Project title:	The epidemiology and management of *Cladosporium* on raspberry
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Industry Representative:	[Name, Organisation, Address]
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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.

Lauren Farwell

PhD Student

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GROWER SUMMARY

Headline

Raspberries appear susceptible to *Cladosporium* skin lesions during the ripening and ripe stages of fruit development, and the stigmata may provide a mechanism for *Cladosporium* to colonise the fruit's surface.

The most frequently isolated species of *Cladosporium* found on raspberries in the UK is *Cladosporium cladosporioides.*

Background

Despite *Cladosporium* being capable of causing considerable crop losses to raspberry growers, little research has investigated the ecology and behaviour of this pathogen to provide potential methods of control. This PhD aims to investigate which environmental and biological factors impact *Cladosporium* growth and presence on fruit. From these studies, we hope to infer husbandry practices and potential methods of control to reduce the risk of *Cladosporium* lesions on raspberries.

Summary

In 2020, a proprietary variety of raspberry was inoculated with either a *Cladosporium* inoculum or a water control inoculum at four different stages of development (flower, green fruit, ripening fruit and ripe fruit). Fruit was assessed for skin lesion development and stigmata infections. It was found that the fruit was susceptible to *Cladosporium* during the ripening and ripe fruit stages. This suggests that agronomists and growers should begin looking for symptoms of *Cladosporium* from the ripening stage onwards.

Samples of raspberries were taken from both supermarkets and directly from growers across the UK, allowed to rot naturally and *Cladosporium* strains were isolated from these fruits. These were then sequenced to provide a molecular identification to the species level. Of 43 isolates collected, the most frequent species was *C. cladosporioides*. As *C. cladosporioides* is one of the most abundant fungal air-borne spores, it indicates that this is probably an important primary inoculum source, and therefore husbandry practices such as venting may impact on the inoculum load of *C. cladosporioides* in the fruit crop canopy.

Financial Benefits

This project on the key windows of opportunity for fruit susceptibility to *Cladosporium* will be beneficial for the development of appropriate management strategies for control. This will allow more precision in the application of appropriate control measures which will prevent the use of ineffective approaches.

Future work will include the screening of potential biological control agents against *Cladosporium,* with the aim of providing advice to growers for more effective and economic management of *Cladosporium* on raspberries.

Action Points

Agronomists and growers should be more aware of the symptoms and visual assessment of *Cladosporium* skin lesions on raspberries from fruit ripening onwards.

Previous research has also shown that the Spotted Winged Drosophila (SWD) can create entry points in ripening raspberries for *Cladosporium* to become established (Swett *et al.* 2019). Therefore, good management practices against SWD will likely decrease the risk of *Cladosporium* infection on ripening fruit.

More effective hygiene to effectively remove senescent foliage and crop debris would be beneficial in reducing the sources of inoculum of *Cladosporium* in raspberry crops.

SCIENCE SECTION

Introduction

Cladosporium is a fungal genus containing several hundred species (Bensch *et al.* 2012) and has been recorded by both growers and academics as causing skin lesions on raspberries, making fruit unmarketable. One study found that *Cladosporium* was present on over 50% of raspberries post-harvest at one trial site alone (O'Neill *et al.* 2012). Even though *Cladosporium* can be ubiquitous on fruit and cause substantial losses, it is an understudied fungus, with only one previous study focusing on *Cladosporium* on raspberry but with a focus more on entomological interactions (Swett *et al.* 2019). To create effective management plans against *Cladosporium*, we require further knowledge on factors that influence the growth, inoculum sources and the infection processes of this fungus.

This PhD is aimed at studying the epidemiology of *Cladosporium* on raspberries to provide information on which biotic factors, abiotic factors and husbandry practices may influence the risk of *Cladosporium* development on raspberries. The project also includes the screening of commercially available biological control agents as a component of the control strategies to reduce *Cladosporium* infection of raspberries.

For this report, I have focused on two experiments conducted in the 2020/2021 seasons which were to determine:

(1) Which species of *Cladosporium* are predominant in UK and Spanish raspberries?
(2) Which stages of raspberry development are most susceptible to *Cladosporium* skin lesions and stigmata infections?

Materials and methods

(1) Which species of *Cladosporium* are present on UK and Spanish raspberries?

Summary: Raspberries were collected from growers across the UK and Spain. The *Cladosporium* strains were all isolated and sequenced in two different gene regions to determine via DNA barcoding the predominant species colonising raspberries.

