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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

- Several new blotch causing *Pseudomonas* species and green mould causing *Trichoderma aggressivum* f. *europaeum* isolates have been obtained from UK farm samples and identified
- New real time PCR assays can identify groups of blotch causing *Pseudomonas* that were not detected using previously developed assays targeting *P. tolaasii* and *'P. gingeri'*
- Bacterial and fungal populations have been sequenced and compared on samples collected from four farms; samples of casing and substrate had distinct populations; *Trichoderma* was detected at a high level in one farm and low level in two other farms
- The detection of *Trichoderma* spp. in farm samples was confirmed by qPCR and MinION sequencing
- Irrigation of casing with a non-pathogenic pseudomonad strain showed promising control levels of three blotch types in a pot bioassay and showed reduction of 40% of blotch on the first flush in a farm experiment
- Bacteriophages isolated from river water and mushroom farm samples have been characterised and shown to reduce blotch in a controlled bioassay

Background

Bacterial blotch is a mushroom disease that has been shown to be caused mainly by the bacterial species *Pseudomonas tolaasii*, *P. costantinii* and several groups of *'P. gingeri'* in the UK. This disease 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. The development of tools that allow early detection of disease and understanding the possible sources of infection should be beneficial to the industry. In this project, we furthered our understanding of the communities of microorganisms involved in mushroom production and developed 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. The tools developed have potential 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, we confirmed that 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 to monitor general farm hygiene.

This project follows on from project M 063 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 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

Summary

Blotch detection and control

Bacterial isolates obtained from mushrooms from several UK farms with symptoms of severe brown blotch, pitting and strong and mild ginger blotch were identified as *Pseudomonas tolaasii*, *P. costantinii* and several groups of *'P. gingeri'* respectively. A Pseudomonad isolated from a mushroom of the brown strain Heirloom with dark brown blotch symptoms was confirmed as being *P. tolaasii* following pathogenicity tests, qPCR and sequencing.

Comparison of whole genome sequences, showed that there are at least five different groups of isolates currently included in *'P. gingeri'* that can cause ginger blotch in UK farms; four

isolates that were not detected by previously developed qPCR assays belonged to three groups of *'P. gingeri'*. Pathogenicity (the ability to cause disease) in mushrooms was confirmed for a range of isolates in cap droplet inoculation tests and pot culture tests. A new method to test the pathogenicity in mushrooms grown in small pots enclosed in plastic bags has been developed and used successfully in experiments to test an antagonist that can reduce the level of disease seen in a crop.

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 only targeted *P. tolaasii* and some groups of *'P. gingeri'* (project M 063).

The counts of background and pathogenic Pseudomonads can be increased by adding a compound to Luria-Bertani broth during incubation of casing extracts. This can help achieve detectable concentrations in samples containing low levels of the Pseudomonad populations.

Commercially available pseudomonads that are used to control pathogens or as growth promoters in other crops, 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.

Non-pathogenic Pseudomonads from culture collections were also tested as potential antagonists to control blotch. Application of inoculum from a *'P. reactans'* isolate P7759 (a non-pathogenic isolate from mushrooms) to pots resulted in an increase in the number of healthy mushrooms compared with water treated pots, except for pots inoculated with *'P. gingeri'*; brown blotch caused by *P. tolaasii* was reduced by the application of P7759**.** In an on-farm experiment, application of P7759 inoculum resulted in a reduction in the number of blotched mushrooms that was not statistically significant.

Bacteriophages (viruses that can infect and destroy bacteria) that target most isolates of *P. tolaasii, P. costantinii* and *'P. gingeri'* were obtained and characterised. The application of bacteriophages resulted in significantly fewer blotched mushrooms in a pot culture bioassay, although there was no corresponding increase in the number of clean healthy mushrooms.

Green mould detection and control

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. PCR assays were selected for the detection of *Trichoderma* spp. at genus, species and subspecies level.

A potential antagonist, *Bacillus subtilis* syn. *B. amyloliquefaciens* AHDB 9849, was tested in pot experiments and was shown to be ineffective in suppressing green mould caused by *T. aggressivum* in compost.

Microbial communities

A study of microbial communities in cropping substrates obtained from four commercial farms, sampled at different cropping stages, was conducted to compare populations in healthy and diseased crops. Microbiome sequencing of bacterial and fungal communities showed differences between substrate and casing and some differences between farms. The methods used did not allow the identification of different *Pseudomonas* species, but successful detection of *Trichoderma* was achieved. A specific qPCR assay developed at Fera for *T. aggressivum* and MinION ITS sequencing detected these pathogens in mushroom casing at concentrations that did not produce visible green mould symptoms.

Financial Benefits

Although it is too early to state and calculate the financial benefits of this work, the development and selection of assays that can detect most blotch causing *Pseudomonas* and *Trichoderma* species can lead to financial benefits if used to make early decisions on disease management.

The identification of potential biocontrol agents including a *Pseudomonas* strain and bacteriophages might lead to significant financial benefits.

Action Points

- A range of diagnostic tests for *Pseudomonas* spp. causing blotch have been developed during this project to include most of the blotch causing pathogens identified in the UK; these tests, available at Fera Science (and planned to be published), are recommended for identification of *P. tolaasii*, *P. constantinii* and most groups of *'P. gingeri'*
- Pathogenicity of other species of *Pseudomonas* that might be present in UK farms should be further investigated (and further assays developed if necessary)
- A *Pseudomonas* sp. strain was shown to have the potential to reduce mushrooms with blotch and increase the number of healthy mushrooms. The application of this

strain as a commercial product should be further tested in different farms in order to assess the potential benefits of the treatments

- Further work is needed to test a range of bacteriophages, individually and/or in cocktails, as potential biocontrol agents and to develop a strategy for their use.
- Diagnostic tests for *Trichoderma* spp. including PCR tests for all *Trichoderma* spp. and a qPCR assay for T*. aggressivum,* the cause of green mould, are published and available (at Fera Science) and are recommended for detection of *Trichoderma* spp. that can be linked to issues in farm hygiene that should then be addressed
- Development of qPCR assays for *Trichoderma* at genus and subspecies level could be useful for quick assessment of farm hygiene
- Further studies involving microbiome sequencing are needed to characterise healthy and disease-linked microbe communities

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 M 063, molecular tests using quantitative real time (TaqMan) polymerase chain reaction have been identified that detected *P. tolaasii* and some groups of *'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 has also been 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 from 5% of fresh casing material at low concentrations and at higher concentrations

(up to 100%) during the mushroom cropping period, using selective plating (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, and so cannot give a reliable estimate of subsequent disease risk (Elphinstone and Noble, 2017). Incubation of casing samples in Luria-Bertani (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, and these products are not currently authorised for use on mushrooms in the UK. Whereas the introduced *Pseudomonas* population in the casing increased during the mushroom cropping, the population of an introduced *Bacillus* population gradually declined.

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.

Bacteriophages (phages for short) are generally very specific viruses that infect and kill bacterial cells without negative effects on human or animal cells. Phages occur naturally in the environment and they tend to persist as long as the host is present. Phage biocontrol has

been studied for a number of bacterial plant diseases (Buttimer et al., 2017). In this project, we have investigated a novel biocontrol strategy involving the use of bacteriophages that specifically target blotch pathogens. 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 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 selection of phages from the environment, including from the River Cam (Cambridge, UK) by our assorted enrichment strategies that now enable simple 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, but the collection of phages needed to be increased to target other pathogenic *Pseudomonas* species and strains. Our data encourages optimism about phage-mediated biocontrol possibilities.

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 sanitisation 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 sanitisation 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), although this product is not currently authorised for use on mushrooms in the UK. 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 were 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 assembled in previous studies (Elphinstone and Noble, 2017) has been used in this work. In addition, new isolates were obtained and added to the collection during this project. 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 sterile loops. Plates

were 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): 0, no symptoms; 1, light mark; 2, intermediate mark; 3 strong brown mark, often expanding.

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 (Appendix Fig. S2). 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. S2). 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 1.2.1) 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).

First, assays were developed for: three groups of *'P. gingeri'* (ANI groups 1, 5 and 14), *P. costantinii* (ANI group 3), P. *NCO_2* (ANI group 2) and *P. yamanorum* (ANI group 10) (Table 1.2). After sequencing additional isolates, new assays were developed for two groups of *'P. gingeri'* (ANI groups 5 and 24).

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 1.2.1. Samples used as input into the find_differential_primers.py script.

ANI Group / species	Sample ID
1 (*'P. gingeri'*)	21614711, 21615525, A6001, C2001, IPO3738**, J1002, J4002, POWE01
5 (*'P. gingeri'*)	A8002, H7001, IPO3754*
5 (*'P. gingeri'*)[a]	FSBactM 033, 050, 059, 060, P7786, H7001b, A8002
14 (*'P. gingeri'*)	C1001, C3001, C4002, D1001, D5001, D8001, E1001, IPO3737, IPO3757, IPO3767, IPO3769, IPO3776, IPO3777, P8018
24 (*'P. gingeri'*)[a]	G9001, FSBactM 035
22 (*'P. gingeri'*)[a,b]	F1001, FSBactM 039
3 (*P. costantinii*)	21815971, 21815972, MDDR01
2 (P. NCO2)	21615526, A4002, B6001, C6002, C8002, CP025624, D3002b, D4002, D5002, D6002, E6002, F1002, F8002, F9001, G1002, G5001, I8001, IPO3774, IPO3775, K5002, P7758, P7779
10 (*P. yamanorum*)	B4002, IPO3753, LT629793

[a] Assays designed after sequencing additional 22 isolates at Fera Science

[b] No assays could be designed for this group

*sample ID for which the files were corrupted during data transfer and unusable in this analysis

Twenty-two UK isolates were selected for whole genome sequencing in 2020. The isolates selected included pathogenic isolates belonging to *P. tolaasii* (isolates FSBactM 061, 063, 064, 082 and 083), *P. constantinii* (FSBactM 029, 040, 057), *'P. gingeri'* ANI group 5 (FSBactM 033, 059) and *'P. gingeri'* ANI group 14 (FSBactM 053). In addition, an isolate (FSBactM 06) of *'P. gingeri'* of unknown ANI group and pathogenic isolates (FSBactM 035, 039, 050, 060) of unknown species that were not detected by existing qPCR assays were included. Non-pathogenic isolates that have been misidentified in the past as *P. tolaasii*

(FSBactM 014 and 027) and *'P. gingeri'* (FSBactM 02), an isolate of the *P. NCO_2* group (FSBactM 031), an unknown isolate (FSBactM 081) and an isolate of '*P. reactans*' (FSBactM 013) used as an antagonist were also sequenced.

Isolates were grown in King's B agar plates for 24-48h and DNA was extracted using a Qiagen DNeasy blood and tissue kit following the manufacturer's instructions for bacteria, using an initial lysis with buffer containing 20mg/ml lysozyme. The DNA samples were sequenced on an Illumina MiSeq at Fera Science. Quality control and trimming was performed by Bbduk, before each sample was assembled by Spades, version 3.14. Coverage was determined using Bbmap. Average nucleotide identity (ANI) was then calculated using the pyani software.

Sequences of isolates confirmed as *'P. gingeri'* were taken forward in an attempt to identify primers for ANI groups 5, 22 and 24 as detailed in Table 1.

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. Samples of casing were also taken from the first flush of a commercial crop showing mushroom cap pitting symptoms caused by *P. costantinii*. Pre-enrichment of *Pseudomonas* species was conducted by incubation of casing samples in LB broth containing compound A or B to try to increase bacterial numbers above detection levels.

Casing samples and broth extracts (pre-and post-incubation) were frozen upon receipt at -20°C until DNA extraction. The samples were thawed at 4°C overnight. Tubes were cleaned using 1% Distel solution, then centrifuged at 14000 xg for 15 minutes. The liquid supernatant was poured off in a sterile 15 ml tubes and frozen again at -20°C until the end of the extraction. The solid pellet was then weighted and transferred into 50 ml tubes using individual sterile spatulas. DNA was extracted from 2 to 4g pellets using the DNeasy PowerMax Soil kit (QIAGEN) following manufacturer's instructions and afterwards tested by TaqMan PCR using universal 16S assays (to confirm that DNA was successfully extracted) and tested 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

1.4.1 *Trichoderma* isolates

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.

A *Trichoderma aggressivum f. aggressivum* isolate was ordered from the Westerdijk Fungal Biodiversity Institute (MB#494487; CBS 100527) to be used in the comparisons.

Fourteen isolates of *Trichoderma* species associated with mushroom substrates taken from the Fera and HRI culture collections and a further ten 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 assays developed previously for the genus *Trichoderma* (Hagn et al. 2007; Kosanovic et al. 2020), for the species *T. aggressivum* (Chen et al., 1999; Kosanovic et al., 2020) and for the subspecies *T. aggressivum* f. *europaeum* (O'Brien et al. 2017) were tested with the collection (Table 1.4.1). All PCRs were in a 25 ul reaction volume with 1x DreamTaq Green PCR Master Mix and 0.5 uM of each primer.

Specificity of the qPCR assay previously developed at Fera for specific detection of *T. aggressivum* as part of AHDB project M 048 (Lane, 2010) was also tested.

Table 1.4.1. Conventional PCR assays tested for *Trichoderma* detection and identification

Reference	**Primers / cycling conditions**	**Targeting (expected size of product)**
Hagn et al. (2007)	uTf: 5'AACGTTACCAAACTGTTG'3 uTr: 5'AAGTTCAGCGGGTATTCCT'3 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 55.5 °C for 30 s and 72 °C for 30 s with a final extension of 72 °C for 10 min.	Genus *Trichoderma* (~540 bp)
Kosanovic et al. (2020)*	EX ITS1: 5'GTA ACA AGG TTT CCG TAG GTG'3 EX ITS4: 5' TTC TTT TCC TCC GCT TAT TGA TAT GC'3 95 °C for 2 min followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. Final amplification was 72 °C for 10 min	Genus *Trichoderma* (~620 bp)
Chen et al. (1999)	Th-F: CGGTGACATCTGAAAAGTCGTG Th-R: TGTCACCCGTTCGGATCATCCG 94°C for 2 min followed by 35 cycles of 94°C for 25 s, 60°C for 35 s and 72°C for 65 s. Final amplification was 72 °C for 5 min	Species *T. aggressivum* (previously T. *harzianum* biotypes 2 / European biotype and 4 / North American biotype) (444 bp)
Kosanovic et al. (2020)*	TH1 INT: 5'CCC CCT CGC GGG TTA TTT TTA CT'3 EX ITS4: 5' TTC TTT TCC TCC GCT TAT TGA TAT GC'3 95 °C for 2 min followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min. Final amplification was 72 °C for 10 min	Species *T. aggressivum* (463 bp)
Mills (1996); O'Brien et al. (2017) [Kosanovic et al. (2020)*]	18S INT: 5'TAA CAA CAC GCC TGC TTA AGA'3 TH1 INT REV: 5'GAG AAG GCT CAG ATA GTA AAA AT'3 95 °C for 3 min followed by 30 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min. Final amplification was 72 °C for 10 min. (O'Brien et al. 2017)	Subspecies *T. aggressivum* f. *europaeum* (802 bp)
* Primers in this reference have some differences (possibly typos) when compared with previous references. These assays were designed in previous work based at Wellesbourne / Warwick HRI.		

1.4.2 Pot pathogenicity test for *Trichoderma* isolates

A pot experiment was set up to determine the threshold at which *Trichoderma aggressivum* and *Trichoderma harzianum* inocula could be detected after application to the casing. Pots containing 550 g of spawn run compost (mushroom strain Sylvan A15) were cased with 300 g casing. Four replicate pots were inoculated with the following 20mL treatments:

1. Control (SDW)
2. *Trichoderma aggressivum* f. *europaeum* (3113) Low, 2 x 10^5 cfu/mL
3. *Trichoderma aggressivum* f. *europaeum* (3113) High, 2 x 10^7 cfu/mL
4. *Trichoderma harzianum* 163 (3107) Low, 2 x 10^5 cfu/mL
5. *Trichoderma harzianum* 163 (3107) High, 2 x 10^7 cfu/m

The pots were assessed for healthy and diseased mushrooms in two flushes. Two replicate samples of casing were taken from each treatment on days 0, 3, 10, 17 and 24 and analysed for the presence of *Trichoderma* on plates with potato dextrose agar.

Samples were sent to Fera Science and were frozen upon receipt at -20°C until DNA extraction. The samples were thawed at 4°C overnight and 10g was weighted from each sample using clean weighting boats and spatulas for each sample. DNA was extracted using the DNeasy PowerMax Soil kit (QIAGEN), following manufacturer's instructions processing seven samples together plus one Extraction Blank.

The samples were analysed at Fera Science with the specific qPCR assay for *T. aggressivum*.

The ITS region of 47 samples were amplified using the ITS5 and ITS4 primers (5'-GGAAGTAAAAGTCGTAACAAGG-3' and 5'-TCCTCCGCTTATTGATATGC-3' respectively) and sequenced on an Oxford Nanopore MinION R9.4 flowcell, over 48 hours. Reads were basecalled with Guppy (version 5.0.11) in high accuracy mode. Reads were trimmed with Cutadapt (Martin, 2011) and then any reads which were too long (greater than 800 bases) or short (fewer than 600 bases) for the expected amplicon size were removed. Subsequent filtering was applied to remove any reads which had a Phred quality score of less than 10. A custom ITS database was built from sequences obtained from Genbank's fungal RefSeq database, and individual reads were subject to a BLASTn (MegaBlast) (Altschul et al, 1990) search against this database. The resulting reads were filtered so that only matches to sequences in the database that had a percentage identity of at least 85% and an alignment length of at least 80% were included. Of these hits, only the hits with a bitscore within 3% of the highest scoring hit were included in the final set of filtered reads. Finally, a lowest common ancestor approach was applied to the dataset, where if at least 75% of the assignments agreed, that taxonomic label was applied to the read. Otherwise, the next highest rank was considered, and the process was repeated until a label was assigned to the read. Where a lowest common ancestor could not be agreed upon, reads were assigned as 'Unresolved'. For each sample, the taxa with the highest number of reads was selected, and then any taxa with a number of reads greater than or equal to 0.5% of this number was also selected. The reads associated with these taxa were extracted and used to build a higher accuracy consensus sequence with Oxford Nanopore's Medaka tool (Oxford Nanopore Technologies Ltd, 2018).

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 four different commercial mushroom farms were sampled in 2020, at different cropping stages from casing to end of third flush. Samples were taken from bed areas that had blotch, green mould or no apparent diseases.

The soil samples were frozen upon receipt at -20°C until DNA extraction. The samples were thawed at 4°C overnight. Then, 10g samples were extracted using the DNeasy PowerMax Soil kit (QIAGEN), following manufacturer's instructions processing seven samples together plus one Extraction Blank.

The communities of bacteria and fungi (including pathogenic *Pseudomonas* and *Trichoderma* spp. and potential beneficial microorganisms such as *Pseudomonas* and *Bacillus* spp.) were assessed using high throughput Illumina MiSeq sequencing based metabarcoding with 16S and ITS rRNA markers. Primers were trimmed using cutadapt and denoised with dada2, before an additional chimera filter was applied with vsearch. Samples were removed if fewer than 3000 reads remained after filtering, and interactive bar plots were generated from the subsequent tables. A sequence alignment was created using mafft, and a phylogenetic tree was created and rooted at the mid-point using fasttree. The alignment was used to generate core phylogenetic diversity metrics.

The population of green mould causing *Trichoderma aggressivum* was also studied through qPCRs previously developed (see 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, 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, 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 3.1a.

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

3.2 Effect of antagonists on blotch

3.2.1 Effect of antagonists on blotch in pot bioassays

Experiment 3.2.1a 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, 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 3.2.1b. 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, 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 3.2.1c. 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, 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.

Experiment 3.2.1d. Effect of PlantWorks produced inoculum of P7759 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, 2018)

Suspensions of the following experimental isolates were applied to pots:

1. Control (SDW)
2. P7759 (FSBactM 013; *'P. reactans'*), produced by PlantWorks

PlantWorks produced suspension of P7759 (10^8 – 10^9 cells/mL) was diluted to 10%v/v and applied in 30 ml immediately after casing, in 16 ml 12 days after set up and after the first flush. Control pots were treated with sterile distilled water.

3.2.3 Effect of antagonists on blotch in commercial farm experiments

Two experiments were conducted at a commercial mushroom farm. Mushroom were grown in wooden trays (cropping area 2.4 m^2) using Phase II compost spawned with the strain Sylvan A15 and cased with Harte casing. Watering of trays followed the commercial farm practice, with additional applications made to the experiment trays as described below.

