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Project leader:	Dr John Elphinstone, Food and Environment Research Agency (Fera)
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The results and conclusions in this report are based on an investigation conducted over an 18-month period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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CONTENTS

GROWER SUMMARY.

Headline

Raising humidity to increase mushroom yield must be balanced with the increased risk of bacterial blotch.

Background

The commercial objectives of this project were:

1. To develop new screening tests for *Pseudomonas* bacteria which may be present in casing materials to help understand how they affect mushroom yield and disease development;
2. To screen casing materials used in the UK for *Pseudomonas* species relate the results to mushroom yield and blotch incidence;
3. To identify practices which reduce the risk of blotch disease and improve mushroom quality.

Summary

- Bacterial blotch can be caused by a variety of fluorescent *Pseudomonas* species, not all of which have been fully characterized. Although two well known bacteria (*Pseudomonas tolaasii* and *Pseudomonas gingeri*) cause severe blotch symptoms under high humidity (>92% at 18°C), a number of other related but less well characterized *Pseudomonas* species can also induce mild blotch symptoms under these conditions, and equally reduce quality and marketability.
- Mushroom yield and potential for bacterial blotch development were apparently not affected by the source of commercial casing material used.
- Under experimental conditions, the disease was completely controlled by limiting the humidity at 88% during production at 18°C. In some experiments, higher yields and mushroom numbers resulted from increasing the humidity above 92% but this also increased the risk of disease.
- A new diagnostic test was developed which can be used to screen for *P. tolaasii* but further assay development is still needed to detect the other blotch-causing bacteria.
- The test was used to show that blotch symptoms developing under experimental conditions using common commercial casing sources was not caused by *P. tolaasii*.

- New DNA sequencing methods are available to help study the balance between blotch-causing and beneficial bacteria but further characterization of the different bacteria is needed before these can be used to maximum effect.

Financial Benefits

- Potential increase in mushroom numbers and yield in response to increased humidity or longer and more frequent watering regimes needs to be carefully balanced with the increased risk of reducing quality and marketability due to bacterial blotch disease. Cost:benefit studies were not been undertaken during this short technical project.

Action Points

- Control bacterial blotch by maintaining relative humidity at a maximum of 88% during typical production at 18°C.
- Look out for bacterial blotch symptoms during trials of new substrates, casings or production practices.
- If bacterial blotch is suspected, have the cause diagnosed using a new test developed through this project.

SCIENCE SECTION

Introduction

The source of casing material, its microbial population as well as watering management, and the growing room environment can have significant influences on mushroom initiation (stimulated by *Pseudomonas* species including *P. putida* and *P. poae*[1]) and the appearance of bacterial blotch (which can be caused by *Pseudomonas* species including *P. tolaasii*, *P. fluorescens, P. reactans* and *P. gingeri* [2-8]). In the same environment and watering regime, some casing materials may stimulate more initials and/or be more prone to blotch disease than others. Some pseudomonad bacteria that stimulate mushroom initiation may also cause blotch, and some that stimulate initiation are more closely related to the blotch causing *P. tolaasii* than to the initiation stimulating *P. putida*[2, 9]. Some casing materials result in over-stimulation of mushroom initials resulting in overcrowded, small and poor quality mushrooms. It is unclear whether this is due to the stimulatory bacterial population.

Laboratory methods have been developed for testing the mushroom initiation stimulatory effect and blotch pathogenicity of pseudomonad isolates[1, 10]. This has involved applying known isolates of pseudomonads to casing, rather than identifying naturally occurring pseudomonads from casing and relating the results to subsequent initiation or blotch incidence. Stimulation of mushroom initiation is usually associated with *Pseudomonas putida*, but other pseudomonads (*P. poae*, *P. veronii*) and possibly other bacterial species have also been shown to have a stimulatory effect[1, 11]. Several phenotypes or biovars of *Pseudomonas fluorescens* have been associated with mushroom disease symptoms[2-8]. Biovars I and II are not known to be pathogenic on mushrooms, biovar III (*P. gingeri*) causes ginger blotch, biovar IV (*P. tolaasii*) causes blotch, biovar V (*P*. 'reactans') is usually non-pathogenic but can cause mild blotch symptoms, biovar VI (*Pseudomonas* sp.) is associated with mummy disease, and biovar VII (*P. agarici*) is associated with drippy gill symptoms. Biovars IV and V produce a positive reaction in the 'white line test' in contact with two different *P. tolaasii* test isolates. Within *P. tolaasii*, there is significant variation between isolates in pathogenicity and severity of blotch symptoms caused[10]. Mushroom initiation stimulatory *Pseudomonas* species such as *P. putida* have also been found to cause blotch symptoms[2, 9].

Molecular taxonomic methods (PCR, 16S rDNA and rRNA gene sequence analysis, DNA:DNA hybridizations, AFLP analysis) have been used for characterising *Pseudomonas*

species [12,13,14], including studies of the genetic diversity within species of *Pseudomonas* stimulating mushroom initiation and/or causing blotch[3-8]. These studies have been conducted in the USA, Canada, South Korea, Belgium, France, Finland and New Zealand.

Diagnostic tests which could be used to screen for mushroom initiation stimulatory and/or blotch causing pseudomonads would provide information on the likely effects of the casing material on initiation and incidence of blotch disease. This could inform casing producers and growers in the selection of peat and other raw materials for use in casing. The commercial objectives of this project were therefore:

1. To develop screening tests for *Pseudomonas* species in mushroom casing materials to assist in understanding their role in stimulation of mushroom initiation and disease development.
2. To screen casing materials used in the UK for *Pseudomonas* species using the above test and relate the results to mushroom pinning and blotch incidence;
3. To facilitate selection of casing materials and management practices which reduce the risk of blotch disease and improve mushroom quality.

Materials and methods

Mushroom cultivation in pots

A series of experiments were conducted to assess the effects of different commercial sources of casing materials on mushroom yield and bacterial disease incidence. Five different casing materials were sourced from McArdle (Ireland), McDon (Ireland); Everris (GB), CNC (Netherlands) and Scott (GB). Mushrooms were grown in plastic pots, 230 mm diameter x 220 mm depth, each containing 3 kg of Hooymans Phase III compost, spawn-run with the mushroom strain Sylvan A15. The pots were cased with 850 g of casing materials (moist mixtures of peat and sugar beet lime, **Table 1**) containing caccing (spawn-run compost of the strain A15) at 1% w/w. The pots were watered and kept in a growing room at 25°C for 6 days. The pots were then transferred to a controlled environment chamber with an air temperature of 18°C and relative humidity of >92%, where the casing surface was inoculated with *Pseudomonas* sp. isolates. The humidity in the June 2011 experiment was lower than in the subsequent experiments. Three of the pots cased with each material in each batch were also transferred to a separate cropping room with an air temperature of 18°C and relative humidity of 88%. Two flushes of mushrooms were picked from the pots.

Table 1. Experimental dates and batches of casing materials used.

Date of experiment	Number of different casing materials used
June 2011	A,B.C
July 2011	A,B,C
October 2011	A,B
January 2012	B
June 2012	B,C,D

Determining bacterial populations in casing materials

Bacterial populations in the casing materials at the start (prior to inoculations) and end of the experiments were determined by preparing suspensions of 1 g casing in 9 mL sterile deionised water[1]. Serial dilutions of the suspension were spread on LB agar plates (Fisher Scientific, Loughborough) and pseudomonad isolation agar (PIA) (Difco Laboratories, Detroit, Michigan) and incubated at 25°C for 48 h to determine the total bacterial populations as colony forming units per gram casing (cfu g^{-1}) and the proportion that were pseudomonads.

