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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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GROWER SUMMARY

Headlines

- New blotch causing *Pseudomonas* spp. and green mould causing *Trichoderma aggressivum* f. *europaeum* have been isolated from UK farm samples
- New real time PCR assays are being developed to identify groups of blotch causing *Pseudomonas* that were not detected using previously developed assays targeting *P. tolaasii* and '*P. gingeri'*
- Irrigation of casing with a non-pathogenic pseudomonad strain showed promising control levels of blotch caused by '*P. gingeri'* and *P. costantinii*, but further tests are needed

Background

Bacterial blotch caused mainly by *Pseudomonas tolaasii*, '*P. gingeri'* and *P. costantinii*, is considered to be the most important disease currently faced by the mushroom industry in the UK and elsewhere in Europe causing losses that can frequently exceed 30% of production. Conditions that favour high yield are also favourable for disease development and transmission, and therefore there is a trade-off between maximising yield and maintaining health and quality of production. Growers would benefit from tools that allow early detection of disease and understanding the possible sources of infection. Practical control measures that can reduce or eliminate spread of pathogenic pseudomonads without having a negative impact on beneficial populations that are necessary for mushroom production, need to be developed to be used across the supply chain to reduce losses and production costs and guarantee sustainable supply.

Although the number of outbreaks of compost green mould caused by *Trichoderma aggressivum* f. *europaeum* has been reduced through the implementation of sanitation measures, this disease still occurs in some farms causing significant losses. The American form of the pathogen (*Trichoderma aggressivum* f. *aggressivum*) so far has not been detected in the UK but constitutes an additional threat to mushroom production. Early detection of *Trichoderma* species has the potential to be used to inform control strategies and also to monitor general farm hygiene.

This project follows on from project M063 and the main aims are to detect, monitor and control blotch-causing pseudomonads and *Trichoderma* species whilst retaining populations of beneficial microorganisms in mushroom cultivation. In particular the aims are:

1. Enable sensitive detection in fresh substrates of all blotch-causing *Pseudomonas* species to determine if the analysis relates to the occurrence of blotch, thereby predicting disease risk
2. Determine the relative abundance of blotch-causing pseudomonads, *Trichoderma* species and other microorganisms in mushroom cropping substrates from different sources and in response to control treatments at different stages of commercial production
3. Estimate degree of control of blotch and/or green mould achieved by irrigating with antagonists, bacteriophages and ionic solutions
4. Make diagnostic tests available and disseminate the results to the mushroom industry

Summary

Severe brown blotch, strong and mild ginger blotch and pitting observed in several UK farms was identified as *Pseudomonas tolaasii*, '*P. gingeri'* and *P. costantinii* respectively. A number of isolates still await identification. Pathogenicity was confirmed for a range of isolates in cap droplet inoculation tests and pot culture tests. Inoculation of casing with *P. costantinii* also resulted in a marked reduction in the number of healthy mushrooms harvested.

A new method to test the pathogenicity of mushrooms in small pots enclosed in plastic bags has been developed and used successfully.

Ten new TaqMan assays have been developed in the current project based on recently obtained whole genome sequences, to detect groups of pathogenic *Pseudomonas* that were not detected by previously developed real-time assays that target *P. tolaasii* and '*P. gingeri'* (project M063).

Results of TaqMan assays targeting *P. tolaasii* and *P. gingeri* (developed previously in project M063) on casing extracts corresponded with the pseudomonad isolates (*P. tolaasii* or '*P. gingeri'*) that were inoculated into the casing, whereas samples inoculated with *P. costantinii* tested negative with these assays. Incubation of the casing samples with compound B increased the pseudomonad count in the extract using a plating method but did not improve the resolution of the TaqMan assay.

Inoculation of the casing with a non-pathogenic pseudomonad (isolate *P*. '*reactans*' P7759) suppressed blotch caused by '*P. gingeri'* and *P. costantinii* in a small pot assay. Commercially

available pseudomonads including *Pseudomonas putida*, *P. fluorescens* and *P. chlororaphis* did not reduce the incidence of blotch.

Irrigation with ionic solutions did not consistently reduce the incidence of blotch in controlled small pot assays.

Results of sequencing of two partial genes of strains of *Trichoderma* species from a culture collection hosted at FERA and from recent farm isolates resulted in changes to the original culture designations. Cultures obtained from mushroom substrates show that *T. aggressivum* f. *europeum* was prevalent on two farms.

Bacteriophages that target *P. tolaasii* and some strains of '*P. gingeri*' were obtained and will be further characterised.

Financial Benefits

Although it is too early to state and calculate the financial benefits of this work, the development of assays that can detect most blotch causing *Pseudomonas* and *Trichoderma* species and also the identification and testing of potential biocontrol agents can potentially lead to significant financial benefits.

Action Points

- Diagnostic tests for *Pseudomonas* causing blotch are available at Fera Science using qPCT assays that target *P. tolaasii* and *'P. gingeri'* (developed in the M063 project) and although these tests do not cover all blotch causing pseudomonads, they are still recommended for these two important groups of pathogens.
- Further work is needed to finish developing tests for diagnostics of other blotch causing pseudomonads and for green mould and to develop and test potential biocontrol agents. These results will then be disseminated to growers.

SCIENCE SECTION

Introduction

Bacterial blotch caused mainly by several species of *Pseudomonas* is considered to be the most important disease currently faced by the mushroom industry in the UK and elsewhere in Europe causing losses that can frequently exceed 30% of production.

In the previous AHDB funded project M063, molecular tests using quantitative real time (TaqMan) polymerase chain reaction have been identified that detected *P. tolaasii* or *'P. gingeri'* strains that caused severe brown or ginger blotch in the UK but did not cross react with other non-pathogenic *Pseudomonas* strains or strains that appeared to cause mild blotch symptoms (Elphinstone and Noble, 2017; Taparia et al., 2020a). Phylotypes of other pathogenic *Pseudomonas* strains have been differentiated at the species level by whole genome analyses.

Surveys of commercial mushroom production in the Netherlands and Belgium have found the same species of bacteria causing severe blotch as in the UK, although other, as yet unnamed (or with unofficial names), species found in some countries are also able to cause either severe or mild blotch or pitting of mushroom caps. Genome sequence of the other blotch causing *Pseudomonas* phylotypes is now available for further diagnostic development (Taparia et al., 2020b).

Fera are currently using high throughput DNA sequence metabarcoding to study total fungal and bacterial communities in soil as part of an AHDB Soil Biology and Soil Health Partnership and a commercial Fera Big Soil Community initiative. The process involves extraction of total DNA using Invitrogen PowerMax soil DNA isolation kits followed by amplification of 16S (Caporaso *et al*., 2011) or ITS (Toju *et al*., 2012) universal rRNA targets for bacteria and fungi respectively. Metabarcoding is then performed using the Illumina MiSeq high throughput sequencing platform (Illumina, 2013) and sequence data is analysed using Qiime2 software (http://qiime2.org). Taxonomic annotation of sequence reads is then performed against the SILVA reference database version 132 (Glöckner et al., 2017) for the 16S reads and the UNITE reference database (Kõljalg et al., 2013) version 7, 01.12.2017 for ITS reads. This approach has enabled comparisons of bacterial and fungal abundances (including *Pseudomonas* and *Trichoderma* species) amongst the entire microbiome across multiple soil types. This pipeline is also being used in the current project across a range of substrates used in mushroom production.

During a survey on a farm in England in the 1980's blotch causing pseudomonads were isolated, using selective plating, from 5% of fresh casing material at low concentrations and

at higher concentrations (up to 100%) during the mushroom cropping period (Wong & Preece 1980). However, the pathogen concentration in fresh casing is usually too low to be detected by selective plating or the molecular tests that have been developed more recently which therefore cannot give a reliable estimate of subsequent disease risk (Elphinstone and Noble, 2017). Incubation of casing samples in LB broth containing 0.02% v/v compound A resulted in a greater pseudomonad population than incubation of casing samples in LB broth alone. This incubation of mushroom casing samples also resulted in a selectively greater increase in the pseudomonad population when *P. tolaasii* or *'P. gingeri'* were present in the samples. Preliminary results suggested that enrichment of inoculated casing samples in LB broth containing 0.02% v/v compound A could improve the detection of *P. tolaasii* or *'P. gingeri'*, and potentially other blotch causing pseudomonads, using the newly developed molecular tests.

The biocontrol products Conquer and Victus, based on the *Pseudomonas fluorescens* biovar V strain NCIB 12089 have been reported to give good control of blotch (Miller & Spear, 1995) but none are currently marketed for this target. Noble & Dobrovin-Pennington (2017) examined the use of Cedemon (*Pseudomonas chlororaphis*) and Serenade QST713 (*Bacillus subtilis* syn. *velezensis*) for control of fungal diseases in mushrooms but the effect of these bacterial biocontrol agents on blotch disease was not examined. Whereas the introduced *Pseudomonas* population in the casing increased during the mushroom cropping, the population of an introduced *Bacillus* population gradually declined.

A novel biocontrol strategy which will be investigated in this project involves the use of bacteriophages (bacterial viruses; phages) that specifically infect and kill the pathogen. Because of the bacterial host specificity of most phages, they are highly unlikely to have any deleterious impacts on other microbes in the mushroom production process (e.g. including other pseudomonads such as *P. putida* isolates that are beneficial for the morphogenetic development of *Agaricus* fruiting body formation).

We have been able to isolate a good selection of phages from the environment, including from the River Cam (Cambridge, UK) by our assorted enrichment strategies that now enable facile and reproducible phage discovery. For example, phages MB55, MB56, JB27, MB8 and TOL1 were independently isolated and show turbid or clear plaque morphologies. Transmission electron microscopy showed icosahedral heads and short tails, classifying them as members of the Podoviridae family. Genome sequencing of these phages and bioinformatic interrogation confirmed all five phages were genetically unique but defined two genetically distinct families of phages, even among this small group of new isolates. Lab-based biocontrol assays for phage biocontrol candidates showed that phages MB55 and MB56 do have some capacity to control (diminish) *P. tolaasii*-induced disease (pitting and

brown blotching) of mushrooms. Given the limited number of phages tested so far in these lab-based bioassays, our preliminary data encourage optimism about phage-mediated biocontrol possibilities. A bank of phages that kill *P. tolaasii* strain NCPPB 2192^{T} has been assembled at the University of Cambridge.

Irrigation of mushrooms with a 0.3% $CaCl_2$ solution was originally developed as a method of improving mushroom whiteness (Beelman et al., 1987). The treatment resulted in significantly fewer blotched mushrooms than irrigating with the same volume of water, both at high and low levels of relative humidity, although it is not registered for use on mushrooms (Noble and Dobrovin-Pennington, 2017). Addition of other sources of Ca^{++} or Cl^{-} ions to the irrigation water may give similar benefits in terms of blotch disease control but may be easier to introduce from a regulatory standpoint. Salt (NaCl) is already an approved commodity substance and calcium hydroxide ($Ca(OH)_2$) is used as a casing ingredient to increase the pH. Leachate or 'tea' prepared from spent mushroom compost is rich in Ca^{++}, K^{+} and Cl^{-} ions and has been shown to be effective in plant disease control such as apple scab (Cronin et al., 1996; St Martin, 2014). Hydrogen peroxide is another treatment which may have a bactericidal effect and rapidly dissociates into water and oxygen.

