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Casing soil microbiome mediates suppression of bacterial blotch of mushrooms during consecutive cultivation cycles

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ABSTRACT

Shifts in the soil microbiome during continuous monoculture cropping coincide with increased suppressiveness against soil-borne diseases, as in the take-all decline of wheat. Here we report a similar phenomenon for bacterial blotch of mushrooms, caused by *Pseudomonas 'gingeri'*, where ginger blotch incidence decreases during consecutive cycles of mushroom cultivation. We explored the infection dynamics of blotch during consecutive cultivation cycles for different casing soil mixtures. We also observed the population dynamics of the pathogen in these casing soils. In addition, the composition of the casing soil microbiome was compared between blotch suppressive and conducive soils. Finally, we studied the transferability of blotch suppressiveness. A consistent decline of bacterial blotch was observed for two consecutive cultivation cycles of mushroom cropping, across ten casing soil mixtures composed of different peat sources and supplements. Blotch suppression occurred without reduction of pathogen populations in the casing soils. Aqueous extracts made from suppressive soils were able to reduce blotch incidence in conducive casing soils, indicating that blotch suppression is transferrable and microbially mediated. Changes in the microbial community composition of the casing soils reflected pathogen invasion, pathogen establishment and disease suppression, in addition to the expected temporal changes across the cultivation cycles. Specific bacterial genera were associated with soil suppressiveness to bacterial blotch, such as, *Pseudomonas* sp., *Dyadobacter* sp., *Pedobacter* sp., and *Flavobacterium* sp. We suggest that the suppression of bacterial blotch is induced due to high pathogen populations in the first cultivation cycle, and mediated by inhibition of virulence factors such as those controlled by quorum sensing in the later cultivation cycles.

1. Introduction

Management of soil-borne diseases via suppressive soils remains a promising yet significant challenge in intensive cropping systems (Gómez Expósito et al., 2017; Stirling et al., 2016). Studies from diverse soils have attributed disease suppression to the composition and the activity of the soil microbial community (Benítez and Gardener, 2009; Penton et al., 2014; Poudel et al., 2016). Changes in the soil microbiome during consecutive growth of crop monocultures that are susceptible to the disease, or inoculation of high pathogen populations into the soil are known to induce soil suppression of fungal pathogens (Hornby, 1983; Pervaiz et al., 2020; Weller et al., 2002). Best-known examples of such induced specific suppression are take-all decline in continuous monocultures of cereals (wheat, barley) and of root rot decline in sugar beet monocultures (Raaijmakers and Mazzola, 2016; Weller et al., 2002). With current developments in -omic technologies, it is now increasingly possible to characterize the microbial composition, networks and activity of disease suppressive soils (De Corato, 2020a; Klein et al., 2013; Mendes et al., 2011). An ideal sector for improving our understanding of disease suppressive soils is controlled horticulture, which are closed cropping systems, independent of agricultural land and where the growth conditions are constantly regulated (Kulak et al., 2013; Marcelis et al., 2007).

Button mushroom, *Agaricus bisporus*, is grown in climate-controlled indoor farms, on a bed of pasteurized compost and a layer of peat-based casing soil, under standardized cultivation conditions. Bacterial blotch is one of its most important soil-borne diseases, economically and globally (Soler-Rivas et al., 1999). Caused by pathogenic *Pseudomonas*

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species prevalent in mushroom beds (Fletcher et al., 1989), the disease is responsible for up to 50% yield loss during cultivation, and reduced shelf life post-harvest (Olivier et al., 1978; Wells et al., 1996). Despite being studied for over a century, only few management strategies exist for the control of blotch in button mushrooms (Elphinstone and Noble, 2018; Tolaas, 1915). Management via regulation of environmental conditions in mushroom farms has been widely explored, although the results are contrasting between studies (Beyer, 2005; Nair and Bradley, 1980; Navarro et al., 2018; Sinden, 1971; Wong and Preece, 1982).

Biological control of bacterial blotch has been explored via nutrient competition, active antagonism, pathogen inactivation, predation, bacteriocin production and phage therapy (Osdaghi et al., 2019). Despite many successful reports under laboratory conditions, no commercial biocontrol products are being marketed. Blotch management is made additionally challenging due to the fact that the genus *Pseudomonas* is both involved in disease expression (Fletcher et al., 1989; Godfrey, 2003) and in formation of the mushroom pinheads (Rainey, 1989). In mushroom beds, fluorescent pseudomonads represent 10% of the culturable cells, which was found to be 10^7-10^8 cells g^{-1} of compost. Generally pseudomonads increase up to 50%–90% of the total of 10^9 culturable cells during the harvest cycles (Samson et al., 1987). Recently, development of soil suppressiveness to bacterial blotch was reported between consecutive cultivation cycles in mushroom cropping systems. Preliminary findings indicate that the disease continues to decline during consecutive harvest cycles even though pathogen populations do not (Taparia et al., 2020a).

Studies on disease suppression of soil-borne plant pathogens have indicated that it is important to explore the dynamics of the soil microbiome, and better understand the role of the microbiome in the pathogen-soil-host nexus (Teixeira et al., 2019). The aim of this study was to increase our knowledge on blotch suppressive soils for possible use in disease management strategies. In this research, we explored soil suppression of blotch over consecutive harvest cycles in different casing soil mixtures. We compared the bacterial and fungal microbiome of conducive and suppressive soils. We also recorded pathogen population dynamics across the cultivation cycles and pathogen inoculum thresholds in the soil for blotch outbreaks. Finally, we performed soil extract transfers from disease suppressive soils into conducive soils, to study the transferability of blotch suppressiveness.

2. Materials and methods

2.1. General set-up of cultivation bioassays

Bacterial blotch suppressiveness in mushroom casing soils was studied in an experimental mushroom cultivation facility (Unifarm, Wageningen University & Research) under conditions that resemble that of conventional mushroom farms. Two different bioassays (Bioassay 1 and 2) were performed, the generic cultivation setup, growing media and growth conditions of which are described in Taparia et al. (2020a). Weight of healthy and diseased mushrooms harvested were registered across three cultivation cycles, called flushes. Disease incidence (%) was calculated as the proportion of diseased harvest weight to the total harvest weight. Mushroom with symptoms unrelated to that of ginger blotch, were categorized as healthlaboy. Disease severity (%) was calculated (Tajalipour et al., 2014) according to the intensity of disease symptoms on the cap surface into four previously defined categories: healthy, mild, moderate and severe symptoms (Supplementary Figure 1) (Geels, 1995).

In bioassay 1, ginger blotch suppression was explored over a range of inoculum densities for *'P. gingeri'*, in different casing soil mixtures, across two cultivation cycles (flushes) and in three independent repetitions of the experiment (Supplementary Table 1). Ten types of casing soil were composed based on peat from three geographic sources, *Friedrichsveen*, *Holriede*, and *Wiesmoor*, at two depths, surface peat and deep-dug peat. They were supplemented with baltic peat, garden peat and sugar beet lime during preparation of casing soil (Supplementary Figure 2). Each experiment comprised of four replicates per treatment. Between the repetition experiments the raw materials were stockpiled and stored at 4 °C, up to a period of 12 months. For bioassay 1, a pathogenic isolate of *'Pseudomonas gingeri'*, IPO3777, was inoculated on the surface of each casing soil at densities of 10^3, 10^4, 10^5 cfu g^{-1} of soil, according to protocol described in Taparia et al. (2020a). Controls were inoculated with tap water instead of pathogen suspension.