Collection of Pseudomonas bacteria

A panel of *Pseudomonas* spp. was assembled from culture collections at EMR and Fera which were previously found to cause blotch or other disease symptoms or to stimulate mushroom initiation (Table 2). Additional fluorescent pseudomonads were isolated on King's medium B from symptomatic mushrooms growing in the various casings and further identified by their LOPAT group by determining levan colony-type, oxidase, pectolytic and arginine dyhydrolase activities and induction of hypersensitive reaction on tobacco (cv. White Burley).

Table 2. Fluorescent *Pseudomonas* isolates used in the study.

Fera Protect Number	Species	Isolate	Effect	LOPAT
7756	*P. tolaasii*	ATCC 51309	Aggressive Blotch	-+-+-
7757	*P. tolaasii*	ATCC 51310	Aggressive Blotch	-+-+-
7544	*P. tolaasii*	NCPPB 2192	Aggressive Blotch	-+-+-
7758	*P. tolaasii**	ATCC 51311	Ginger Blotch	-+-+-
7779	*P. tolaasii**	ATCC 51312	Ginger Blotch	-+-+-
7548	*P.gingeri*	NCPPB 3637	Ginger blotch	-+-+-
7549	*P.gingeri*	NCPPB 3636	Ginger blotch	-+-+-
7760	*P. agarici*	CH6	Drippy gill	---+-
7761	*P. agarici*	NCPPB 2289	Drippy gill	-+--+
7762	*P. agarici*	NCPPB 2472	Drippy gill	-+--+
7547	*P. reactans*	NCPPB 3149		-+-+-
7763	*P. syringae*	P.syrin		-+---
7764	*P. putida*	T6/6	Unknown	
7765	*P. putida*	T2/6	Stimulates initiation	-+-+-
7766	*P. putida*	4A lux 1	Stimulates initiation	
7767	*P. putida*	4A lux 2	Stimulates initiation	
7768	*P. putida*	T1/4 1	Stimulates initiation	
7769	*P. putida*	T1/4 2	Stimulates initiation	
7770	*P. putida*	NP34	Stimulates initiation	
7771	*P. putida*	WB1	Unknown	
7772	*P. veronii*	mar-12	Stimulates initiation	++-+-
7773	*P. veronii*	mar-2	Stimulates initiation	++-+-
7774	*P. poae*	n12	Stimulates initiation	-+-+-
7775	*'P. psl'*	NSC4	Stimulates initiation	-+-+-
7776	*'P. psl'*	NSC6	Stimulates initiation	
7777	Unidentified	mar+1	Stimulates initiation	-+-+-
7778	Unidentified	mar+13	Stimulates initiation	-+-+-
Isolates collected from symptomatic mushrooms during the course of the project				
7545	*P. fluorescens*	2007974	Mild blotch	-+-+-
7546	*P. fluorescens*	2007941	Mild blotch	-+-+-
7785	Unidentified		Mild blotch	-+++-
7786	Unidentified		Mild blotch	-+-+-
7787	Unidentified		Mild blotch	-+++-
7788	Unidentified		Mild blotch	-+++-
8015	Unidentified		Mild blotch	-+-+-
8016	Unidentified		Mild blotch	-+-+-
8018	Unidentified		Ginger blotch	-+++-
8020	Unidentified		Mild blotch	-+++-
8021	Unidentified		Mild blotch	-+-+-
8134	Unidentified		Mild blotch	-+++-

*Subsequently re-classified as *Pseudomonas gingeri*

Verification of pathogenicity of Pseudomonas tolaasii isolates on cut mushroom caps.

To verify pathogenicity of selected isolates of *P. tolaasii* (7544, 7756 and 7779), a cut-cap bioassay was conducted based upon the method of Godfrey *et al*., 2001. In brief, white button mushrooms were dissected to produce 1cm^3 pieces of inner cap tissue (excluding the gills and outer skin). A disc of Whatman filter paper was placed into the bottom of a sterile Petri dish (9cm) and damped with 800 µl of sterile distilled water. Four cubes of tissue were placed into each dish well spaced apart. Bacterial cultures were grown on nutrient agar for 24 hours at 28ºC and a suspension of 10^9 cells per ml was prepared in sterile distilled water. Drops (50 µl each) of inoculum were carefully placed onto each tissue cube. The plates were then sealed with Parafilm® (to maintain a humid environment) and left at room temperature (approx 20 °C, 16 hours daylight). Symptom development was visually assessed after 24 and 48 hours and photographed. Negative controls were either uninoculated or inoculated with sterile distilled water. Non-pathogenic isolates of *Pseudomonas syringae* (P7763) and *Pseudomonas putida* (P7771) were also used as inoculum in the same way.

Inoculation of initiating mushroom cultures

To assess the effect of the various *Pseudomonas* spp. on disease incidence, pots were inoculated at the initiation stage by evenly flooding the casing in each pot with 70ml of an aqueous suspension of each bacterial isolate containing 1x10^6 colony-forming units per ml. Controls were flooded with 70 ml sterile distilled water. The pots were then watered daily from above to maintain humidity around the developing mushrooms. Inoculations were performed only in the disease-conducive high humidity chamber. In January 2012, 18 different pseudomonad isolates were inoculated individually into pots, all with a single casing material (McDon). In June 2012, the 4 most aggressive pseudomonad isolates were inoculated individually into replicated pots containing casing materials from three different sources (McDon, Everris or CNC). Disease development was recorded daily after inoculation.

Development and evaluation of a real-time PCR test for detection of Pseudomonas tolaassi

To produce an assay specific for pathogenic strains of *P. tolaasii*, BLAST searches of the NCBI database identified four potential target regions of the tolaasin toxin gene (see **Table 3**). These regions were further analysed by BLAST to check for homology to other bacterial species, in particular other pseudomonads. One of the identified regions (target number 4)

was previously used in the development of a conventional PCR method for specific detection of *P. tolaasii*[15].

Table 3. DNA sequences from the NCBI database identified as tolaasin gene

Target number	Accession number	Size (bp)	Source	Description	Authors
1	AY291584	1072	*Pseudomonas tolaasii* NCPPB 2192	*Pseudomonas tolaasii* tolaasin gene cluster, partial sequence	Godfrey,S.A.C. and Bulman,S.R.
2	AY228241	1042	*Pseudomonas tolaasii* (unspecified)	*Pseudomonas tolaasii* tolaasin gene, partial cds.	Godfrey,S.A.C. and Bulman,S.R.
3	U16024	492	*Pseudomonas tolaasii* (unspecified)	*Pseudomonas tolaasii* tolaasin-resistance gene, partial cds.	Hutchison,M.L. and Johnstone,K.
4	AF291753	2318	*Pseudomonas tolaasii* (unspecified)	*Pseudomonas tolaasii* tolaasin biosynthesis gene sequence.	Lee,H.-I., Jeong,K.-S. and Cha,J.-S.

After analysis of the four identified sequences a TaqMan real-time PCR assay was designed to each using Primer Express v3 (**Table 4**). All primers and probes were synthesized by Eurofins MWG, with the probe reporter/quencher of FAM-BHQ-1.