Sample Collection. Raspberries were either bought from supermarkets in punnets (all the Spanish fruit were derived in this way) or sent to us directly by growers. The raspberries were incubated in a bagged tray in ambient room conditions for 4 days to accelerate fungal growth. Fruit with *Cladosporium* lesions were dissected and diseased parts were directly plated onto a Potato Dextrose Agar in 9 cm Petri dish (supplemented with NaCl to inhibit bacterial growth). The fungal species considered to be *Cladosporium* species were then sub-cultured onto fresh PDA medium to obtain pure cultures, and these were subsequently used for molecular identification.

DNA Extraction and Amplification. DNA from the pure isolates were extracted using a rapid fungal DNA extraction method utilising Sigma extraction and dilution buffers. Once the DNA was extracted, a PCR was performed on the Trans Elongation Factor α (TEF1α) and Actin (ACT) regions (shown in previous taxonomic studies to be the best regions at identifying *Cladosporium* to the species level; Bensch *et al.* 2012) using the TEF1α primers (EF1-728F and EF-2R), and the ACT primers (ACT-512F and ACT-783R). The PCR cycler was set to the same programme as described by Swett *et al.* (2019). The PCR products were checked by running them on a 1.5% agarose gel for 45 minutes at 100V with a 1kb base pair ladder. If strong bands were seen, products were diluted 1:10 for the final PCR product to be sent for sequencing.

- **DNA Sequencing and Isolate I.D.** The PCR products were then sent to Eurofins via their lightrun service for Sanger sequencing of the forwards and reverse of the ACT and TEF1α regions. The chromatograms were trimmed to remove poor end regions and any sequences with signs of contamination were removed. Each gene region then had the forwards and reverse combined into a consensus sequence using the software Geneious (v. 2019.2.1). The consensus sequences were then run through the BLASTmega database (v. 2.12.0+) to look for a potential species I.D. based on the e-value and the percentage identity. If multiple species showed similar scores in the search results, the identity was resolved as ambiguous.

(2) Which stages of raspberry development are susceptible to *Cladosporium* skin lesions and stigmata infections?

Summary: A proprietary variety of raspberry was inoculated with either a *Cladosporium* inoculum or a control (0.1% tween 20 + 0.5% NaCl) to determine which stages of fruit development are more susceptible to visible *Cladosporium* skin lesions and stigmata infections. As *Cladosporium* has mainly been previously reported as occurring post-harvest, we expected to see *Cladosporium* skin lesions present at ripening but not during earlier development. We also expected to see more *Cladosporium* growth on stigmata that had not been sterilised (indicating post-harvest growth) vs those that had been sterilised (indicating pre-harvest growth).

Two separate hypotheses were examined in this study:

(2.1) Is there a difference in the post-harvest *Cladosporium* skin lesions scores across age?

This would help to evaluate what stages of fruit development are susceptible to *Cladosporium* skin lesions and therefore when best to apply control measures.

(2.2) Is there a difference in the stigma infection scores across fruit ages and pre- vs post-harvest?

To elucidate if the stigma can provide a nutritional source for *Cladosporium* colonisation, and if colonisation changed across fruit development and pre- vs post-harvest.

-**Experimental Design and Set-up.** The experiment was conducted from July 2020 to October 2020 at NIAB EMR in a non-commercial raspberry polytunnel. In total, 32 pots of a proprietary variety of raspberries, with 3 canes per pot, were arranged into a randomised block design: there were 2 rows, each with 4 blocks (8 blocks in total). Three inoculations were performed over time. This study was focused on two factors: the stage of fruit development (the four stages of development were flowering, green, ripening, and ripe fruit as shown in Figure 1) and whether raspberries were sterilised or unsterilised (indicating pre- vs post-harvest susceptibility to colonisation by *Cladosporium* of raspberries).

Figure 1. From left to right: the four stages of fruit development assessed for susceptibility to *Cladosporium* skin rot.

Fruit was inoculated with one of three *Cladosporium cladosporioides* strains or a control using 0.1% Tween-20 and 0.5% KCl (used to produce the *Cladosporium* spore suspensions). These were considered as co-variables as *Cladosporium* was present across all treatments (with the control showing the naturally occurring *Cladosporium* present without *Cladosporium* inoculations).

Each pot was inoculated with only one inoculum, and when branches were inoculated the bag used to incubate the branch was held over to prevent drift of inoculum. Each inoculation was performed on one row of the polytunnel, and then alternated to avoid cross contamination (as infection – any visible rotting – would be removed before the next inoculation).