Inoculum of *'P. reactans'* P7759 containing 10^9 to 10^{10} cfu/mL was prepared by PlantWorks. In the first experiment, 150 mL of inoculum was diluted to make 2 litres of solution per tray, applied 9 days after casing. In the second experiment, 200 mL of inoculum was diluted to make 2 litres of solution, applied two days after casing. Control trays were irrigated with 2 litres of water at the same time as inoculum applications.

In each experiment, there were five replicate trays of each treatment, with treatments applied to the top layer of trays in an alternating arrangement.

The numbers of blotched mushrooms on each tray was recorded for three flushes. At the end of the second experiment, samples of casing were tested for the presence of *P. costantinii*.

DNA was extracted from samples as previously described and the rpoD gene sequenced on an Oxford Nanopore MinION R9.4 flowcell. Bioinformatics analysis was performed. Reads were trimmed with Cutadapt and then any reads which were too long (greater than 500 bases) or short (fewer than 800 bases) for the expected amplicon size were removed. Subsequent filtering was applied to remove any reads which had a Phred quality score of less than 7. A targeted database was built using *P. constantinii, 'P. reactans'* and *P. fluorescens* sequences, as these were identified as the taxa of interest. The resulting reads were filtered so that only matches to sequences in the database that had a percentage identity of at least 80% and an alignment length of at least 80% were included. Of these hits, only the hits with a bitscore within 1% of the highest scoring hit were included in the final set of filtered reads. Finally, a lowest common ancestor approach was applied to the dataset, where if at least 75% of the assignments agreed, that taxonomic label was applied to the read. Otherwise, the next highest rank was considered, and the process was repeated until a label was assigned to the read. Where a lowest common ancestor could not be agreed upon, reads were assigned as 'Unresolved'. For rpoD, due to the small number of taxa in the database, reads assigned to all taxa were selected. The reads associated with these taxa were extracted and used to build a higher accuracy consensus sequence with Oxford Nanopore's Medaka tool.

3.2.3 Effect of antagonist on *Trichoderma* green mould in pot bioassays

Two pot experiments were conducted to investigate the effects of *Trichoderma aggressivum* inocula and antagonist product AHDB 9849 on mushroom cropping and green mould. Pasteurised (Phase II) compost was spawned at 0.8%w/w with spawn strain Sylvan A15. In both experiments, treatments were applied to four replicate pots of each treatment. Pots were filled with 550 g compost and cased with 300 g casing. The number of healthy and diseased mushrooms and the presence of green mould in each pot was recorded for two flushes of mushrooms.

Experiment 3.2.3a. Effect of Trichoderma aggressivum isolates applied to Phase II compost

The following treatments were applied to batches of spawned compost:
Trichoderma aggressivum f. *europaeum* inoculum (suspension of 3 x 10^5 cfu/mL applied as 3.5 mL per kg compost)

1. Control (SDW)
2. 23443B (collection strain 3098)
3. 3115 (obtained from a farm in 2020)
4. 3113 (obtained from a farm in 2020)

Antagonist treatment *Bacillus subtilis* syn. *B. amyloliquefaciens* AHDB 9849

1. Control (None)
2. AHDB 9849 (0.1 g per kg compost).

Experiment 3.2.3b. Effect of AHDB 9849 inoculum in compost on Trichoderma aggressivum

The following treatments were applied to batches of spawned compost:
Trichoderma aggressivum isolate 3113 inoculum applied:

1. Control (none)
2. Spore suspension (3 x 10^5 cfu/mL applied as 3.5 mL per kg compost)
3. Infected compost, 0.1% w/w

Antagonist treatment *Bacillus subtilis* syn. *B. amyloliquefaciens* AHDB 9849

1. Control (none)
2. AHDB 9849, 1g/kg compost
3. AHDB 9849, 10g/kg compost

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.

3.3.4 Effect of phages on blotch in a pot experiment

A mushroom blotch pot bioassay was set up using the culture methods described in Section 1.1.2. Pots were inoculated with the following pathogen isolates, 8 days after set-up in 20 ml suspensions containing 10^{8} cells/ml:

1. Control (water)
2. *Pseudomonas tolaasii* FSBactM 061 (TRF42; UK, 1980s)
3. *'Pseudomonas gingeri'* FSBactM 005 (P8018; UK, 2011)
4. *Pseudomonas costantinii* FSBactM 037 (J5 (21815972 Blotch Pred – 10); UK, 2018)

The following phage treatments were applied, 12 days after set-up in a 30 ml suspension:

1. Control (water)
2. JW26
3. JB56
4. JW26+JB56.

The pots were assessed for the number of clean healthy mushrooms and blotched mushrooms (brown and ginger blotch and pitting symptoms).

Results

1. Improved pathogen detection in mushroom cropping substrates

New isolates obtained from five commercial UK mushroom farms are listed in Table 1.1A and B together with isolates used for comparisons in various experiments.

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 December 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. The results of the assays are presented in Table 1.1 A and B and Appendix Fig. S3.

The control isolates of *P. tolaasii* and *P. costantinii* generally produced strong symptoms on caps. The symptoms observed with *'P. gingeri'* isolates were more variable including some very weak reactions (isolates FSBactM 001, 006, 007, 077, 080 produced very weak symptoms on caps).

From the most recent isolations from UK farms (from 2018 to 2020), three isolates (FSBactM 033, 035, 059) were weak on caps, whilst the others 17 were clearly pathogenic (Table 1.1.1A).

Fourteen isolates from the UK, USA and the Netherlands were very weak or non-pathogenic. Six isolates from other *Pseudomonas* species were also confirmed as non-pathogenic on caps. Twenty-six isolates recently obtained from UK farms (from 2018-2020) were considered non-pathogenic or very weak on the cap assay (Table 1.1.1B).

Table 1.1.1A Blotch pathogenicity observed in mushroom cap droplet tests and results of TaqMan assays targeting *'P. gingeri'* and *P. tolaasii* of pathogenic *Pseudomonas* isolates

Isolate ID		Species Identity (ANI group[1])	Origin	Cap test symptom scores[2]	TaqMan[3] C_T values		
FSBactM	Other identifier				Pg2	Pg6	Ptol
Control isolates from the UK							
001	NCPPB 3146	*'P. gingeri'* (1)	UK, 1981	0, 1	18	17	40
005	P8018	*'P. gingeri'* (14)	UK, 2011	2	18	17	40
006	21614711	*'P. gingeri'* (14)	UK, 2016	1	17	17	40
007	21615525	*'P. gingeri'* (1)	UK, 2016	1	17	17	40
024	P7786	*'P. gingeri'* (5)	UK, 2011	2	40	40	40
009	P7544, NCPPB 2192	*P. tolaasii*	UK, 1965	3	40	40	17
061	TRF 42 col A	*P. tolaasii* (6)	UK, 1980s	3	40	40	17
062	TRF 42 col B	*P. tolaasii*	UK, 1980s	3	40	40	17
063	TRF 59	*P. tolaasii* (6)	UK,1980s	3	40	40	17
Control isolates from other countries							
015	NCPPB 2192 T	*P. costantinii*	Finland, 1997	2,3	40	40	40
073	C2001	*'P. gingeri'* (1)	Netherlands, 2016	1, 2	Nt	22	40
080	IPO3777	*'P. gingeri'* (14)	Netherlands, 2015	0, 1	Nt	21	40
077	H7001	*'P. gingeri'* (5)	Netherlands	0, 1	Nt	40	40
Isolates obtained 2018-2020 from UK commercial farms							
029	21815970 Blotch 2 I7	*P. costantinii* (3)	F, 2018	2 pit	40	40	40
033	21815970 J1	*'P. gingeri'* (5)	F, 2018	0, 1	40	40	40
035	21815971 Pit 8 J3	*'P. gingeri'* (24)	F, 2018	1	40	40	40
036	21815971 Pit 9 J4	*P. costantinii* (3)	F, 2018	3 pit	40	40	40
037	21815972 Blotch 10 J5	*P. costantinii* (3)	F, 2018	3 pit	40	40	40
039	21815973 Pit 12 J7	*'P. gingeri'* (22)	F, 2018	2	40	40	40
040	21815973 Pit 13 J8	*P. costantinii* (3)	F, 2018	2	40	40	40
050	22000021 mr1	*'P. gingeri'* (5)	B, Jan. 2020	Nt	Nt	40	40
051	22000021 mr2	(like 050)	B, Jan. 2020	3	Nt	40	40
053	22000021 mr3B	*'P. gingeri'* (14)	B, Jan. 2020	2	Nt	23	40
057	22007191	*P. costantinii* (3)	F, Feb. 2020	2	Nt	40	40
059	Mr2 Hut 7	*'P. gingeri'* (5)	F, Mar. 2020	1	Nt	40	40
060	Mr3 Hut 7	*'P. gingeri'* (5)	F, Mar. 2020	2	Nt	40	40
064	-	*P. tolaasii* (6)	G, May 2020	3	Nt	40	17
065, 066,	A, B	*P. tolaasii*	F, Sept. 2020	3	Nt	40	21
068, 070	D, E clear	*P. tolaasii*	F, Sept. 2020	3	Nt	40	22
082	Chestnut mushroom	*P. tolaasii* (6)	L, Oct 2020	3	Nt	40	21
083	Dark blotch ex cap	*P. tolaasii* (6)	F, Nov 2020	3	Nt	40	17
STW	Control	-	-	clean	-	-	-

[1]According whole sequence analyses from Taparia et al. (2020b) and/or this study (underlined)
[2]Scale of 0 to 3: 0 no symptoms; 1, weak mark; 2; intermediate; 3, strong positive
[3]Assays Pg2 and Pg6 target some groups of *'P. gingeri'* (ANI 1 and 14) and Ptol targets *P. tolaasii* as described in Elphinstone and Noble (2017) and Taparia et al. (2020a) Ct of 40 = target was not detected. Ct below 40 = target was detected; Nt = not tested
Isolates from the Netherlands were received from the lab of Jan Van Der Wolf (Taparia et al., 2020b)

Table 1.1.1B Blotch pathogenicity observed in mushroom cap droplet tests and TaqMan results of non-pathogenic or very weak *Pseudomonas* isolates

Isolate ID		Species Identity[1]	Origin	Cap test symptom scores[2]	TaqMan[3] C_T values		
FSBactM	Other identifier				Pg2	Pg6	Ptol
Control isolates							
002	P7548	(*P.'gingeri'*) *P. edaphica*	UK, 1989	0	40	40	40
003	P7758, ATCC 51311	(*'P. gingeri'*) P. NCO_2	USA	0	40	40	40
004	P7779, ATCC 51312	(*'P. reactans'*) P. NCO_2	USA	0, 1	40	40	40
008	21615526	(*'P. gingeri'*) *P. edaphica*	UK, 2006	0	40	40	40
013	P7759, NCPPB 387	(*P. tolaasii*) *'P. reactans'*	UK, 1957	0	40	40	40
014	NCPPB 1311	(*P. tolaasii*) *P. edaphica*	UK, 1962	Nt	40	40	40
026	P8021	(*'P. reactans'*) *P. edaphica*	UK, 2016	0	40	40	40
027	NCPPB 2193	(*P. tolaasii*) *P. edaphica*	UK, 1968	0	40	40	40
078	F8002	P. NCO_2 (ANI 2)	Netherlands	1	Nt	40	40
079	G1002	P. NCO_2 (ANI 2)	Netherlands	1	Nt	40	40
075	A5002	*P. yamanorum* (ANI 4)	Netherlands, 2016	0, 1	Nt	40	40
074	B4002	*P. yamanorum* (ANI 10)	Netherlands, 2016	1	Nt	40	40
076	IPO3753	*P. yamanorum* (ANI 10)	Netherlands, 2014	0, 1	Nt	40	40
072	IPO3778	*P. yamanorum* (ANI 26)	Netherlands, 2015	0	Nt	40	40
071	K7002	*P. edaphica* (ANI 12)	Netherlands, 2018	0	Nt	40	40
Other non-pathogenic species							
016	NCBBP 2289	(*P. agarici*)	New Zealand	Nt	40	40	40
017	P7760, HRI CH6	(*P. agarici*)		0	40	40	40
018	P7772, HRI mar-12	(*P.s. veronii*)		0	40	40	40
019	P7774, HRI n12	(*P. poae*)		0	40	40	40
020	NCPPB 4617	(*P. protegens*)	Switzerland, 1986	0	40	40	40
021	P7771, HRI WB1	(*P. putida*)		Nt	40	40	40
022	P7765, HRI S.Lincoln T2/6	(*P. putida*)	UK, 1980s	0	40	40	40
023	P7753, HRI -	(*P. syringae*)		0	40	40	40
025	P7787	(*Pseudomonas* sp.)		Nt	40	40	40
Isolates obtained 2018-2020 from UK commercial farms							
028	21815970 Blotch 1 I6	?	F, 2018	0	40	40	40
030 032 034	21815970 I8 to J2	?	F, 2018	0	40	40	40
031		P. NCO_2	F, 2018	0	40	40	40
038	21815972 J6	?	F, 2018	0	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
052	22000021 mr3A	?	B, Jan. 2020	0, 1	Nt	40	40
054-055	22007191	?	F, Feb. 2020	0	Nt	40	40
056	22007191	*P. edaphica*	F, Feb, 2020	0	Nt	40	40
058	Mr1 Hut 7	?	F, Mar. 2020	0	Nt	40	40
067, 069		?	F, Sept, 2020	0	Nt	40	40
081		*'P. reactans'*	M, Oct. 2020	1	Nt	40	40
084		?	F, Nov 2020	0, 1	Nt	40	40
085-086		?	F, Dec, 2020	0	Nt	40	40

[1] Original identification in brackets; According whole sequence analyses from Taparia et al. (2020b) and/or this study (underlined)
[2]Scale of 0 to 3: 0 no symptoms; 1, weak mark; 2; intermediate; 3, strong positive
[3]Assays Pg2 and Pg6 target some groups of *'P. gingeri'* (ANI 1 and 14) and Ptol targets *P. tolaasii* as described in Elphinstone and Noble (2017) and Taparia et al. (2020a) Ct of 40 = target was not detected. Ct below 40 = target was detected; Nt = not tested
Isolates from the Netherlands were received from the lab of Jan Van Der Wolf (Taparia et al., 2020a)

1.1.2. Mushroom pot culture test

Twenty-three isolates listed in Table 1.1.2, including 13 isolates obtained from commercial mushroom farms from 2018 until January 2020, maintained in the Fera Science collection, were selected for a pot culture pathogenicity test. Symptoms observed with selected isolates are presented in Appendix Fig. S3.

The most pathogenic isolates belong to *'P. gingeri'* (ANI groups 1, 14 and 5) and *P. tolaasii* and produced symptoms in 62% to 80% of mushrooms grown over two flushes.

Blotch symptoms caused by the control *P. tolaasii* strain (FSBactM 009) were dark brown as expected, and appear in 70% of the mushrooms, which was not as strong as seen before (M063 project).

Four *'P. gingeri'* isolates (FSBactM 005, 006, 007 and 053) produced strong ginger symptoms in over 60% of the mushrooms. The isolate FSBactM 001 produced weaker ginger symptoms that were not as widespread. Isolates FsBactM 001 and 006 also reduced the number of mushrooms harvested compared with the untreated control pots.

The *'P. gingeri'* isolates FSBactM 024, 035, 039, 050 and 051 generally produced milder ginger symptoms and some flecks, with 49 to 65% of mushrooms showing symptoms. Pots inoculated with isolate FSBactM 039 had a marked reduction in the number of harvested mushrooms compared with the untreated control pots.

The *P. costantinii* isolates FSBactM 029, 036, 037 and 040 produced pitting symptoms in 24 to 55% of the mushrooms. Pots inoculated with isolate 036 had a marked reduction in the number of harvested mushrooms compared with the untreated control pots, but these pots dried considerably more than others due to their location in the room, so more than one factor might have contributed to the reduction of yield.

Seven isolates (FSBacM 002, 013, 026, 027, 028, 047, 052) produced mainly clean mushrooms in similar proportions and numbers to the untreated control pots. The results for one isolate, FSBactM 042 indicate some weak pathogenicity.

Table 1.1.2 Blotch pathogenicity and average number of mushrooms per pot in two flushes of mushrooms grown in large pots inoculated with twenty-three UK *Pseudomonas* isolates and two water controls. Each value is the mean of two replicates. Isolates are ordered from highest to lowest percentage of symptoms.

Isolate ID		Species Identity (ANI group*)	Cap test	Total (over two flushes)		1st flush		2nd flush		Main type of symptom
FS BactM	Other identifier			Mush /pot	% symp-toms	Mush /pot	% symp-toms	Mush /pot	% symp-toms	
007	21615525	*'P. gingeri'* (1)	1	107.5	79.5	48.5	94.8	59.0	66.9	Strong ginger
005	P8018	*'P. gingeri'* (14)	2	112.0	77.2	64.0	89.1	48	61.5	Strong ginger
053	22000021 mr3B	*'P. gingeri'* (14)	2	94.5	76.2	48.0	90.6	46.5	61.3	Strong ginger
009	P7544	*P. tolaasii* (6)	3	108.5	70.0	48.0	84.4	60.5	58.7	Dark brown
050	22000021 mr1	*'P. gingeri'* (5)	Nt	105.5	64.5	45.0	70.0	60.5	60.3	Mild ginger
006	21614711	*'P. gingeri'* (14)	1	68.5	62.0	38.5	80.5	30.0	38.3	Strong ginger
024	P7786	*'P. gingeri'* (5)	2	79.5	59.7	41.0	79.3	38.5	39.0	Mild ginger
051	22000021 mr2	*'P. gingeri'* (5)	3	100.0	59.0	51.0	80.4	49.0	36.7	Mild ginger
037	21815972 Blotch 10, J5	*P. costantinii* (3)	3	81.5	54.6	48.0	60.4	33.5	46.3	Pit, flecks
029	21815970 Blotch 2, I7	*P. costantinii* (3)	2	78.0	51.9	32.0	46.9	46.0	55.4	Pit, flecks
039	21815973 Pit 12, J7	*'P. gingeri'* (22)	2	49.5	51.5	25.0	70.0	24.5	32.7	Mild ginger, flecks
035	21815971 Pit 8, J3	*'P. gingeri'* (24)	1	102.5	48.8	51.5	71.8	51.0	25.5	Mild ginger. flecks
040	21815973 Pit 13, J8	*P. costantinii* (3)	2	66.5	36.1	28.0	39.3	38.5	33.8	Pit
001	NCPPB 3146	*'P. gingeri'* (1)	0, 1	69.5	33.8	29.0	36.2	40.5	32.1	Weak ginger
042	JV1A	?	0, 1	97.5	26.2	42.5	14.1	55.0	35.5	Very weak ginger, pit
036	21815971 Pit 9, J4	*P. costantinii* (3)	3	31.0	24.2	18.9	16.7	13.0	34.6	Pit (dried**)
002	P7548	(*'P. gingeri'*) *P. edaphica*	0	82.0	16.5	32.0	15.6	50.0	17.0	Clean
027	NCPPB 2193	(*P. tolaasii*) *P. edaphica*	0	86.5	12.7	37.0	18.9	49.5	8.1	Clean
028	21815970 Blotch 1, I6	?	0	97.0	12.4	37.5	9.3	59.5	14.3	Clean
STW1	Control	-	-	88.5	11.3	44.5	10.1	44.0	12.5	Clean
047	JV3B	?	0, 1	93.5	10.7	37.0	10.8	56.5	10.6	Clean
052	22000021 mr3A	?	0, 1	113.0	7.1	56.0	4.5	57.0	9.6	Clean
026	P8021	*('P. reactans')* *P. edaphica*	0	96.0	4.7	45.5	3.3	50.5	5.9	Clean
013	P7759	(*P. tolaasii*) *'P. reactans'*	0	86.5	3.5	45.5	4.4	41.0	2.4	Clean
STW2	Control	-	-	106.5	2.8	46.5	0.0	60.0	5.0	Clean

*Based on results of qPCR assays and sequencing done in the current project.

**Pots dried more than others and that could have reduced the number of mushrooms per pot.

The first flush generally had higher percentages of mushrooms with symptoms following inoculation with most of the pathogenic isolates. For some of the *'P. gingeri'* isolates (e.g. FSBactM 006 and 051) the second flush had a much lower percentage of diseased mushrooms. This was not the case with two *P. costantinii* isolates (FSBactM 029 and 036) that had a slightly higher percentage of mushrooms showing disease in the second flush.