Compost green mould caused by *Trichoderma aggressivum* f. *europaeum* resulted in large UK mushroom crop losses during the 1990s and early 2000s. Improvements in the design and sanitization of mushroom composting facilities means that outbreaks of green mould have been reduced, although the disease remains a threat, particularly following the recent report of the American form of the pathogen (*Trichoderma aggressivum* f. *aggressivum*) in Europe (Hatvani et al., 2017). Early detection of the pathogen in commercial size batches of compost using diagnostic volatiles or real time PCR has not proved successful to date due to the very small amount of inoculum that can produce a green mould disease outbreak (Radvanyi et al., 2015; O'Brien et al., 2017), analogous to 'finding a needle in a thousand haystacks without a metal detector'. Potentially more useful would be to continually monitor the presence of all *Trichoderma* species (not just *T. aggressivum*) in locations on a composting or casing production site or mushroom farm, since they are widespread, can harbour in organic debris, are moderately tolerant of chemical and thermal disinfection methods and are good indicators of farm hygiene (Fletcher & Gaze, 2008). They provide an indication of disease risk and vulnerability, not only to green mould but other fungal and bacterial diseases that may be recirculating on a mushroom farm, composting site or casing production facility. A similar approach has been used to monitor sanitization of green waste composts using naturally occurring *E. coli* and plant pathogens as indicator organisms (Anon. 2011; Noble et al 2011). Treatment of compost at spawning with the biocontrol product Serenade QST713 has been reported to give suppression of green mould in France where it

has been used for more than 10 years (Pardin et al., 2018). However, tests in the project MushTV showed it to be ineffective against *T. aggressivum* and other fungal pathogens (Noble et al., 2011).

The main aims of this project are to detect, monitor and control blotch-causing pseudomonads and *Trichoderma* species whilst retaining populations of beneficial microorganisms in mushroom cultivation. The project objectives are to:

1. Enable sensitive detection in fresh substrates of all blotch-causing *Pseudomonas* species to determine if the analysis relates to the occurrence of blotch, thereby predicting disease risk
2. Determine the relative abundance of blotch-causing pseudomonads, *Trichoderma* species and other microorganisms in mushroom cropping substrates from different sources and in response to control treatments at different stages of commercial production
3. Estimate degree of control of blotch and/or green mould achieved by irrigating with antagonists, bacteriophages and ionic solutions
4. Make diagnostic tests available and disseminate the results to the mushroom industry

Materials and methods

1. Improved pathogen detection in mushroom cropping substrates

A collection of isolates from mushrooms that were included in previous studies (Elphinstone and Noble, 2017) has been used in this work. In addition, new isolates were obtained and added to the collection. Mushrooms with a range of blotch symptoms were collected at five commercial farms in 2019/20. Isolations were done from excised small portions (approx. 5 x 4mm) of the edge of lesions in mushroom caps with one quarter of the excised tissue displaying symptom and the other three quarters appearing healthy. The excised tissue was placed into a couple of drops of 0.1% peptone solution and cut up to allow any bacteria present to exude into solution. Drops (10 µL) of the solution were then streaked onto King's B (KB) and Sucrose Nutrient Agar (SNA) using a sterile loop. Plates are incubated for 48 hours at 25°C before examination for typical colony growth. Typical colonies (small and fluorescent on KB and small, whitish, round and non-levan on SNA) were sub-cultured for purity. Fluorescence was checked under UV light and LOPAT testing was generally carried out on isolated *Pseudomonas* spp., with most mushroom isolates providing the following profile -+-+- (Levan -ve, Oxidase +ve, Pectate -ve, Arginine +ve, Tobacco -ve). The new

Pseudomonas isolates were preserved in Protect® and stored at -80°C following standard procedures at Fera Science Ltd.

1.1. Pathogenicity of *Pseudomonas* isolates

Blotch pathogenicity of newly isolated pseudomonads and control isolates were tested in mushroom pathogenicity bioassays. Two bioassay methods for determining blotch pathogenicity of *Pseudomonas* spp. isolates were used: a mushroom cap droplet test and a mushroom pot culture test.

1.1.1. Mushroom cap droplet test

Cut-cap bioassays based on the method of Godfrey et al. (2001), were performed in several rounds. Reference isolates were used as controls in the bioassays including *P. tolaasii*, *'P. gingeri'* and *P. costantinii* isolates known to be pathogenic from previous cap tissue and/or pot culture bioassays. The stipes of white button mushrooms were removed, and the caps were placed in large square Petri dishes on damp filter paper. Drops (10 µL) of bacterial inoculum (aqueous suspensions containing approximately 10^7 cfu per mL) were placed onto two small caps per *Pseudomonas* isolate. The experiment was repeated using bigger caps inoculated with four drops per cap (Appendix Fig. S1). Drops of sterile distilled water were used for controls. The dishes were incubated at 21ºC and photographed up to 72 hrs following inoculation; symptom development was visually assessed daily using a 0 to 3 scale as described in Taparia et al. (2020b).

1.1.2. Mushroom pot culture test

To assess the effect of 23 isolates of *Pseudomonas* species on disease incidence, mushrooms were grown in large plastic pots, 230 mm diameter x 220 mm depth, each containing 4 kg of Phase III compost, spawn-run with the mushroom strain Sylvan A15. The pots were cased with 1.3 L of casing (moist mixture of peat and sugar beet lime). The pots were watered and covered with black plastic sheet and kept in a growth room at 25°C at high humidity (over 85%); the plastic sheets were removed after four days and the pots kept for three more days in those conditions. The room was then 'aired' and the air temperature reduced to 18°C with relative humidity maintained at 85-95% for three more days before inoculation. Plates of bacterial cultures were washed with sterile distilled water (SDW) into a Universal giving a concentrated suspension containing between 10^8 to 10^9 cfu/mL. Each suspension was diluted 100x into two universals (each with 0.5 mL concentrated suspension

into 49.5 mL SDW) to contain between 10^7 and 10^8 cfu/mL. Two pots for each isolate were inoculated by evenly watering the casing with 50 mL of aqueous suspensions per pot. Concentration was checked for a couple of isolates by dilution plating. The two replicate pots of each isolate were randomly positioned on two shelves in the growing room (Appendix Fig. S3). Two negative controls on each shelf were watered with sterile distilled water only (four pots in total). Disease development on the mushrooms was recorded over two flushes as severe or mild ginger, brown blotch or other symptoms like pitting, or no disease. Mushroom harvest and scoring were done 7 to 9 days after inoculation for the first flush and up to 18 days after inoculation for the second flush.

1.2. Comparative genomics between blotch-causing Pseudomonas isolates

The aim of this work was to design and test real-time PCR assays which could be used to indicate the presence of a range of disease-causing *Pseudomonas* bacteria and also distinguish the different strains / species.

Sequences of 53 genomic isolates belonging to six different Average Nucleotide Identity (ANI, a measure of genomic similarity) groups (Table 1.2) were obtained, either from GenBank or from Tanvi Taparia, Wageningen University and Research, Netherlands (Taparia et al., 2020b). Where necessary, the genomic data was assembled using Sickle (quality control and trimming) and Spades (genomic assembly). Samples had previously been grouped by ANI analysis, and these groups were used as the input to "find_differential_primers.py" script (https://github.com/widdowquinn/find_differential_primers). This software was designed and previously used to develop PCR primer sets to distinguish *E. coli* O104:H4 outbreak strains (Pritchard et al., 2012) and plant pathogenic *Dickeya* species (Pritchard et al., 2013).

Assays were developed for: three groups of '*P. gingeri*' (ANI groups 1, 5 and 14), *P. costantinii* (ANI group 3), P. *NCO2* (ANI group 2) and *P. yamanorum* (ANI group 10) (Table 1).

All input assemblies were checked and any ambiguous nucleotides other than 'N' were replaced. Assemblies that were fragmented were stitched into one sequence, with 'N' characters placed at the sequence boundaries. The EMBOSS ePrimer3 package (Rice et al., 2000) was then used to design primers for each input genome. As genomes were treated individually, any identical primers designed were deduplicated to create a more streamlined dataset of potential primers. The EMBOSS primersearch tool (Rice et al., 2000) was then used to predict which genomes produce amplicons for each of the previously created primer sets. Finally, each primer set was then assessed for specificity to determine whether it can amplify a specific ANI group.

Table 1. Samples used as input into the find_differential_primers.py script. An asterisk following a sample ID means that the file was corrupted during data transfer and unusable in this analysis

ANI Group	Sample ID
1 (gingeri)	21614711, 21615525, A6001, C2001, IPO3738*, J1002, J4002, POWE01
2 (NCO2)	21615526, A4002, B6001, C6002, C8002, CP025624, D3002b, D4002, D5002, D6002, E6002, F1002, F8002, F9001, G1002, G5001, I8001, IPO3774, IPO3775, K5002, P7758, P7779
3 (costantinii)	21815971, 21815972, MDDR01
5 (gingeri)	A8002, H7001, IPO3754*
10 (yamanorum)	B4002, IPO3753, LT629793
14 (gingeri)	C1001, C3001, C4002, D1001, D5001, D8001, E1001, IPO3737, IPO3757, IPO3767, IPO3769, IPO3776, IPO3777, P8018

1.3. Enrichment of pseudomonad populations in fresh substrate samples using compound A or B to improve the detection limit of subsequent real time TaqMan PCR analysis for blotch-causing *Pseudomonas* species

Casing samples taken from culture pots after the second flush of mushrooms from Experiments 1.1 and 3.1b were used for the tests. Samples were taken from pots inoculated with '*P. gingeri*', *P. tolaasii*, or *P. costantinii* isolates or with sterile distilled water at the start of the experiments. Pre-enrichment of *Pseudomonas* species was conducted by incubation of casing samples in LB broth containing compound A or B. Casing samples and broth extracts (pre-and post-incubation) were then tested by TaqMan PCR for the presence and concentration of blotch-causing *Pseudomonas* species (*P. tolaasii* and *'P. gingeri'*).

1.4. Development of a detection system for *Trichoderma* species as a hygiene indicator of *T. aggressivum* and other mushroom pathogens

Collections of *Trichoderma* cultures maintained at Microbiotech and Fera were recovered from storage conditions. From approximately 55 cultures maintained in a fridge at Fera

Science, only 20 grew well. Sixteen cultures from previous projects maintained at Microbiotech grew well and were transferred to the Fera collection.

In addition, samples of cultures obtained from mushroom substrates with green mould symptoms were collected at two commercial UK farms in 2019 and 2020. New isolations of *Trichoderma* were performed at Microbiotech.

Isolates of *Trichoderma* species (14) associated with mushroom substrates taken from the Fera and HRI culture collections and a further 10 isolates from current green mould outbreaks on UK mushroom farms were grown and DNA was extracted using the Macherey-Nagel NucleoSpin Plant II Mini kit for DNA from plants following the manufacturer's instructions.

Conventional ITS PCR was performed for all DNA samples using the primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') from White et al. (1990) according to the method described in the EPPO Bulletin (2016). Conventional PCR was also performed to amplify the EF-1a (translation elongation factor 1 alpha) gene with primers EFCF1 (AGTGCGGTGGTATCGACAAG) and EFCF2 (TGCTCACGGGTCTGGCCAT) from Oliveira et al. (2015) according to the method also described in the EPPO Bulletin (2016). Sequences were aligned and compared with available sequences through BLAST and trees were constructed.

Conventional PCR with primers Th-F: CGGTGACATCTGAAAAGTCGTG and Th-R: TGTCACCCGTTCGGATCATCCG that were developed for detection of *T. aggressivum* previously *T. harzianum* biotypes 2 (European biotype) and 4 (North American biotype) by Chen et al. (1999) was tested with the existing collection of *Trichoderma*.

Specificity of qPCR assays previously developed at Fera for specific detection of *T. aggressivum* as part of AHDB project M048 (Lane, 2010) will be compared with other previously published *Trichoderma* subspecies, species and genus-specific assays (Friedl and Druzhinina, 2012; Kredics et al., 2018; Kosanovic et al., 2020). The selected assays will be tested on DNA from reference strains to validate their use for pathogen detection and quantification purposes.

2. Study of microbial communities in cropping substrates

2.1 Comparison of relative abundancies and populations of pathogenic and beneficial microorganisms in cropping substrates from different sources and following different control treatments

Casing and compost materials from different commercial farms have been sampled and additional farms will be sampled towards the end of 2020. The communities of bacteria and

fungi (including pathogenic *Pseudomonas* and *Trichoderma* spp. and potential beneficial microorganisms such as *Pseudomonas* and *Bacillus* spp.) will be assessed using high throughput sequencing based metabarcoding with 16S and ITS rRNA markers. Population dynamics of blotch-causing *Pseudomonas* spp. and green-mould causing *Trichoderma* spp. will be further analysed using quantitative qPCR assays previously developed at Fera and new assays developed in 1.2 and 1.4.

