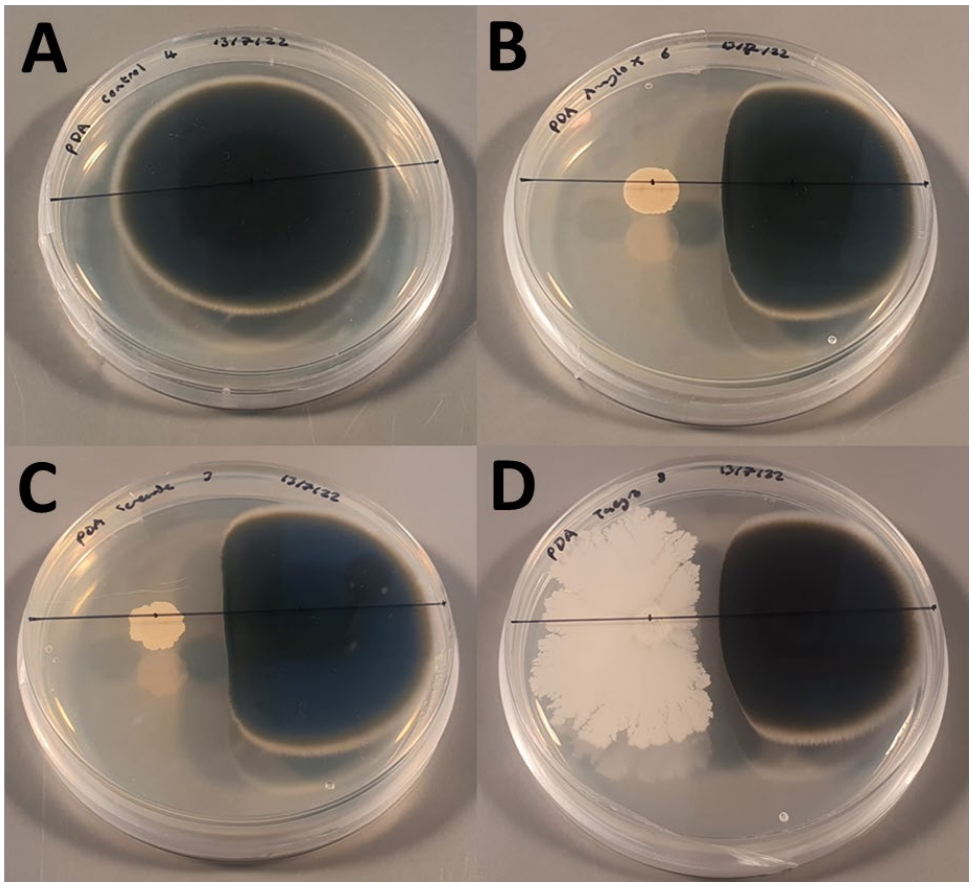

Figure 9. Dual plate screening images of BCAs against *Cladosporium* on potato dextrose agar. A) *Cladosporium* control, B) *Bacillus amyloliquefaciens* strain D747, C) *B. subtilis,* and D) *B. amyloliquefaciens* strain FZB24.

4.5.2. Field Applications of BCAs

In preventative BCA treatments, all products significantly reduced the number of fruit infected with *Cladosporium*, with the *Trichoderma* sp. being the most successful BCA, reducing infections by 28% (z = -10.89, p < 0.001). This was followed by *B. amyloliquefaciens* strain FZB24 reducing infections by 19% (z = -7.26, p < 0.001), and finally, *B. subtilis* reducing infections by 15% (z = -5.33, p <0.001; Fig. 10).

