Project title:	**Engineering tolerance to *Botrytis cinerea* in soft fruit crops**
Project number:	CTP_FCR_2020_11
Project leader:	Dr Charlotte Nellist (NIAB), Dr Helen Cockerton (NIAB EMR), Prof Matthew Dickinson (University of Nottingham), Assoc Prof Tim Robbins (University of Nottingham)
Report:	Annual report, October 2021
Previous report:	NA
Key staff:	Finlay Bourquin
Location of project:	NIAB
Industry Representative:	Harriet Duncalfe, Berry Gardens
Date project commenced:	01/10/2020

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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.

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CONTENTS

GROWER SUMMARY

Headline

Investigating the molecular mechanisms behind *Botrytis cinerea* infections of different strawberry organs. The results obtained during this PhD will increase our understanding of this pathosystem and could lay the groundwork for producing *B. cinerea* resistant strawberry plants.

Background

Botrytis cinerea is an extremely virulent necrotrophic plant pathogen. It is responsible for causing fruit rot in strawberries. Strawberries are an exceedingly valuable soft fruit crop with almost 9 million tonnes being produced globally in 2019[1]. In the same year, the value of strawberry production exceeded $2.5 billion in the US[2] and £350 million in the UK[3]. Not only are strawberries a high value crop, they also have numerous benefits for human health; they contain high levels of vitamin C and phenolics like anthocyanins[4], which have been shown to reduce the risk of many disorders including cancers, cardiovascular diseases and diabetes[5,6].

B. cinerea is particularly concerning since there are a myriad of factors that make it difficult to control. It can initiate disease through primary infection (infecting the flowers) and secondary infection (infecting the fruit receptacles)[7]. It can infect all of the above ground parts of a plant, including the seeds[8], through various sources of inoculum such as overwintering sclerotia (hardened mass of mycelia), mycelia from neighbouring plants and conidia[9]. It has a quiescent stage of infection where it appears asymptomatic[10], enabling it to become established in plants and ultimately leading to pre- and post-harvest crop losses. Currently, the most effective control methods are fungicides. Unfortunately, *B. cinerea* is able to recurrently develop resistance to different fungicides, with the number of multi-pesticide resistance phenotypes increasing in *B. cinerea* populations across different countries[11,12]. Moreover, there are growing concerns from the public about fungicide residues on crops and in the environment[13]. It is clear that novel control methods are paramount to prevent the spread of this fungus.

In order to develop novel control methods, it is crucial to understand the mechanisms behind the *B. cinerea*-strawberry pathosystem. This project aims to examine susceptibility and resistance factors in strawberry and virulence factors in *B. cinerea.*

Summary

This project aims to elucidate some of the mechanisms behind *B. cinerea's* success as a strawberry pathogen. To do this pathogenicity tests are being developed to examine differences in virulence between 31 *B. cinerea* isolates, these tests will be conducted on strawberry leaves, fruits and flowers to capture the mechanisms this fungus uses for infection. To date, preliminary pathogenicity tests have revealed 5 x 10^5 spores/ml is the optimum spore concentration for discerning differences in virulence between isolates. Optimising these pathogenicity tests on the different strawberry plant organs is currently ongoing.

To study how *B. cinerea* causes disease, isolates will be subjected to UV radiation, inducing random mutations throughout their genomes. The isolates will then undergo pathogenicity tests to examine changes in virulence; any isolates that exhibit a change in virulence will be genome sequenced in order to identify the responsible genomic loci. This will reveal certain mechanisms that *B. cinerea* is using to cause infection.

In order to examine strawberry susceptibility or resistance factors to *B. cinerea,* an ethyl methanesulfonate (EMS) population of *Fragaria vesca* plants will be produced; this is a population of plants that will have random mutations in their genomes. *F. vesca,* unlike *Fragaria* x *ananasssa,* is a diploid and therefore is a less complex organism to study. These plants will be screened for changes in susceptibility or resistance to *B. cinerea* using the developed pathogenicity tests; plants of interest will have their genomes sequenced to identify genomic areas involved in successful infection. This will reveal targets to examine in the cultivated strawberry (*Fragaria* x *ananassa*). This knowledge will lay the groundwork for future research to produce *B. cinerea* resistant strawberry plants. Since *F. vesca* is the dominant subgenome of cultivated strawberries[14], this work may have implications for the horticultural industry. This could occur if desirable genomic loci detected in *F. vesca* are conserved in the cultivated strawberry. A potential avenue could be the production of selective markers for breeding or genetic modification depending on legislations. Either of these avenues could lead to the future production of *B. cinerea* resistant strawberry cultivars.