3. Comparing blotch control efficacy by irrigating with antagonists, bacteriophages or ionic solutions

To assess the effect of control treatments on blotch, mushrooms were grown in small 1 L plastic pots, each containing 550 g of Phase III compost, spawn-run with the mushroom strain Sylvan A15. The pots were cased with 300 g casing. The pots were watered with sterile distilled water (SDW) or other irrigation treatments, placed inside tie-handle polythene bags and positioned on shelves in an aerated incubator at 25°C for five days. The air temperature was then reduced to 18°C and the pots periodically aired by removing them from the cabinet and opening the bags for 6 hours at 2-3 day intervals. Inocula of bacterial pathogens were applied to the pots nine days after the start. Plates of bacterial cultures were washed with 20 mL SDW into a Universal using a Pasteur pipette and a spreader giving a concentrated suspension containing between 10^8 to 10^9 cfu/mL. Each suspension (1 mL) was added to 50 mL SDW to produce a dilute suspension containing between10^7 and 10^8 cfu/ml. Pots were inoculated by pouring 20 mL dilute suspension evenly over the surface of the casing. Control pots were treated with 20 mL of SDW. Further applications of 20-30 mL SDW or other irrigation treatments were applied to the pots at 7-10 day intervals to maintain a moist casing.

3.1. Effect of irrigating with ionic solutions on blotch

Experiment 3.1a. Effect of solutions and compost tea on blotch

The following pseudomonad suspensions were applied to pots:

1. No pathogen control (SDW)
2. *Pseudomonas tolaasii* P7544 (FSBactM 009; NCPPB2192 type strain, UK, 1965)
3. *'Pseudomonas gingeri'* P8018 (FSBactM 005; UK, 2011)
4. *Pseudomonas costantinii* J5 (FSBactM 037; 21815972-10; UK, Flixton farm, 2018)

Pots were irrigated with the following treatments:

1. Control (SDW)
2. Calcium chloride 0.3% (3g/L)

3. Potassium chloride 0.3% (3g/L)
4. Potassium bicarbonate 0.3% (3g/L)
5. SMC Compost tea (1:4 w/v) Compost pasteurised at 60˚C for 4 hours. Water added and left to 'brew' for 4 days before use. Tea filtered through cloth.

There were three replicate pots of each pseudomonad x irrigation treatment.

Experiment 3.1b. Effect of solutions and compost tea on blotch

The following pseudomonad suspensions were applied to pots:

1. No pathogen control (SDW)
2. *Pseudomonas tolaasii* P7544 (FSBactM 009, NCPPB2192 type strain; UK, 1965)
3. *'Pseudomonas gingeri'* P8018 (FSBactM 005; UK, 2011)
4. *Pseudomonas costantinii* J5 (FSBactM 037; 21815972-10; UK, Flixton farm, 2018)
5. *Pseudomonas tolaasii* TRF 42 (FSBactM 061; UK, 1980s)

Pots were irrigated with the following treatments:

1. Control (SDW)
2. Calcium chloride 0.3% (3g/L)
3. Hydrogen peroxide solution 1% v/v
4. SMC Compost tea (1:4 w/v) prepared as in Experiment 1a.

There were three replicate pots of each pseudomonad x irrigation treatment.

3.2 Effect of antagonists on blotch

Experiment 3.2a Effect of commercial pseudomonad inocula on blotch

The following pseudomonad pathogen suspensions were applied to pots:

1. No pathogen control (SDW)
2 *Pseudomonas tolaasii* TRF 42 (FSBactM 061; UK, 1980s)
3 *'Pseudomonas gingeri'* P8018 (FSBactM 005; UK, 2011)
4 *Pseudomonas costantinii* J5 (FSBactM 037; 21815972-10; UK, Flixton farm, 2018)

Suspensions of the following commercial products were applied to pots:

1. Control (SDW)
2. *Pseudomonas fluorescens* (Plantworks) 10%v/v in water
3. *Pseudomonas putida* (Plantworks) 10%v/v in water
4. *Pseudomonas chlororaphis* (Cedress, Lantmannen) 10%v/v in water.

The diluted product suspensions contained 10^8 to 10^9 cells/mL.

There were four replicate pots of each Pathogen x Product treatment.

Experiment 3.2b. Effect of experimental pseudomonad isolates on blotch

The following pseudomonad pathogen suspensions were applied to pots:

1. No pathogen control (SDW)
2. *'Pseudomonas gingeri'* P8018 (FSBactM 005; UK, 2011)
3. *Pseudomonas costantinii* J5 (FSBactM 037; 21815972-10; UK, Flixton farm, 2018)

   P. tolaasii was not included in the experiment due to insufficient space in the incubator.

Suspensions of the following experimental isolates were applied to pots:

1. Control (SDW)
2. P7758 (FSBactM 003; *Pseudomonas* sp.)
3. P7759 (FSBactM 013; *P. 'reactans'*)
4. 21615527 (FSBactM 008; *Pseudomonas* sp.)
5. NCPBB 3149 (FSBactM 012; *P. 'reactans'*)
6. NCPBB 1311 (FSBactM 014; *P. 'reactans'*)
7. NCPBB 2193 (FSBactM 027; *Pseudomonas* sp.)

The diluted isolate suspensions contained between 10^7 and 10^8 cfu/ml. There were three replicate pots of each pathogen x isolate treatment.

Experiment 3.2c. Effect of experimental pseudomonad isolates on blotch

The following Pseudomonad pathogen suspensions were applied to pots:

1. No pathogen control (SWD)
2. *Pseudomonas tolaasii* TRF 42 (FSBactM 061; UK, 1980s)
3. *'Pseudomonas gingeri'* P8018 (FSBactM 005; UK, 2011)
4. *Pseudomonas costantinii* J5 (FSBactM 037; 21815972-10; UK, Flixton farm, 2018)

Suspensions of the following experimental isolates were applied to pots:

1. Control (SWD)
2. P7759 (FSBactM 013; *P. 'reactans'*)
3. NCPBB 1311 (FSBactM 014; *P. 'reactans'*)
4. NCPBB 2193 (FSBactM 027; *Pseudomonas* sp.)

The diluted isolate suspensions contained between 10^7 and 10^8 cfu/ml. There were three replicate pots of each pathogen x isolate treatment.

3.3. Control of blotch using bacteriophages

3.3.1. Extension of phage library

Using standard phage enrichment methods previously used successfully by the Cambridge group to find new phages in other Gram-negative bacteria (Zheng and Salmond, 2020), multiple enrichments with *P. tolaasii* strain NCPPB 2192^T (FSBactM 009; P7544) as host were used for the isolation of new virgin environmental phage isolates from the river Cam.

A series of new enrichments using alternative environmental sources (including soil and wild mushroom samples) has been initiated. Multiple new enrichments were performed using:

a) alternative *P. tolaasii* and '*P. gingeri'* strains supplied by Fera

b) using material samples from different locations in the production process, provided by several commercial mushroom producers.

3.3.2. Host range determination and Electron Microscopy (EM)

The phages isolated on strain NCPPB 2192^{T} were plaque purified and amplified to high titre and used for transmission electron microscopy (EM). EM analysis was used to determine the morphologies of a selection of environmental phages. These phages were then used in screening assays against multiple *P. tolaasii* and '*P. gingeri'* strains provided by Fera, plus a *P. fluorescens* isolate (used as a control).

3.3.3. Phage genomics

Full genome sequencing and further bioinformatic comparisons were performed for a large selection of new phages.

Results

1. Improved pathogen detection in mushroom cropping substrates

New isolates obtained from five commercial UK mushroom farms are listed in Table 1.1.

1.1. Pathogenicity of *Pseudomonas* isolates

Two bioassay methods for determining beneficial blotch pathogenicity of *Pseudomonas* spp. isolates were used: a mushroom cap droplet test and a mushroom pot culture test.

1.1.1. Mushroom cap droplet test

Cut-cap bioassays were performed in several rounds including isolates obtained from 2018 up until May 2020. Reference isolates were used as controls in the bioassays including *P. tolaasii*, *'P. gingeri'* and *P. costantinii* isolates known to be pathogenic from previous cap tissue and/or pot culture bioassays (Appendix Fig. S1 and S2). The results of the assays are presented in Table 1.1. From the most recent isolations, eleven isolates were strongly pathogenic, five isolates were weakly pathogenic and other isolates did not produce symptoms on caps.

Table 1.1. Blotch pathogenicity observed in mushroom cap droplet tests and TaqMan results of *Pseudomonas* isolates

Isolate ID		**Species identity**	**Origin**	**Symptom Scores**	**TaqMan C_T values**		
FSBactM	**Other identifier**				**Pg2**	**Pg6**	**Ptol**
Control isolates							
001	NCPPB 3146	*'P. gingeri'*	UK, 1981	0, 1	18	17	40
002	P7548	*'P. gingeri'*	UK, 1989	0	40	40	40
003	P7758	*'P. gingeri'*	USA	0	40	40	40
005	P8018	*'P. gingeri'*	UK, 2011	2	18	17	40
006	21614711	*'P. gingeri'*	UK, 2016	1	17	17	40
007	21615525	*'P. gingeri'*	UK, 2016	1	17	17	40
009	P7544	*P. tolaasii*	UK, 1965	3	40	40	17
013	P7759	*P. 'reactans'*	UK, 1957	0	40	40	40
015	NCPPB 2192 T	*P. costantinii*	Finland, 1997	2,3	40	40	40
024	P7786	*'P.* sp. *gingeri'*?	UK, 2011	2	40	40	40
026	P8021	*P.* sp. *reactans*?	UK, 2016	0	40	40	40
027	NCPPB 2193	*P. tolaasii*?	UK, 1968	0	40	40	40
061-062	TRF 42	*P. tolaasii*	UK, 1980s	3	40	40	17
063	TRF 59	*P. tolaasii*	UK,1980s	3	40	40	17
Isolates obtained 2018-2020 from UK commercial farms							
028	21815970 Blotch 1 I6	?	F, 2018	0	40	40	40
029	21815970 Blotch 2 I7	?	F, 2018	2 pit	40	40	40
030 to 034	21815970 I8 to J2	?	F, 2018	0	40	40	40
035	21815971 Pit 8 J3	?	F, 2018	1	40	40	40
036	21815971 Pit 9 J4	*P. costantinii*	F, 2018	3 pit	40	40	40
037	21815972 Blotch 10 J5	*P. costantinii*	F, 2018	3 pit	40	40	40
038	21815972 J6	?	F, 2018	0	40	40	40
039	21815973 Pit 12 J7	?	F, 2018	2	40	40	40
040	21815973 Pit 13 J8	?	F, 2018	2	40	40	40
041	21815973 J9	?	F, 2018	0	40	40	40
042	JV1A	?	F, Nov. 2019	0, 1	Nt	40	40
043-046 048-049		?	F, Nov. 2019	0	Nt	40	40
047	JV3B	?	F, Nov. 2019	0, 1	Nt	40	40
050	22000021 mr1	?	B, Jan. 2020	3	Nt	40	40
051	22000021 mr2	?	B, Jan. 2020	3	Nt	40	40
052	22000021 mr3A	?	B, Jan. 2020	0, 1	Nt	40	40
053	22000021 mr3B	*'P. gingeri'*	B, Jan. 2020	2	Nt	23	40
054-056	22007191	?	F, Feb. 2020	0	Nt	40	40
057	22007191	?	F, Feb. 2020	2	Nt	40	40
058	Mr1 Hut 7	?	F, Mar. 2020	0	Nt	40	40
059	Mr2 Hut 7	?	F, Mar. 2020	1	Nt	40	40
060	Mr3 Hut 7	?	F, Mar. 2020	2	Nt	40	40
064		*P. tolaasii*	G, May 2020	3	40	40	17
065 to 070		?	F, Sept. 2020	Nt	Nt	Nt	Nt
081		?	M, Oct. 2020	Nt	Nt	Nt	Nt
082		?	L, Oct 2020	Nt	Nt	Nt	Nt
STW	Control	-		clean			

1.1.2. Mushroom pot culture test

Blotch symptoms caused by '*P. gingeri'* and *P. tolaasii* isolates corresponded with their known pathogenicity (Appendix Fig. S4) and TaqMan C_T values (shaded cells in Table 1.2). '*P. gingeri'* isolates FsBactM 001 and 006 also reduced the number of mushrooms harvested compared with the untreated control pots. One newly obtained isolate (053) produced ginger symptoms (Appendix Fig. S4) and was positive in the TaqMan assays that target '*P. gingeri'*.