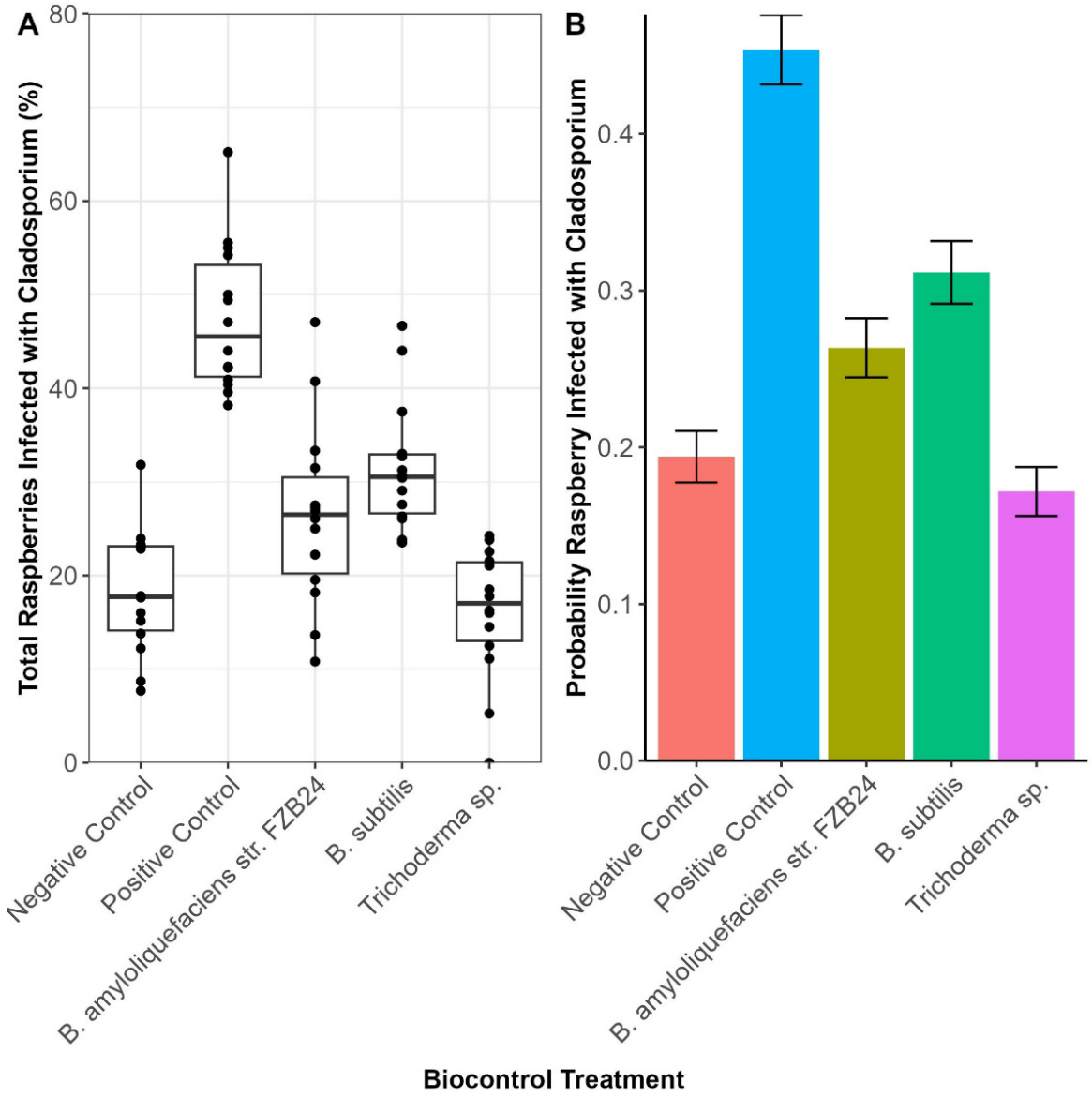

Figure 10. The A) percentage of raspberries infected with *Cladosporium* skin lesions, and B) probability that raspberry fruits are infected with *Cladosporium* skin lesions, after being preventatively treated with a biocontrol, compared to raspberries inoculated with only *Cladosporium* (positive control), and to those that have undergone no inoculation (negative control). BCAs were applied on the 14th of August 2022 and 11th of August 2023.

In curative BCA treatments, all treatments significantly reduced the number of infected fruit; once again, the *Trichoderma* sp. was the most effective, reducing infections by 27% (z = -9.88, p < 0.001), this time followed by *B. subtilis* reducing infections by 14% (z = -4.82, p < 0.001), and *B. amyloliquefaciens* strain FZB24 by 13% (z = -4.37, p < 0.001; Fig. 11).