- **Inoculations.** At each inoculation timing, one row (four blocks) was inoculated. Each block contained seven pots, with the four middle pots being inoculated. Between two to three branches were selected for inoculation and sprayed with a hand sprayer with one of the three isolate inoculums (adjusted to 10^7 spores) or the control. They were then covered with a polythene bag, plugged with a damp cotton bung which was held on with a cable tie. The bag was left for 24 hours and then removed, and all the fruit on the branch was removed into their own punnets. Afterwards branches were selected to either be sterilized or left unsterilized, and fruit then placed into punnets covered with a polythene bag and incubated for a further four days.

- **Assessments.** After four days fruit were scored for the number of stigmata with *Cladosporium* and the number of drupelets with *Cladosporium* (Table 1).

Table 1. The scoring system used to assess the raspberry fruit in the experiments.

	Definition of Score					
Score	0	1	2	3	4	5
Number of stigma infected with *Cladosporium*	No stigma infected	1-20% of stigma infected	21-40% of stigma infected	41-60% of stigma infected	61-80% of stigma infected	81-100% of stigma infected
Number of drupelets infected with *Cladosporium*	No drupelets infected	1 drupelet infected	2 drupelets infected	3 drupelets infected	4 drupelets infected	5+ drupelets infected

- **Statistical Analysis.** The results were analysed using ordinal regression models in r (v. 4.0.3) using the ordinal package (v. 2019.12-10). As green fruit all scored 0 for skin lesions, they were redacted from the ordinal model for hypothesis 2.1.

Results

(1) Which species of *Cladosporium* are present on UK and Spanish raspberries?

We successfully sequenced the TEF1α and ACT genes of 43 UK isolates and 14 Spanish isolates of *Cladosporium* (Figure 2).

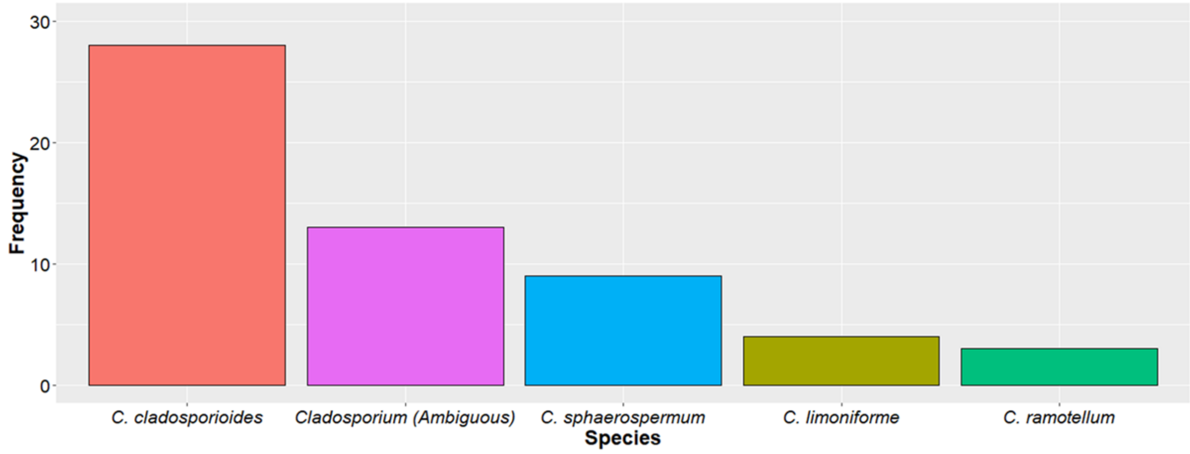

Figure 2. The frequency of isolation of different *Cladosporium* species from UK and Spanish raspberries. A total of 57 isolates were sequenced in total, of which 14 were from Spanish and 43 from UK fruit.

The most frequent species was *C. cladosporioides* (28 isolates), with the next most abundant group being ambiguous species (13 isolates). The less frequently found species included *C. sphaerospermum* (9 isolates), *C. limoniforme* (4 isolates) and *C. ramotellum* (3 isolates).

Of the Spanish isolates, 11 were *C. cladosporioides,* and 3 were *C. sphaerospermum*.

(2) Which stages of raspberry development are susceptible to *Cladosporium* skin lesions and stigmata infections?

2.1 Which stages of raspberry development are susceptible to *Cladosporium* skin lesions and stigmata infections?

An ordinal regression analysis revealed that (a) inoculation date (df=2, $p<0.05$), inoculum (df=3, $p<0.01$) and (b) fruit stage (df=1, $p<0.00$) contributed towards explaining variation in skin lesions scores in a Wald test.

As fruit progressed from the ripening to the fully ripe stage of development, the chances of developing skin lesions increased with an odds ratio of 2.04, Wald $X^2(1)= 35.46$, $p<0.00$.