Financial Benefits

There are no financial benefits to report. However, the potential results from this work could aid in developing more resistant strawberry plants and hence reduce harvest losses for growers and consumers.

Action Points

This is the first year of this project, currently there are no action points to suggest.

SCIENCE SECTION

Introduction

Botrytis cinerea is a fungal plant pathogen which causes fruit rot in strawberries. Strawberries are a high value crop with a market value exceeding £350 million in the UK in 2019[3]. Therefore, the ability to control pathogens, such as *Botrytis*, which reduce yields will be extremely beneficial for the economy, farmers, and consumers. Gaining insight into how *B. cinerea* causes disease in strawberries and the molecular interactions that are occurring during infection is one of the first steps in the production of new targeted control methods.

There have been many technological advances which will aid in investigating the *B. cinerea*-strawberry pathosystem. For instance, the genomes of 37 *B. cinerea* isolates[15–20], *Fragaria* x *ananassa*[14] and *Fragaria vesca*[21] have been sequenced. Additionally, transcriptome analyses have been conducted for several host species of *B. cinerea,* ranging from fruits (including strawberries) and vegetables, to ornamental plants and mosses[22–31]. This genomic and transcriptomic data is useful for comparative and functional studies and allows us to investigate *B. cinerea* virulence factors and strawberry susceptibility factors. Virulence factors are molecules that help a pathogen cause disease, whereas susceptibility factors are genetic elements that increase the likelihood of disease in the host.

This project aims to develop pathogenicity assays for strawberry leaves, fruits, and flowers; these assays are crucial for observing differences in virulence and susceptibility. An optimum spore concentration of 5 x 10^5 has been ascertained through preliminary pathogenicity assays, a leaf pathogenicity test had been developed and producing reproducible assays on fruits and flowers is underway. Through these assays it can be determined which isolates are the most pathogenic and hence which isolates to use for further study.

To investigate *B. cinerea* virulence factors, isolates will be mutated using UV radiation. The mutated spores will be subjected to pathogenicity tests to observe if there are any changes in virulence between isolates and between strawberry plant organs. A change in virulence following mutation implies an area of the genome that is involved in pathogenicity was mutated. Therefore, isolates that exhibit this change in phenotype will have their genomes sequenced to identify the genomic area of interest.

A method of similar design will be employed to test for strawberry susceptibility factors. Here a population of *F. vesca* plants will be exposed to the chemical ethyl methanesulfonate (EMS), inducing random mutations throughout the genomes. Pathogenicity tests using these mutated plants will be carried out to discern any changes in susceptibility. Like with mutating *B. cinerea*

isolates, plants that display changes in susceptibility will be sequenced to evaluate the area of the genome responsible for said change in phenotype.

So far, pathogenicity tests are being developed to aid the discovery of novel virulence and susceptibility factors with the hope of enhancing our understanding of how *B. cinerea* is causing disease in strawberries.

Materials and methods

B. cinerea isolates

Isolates were obtained from various locations and host plants (Table 1). Isolates were single-spored to limit genetic variation. Single spores were isolated by dislodging the spores, with a spreader from sporulating cultures with 10 ml of sterile distilled water (SDW), producing a spore suspension. The spore suspension was filtered through a Mira cloth to remove mycelial fragments and the spore concentration was checked using a hemocytometer. Serial dilutions were produced and 100 µL of each dilution was plated onto 1.5 % water agar (WA) and left to incubate at room temperature. Once spores had germinated, they were isolated, sub-cultured onto potato dextrose agar (PDA) and incubated at room temperature. Once sporulated, spore suspensions were produced as described above, except using 5 mL SDW. These spore suspensions were then used to create 50 % glycerol stocks maintained at –80 ˚C.