Table 1.2. Blotch pathogenicity in two flushes of mushrooms in large pot experiment and TaqMan results of *Pseudomonas* isolates. Each value is the mean of two replicates (four replicates of controls)

Isolate ID		**Original**	**Mushrooms**	**Symptoms**		**TaqMan C_T values***		
FSBactM	**Other identifier**	**identity**	**/pot**	**%**	**Main type**	**Pg2**	**Pg6**	**Ptol**
001	NCPPB 3146	*'P. gingeri'*	69.5	33.8	ginger	18	17	40
002	P7548	*'P. gingeri'*	82.0	16.5	clean	40	40	40
005	P8018	*'P. gingeri'*	112.0	77.2	ginger	18	17	40
006	21614711	*'P. gingeri'*	68.5	62.0	ginger	17	17	40
007	21615525	*'P. gingeri'*	107.5	79.5	ginger	17	17	40
009	P7544	*P. tolaasii*	108.5	70.0	brown	40	40	17
013	P7759	*P. 'reactans'*	86.5	3.5	clean	40	40	40
024	P7786	*'P.* sp. *gingeri'*?	79.5	59.7	mild ginger	40	40	40
026	P8021	*P.* sp. *reactans*?	96.0	4.7	clean	40	40	40
027	NCPPB 2193	*P. tolaasii*?	86.5	12.7	clean	40	40	40
028	21815970 Blotch 1	?	97.0	12.4	clean	40	40	40
029	21815970 Blotch 2	?	78.0	51.9	pit	40	40	40
035	21815971 Pit 8	?	102.5	48.8	pit	40	40	40
036	21815971 Pit 9	*P. costantinii*	31.0	24.2	pit	40	40	40
037	21815972 Blotch 10	*P. costantinii*	81.5	54.6	pit	40	40	40
039	21815973 Pit 12	?	49.5	51.5	pit	40	40	40
040	21815973 Pit 13	?	66.5	36.1	pit	40	40	40
042	JV1A	?	97.5	26.2	pit	Nt	40	40
047	JV3B	?	93.5	10.7	clean	Nt	40	40
050	22000021 mr1	?	105.5	64.5	pit	Nt	40	40
051	22000021 mr2	?	100.0	59.0	pit	Nt	40	40
052	22000021 mr3A	?	113.0	7.1	clean	Nt	40	40
053	22000021 mr3B	?	94.5	76.2	ginger	Nt	23	40
STW	Control	-	97.5	7.1	clean			

*Nt= not tested; TaqMan results from assays developed in AHDB M063 project (Elphinstone and Noble, 2017).

Isolate 024 produced mild ginger blotch symptoms and nine isolates (029 to 042, 050, 051) caused pitting previously observed with *P. costantinii* isolates and were negative on TaqMan assays that target '*P. gingeri'* and *P. tolaasii*. Pots inoculated with isolates 036 and 039

resulted in a marked reduction in the number of harvested mushrooms compared with the untreated control pots.

Seven isolates (002, 013, 026, 027, 028, 047, 052) produced mainly clean mushrooms in similar proportions and numbers to the untreated control pots.

1.2. Comparative genomics between blotch-causing *Pseudomonas* isolates

Newly available genome sequences obtained at Fera and at the University of Wageningen and reported in Taparia et al. (2020b) have been used to select unique regions for development of TaqMan qPCR primers for detection of several groups of isolates.

find_differential_primers outputted 29 100bp regions which were ANI specific and a primer set which would amplify each region. As an initial screen the regions were subjected to a BLASTn search against a database of Illumina adapters to remove any regions containing artificial artefacts in the sequences. The remaining regions were compared to GenBank nt database, in order to check the taxonomic specificity, with any poor or non-specific regions removed. The software package Primer Express 2 (thermo, UK) was then used to design real time PCR assays to the regions using the software defaults for standard TaqMan assays. Where possible, the original primers designed by *find_differential_primers* were used and a new TaqMan probe added. Where this was not possible, the primers were adjusted (lengthened to increase melting temperature) or a completely new primer and probe set designed. Twelve primer and probe sets were designed using approach. Ten were taken forward for screening against bacterial DNA:

- One assay for '*P. gingeri'* ANI group 1
- One assay for '*P. gingeri'* ANI group 14
- Two assays for '*P. gingeri'* ANI group 5
- Two assays for *P. costantinii* ANI group 3
- Three assays for *P. NCO2* ANI group 2
- One assay for *P. yamanorum* ANI groups 4, 10 and 26

These ten qPCR assays were tested with eight selected isolates from the Fera Science collection and the results are presented in Table 1.3.

Table 1.3. Results of C_T values of preliminary tests of ten new qPCR assays with eight isolates of *Pseudomonas* spp.

Isolate FSBactM	Species, ANI group and origin	qPCR assays C_T values*									
		1a2 Gingerii	14a25 Gingerii	5a16 Gingerii new	5a18 Gingerii new	3a10 Cost.	3a12 Cost.	2a4 NC02	2a6 NC02	2a7 NC02	10a24 Yama
001	'*P. gingeri*', (ANI 1) UK	**25.4** **25.0**	U	U	U	U	U	U	U	U	U
007	'*P. gingeri*' (ANI 1) UK	**23.6** **23.2**	U	U	U	U	U	U	U	U	U
005	'*P. gingeri*' (ANI 14) UK	U	**21.5** **20.3**	U	U	U	U	U	U	U	U
024	'*P. gingeri*' (ANI 5) UK	U	U	**24.4** **24.1**	**22.1** **23.4**	U	U	U	U	U	U
015	*P. costantinii* (ANI 3) Finland	U	U	U	U	**20.0** **19.6**	**19.8** **20.1**	U	U	U	U
037	*P. costantinii* (ANI 3) UK	U	U	U	U	**21.9** **21.2**	**18.8** **18.0**	U	U	U	U
003	NC02? (ANI 2) USA	U	U	U	U	U	U	**23.5** **23.5**	**22.0** **21.9**	**21.3** **22.1**	U
009	*P. tolaasii* (ANI 6) UK	U	U	U	U	U	U	U	U	U	U

* U= undetected

All assays detected the predicted isolates. The assays 1a2 and 14a25 correctly differentiated the two groups of '*P. gingeri*' isolates; both these groups of isolates were detected by the '*P. gingeri*' assays developed previously (Taparia et al., 2020a). We did not have a *P. yamanorum* isolate available at the time of testing and therefore assay 10a24 will need to be tested further.

A selection of these qPCR assays will be tested with a large collection of strains including the unidentified strains in tables 1.1 and 1.2.

1.3. Enrichment of pseudomonad populations in fresh substrate samples using compound A to improve the detection limit of subsequent real time TaqMan PCR analysis for blotch-causing *Pseudomonas* species

TaqMan assay results on casing extracts corresponded with the pseudomonad isolates (*P. tolaasii* or '*P. gingeri*') that were inoculated into the casing. Samples inoculated with *P. costantinii* tested negative in these assays (Table 1.4) as predicted from previous tests. The

control samples in water extracts were weakly positive for Pg-2 and Pg-6 indicating a low level of natural inoculum in the casing or cross-contamination in the pot experiment (small numbers of blotched mushrooms were detected, Table 1.2). Incubation of casing samples in compound A did not increase the pseudomonad counts in the extracts (Fig. 1.1) nor improve the resolution of the assays (Table 1.4).

Table 1.4. TaqMan analysis of casing extract samples from Experiment 1.1 treated with water or compound A. Each C_T value is the mean of two replicate samples

Casing sample	Ptol		Pg-2		Pg-6	
	Water	Compound A	Water	Compound A	Water	Compound A
Control	38.6	39.7	36.6	38.8	34.7	37.9
P. tolaasii 009 (P7544)	22.9	20.1	39.8	40.0	40.0	40.0
'P. gingeri' 007 (21615525)	38.1	40.0	25.9	37.8	26.5	38.2
'P. gingeri' 006 (21614711)	40.0	39.3	33.7	33.2	35.2	32.8
'P. gingeri' 001 (NCPPB 3146)	39.2	40.0	33.1	40.0	31.4	40.0
P. costantinii 037 (21815972)	40.0	40.0	39.5	40.0	39.4	40.0

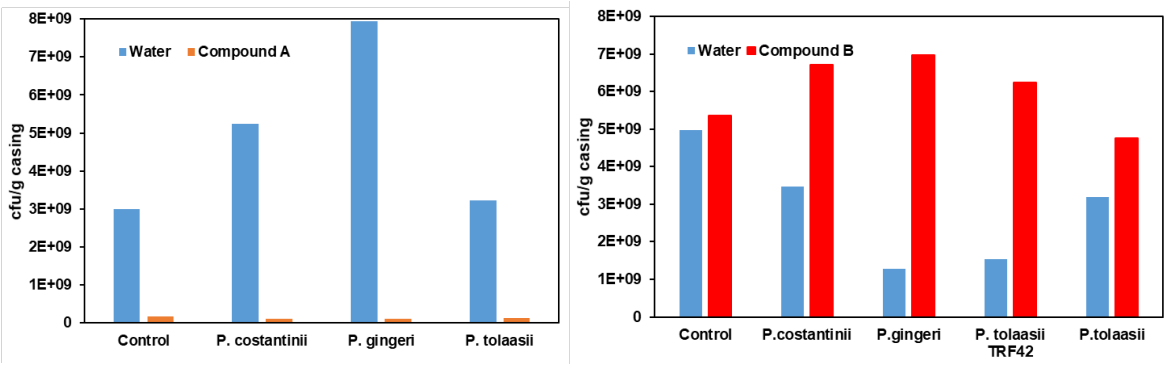


Figure 1.1. Pseudomonad counts in casing extracts treated in water or compound A (Expt. 1.1 left) or water and compound B (Expt. 3.1b right)

Casing samples treated in compound B produced higher pseudomonad counts in the extracts than water treated samples (Fig. 1.1). However, subsequent TaqMan assays on extracts showed no difference in C_T values between water and compound B extracts (Table 1.5). Casing inoculated with *P. tolaasii* or '*P. gingeri'* produced strongly positive results for the Ptol and Pg TaqMan assays respectively. All other assay results for the inoculated and control samples were weakly positive or negative (Table 1.5).

Table 1.5. TaqMan analysis of casing extract samples from Experiment 1.1 treated with water or compound B. Each value is the mean of three replicate samples

Casing sample	Ptol		Pg	
	Water	Compound B	Water	Compound B
Control	32.7	26.1	31.8	36.4
P. tolaasii 061 (TRF42)	18.5	20.0	37.0	37.1
'P. gingeri' 005 (P8018)	28.5	29.5	22.1	23.5
P. costantinii 037 (21815972)	32.0	31.6	33.8	37.5

1.4. Development of a detection system for *Trichoderma* species as a hygiene indicator of *T. aggressivum* and other mushroom pathogens

Results of sequence comparisons of two genes (ITS and EF) of *Trichoderma* species isolates in the FERA culture collection are shown in Table 1.6 and Appendix Fig. S5. Changes to the original culture designations are shown in red text. Analysis of cultures obtained from mushroom substrates show that *T. aggressivum* f. *europeum* was prevalent on two farms (Table 1.6).