Bioassay 2 was performed to examine if soil suppressiveness to ginger blotch could be transferred to fresh casing soil (Supplementary Table 2). 'Suppressive soils' were identified as those having less than 5% disease incidence at the highest inoculated pathogen density, i.e., casing soil from the second and third harvest cycle (Gómez Expósito et al., 2017). Aqueous extract was made from mixed samples of 'suppressive soils' from bioassay 1. It was inoculated with and without *'P. gingeri'* in fresh casing soil. Bioassay 2 contained four treatments (i) water, (ii) soil extract (iii) pathogen and (iv) pathogen + soil extract. For bioassay 2, 100g of suppressive soils of the 2nd flush of bioassay 1 were sampled and suspended in 1L of sterile 'Ringers solution'. The soil suspension was filtered through cheese-cloth first and then sequentially vacuum-filtered through 270 mm and 80 mm membranes. 200 ml of filtrate was used to inoculate the surface of fresh casing soil (2.5 L), together with 10^6 cfu g^{-1} of *'P. gingeri'* suspension. After inoculation, the remaining soil extract was serially diluted and plated on KingsB media (King et al., 1954), to quantify the fluorescent *Pseudomonads* population.

2.2. Soil sampling & DNA extraction

1 g of casing soil was sampled with a spatula, from one spot per box, at the surface of the casing soil during "cac-ing" (T0), pinhead formation in 1st flush (T1), pinhead formation in 2nd flush (T2) and pinhead formation in 3rd flush (T3). Mycelium adhering to mushrooms was avoided during soil sampling. 250 mg of the homogenized soil was used for DNA extraction using a Soil PowerMag DNA Extraction kit (Qiagen, Germany) according to manufacturer's protocol. A suspension of 10^5 cfu of *Xanthomonas campestris* pv. *campestris* was added to each soil sample as an extraction control. The soil DNA was quantified fluorometrically using a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol on the Infinite M200 PRO (Tecan, Switzerland) and then diluted to a concentration of 2 ng/µl.

2.3. Quantification of pathogen populations

Detection of bacterial DNA in soil samples was performed using the TaqMan™ probe technology, on a QuantStudio™ 12K Flex from Applied Biosystems (Thermo Fisher Scientific, USA). Previously developed qPCR assays Pg2 and Pg6 were used to detect *'P. gingeri'* and assay Xcc was used to detect the extraction and amplification control, *X. campestris* (Taparia et al., 2020b). The sequence of the primers and probes are described in Taparia et al. (2020b). qPCR reactions were performed with 2 ng of soil DNA, according to previously reported protocol and reaction conditions (Taparia et al., 2020b). Pathogen populations in the casing soil were quantified relative to known densities of *X. campestris*, as described in Taparia et al. (2020b).

2.4. In-vitro pathogenicity cap test

Two strains of *'P. gingeri'*, IPO3777 and LMG5327, referred to as strain A and B respectively, were cultured in KingsB medium at 25 °C for 24 h. Similarly sized caps(~4 cm in diameter) of healthy mushrooms harvested from the first and second flush were placed on damp filter paper and inoculated with 20 µl of aqueous *'P. gingeri'* suspension of 10^6 cfu per ml, and tested in replicates of three. The mushrooms were incubated under high humidity conditions for 72 h at 20 °C. The development of blotch symptoms on the cap surface was observed visually and photographed (Supplementary Figure 3). The mushroom

caps were scored, between 0 and 3, with the ascending numbers referring to non-pathogenic (healthy), mild, moderate, and severe symptoms for bacterial blotch. Negative controls consisted of uninoculated mushroom caps and sterile water inoculated mushroom caps.

2.5. Sequencing of the casing soil microbiome

The microbiome was determined for 42 casing soil samples, comprising of three replicates each. Microbial community composition of the casing soil was observed from targeted sequencing of the V3–V4 regions of bacterial 16S rRNA gene and the fungal ITS2 gene region (Supplementary Table 3). Target regions were amplified via PCR using gene-specific primers (10 μM), Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs, USA) (1x), reaction buffer (1x), dNTP (5 mM), 6 ng of template DNA and nuclease-free water up to 30 μl. Reactions conditions for 16S and ITS PCRs included a hot start of 98 °C for 30s, followed by 20 or 25 cycles respectively, of 98 °C for 10s, 55 °C for 30s, 72 °C for 30s and a final extension at 72 °C for 2 min, on a Veriti Thermo Cycler from Applied Biosystems (Thermo Fisher Scientific, USA). PCRs were performed with randomized plate design in replicates of three. Replicate PCR products were pooled and purified with AMPure XP beads (Beckman Coulter, USA). Library construction was performed with Illumina Nextera XT Index kit (Illumina, USA) and KAPA HiFi Hotstart ReadyMix (Roche, Switzerland). The quality and quantity of the libraries were evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The libraries for 16S and ITS2 respectively, were sequenced on Illumina MiSeq using MiSeq v2 or v3 reagents (Illumina, USA) to generate 250 or 300bp paired-end reads that overlap.

Raw sequences were split according to unique barcode combinations per sample and subsequently analysed with QIIME2 (Bolyen et al., 2019), version qiime2-2020.2. For the ITS2 reads, cutadapt (Martin, 2011) was used as a plugin in QIIME2 to remove primers and possible read-through into opposite primers or adapters. For all data, Dada2 (Callahan et al., 2016) was used to run specific quality control and filtering, merging of paired-end reads and chimera filtering, resulting in a set of unique sequences and an abundance table of amplicon sequence variants (ASVs) (Callahan et al., 2017). Very low abundant ASVs with total abundance below 10 were removed from both datasets. Taxonomy was assigned to the representative unique sequences for each ASV with Naive Bayes classifiers (Bokulich et al., 2018; Pedregosa et al., 2011). The 16S classifier was pretrained on extracted 16S V3–V4 region of the Silva 16S/18S database release 132 (Quast et al., 2012) and the ITS2 classifier was pretrained on full length ITS of Unite database, QIIME release, for all Eukaryotes, version 8.0 with dynamic use of clustering thresholds (Nilsson et al., 2019). ASV tables were additionally filtered based on the taxonomy assignments. ASVs that were classified as plant chloroplast or mitochondria were removed, as well as ASVs that lacked assignment at Phylum level. The highest taxonomic resolution for identification of microbial communities was at the Genus level, although it is possible to distinguish some bacteria on a sub-genus level. All raw sequences for soil fungi and bacteria were deposited in NCBI under Bioproject numbers PRJNA657168 and PRJNA657276respectively.

