DETECTION OF SOURCES OF *LECANICILLIUM* (*VERTICILLIUM*) *FUNGICOLA* ON MUSHROOM FARMS.

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ABSTRACT

The objective of this work was to identify sources of *L. fungicola* on commercial mushroom farms. For detection of *L. fungicola* two methods were used: selective media and Real Time PCR (RT PCR). Two selective media were compared using 438 samples and an RT PCR method was used with 375 samples. There was no difference in the success rate of the two selective media but *L. fungicola* grew faster and was more easily detected on the novel PDA media than on MRSM. RT PCR detected *L. fungicola* more frequently than the selective media. This might indicate a high level of dead *L. fungicola* on farms. Living *L. fungicola* was detected at most locations on farms but was much higher in growing rooms where 14, 26 and 47 % of samples from 1st, 2nd and 3rd flush, respectively, tested positive. Live *L. fungicola* was detected in 19 % of samples taken from other locations (outdoors and canteen area). When data were organised by type of sample *L. fungicola* was not detected on ruffling, filling or empting machines by either method, but in other places (inside, floor of growing room, crates, flies, door handle) the pathogen was detected by both methods.

Keywords: *Lecanicillium fungicola*, mushroom farms, selective medium, Real Time PCR

INTRODUCTION

Today *Agaricus bisporus* is cultivated in more than 70 countries in the world [1]. This monoculture is affected by many pathogens and pests. The most important pathogen of *A. bisporus* is *L. fungicola* which causes the disease called "dry bubble". The symptoms of dry bubble disease are un-differentiated masses of mushroom tissue, split stipes and cap spotting.

Lecanicillium fungicola produces large numbers of conidia which are held in sticky mucilage and these conidia can be very easily spread around the mushroom farm. Conidia are spread and dispersed in many ways via water, flies, humans and machinery [2, 3]. The conidia can also survive for a long time (7-12 month) in dry or moist casing soil mixture [4, 5]. All these factors make *L. fungicola* a very serious pathogen of the mushroom industry that is difficult to eliminate.

The first comprehensive study to search for sources of *L. fungicola* on mushroom farms was presented by Wong and Preece [6]. They used two different microbiological media for the detection of *L. fungicola* in samples from a large mushroom farm in the UK which was seriously affected by dry bubble disease.

Nair and Macauley [7] reported that the most common source of *L. fungicola* var. *fungicola* was soil from around mushroom farms. The peat moss and water which were tested were not a source of the pathogen. They detected *L. fungicola* by preparing a serial dilution of a sample and plating it out on potato dextrose agar with antibiotics.

Information about sources of *L. fungicola* on mushroom farms was also reported by Rinker *et al.* [8], who designed a selective medium for *L. fungicola* to test samples from mushroom farms.

Grogan [9] confirmed Gandy's [10] information about the spread of *L. fungicola* by the debris-dust fraction which is present on a mushroom farm. Debris samples collected from inside and outside mushroom houses that were added to casing soil, caused dry bubble symptoms to occur.

The objectives of this work were to detect *L. fungicola* on commercial mushroom farms in Ireland using two methods – one microbiological and one molecular. Identification of the possible sources of *L. fungicola* on mushroom farms could provide useful information for managing dry bubble disease.

MATERIALS AND METHODS

Mushroom Farms Visits. Between 2008 and 2010 samples were collected during 18 visits to 9 mushroom farms with different levels of dry bubble disease. In total 438 samples were collected from different stages of the crop cycle from spawn running to 3rd flush, as well as from other locations. Samples were categorised (1) by location or (2) by crop stage. Samples were examined using microbiological (selective medium) and molecular (Real Time PCR) methods.

Sample Collection and Preparation. Most samples from mushroom farms were collected by passing a sterile wet swab over the selected surface. The swab was then put into a 50 ml centrifuge tube. Other samples were collected directly (e.g. 5-10 flies, few gram soil, discarded gloves, hair net etc.) into either a 50 ml centrifuge tube or a clean plastic bag. The samples were stored at 4°C overnight and sample preparation started on the following day. Centrifuge tubes with samples were filled with sterile water up to 50 ml and shaken vigorously for 30 min. at 120 rpm. After that the sample was filtered through a square of UV sterilised Miracloth. Sample filtrate was concentrated by centrifugation (GS-6 Centrifuge, Beckman) for 10 min at 3,000 × g. After centrifugation samples were left overnight to sediment. The following day the upper layer was removed using a disposable transfer pipette and the debris pellet plus a small amount of water was left in the bottom of the tube to give a final sample volume of around 3 ml. Of this, 600 µl was used for selective media tests and the remainder of the sample was transferred into a 2 ml Eppendorf for molecular tests.