2.2 Is there a difference in the stigmata infection scores across fruit ages and pre vs post-harvest?

An ordinal regression analysis determined the factors (a) inoculation date (df= 2, $p<0.00$), (b) inoculum (df= 3, $p<0.00$), and (c) fruit sterilisation (df=1, $p<0.00$) all contributed to explaining variation in stigmata infection scores. However, the fruit age did not significantly account for variation in stigmata infections (df= 3, $p=0.069$). There was a significant interaction between sterilisation and fruit stage (df= 3, $p<0.00$), and a post-hoc test revealed that there were much higher stigmata infection scores in unsterile flowers versus sterile flowers (as shown in Figure 3).

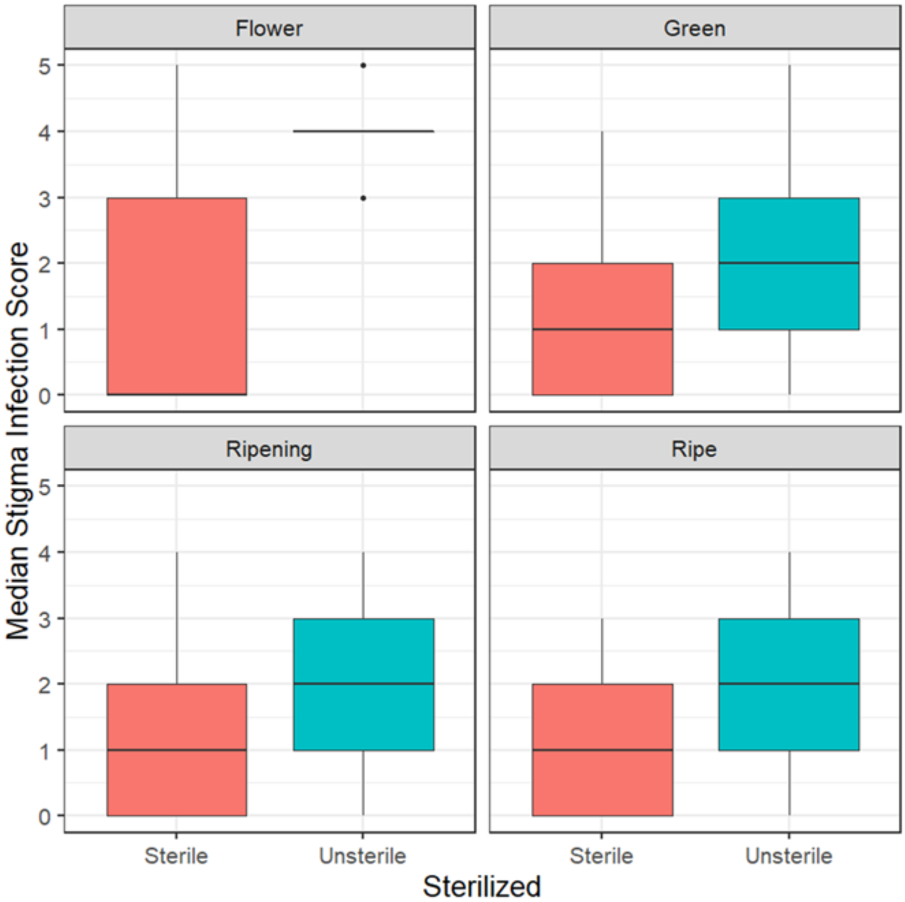

Figure 3. The median stigmata infection scores across fruit ages and between sterile (pre-harvest) and unsterile (post-harvest) stigmata.

Discussion

This project has identified that *C. cladosporioides* is the most frequent species on UK fruit. This correlates with knowledge of fungal air spora studies that have found the *C. cladosporioides* is a predominant component (Aira *et al.* 2012) and implies that air-borne spores may be an important inoculum source. Many studies have discussed the impact of environmental factors on the abundance of *Cladosporium* spores in the general air outside. However, to my knowledge none have investigated if these factors similarly impact on agricultural environments such as polytunnels. Hence it is vital for future work to investigate if the inoculum load is similarly impacted in polytunnels as to outdoor environments, as the disease risk will vary accordingly. Interestingly, several isolates could not be identified using the ACT and TEF1α genes. Potentially these are isolates with few records in the BLAST database or may be unrecorded species leading to ambiguous results. Recent research has also found there to be uncertainty in the BLAST results, with some *C. cladosporioides* isolates not being updated to their new species name, *C. anthropophilum* (Swett *et al.* 2019). Due to these ambiguities, additional phylogenetic analysis will be performed to determine if the species labels for the isolates are correct and to try and solve the ambiguous sequences.