Table 4. Primer and probe sequences for the four assays designed

Primer/probe name	Accession used for design	Sequence (5' to 3')	Use
Tol-1-F		TGTTGTGCGCCTCGTTTTTA	Forward primer
Tol-1-R	AF291753	AATGCGAGGGTCACTTTGGT	Reverse primer
Tol-1-P		CCGCCGCACAGGCTCAGGA	Probe
Tol-2-F		AGGCCGAAGGGCAAGGT	Forward primer
Tol-2-R	AY291584	TGTCAGCGAGCAGGAGCAT	Reverse primer
Tol-2-P		TGTCGATATCCCCGAGCAACTCGC	Probe
Tol-3-F		CGCCCAGTTCGAAGAAGTG	Forward primer
Tol-3-R	AY228241	CGCAGCTGTGGCAAACG	Reverse primer
Tol-3-P		TCATGACGCCCCACGCGT	Probe
Tol-4-F		AGAACGAAAACCCCGAATACAA	Forward primer
Tol-4-R	U16024	CCCGCCACATACCCTTTG	Reverse primer
Tol-4-P		CGCTCGAAGAAAGAGCGGCGG	Probe

DNA extraction from pure cultures was performed using the QIAGEN QIAamp DNA Mini Kit, and from mushroom cap tissue using the QIAGEN DNeasy Plant Mini Kit, following the manufacturers protocol. DNA was extracted from three healthy mushrooms to be used as negative controls, one button mushroom (*Agaricus bisporus*) and two species of UK native wild mushrooms (species unknown).

Real-time PCR was performed using Applied Biosystems Prism 9700HT instrument, and data were analyzed with sequence detection system V. In all cases, 1 µl of DNA extract (concentration as extracted) was used in 24 µl of master mix, and all samples were tested in

duplicate. Negative controls containing nuclease-free water in the place of DNA were included in all runs. Real-time PCR was conducted using Taq-Man core reagents (Applied Biosystems) consisting of 1 x buffer A (50 mM KCl, 10 mM Tris-HCl, pH 8.3, carboxy-X-rhodamine passive reference dye), 5.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, and 0.625 U Ampli*Taq* Gold. All primers were used at a final concentration of 300 nM and all probes at a final concentration of 100 nM. Universal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles each consisting of 15 s at 95°C and 1 min at 60°C. Results were analyzed in terms of the average cycle threshold (C_T) values (cycle that produces a positive PCR signal as determined by the algorithm built into the software).

Analysis of bacterial populations in casing materials using next generation pyrosequencing.

The value of next generation pyrosequencing was explored as a potential tool to investigate population dynamics of different pseudomonads amongst the total bacterial populations in casing materials from different sources. Casing material was analysed from three sources (CNC from The Netherlands, McDon from Ireland and Scott from the UK) to represent the potential range of diversity. DNA was extracted from 10g of mushroom casing using the PowerMax Soil DNA isolation kit as per the manufacturer's instructions (MoBio, USA). The resulting DNA was then PCR amplified in triplicate using M13 tagged bacterial 16S primers[17] in the presence of a unique M13 tagged multiplex identifier primer[18]. The resulting amplicons were then sequenced on $^1/_8{}^{th}$ of a pico titre plate in a Roche 454 GS-FLX sequencer. The amplicon sequence reads were analysed in the QIIME package[19] after standard quality filtering. The sequences were grouped into operational taxonomic units (OTUs) with a threshold of 97% and taxonomic placement of each OTU was carried out using the Ribosomal Database Project (RDP) classifier. Each OTU is assumed to be approximately equivalent to a different species.

Results

Effect of casing materials and humidity on mushroom yield and numbers

Mushroom yields from pots kept in the lower humidity growing room were consistent between experiments and there were no clear differences in mushroom yield or numbers between casing materials at either humidity (**Table 5**). There were no consistent effects of growing room humidity on either mushroom yield or numbers; these figures were lower at the higher humidity in the June 2011 experiment, but were higher when the humidity was further increased in the January 2012 and June 2012 experiments.

Effect of casing materials on recoverable bacterial populations and blotch incidence/severity in uninoculated mushrooms.

Bacterial blotch was not observed in any of the mushrooms grown in the lower humidity cultivation room, or in the June 2011 experiment at either humidity level. In subsequent experiments, when the humidity of the higher RH room was increased, blotch symptoms occurred on all uninoculated mushrooms except on one occasion (October 2011) with one of the casing samples (**Table 5**). In almost all cases mild blotch symptoms were observed (Figure 1) compared with those induced following inoculations with known *P. tolaasii* isolates (Figure. 2). A single case of ginger blotch was also recorded on one occasion (July 2011) on uninoculated mushrooms growing on one casing source (Figure 3), where the source of inoculum was not identified. There were no consistent differences in blotch incidence between casing materials, with greater differences in blotch occurrence between experiments than within experiments.

Table 5. Mushroom yield, total number and diseased number from different casing materials in different experiments.

Experiment	Casing Material supplier	Yield, g/kg		Number/pot		Diseased/pot	
		Low RH[1]	High RH[2]	Low RH[1]	High RH[2]	Low RH[1]	High RH[2]
June 2011	A	353	321	72	45	0	0
	B	351	327	99	49	0	0
	C	337	277	80	40	0	0
July 2011[3]	A	_	382	_	125	_	22
	B	_	375	_	124	_	10
	C	_	233	_	119	_	12
October 2011	A	350	381	67	57	0	0
	B	379	369	74	61	0	1
January 2012	B	313	451	63	91	0	24
June 2012	C	310	486	49	80	0	7
	B	311	451	53	76	0	8
	D	285	473	62	77	0	2

[1] Low RH: 88% at 18°C.

[2] High RH: 88-92% at 18°C in June 2011; >92% at 18°C in subsequent experiments.

[3] Experiment not performed at low RH in July 2011.

Figure 1. Mild blotch symptoms caused by unknown fluorescent pseudomonad species.

Figure 2. Severe blotch symptoms after inoculation with *Pseudomonas tolaasii*

Figure 3. Ginger blotch symptoms caused by *Pseudomonas gingeri*

Following isolation of recoverable total pseudomonad populations from uninoculated casing materials during the January 2012 experiment, large increases in the populations of total culturable bacteria and pseudomonads were observed between the application of casing and the end of the second flush (x100 and x1000 respectively) (**Table 6**). The percentage of bacteria that were pseudomonads also increased during the mushroom culture. These figures were slightly higher in the high humidity chamber than in the lower humidity room (**Table 6**).

Table 6. Casing population counts of bacteria on LB agar and *Pseudomonas* spp. on pseudomonad isolation agar (cfu x 10^8/g casing) in fresh casing (supplier B) or after the second flush of mushrooms, following cultivation at 88% or >92% relative humidity. Each value is the mean of three replicate samples from uninoculated casing in the January 2012 experiment.

Casing sample	LB agar	PIA	% Pseudomonads
Fresh	1.30×10^6	1.51×10^5	11
2nd flush, 88% RH	2.50×10^8	0.60×10^8	24
2nd flush, >92% RH	4.83×10^8	2.00×10^8	41

Pathogenicity of Pseudomonas tolaasii isolates on cut mushroom caps.

All samples inoculated with the *P. tolaasii* reference strains (P7756 and P7544) developed symptoms of dark brown pigmentation (see **Figures 4** and **5**). However, the *P. gingeri* isolate (P7779) induced a paler discolouration of the tissue. Colouration intensified between 24 and 48 hours and was consistent within the four replicated cubes for each inoculums treatment. The non-blotch inducing pseudomonads (*P.putida* P7771 and *P.syringae* P7763) and the untreated and dummy inoculated controls did not demonstrate any colour2ation over the course of the experiment. Pathogenicity of the *P. tolaasii* strains was confirmed and these isolates were selected for further use in inoculation experiments.