Selective Media. Two media were used for the detection of viable *L. fungicola* in mushroom farm samples: a "Modified Rinkers Selective Medium" (MRSM) and a "Novel PDA based Selective Medium" (NPDASM). The MRSM is based on a selective medium for *L. fungicola* described by Rinkers *et al.* [8]. Both media are described in Piasecka [11]. A 100 µl aliquot of sample was spread onto 3 replicate Petri dishes for each medium, which were then incubated for 6-7 days at 20°C.

DNA Extraction. DNA extraction was performed using Wizard Magnetic DNA Purification System for Food (Promega) and QuickGene Mini 80 device with QuickGene DNA tissue DT-S DNA isolation kits (Fujifilm) following the extraction protocol.

Real Time PCR. Real Time PCR was done using the primers and probe designed by Zijlstra *et al.* [12, 13, and 14]. A result of Real Time PCR was recorded as positive when 6-FAM signal was present after 45 cycles or less. If 6-FAM signal was present after more than 45 cycles and/or was negative the sample result was recorded as negative.

Data Analysis. Results from the microbiological and molecular detection methods were compared using McNemar's test for comparison of proportions from paired binary outcomes. This is a nonparametric test for a 2 × 2 contingency table with matched subjects where the outcomes are not independent. McNemar's Test statistic was calculated using SAS Software (SAS Institute Inc. 2004. SAS/STAT® 9.1, Cary, NC: SAS Institute Inc.).

RESULTS AND DISCUSSION

When the effectiveness of detection of *L. fungicola* on NPDASM and on MRSM selective medium was compared there was no significant difference between the media, but *L. fungicola* grew better on NPDASM compared to MRSM (see Fig. 1) making detection easier.

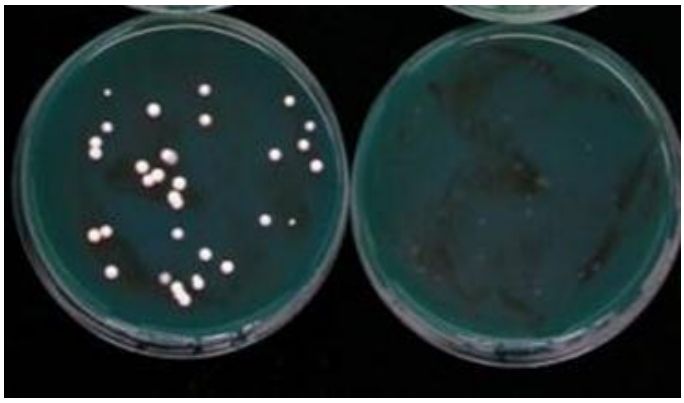

Figure 1: Growth of *L. fungicola* on NPDASM (left) and on MRSM (right) selective medium.

The Real Time PCR method gave significantly more positive results (63 %) for presence of *L. fungicola* compared with the selective media (18 %) at $P \leq 0.05$ except for three sample categories (machinery, watering equipment and worker clothes) where there were too few samples for an accurate comparison (see Fig. 2). This high detection rate by Real Time PCR may reflect the detection of dead *L.fungicola* by the Real Time PCR method as 50-90% of samples in all categories tested positive, while the corresponding selective media results had fewer positives.

When data were analysed and organised by location of samples *L. fungicola* was not detected on machinery by either method, but in in samples from other locations the pathogen was detected by both the selective media and Real Time PCR. Samples containing casing soil (e.g. growing room floors, pickers trolleys and platform) often gave more positive results on selective media compared to other samples. Samples which did not contain polymerase inhibitors (flies, door handle, water equipment, crates, etc.) gave more positive results with Real Time PCR compared with selective media (see Fig. 2).

When data were analysed and organised according to crop stage, viable *L. fungicola* was detected by selective media at all crop stages except during the spawn running stage (see Fig. 3). The number of samples testing positive for viable *L. fungicola* increased from 14 to 47% between the 1st and 3rd flush while 19% of samples from non-crop locations (e.g. outdoors and canteen areas) were positive for viable *L. fungicola*. Real Time PCR testing detected *L. fungicola* in 50-80% of samples for all crop stages which is a cause for concern. However it is unlikely that all Real Time PCR positive results reflect live *L. fungicola* therefore its use as a diagnostic tool on mushroom farms might be limited. There is likely to be a high level of dead

L. fungicola on farms that use steam to kill off diseased crops increasing the likelihood of false positives with Real Time PCR.