2.6. Soil physico-chemical analysis

Five L of each casing soil was sampled during preparation, for assessment of physical properties of the ten casing soil mixtures, before the first experiment, and again after storage for 12 months. Dry matter content, organic matter content, bulk density were measured according to EN 13039 protocols. Water holding capacity, soil moisture retention (pF curves), pore fraction, water fraction, and air fraction measured according to EN 13041 protocols (BVB substrates, Netherlands). Chemical properties such as electrical conductivity, cation exchange capacity and pH were measured according to EN 13038 and EN 13037 protocols (BVB Substrates, Netherlands). The casing soils were evaluated for presence of minerals and trace elements by measurement of $N0_3$, NH_4, H_2P0_4, K Ca, Mg, $S0_4$, Si, $HC0_3$, Na, Cl, Fe, Mn, Zn, B, Cu and Mo levels (Groen Agro Control, Netherlands).

2.7. Statistical data analyses

All statistical analysis was performed on RStudio with R version 3.4.0 (Team, 2013). Bioassay data was transformed using *tidyverse* (Wickham et al., 2019). Analysis of variance, generalized linear regression and zero-inflated beta regression was performed using packages, *glm* and *betareg* (Cribari-Neto and Zeileis, 2009) respectively. Widely applicable information criteria (WAIC) and adjusted R squared values were compared to arrive at a final minimally adequate model (Johnson and Omland, 2004). Model assumption on normality of data and homogeneity of residuals were verified using diagnostic plots and statistical tests (Fox et al., 2012). Sequence data transformation, downstream analysis of ASV tables and compositional graphics were made with packages *vegan* (Oksanen et al., 2007) and *phyloseq* (McMurdie and Holmes, 2013). Core microbiome analysis was performed using *microbiome* (Lahti et al., 2017). Co-occurrence network analysis was performed using packages, *igraph* (Csardi and Nepusz, 2006) and *speic-easi* (Kurtz et al., 2015).

3. Results

3.1. Physical and chemical characteristics vary between the casing soil mixtures

The physical and chemical properties were evaluated for the casing soil samples at the start of the experiment (Table 1, Table 2 and Table 3). The $S0_4$ ($P = 0.001$), Si ($P = 0.095$) and Mn ($P = 0.041$) content varied significantly based on the geographical source of the black peat used. Supplementation with baltic peat and garden peat further increased the $S0_4$ content ($P = 0.018$) of the casing soil. Casing soil composition based on peat source, peat depth and supplementation had no significant impact on other chemical properties such as pH, cation exchange capacity, electrical conductivity and other mineral nutrient content. The soil physical properties such as moisture content ($P = 0.030$) and water holding capacity ($P = 0.078$) varied with casing soil composition. The soil moisture retention curves also varied with peat source ($P = 2.3 \times 10^{-8}$), peat depth ($P = 0.011$) and supplementation ($P = 7.9 \times 10^{-5}$).

Casing soil characteristics were also compared between the three repetitions of the bioassay, at the beginning of the experiment, and after one year of cold storage (Tables 2 and 3). The water holding capacity of all casing soils decreased slightly ($P = 0.055$) with storage period, from 70.8 during procurement to 69.0 after one year of storage. The overall soil moisture retention also decreased specific to the peat source ($P = 2.3 \times 10^{-8}$), depth ($P = 0.011$) and supplementation ($P = 0.011$). Storage period had no impact on other physical and chemical parameters such as moisture content, organic matter content, bulk density or soil swelling characteristics such as shrinkage. Soil properties could not be compared between the consecutive cultivation cycles, in order to preserve the cultivation setup.

3.2. Ginger blotch dynamics during consecutive cultivation cycles

The disease pressure of ginger blotch in the mushroom cropping system was determined for ten casing soil mixtures, during two consecutive harvest cycles (flushes) in three independent replicate experiments. In the 1st flushes, ginger blotch incidence (Fig. 1A) and severity (Fig. 1B) strongly increased with the inoculation density of *'P. gingeri'* in the casing soil. Instead, in the 2nd flushes only limited effects were seen from increasing inoculation densities. In mushroom beds inoculated with *'P. gingeri'*, the mean blotch incidence declined from 15.3% in the 1st flush to 2.5% in the 2nd flush, and the mean blotch severity also reduced similarly. In mock-inoculated mushroom beds, blotch incidence and blotch severity remained very low but consistent

Table 1
Mean chemical properties and mineral content of ten casing soils, which comprise peat from different sources, at different depths of excavation and supplemented with different additives. A, B and C refer to peat from the geographic locations of friedrichsveen, wiesmoor and holriede respectively.

Casing soil nr	1	2	3	4	5	6	7	8	9	10
Peat source	A	A	B	B	A	A	B	B	C	C
Additives	no	no	no	no	yes	yes	yes	yes	no	yes
Peat depth	surface	deep dug	surface	deep dug	surface	deep dug	surface	deep dug	surface	surface
EC (mS/cm)	0.49	0.5	0.5	0.47	0.52	0.55	0.49	0.48	0.49	0.48
pH	7.5	7.4	7.3	7.3	7.2	7.3	7.5	7.3	7.2	7.3
NO_3 (mmol/l)	0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
NH_4 (mmol/l)	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
H_2PO_4 (mmol/l)	0.25	0.3	0.3	0.3	0.35	0.3	0.15	0.15	0.4	0.35
K (mmol/l)	0.2	0.2	0.2	0.3	0.3	0.3	0.2	0.2	0.2	0.2
Ca (mmol/l)	1.3	1.5	1.4	1.4	1.8	1.8	1.3	1.2	1.8	1.7
Mg (mmol/l)	0.7	0.7	0.8	0.7	0.9	0.8	0.8	0.8	0.8	0.8
SO_4 (mmol/l)	0.8	0.6	0.4	0.4	0.8	0.8	0.6	0.5	0.4	0.5
Si (mmol/l)	0.4	0.5	0.6	0.6	0.5	0.5	0.5	0.5	0.6	0.6
HCO_3 (mmol/l)	2.5	3	3.2	3	3.2	3.4	2.9	3.1	3.3	3.1
Na (mmol/l)	0.6	0.9	0.8	0.7	0.7	0.9	0.8	0.6	1	0.8
Cl (mmol/l)	0.4	0.4	0.5	0.4	0.4	0.4	0.5	0.4	0.5	0.4
Fe (μmol/l)	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4
Mn (μmol/l)	0.1	0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.3	0.3
Zn (μmol/l)	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	0.1
B (μmol/l)	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
Cu (μmol/l)	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	<0.1
Mo (μmol/l)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	<0.1
CEC (meq/g)	95	97	91	81	83	96	94	93	92	98

Table 2
Mean physical properties of ten casing soils across the first and third repetition experiments, spanning 12 months of storage.