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

The results of qPCR targeting *'P. gingeri'* (Pg2 and Pg6) and targeting *P. tolaasii* (Ptol), developed in a previous project (M063) and described in Taparia et al. (2020a), are presented in Tables 1.1A and B. Most pathogenic control isolates were detected by the *'P. gingeri'* assays (six isolates) or by the *P. tolaasii* assay (four isolates), but three isolates were not detected by these assays. From the pathogenic isolates recently obtained from UK farms, more than half of the isolates (12/20) were not detected by these assays, whilst seven isolates were detected by the *P. tolaasii* assay and one was detected by the *'P. gingeri'* assays. This showed the need to develop new assays to detect *P. costantinii* and different groups of *'P. gingeri'*.

The genome sequences previously 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. Fifteen primer and probe sets were designed using approach. Thirteen 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 followed by two new assays based on additional genome sequences
- Following additional genome sequencing, one assay for *'P. gingeri'* ANI group 24
- For *'P. gingeri'* ANI group 22, suitable primers were not found (no assay developed)
- Two assays for *P. costantinii* ANI group 3
- Three assays for *P. NCO$_2$* ANI group 2
- One assay for *P. yamanorum* ANI groups 4, 10 and 26

Ten of these qPCR assays were preliminary tested with eight selected isolates from the Fera Science collection and the results are presented in Table 1.2.1.

Table 1.2.1. 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*									
		Pg1a2 *'P. gingeri'* (ANI 1)	Pg14a25 *'P. gingeri'* (ANI 14)	Pg5a16 *'P. gingeri'* (ANI 5)	Pg5a18 *'P. gingeri'* (ANI 5)	Pc3a10 *P. costant.*	Pc3a12 *P. costant..*	Pn2a4 *P. NC0$_2$*	Pn2a6 *P. NC0$_2$*	Pne2a7 *P. NC0$_2$* *P. edaphica*	Py10a24 *P. 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	P. 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 (Ct =40); Ct values below 40 mean that the pathogen was detected.

All assays detected the predicted isolates. The assays Pg1a2 and Pg14a25 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.

Five qPCR assays were selected to be tested with all 86 isolates listed in tables 1.1A and B; assay 2a6 for *P. NCO2* was tested with a selection of 15 isolates. The results presented in Table 1.2.2. show that:

- Assay Pg1a2 detected the three *'P. gingeri'* ANI 1 isolates tested
- Assay Pg14a25 detected three *'P. gingeri'* ANI 14 isolates, but did not detect isolate 006 (that was detected by Pg2/Pg6 assays previously developed)
- Assay Pg5a16 detected four isolates of *'P. gingeri'* ANI 5 but did not detect three isolates subsequently identified as ANI 5 (050, 051 and 060); therefore, new assays were designed for this group. New assays Pg5-1 and Pg5-2 detected all seven *'P. gingeri'* ANI 5 isolates
- New assay Pg24 detected the only *'P. gingeri'* ANI 24 isolate tested
- Assay Pc3a12 detected the six *P. costantinii* isolates tested
- Assay Pn2a6 detected four isolates from group *P. NCO₂*
- Assay Pne2a7 detected 11 isolates (including a cross reaction with a *P. costantinii* isolate); six isolates (008, 014, 026, 027, 056, 071) that were detected with this assay and not detected with Pn2a6 are most likely *P. edaphica*
- Assay Py10a24 detected two *P. yamanorum* ANI 10 isolates, but not *P. yamanorum* from ANI groups 4 and 26

Table 1.2.2. Isolates detected with new qPCR assays

TaqMan Assay*	**Targeting species / ANI group**	**Control isolates from other countries**	**Control isolates from the UK**	**Isolates obtained 2018-2020 from UK farms**
Pg1a2	*'P. gingeri'*/ ANI 1	073	001, 007	-
Pg14a25	*'P. gingeri'*/ ANI 14	080	005	053
Pg5a16	*'P. gingeri'*/ ANI 5	077	024	033, 059
Pg5-1 and Pg5-2	*'P. gingeri'*/ ANI 5	077	024	033, **050, 051**, 059, **060**
Pg24	*'P. gingeri'*/ ANI 24	-	-	035
Pc3a12	*P. costantinii* ANI 3	015	-	029, 036, 037, 040, 057
Pn2a6	P. NCO_2 / ANI 2	078	003, 004	031
Pne2a7	P. NCO_2 / ANI 2 and *P. edaphica* / ANI 12	071, 078	003, 004, 008, 014, 026, 027	(029 cross reaction) 031 056
Py10a24	*P. yamanorum* / ANI 10	074, 076	-	-

* Assays Pg5-1, Pg5-2 and Pg24 were tested with a selection of 23 isolates; assay Pn2a6 was tested with a selection of 15 isolates; the other six assays were tested with all the 86 isolates.

Sequences of 22 isolates generated on Illumina MiSeq, had a total of 28.7 million reads. The average coverage was 28.4x (ranging from 15x to 47x). Average nucleotide identity (ANI) comparisons confirmed the ID of five *P. tolaasii* and three *P. costantini* isolates and resulted

in isolates 6, 33, 35, 39, 50, 53, 59 and 60 being identified as *'P. gingeri'*, including four ANI groups (14, 5, 22 and 24). Three non-pathogenic isolates from the National Collection of Plant Pathogenic Bacteria (NCPPB) identified as *'P. gingeri'* or P*. tolaasii* were re-identified as *P. edaphica*. One isolate from a UK farm was identified as *P. NCO₂*, the antagonist isolate (FSBactM 013) and a new isolate from a UK farm were identified as *'P. reactans'* (Table 1.2.3. and Appendix Fig. S4).

The comparison of genome sequences of the *'P. gingeri'* complex supports the idea that this group includes more than one species.

Table 1.2.3. Sequences of UK *Pseudomonas* spp. strains

Isolate ID		**Species Identity (from original Ptol and Pg2/Pg6 qPCR assays)**	**Species Identity with qPCRs developed in this study**	**Origin**	**Sequence details**		
FSBactM	**Other identifier (original species identity)**				**Cover**	**ANI group**	**Final ID**
Pathogenic isolates							
061	TRF 42	*P. tolaasii*	-	UK, 1980s	39.737	6	*P. tolaasii*
063	TRF 59	*P. tolaasii*	-	UK,1980s	29.006	6	*P. tolaasii*
064	-	*P. tolaasii*	-	Farm G, 2020	22.243	6	*P. tolaasii*
082	Chestnut mushroom	*P. tolaasii*	-	Farm L, 2020	36.092	6	*P. tolaasii*
083	Dark blotch	*P. tolaasii*	-	Farm F, 2020	32.85	6	*P. tolaasii*
029	21815970 Blotch 2 I7	Nd	*P. costantinii*	Farm F, 2018	26.846	3	*P. costantinii*
040	21815973 Pit 13 J8	Nd	*P. costantinii*	Farm F, 2018	29.897	3	*P. costantinii*
057	22007191	Nd	*P. costantinii*	Farm F, 2020	42.617	3	*P. costantinii*
006	21614711	*'P. gingeri'*	Nd	UK, 2016	16.865	14	*'P. gingeri'*
053	22000021 mr3B	*'P. gingeri'*	*'P. gingeri'*	Farm B, 2020	27.938	14	*'P. gingeri'*
033*	21815970 I8 to J2	Nd	*'P. gingeri'*	Farm F, 2018	24.64	5	*'P. gingeri'*
059	Mr2 Hut 7	Nd	*'P. gingeri'*	Farm F, 2020	23.916	5	*'P. gingeri'*
050	22000021 mr1	Nd	*'P. gingeri'*	Farm B, 2020	47.054	5	*'P. gingeri'*
060	Mr3 Hut 7	Nd	*'P. gingeri'*	Farm F, 2020	40.048	5	*'P. gingeri'*
035	21815971 Pit 8 J3	Nd	*'P. gingeri'*	Farm F, 2018	26.3	24	*'P. gingeri'*
039	21815973 Pit 12 J7	Nd	*'P. gingeri'*	Farm F, 2018	30.858	22	*'P. gingeri'*
Non-pathogenic isolates							
002	P7548, NCPPB3637 (*'P. gingeri'*)	Nd	Nd	UK, 1989	18.793		*P. edaphica*
014	NCPPB 1311 (*P. tolaasii*)	Nd	*P. edaphica*	UK, 1962	24.589		*P. edaphica*
027	NCPPB 2193 (*P.tolaasii*)	Nd	*P. edaphica*	UK, 1968	21.121		*P. edaphica*
031	21815970 (I9)	Nd	*P. NCO₂*	Farm F, 2018	17.157		*P. NCO₂*
013	P7759 (*'P. reactans'*)	Nd	Nd	UK, 1957	15.284		*'P. reactans'*
081	-	Nd	Nd	Farm M, 2020	31.363		*'P. reactans'*

Nd – Not detected

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

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 1.3.1.) as predicted from previous tests. The control samples in water extracts were weakly positive for assays 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 1.6).

Table 1.3.1. 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

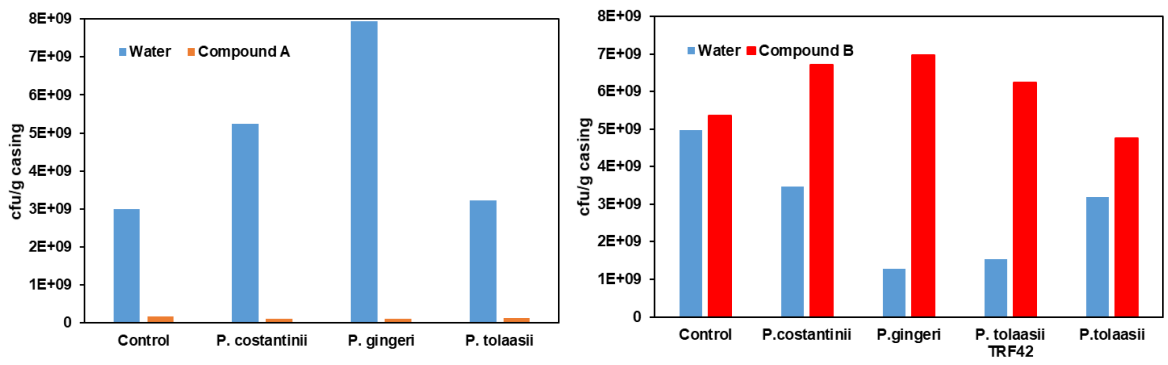

Figure 1.1. Pseudomonad counts in casing extracts treated in water or compound A (Expt. 1.1 left) or water and compound B (Expt. 3.1b right)

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 1.7). 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 1.3.2).

Table 1.3.2. 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

Taqman analysis of casing extract samples taken from the end of Experiment 3.2b showed that the qPCT assays correctly identified the pathogens that were inoculated on to the casing. The Ptol, Pg6 and Pc13a12 Taqman tests produced C_T values of <23 for *P. tolaasii*, *'P. gingeri'* and *P. costantinii* respectively but C_T values of >32 for the other pathogens (Fig. 1.2) or extracts from uninoculated casing. All three tests were negative for casing inoculated with the Pseudomonad antagonist (P7759).

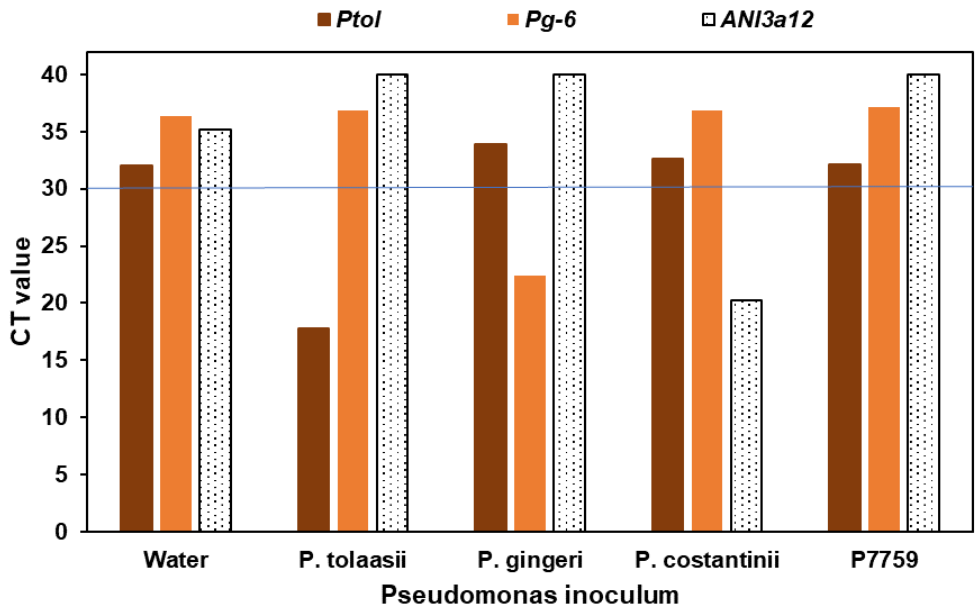

Figure 1.2. TaqMan analysis of casing extract samples from Experiment 3.2.1b (lower Ct values, below 30, indicate higher presence of DNA of the respective pathogen)

Taqman Pc3a12 for *P. costantinii* analysis of casing extract samples taken from the first and third flushes of mushrooms from a commercial crop are shown in Table 1.3.3. Casing extracts from the first flush, which showed pitting symptoms, tested positive using the Pc3a12 assay for *P. costantinii* (C_T values <28). All replicate samples incubated with compound B tested positive; 50% of samples incubated in water tested positive. Casing extracts from the third flush, which showed no pitting symptoms, tested negative using the Pc13a12 assay for *P. costantinii* (C_T values 40).

Table 1.3.3. TaqMan Pc3a12 targeting *P. costantinii* analysis of casing extract samples taken from commercial crops. Samples were treated with water or compound B. Each value is the mean of three replicate samples

Sample	Extract	Sample A		Sample B	
		Rep 1	Rep 2	Rep 1	Rep 2
First flush	Water	40	26.67	27.83	40
First flush	Compound B	27.35	27.14	27.10	26.98
Third flush	Water	40	40	40	40
Third flush	Compound B	40	40	40	40
Water		40	40	40	40
Positive		19.25	19.20	17.68	18.44

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 1.4.1. and Appendix Fig. S5. More than half the isolates were re-identified (highlighted in the table) and only 5 of the original species identifications were confirmed. Analysis of cultures obtained from mushroom substrates show that *T. aggressivum* f. *europeum* was prevalent on two farms (Table 1.4.2).

Table 1.4.1. 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 1.4.2. Results of analysis of partial sequences of ITS and TEF of *Trichoderma* species isolates from green mould infected mushroom substrates collected in 2019 and 2020

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*

The assays from Kosanovic et al. (2020) for genus and species level also amplified DNA from other genera (including *Agaricus*) and species of *Trichoderma* and therefore were not selected (Table 1.4.3). The assays from Hagn et al. (2007) for genus, Chen et al. (1999) for species and O'Brien et al. (2017) for subspecies gave promising results (Appendix Fig. S6). The specificity of these three conventional PCR assays was tested again (Appendix Fig. S7) including *T. aggressivum* f. *aggressivum* as a control:

- The Hagn et al. (2007) assay was the most promising for detecting *Trichoderma spp.* All *Trichoderma* species tested were detected on the gel, although some produced weaker bands than others. One out of the ten isolates of *Trichoderma atroviride* (3110) produced no band. One out of the nine isolates of *Trichoderma harzianum* (175) also produced no band (for unknown reason). None of the non-target soil fungi produced a band. It was able to successfully detect *T. aggressivum f. aggressivum* when tested with the new isolate.
- The Chen et al. (1999) assay was the most promising for detecting *T. aggressivum,* in the initial tests it detected all the *T. aggressivum f. europaeum* isolates [five strains including a collection strain (3098=23443) and strains obtained in 2020 from two farms (3113, 3114, 3115)]; these results agreed with results of ITS and EF gene sequencing (Appendix Fig. S6). When tested with *T. aggressivum f. aggressivum* isolate, it also successfully detected this isolate.
- There was only one assay for *T. aggressivum f. europaeum* [Mills (1996); O'Brien et al. (2017); Kosanovic et al. (2020)]. This assay successfully detected all *T. aggressivum f. europaeum* isolates and had no cross reactions with any other *Trichoderma spp*. including the *T. aggressivum f. aggressivum* isolate.

The Fera qPCR assay (Lane, 2010) also successfully detected all *T. aggressivum* isolates including *T. aggressivum* f. *europaeum and* f. *aggressivum.*

Table.1.4.3. Detection of *Trichoderma* spp. at genus, species and subspecies level, and other fungi associated with mushrooms, using five PCR assays and one qPCR assay

	Genus		**Species**			**Subspecies**
	Hagn et al. (2007)	Kosanovic et al. (2020)	Chen et al. (1999)	FERA commercial qPCR	Kosanovic et al. (2020)	O'Brien et al. (2017)
T. aggressivum f. *europaeum*	+	+	+	+	+	+
T. aggressivum f. *aggressivum*	+	+	+	+	+	-
Other *Trichoderma* spp. detected	+	+	-	-	*T. sinuosum, T. virens, T. viride, T. viridescens*	-
Other genera detected	-	*Mucor, Penicillium, Verticillium, Agaricus*	-	-	-	-
Trichoderma spp. isolates not detected	*T. harzianum* (one strain)	*T. harzianum* (one strain)				

1.4.2 Pot pathogenicity test for *Trichoderma* isolates

No green mould symptoms were observed on the casing. Increasing the inoculum concentration of *T. aggressivum* reduced the number of healthy mushrooms and produced mushrooms with spotting symptoms (Figs. 1.4a and b). Both inoculum concentrations of *T. harzianum* resulted in a reduction in healthy mushrooms and a corresponding increase in diseased mushrooms.

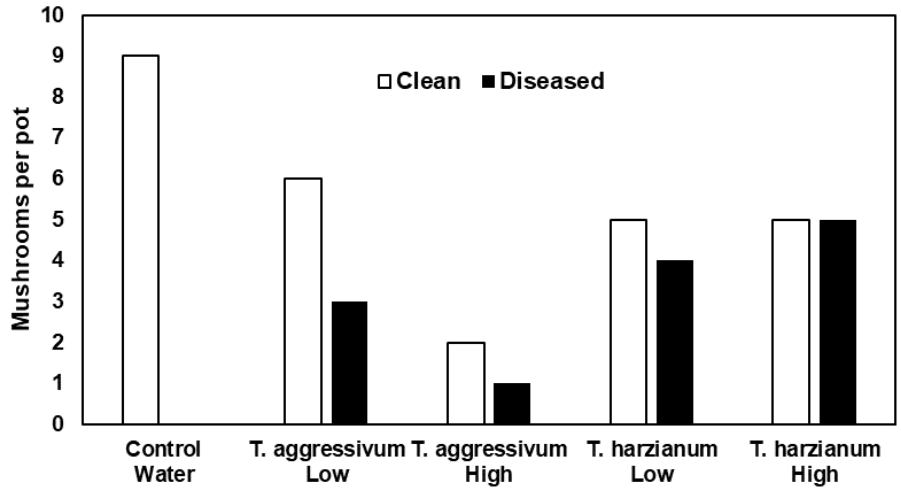

Fig. 1.4a Effect of inoculum concentration of *Trichoderma aggressivum* and *Trichoderma harzianum* applied to the casing on the number of healthy and diseased mushrooms. Each value is the mean of four pots

Disease symptoms on mushrooms were more severe with *T. harzianum* than with *T. aggressivum* (Fig. 1.4b). The populations of *Trichoderma* colony forming units detected in casing samples corresponded with the initial inoculations levels and remained stable during the experiment (Fig. 1.4c).

Fig. 1.4b. Uninoculated pots (top left) and pots inoculated with *Trichoderma aggressivum*, High (top right) and *Trichoderma harzianum* Low (bottom right) and High (bottom right)

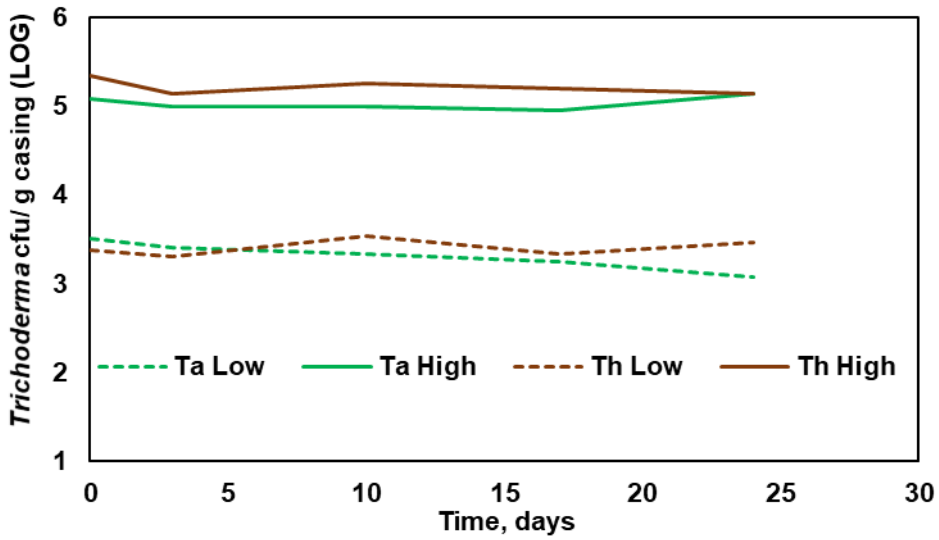

Fig. 1.4c Trichoderma colony forming units detected in casing samples at different times after inoculation of *T. aggressivum* (Ta) and *T. harzianum* (Th) on to casing at two concentration levels (Low $2x10^5$ cfu/mL; High $2x10^7$ cfu/mL)

The results of qPCR assays for the detection of *T. aggressivum* are presented in Table 1.4.4. The assay detected the pathogen from day 3 to day 24 in the pots that were inoculated with a high concentration of *T. aggressivum* f. *europaeum*, but only had some (weak) positive detections at day 10 and 17 in the pots inoculated with lower concentration. Samples from pots inoculated with *T. harzianum* were negative as the assay does not target this species.