To ensure the isolates were *B. cinerea* DNA was extracted from single-spored isolates and the internal transcribed spacer (ITS) region was sequenced. DNA was extracted as described in Lui *et al.*[32]. ITS1 (5’ – TCCGTAGGTGAACCTGCGC – 3’) and ITS4 (5’ – TCCTCCGCTTATTGATATGC – 3’) primers were used to amplify the ITS region as described in Walch *et al.*[33], without using bovine albumin serum. The PCR products were then purified using the Monarch® PCR & DNA Cleanup Kit and sent for sequencing following Eurofins specifications. PCR was also performed using *B. cinerea* specific primers, again ensuring the isolates were in fact *B. cinerea*, as described in Rigotti *et al*.[34].

Table 1. *Botrytis cinerea* isolates investigated in this study.

Isolate	Host plant isolated from	Date isolated	Source
R59/12	Jazz apple	02.02.2012	Newmafruit coldstore
R36/19	Cox apple	11.04.2019	Mansfields (FWM) Chartham coldstore
R3/20	Strawberry	26.01.2020	South Park, East Malling Research
R4/20	Strawberry	27.08.2020	Ditton Rough, East Malling Research
NHF	Strawberry	n/a	n/a
CHF	Strawberry	n/a	n/a
B101	n/a	18.05.1994	n/a
B102	n/a	18.05.1994	n/a
B103	n/a	18.05.1994	n/a
B104	n/a	18.05.1994	n/a
B105	n/a	18.05.1994	n/a
B106	n/a	18.05.1994	n/a
B107	n/a	18.05.1994	n/a
21009	Apple	24.07.1998	n/a
21010	Apple	24.07.1998	n/a
21012	Pear	24.07.1998	East Malling Research
21013	Pear	24.07.1998	East Malling Research
21016	n/a	12.07.2005	n/a
21018	Raspberry cane	25.11.2005	East Malling Research
21019	Raspberry fruit	25.11.2005	East Malling Research
21020	Raspberry cane	25.11.2005	ADAS
21022	Raspberry cane	25.11.2005	ADAS
21023	Raspberry cane	25.11.2005	Rectory Farm, Oxford

21024	Tomato	10.02.2006	n/a
Bc14	Tomato	n/a	n/a
Bc15	Tomato	n/a	n/a
Bc19	Tomato	n/a	n/a
Bc20	Lettuce	n/a	n/a
Bc66	Pepper	n/a	n/a
Nos6	Rose	n/a	n/a
Nos7	Rose	n/a	n/a

n/a = not available

Fragaria vesca plants

F. vesca accession 'Hawaii 4' tissue culture plants were obtained from callus regeneration. When plant roots were approximately 5 cm in length, they were weaned onto vermiculite in a module tray and placed in a propagator, ensuring to not saturate the plants with water or to bury the crown when planting, to limit fungal growth. The plants were placed in an incubator at 21 ˚C with a 16 h day / 8 h night cycle. The vents of the propagator were closed for one week, opened the following week and the lid removed on the third week. The plants were then transferred onto M2 compost once the roots were growing at least 1 – 2 cm out of the bottom of the module. After two weeks the plants were incubated at room temperature under natural day/ night cycles.

Pathogenicity tests

Preliminary pathogenicity test on *F. vesca* leaves

Two-week-old *F. vesca* leaves were surface sterilised by soaking in 0.5 % sodium hypochlorite for 3 min, washing in 70 % ethanol, washing in SDW twice and then left to dry in a laminar flow hood. Spore suspensions were produced as mentioned previously with 5 mL SDW and adjusted to spore concentrations of 5 x 10^5, 5 x 10^4 and 5 x 10^3 for isolates R3/20, CHF and 21013. Ten µL of each spore concentration for every isolate, or SDW for the control, was inoculated onto a 1 cm leaf disc. The leaf discs were wounded at the centre using a sterile 16 G needle and placed onto 1.5 % WA, three replicates were produced per treatment. The inoculated leaves were incubated at 25 ˚C with a 16 h day / 8 h night cycle. Every 24 h for ten days the leaf discs were imaged using a BioRad ChemiDoc MP Imaging System as described in Villanueve *et al*.[35], with an exposure setting of 8 s and cy3 as a filter. This method measures the red-light intensity emitted from leaves as a measure of cell death and therefore

was used as a proxy for *B. cinerea* infection. The images were analysed using ImageJ to measure the mean red-light intensity the lesions were producing per leaf disc.