Table 1.6. Results of analysis of partial sequences of ITS and TEF of *Trichoderma* species isolates from culture collections

Number	Original Culture Label	ITS Result	TEF Result
3098	*T. aggressivum* 23443 B Tafe	*T. aggressivum*	*T. aggressivum f. europaeum*
3099	*T. asperellum* T 34	*T. asperellum*	*T. asperellum*
3100	*T. atroviride* C52 (TENET) R654	*T. atroviride*	*T. atroviride*
3101	*T. harzianum* 278 (Th1)	*T. harzianum / T. lixii*	*T. harzianum /T. lixii*
3102	*T. harzianum* 24651 (Th1)	*Hypocrea lixii*	*Hypocrea lixii*
3103	*T. harzianum* IMI 275950	*T. atroviride*	*T. atroviride*
3104	*T. harzianum* IMI 284726	*T. atroviride*	*T. atroviride*
3105	*T. harzianum* T5	*T. harzianum*	*T. harzianum*
3106	*T. harzianum* T 40 (Th2)	*T. longibrachiatum / T. viride*	*T. longibrachiatum / T. bissettii*
3107	*T. koningii* 163	*T. harzianum*	*T. harzianum*
3108	*T. pseudokoningii* 17	*T. harzianum*	*T. harzianum*
3109	*T. viride* S17A	*T. viridescens / T. atroviride*	*T. paraviridescens / T. viridescens*
3110	*T. viride* 194 Ci	*T. atroviride*	*T. atroviride*
3111	*T. viride* 237 A	*T. trixiae / T. viridarium*	*T. viridarium*

Table 1.7. Results of analysis of partial sequences of ITS and TEF of *Trichoderma* species isolates from green mould infected mushroom substrates

Number	Source and date of culture	ITS Result	TEF Result
3112	Farm 1, spawn-run compost, Dec 2019	*T. viridescens / T. atroviride*	*T. viridescens*
3113	Farm 1, swab, Jan 2020	*T. aggressivum*	*T. aggressivum f. europaeum*
3114	Farm 2, spawn-run compost, May 2020	*T. aggressivum*	*T. aggressivum f. europaeum*
3115	Farm 2, casing, May 2020	*T. aggressivum*	*T. aggressivum f. europaeum*
3116	Growing media, Nov 2019	*T. atroviride*	*T. atroviride*
3117	Growing media, Nov 2019	*T. harzianum*	*T. harzianum*

A conventional PCR assay (Chen et al., 1999) showed that five strains including a collection strain (3098=23443) and strains obtained in 2020 from two farms (3113, 3114, 3115) are *T. aggressivum*; these results agreed with results of ITS and EF gene sequencing (Appendix Fig. S6).

All Trichoderma cultures will be further tested with the Fera qPCR assay that targets *T. aggressivum* and PCR assays that are genus, species and subspecies specific.

2. Study of microbial communities in cropping substrates

2.1 Comparison of relative abundancies and populations of pathogenic and beneficial microorganisms in cropping substrates from different sources and following different control treatments

Casing and compost materials has been sampled from three different commercial farms in cropping rooms at different stages of production. Additional farms will be sampled. The following samples have been stored in a freezer.

1. Farm G, collected 19th of May 2020: 18 samples (from fresh casing to end of 3rd flush)
2. Farm F, collected 9th of September 2020: 14 samples (as above)
3. Farm G, collected 9th of September 2020: 12 samples (as above)
4. Farm L, collected 13th of October 2020: 14 samples (as above)

DNA will be extracted from samples and the communities of bacteria and fungi (including pathogenic *Pseudomonas* and *Trichoderma* spp. and potential beneficial microorganisms such as *Pseudomonas* and *Bacillus* spp.) will be assessed using high throughput sequencing based metabarcoding with 16S and ITS rRNA markers.

Presence or absence of blotch or green mould associated with each sample was recorded. Results obtained from these experiments will be related to levels of disease encountered during assessments at different stages of commercial production (first and second flush) for each source of casing in which pathogens were detected and in the bioassay experiment described in 2.2. Observed disease levels will be related to changes in the communities of pathogenic as well as beneficial microorganisms indicated by the metabarcoding analyses. This will allow the progression of the disease and effects of potential control measures to be assessed. With an in depth understanding of the microbiology of the disease development it may be possible to identify further control measures based on manipulation of beneficial microbial communities. It should also be possible to identify potential indicator species of healthy and diseased mushroom cultivation which may be targeted for improved diagnostic tests.

3. Comparing blotch control efficacy by irrigating with antagonists, bacteriophages or ionic solutions

3.1. Effect of irrigating with ionic solutions on blotch

Experiments 3.1a and 3.1b: Effect of ionic solutions and compost tea on blotch

Blotch symptoms on inoculated pots were predominantly as expected: *P. tolaasii* isolates caused mainly brown blotch, the '*P. gingeri*' caused mainly ginger blotch and cap splitting in severe cases, and *P. costantinii* caused mainly pitting and some brown spotting (Fig. 3.1). In the absence of pathogen inoculum, none of the ionic solutions caused blotch (Figs. 3.1a and 3.1b). In both Experiments 3.1a and 3.1b, the *P. tolaasii* isolate P7544 (FSBactM 009) caused a small amount of brown blotch and a corresponding reduction in the number of healthy mushrooms. *P. tolaasii* isolate TRF42 (FSBactM 061) was more pathogenic than isolate P7544 and caused a greater amount of brown blotch and almost eliminated any healthy mushrooms. '*P. gingeri*' resulted in more mushrooms having ginger blotch than being without symptoms. *P. costantinii* produced mainly pitting and reduced mushroom numbers more severely than the *P. tolaasii* or '*P. gingeri*' isolates (Fig. 3.1b). None of the ionic solutions reduced the incidence of brown or ginger blotch or pitting compared with the water irrigation treatment.

Figure 3.1. Uninoculated mushroom culture pots (top row) and pots inoculated with *P. tolaasii* (middle row) isolates P7544 (left) and TRF42 (right), '*P. gingeri*' (bottom row left) and *P. costantinii* (bottom row right)

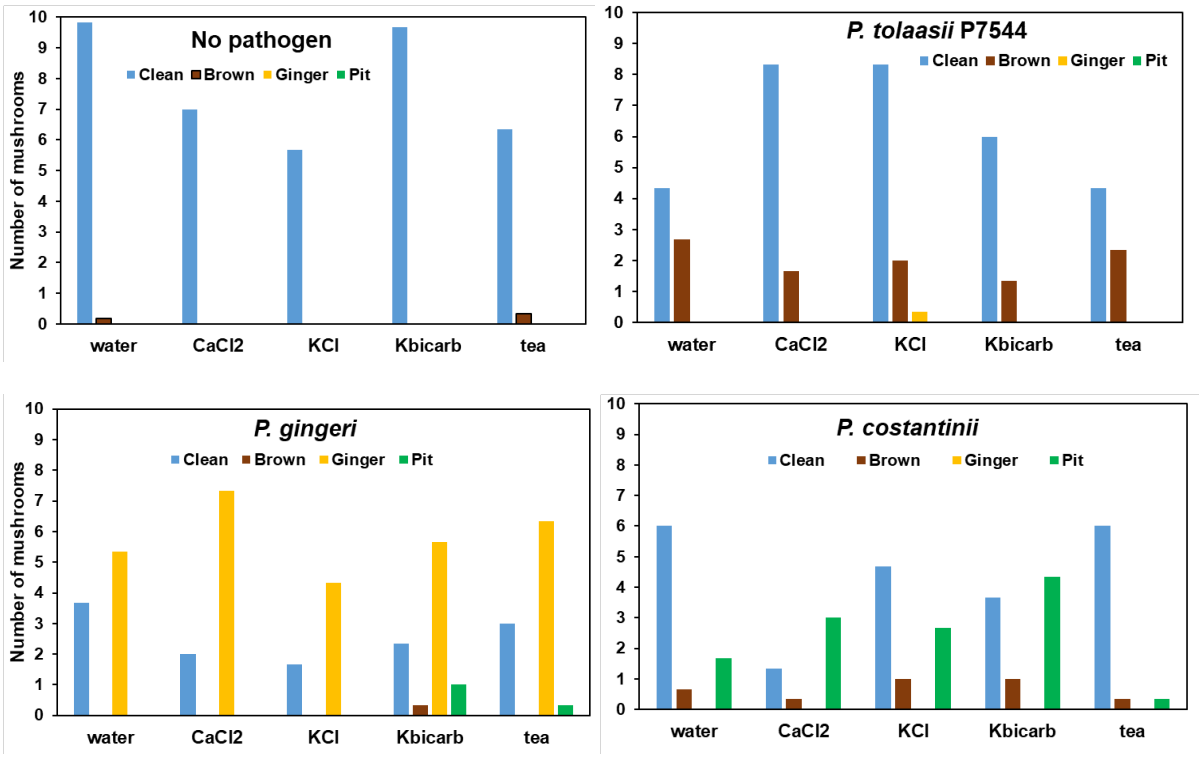


Figure 3.1a. Experiment 3.1a: Effect of ionic solutions on different types of blotch. Each treatment value is the mean of four replicate pots

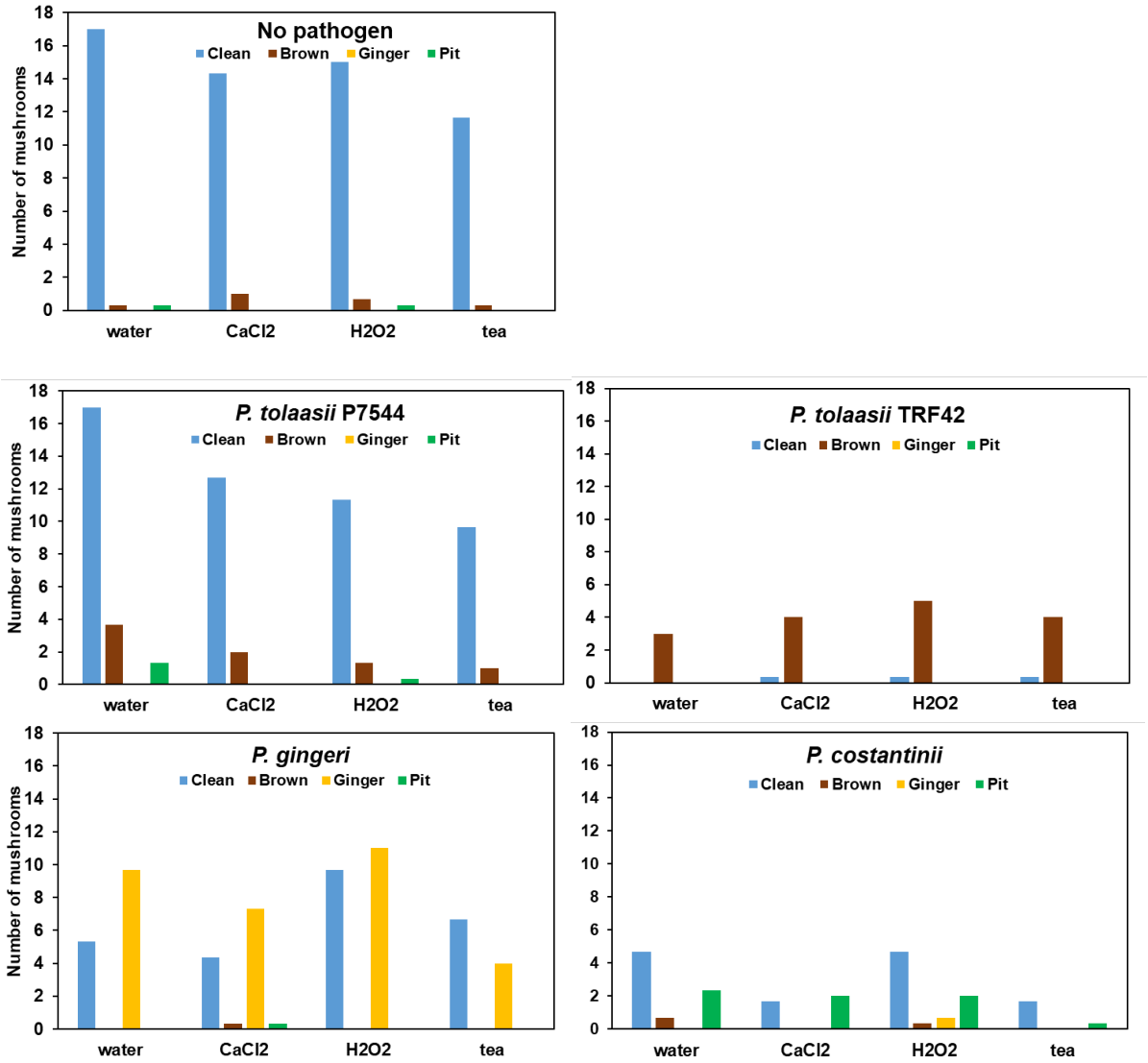


Figure 3.1b. Experiment 3.1b: Effect of ionic solutions on different types of blotch. Each treatment value is the mean of four replicate pots

3.2. Effect of antagonists on blotch

Experiment 3.2a. Effect of commercial Pseudomonad inocula on blotch

The effects of pathogen treatments were similar to those in Experiments 3.1a and 3.1b, with the uninoculated pots producing mainly healthy mushrooms and the inoculated pots producing mushrooms with blotch or pitting symptoms corresponding with the pathogen treatment applied (Fig. 3.2a). *P. tolaasii* isolate TRF42 resulted in almost all mushrooms showing brown blotch. None of the commercial pseudomonad suspensions caused or supressed blotch compared with the water control treatment.