We have confirmed that the stages of raspberry fruit development susceptible to skin lesions are from the ripening stage of development onwards. This complements previous research that discovered *Cladosporium* can grow pre-harvest on raspberries (Swett *et al.* 2019). However, the present study has now confirmed that green fruit are not susceptible to *Cladosporium* and it is likely to be more likely to occur close to harvest. This means growers and agronomists should search for skin lesions only from the ripening stage onwards, and any future work into control methods for *Cladosporium* should also focus on appropriate strategies around this growth stage. Despite finding no evidence of *Cladosporium* lesions on green fruit in this study, skin lesions may occur under specific circumstances. For instance, *Cladosporium* has been recorded as a sooty mould on other crops (Tashiro *et al.* 2013), and potentially when outbreaks of insects such as aphids occur, it may cause an increase in honeydew on the surface of green fruit allowing phyllosphere fungi such as *Cladosporium* to grow and result in visible lesions on the fruit surface. It is also important to note that a study focusing on the impact of SWD on *Cladosporium* demonstrated that when fruit are damaged by this pest, it produces openings for fungal pathogens to gain easy access to the rich fruit nutritional source (Swett *et al.* 2019). It is therefore important when controlling plant pathogens, to consider a more holistic approach to plant health to aid in disease prevention.

As inoculums were produced using different isolates of *Cladosporium*, the inoculum was a co-variable in the analysis which was shown to be significantly different in causing skin lesions and stigmata infections. This implies further work to investigate the pathogenicity of *Cladosporium* isolates may be warranted.

In addition, this study has shown that *Cladosporium* can grow on the stigmata of raspberries both pre- and post-harvest. However, *Cladosporium* appears to predominantly grow on stigmata post-harvest. *Cladosporium* has been previously documented as growing on the surface of plant material, rather than producing hyphae that are able to enter the fruit (Briceño & Latorre, 2008). Hence it would be plausible to assume that the *Cladosporium* growing on stigmata are primarily growing on the surface of the decaying/senescing material. Consequently, the stigmata may provide a means for *Cladosporium* to colonise from an earlier stage of fruit development, perhaps surviving as an endophyte, and then subsequently occurring as visible symptoms later when the fruit is more susceptible.

It was observed that there were significantly higher stigmata infection scores in flowers that were unsterilised vs sterilised. This is likely due to the architecture of the flower creating an ideal microclimate that can retain moisture more than raspberry fruits, therefore aiding fungal development. *C. cladosporioides* has been found to cause strawberry blight in strawberry plants in Korea (Nam *et al.* 2015), but it is currently unclear if similar infections occur in raspberry flowers.

Potentially, with developments in fruit breeding, the stigmata on raspberries could be bred to abscise once fruit development is underway to decrease potential plant material for pathogens to colonise. The application of control measures such as biocontrol products earlier in plant development may decrease colonisation by *Cladosporium* species. However, the timing of such applications would need to be carefully considered in the context of the cost-benefit analysis of applying such products for controlling this pathogen and the reduction in losses. Thus, studies will be carried out to screen commercially available biocontrol products, both on raspberry-based media and on fruit to identify the potential for controlling *Cladosporium* on raspberry fruit. With the best candidates, pilot scale experiments of these will be carried out to develop appropriate recommendations to growers.

Conclusions

C. cladosporioides is the most frequently found species of *Cladosporium* on UK fruit, indicating that air-borne spores may be an important primary source of *Cladosporium* on raspberries. Potential husbandry methods such as venting and improved hygiene may influence the inoculum load present. However, further work is needed to determine how raspberry agronomy and harvesting may influence the inoculum in the canopy of raspberry plants, especially in polytunnels.

We have determined that *Cladosporium* can only cause skin lesions of raspberries from the ripening stage onwards and is therefore a close to harvest pathogen. Agronomists and growers should thus have knowledge and be able to identify *Cladosporium* skin lesions from these stages onwards. *Cladosporium* predominantly grows on stigmata post-harvest but is capable of growing pre-harvest from flowering onwards. Therefore, *Cladosporium* may be able to colonise stigmata to then subsequently cause skin lesions. Potentially, effective biocontrol products could be used to reduce the colonisation of raspberries by *Cladosporium.* The economics of such control strategies would need to be evaluated.

Knowledge and Technology Transfer

Presentations:

2021 CTP Summer Event

2021 Berry Gardens Autumn Event

2021 NIAB EMR Soft Fruit Technical Day

Poster Presentations:

2021 CTP Autumn Event

2021 BCPC Congress

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Appendices

Raw data for experiments shown available on request to: Lauren.Farwell@NIAB.com.