Storage (months)	Casing soil nr	Peat source	Peat depth	Additives	Moisture content (%)	Organic matter (%)	Bulk density (kg/m^3)	Shrinkage (%)
0	1	A	surface	no	81.2	61.4	171	65
0	2	A	deepdug	no	83.7	71.4	166	70
0	3	B	surface	no	80.3	59.1	194	63
0	4	B	deepdug	no	84.5	69.3	175	66
0	5	A	surface	yes	82.4	71.1	172	31
0	6	A	deepdug	yes	81.9	67.8	175	63
0	7	B	surface	yes	79.9	61.9	178	61
0	8	B	deepdug	yes	82.3	66.5	169	63
0	9	C	surface	no	82	71.2	154	72
0	10	C	surface	yes	80.8	59.7	188	67
12	1	A	surface	no	84.7	68.9	161	66
12	2	A	deepdug	no	83	69.4	158	68
12	3	B	surface	no	81.8	69.2	177	62
12	4	B	deepdug	no	82.2	65.5	164	65
12	5	A	surface	yes	81.6	60.8	167	62
12	6	A	deepdug	yes	82.3	69.4	161	67
12	7	B	surface	yes	82.4	69.8	161	59
12	8	B	deepdug	yes	82.1	67.6	170	63
12	9	C	surface	no	82.5	64.8	169	72
12	10	C	surface	yes	83.1	71	172	66

between both flushes at 0.8% and 0.3% respectively.

Similar patterns in temporal decline of ginger blotch were observed for all ten different casing soil mixtures (Supplementary Table 4). Longer storage period of casing soil increased the overall ginger blotch incidence ($P = 2 \times 10^{-16}$) and severity ($P = 2 \times 10^{-16}$) of the cropping system (Supplementary Table 4 and Supplementary Table 5). However the blotch decline in the 2nd flush also decreased with the increased storage period of raw materials used in casing soil preparation. To test the difference in susceptibility of mushroom caps from the first and second flush to the pathogen, an *in-vitro* pathogenicity cap test was performed. Freshly harvested mushroom caps from both flushes showed similar blotch symptoms, when inoculated with two different strains of *'P. gingeri'* (Supplementary Figure 3).

3.3. Population dynamics of endemic and inoculated pathogens

Endemic and inoculated *'P. gingeri'* populations had unique temporal dynamics ($P = 2 \times 10^{-16}$) across the cultivation cycle when tracked via diagnostic Taqman™ assays (Supplementary Table 6). Endemic pathogen populations in uninoculated mushroom beds were undetectable at the beginning of the experiment (T0), but they increased steeply from 1.8×10^3 cells g^{-1} in the first flush (T1) to 1.5×10^5 cells g^{-1} in the second flush (T2) (Fig. 2A). The overall abundance of *'P. gingeri'* was higher in pathogen-inoculated mushroom beds. Inoculated pathogen populations in the casing soil, continued to increase ($P = 7 \times 10^{-7}$) between the consecutive flushes, in the three experiments (Fig. 2A), despite declining disease incidence. Among the inoculated mushroom beds, *'P. gingeri'* populations increased from 7.5×10^5 cells g^{-1} in the first flush to 2.5×10^6 cells g^{-1} in the second flush. This increase varied with the initial inoculum density of the pathogen in the soil ($P = 3 \times 10^{-4}$) (Fig. 2B). At inoculation densities of 10^3 and 10^4 cfu g^{-1}, population dynamics of *'P. gingeri'* in inoculated mushroom beds were similar, and the pathogen densities in the soil also remained consistent between the first and second flush. At an inoculum density of 10^5 cfu g^{-1}, the pathogen populations increased 100x between the flushes. Densities of both endemic and inoculated pathogens differed

Table 3
Mean values of the pore, water and air fractions of ten casing soils across the first and third repetition experiments, spanning 12 months of storage.

Storage (months)	Casing soil nr	Pore fraction (%)	Water fraction (%)					Air fraction (%)				
		pF	0.5	1.0	1.5	1.7	2.0	0.5	1.0	1.5	1.7	2.0
0	1	91	85	81	73	71	68	6	10	18	20	23
0	2	91	87	82	77	74	72	4	8	14	16	18
0	3	90	89	84	79	76	74	1	6	11	14	16
0	4	90	85	81	77	74	71	6	9	14	16	19
0	5	90	88	80	73	71	69	2	11	17	19	21
0	6	90	84	78	73	71	68	6	12	17	20	22
0	7	90	87	80	72	69	67	4	11	18	21	23
0	8	91	87	83	77	75	73	4	8	14	16	18
0	9	91	89	84	78	74	72	3	7	14	17	19
0	10	90	88	84	79	76	74	2	6	11	14	16
12	1	91	88	83	76	74	69	3	8	15	17	22
12	2	91	87	82	76	73	70	4	9	16	18	21
12	3	90	86	80	73	71	67	5	10	17	19	23
12	4	91	87	82	76	74	69	4	9	15	18	22
12	5	91	87	81	74	72	68	4	10	17	19	23
12	6	91	88	82	76	73	70	3	9	15	18	21
12	7	91	84	78	71	69	65	7	13	20	22	26
12	8	91	88	84	77	74	70	2	7	14	16	21
12	9	91	88	84	78	75	71	2	7	13	16	20
12	10	90	88	84	78	75	71	2	6	13	15	19

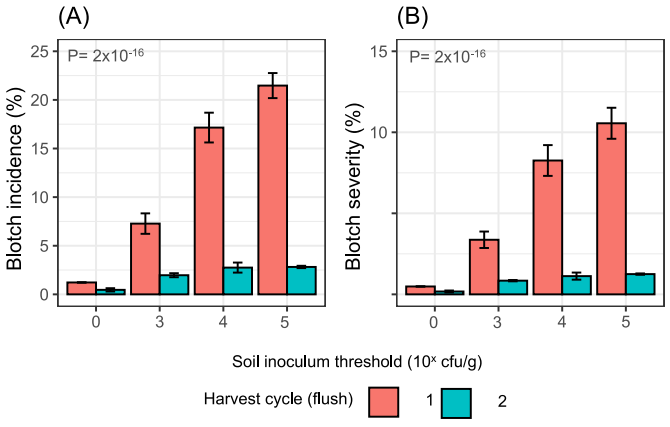

Fig. 1. Temporal decline in ginger blotch between the first and second harvest cycles (flushes) in pathogen-inoculated casing soil from bioassay 1. (A) Disease incidence and (B) disease severity in the cropping system.

significantly between casing soil mixtures (P = 0.0007), and the temporal dynamics of the pathogen populations also varied between casing soils (P = 0.008) (Supplementary Table 6). Correlation analysis between pathogen populations and disease pressure indicated that reduced blotch incidence (P = 0.035) and severity (P = 0.07) in the second flush coincided with increasing pathogen populations according to Pearson's coefficients (Supplementary Figure 4).

3.4. Transferability of blotch suppressiveness by soil extract

An aqueous microbial extract made from mixed casing soils in the 2nd flush of bioassay 1, was found to moderately suppress ginger blotch when inoculated in fresh casing soils in bioassay 2 (Fig. 3 and Supplementary Table 7). Control mushroom beds with no microbial inoculation had a ginger blotch incidence of 4.8%. When *'P. gingeri'* was inoculated in the casing soil at a density of 10^6 cfu g^{-1}, a blotch incidence of 51.4% was observed. Addition of a microbial extract made from blotch suppressive soils in previous experiments, together with the inoculated pathogen, reduced the ginger blotch to 34.5% (P = 0.0304), suggesting that blotch suppressiveness is transferable. However, mushroom beds inoculated with only microbial extract, in the absence of additional pathogen, also had a blotch incidence of 15.8%, suggesting that former pathogen populations were also carried over.