The same method will be used to conduct pathogenicity tests on all 31 isolates (Table 1), however square plates with inserts will be used to prevent cross-contamination (Figure 1). Due to the large number of isolates, treatments will have to be spread across two plates, the statistics and power analyses required for such a large number of isolates and this type of experimental set-up are being investigated.

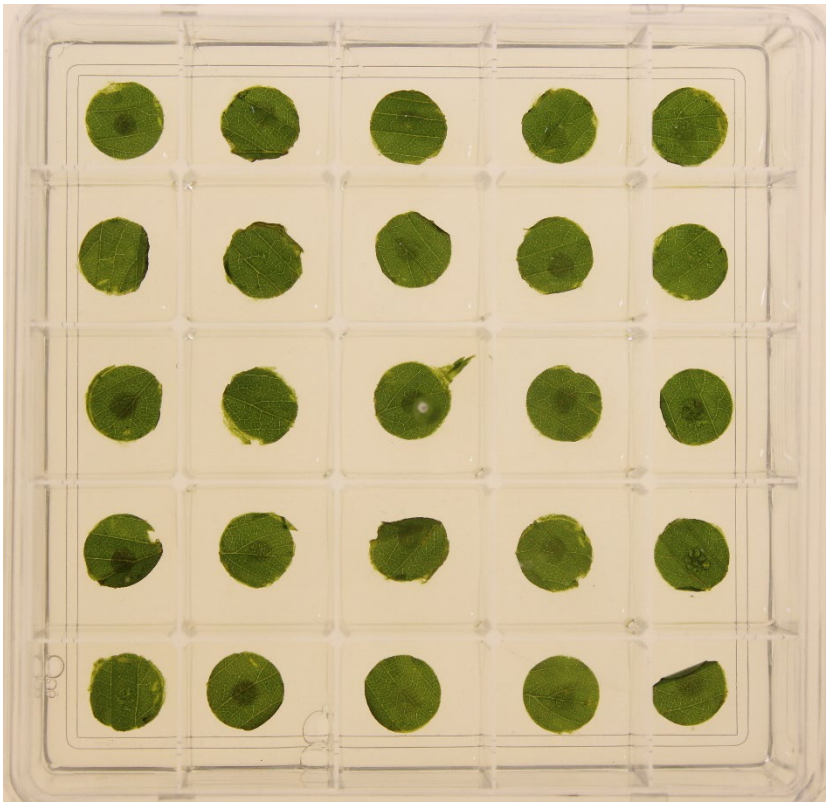

Figure 1. Leaf pathogenicity assay set-up.

Preliminary pathogenicity test on *F. x ananassa* fruit

The pedicels and sepals were cut off from *F.* x *ananassa* cv. 'Murano' fruits purchased from the supermarket. The fruits were surface sterilised in 0.5 % sodium hypochlorite for 3 min, washed twice with SDW and dried in a laminar flow hood. The strawberries were placed on sterilised coffee filter paper in a seed tray and inoculated with 10 µL of each spore suspension used for the leaf discs or SDW for the control, ensuring not to damage the exocarp. The treatment of spore suspension was randomised, and the experiment was replicated three times. The tray of inoculated fruit was placed in an autoclave bag to maintain humidity and incubated at 25 ˚C with a 16 h day / 8 h night cycle. Each fruit's height and diameter were measured with calipers prior to setting up the experiment and their surface area was

calculated under the assumption that their shape was a cone. For ten days the height and width of the lesions were measured using calipers and averaged to produce a representative diameter of the lesion. The surface area of the lesion was then calculated under the assumption that the lesion was circular. The disease incidence was then calculated by dividing the surface area of the lesion by the surface area of the fruit.

Optimising the *F. x ananassa* fruit pathogenicity tests

The following changes have been attempted to optimise the assay. The strawberries were cut in half, so every fruit has its own control and a range of temperatures, 10, 18 and 22 °C, have been trialled.

Results

B. cinerea isolates

The sequencing results of the ITS regions were searched against the NCBI database with *B. cinerea* appearing as the top search result. Additionally the PCR using *B. cinerea* specific primers amplified a product of approximately 480 bp as expected from Rigotti *et al.*[34]. Therefore, it can be confidently concluded that all 31 isolates in Table 1 are *B. cinerea*.