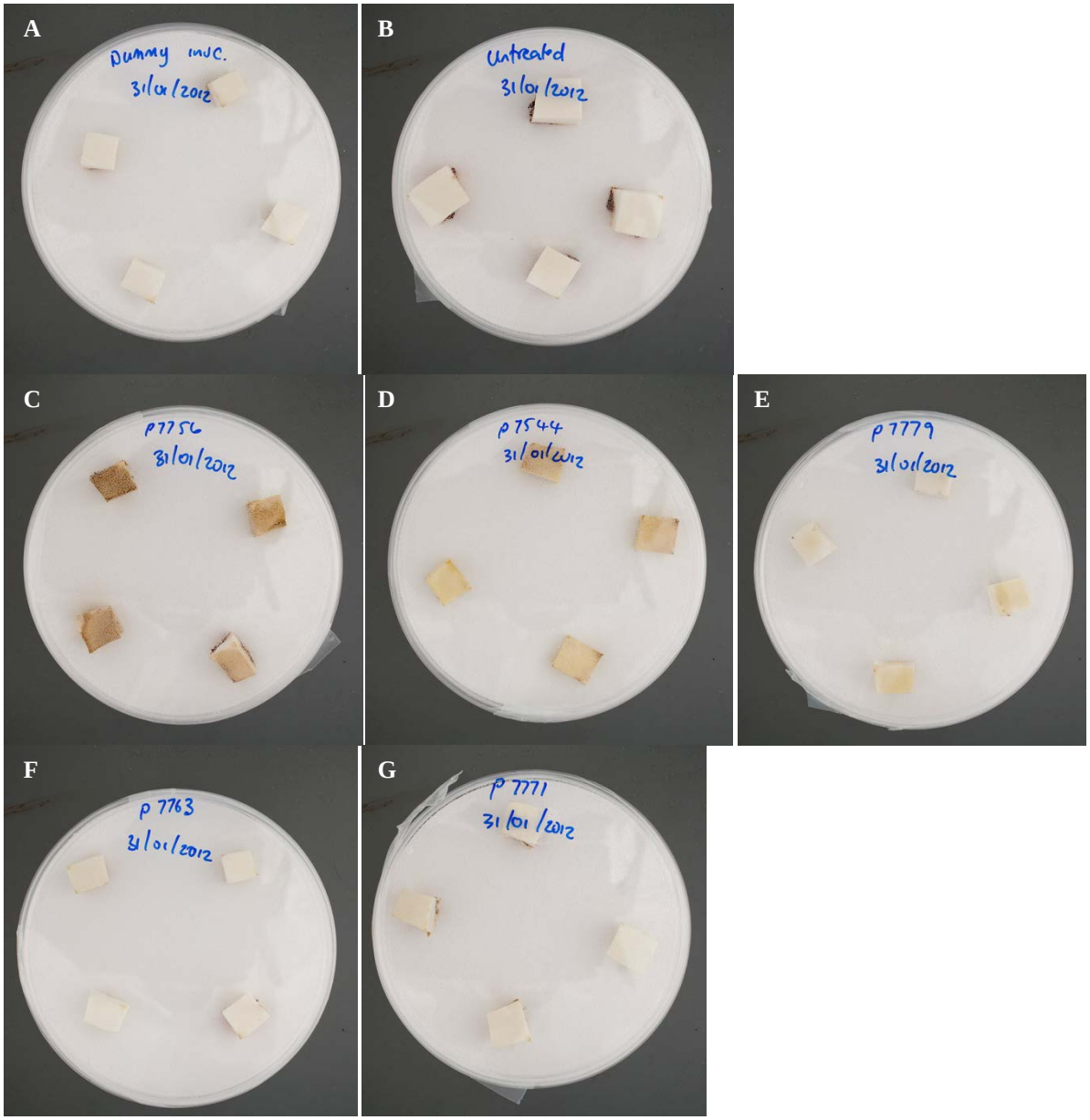

Figure 4. Bioassay pathogenicity samples after 24 hours. A) water inoculated, B) untreated, C) *P. tolaasii* (P7756) D)), *P. tolaasii* (P7544) E), *P. gingeri* (P7779), F) *P. syringae* (P7763) and G) *P. putida* (P7771).

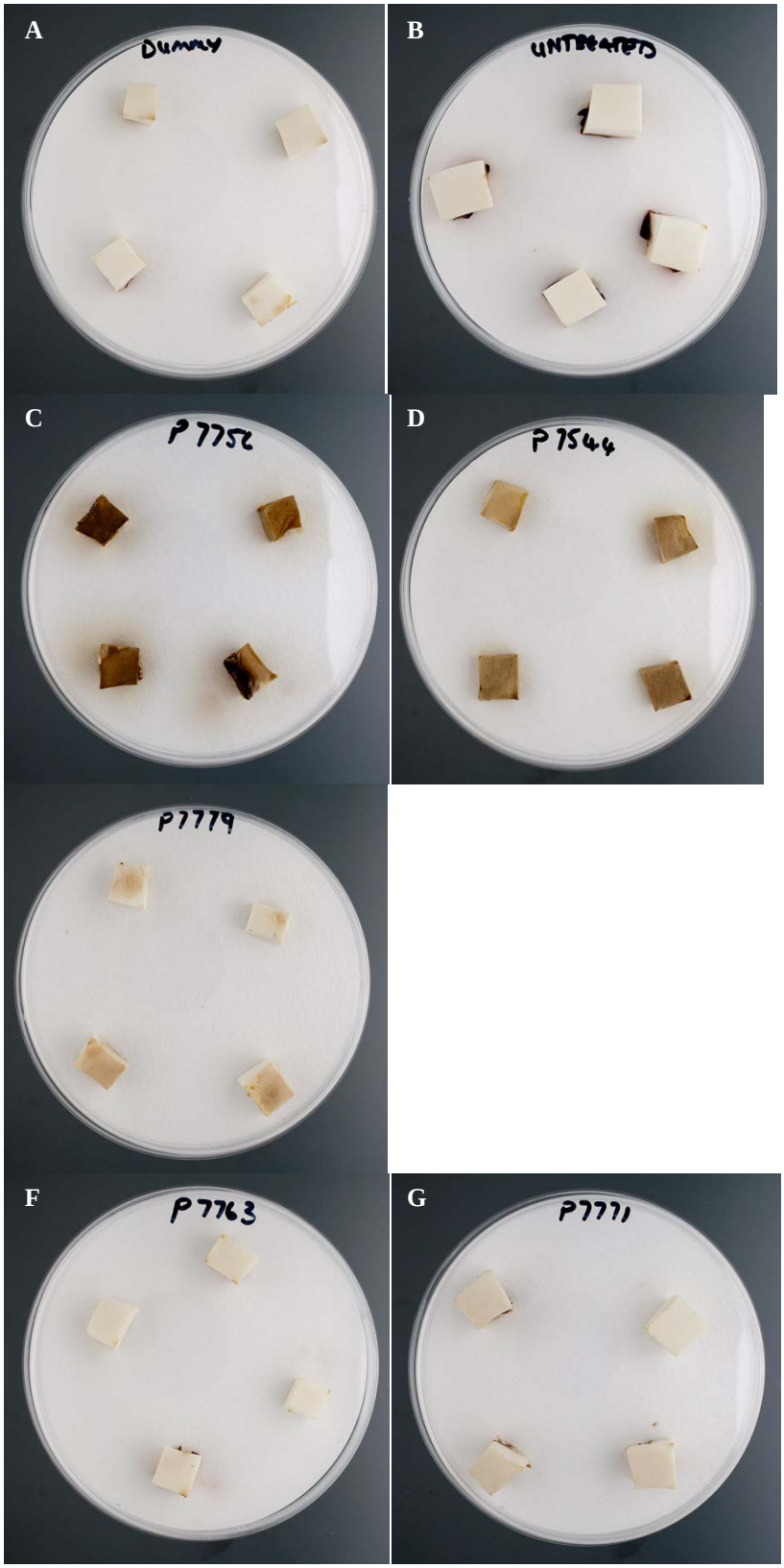

Figure 5. Bioassay pathogenicity samples after 48 hours. A) water inoculated, B) untreated, C) *P. tolaasii* (P7756) D), *P. tolaasii* (P7544) E), *P. gingeri* (P7779), F) *P. syringae* (P7763) and G) *P. putida* (P7771).