Table 1.4.4. Results of qPCR targeting *Trichoderma aggressivum* on samples from the *Trichoderma* pot experiment (lowest Ct lowest value of two reactions presented)

Treatment	Day 3		Day 10		Day 17		Day 24	
Replicate	1	2	1	2	1	2	1	2
1 Control	U	U	U	U	U	U	U	U
2 *T. aggressivum* Low	U	U	37.6	U	36.6	U	U	U
3 *T. aggressivum* High	33.3	33.6	33.7	33.6	33.0	32.7	33.1	34.7
4 *T. harzianum* Low	U	U	U	U	U	U	U	U
5 *T. harzianum* High	U	U	U	U	U	U	U	U

U = undetected

Oxford Nanopore MinION sequencing of the ITS region using ITS5 and ITS4 primers of 47 samples from this experiment generated a total of 7,118,329 reads were generated, equating to roughly 5.7Gb, with an average quality score of 10.9 (~8% error rate) and a median read length of 760. The sequences were analysed and a matrix detailing the number of reads

assigned to each taxon per sample was produced. A large proportion of the reads were assigned to *A. agaricus*, the mushroom species, as would be expected. However, notable levels of *Trichoderma* were identified in all non-control samples at day 0, and in four samples at day 3 (Figure 1.4d). Samples from days 10, 17 and 24 did not have a level of *Trichoderma* above 'noise' level. Where *Trichoderma* consensus sequences were generated, blast searches suggest that both *T. aggressivum* and *T. harzianum* were likely the origins of the sequence – a result that agreed with the treatments applied.

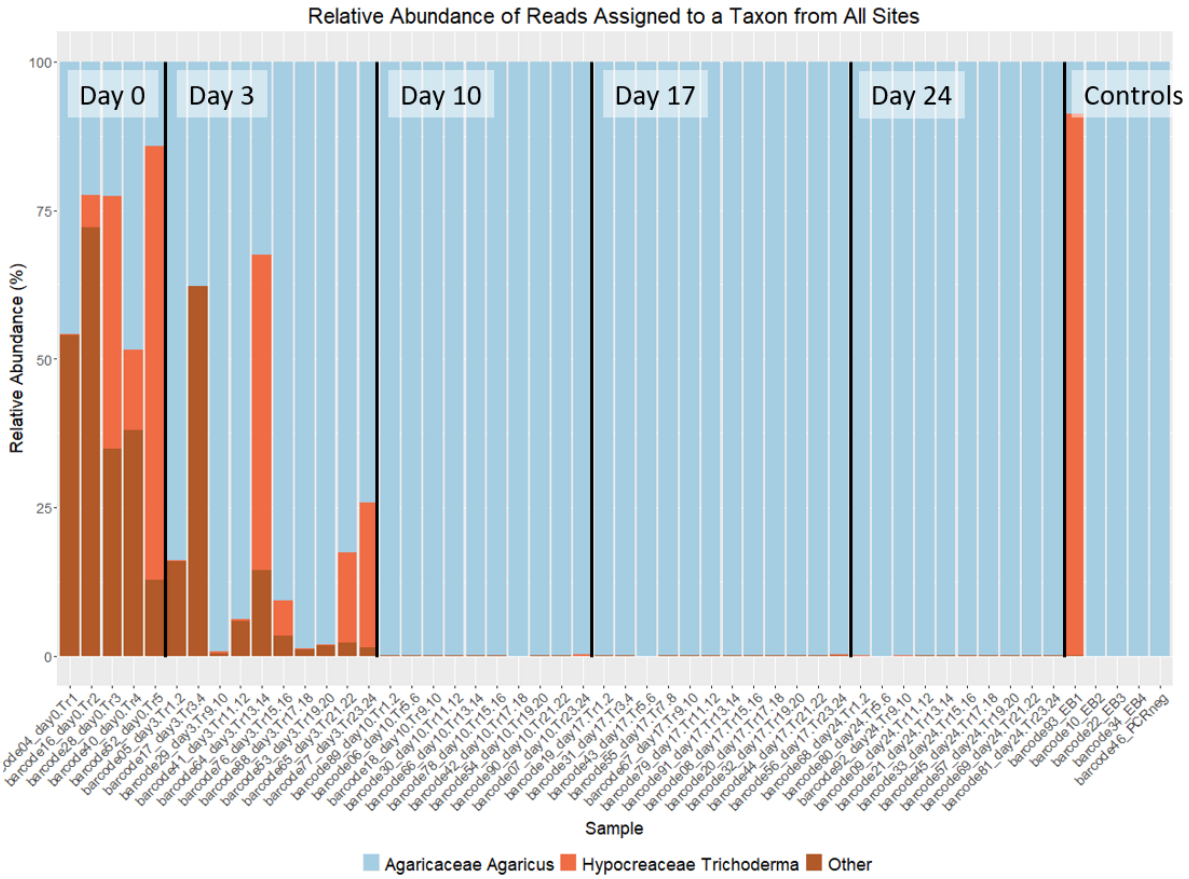

Figure 1.4d. Relative abundance of reads of *Agaricus* (mainly mushroom) and *Trichoderma* in samples collected at days 0, 3, 10, 17 and 24 of the pot pathogenicity experiment

The results show that qPCR targeting *T. aggressivum* was more successful in the detection than ITS sequencing possibly due to the high level of mushroom DNA present in the samples from day 10 to 24. A qPCR for all *Trichoderma* species would be a useful tool to assess possible issues linked to farm hygiene.

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 were sampled from four different commercial farms in cropping rooms at different stages of production. Presence or absence of blotch or green mould associated with each sample was recorded (Table 2.1).

Table 2.1 Casing and compost substrates sampled in duplicate from four different mushroom farms

Farm	Day in cycle	Date of collection	Cropping stages	Disease observed	Casing and compost pairs of samples
A	1 – 29	19 May 2020	Casing to Second flush	None	11
A	18, 22	19 May 2020	First flush, Post first flush	Blotch	2
B	1 – 35	13 Oct 2020	Casing to Third flush	None	6
B	29	13 Oct 2020	Second flush	Blotch	1
C	1 – 35	19 May & 8 Sep 2020	Casing to Third flush	None	12
C	35	19 May 2020	Third flush	Blotch	1
C	31, 35	19 May 2020	Third flush	Green mould	2
D	1 – 29	18 Dec 2020	Casing to Second flush	None	5
TOTAL	-	-	-	-	40 (x2)

The communities of bacteria and fungi was assessed using high throughput DNA sequencing based metabarcoding with 16S and ITS rRNA markers. A total of 80 samples were sequenced on an Illumina MiSeq, for both 16S and ITS amplicons, generating a total 10,155,834 paired end reads (6,432,030 for 16S and 3,723,804 for ITS).

Faith's phylogenetic diversity measures the amount of the phylogenetic tree covered by the community and is therefore a measure of richness, i.e. the larger the Faith pd value, the richer (more different species present) and more diverse the sample/dataset is. No significant differences were observed between farms or the stage of cultivation, however a significant difference was observed when inspecting the 'Casing or Substrate' variable for both bacterial and fungal communities (Kruskal-Wallis test, p-value: 3.154613e-10 for 16S and 1.514007e-10 for ITS) (Figure 2.1).

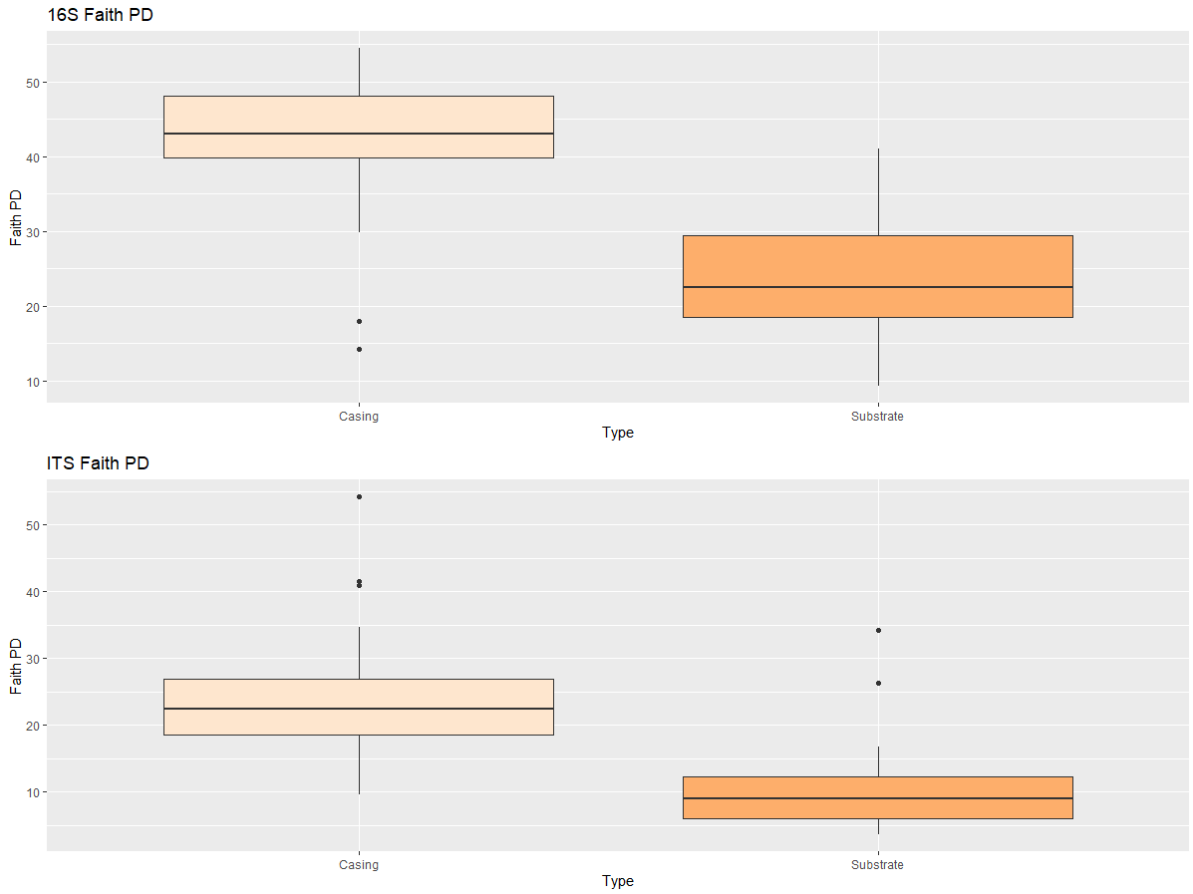

Figure 2.1. Box and whisker plots showing the differences observed between substrate and casing types, across both 16S and ITS datasets. Faith phylogenetic diversity is displayed on the y axis, with higher numbers on casing samples representing greater diversity.

Pielou's evenness was used to assess how even the distribution of species was in a sample across all samples in the dataset. The value can range from 0 to 1 – from no evenness to complete evenness. In the 16S dataset, no significant differences were observed between farms or the stage of cultivation. In the ITS dataset, however, a significant difference was observed between farms, with an all group Kruskal-Wallis p-value of 0.009 observed. When inspecting the Kruskal-Wallis pairwise differences, it becomes apparent that farm B is significantly different to both A (p-value 0.011, adjusted p-value 0.034) and C (p-value 0.002, adjusted p-value 0.013) farms, but not farm D, with an overall lower evenness score (Figure 2.2) indicating that in farm B the relative abundance of species is more variable. With regards to the 'Casing or Substrate' category, no significant differences were observed in the ITS dataset with a Kruskal-Wallis test, however a significant difference was observed in the 16S dataset (p-value 1.526755e-09, Figure 2.3) indicating that the relative abundance of bacterial communities is more even in casing samples.

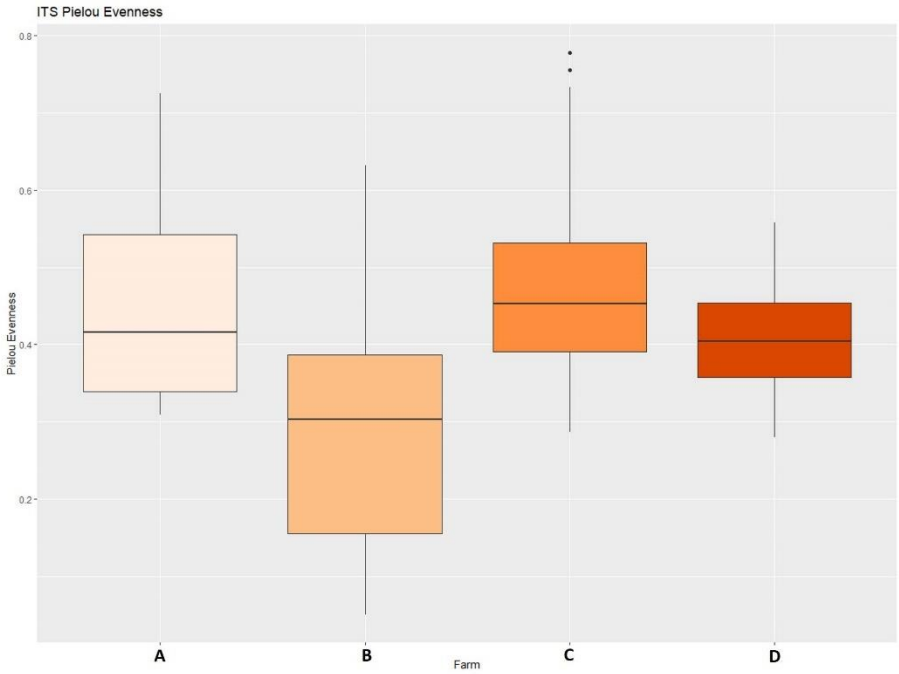

Figure 2.2. Box and whisker plots showing the differences observed between farms, across the ITS dataset. Pielou's Evenness is displayed on the y axis, with higher numbers representing more evenness of distribution of abundance across species in the community

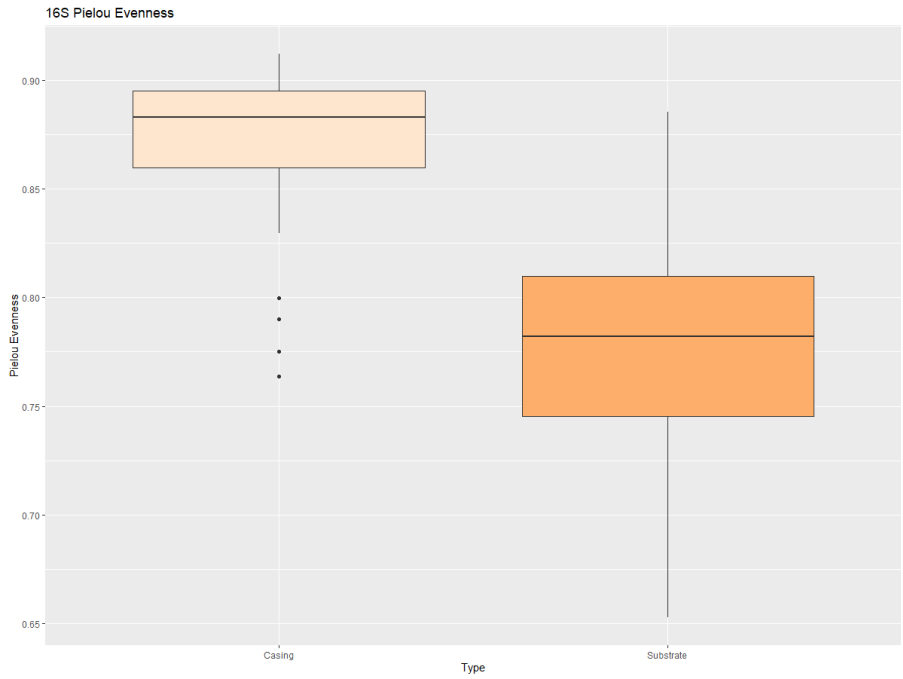

Figure 2.3. Box and whisker plots showing the differences observed between substrate and casing types, across the 16S dataset. Pielou's Evenness is displayed on the y axis, with higher numbers representing more evenness of distribution of abundance across species in the community

The analysis of results from this study show that:

- There was no difference in richness of the bacterial and fungal communities between farms and between stage of cultivation, but both communities are richer in the casing than in the substrate
- The evenness of distribution of bacterial species in the samples had no significant differences between farms or between stages of cultivation, but there was a significant difference between farms with regards the fungal communities. There were no significant differences in the fungal communities between casing and substrate samples, but there was a significant difference observed in the bacterial communities, with the relative abundance of species being more even in casing samples.

In the 16S dataset, *Pseudomonas* spp. can be detected, but only at genus level and not species level; therefore, it was not possible to differentiate between pathogenic, beneficial or harmless *Pseudomonas* species. The non-specific nature of targeting 16S, could have the benefit of pointing towards other possible causes of disease, so it is still useful as a screening tool. From the available genomes of beneficial *Pseudomonas* species, it may be possible to identify unique marker sequences in the genome which we can design assays for. This would then enable levels of beneficial *Pseudomonas* species to be estimated without the need for expensive sequencing steps.

Trichoderma was detected in eight samples from three different farms (B, C and D): four samples from the three farms were not associated with visible *Trichoderma* at the time of collection whilst four samples from farm C (MC25T samples) were identified as being associated with *Trichoderma* at the time of collection (Figure 2.4). This indicates that ITS microbiome sequencing can lead to the detection of *Trichoderma* in samples where symptoms were not visible. The detection of pathogenic fungi would greatly benefit from mushroom blocking primers, as *Agaricus* dominated samples – most samples contained over 50% *Agaricus*.

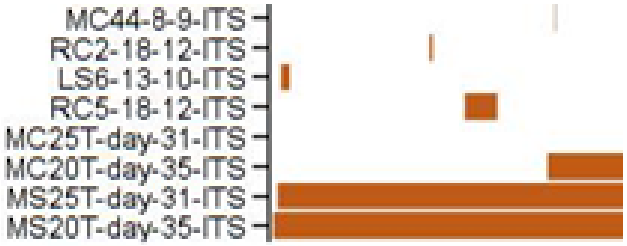

Figure 2.4. Detection of *Trichoderma* on ITS sequences obtained from samples from four farms.

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

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; 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 consistently reduced the incidence of brown or ginger blotch or pitting compared with the water irrigation treatment.