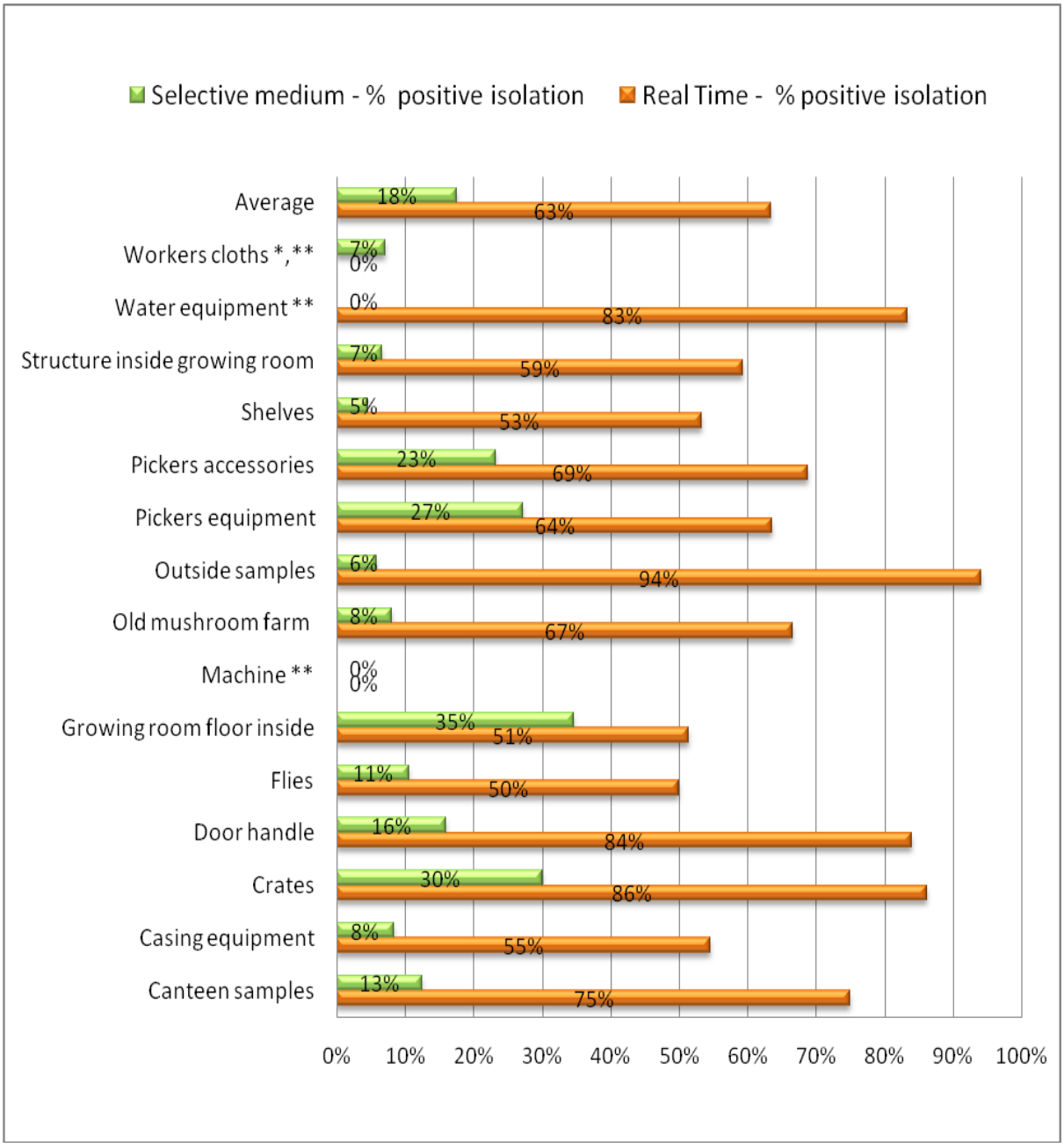

Figure 2: Detection of *L. fungicola* in samples from different locations. * Only selective medium tested, ** not enough samples for comparison.