Effect of inoculation with selected pseudomonad isolates on mushroom yield and numbers

Data from inoculation treatments with multiple pseudomonad isolates at the high RH (>92%) level in January 2012 are shown in **Table 7**. Since each isolate was inoculated into a single pot, it is not possible to statistically determine whether individual isolates had significant effects on mushroom yield or numbers. However, all except three of the inoculated pots produced mushroom yields that were within 10% of the water control treatment. Furthermore, inoculation of replicated pots with four selected pseudomonads in June 2012 also indicated no apparent effect on mushroom yields or numbers (**Tables 8** and **9**).

Effect of inoculation with selected pseudomonad isolates on casing bacterial population

Inoculation with various pseudomonad isolates in the January 2012 experiment had no apparent effect on total bacterial or pseudomonad populations recovered from the casing material after the second flush (**Table 7**). In fact, the highest bacterial and total pseudomonad populations measured were found in the uninoculated negative control. Inoculation with only one isolate (8015) resulted in a higher percentage of total bacteria that were pseudomonads compared with the uninoculated control.

Effect of inoculation with selected pseudomonad isolates on bacterial blotch

In **Table 7**, all the pots in the high humidity chamber produced blotched mushrooms, whether or not pseudomonad isolates were applied as additional inoculum. Inoculation with pseudomonad isolates P7756, P7757, P7786, P8018 and P8021 resulted in the most blotched mushrooms. In **Table 10**, where four of these isolates were applied to three different casing materials, all the inoculated pots produced more blotched mushrooms than the untreated or negative water treated pots. There were no correlations between the bacterial populations in casing or mushroom yield and the numbers of blotched mushrooms.

Table 7. Casing population counts of bacteria on LB agar and *Pseudomonas* spp. on pseudomonad isolation agar (cfu x 10^8/g casing) after the second flush of mushrooms, mushroom yield (g/kg compost) and numbers of total and diseased mushrooms per pot, with and without inoculation of different pseudomonad isolates (January 2012).

Isolate applied	LB agar	PIA	% *Pseudomonas*	Yield	Number	Diseased
Control (none)	4.83	2.00	41	451	91	24
Control (water)	13.22	3.83	29	413	95	8
P. tolaasii P7544	2.33	0.35	15	371	84	18
P. tolaasii P7756	10.00	1.33	13	376	89	59
P. tolaasii P7757	10.20	2.67	26	435	85	67
P. gingeri P7548	10.00	1.33	13	473	93	19
P. gingeri P7758	6.50	0.83	13	445	95	11
P. agarici P7760	3.50	0.50	14	427	90	29
P. fluorescens P7545	6.16	0.67	11	332	79	13
P. putida P7771	6.17	1.67	27	372	75	9
Pseudomonas sp. P7785	0.88	0.27	31	438	90	27
Pseudomonas sp. P7786	5.50	1.33	24	425	97	54
Pseudomonas sp. P7787	5.00	0.92	18	380	84	7
Pseudomonas sp. P7788	5.17	1.83	35	424	102	21
Pseudomonas sp. P8015	3.83	2.17	57	372	74	12
Pseudomonas sp. P8016	5.67	0.58	10	343	84	11
Pseudomonas sp. P8018	4.50	0.63	14	406	100	30
Pseudomonas sp. P8020	5.67	0.68	12	454	96	20
Pseudomonas sp. P8021	6.00	0.65	11	378	90	55
Pseudomonas sp. P8134	2.83	0.43	15	427	84	12
Mean of isolates	5.55	1.05	20	406	88	26

Table 8. Yield of mushrooms (g/kg) from pots cased with different peat casings and inoculated with different *Pseudomonas* spp. isolates. Each value is the mean of two replicate pots (June 2012).

Isolate applied	Casing C	Casing D	Casing B
Control (none)	520	473	451
Control (water)	515	444	461
P. tolaasii P7756	471	483	438
P. tolaasii P7757	500	480	456
Pseudomonas sp. P7786	500	463	476
Pseudomonas sp. P8018	508	475	452

Table 9. Total number of mushrooms from pots cased with different peat casings and inoculated with different *Pseudomonas* spp. isolates. Each value is the mean of two replicate pots (June 2012).

Isolate applied	Casing C	Casing D	Casing B
Control (none)	80	77	76
Control (water)	82	65	72
P. tolaasii P7756	74	90	88
P. tolaasii P7757	88	88	80
Pseudomonas sp. P7786	75	85	62
Pseudomonas sp. P8018	84	82	66

Table 10. Number of diseased mushrooms from pots cased with different peat casings and inoculated with different *Pseudomonas* spp. isolates. Each value is the mean of two replicate pots (June 2012).

Isolate applied	Casing C	Casing D	Casing B
Control (none)	8	2	7
Control (water)	6	6	6
P. tolaasii P7756	65	79	80
P. tolaasii P7757	78	73	68
Pseudomonas sp. P7786	25	25	25
Pseudomonas sp. P8018	52	62	38

Development and evaluation of a real-time PCR test for detection of Pseudomonas tolaassi

Initial assessment of the four assays was performed on a small panel of reference isolates representing the range of pseudomonad species commonly found associated with mushroom disease. Samples of healthy mushrooms, healthy plant and *Pseudomonas syringae* were used as negative controls (see **Table 11** and **Figure 6**). Three assays (Tol-1, Tol-2 and Tol-3) reacted in a very similar manner, with the *P. tolaasii* reference isolate giving a strong positive signal with early DNA target amplification detected (denoted by C_T values of 19-20). The other five reference isolates from three different species tested negative. However for Tol-1 two isolates (*P. fluorescens* P7545 and *P. gingeri* P7548) gave unexpected slight false positive results characterized by very late detection of target DNA with C_T values of >39. Assay Tol-4 failed to show any amplification.

Table 11. Real-time PCR results from preliminary testing of the four assays, figures in red show amplification. All C_T values are the average from duplicate wells.

Sample	**Fera Protect Number**	**Average C_T value**			
		Tol-1	**Tol-2**	**Tol-3**	**Tol-4**
P. tolaasii	7544	19.7	20.9	20.0	40.0
P. fluorescens	7545	39.4	40.0	40.0	40.0
P. fluorescens	7546	40.0	40.0	40.0	40.0
P. reactans	7547	40.0	40.0	40.0	40.0
P. gingeri	7548	39.2	40.0	40.0	40.0
P. gingeri	7549	40.0	40.0	40.0	40.0
Healthy mushroom A		40.0	40.0	40.0	40.0
Healthy mushroom B		40.0	40.0	40.0	40.0
Healthy mushroom C		40.0	40.0	40.0	40.0
Infected mushroom A (*P. tolaasii* P7756)		20.3	22.6	NT	NT
Infected mushroom B (*P. tolaasii* P7756)		19.1	22.2	NT	NT
Infected mushroom C (*P. tolaasii* P7756)		17.9	19.1	NT	NT
Infected mushroom D (*P. tolaasii* P7757)		19.8	23.4	NT	NT
Infected mushroom E (*P. tolaasii* P7757)		16.9	19.3	NT	NT
Infected mushroom F (*P. tolaasii* P7757)		20.6	24.5	NT	NT
Healthy plant tissue (*Catharanthus* sp.)		40.0	40.0	40.0	40.0
Pseudomonas syringae		40.0	40.0	40.0	40.0
Negative control (water)		40.0	40.0	40.0	40.0

NT= Not tested

Symptomatic mushrooms infected with *P. tolaasii* during the June 2012 experiment all tested positive with assays Tol-1 and Tol-2 with early amplification of the target DNA indicated by low CT values (16.9-24.5). None of the assays cross-reacted with three samples of healthy mushrooms, healthy plant DNA or *Pseudomonas syringae* (no amplification detected, designated as C_T=40).