Figure 3.1a. Uninoculated mushroom culture pots (top row) and pots inoculated with *P. tolaasii* (middle row) isolates P7544 (left) and TRF42 (right), *'P. gingeri'* P8018 (bottom row left) and *P. costantinii* J5 (bottom row right)

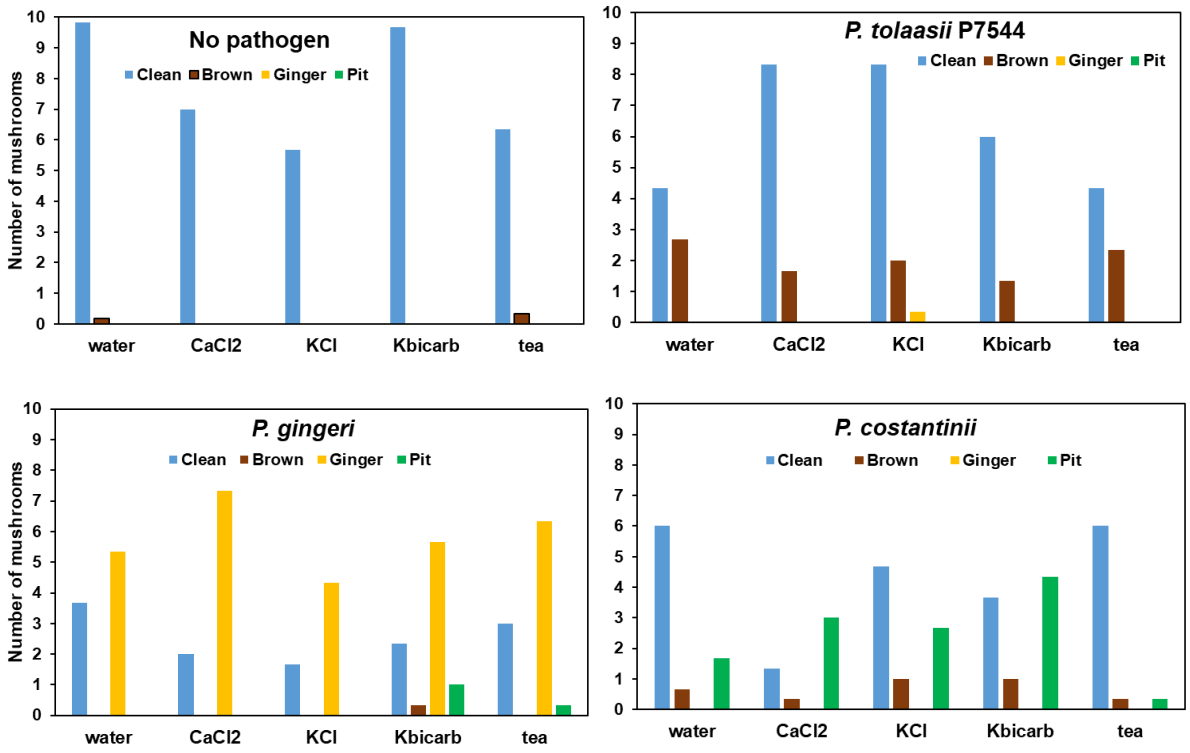

Figure 3.1b. Effect of ionic solutions on different types of blotch (Experiment 3.1a). Each treatment value is the mean of four replicate pots

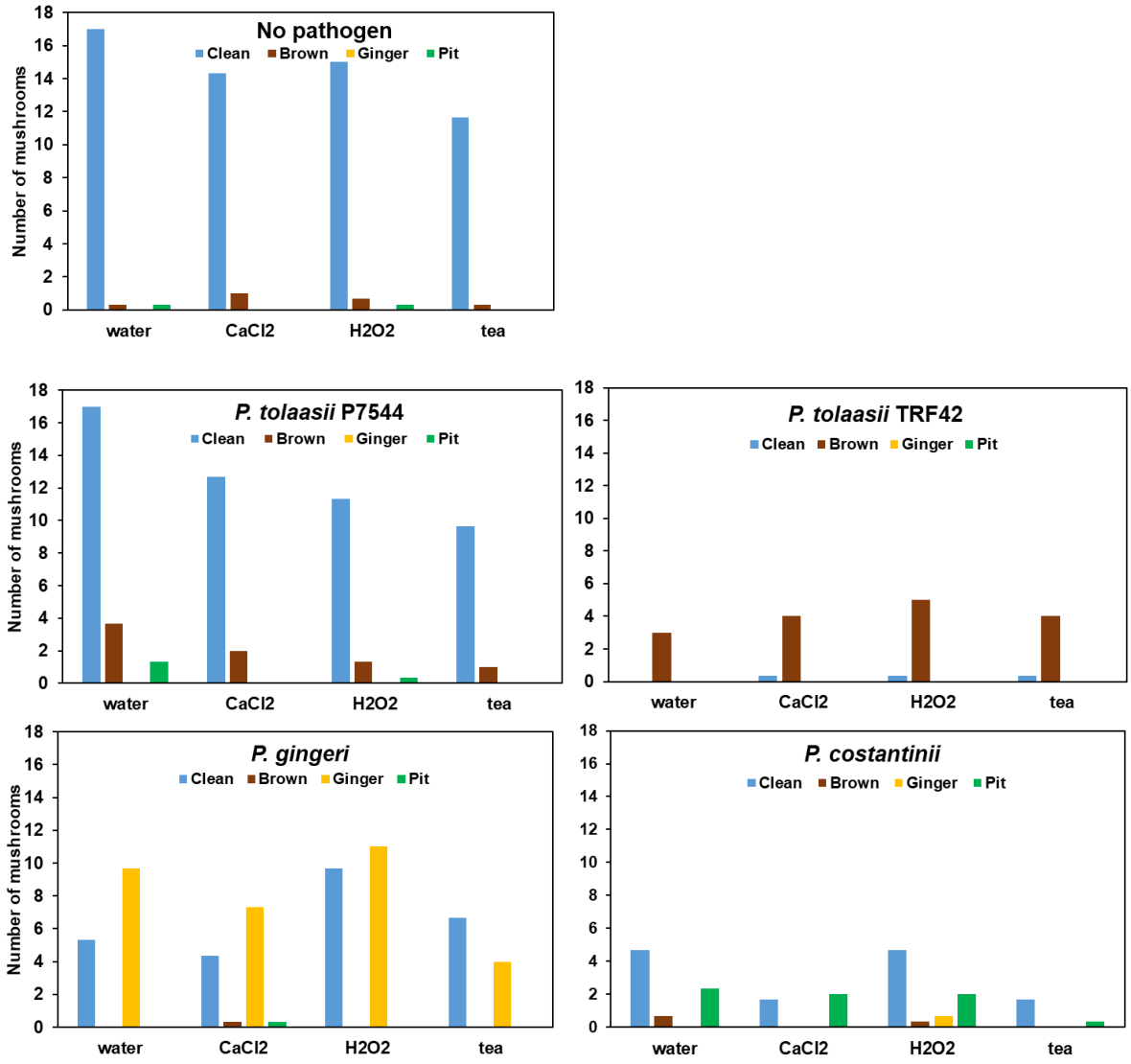

Figure 3.1c. Effect of ionic solutions on different types of blotch (Experiment 3.1b). Each treatment value is the mean of four replicate pots

3.2. Effect of antagonists on blotch

3.2.1 Effect of antagonists on blotch in pot experiments

Experiment 3.2.1a. 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.

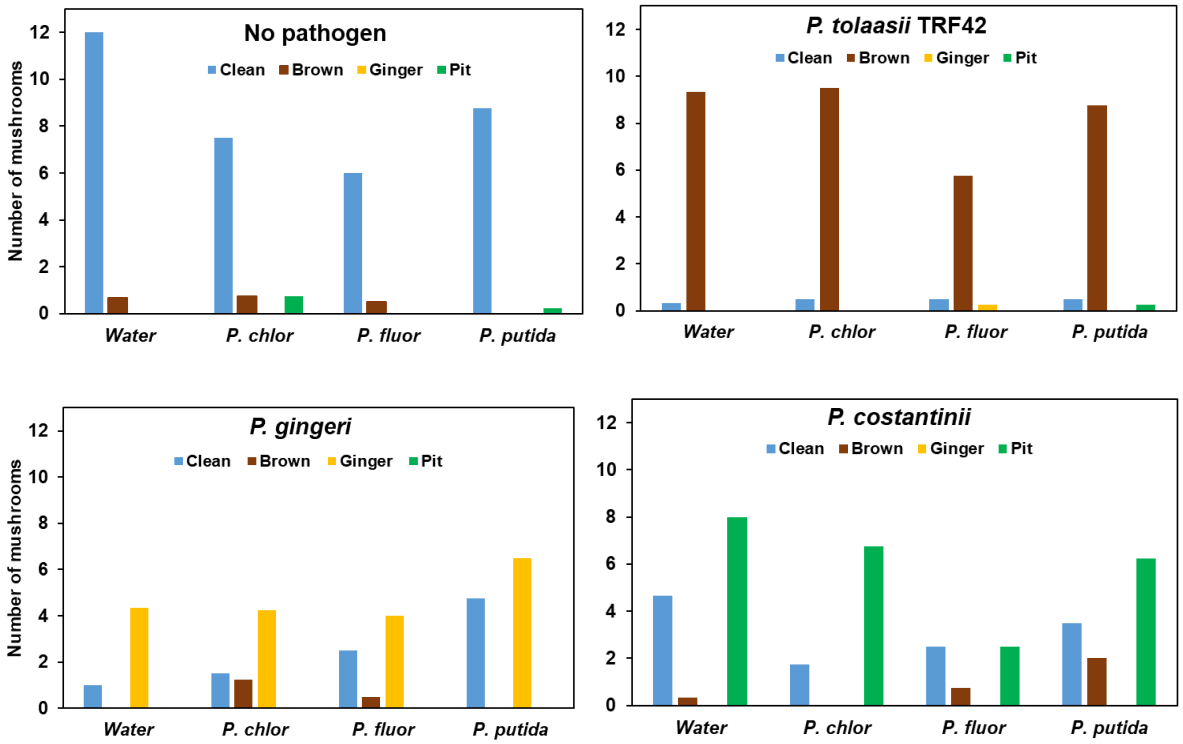

Figure 3.2a. Effect of commercial pseudomonad inocula on different types of blotch. Each treatment value is the mean of three replicate pots

Experiment 3.2.1b: Effect of experimental pseudomonad isolates on blotch

None of the experimental pseudomonad isolate suspensions resulted in reduction blotch symptoms in comparison with the water treated control (Fig. 3.2b). Ginger blotch was observed in all 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.

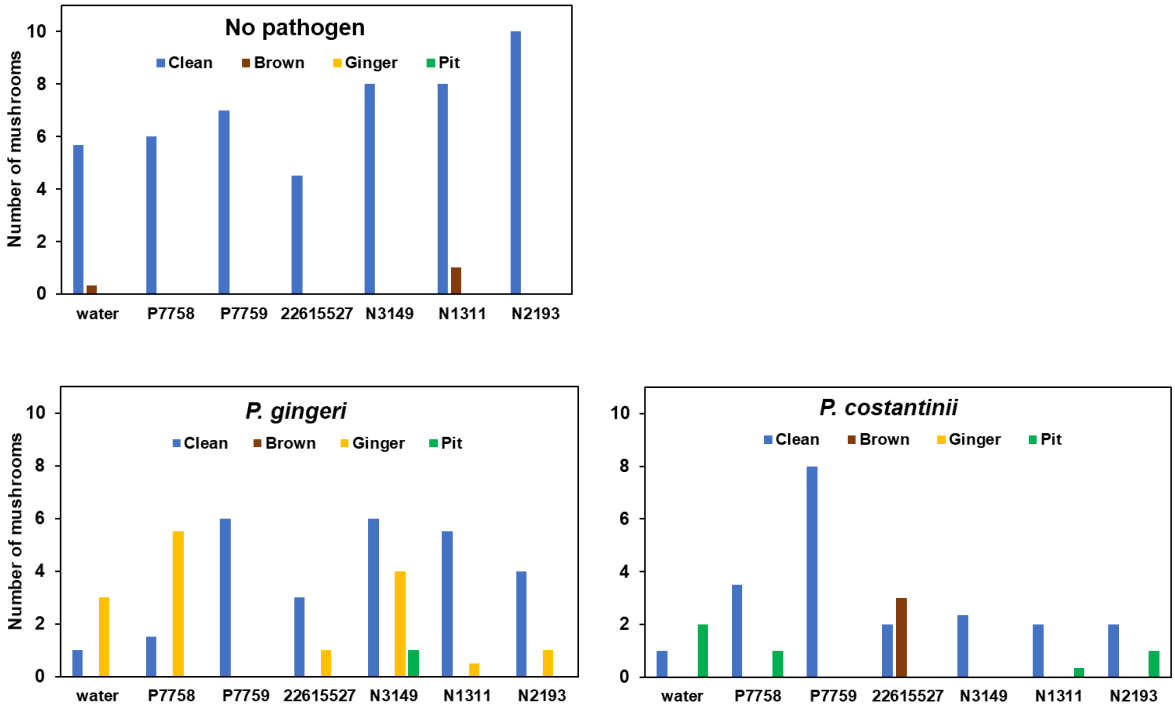

Figure 3.2b. Effect of six experimental pseudomonads on ginger blotch and pit. Each treatment value is the mean of two replicate pots

Experiment 3.2.1c. Effect of experimental pseudomonad isolates on blotch

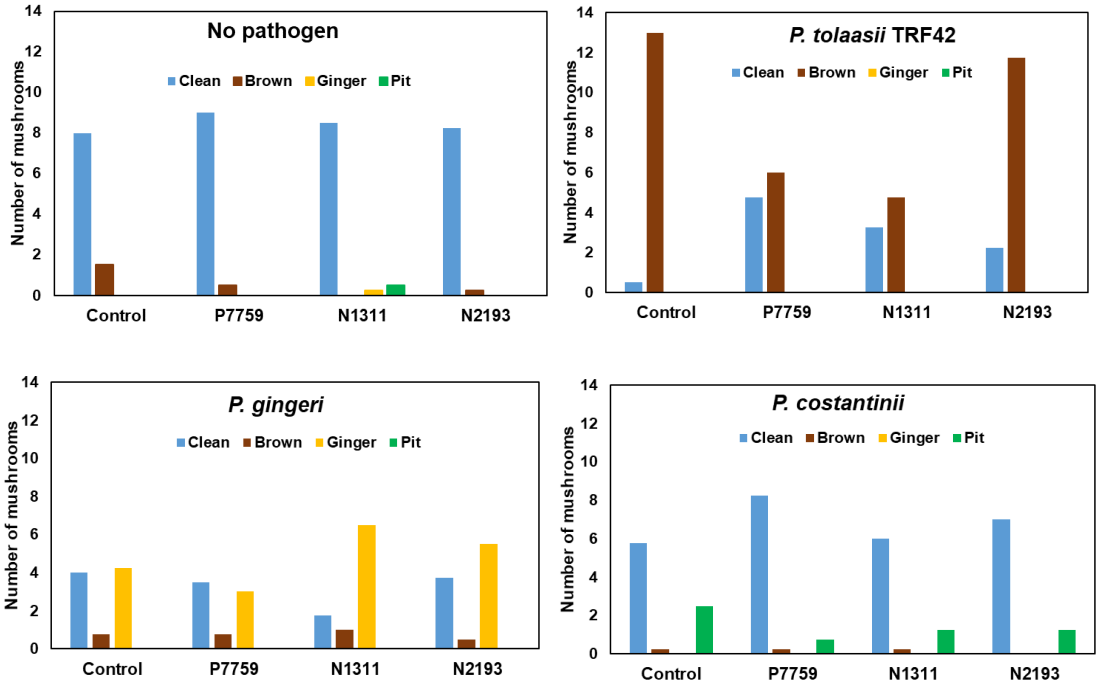

Figure 3.2c Effect of experimental pseudomonads on different types of blotch. Each treatment value is the mean of two replicate pots

Blotch disease symptoms corresponded with the pathogen isolates that were applied to pots, except for a small amount of brown blotch which was present on the 'no pathogen' pots (Fig. 3.2c). The number of mushrooms with symptoms on pots inoculated with *P. costantinii* was smaller than on pots inoculated with *P. tolaasii* or *'P. gingeri'* isolates. Overall, the number of blotched mushrooms was reduced by application of the antagonist P7759 although the effect was only significant for *P. tolaasii*. Isolate N1311 also reduced brown blotch caused by TRF42 but did not significantly affect blotch caused by *'P. gingeri'* or *P. costantinii*. Isolate N2193 did not affect blotch and none of the antagonist treatments caused blotch.

The following trends were observed in the Pseudomonad counts in casing samples taken after the second flush of mushrooms:

- Without P7759 inoculum, total Pseudomonad counts were higher in casing samples incubated in LB broth with Compound B than without (Fig. 3.2d)
- Where both P7759 and *P. tolaasii* or *'P. gingeri'* inocula were added, total Pseudomonad counts were also higher in casing samples incubated in LB broth with Compound B than without
- Where P7759 was added alone or with *P. costantinii* inoculum, total Pseudomonad counts were lower in casing samples incubated in LB broth with Compound B than without.

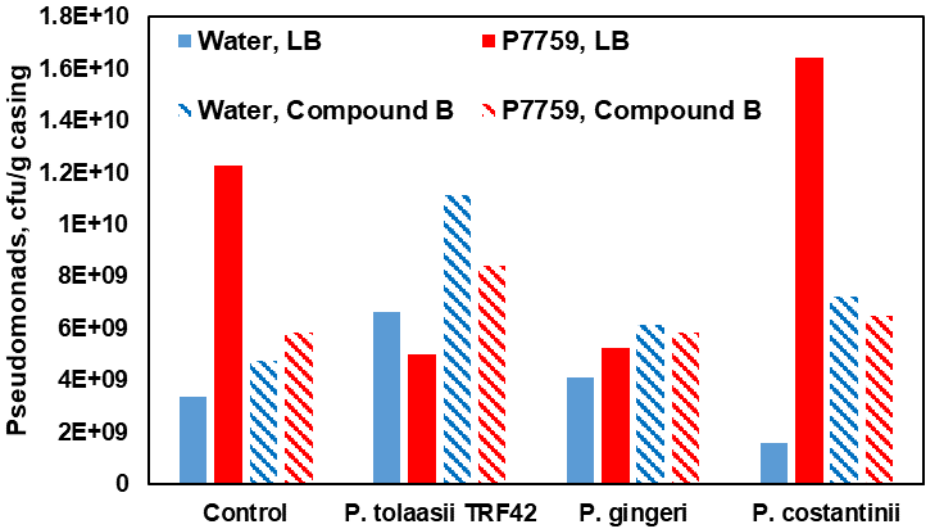

Figure 3.2d Pseudomonad counts of casing inoculated with or without different blotch pathogens and/or *'Pseudomonas reactans'* P7759. Samples were taken after the second flush and incubated in LB broth, with or without Compound B.

Experiment 3.2.1d. Effect of PlantWorks produced inoculum of P7759 on blotch

Pots that were not inoculated with pathogen inoculum produced only clean healthy mushrooms (Fig. 3.2e). Blotch symptoms on other pots corresponded with the pathogen inocula applied, although in this experiment very little pitting was observed on pots inoculated with *P. costantinii*. Application of P7759 inoculum to pots resulted in an increase in the number of healthy mushrooms compared with water treated pots, except for pots inoculated with *'P. gingeri'*. Brown blotch caused by *P. tolaasii* was reduced by the application of P7759.

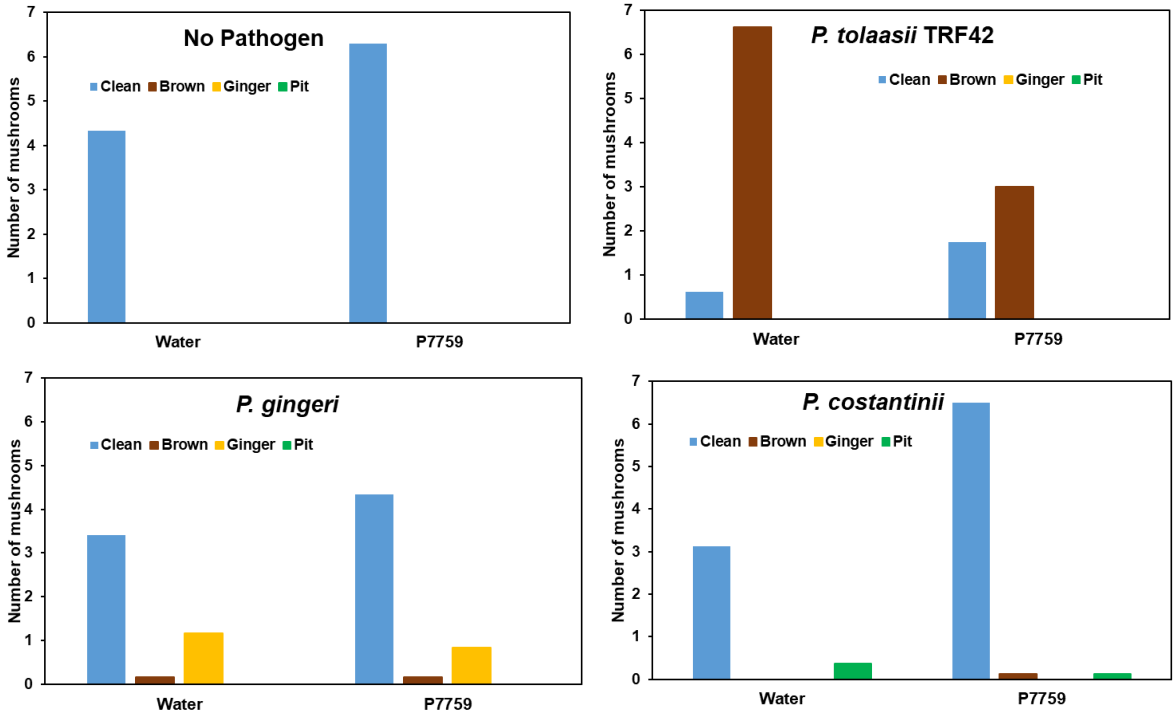

Figure 3.2e Effect of PlantWorks produced P7759 inoculum on different types of blotch. Each treatment value is the mean of two experiments, each with four replicate pots

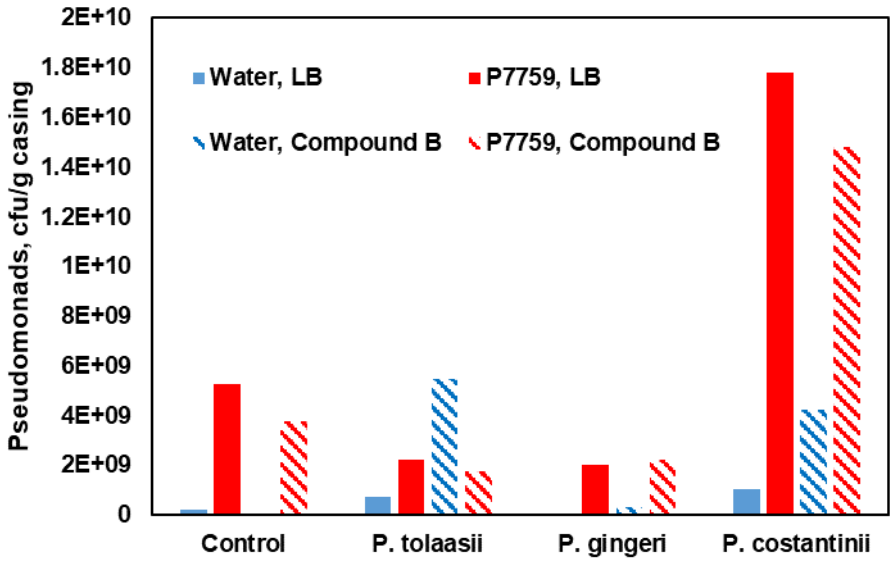

Figure 3.2f Pseudomonad counts of casing inoculated with or without different blotch pathogens and/or with PlantWorks produced *'Pseudomonas reactans'* P7759. Samples were taken after the second flush and incubated in LB broth, with or without Compound B.