3.5. Casing soil microbiome reflects pathogen invasion and establishment

The total bacterial microbiome of casing soils comprised of 10860 amplicon sequence variants (ASVs) or taxa originating from 42 casing soil samples. The fungal microbiome of the casing soils was less diverse, and comprised of only 294 taxa. Both the bacterial and fungal microbiome of the casing soil underwent significant changes due to pathogen inoculation, consecutive harvest cycles and casing soil storage period based on non-metric multidimensional scaling analyses (Fig. 4A and B). The bacterial community composition of the casing soil varied significantly between mock-inoculated and pathogen inoculated mushroom beds (R^2 = 0.022, P = 0.023), based on a PERMANOVA. Overall species richness calculated from inverse Simpson (P = 0.831) and Shannon (P = 0.787) diversity indices, were unaffected by pathogen inoculation. The effect of pathogen inoculation was observed until the end of the experiment, independent of changes across the cultivation cycle, as many taxa were differentially abundant between mock-inoculated and pathogen-inoculated casing soils, across the first and the second flush (Fig. 4C). The soil fungal community composition was unaffected by pathogen inoculation. Species richness of the casing soil bacteria also reduced significantly during the year-long storage period between the replicate experiments (P = 0.0161).

3.6. Specific bacteria and fungi are associated with blotch decline

The alpha diversity of the bacterial microbiome varied across different time points in the cultivation cycle (P = 4.1 × 10^{-6}). Casing soils from the second and third harvest cycles (T2 and T3) had higher species richness than that of the first harvest, as indicated by inverse Simpson (P = 4.1 × 10^{-6}) and Shannon (P = 0.0003) diversity indices (Fig. 5A). The bacterial profiles of casing soils from the consecutive flushes also clustered differently from each other, on the basis of Bray-Curtis distances, and had significantly (R^2 = 0.435, P = 0.001) different beta diversity (Fig. 4A). The community composition of the fungal microbiome did not vary significantly (P = 0.051) across the harvest cycles based on Bray-Curtis distances, and casing soils from consecutive flushes did not cluster differently from each other (Fig. 4B). More than 200 bacterial taxa that are potentially associated with ginger blotch suppression were identified, which contribute to 70% of the overall differences in beta-diversity between soils from the first and second flush (Supplementary Figure 5). Two fungal taxa, *Saitozyma*

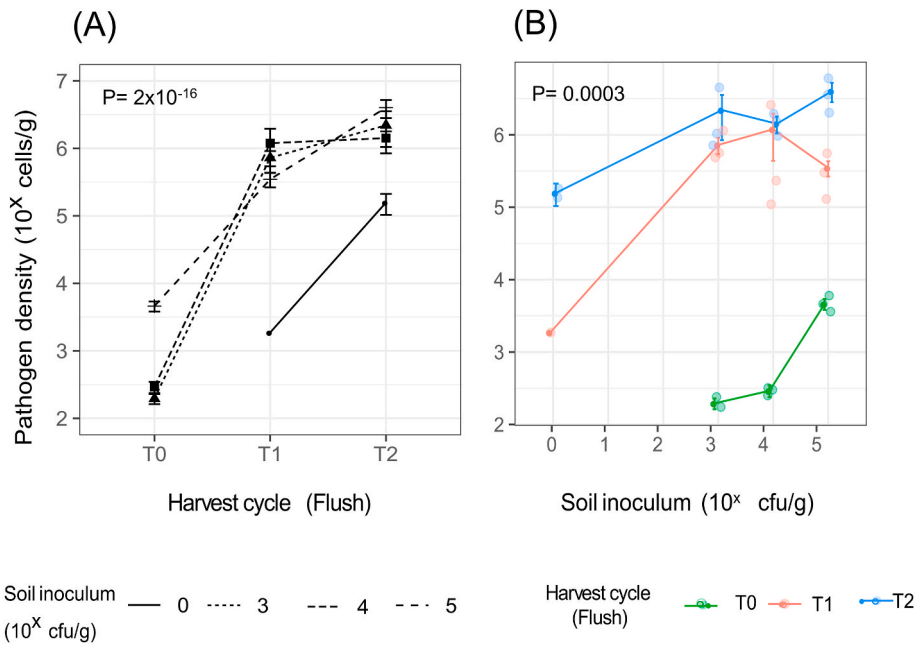

Fig. 2. qPCR-based diagnostics of *"P. gingeri"* densities in the mushroom beds. Endemic and inoculated pathogen populations vary over (A) timepoints in the cropping cycle and (B) soil inoculum densities.

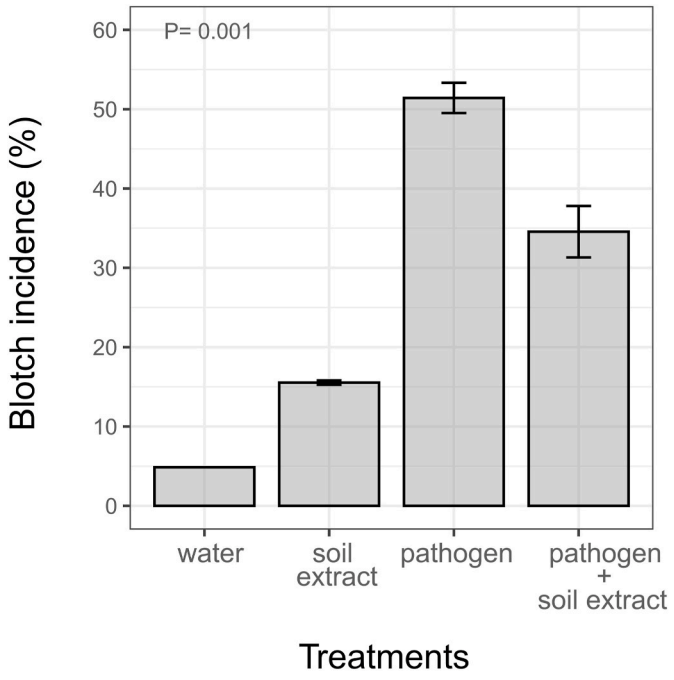

Fig. 3. Transferability of ginger blotch decline via microbial extract from suppressive soils to conducive soils, performed in bioassay 2.

podzlica (P = 0.039) and *Trichoderma sp.* (P = 0.029) were found to be associated with ginger blotch decline. 26 bacterial genera were also found to be significantly differentially abundant (P<0.05, −2> fold change >2) between suppressive and conducive soils after correcting for multiple comparisons (Fig. 5B). Several of these genera also form clusters in co-occurrence networks of casing soil microbiome (Supplementary Figure 6), indicating that they are likely to interact with or influence other members of the microbiome.