All of the assays were further assessed for specificity against the full panel of isolates available, including *P. tolaasii* and other psuedomonads pathogenic or stimulatory to mushrooms (see **Table 12**). Assay Tol-4 again failed to show any amplification with any samples, and was not used further. Assays Tol-1, Tol-2 and Tol-3 all appeared to have very similar characteristics. Three out of the five *P. tolaasii* reference isolates were strongly detected by all three assays. Further investigation of the two isolates which tested negative (ATCC51311 and 51312) revealed that these had now both been re-classified as *P. gingeri*. All available reference isolates of *P. tolaasii* were therefore detected by the three assays.

A number of cross-reactions with several with non-*tolaasii* isolates of other *Pseudomonas* species were observed as late detection of target DNA. These were characterized by high C_T values >34.5 for Tol-1 and >37.5 for Tol-2 and Tol-3 for at least one replicate of each isolate tested. This kind of very late amplification with high C_T is not uncommon when using very concentrated DNA generated from pure cultures and may result from partial homology of the fluorescent probe used in the real time PCR reaction to the very high levels of DNA in the sample. False positive results would not be expected when testing for lower levels of the bacteria typically present in environmental samples. Assay Tol-1 appeared approximately 10-fold more sensitive that Tol-2 and Tol-3, and a correspondingly higher number of cross-reacting results were observed with this assay. Assays Tol-2 and Tol-3 appeared to have similar sensitivity.

The Tol-1 assay was also used to test all isolates obtained from mushrooms observed with mild blotch symptoms which developed at high humidity in the non-inoculated pot experiments described above. None of the isolates obtained tested positive for *P. tolaasii* with this assay, irrespective of the commercial casing material used. One of these isolates (P8018) had been found to induce symptoms of ginger blotch after inoculation onto healthy mushrooms.

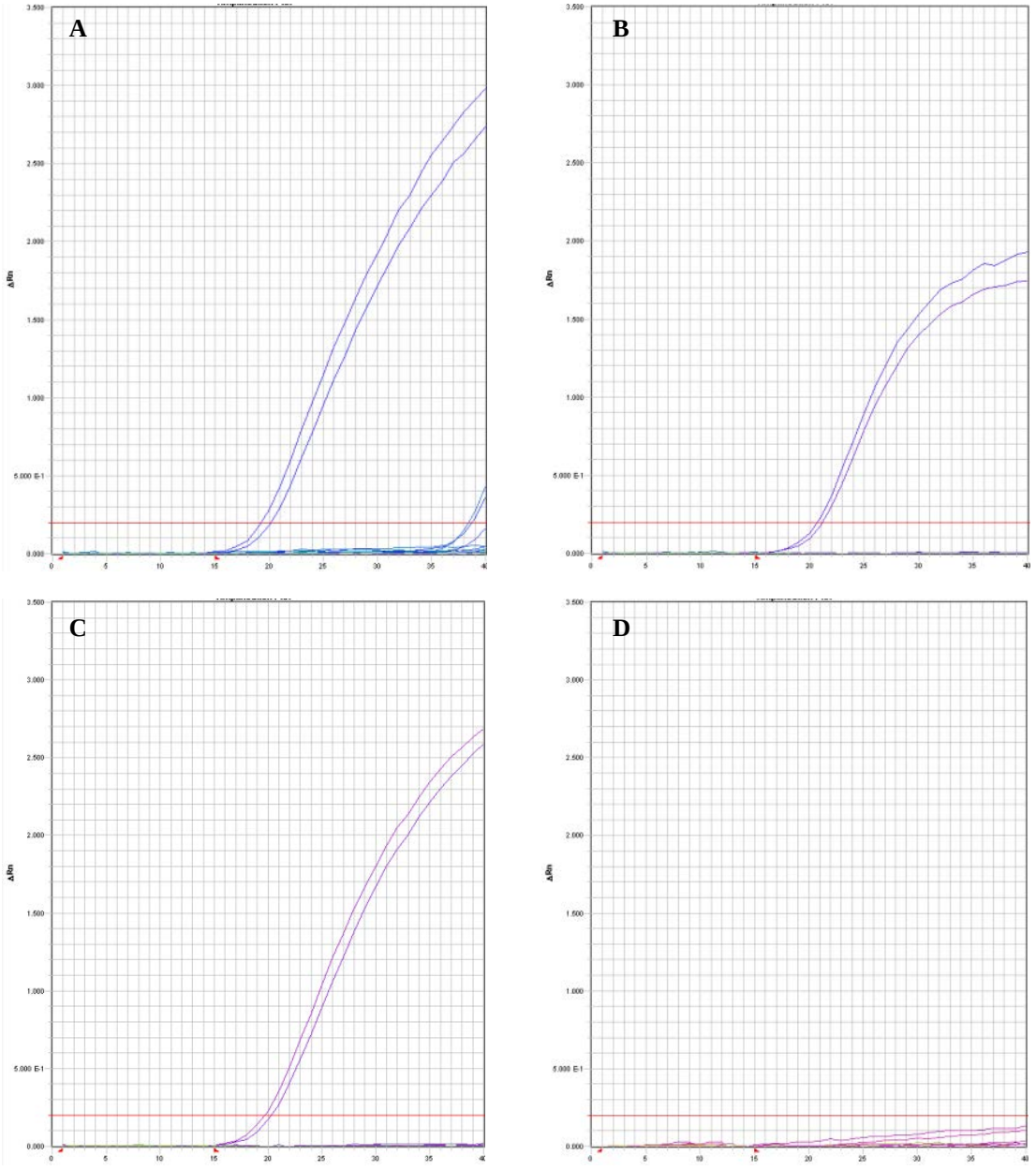

Figure 6: Amplification profiles of the four TaqMan assays with the initial panel of samples. A) Tol-1, B) Tol-2, C) Tol-3 and D) Tol-4.

Table 12: Full specificity testing of all four assays, with *P. tolaasii* isolates shaded in grey, genuine positive samples in red and cross-reacting samples in blue. All C_T values are the average from duplicate tests.