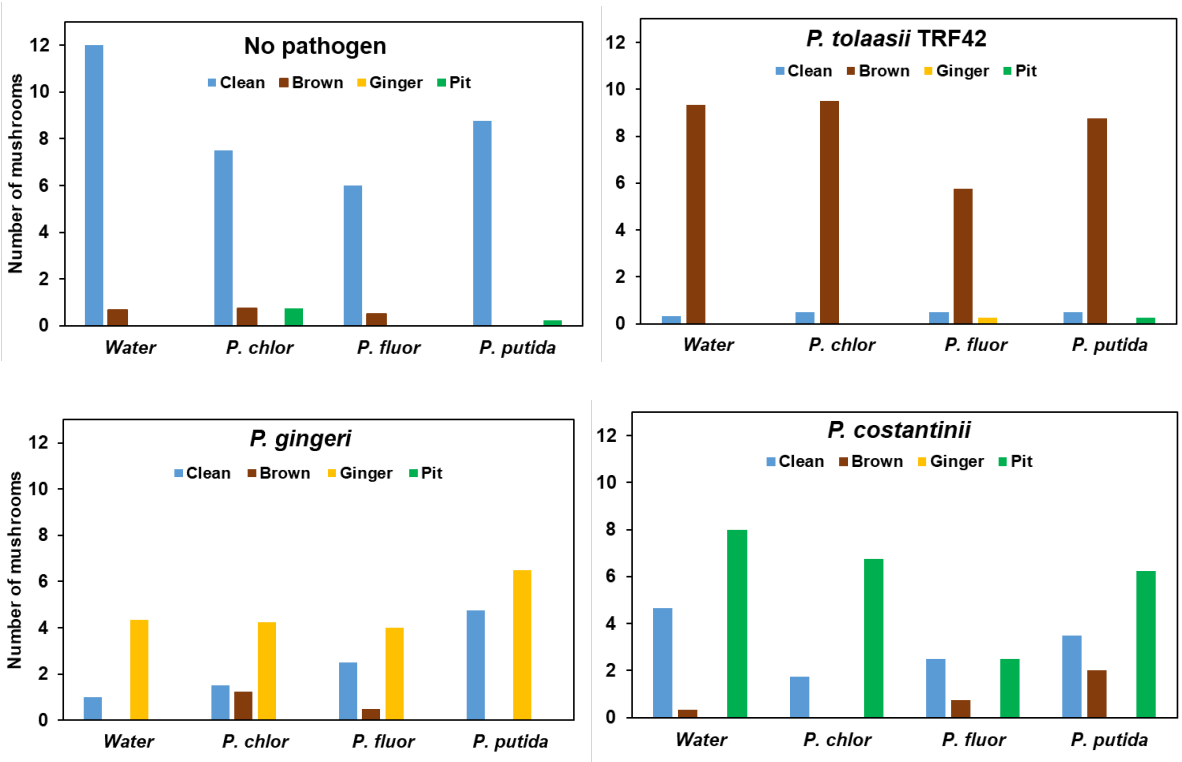


Figure 3.2a. Effect of commercial pseudomonad inocula on different types of blotch. Each treatment value is the mean of three replicate pots

Experiment 3.2b: Effect of experimental pseudomonad isolates on blotch

None of the experimental pseudomonad isolate suspensions resulted in more blotch symptoms than the water treated control (Fig. 3.2b). Ginger blotch was observed in all of the '*P. gingeri*' inoculated treatments with the exception of pots also inoculated with isolate FSBactM 013 (P7759). Inoculation of pots with *P. costantinii* again resulted in pitting and a reduction in mushroom numbers compared with uninoculated pots, with the exception of pots also inoculated with isolate P7759.

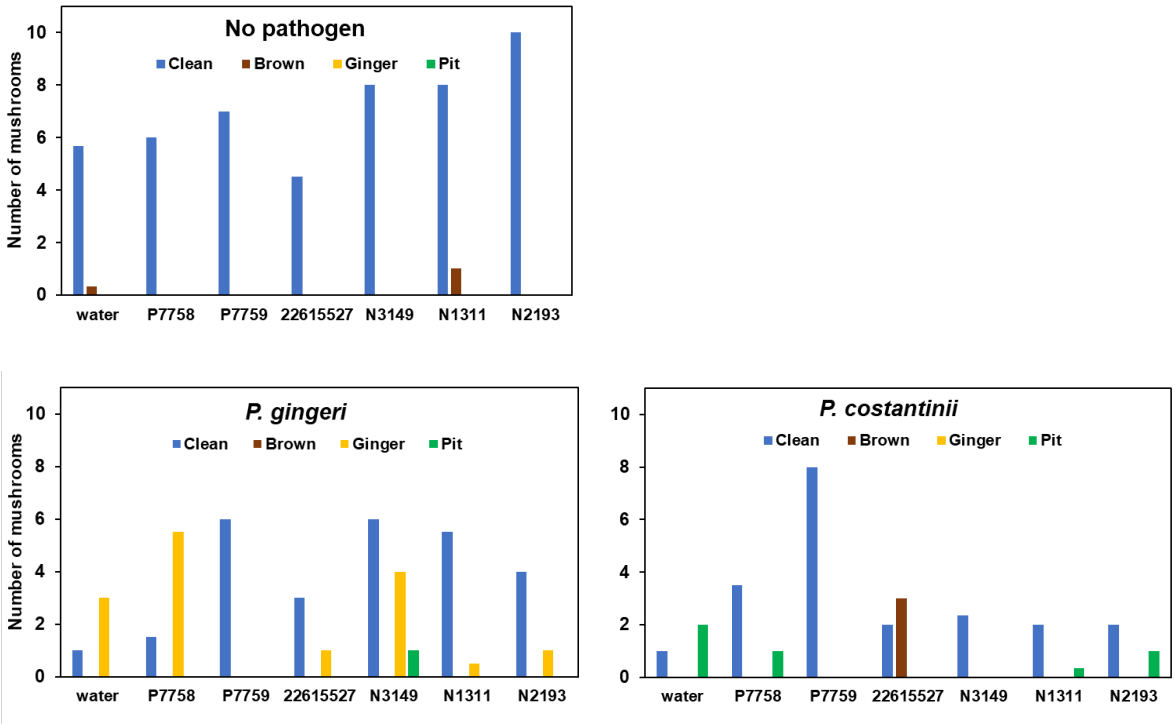


Figure 3.2b. Effect of experimental pseudomonads on ginger blotch and pit.
Each treatment value is the mean of two replicate pots

3.3. Control of blotch using bacteriophages

3.3.1. Extension of phage library

The river Cam proved to be a reliable and reproducible source for facile phage isolation using enrichment methods with the *P. tolaasii* type strain NCPPB 2192^{T} (FSBactM 009; P7544). Independent isolates of the phages produced different plaque morphologies on this strain; clear or turbid and of varying plaque sizes (Fig 3.3) suggesting viral heterogeneity. Two phages were used along with strain 2192^{T} in preliminary lab-based mushroom blotch/pitting experiments. These lab experiments involved cut mushroom caps challenged with *P. tolaasii*, with or without phage added. The extent of pitting/browning of the infected zones on mushroom caps was measured. These lab-based assays provided an indication of potential biocontrol capacity using the phages, acting in a “dose-dependent” fashion.

Preliminary results from the new enrichments using river Cam water, and water and blotched mushrooms from commercial producers were promising because recently we have been able to isolate new phages and increase the library. Among the new isolates we now have phages that can infect particular *P. tolaasii* or ‘*P. gingeri*’ strains (Table 3.1). The analysis of these new phages is at an early stage but they will be investigated in detail along the lines of our

previous *P. tolaasii* phages. Further enrichments will be performed with the aspiration of building a wider bank of phages.

Table 3.1. Phages from enrichments of new environmental samples and commercial sources

Phage	Host Strain	Enrichment Source
JB50	*P. gingeri* 3 smooth colony	River Cam
JB51	*P. gingeri* 3 mucoid colony	River Cam
JB52	*P. tolaasii* 2192T	Suffolk Mushrooms**
JB53	*P. tolaasii* 2192T	Suffolk Mushrooms**
JB54	*P. tolaasii* 2192T	Suffolk Mushrooms**
JB55	*P. gingeri* 1	Suffolk Mushrooms**
JB56	*P. gingeri* 6	Suffolk Mushrooms**
JB57	*P. gingeri* 1	Bressingham Mushrooms*
JB58	*P. gingeri* 6	Bressingham Mushrooms*

Enrichment of new phages on different host strains. ** from water and blotched mushroom samples; * from blotched mushroom samples

3.3.2. Host range determination and Electron microscopy (EM)

Phages isolated on *P. tolaasii* strain NCPPB 2192^T were plaque purified and amplified to high titre and used for transmission electron microscopy. These phages were then used in screening assays against multiple *P. tolaasii* and '*P. gingeri'* strains provided by Fera, plus a *P. fluorescens* isolate as a control strain. Although the independent phage isolates produced plaque heterogeneity on strain 2192^T, they showed a very strong specificity towards that enrichment strain rather than other bacterial strains originally isolated from mushrooms. These results, plus the genomic data (see below) suggested strong enrichment bias of the phages when using strain 2192^T. This type strain is clearly susceptible to many different phages, enabling facile enrichment and isolation of phages from both the natural environment and commercial sources. However, to date the results suggest that the phages isolated by enrichment on this particular host strain do not appear to infect many alternative strains.

EM analysis was used to determine the morphologies of a selection of environmental phages and this confirmed that most were members of the phage *Podoviridae* family (e.g. Fig 3.3).

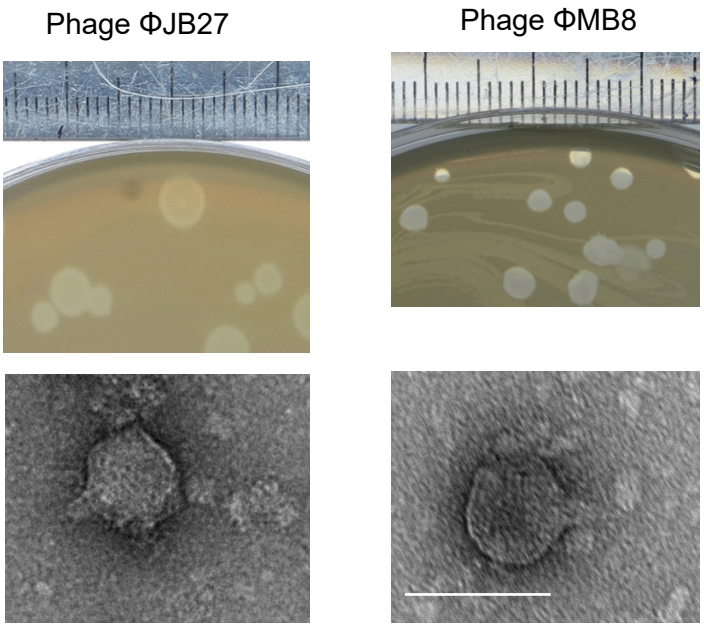


Figure 3.3. Example of *Pseudomonas tolaasii* phages

Top: Phages JB27 and MB8 produce turbid and clear plaques on a lawn of *P. tolaasii* strain NCPPB 2192^{T} (scale bars 100 nm); Bottom: TEM images showing morphology characteristic of *Podoviridae* family phages displaying a short “stumpy” tail.