3.7. The core players of the casing soil microbiome are stable

The core microbiome of the casing soil comprised of 69 bacterial genera and 9 fungal genera (Fig. 6A and B), that were present in 99% of the casing soil samples above a detection threshold of 1%. Genera from the core microbiome were relatively conserved between the treatments, and their relative abundances differed between consecutive harvest cycles (R^2 = 0.435, P = 0.001) and storage period (R^2 = 0.194, P = 0.001), but they were not influenced by pathogen inoculation (P = 0.056), according to PERMANOVA. The core bacterial community was largely dominated by Proteobacteria. Other phyla in decreasing order of relative abundance included, *Bacteroidetes, Firmicutes, Gemmatimonadetes, Verrucomicrobia, Chloroflexi, Actinobacteria, Epsilonbacteraeota, Acidobacteria* and *Patescibacteria* (Fig. 6A). Relative abundances of the core fungal genera differed between storage periods (R^2 = 0.215, P = 0.001), but not due to pathogen inoculation (P = 0.516) or consecutive harvest cycles (P = 0.061). The core fungal community in the casing soil was dominated by *A. biporus,* although it's relative abundance in the casing soil did not increase with consecutive harvest cycles. Other fungal genera included *Apiotrichum, Meliniomyces, Mycothermus, Pseudallescheria, Candida, Pseudeurotium, Solicoccozyma* and *Saitozyma* in decreasing order of abundance (Fig. 6B).

4. Discussion

4.1. Temporal casing soil suppressiveness to ginger blotch

A consistent decline in ginger blotch incidence and severity was observed during consecutive harvest cycles (flushes) for ten different casing soil compositions, in three independent experiments. Given the relative increase in abundance of pathogen populations during consecutive flushes, this reduction in disease pressure cannot be attributed to declining pathogen populations. Similar results were obtained during a preliminary investigation of the pathogen densities in the casing soil across multiple flushes (Taparia et al., 2020a). Mushroom caps from the second flush can have less yellow discolorations from physical bruising than those of the first flush, due to the post-harvest physiology of the mushroom (Burton and Noble, 1993). However, in our *in-vitro* pathogenicity assay, mushroom caps from first and second flush had the same

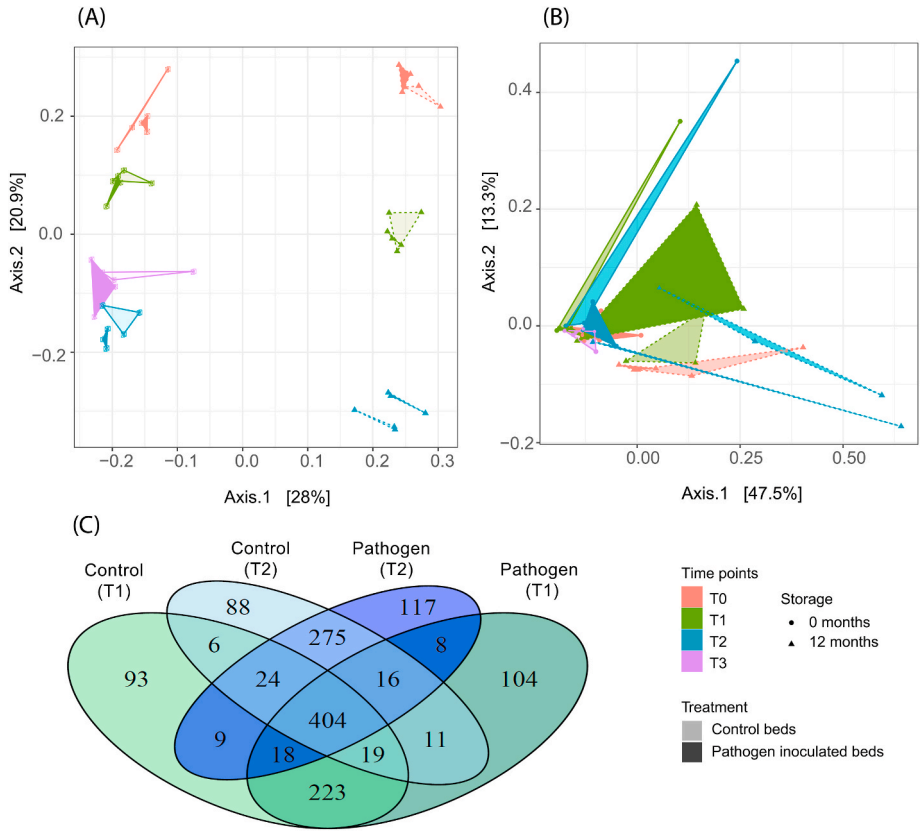

Fig. 4. Principal component analyses showing the variation in composition of casing soil microbiomes for (A) bacteria and (B) fungi as affected by pathogen inoculation, time points during cultivation cycles, and storage period of casing soil. (C) Differential presence of bacterial taxa (ASVs) between the cultivation cycles due to pathogen inoculation are shown as a Venn diagram.

Fig. 5. Changes in casing soil microbiome between conducive (T1) and suppressive (T2) soils are visualized as (A) species richness based on Inverse Simpson diversity index. Differentially abundant genera between T1 and T2 with fold-change greater than 2, are described in (B) mock-inoculated beds and (C) pathogen-inoculated beds.

symptom development for ginger blotch, when tested against two different strains of *'P. gingeri'*. This indicates that the reduced blotch incidence and severity in the later flushes cannot be attributed to differences in post-harvest physiology or induced resistance in *A. bisporus*. This is in line with findings of another study, where infection of mushrooms with dry bubble pathogen, *Lecanicillium fungicola*, failed to induce systemic resistance in *A. bisporus* (Berendsen et al., 2013).

Filtered soil extract from the second flush, when inoculated into fresh casing soil, led to significantly reduced ginger blotch incidence. This indicates that the temporal decline in ginger blotch may be attributed to the development of soil suppressiveness to *'P. gingeri'*, and this blotch suppressiveness can be partially transferred to fresh casing soil via a soil microbial extract. Transferability is an important characteristic of biological soil suppressiveness (Baker and Cook, 1974) and has been proven for several soil-borne plant pathogens (Westphal and Becker, 2000; Jambhulkar et al., 2015). Across the three replicate experiments, soil suppressiveness to bacterial blotch reduced slightly. Since, the conditions of the experimental farm were strictly regulated, and did not differ between experiments, this reduced soil suppression may be due to changes in the soil physico-chemical characteristics, or activity and composition of soil microorganisms during the cold storage period between the experiments. However, it is also possible that this effect is related to differences in the compost microbiomes between the replicates, as freshly prepared compost is required for mushroom cultivation, which could differ between experiments.

Remarkably, similar disease suppression patterns over continuous cultivation cycles, have been observed for plant crops grown in monoculture. Take-all disease caused by soil-borne fungus *Gaeumannomyes graminis* var. *tritici* in wheat or barley monocultures, is also known to first increase and then decrease during consecutive harvest cycles, a phenomenon known as take-all decline (Schreiner et al., 2010). This suppression is commonly ascribed to the increase of antibiotic (2, 4-diacetylphloroglucinol) producing *Pseudomonas* species (de Souza et al., 2003), but several other rhizosphere bacteria are also speculated to be involved (Sanguin et al., 2009). Another example includes the continuous cultivation of sugar beet seedlings on soils infested with *Rhizoctonia solani*, which also undergo a temporal decline of root rot (Expósito, 2017). This has been attributed to increased abundance of *Lysobacter* sp. (Gómez Expósito et al., 2015; Postma and Schilder, 2015). It is also important to note, that in case of take-all decline, pathogen populations decline over continuous cultivation (Lebreton et al., 2004), but this is not in the case for blotch suppression. Hence, different mechanisms may operate towards the decline of ginger blotch in mushroom cultivation. It can be speculated that inhibition of virulence factors such as those controlled by quorum sensing can be responsible, as have been observed for other *Pseudomonas* species (Adonizio et al., 2008; El-Mowafy et al., 2014; Vandeputte et al., 2011). The temporal scale also varies significantly between the cultivation systems, as wheat and barley grow over 7–8 months, and mushroom cropping cycles typically last 4–6 weeks.