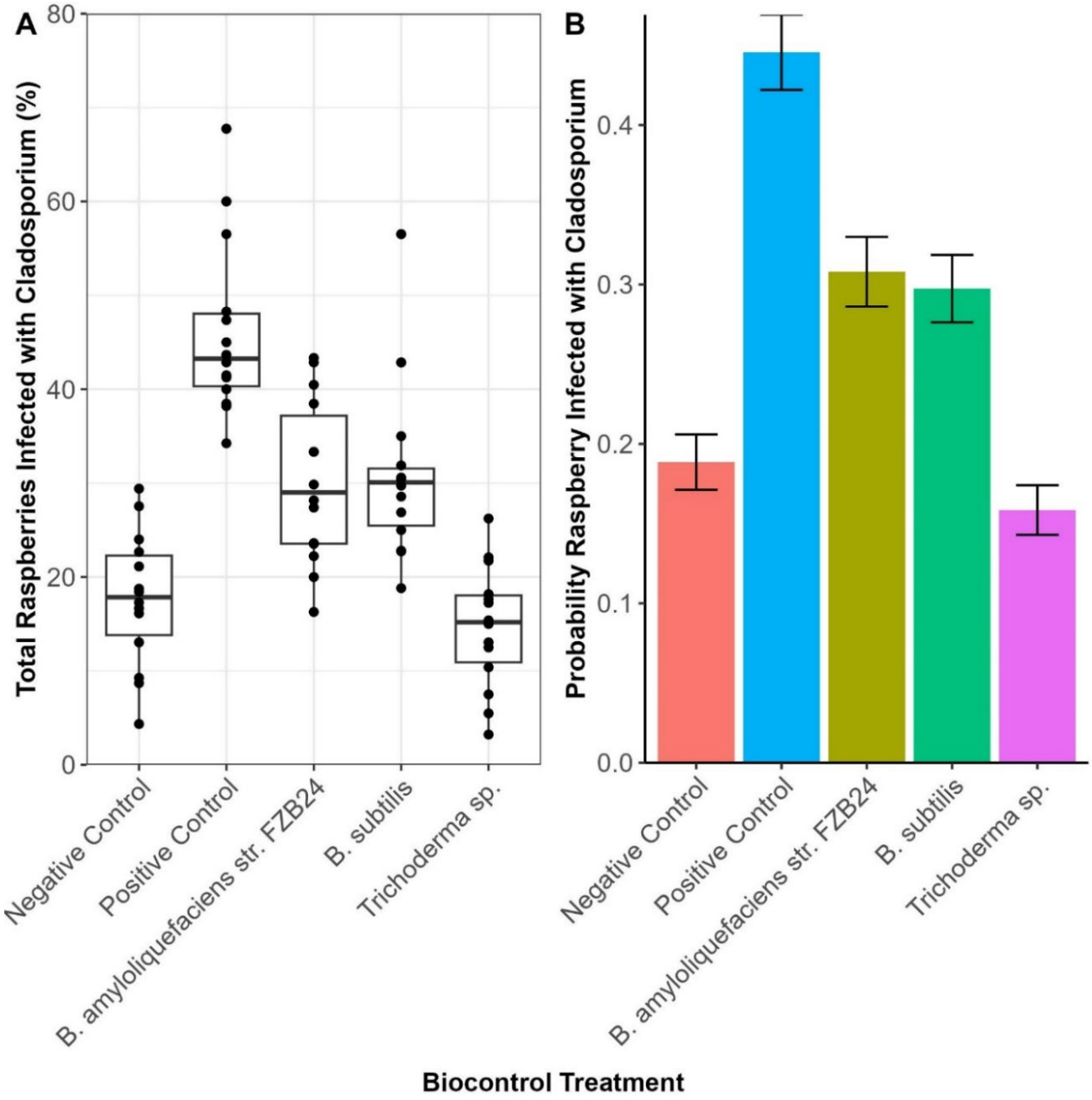

Figure 11. The A) percentage of raspberries infected with *Cladosporium* skin lesions and B) probability that raspberry fruits are infected with *Cladosporium* skin lesions after being curatively treated with a biocontrol treatment compared to raspberries inoculated with only *Cladosporium* (treated control), and to raspberries that have undergone no inoculation (untreated control). BCAs were applied on 14th of August 2022 and 11th of August 2023.