3.3.3. Phage genomics

Full genome sequencing of 14 phages and further bioinformatic comparisons revealed that the environmental phages isolated on *P. tolaasii* 2192^{T} fell into two genetically distinct groups (Luz24-like and T7-like). Despite the obvious plaque heterogeneity exhibited among the phage isolates, there was clear genomic variation indicative of evolution of the phages within the distinct groups. A comprehensive genome sequence interrogation of 12 isolates defined the locations of mutations in the phage genomes of the Luz24-like isolates (Fig. 3.4). We do not know if any of these mutations play roles in phage virulence or host specificity.

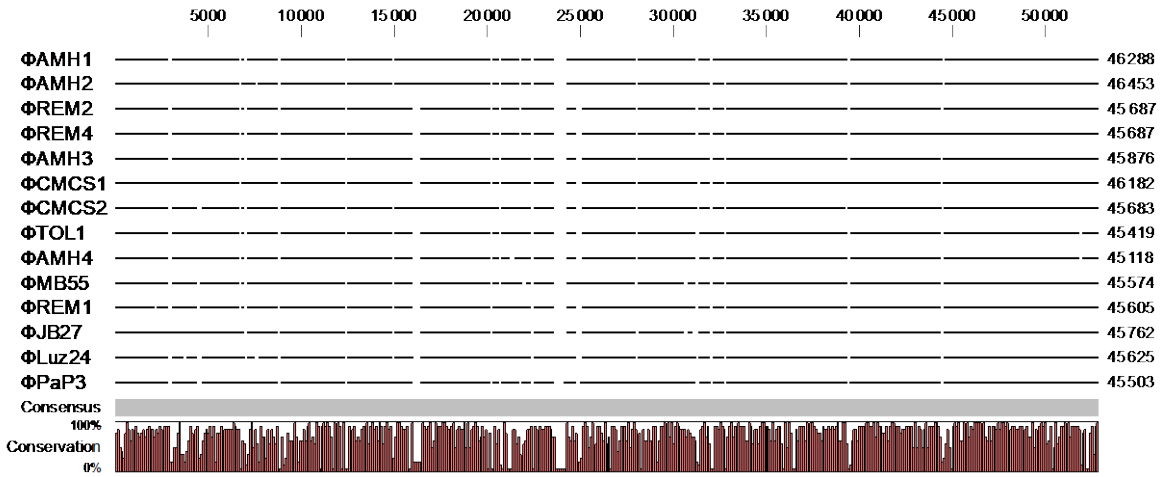


Figure 3.4. Summary of the genomic comparisons of Luz24-like phages. All phages, listed in left hand column, are aligned and had genomes sizes of around 45 kbp. The red bars depict sequence conservation and blank areas represent regions of sequence variation between the phage genomes.

Notwithstanding the ease of phage isolation on the *P. tolaasii* 2192^T strain, from a practical perspective these results suggested that isolation of new phages from the local river using this particular bacterial host strain created a substantial enrichment bias on the classes of phages that were enriched.

Because of the strong phage enrichment bias engendered by use of strain 2192^T, we initiated a series of new enrichments using alternative environmental sources including soil and wild mushroom samples and samples from mushroom farms. We now hope to develop a wider selection of new phages through further enrichments from commercial production plants to then build potentially useful phage cocktails that ideally will show wider strain coverage of *P. tolaasii* hosts or '*P. gingeri*' hosts. We also aim to test some of the phages in experiments using the new pot growth protocols described earlier, initially as a proof of principle that some phages will have biocontrol capacity under such mushroom growth conditions.

Conclusions

- Strong brown blotch, strong and mild ginger blotch and pitting observed in several UK farms was identified as *Pseudomonas tolaasii*, '*P. gingeri*' and *P. costantinii*. Inoculation of casing with *P. costantinii* also resulted in a marked reduction in the number of healthy mushrooms harvested

- A new method to test the pathogenicity of mushrooms in small pots enclosed in plastic bags has been developed and used successfully
- Ten new TaqMan assays have been developed based on recently obtained whole genome sequences, to detect groups of pathogenic *Pseudomonas* that were not detected by previously developed real-time assays that target *P. tolaasii* and '*P. gingeri*' (project M063)
- TaqMan assay results on casing extracts corresponded with the pseudomonad isolates (*P. tolaasii* or '*P. gingeri*') that were inoculated into the casing, although samples inoculated with *P. costantinii* were negative for the assays
- Incubation of the casing samples with compound B increased the pseudomonad count in the extract but did not improve the resolution of the TaqMan assay
- Inoculation of the casing with a non-pathogenic pseudomonad (isolate *P*. '*reactans*' P7759) suppressed blotch caused by '*P. gingeri*' and *P. costantinii* in a small pot assay
- Commercially available pseudomonad biocontrol agents, including *Pseudomonas putida*, *P. fluorescens* and *P. chlororaphis*,did not reduce the incidence of blotch
- Irrigation with ionic solutions did not consistently reduce the incidence of blotch in controlled assays
- The analysis of partial sequences of two genes (ITS and TEF) of culture collection and recent farm isolates of *Trichoderma* species resulted in changes to the original culture designations
- Cultures obtained from mushroom substrates show that *T. aggressivum* f. *europeum* was prevalent on two farms
- Phages infecting *P. tolaasii* and *P. gingeri* have been isolated and some have been genomically sequenced. Further phages are being enriched, purified, and assessed for host range against different strains of both mushroom pathogens. Some phages will be assessed for biocontrol capacity using pot-grown mushroom assays

Knowledge and Technology Transfer

Presentation at the Mushroom Grower Group meeting, 3rd of September 2020, by Ralph Noble.

Glossary

Bacteriophage, shortened to phage: virus that infects and replicates within bacteria and archaea.

Microbiome: genetic material of all microbes (bacteria, fungi, protozoa and viruses) that live in complex communities in a certain environment.

HRI: Horticulture Research International (now part of the School of Life Sciences, Wellesbourne Campus, University of Warwick.

Trichoderma biotypes: there are four biotypes that are now included in different species and subspecies as detailed in Table S1.

Table S1. *Trichoderma* biotypes, presence in the UK and economic damage.

Biotype	Name	Present in UK	Economically Damaging
Th1	*Trichoderma harzianum*	Yes	No
Th2	*Trichoderma aggressivum f. europaeum*	Yes	Yes
Th3	*Trichoderma atroviride*	Yes	No
Th4	*Trichoderma aggressivum f. aggressivum*	No*	Yes
*In Europe, it has been detected in Hungary in 2015 (Hatvani et al., 2017)			

References

Anonymous (2011) Specification for composted materials. PAS100: 2011. British Standards Institution.

Beelman RB et al (1987) Treatments on quality and shelf-life of fresh mushrooms. Developments in Crop Science 10: 271-282.

Caporaso, J.G. et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences 108, 4516-4522.

Chen X., Romaine CP, Ospina-Giraldo MD and Royse DJ (1999) A polymerase chain reaction-based test for the identification of *Thrichoderma harzianum* biotypes 2 and 4, responsible for the worldwide green mold epidemic in cultivated *Agaricus bisporus*. Appl. Microbiol. Biotechnol. 52:246-250.

Cronin MJ et al (1996) Putative mechanism and dynamics of inhibition of the apple scab pathogen Venturia inequalis by compost extracts. Soil Biol Biochem 28: 1241-1249.

Elphinstone J., Noble R (2017) M 63 – Mushrooms: Identification, detection and control of *Pseudomonas* species causing different types of bacterial blotch symptoms. AHDB Horticulture.

EPPO Bulletin (2016) PM 7/129 (1) DNA barcoding as an identification tool for a number of regulated pests. 46: 501-537, ISSN 0250-8052. DOI: 10.1111/epp.12344

EU (2015) European Union Seventh Framework Programme (FP7/2007-2013) grant agreement no. 286836 – MushTV.

Fletcher J, Gaze RH (2008) Mushroom Pest and Disease Control. UK. Mason Publishing.

Friedl MA, & Druzhinina IS (2012) Taxon-specific metagenomics of Trichoderma reveals a narrow community of opportunistic species that regulate each other's development. Microbiology, 158, 69–83. Doi:10.1099/mic.0.052555-0

Glöckner FO et al. (2017) 25 years of serving the community with ribosomal RNA gene reference databases and tools. Journal of Biotechnology, 261, pp.169-17.

Hatvani L et al (2017) First report of *Trichoderma aggressivum* f. *aggressivum* green mold on *Agaricus bisporus* in Europe. Plant Disease 101: 1052.

Illumina (2013) 16S Metagenomic Sequencing Library Preparation Guide [Online]. http://emea.support.illumina.com/content/dam/illuminasupport/documents/documentation/chemistry_documentation/16s/16s-metagenomiclibrary-prep-guide-15044223-b.pdf.

Kõljalg U. et al. (2013) Towards a unified paradigm for sequence-based identification of Fungi. Molecular Ecology, 22, pp. 5271-5277.

Kosanovic D, Grogan H and Kavanagh K (2020) Exposure of *Agaricus bisporus* to *Trichoderma aggressivum* f. *europaeum* leads to growth inhibition and induction of an oxidative stress response. Fungal Biology 124: 814-820.

Kredics, L., Chen, L., Kedves, O., Büchner, R., Hatvani, L., Allaga, H., … Vágvölgyi, C. (2018) Molecular Tools for Monitoring Trichoderma in Agricultural Environments. Frontiers in Microbiology, 9, 1599. Doi:10.3389/fmicb.2018.01599.

Lane C (2010) Trichoderma green mould – Diagnostic assay for improved disease management. AHDB project M48. Final report. 20pp.

Miller FC and Spear M (1995) Very large commercial trial of a biological control for mushroom blotch disease. Mushroom Sci14: 635-642.

Noble R and Dobrovin-Pennington A (2017) M62 – Mushrooms: Inhibiting fungicide degradation in casing, and evaluating fungicides, biopesticides and diseased area covering methods for fungal disease control. AHDB Horticulture.

Noble R et al (2011) Indicator organisms for assessing sanitization during composting of plant wastes. Waste Mangmnt 31: 1711-1719.

O'Brien M et al (2017) Detection of Trichoderma aggressivum in bulk phase III substrate and the effect of T. aggressivum inoculum, supplementation and substrate-mixing on Agaricus bisporus yields. Eur J Plant Pathology 147: 199-209.

Oliveira LSS, Harrington TC, Ferreira MA, Damacena MB, Al-Sadi AM, Al-Mahmooli HIS and Alfenas AC (2015) Species or genotypes? Reassessment of four recently described species of the Ceratocystis wilt pathogen, *Ceratocystis fimbriata*, on *Mangifera indica*. Phytopathology 105: 1229-1244.

Pardin C et al (2018) Dynamics of compost microbiota during the cultivation of Agaricus bisporus in the presence of Bacillus velezensis QST713 as biocontrol agent against Trichoderma aggressivum. Biological Control 127: 39-54.

Pritchard L., Holden N.J., Bielaszewska M., Karch H., and Toth I.K. (2012). Alignment-Free Design of Highly Discriminatory Diagnostic Primer Sets for Escherichia coli O104:H4 Outbreak Strains. *PLOS ONE* 7**,** e34498.