The results suggest that selective medium may be a more reliable method for routine detection of *L. fungicola* on mushroom farms. It is not an expensive method for detection, but it requires a few days to give results. It detects only live material such as spores and mycelium so that a positive result identifies a very real disease risk for a farm. Real Time PCR is a fast but

expensive method for detection of *L. fungicola* from samples from mushroom farms however in these experiments detection levels were very high suggesting that non-viable background levels of *L. fungicola* were being detected. In order for Real Time PCR to be more useful in *L. fungicola* diagnostics it needs to be more specific for living material.

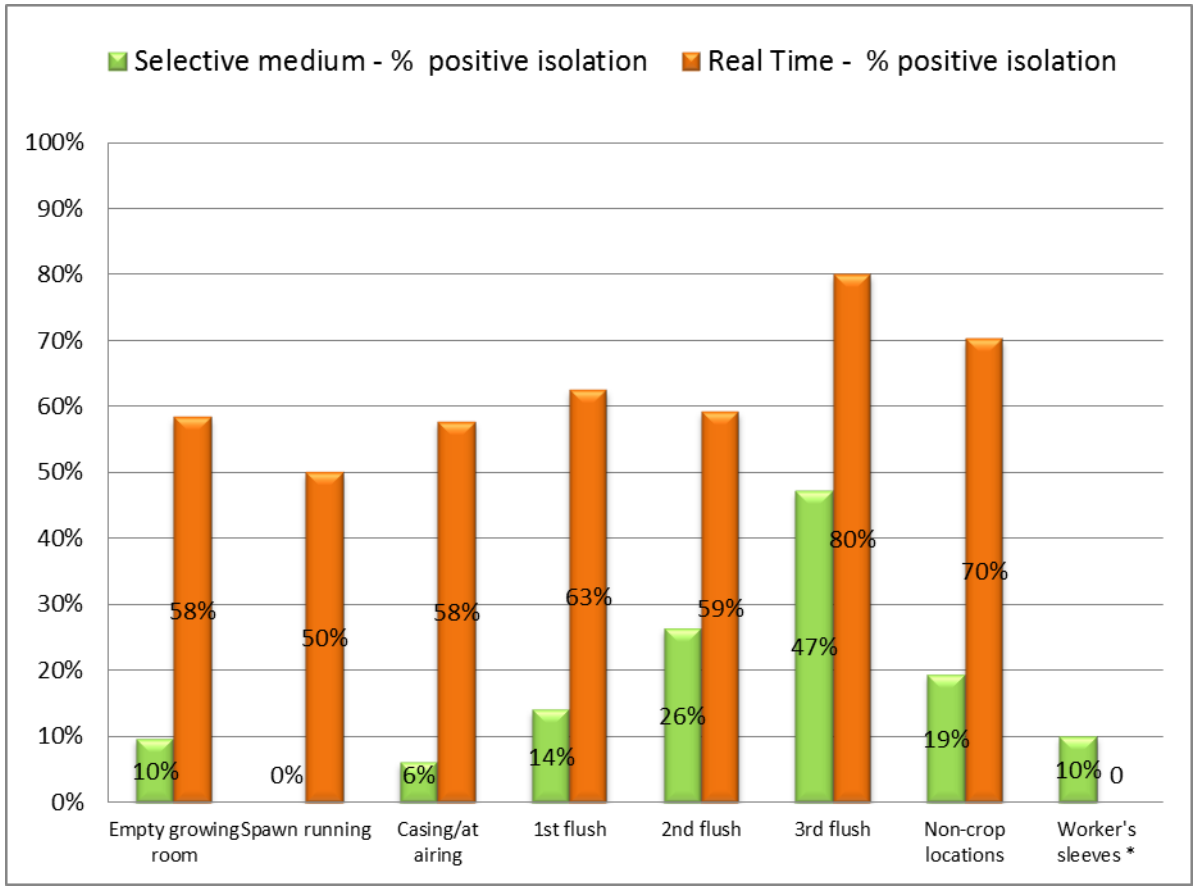

Figure 3: The percentage detection of *L. fungicola* using selective media and Real Time PCR after 45 cycle. * Only selective media tested.

CONCLUSION

Live *L. fungicola* was detected at most locations on mushroom farms but was most abundant in 2nd and 3rd flush growing rooms and outdoor samples. Real Time PCR detected four times more *L. fungicola* than selective media but it is likely that Real Time PCR is also detecting non-viable *L. fungicola*.

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