4.6. Screening Raspberry Varieties for Resistance to *Cladosporium* Skin and Stigmata Lesions

Due to significant plant losses and extreme weather conditions in the two years this experiment was conducted, we advise reading the discussion to aid with the interpretation of these results. The

omnibus statistical test (ANOVA) found significant differences between varieties in the incidence of skin lesions ($\chi^2(4) = 9.64$, $p < 0.05$), however post-hoc testing could not significantly differentiate varieties ($p > 0.05$; Fig. 12).

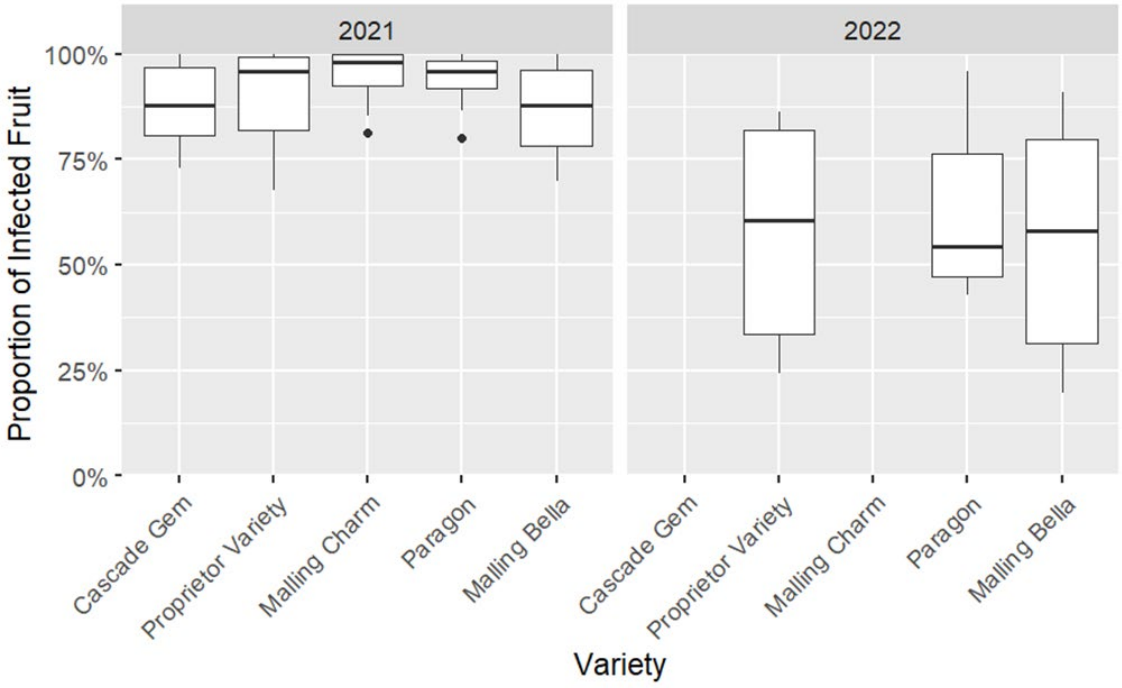

Figure 12. The proportion of fruit infected with *Cladosporium* skin lesions across five varieties (Cascade Gem, a proprietor variety, Malling Charm, Paragon and Malling Bella). Cascade Gem and Malling Charm are absent in 2022 due to plant losses.

Varieties had significant differences in the proportion of infected stigmata ($\chi^2(4) = 33.09$, $p < 0.001$), with post-hoc testing showing that Malling Charm had significantly more infected stigmata than Cascade Gem ($\chi^2(2) = 7.08$, $p < 0.001$) and Malling Bella ($\chi^2(2) = 17.0864$, $p < 0.001$; Fig. 13).