4.2. Disease suppressive microbiomes, dynamics and targets

The overall bacterial profile of the casing soil was dynamic and

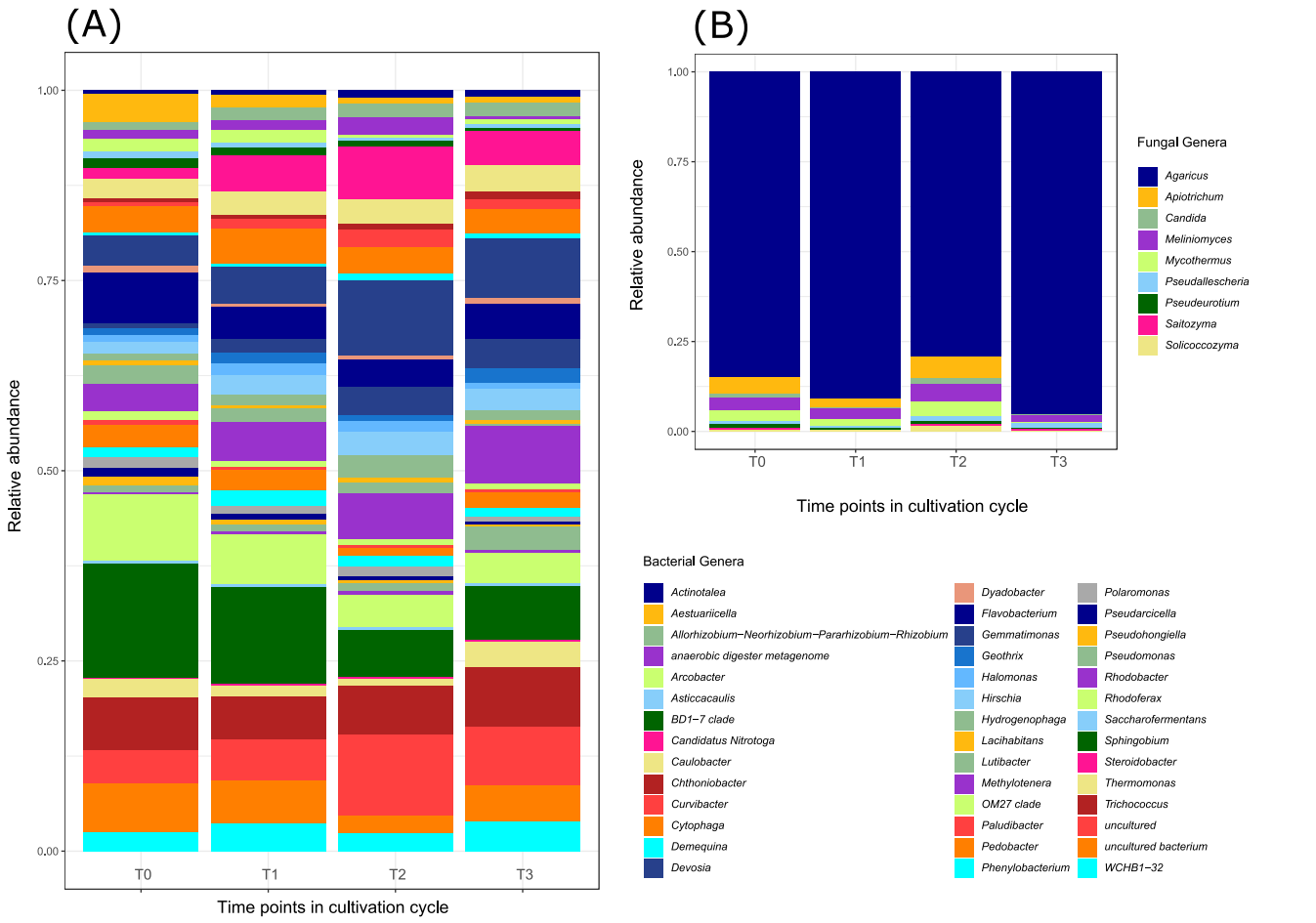

Fig. 6. Bar plots representing core microbiome of casing soil across all treatments. (A) Bacterial and (B) fungal genera, which are present in 99% of the soils above a detection limit of 1%.

varied temporally during the cropping cycle, while the core microbiome remained relatively stable. Changes at the genus level were marginal, although many taxa (ASVs) were differentially abundant across the cropping cycle, indicating that more differences are expected at lower taxonomic levels. This is also supported by earlier investigations where the bacterial community of the casing soil changed with the growth of *Agaricus* mycelium into the casing soil along the cropping cycle (Pecchia et al., 2014), although the overall composition remained relatively stable (Carrasco et al., 2019). The mycelium from *A. bisporus* is known to migrate upwards from the compost, and colonize the casing soil along the cultivation cycle. However, the relative abundance of *A. bisporus* in the casing soil microbiome did not increase across the cropping cycle. Given that very few fungal genera were detected in the casing soil microbiome, it can be speculated that the absolute abundance of fungi is quite low in peat, and *A. bisporus* is predominantly present from T0 itself, due to inoculation of the casing soil with small amounts of compost by a process called 'cac-ing'. This can also be an artefact of the soil sampling technique, which ensured that bulk soil was collected instead of soil with mycelium adhering to it.

In the current experiments, pathogen inoculation on the casing soil led to an additional shift in the soil microbiome, the effect of which lasted throughout the cultivation cycle. This can be compared with known changes in the rhizosphere microbiome that occur due to pathogen invasion. For instance, invasion by the bacterial plant pathogen *Ralstonia solanacearum*, led to simplification of soil microbiome networks (Wei et al., 2018), whereas inoculation of fungal pathogen *R. solani* coincided with increase of specific bacterial taxa in the soil such as, *Burkholderiaceae*, *Sphingobacteriaceae* and *Sphingomonadaceae* (Chapelle et al., 2016). These changes can also be indirectly mediated by exudate patterns of the plant host itself (Gu et al., 2016). It is speculated that brown blotch pathogen, *P. tolaasii*, can potentially inhibit the growth of other soil microorganisms (Martins et al., 2020), based on the broad-spectrum volatile organic compounds it produces (Lo Cantore et al., 2015). This may be the reason why abundant genera for both the 1st flush and 2nd flush varied between mock-inoculated and pathogen-inoculated mushroom beds. However, it is also important to note that the overall species richness did not vary significantly between mock-inoculated and pathogen-inoculated mushroom beds. On the other hand, blotch suppressive soils of the second flush were found to be more diverse than conducive soils from the first flush. The shift in abundant genera between the 1st and 2nd flush of pathogen inoculated casing soils could contribute to blotch suppressiveness, as disease suppression is known to be induced by soil-borne pathogens (Carrión et al., 2019).