The following trends were observed in the Pseudomonad counts in casing samples taken after the second flush of mushrooms:

- Background Pseudomonad and *'P. gingeri'* counts in the casing extracts were very low (Fig. 3.2f)

- Where *P. tolaasii* or *P. costantinii* inocula were added, the Pseudomonad counts were increased by adding Compound B to casing extracts
- Addition of P7759 inoculum to the casing increased the Pseudomonad count; this increase was slightly less when Compound B was added to the casing extract

The results therefore indicate that Compound B is effective in increasing the counts of *P. tolaasii* and *P. costantinii* during incubation of casing extracts. However, Compound B is slightly inhibitory to P7759 during incubation of casing extracts.

3.2.2 Effect of antagonists on blotch in commercial farm experiments

In the first farm experiment, there was a high level of blotch in the first flush, with lower numbers of blotched mushrooms in the second and third flushes (Fig. 3.2.2a). Blotch disease symptoms (pitting of caps and discolouration between touching mushrooms) was typical of that caused by *P. costantinii* (Fig. 3.2.2b). Application of P7759 inoculum did not significantly affect the number of blotched mushrooms.

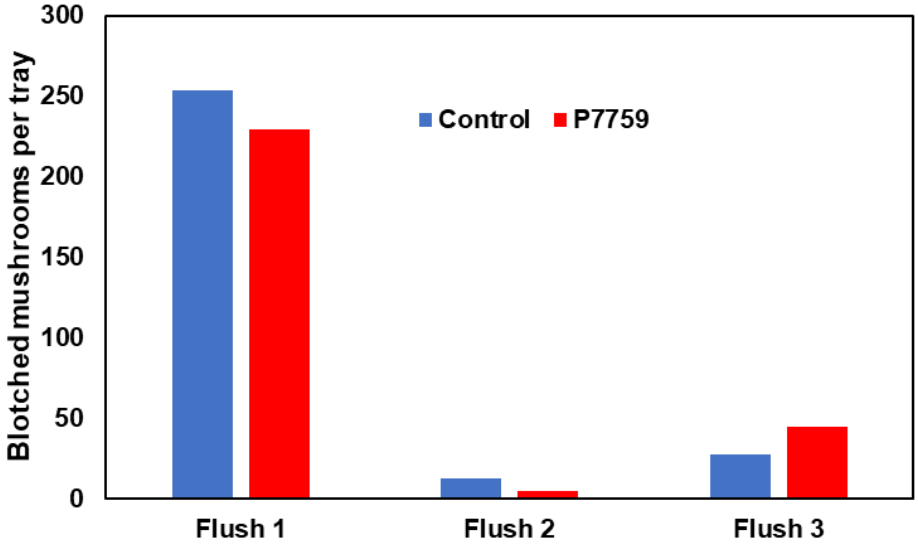

Figure 3.2.2a Effect of PlantWorks produced inoculum of P7759 on the number of blotched mushrooms per tray In Experiment 1. Each value is the mean of five replicate trays.

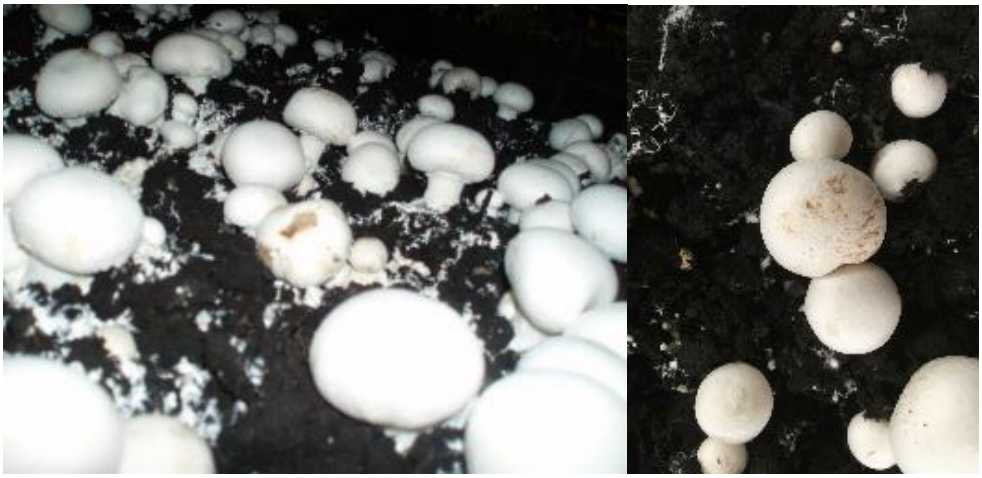

Fig. 3.2.2b Blotch and pitting symptoms observed in the commercial farm trial

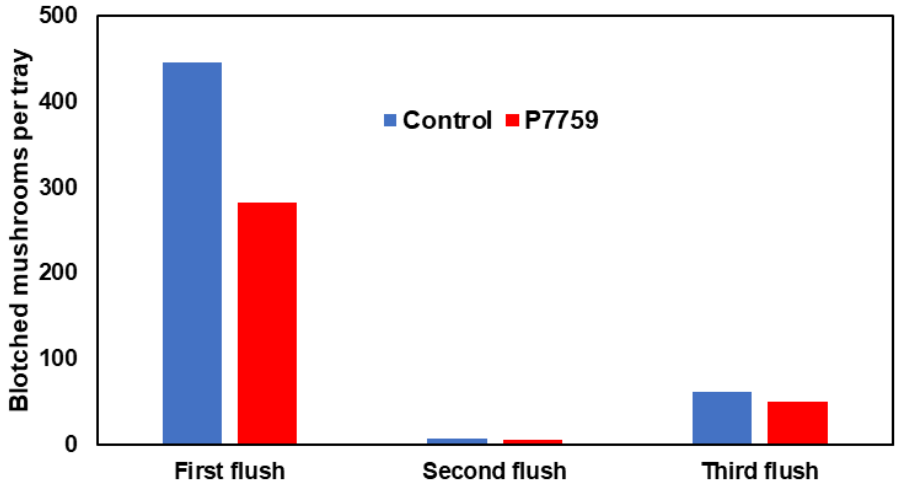

Figure 3.2.2c Effect of PlantWorks produced inoculum of P7759 on the number of blotched mushrooms per tray in Experiment 2. Each value is the mean of five replicate trays.

In the second farm experiment, there was also a very high level of blotch in the first flush, with lower numbers of blotched mushrooms in the second and third flushes (Fig. 3.2.2c). Blotch disease symptoms were again typical of that caused by *P. costantinii*. Application of P7759 inoculum resulted in a reduction in the number of blotched mushrooms which was not quite statistically significant with five replicates ($P = 0.064$).

The results of qPCR for detection of *P. costantini* (assay Pc3a12) on 18 samples, failed to detect the pathogen in all samples except for one sample that had a high Ct (of 35). Analysis of RpoD nanopore sequencing did not allow to detect *P. constantinii* or *'P. reactans'* at reliable levels with the bioinformatics method used, with the vast majority of sequences assigned to *P. fluorescens* or *P. syringae* (control). The inability to detect *P. constantinii* is in line with the

qPCR results, where Ct values for *P. constantinii* were 40 for most samples (indicating a very low-level present). The level of *P. costantinii* present in these samples might have been too low for this sequencing method to detect it.

In a separate crop, dark brown blotch symptoms were observed on the brown strain Heirloom (Fig, 3.2.2d). The Pseudomonad isolate tested positive for *P. tolaasii* using the Pt Taqman assay. Similar symptoms on the same brown mushroom strain were also observed at a different commercial farm.

Fig. 3.2.2d Dark brown blotch on the brown mushroom strain Heirloom observed at a commercial farm

3.2.3 Effect of antagonist on Trichoderma green mould in pot bioassays

Experiment 3.2.3a. Effect of Trichoderma aggressivum isolates applied to Phase II compost

There were no green mould symptoms on uninoculated control pots. Spore suspensions of *T. aggressivum* isolates 23343B and 3115 did not significantly affect the number of healthy mushrooms or result in green mould symptoms (Fig. 3.2.3a). Isolate 3113 resulted in green mould in the compost and reduced the number of healthy mushrooms. Application of inoculum of AHDB 9849 at 0.1 g /kg compost did not significantly affect green mould or mushroom numbers.

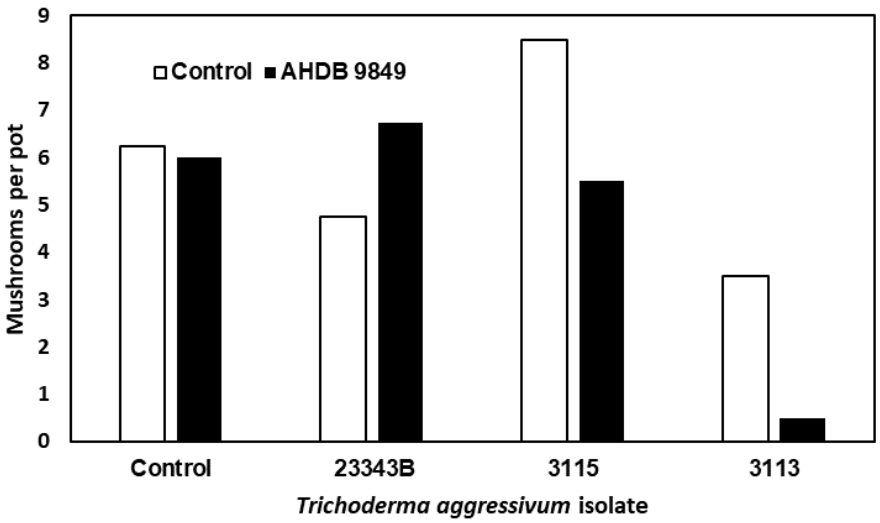

Figure 3.2.3a. Effect of *Trichoderma aggressivum* isolates and AHDB 9849 inoculum on the number of mushrooms. Each value is the mean of four pots.

Experiment 3.2.3b. Effect of Bacillus inoculum in compost on Trichoderma aggressivum

There were no green mould symptoms on uninoculated pots. Inoculation of pots with *T. aggressivum* spores reduced the number of mushrooms per pot and produced moderate green mould symptoms on some of the pots (Figs. 3.2.3b and 3.2.3c). Addition of *T. aggressivum* infected compost inoculum at 0.1%w/w resulted in severe green mould infection of compost and casing, and no mushrooms were produced on these pots (Fig. 3.2.3b). The effect of AHDB 9849 on mushroom numbers was small and inconsistent.

Figure 3.2.3b Uninoculated control pot (top left) and pots inoculated with *Trichoderma aggressivum* spores (top right) or infected compost (bottom pots)

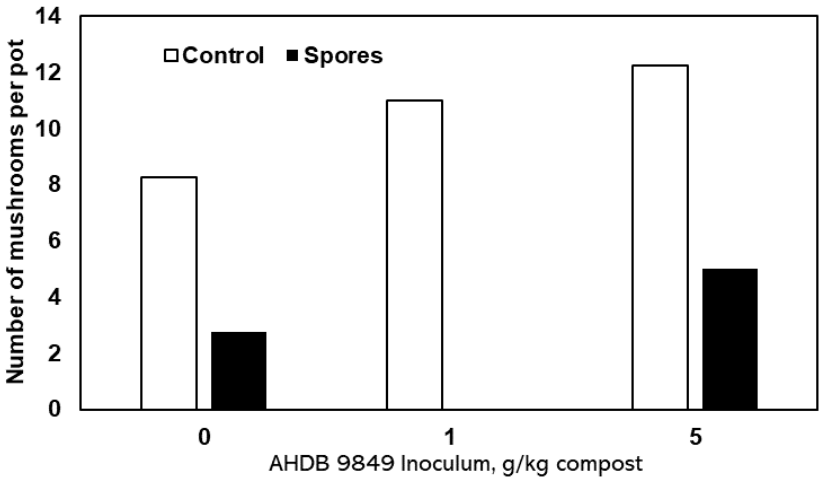

Figure 3.2.3c. Effect of *Trichoderma aggressivum* spores and AHDB 9849 inoculum on the number of mushrooms. Each value is the mean of four pots.

3.3. Control of blotch using bacteriophages

3.3.1. Extension of phage library

The river Cam was a reliable source for phage isolation using enrichment methods (Table 3.1; Appendix Fig. S8) with the *P. tolaasii* strain NCPPB 2192^T (FSBactM 009; P7544) as the "bait" strain for initial phage enrichments. Independent isolates of the phages produced different plaque morphologies on this strain; clear or turbid and of varying plaque sizes (Fig. 3.3 and Fig. S9 for examples) suggesting viral heterogeneity. Two phages (ϕMB55 and ϕMB56) were then used along with strain NCPPB 2192^T in mushroom blotch/pitting assays. These lab-based experiments involved cut mushroom caps challenged with *P. tolaasii* with, or without, phage added at different multiplicity of infection (MOI; 1 or 10) with Luria broth (LB) as a negative control. The extent of pitting/browning of the infected zones on mushroom caps was measured. The assays provided a positive indication of potential biocontrol capacity using these phages, acting in a "dose-dependent" fashion on the NCPPB 2192^T host strain (Fig. S10).

Results from further enrichments using river Cam water, and water and blotched mushrooms from commercial producers were also promising (Table 3.1). Increasing the diversity of bacterial host strains (supplied from the Fera collection) in several enrichments helped avoid an extreme enrichment bias seen when using strain NCPPB 2192^T. These further enrichments enabled isolation of new phages that increased the library diversity. Among the new isolates we discovered phages that infect multiple *P. tolaasii, P. costantinii* or *'P. gingeri'* strains (Table 3.3.1 and Appendix Table S1). The analysis of these new phages (genomics, host range, TEM analysis etc) helped reveal the best candidates for potential biocontrol. Further enrichments could be performed with the aspiration of building an even wider bank of phages.

Table 3.3.1. 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**

* 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 (TEM). These phages were then used in screening assays against multiple *P. tolaasii, P. costantinii* and *'P. gingeri'* strains provided by Fera, plus a range of other species including *P. fluorescens* and *P. putida* isolates as control strains. The phages did not infect *P. fluorescens* or *P. putida*. Although the independent phage isolates produced plaque heterogeneity on strain 2192^T, multiple enrichments 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 strain is clearly susceptible to many different phages, enabling easy enrichment and isolation of phages from both the natural environment and commercial sources. However, the results suggested that phages isolated by enrichment on this particular host strain do not appear to infect many alternative strains and so this restricted host range created limitations for wider biocontrol utility. Consequently, a series of additional enrichments using alternative bacterial strains of *P. tolaasii, P. costantinii* and *P. gingeri* were performed using multiple Fera hosts. This led to the isolation of a wider selection of new phages (Tables 3.1 and Appendix Table S1) with extended host ranges. These may have potential for building promiscuous phage cocktails exhibiting wider strain coverage of the mushroom-pathogenic species. One such phage was ϕJW26 which was effective in biocontrol of strong pitting seen in lab-based cut cap assays with *P. tolaasii* strain TRF42 – a very virulent strain (Appendix Fig. S11 and S12). Phages ϕJW26 and ϕJB56 were tested in experiments using the new pot growth protocols described earlier, initially to test a proof of principle that some phages will have biocontrol capacity under such mushroom growth conditions.

TEM 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 (Fig. 3.3 and Appendix Fig. S9).

3.3.3. Phage genomics

Full genome sequencing of multiple phages was performed and bioinformatic comparisons revealed that the environmental phages isolated on *P. tolaasii* 2192^T fell into two genetically distinct groups (Luz24-like and T7-like phages). 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. For example, a comprehensive genome sequence interrogation of independently-isolated phages 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.

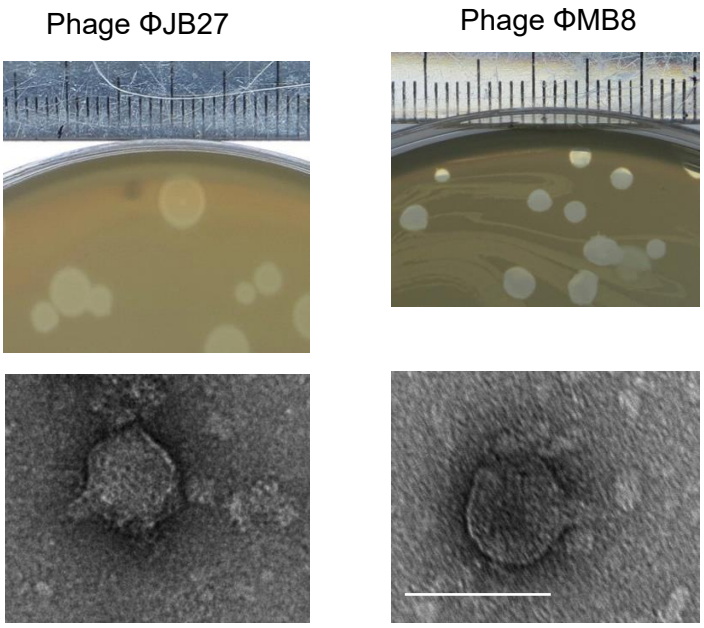

Figure 3.3. Example of *Pseudomonas tolaasii* phages. Top: Phages ϕJB27 and ϕMB8 produce turbid and clear plaques on a lawn of *P. tolaasii* strain NCPPB 2192^{T}. Bottom: TEM images showing morphology characteristic of *Podoviridae* family phages displaying a short "stumpy" tail extending from the phage heads (scale bar 100 nm).