Fera #	Identification	Isolate	Effect	Tol-1	Tol-2	Tol-3	Tol-4
P7544	*P.tolaasii*	NCPPB2192	Unknown	18.9	21.7	21.6	40.0
P7756	*P.tolaasii*	ATCC 51309	Blotch	20.6	22.9	23.0	40.0
P7757	*P.tolaasii*	ATCC 51310	Blotch	16.6	19.3	19.3	40.0
P7758	*P.tolaasii**	ATCC 51311	Ginger Blotch	37.1	40.0	38.9	40.0
P7779	*P.tolaasii**	ATCC 51312	Ginger Blotch	38.8	40.0	37.5	40.0
P7545	*P. fluorescens*	Diagnostic sample	Unknown	39.0	40.0	40.0	40.0
P7546	*P. fluorescens*	Diagnostic sample	Unknown	40.0	40.0	40.0	40.0
P7547	*P. reactans*	NCPPB3149	Unknown	40.0	40.0	40.0	40.0
P7759	*P.reactans*	ATCC 14340	Blotch	38.8	40.0	39.1	40.0
P7548	*P. gingeri*	NCPPB3637	Ginger Blotch	40.0	40.0	40.0	40.0
P7549	*P. gingeri*	NCPPB3636	Ginger Blotch	39.5	40.0	40.0	40.0
P7760	*P.agarici*	CH6	Drippy gill	40.0	40.0	40.0	40.0
P7761	*P.agarici*	NCPPB 2289	Drippy gill	35.9	39.6	38.2	40.0
P7762	*P.agarici*	NCPPB 2472	Drippy gill	39.1	40.0	39.1	40.0
P7763	*P.syringae*	Ex-Warwick	Non-pathogenic	38.6	39.0	39.6	40.0
P7764	*P.putida*	T6/6	Unknown	40.0	40.0	40.0	40.0
P7765	*P.putida*	T2/6	Stimulates initiation	38.6	40.0	40.0	40.0
P7766	*P.putida*	4A lux 1	Stimulates initiation	38.5	39.4	39.4	40.0
P7767	*P.putida*	4A lux 2	Stimulates initiation	37.3	38.5	40.0	40.0
P7768	*P.putida*	T1/4 1	Stimulates initiation	40.0	40.0	40.0	40.0
P7769	*P.putida*	T1/4 2	Stimulates initiation	37.9	37.4	38.7	40.0
P7770	*P.putida*	NP34	Stimulates initiation	35.3	38.7	38.0	40.0
P7771	*P.putida*	WB1	Unknown	34.8	36.6	38.2	40.0
P7772	*P.veronii*	mar-12	Stimulates initiation	40.0	39.1	40.0	40.0
P7773	*P.veronii*	mar-2	Stimulates initiation	37.4	35.6	35.9	40.0
P7774	*P.poae*	n12	Stimulates initiation	40.0	40.0	39.6	40.0
P7775	*P.psl*	NSC4	Stimulates initiation	34.7	37.1	36.3	40.0
P7776	*P.psl*	NSC6	Stimulates initiation	37.9	40.0	37.8	40.0
P7777	*Unidentified*	mar+1	Stimulates initiation	37.2	40.0	38.7	40.0
P7778	*Unidentified*	mar+13	Stimulates initiation	40.0	40.0	40.0	40.0

*Subsequently re-classified as *Pseudomonas gingeri*

Bacterial populations in casing materials analysed by next generation pyrosequencing.

After quality filtering, 30,001 sequence reads were clustered into 2987 operational taxonomic units (OTUs), representing different taxa of bacteria. Of these only around 10.8-18.1% were associated with bacteria which have been taxonomically classified with a recognized name. OTUs taxonomically placed as having originated from the order *Pseudomonadales* comprised only 0.045% of the amplicon sequences produced in total.

The most abundant taxa present in the data are shown in **Table 13**. **Figure 7** shows the relative abundance of each taxon identified in the casing samples as amplified by three technical PCR replicates and **Figure 8** the cumulative analysis for each sample.

Table 13: Breakdown of most prevalent OTUs in each of the casing samples after pyrosequencing

OTU taxonomic placement	Casing sample		
	Casing D	Casing B	Casing E
Bacteria; Bacteroidetes;	18.1%	19.0%	10.8%
Bacteria;	10.5%	5.7%	6.7%
Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;	10.4%	2.3%	9.2%
Bacteria; Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae;	3.6%	7.7%	8.7%
Bacteria; Proteobacteria; betaproteobacteria; Burkholderialse; Comamonadaceae; Curvibacter	4.4%	7.9%	3.2%
Bactera; Proteobacteria;	5.0%	3.1%	3.0%
Bacteria; Proteobacteria; Betaproteobacteria;	2.4%	1.8%	6.0%

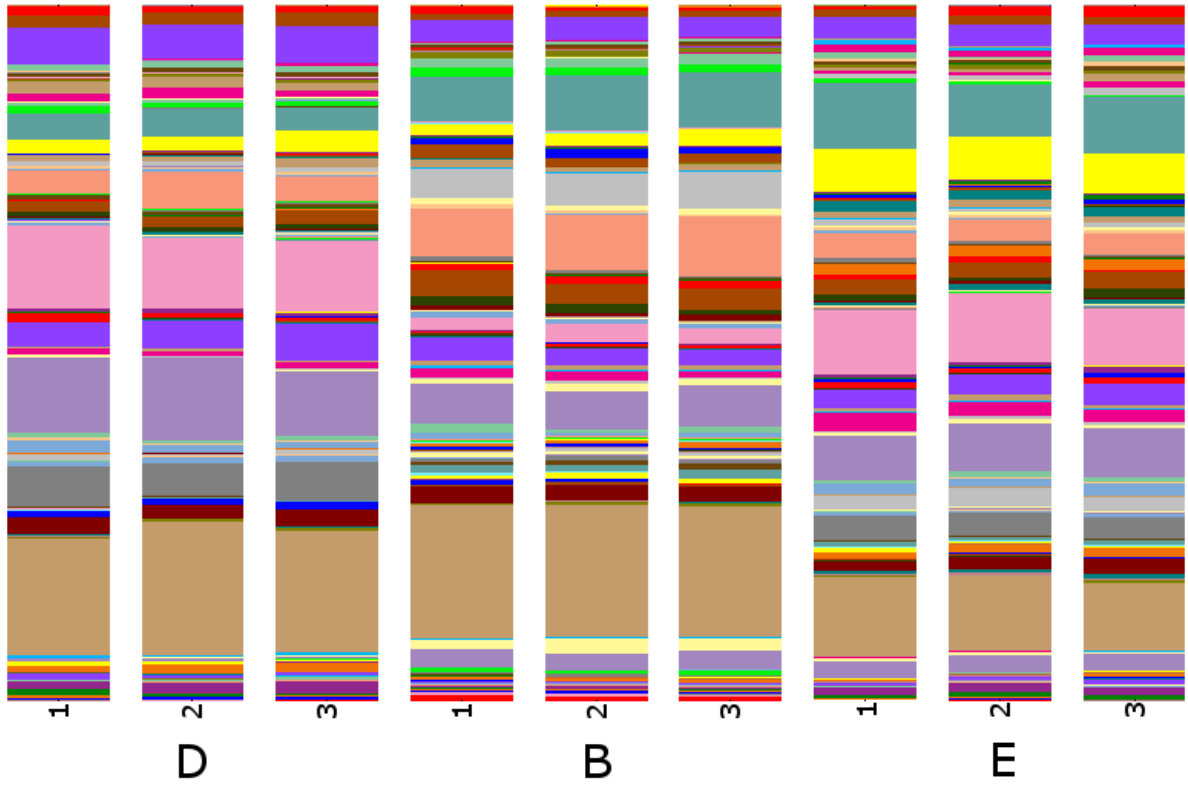

Figure 7. Stacked bar charts diagrammatically reflecting the relative abundances of 2987 bacterial OTUs present in casing samples from different sources (D, B and E) after taxonomic placement. Data from 3 PCR and sequencing replicates shown for each casing source. The full range of OTUs is too numerous to list in a key but the most prevalent are shown in **Table 13**.