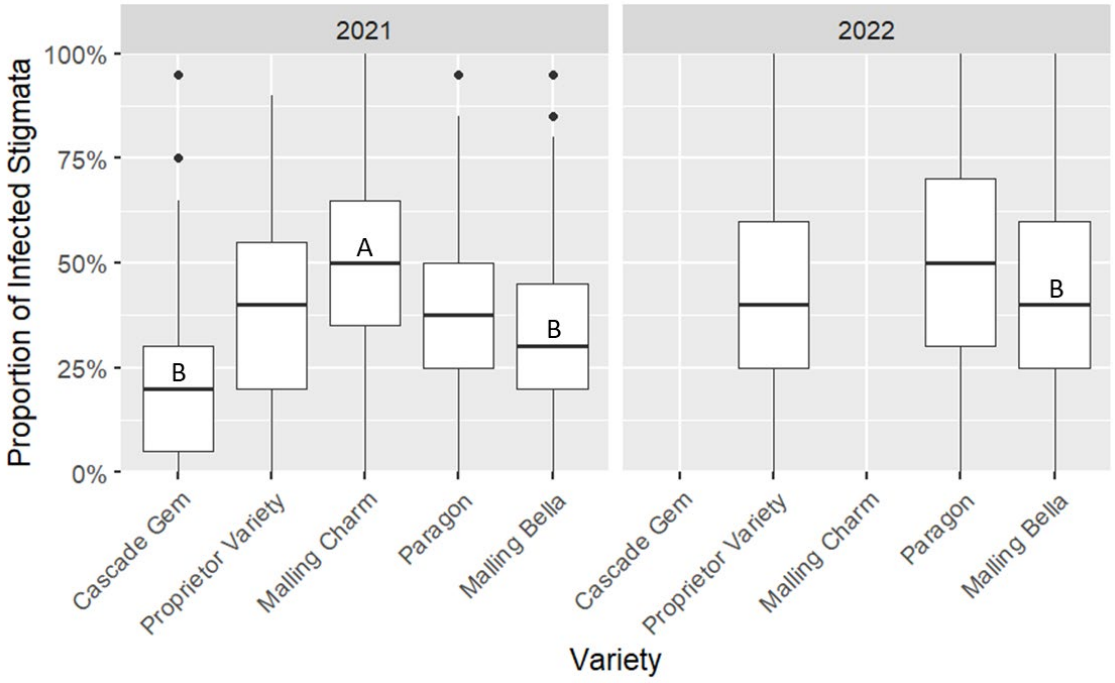

Figure 13. The proportion of stigmata infected with *Cladosporium* across five varieties (Cascade Gem, a proprietor variety, Malling Charm, Paragon and Malling Bella). Cascade Gem and Malling Charm are absent in 2022 due to plant losses. Letters denote varieties with a significant difference in Benjamini-Hochberg corrected analysis of deviance ($p < 0.001$).

5. Discussion

As previously mentioned, for a more detailed description of the results of these studies, please refer to the relevant sections outlined in the thesis.

5.1. Determining the Predominant *Cladosporium* Species on UK Fruit

A complex of *Cladosporium* species was found on raspberries collected from the UK, with the predominant species being *Cladosporium cladosporioides*. This species is abundant within airborne spore samples (Harvey, 1967; Rodríguez-Rajo *et al.*, 2005), indicating that airborne spores are likely an important primary inoculum source. Further investigation into how airborne inoculum is affected by husbandry practices such as venting are warranted in the future. For the purpose of our future experiments, *C. cladosporioides* was the focus species; further investigations into the other *Cladosporium* species found is therefore needed in the future. This study, to the authors' knowledge, is the first to isolate *C. fusiforme* from plant material, suggesting it may be present on other crops. Three *C. fusiforme* isolates were collected from one farm in Kent,

indicating that a particular niche within that farm may have been suitable for this species. For more information, refer to section 3.5. of the thesis.