Pathogenicity tests

Preliminary pathogenicity test on *F. vesca* leaves

Red-light intensity measurements were analysed to determine if different spore concentrations produced statistically significant differences regarding the amount of cell death induced 10 days post inoculation. Data was normalized by dividing the red-light intensity reading of the inoculated leaf discs with that of the control leaf discs. To incorporate the time element of the study into the analysis, the area under the red-light intensity curve (AURLIC) was calculated (Figure 2). A two-way ANOVA was conducted to examine the effect isolates and spore concentration had on the AURLIC. A statistically significant difference was observed between isolates ($F_{2,15}$ = 6.559, p = 0.009). There was not a statistically significant difference between concentrations and there was no statistically significant interaction between isolates and concentrations ($F_{2,15}$ = 1.886, p = 0.186) and ($F_{4,15}$ = 2.023, p = 0.143) respectively. A post-hoc pairwise comparison was carried out between isolates at the different spore concentrations using the R[36] package emmeans[37] to identify a discriminatory concentration (Table 2).

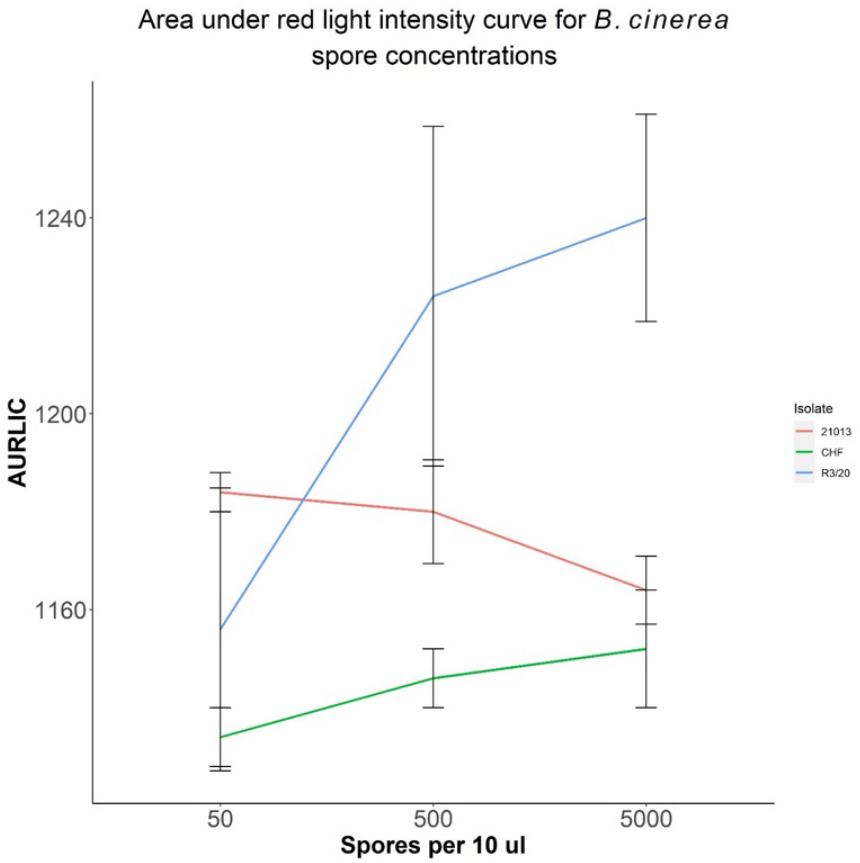

Figure 2. Area under the red-light intensity curve for three *Botrytis cinerea* isolates at three different spore concentrations 5 x 10^3, 5 x 10^4 and 5 x 10^5 which correspond to 50, 500 and 5000 spores per 10 µL of inoculum.

Table 2. Pairwise comparisons of isolates at different spore concentrations using emmeans.