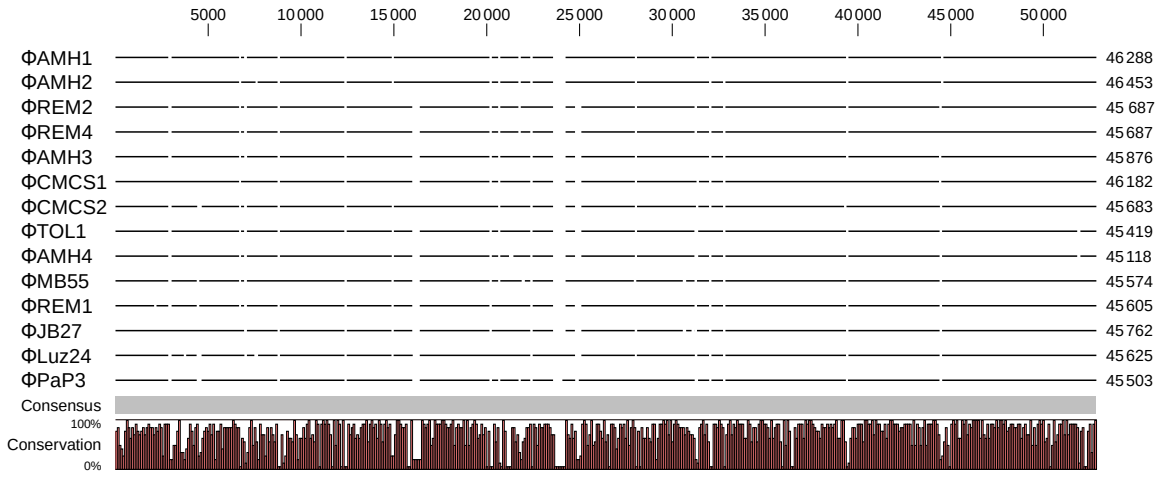

Figure 3.4. Summary of genomic comparisons of the Luz24-like family phages isolated on *P. tolaasii* strain NCPPB 2192^{T}. The genome sequences of all phages (listed down the left-hand column) are aligned and had genomes sizes of around 45 kbp. The red bars depict sequence conservation throughout the genomes and the blank areas represent regions of sequence variation between the phage genomes.

Notwithstanding the ease of phage isolation on the *P. tolaasii* 2192^T strain and proof of principle, from a practical perspective the genomic results confirmed that isolation of new phages from the local river using this particular bacterial host strain created a substantial bias on the classes of phages that were positively enriched. The new series of enrichments using several Fera strains of *P. tolaasii, P. costantinii* and *'P. gingeri'* challenged with environmental samples - including soil and wild mushroom samples and samples from commercial mushroom production plants – have produced new bacteriophages that extend the possible biocontrol range.

3.3.4 Effect of phages on blotch in a pot experiment

Two bacteriophages were selected as treatments for this experiment, ϕJW26 and ϕJW56 (Appendix Table S1), individually or in combination.

The control pots inoculated with *P. tolaasii* isolate TRF42 (FSBacM 061) resulted in severe blotch symptoms with almost no clean mushrooms (Fig. 3.5). However, this isolate stimulated the production of mushrooms, i.e. there were more blotched mushrooms with *P. tolaasii* TRF42 than clean mushrooms without the inoculum (Fig. 3.6). The *'P. gingeri'* (FSBacM 005) and *P. costantinii* (FSBacM 037) isolates were not pathogenic in this experiment and did not cause more blotched mushrooms than the background level of blotch in the uninoculated control treatment. Across all pathogen treatments, the application of phages resulted in significantly less blotched mushrooms, although there was no corresponding increase in the number of clean healthy mushrooms.

Fig. 3.5 Severe brown blotch on mushrooms resulting from inoculation of casing with *P. tolaasii* TRF 42.

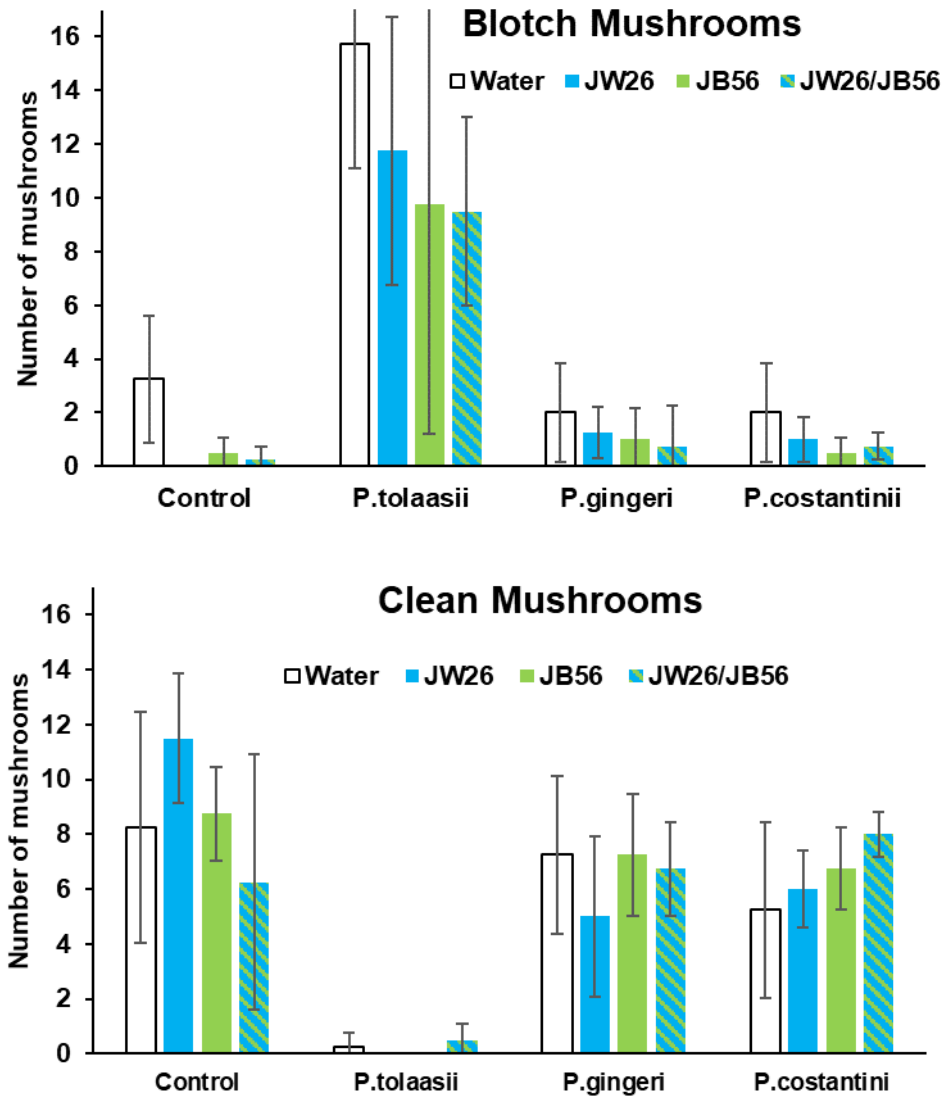

Fig. 3.6 Effect of phages ϕJW26 and ϕJB56 and different blotch pathogens on the number of clean and blotched mushrooms in pot tests. Each value is the mean of four replicate pots (± SE).

Conclusions

Bacterial blotch detection and control

- 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*
- A new method to test the pathogenicity of mushrooms in small pots enclosed in plastic bags has been developed and used successfully
- 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 two groups of *'P. gingeri'* (in project M063)

- TaqMan assay results on casing extracts corresponded with the pseudomonad isolates (*P. tolaasii*, *'P. gingeri'* and *P. costantinii*) that were inoculated into the casing. The assays were negative for the Pseudomonad antagonist P7759
- A new Taqman assay targeting *P. costantinii* detected the pathogen in casing extracts taken from a crop showing pitting symptoms typical of *P. costantinii*
- Compound B added to LB broth was effective in increasing the counts of background and pathogenic Pseudomonads during incubation of casing extracts but did not improve the resolution of the TaqMan assay
- In an on-farm experiment, application of P7759 inoculum resulted in a reduction in the number of blotched mushrooms which was not quite statistically significant with five replicates
- 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
- Phages infecting a range of *P. tolaasii, P. costantinii* and *'P. gingeri'* have been isolated and some have been genomically sequenced
- Across all pathogen treatments, the application of phages resulted in significantly fewer blotched mushrooms in a pot culture experiment. However, there was no corresponding increase in the number of clean mushrooms

Green mould detection and control

- 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
- Specific qPCR assays developed at Fera for *T. aggressivum* and *T. harzianum* detected these pathogens in mushroom casing at concentrations that did not produce visible green mould symptoms
- Application of an antagonist treatment *Bacillus subtilis* syn. *B. amyloliquefaciens* AHDB 9849 was ineffective in suppressing green mould in compost caused by *Trichoderma aggressivum*

Microbial communities

- Microbiome sequencing of bacterial and fungal communities showed differences between substrate and casing and some differences between farms
- The methods used did not allow the identification of different *Pseudomonas* species, but successful detection of *Trichoderma* was achieved
- Specific qPCR assays developed at Fera for *T. aggressivum* and MinION ITS sequencing detected *Trichoderma* in mushroom casing at concentrations that did not produce visible green mould symptoms.

Knowledge and Technology Transfer

- Webinar 'Detection and control of pathogens causing blotch and green mould in mushroom cultivation', 16th of February 2021, by Joana Vicente, Ralph Noble and George Salmond. https://ahdb.org.uk/events/detection-and-control-of-pathogens-causing-blotch-and-green-mould-in-mushroom-cultivation

  Available on YouTube https://www.youtube.com/watch?v=_E8C3FQiHMA

- Presentation at the e-Congress of The International Society of Mushroom Science (ISMS) 'Diversity of Pseudomonas spp. associated with mushroom blotch in the UK, development of qPCR assays for quantitative diagnostics and disease control using antagonists and bacteriophages', 14-17 September 2021 (pre-recorded talk that is still available to members that registered) by Joana Vicente.

  Available at: https://event.isms2021.exordo.com/session/10/pests-and-diseases

- Presentation with M065 project update at the online Mushroom Grower Group meeting, 6th of September 2021, by Joana Vicente.

Glossary

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

Enrichment with compounds A and B: Increasing the population (of Pseudomonads) in a sample (to a detectable concentration) by addition of a selective nutrient source

Isolate: pure microbial culture obtained by separation from a mixed population of living microbes present in a sample or environment

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

MinION (Nanopore) sequencing: technology that enables direct, real-time analysis of long molecules of DNA (or RNA). The result is decoded to provide DNA or RNA sequences that can be analysed (bioinformatically).

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

Pathogenicity: the potential ability (of a microbial isolate) to produce disease in mushrooms

PCR Assay: test to detect the presence of genetic material from a specific organism or organisms. Polymerase chain reaction technique for rapidly produces (amplifies) millions to billions of copies of a specific segment of DNA that can then be visualised on a gel and/or sequenced.

Phylotype: biological type that classifies an organism by its phylogenetic relation to other organisms. It groups organisms with DNA sequences sharing more than an arbitrarily chosen level of similarity.

Pot bioassay: assessment of the pathogenicity of an isolate or the effect of a treatment in a small pot where mushrooms are cultivated.

qPCR (or real-time PCR) Assay: laboratory technique based on the polymerase chain reaction (PCR) that monitors the amplification of a target DNA molecule in real-time and can be used quantitatively.

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**	**Potential Economic Damage**
Th1	*Trichoderma harzianum*	Yes	Low
Th2	*Trichoderma aggressivum f. europaeum*	Yes	High
Th3	*Trichoderma atroviride*	Yes	Low
Th4	*Trichoderma aggressivum f. aggressivum*	No*	High
*In Europe, it has been detected in Hungary in 2015 (Hatvani et al., 2017)			

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Appendices

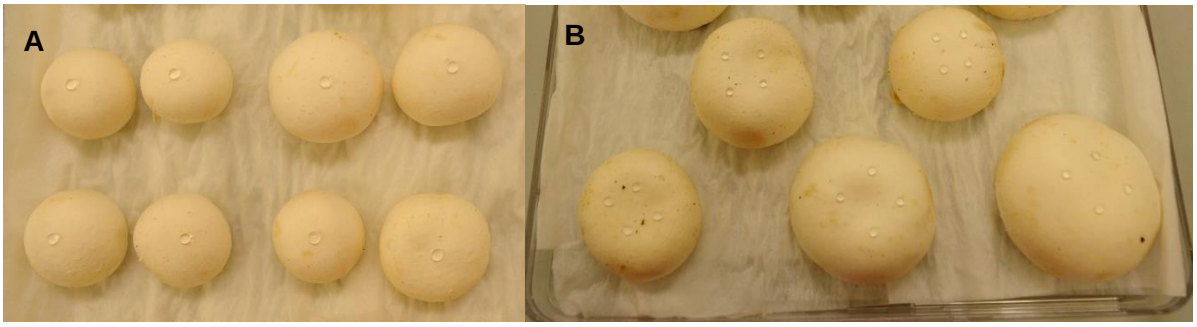


Figure S1. Cut cap pathogenicity assays in large plates after drop inoculation with bacterial suspensions from a range of isolates. A: Small mushroom caps inoculated with single drops of *Pseudomonas* spp. suspension. B: larger caps inoculated with four drops of suspension.

Figure S2. Growth room set up with mushrooms starting to appear in pots four days after inoculation.

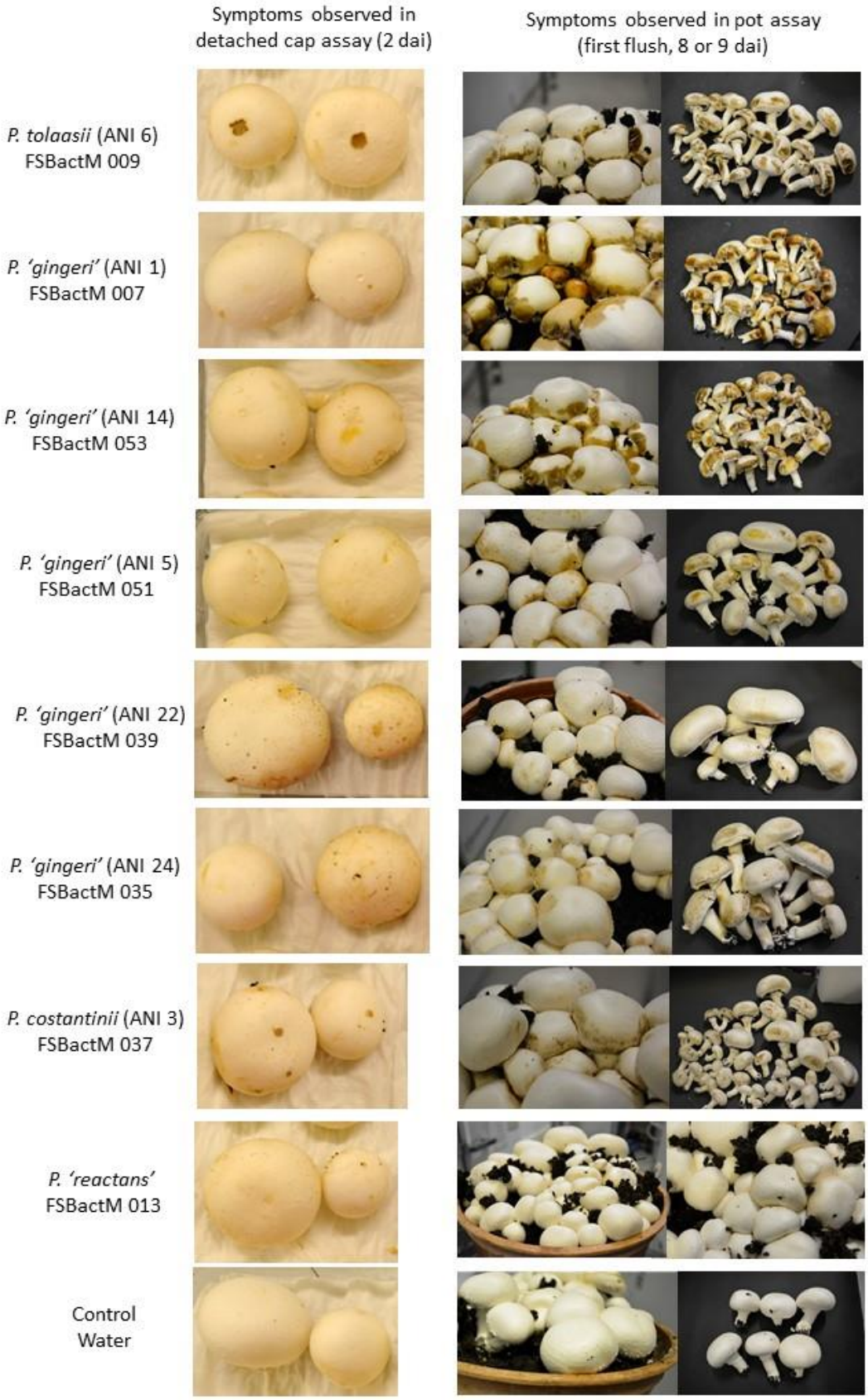
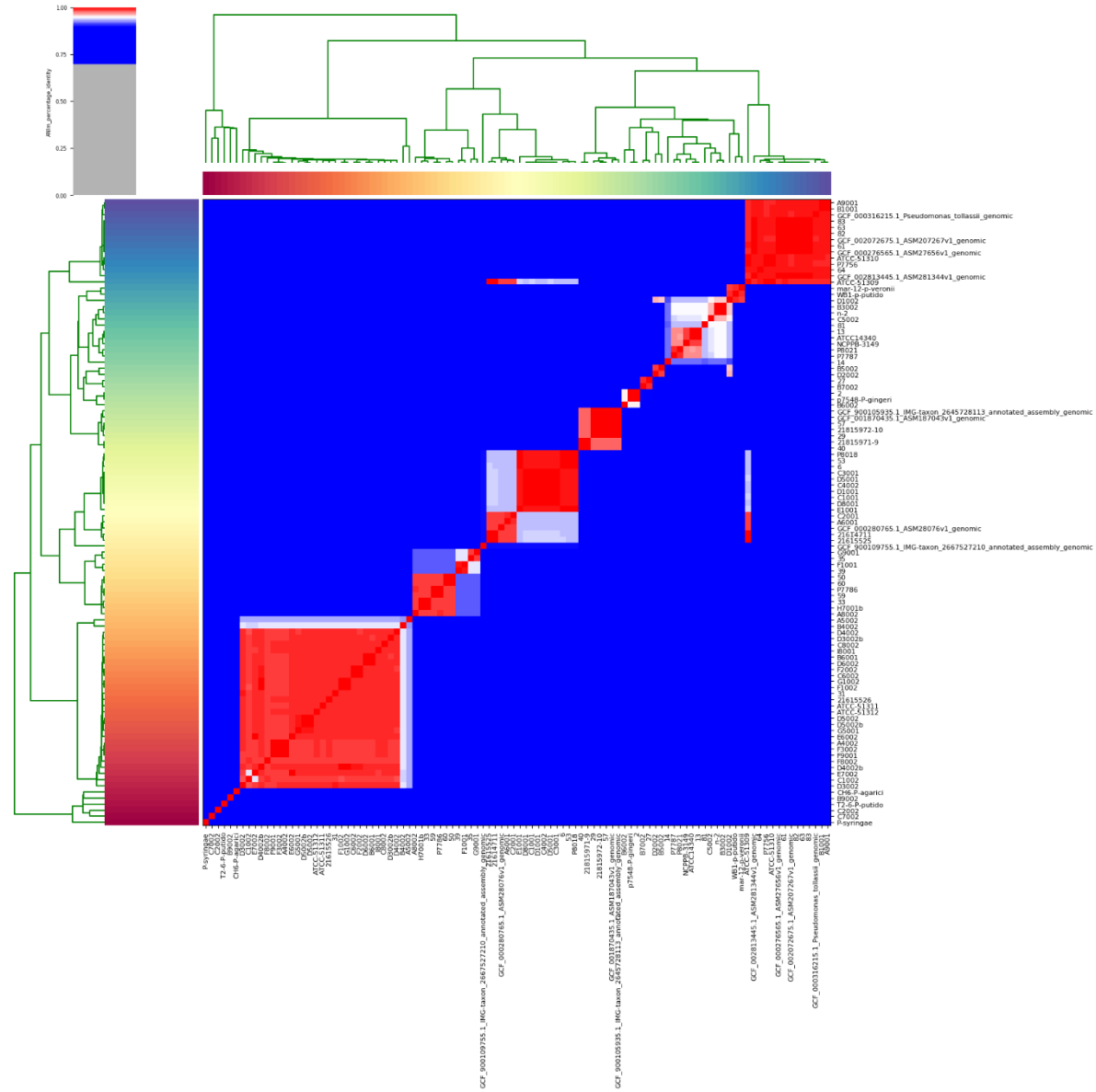


Figure S3. Symptoms observed in the detached cap assay and in the pot assay for isolates representative of *P. tolaasii* (brown blotch), *'P. gingeri'* (two isolates causing strong ginger blotch and three isolates causing mild ginger blotch), *P. costantinii* (pitting) and *'P. reactans'* (not pathogenic).

A



B.