5.2. When are Raspberries Susceptible to *Cladosporium* Skin Lesions and Stigmata Infections?

Raspberries were susceptible to skin lesions from the ripening stage of fruit development onwards, with ripe fruit being more susceptible than ripening fruit. It is, however, important to note that during this experiment there were no outbreaks of aphids. Excess honeydew from aphids can provide a food source for sooty moulds like *Cladosporium* to colonise. The pest SWD has also been shown to prefer ripening fruit to green fruit, due to the contrast in colour with the surrounding foliage (Little *et al.*, 2017). The damage from SWD oviposition provides entry points for saprophytic fungi to colonise (Lewis *et al*., 2019). Despite the increased susceptibility to skin lesions over time, stigmata were susceptible to infection from the green stage of fruit development. More research is needed into *Cladosporium* spore survival to determine if spores that have germinated on the stigmata earlier in development can survive long enough to cause subsequent skin infections when conditions are right, such as when fruit are placed into punnets with poor ventilation and stigmata are pushed into the delicate skin surface.

As raspberries are continually fruiting, it may be difficult to time the application of chemical fungicides safely to prevent excess residue build-up. The use of BCAs may be a more effective measure for controlling pre-harvest outbreaks of *Cladosporium.* For more information, refer to section 3.5. of the thesis.

5.3. Investigating the Inoculum Load on the Surface of Raspberries

Cladosporium was the predominant genus of fungi present on the surface of raspberries. These were healthy raspberries with no visual symptoms of *Cladosporium* skin lesions; *Cladosporium* can therefore be dominant on the fruit surface without causing disease. Fungal diversity on the fruit surface increased during ripening, whereas bacterial diversity decreased, perhaps explained by more fungi landing on the fruit surface over time via airborne dispersal, so then bacteria may have been outcompeted on the ripe fruit surface. It is important to remember that these are dynamic communities of organisms, and therefore many factors not recorded in the experiments will be impacting diversity.

Interestingly, *Cladosporium* decreased in abundance as fruit ripened, the opposite of what we had originally predicted. It is important to note that the fruit we collected from were disease free (not displaying visible symptoms of any pathogens), perhaps suggesting *Cladosporium* will struggle to become pathogenic in the absence of wounding from SWD and when plant husbandry is managed

effectively. While *Cladosporium* was less prevalent on ripening and ripe fruit, a bacterial genus called *Rouxiella* displayed the opposite trend. A member of this genus (*R. badensis)* has been previously tested as a biocontrol for strawberries, where effective control of common pathogens such as *Botrytis cinerea* was achieved in detached fruit assays (Morales-Cedeño *et al.*, 2021). In the present study, bacteria could only be identified to the genus level due to the primers and database used in bioinformatics. More targeted sequencing in the future may allow identification of the *Rouxiella* species present, which may provide a new source of biocontrols for future commercialisation. For more information, refer to section 4.5.1. of the thesis.

5.4. Variations in Airborne *Cladosporium* Inoculum in a Polytunnel vs. an Open Field

Across both experiments, *Cladosporium* was significantly higher inside the raspberry polytunnel than the open field 100 m away. Potentially, the increased number of plants with decayed leaves, receptacles, and other materials, may have provided sources for *Cladosporium* to colonise and sporulate. Further research into how polytunnel infrastructure impacts pathogen dispersal is needed, particularly to understand how to safely reduce the inoculum load within tunnels through venting. *Cladosporium* airborne spores peaked in number in the daytime, specifically in the afternoon period, and overall fungal DNA was lower in the night. This is likely due to *Cladosporium* requiring low humidity for spores to be removed from conidial chains; in contrast, many fungal species require high humidity to release spores. It is important to note that while low humidity can aid spore release for *Cladosporium*, it is high humidity that aids spore germination and subsequent visible mycelial development (Dickinson and Bottomley, 1980). By building a larger dataset of how *Cladosporium* spores disperse within polytunnels, existing *Cladosporium* spore models used for allergen warnings could be modified and used for disease risk within tunnels. For more information, refer to section 4.5.2. of the thesis.

5.5. Assessing the Efficacy of Biocontrol Agents Against *Cladosporium*

All BCAs tested were effective at reducing the mycelial growth of *Cladosporium* in plate assays. This was only performed on one strain of *C. cladosporioides*, hence further investigations on more species and strains of *C. cladosporioides* are needed to ensure BCAs are effective across multiple isolates. The bacterial BCAs grew poorly on the 3% raspberry media, perhaps due to the low acidity of the raspberries. This may raise concerns for using bacterial BCAs when SWD is also present and causing wounding of the fruit, as the high acidity juice may make bacterial BCAs less effective than fungal BCAs (Rousk *et al.*, 2009).