More than 200 taxa were found to be associated with blotch suppression, based on either high relative abundance in 2nd flush soils or high differential abundance from conducive casing soils. Differentially abundant genera between 1st and 2nd flush were specific for pathogen-inoculated mushroom beds. Organisms of interest, that were associated with blotch decline include *Burkholderia* sp., *Metylophilus* sp., *Pir4* lineage, *Pseudomonas* sp., *Pseudaminobacter* sp., *Pseudolabrys* sp., *Bdellovibrio* sp., *Haliangium* sp., *Sphingobacterium* sp., *Dyadobacter* sp., *Pedobacter* sp., and *Flavobacterium* sp. A few of these genera have been previously identified for their biocontrol activity against brown blotch. Non-pathogenic *Pseudomonas* species recovered from casing soil, such as, *P. putida, P. reactants, P. fluorescens*, have been shown to significantly

reduce blotch incidence in laboratory and commercial trails (Aslani et al., 2018; Fermor et al., 1991; Tajalipour et al., 2014). *Sphingobacterium multivorum* and a *Pedobacter* sp., isolated from wild agricales, were also found to strongly suppress brown blotch development in vitro by deactivating the blotch toxin (Tsukamoto et al., 2002). *Burkholderia cepacia* was found to be antagonist to brown blotch pathogen, *P. tolaasii* (Nair, 1974). Predatory bacteria, *Bdellovibrio bacteriovorus*, was found to be effective against bacterial blotch, however, due to its broad host range, it could also affect the endemic beneficial microbiome of mushroom beds (Saxon et al., 2014).

4.3. Soil and microbiome interactions for blotch suppression

For successful use of biocontrol agents a repeated application is often required as populations of the beneficials in soil decline due to competition for space and nutrients, presence of inherent predators and specific physico-chemical features of the soil matrix. Experimental trials of blotch biocontrol agents tested *in-vitro*, resulted in reduced effectiveness *in-vivo* and an inconsistent field performance (Tsukamoto et al., 1998). In this context, blotch management via stable changes of the endemic soil microbiome presents better opportunities, as it preserves community level interactions, and these microorganisms are better adapted to the local conditions (Ab Rahman et al., 2018; Kim et al., 2011; Mueller and Sachs, 2015). Many of the genera that were found to be associated with blotch suppression, also co-occurred with each other in the microbial soil networks. For example, *Burkholderiaceae* interacted with *Pedobacter*, *Flavobacterium* and *Brevundimonas*. *Dyadobacter* also co-occurred with *Brevundimonas* and *Pedobacter*. And *Pedobacter* was directly linked with *Pseudomonas*. Co-habitation of *Pedobacter* with other bacterial strains in *in-vitro* assays, was found to suppress several plant fungal pathogens due to interspecific interactions (De Boer et al., 2007). These microbes would hence make good targets for further research on blotch management.

Organic amendments have been shown to increase disease suppressiveness characteristics of soils in both agricultural and horticultural crops (Postma et al, 2003, 2014; Postma and Schilder, 2015; De Corato, 2020b). Compost, biochar and animal manure have been used to control common soil-borne bacterial and fungal pathogens such as *Ralstonia solanacearum, Fusarium sp. and Rhizoctonia solani*, by promoting the survival and activity of beneficial microbes (Bonanomi et al., 2018). Similarly, temporal suppression of ginger blotch could be associated to the increased contact period of the casing soil with the underlying compost in later cultivation cycles. The bed of compost, which has a microbial community different from that of peat (Carrasco et al., 2020), may provide beneficial microbes to the casing soil with time, leading to the increased bacterial diversity observed in later cultivation cycles (Carrasco et al., 2020).

In our experiments, long-term storage of the casing soil led to reduced soil suppressiveness against ginger blotch. But we did not observe changes in the overall organic matter content of casing soil due to storage. However, the bacterial diversity reduced and composition of casing soil microbiome did change during storage of the casing soil between experiments. It can be speculated that these changes are instead associated to the quality of soil organic matter. In particular for peaty substrates, similar to that of mushroom casing soils, disease suppressive activity has been previously associated to the quantity and quality of soil organic matter (Hoitink and Boehm, 1999). Edaphic carbon sources are essential for the survival of biological control agents such as *Pseudomonas* and *Pantoea* spp, especially when rhizosphere deposits and root exudates processes do not provide enough resources for sustained biocontrol activity against the pathogen (Boehm et al., 1997).

Unfortunately, we were not able to measure soil chemical and physical characteristics during the consecutive cultivation cycles, in order to preserve the experimental setup. Even though the nutrient conditions of the casing may change during the cultivation cycle, due to growth of *A. bisporus* in the casing soil, and this can have an effect on the developing disease suppressiveness, our transfer experiment supports that suppression of bacterial blotch is brought about, at least partly, by changes in the microbial community composition. The invasion resistance of a microbial community is often attributed to increased diversity, as the endemic microbiome can better exploit available resources (van Elsas et al., 2012), however, this is complicated by the fact that addition of resources can also temporarily make the community more susceptible to invasion (Mallon et al., 2015). This suggests that the casing soil microbiome can also be steered by other factors, such as organic amendments, which are a rich source of beneficial microbiota, towards blotch suppression. Better knowledge of the pre-treatment, composition, supplementation, physical and chemical characteristics of the casing soils, is thus required to induce suppressiveness against soil-borne pathogens. A combined effort using a mixture of microbial inoculants and organic amendments to induce stable changes in the casing soil microbiome presents a better opportunity for the management of blotch diseases.

5. Conclusions

This study reported induced soil suppressiveness to bacterial blotch between consecutive cultivation cycles of mushroom cropping systems. Blotch suppression was induced due to high pathogen populations in the casing soils, and was observed consistently across ten casing soil mixtures. The soil suppressiveness was found to be partially transferable to conducive soils via an aqueous soil extract. We hyposethize that blotch suppression was microbially mediated and involved the inhibition of virulence factors such as those involved in quorum sensing. Pathogen invasion, pathogen establishment and disease suppression were also reflected in the community composition of the casing soil microbiome. Specific bacterial and fungal genera were associated with blotch suppression and they also interacted with each other in co-occurrence microbial networks. These results provide a platform for further applied studies on the design of blotch management strategies.

Future research on ginger blotch suppression should address the functional activity of the casing soil and possible mechanisms behind this induced suppressiveness. Potential targets for suppressiveness identified in this study, should be tested either as individual strains or microbial mixtures for their biocontrol activity against blotch. Their survival and efficacy in the casing soil, and their effect on the composition and activity of the soil microbiome, also requires further investigation. Additionally, the use of organic amendments to steer the chemical composition of casing soil towards blotch suppression should be explored. This knowledge is not only instrumental for successful biological control of bacterial blotch in mushroom cultivation, it also improves our general understanding of induced disease suppressiveness in soils.

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Author contributions

TT, EH, and JW designed the experiments. TT, EH, MK, and MH performed the bioassay. TT, MH and EN did the molecular analyses. TT, WB and JW wrote the manuscript. All authors contributed to subsequent manuscript revision, read and approved the submitted version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2021.108161.

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