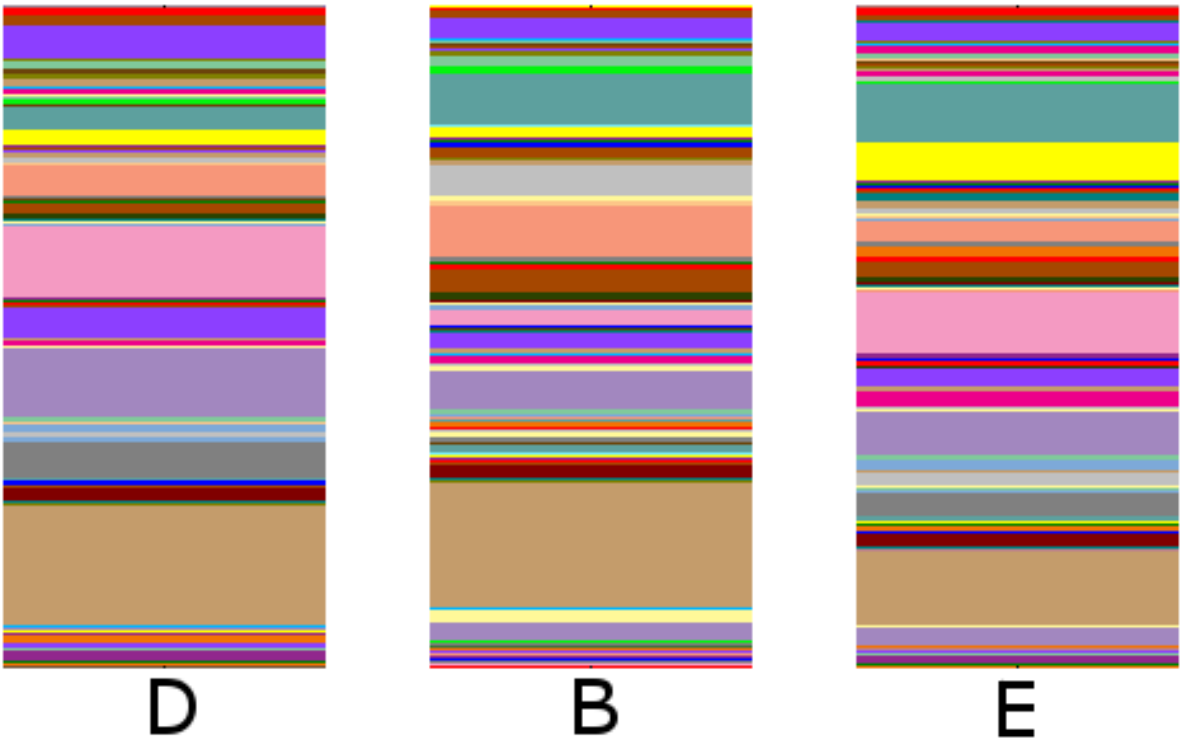

Figure 8: Stacked bar charts diagrammatically reflecting the relative abundances of 2987 bacterial OTUs present in casing samples from different sources (D, B and E) after taxonomic placement. The full range of OTUs is too numerous to list in a key but the most prevalent are shown in **Table 13**.

Three OTUs were originally classified by the RDP as pseudomonads. After further sequence similarity searches using BLAST, two of these OTUs had their classification refined as *Acinetobacter* spp. and *Cellvibrio* spp.. The remaining OTU had closest homology to *Pseudomonas luteola*, however with only 96% identity this is unlikely to be a species-level taxonomic placement and likely represents a new species which has yet to be sequenced conclusively and submitted to Genbank.

Discussion

The findings of this study confirm that bacterial blotch disease can be caused by a variety of *Pseudomonas* species. Although the most severe symptoms observed were associated with *P. tolaasii* and *P. gingeri,* high incidences of milder blotch symptoms were also associated with presence of other fluorescent *Pseudomonas* spp. Further characterization of these bacteria should help to distinguish those able to cause blotch from closely related *Pseudomonas* species which were also found to be abundant in the casing materials during mushroom cultivation.

Although blotch incidence itself did not necessarily reduce yield or number of mushrooms, even mild symptoms can contribute significantly to reduced quality and marketability. Blotch symptoms were only induced when humidity was maximized, emphasizing the importance of environmental control in disease avoidance. At the highest humidity regimes (>92% at 18°C), both number and yield of mushrooms were higher than in the low humidity (88% at 18°C), indicating blotch disease to be a potential limiting factor to increasing yield.

Blotch incidence did not appear to be influenced by the source of different commercially available casing materials and no evidence was found in this study that *P. tolaasii* was introduced with any of the casing materials or compost used in the experiments described. It was not determined whether *P. gingeri* or other *Pseudomonas* species associated with blotched mushrooms observed under the highly humid experimental conditions had originated from the compost or casing materials or from the surrounding environment.

New real-time PCR assays were developed which detected and identified *P. tolaasii* in infected but not in healthy mushrooms. Despite some low level cross-reaction observed with related bacteria, the assays demonstrated sufficient specificity and sensitivity to be useful as screening tests to indicate presence of *P. tolaasii* during production. The assays were successfully used to demonstrate that blotch induced at high humidity in a number of experimental conditions with varying commercially available casing materials was all caused by fluorescent pseudomonads other than *P. tolaasii*. Such screening tests may help to optimize production conditions for increased yield whilst simultaneously indicating the risk of increased disease. Since the new assays only detect *P. tolaasii*, further assay development will be required for detection of the other blotch-causing bacteria, such as *P. gingeri* and other fluorescent pseudomonads.

Methods were also optimized for purification of bacterial DNA from casing materials, thus enabling profiling of bacterial populations using next generation pyrosequencing methods. In this first attempt to assess the usefulness of this technology, generic bacterial primers were used for the first time to investigate the total bacterial population structure in commercially available casing materials. The results indicated high levels of similarity in the population structures within and to a lesser extent between casing samples from different sources. Unfortunately, the method used was not sufficiently sensitive to permit detailed analysis of fluorescent *Pseudomonas* genotypes within the total bacterial population in each casing type. Such an approach will require further analysis using primer sets specific for these bacteria once they have all been fully characterized.

Conclusions

- Bacterial blotch can be caused by a variety of fluorescent *Pseudomonas* species, not all of which have been fully characterized.
- The disease was completely controlled by limiting the humidity at 88% during production at 18°C.
- *Pseudomonas tolaasii* and *Pseudomonas gingeri* can cause severe blotch symptoms under high humidity (>92% at 18°C) but a number of other fluorescent *Pseudomonas* species can also induce mild blotch symptoms under these conditions which equally reduce quality and marketability.
- Mushroom yield and potential for bacterial blotch development were apparently not affected by the source of commercial casing material used.
- A new real-time PCR diagnostic test was developed which can be used to screen for *P. tolaasii* but further assay development will be needed to detect the other blotch-causing pesudomonads.
- The test was used to show that blotch symptoms developing under experimental conditions using common commercial casing sources was not caused by *P. tolaasii*.
- Whole bacterial population structures of mushroom casing were analysed using next generation pyrosequencing methods but study of the variation in *Pseudomonas* genotypes present within the total bacterial populations will require further characterization of the blotch-causing psedomonads so that more specific primer sets can be developed.

Knowledge and Technology Transfer

- Results have been shared at HDC mushroom panel meetings throughout the course of the project.
- The details of the real-time PCR assays will be made available through this final report and are intended for submission for publication in a refereed scientific journal.
- Use of the assays as required in commercial diagnostic services is also intended through the Fera Plant Clinic.

Glossary

ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
C_T	Critical Threshold (PCR cycle at which target amplification is first detected)
EMR	East Malling Research
Fera	Food and Environment Research Agency
NCPPB	National Collection of Plant Pathogenic Bacteria
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
RDP	Ribosomal Database Project

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