Both preventative and curative field applications of BCAs were effective at reducing the number of fruit with *C. cladosporioides* skin lesions. The most effective species in both preventative and

curative applications was the *Trichoderma* sp.; this is likely due to these species using multiple mechanisms, such as volatile release and hyphae that hyper-parasitise other fungi (Asad, 2022). The *B. amyloliquefaciens* strain FZB24 and *B. subtilis* strain QST 713 were also effective at reducing the number of infected fruit. In these experiments, fruit quality measurements were not taken, but are essential in future experimental applications to ensure that BCAs will not affect fruit quality measures on commercial farms. While this isn't important for already licensed products, other products that have yet to be approved on raspberries will require such experiments for future licensing.

For curative applications, the BCAs were only applied 24 hours post *Cladosporium* inoculation. Applying the BCAs for a longer period post-inoculation will provide a better understanding of the efficacy of these products. These products were also applied with a handheld sprayer, and therefore coverage was likely better than what can be achieved with commercial tractor sprayers. Repeats testing both commercial sprayers and reduced doses of BCAs will provide more realistic applications of products to determine if efficacy of control can be maintained in commercial conditions. For more information, refer to section 5.5. of the thesis.

5.6. Screening Raspberry Varieties for Resistance to *Cladosporium* Skin and Stigmata Lesions

Unfortunately, due to both extreme temperature stress (in excess of 35 °C in 2022) and plant losses, we could not determine which varieties of raspberries were significantly more or less susceptible to *Cladosporium* skin lesions. Further repeats of this experiment are needed with more plants and more repeats in time. We would also suggest obtaining more varieties that fruit at similar periods, and targeting inoculations to the periods these varieties fruit in the growing season (early, mid or late season). This is due to the changes in unique pest pressures and environmental conditions plants will be subjected to across the season, so separating inoculations into different sections of the season will better represent the conditions each variety is subjected to commercially.

Some varieties did show significant differences in their susceptibility to stigmata infections, with Malling Charm being more susceptible than Cascade Gem and Malling Bella. This variety, however, was likely infected asymptomatically with *Phytophthora* in the 2021 growing season, as the plants were subsequently killed by *Phytophthora* in 2022. If this disease was afflicting plants and their ability to uptake water, parts of the plants that senesce (such as the stigmata) were likely decaying at an accelerated rate due to stress. This could then have aided subsequent *Cladosporium* colonisation. This highlights the need for plant material obtained for use to be

healthy, and to view plant health holistically. For more information, refer to section 6.5. of the thesis.

5.7. Action Points for Growers

- Reduce the amount of dead material in crops for *Cladosporium* to colonise, for example, remove pruned canes and leaves as soon as possible.
- Control SWD effectively throughout the season to prevent wounds on fruit for saprophytic fungi to colonise. This can include hygiene picks to remove affected fruit, using available spray products, deterrents and traps currently available.
- Try to control aphids in a timely manner to prevent honeydew buildup. If using plants for two years and an aphid outbreak has occurred in year one, ensure plants are sprayed with an aphicide in the autumn to reduce over-wintering aphids.
- If a severe aphid outbreak has occurred with honeydew present, discuss with a BASIS qualified agronomist about spraying with water (and an adjuvant) to wash off excess honeydew and prevent sooty moulds colonising.
- Ventilate your polytunnels to prevent humidity build-up. While venting may allow *Cladosporium* spores to spread, high humidity will encourage spore germination and mycelial growth, resulting in skin lesions.
- Monitor for visible symptoms of *Cladosporium* when its food sources are available (e.g. honeydew after aphids, excess nectar production in flowers, or excess dead material in the crop canopy). In the absence of these factors, look for signs of skin lesions from the ripening stage of fruiting onwards.
- Some of the products applied in the BCA screens are licensed for use on raspberries in the UK. However, we advise you to consult with a BASIS qualified agronomist to discuss the availability and licensing of such products, as these were experimental applications at the maximum label recommended dose.

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