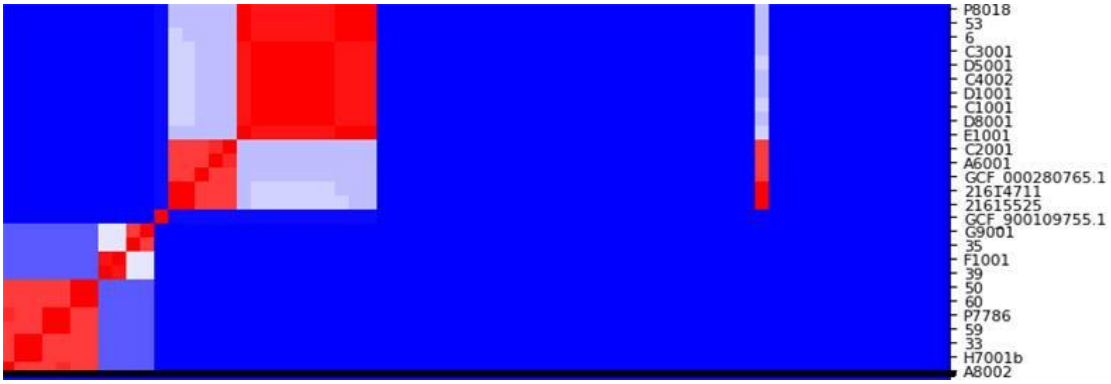


Figure S4 (A and B). ANI comparisons of available whole genome sequences of mushroom associated *Pseudomonas spp*. isolates including 22 isolates sequenced during this project. A. Complete figure with 102 sequences B. highlight of *'P. gingeri'* groups (27 sequences).

EF ITS

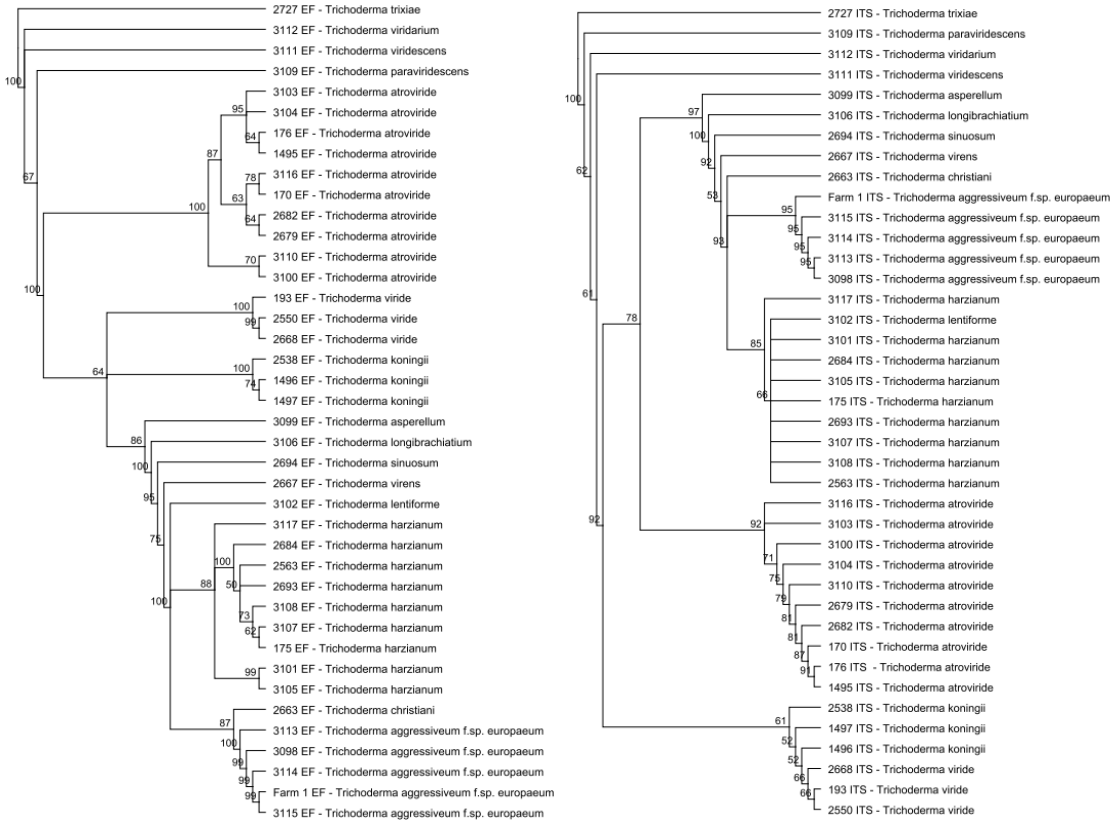
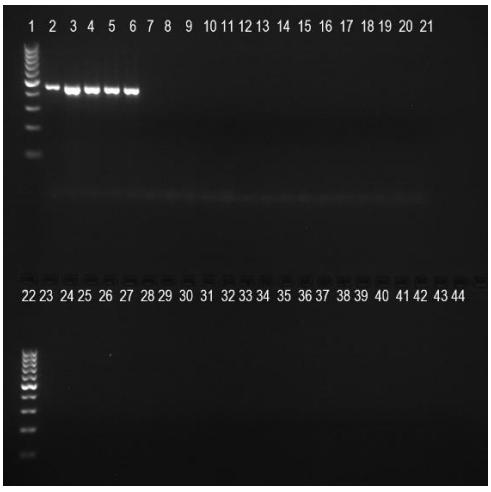


Figure S5. Trees based on the alignments of EF and ITS partial gene sequences for *Trichoderma* strains



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

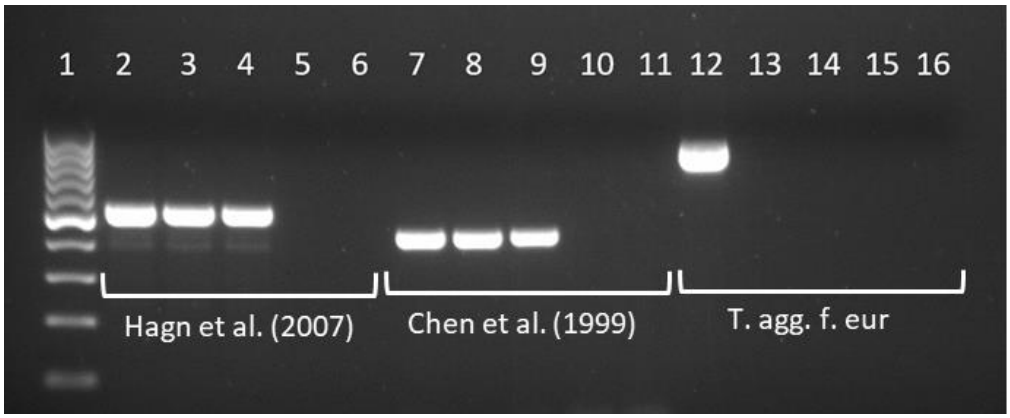


Figure S7. Gel with PCR products of assays targeting the genus *Trichoderma* (Hagn et al, 2007), the species T*. aggressivum* (Chen et al., 1999) and the subspecies *T. aggressivum* f. *europaeum* (Kosanovic et al., 2020). Lanes 2, 7, 12 *Trichoderma aggressivum f. europaeum.* Lanes 3, 4, 8, 9, 13, 14 *Trichoderma aggressivum f. aggressivum.* Lanes 5, 10, 15 Extraction blank control. Lanes 6, 11, 16 negative PCR control

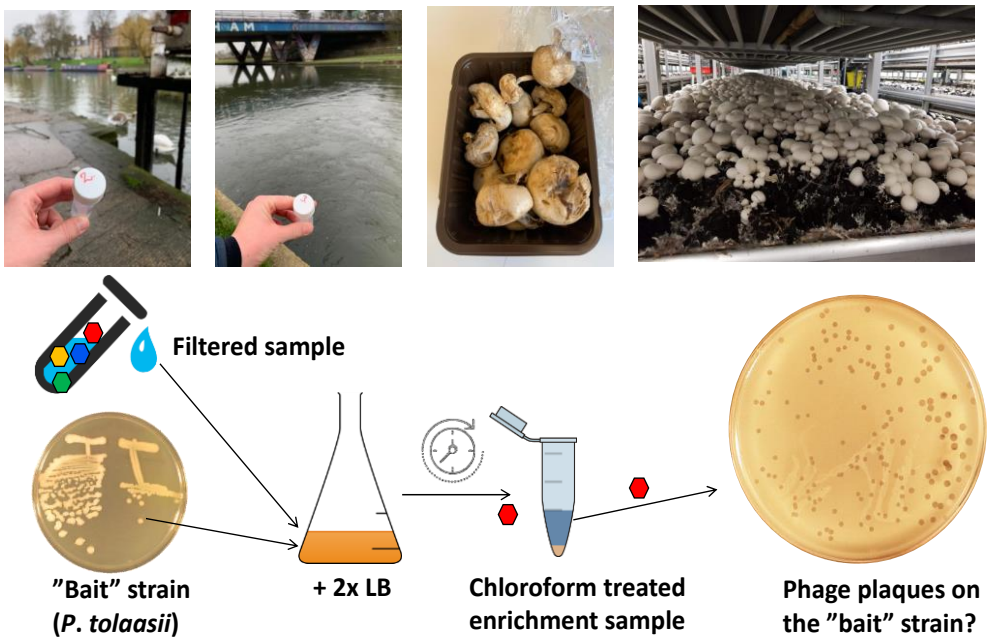


Figure S8. General phage enrichment and isolation strategy from river water, wild mushrooms and soil samples and mushroom production plant samples

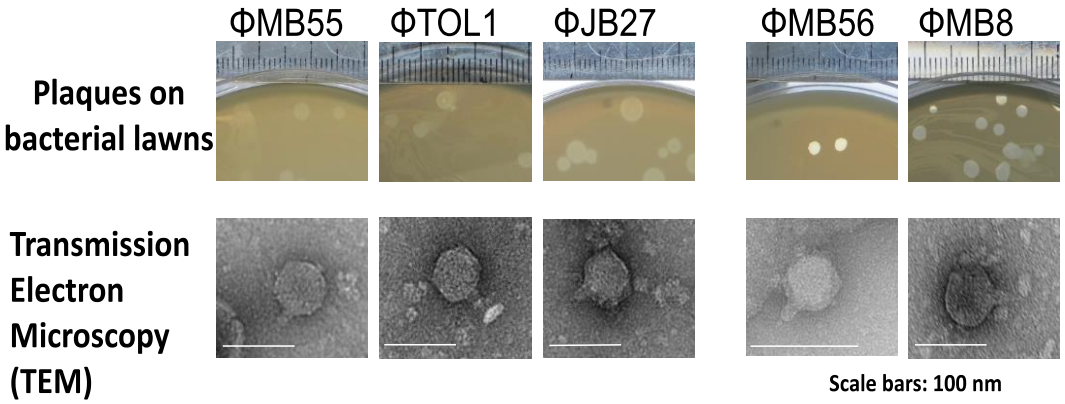


Figure S9. Examples of environmental phages with different morphologies infecting *P. tolaasii* strain 2192^{T}. Phage plaques on bacterial lawns and transmission electron microscopy (TEM) images. Plaques can be turbid or clear; short tails indicate that the phages are part of the *Podoviridae* family

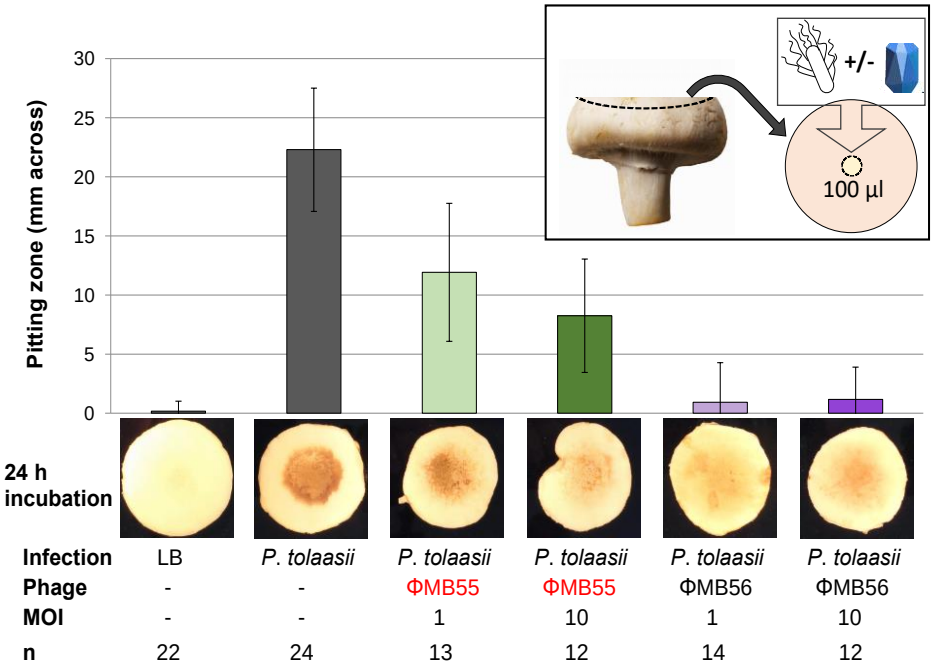


Figure S10. Lab-based biocontrol tests on mushroom cut-caps inoculated with *P. tolaasii* strain 2192^{T} and two phages

Table S1. Host ranges and infection efficiency of three phages (ϕJW3, ϕJW26, ϕJB56) on different *Pseudomonas* species pathogenic and non-pathogenic to mushrooms*

FSBactM strains	Identity originally assigned	This study identity (ANI group)	Pathogenicity cap / pot	ϕJW3	ϕJW26	ϕJB56
'Pseudomonas gingeri'						
001	*'P. gingeri'*	*'P. gingeri'* (1)	- cap / weak pot	**2+**	-	**+**
005	*'P. gingeri'*	*'P. gingeri'* (14)	+ / +	-	-	**+**
006	*'P. gingeri'*	*'P. gingeri'* (14)	+ / +	-	-	**+**
007	*'P. gingeri'*	*'P. gingeri'* (1)	+ / +	-	-	**+**
024	*'P. gingeri'*	*'P. gingeri'* (5)	+ / +	**2+**	**2+**	**+**
033		*'P. gingeri'* (5)	-(+)	-	-	-
035		*'P. gingeri'* (24)	- / +	-	-	**+**
039		*'P. gingeri'* (22)	+ / +	-	**2+**	-
050		*'P. gingeri'* (5)	+ / +	**2+**	**2+**	**+**
051		*'P. gingeri'* (5)	+ / +	**2+**	**2+**	**+**
053		*'P. gingeri'* (14)	+ / +	-	-	-
059		*'P. gingeri'* (5)	+	**2+**	**2+**	**+**
060		*'P. gingeri'* (5)	+	**2+**	**2+**	**+**
Pseudomonas costantinii						
015	*P. costantinii*	*P. costantinii* (3)	+ / +	-	**(2+)**	**+**
029	*P. costantinii*	*P. costantinii* (3)	+ / +	-	**2+**	-
036	*P. costantinii*	*P. costantinii* (3)	+ / weak	-	**2+**	-
037	*P. costantinii*	*P. costantinii* (3)	+ / +	**2+**	**2+**	-
040		*P. costantinii* (3)	+ / weak	-	-	**+**
057		*P. costantinii* (3)	+	-	**2+**	**+**
Pseudomonas tolaasii						
009	*P. tolaasii*	*P. tolaasii* (6)	+ / +	-	-	-
010	*P. tolaasii*	*P. tolaasii* (6)	+ / +	-	-	-
011	*P. tolaasii*	*P. tolaasii* (6)	+ / +	-	-	-
061	*P. tolaasii*	*P. tolaasii* (6)	+	**2+**	**(2+)**	**+**
062	*P. tolaasii*	*P. tolaasii* (6)	+	**2+**	-	**+**
063	*P. tolaasii*	*P. tolaasii* (6)	+	-	-	**+**
064		*P. tolaasii* (6)	+	-	-	**+**
065		*P. tolaasii* (6)	+	-	-	**+**
066		*P. tolaasii* (6)	+	-	-	**+**
068		*P. tolaasii* (6)	+	-	-	**+**
070		*P. tolaasii* (6)	+	-	-	**+**
082		*P. tolaasii* (6)	+	-	-	**+**
083		*P. tolaasii* (6)	+	-	-	-
Other *Pseudomonas spp.*						
002	*P. gingeri*	*P. edaphica*	- / weak	**2+**	-	-
003	*P. gingeri*	*P.* NCO_2 (2)	- / -	-	-	-
013	*'P. reactans'*	*'P. reactans'*	- / -	**-**	-	-

017	*P. agarici*			-	-	-
018	*P.s veronii*			-	-	-
019	*P. poae*			-	-	-
020	*P.s protegens*			-	-	-
022	*P. putida*			-	-	-
023	*P. syringae*			-	-	-
027	*P. tolaasii*	*P. edaphica* (12)	- / -?	-	-	-
028			- / -	-	-	-
031		*P.* NCO_2 (2)	-/-	-	-	-
042			- / weak	-	-	-
069			-	-	-	-
081		*'P. reactans'*	(+)	-	-	-
084			/(+)?	-	-	-

*Strain numbers are original Fera strain designations; efficiency of infection (infects) is reflected in score e.g. from weak (+) up to 2+. Shading represents host used for high titre production. The evolution of some spontaneous candidate host range mutants of a few phages was also noted, potentially useful for extending biocontrol capacity.

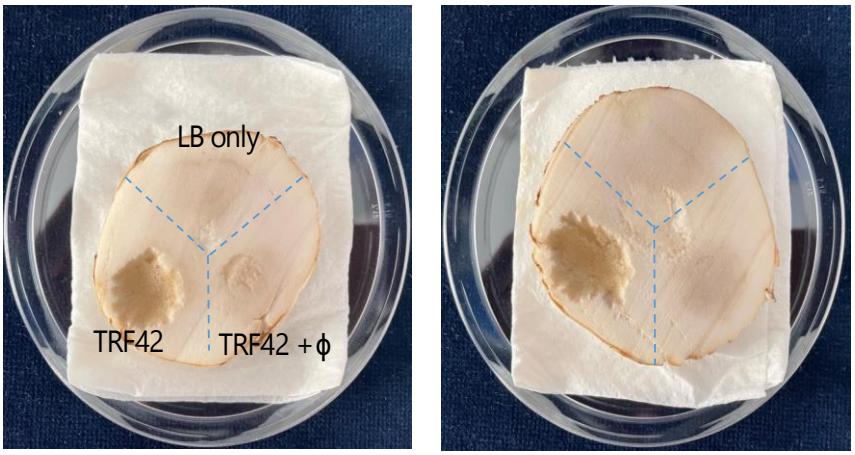


Figure S11. Example of pitting assays on cut caps with strain TRF42 (FSBactM 061; UK, 1980s) with and without phage ϕJW26 at two phage multiplicities of infection (MOI).

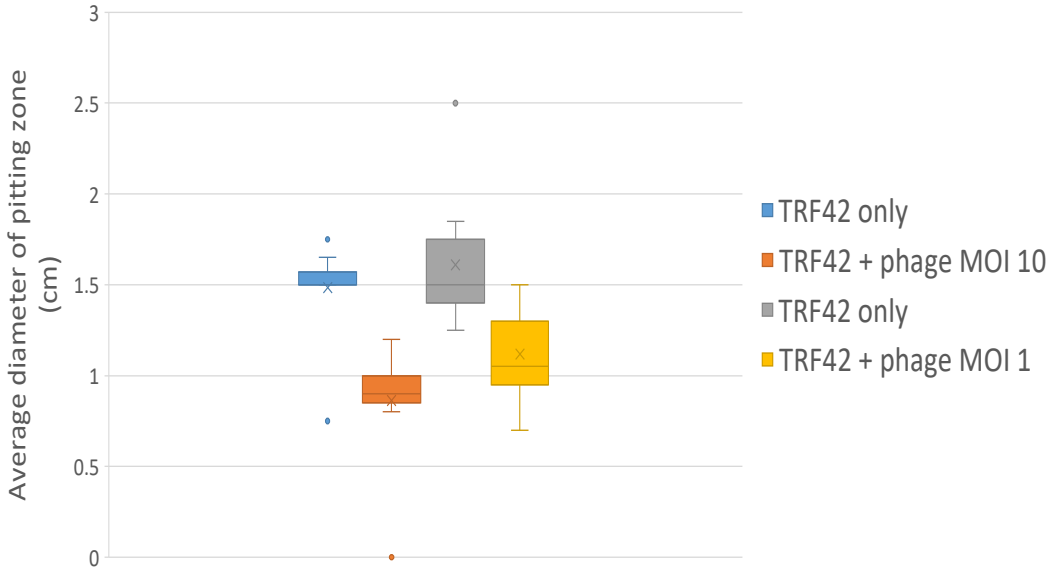


Figure S12. Cut cap biocontrol (pitting) assay with and without phage ϕJW26 on *P. tolaasii* TRF42 (FSBactM 061; UK, 1980s) at two multiplicities of infection (MOI)