Pritchard L, Humphris S, Saddler GS, Parkinson NM, Bertrand V, Elphinstone JG, and Toth IK (2013). Detection of phytopathogens of the genus Dickeya using a PCR primer prediction pipeline for draft bacterial genome sequences. *Plant Pathology* 62**,** 587-596.

Radvanyi D et al (2015) Rapid evaluation technique to differentiate mushroom disease related moulds by detecting microbial volatile organic compounds using HS-SPME-GC-M. Annals Bioanal Chem 407: 537-545.

Rice P., Longden I. and Bleasby A. (2000). EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet* 16**,** 276-277.

St Martin CCG (2014) Potential of compost tea for suppressing plant diseases. CAB Reviews 9, No. 032: 1-37.

Taparia T, Krijger M, Hodgetts J, Hendriks M, Elphinstone JG and van der Wolf J (2020a) Six multiplex TaqManTM-qPCR assays for quantitative diagnostics of *Pseudomonas* species causative of bacterial blotch diseases of mushrooms. Frontiers in Microbiology 11:989. doi: 10.3389/fmicb.2020.00989.

Taparia T, Krijger M, Haynes E, Elphinstone JG, Noble R and van der Wolf J (2020b) Molecular characterization of *Pseudomonas* from *Agaricus bisporus* caps reveal novel blotch pathogens in Western Europe. BMC Genomics 21: 505.

Toju H. et al. (2012). High-Coverage ITS Primers for the DNA-Based Identification of Ascomycetes and Basidiomycetes in Environmental Samples. PloS ONE 7,e40863.

White TJ, Bruns T, Lee S and Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A guide to methods and Applications. 315-322.

Wong WC, Preece TF (1980) Pseudomonas tolaasii in mushroom crops: A note on primary and secondary sources of the bacterium on a commercial farm in England. J Appd Bacteriology 49: 305-314.

Zeng Z and Salmond G (2020) Bacteriophage host range evolution through engineered enrichment bias, exploiting heterologous surface receptor expression. *Environmental Microbiology*, https://doi.org/10.1111/1462-2920.15188

Appendices

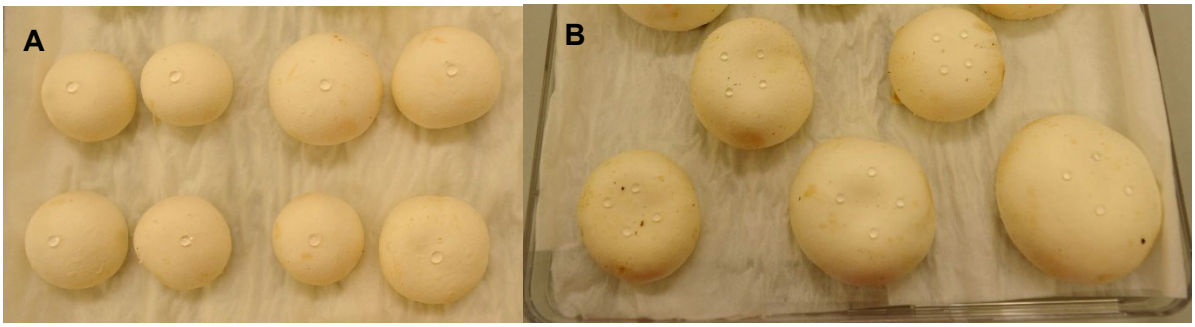


Figure S1. Cut cap pathogenicity assays in large plates after drop inoculation with bacterial suspensions from a range of isolates. A: Small mushroom caps inoculated with single drops of *Pseudomonas* spp. suspension. B: larger caps inoculated with four drops of suspension.

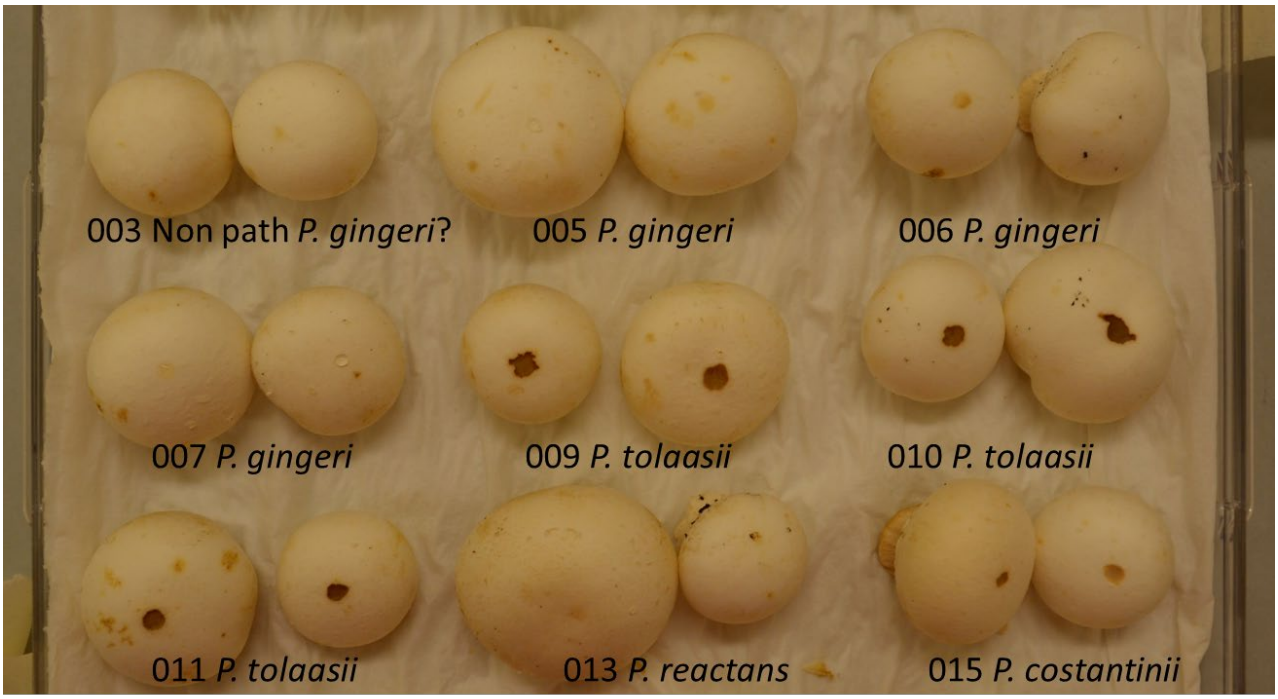


Figure S2. Cut cap assay with nine *Pseudomonas* isolates (48hrs after inoculation, single drop inoculations in each cap).

Figure S3. Growth room set up with mushrooms starting to appear in pots four days after inoculation.

Figure S4. Some of the harvested mushrooms from the first flush (7 dai) from pots inoculated with: top left, FSBactM 053 (‘*P. gingeri*’); top right, 037 (*P. constantinii*); bottom left, 009 (*P. tolaasii*); bottom right, sterile water control.

EF ITS

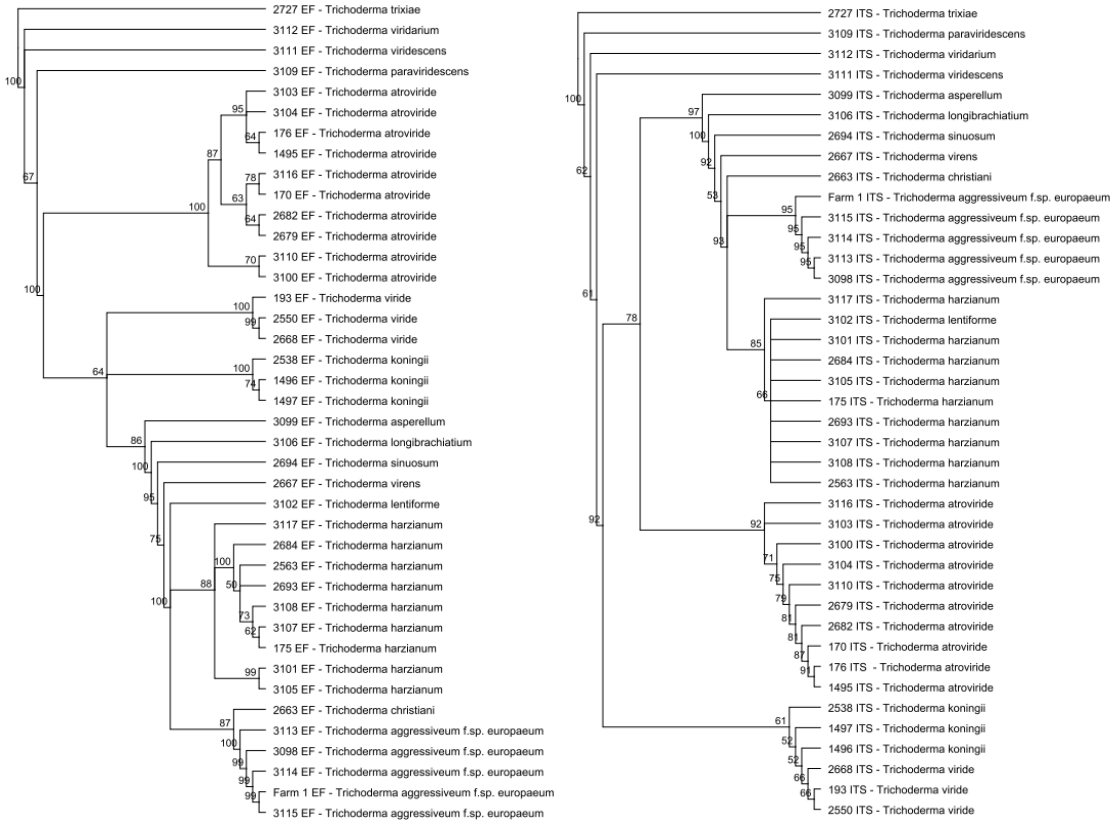
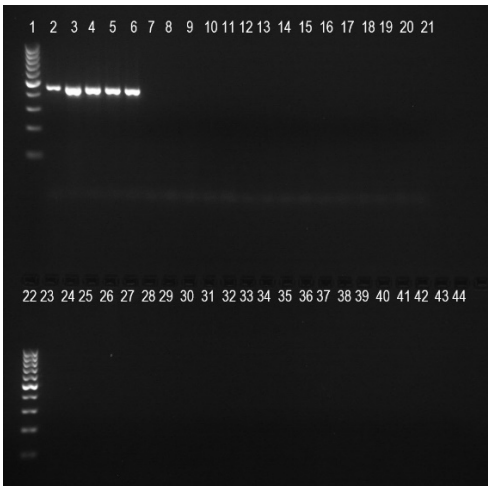


Figure S5. Trees based on the alignments of EF and ITS partial gene sequences for *Trichoderma* strains



1) Ladder;
2) Farm 1; 3) 3098; 4) 3113; 5) 3114; 6) 3115 (*T. aggressivum*)
7) 3099 (*T. asperellum*);
8) 2682; 9) 170; 10) 2679; 11) 176; 12) 1495; 13) 3100; 14) 3103; 15) 3104; 16) 3110; 17) 3116 (*T. atroviride*)
18) 2663 (*T. christani*)
19) 1497; 20) 1496; 21) 2538 (*T. koningii*)
22) Ladder
23) 2684; 24) 175; 25) 2563; 26) 3101; 27) 3105; 28) 3107; 29) 3108; 30) 3117; 31) 2693 (*T. harzianum*)
32) 3102 (*T. lentiforme*); 33) 3106 (*T. longibrachiatium*)
34) 3109(*T. paraviridescens*); 35) 2694 (*T. sinuosum*)
36) 2727 (*T. trixiae*); 37) 2667 (*T. virens*)
38) 3112 (*T. viridarium*)
39) 2550; 40) 193; 41) 2668 (*T. viride*)
42) 3111(*T. viridescens*)
43) EB; 44) MM

Figure S6. Gel with PCR products amplified using the assay from Chen et al. (1999) targeting *T. aggressivum* (previously *T. harzianum* biotypes 2 and 4) from a range of different *Trichoderma* species.