Spore concentration	Pairwise comparisons	*p* value
5 x 10^3	21013 - CHF	0.2531
	21013 – R3/20	0.5652
	CHF – R3/20	0.7501
5 x 10^4	21013 - CHF	0.5126
	21013 – R3/20	0.2635
	CHF – R3/20	0.0510
5 x 10^5	21013 - CHF	0.9169
	21013 – R3/20	0.0328
	CHF – R3/20	0.0270

Preliminary pathogenicity test on *F. x ananassa fruit*

Unfortunately, six fruits had to be discarded from the study since they had succumbed to post-harvest infection rather than infection caused by the inoculum. This meant there was not enough data to conduct statistical analyses.

Optimising the *F. x ananassa* fruit pathogenicity tests

Despite the changes to the experimental design, the control halves of the strawberry were still appearing infected, therefore no conclusion regarding isolate virulence could be drawn.

Discussion

The aim of this PhD is to gain an increased understanding of the interaction between *B. cinerea* and strawberries. One way to do this is to explore how *B. cinerea* causes disease and what factors make strawberries susceptible. The preliminary pathogenicity tests on *F. vesca* leaves and *F.* x *ananassa* fruit were conducted to see if there was an ideal spore concentration for infecting strawberries, as there does not seem to be a consistent spore concentration that is used in the literature when conducting pathogenicity tests. The results of the two-way ANOVA suggested there was no statistically significant difference between spore concentrations nor was there a significant interaction between spore concentrations and isolates. However, when looking at Figure 1, it does appear that the different concentrations are causing a difference in the AURLIC depending on the isolate. Therefore, a pairwise comparison of isolates within the different spore concentrations was conducted. It is clear looking at Table 2 that at concentration 5 x 10^5 there was a significant difference between isolate R3/20 and 21013 and between R3/20 and CHF. At 5 x 10^4 there was only a significant difference between R3/20 and CHF and at 5 x 10^3 there were no differences between isolates. Therefore, the spore concentration that revealed the most variation between isolates was 5 x 10^5 and hence this concentration will be used when performing a future pathogenicity test on all 31 isolates listed in Table 1; this will be carried out to determine which isolates are the most virulent for further study.

Despite changing the temperature, the fruit pathogenicity test is still not appropriate to determine differences in virulence between *B. cinerea* isolates. It is possible that 3 min in 0.5 % sodium hypochlorite is too damaging for the exocarp of the strawberries, causing the fruit to turn prematurely brown and mimic signs of infection. In future assays fruit will be dipped in 0.5 % sodium hypochlorite instead of soaking for 3 min. Furthermore, more replicates will be introduced for fruit pathogenicity tests to overcome the potential loss of samples due to post-harvest infection. For future pathogenicity tests, methods for inoculating strawberry flowers

and how to measure disease progression will be studied. Investigating *B. cinerea* virulence on flowers, or susceptibility and resistance mechanisms that may occur in flowers, is pivotal for understanding this pathosystem. Floral infection is how *B. cinerea* causes post-harvest infection, therefore it is extremely beneficial for growers and consumers that this stage of infection is well researched.

These pathogenicity tests should reveal if there any differences in virulence between isolates. They will be conducted on multiple plant organs as the mechanisms that *B. cinerea* uses to cause disease, and the mechanisms strawberry uses to defend itself will differ depending on the organ in question. This will provide us with a more comprehensive overview of exactly how virulence differs between isolates. It is important to know if virulence varies between the isolates in Table 1 as these will be utilised for future pathogenicity experiments, for instance, they will be mutated by UV radiation to investigate virulence factors and they will be used as inoculum on *F. vesca* mutated plants to study strawberry susceptibility or resistance factors.

Conclusions

The aim of this project is to learn about how *B. cinerea* causes disease in strawberry plants. This will be attempted through mutation experiments on both *B. cinerea* and *F. vesca,* and the development of pathogenicity assays. The conclusions drawn from this project so far are as follows:

- The *B. cinerea* isolates obtained are all in fact *B. cinerea.*
- The optimal spore concentration for pathogenicity assays is 5×10^5.

The next stages of this project are:

- To investigate the appropriate statistical tests that will provide accurate results and how many replicates to consider.
- To continue to develop pathogenicity assays for strawberry leaves, fruits and flowers.
- To begin the process of mutating both *B. cinerea* and *F. vesca.*

Knowledge and Technology Transfer

- Autumn CTP presentation - 25/11/2020
- AHDB Crops PhD conference – 25/01/21
- Summer CTP presentation - 6-7/07/21

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