Project title:	*Trichoderma aggressivum f. europaeum* (Th2): Epidemiology in bulk Phase III systems
Project number:	M 50
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Report:	Final Report, February 2011
Previous report:	None
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Location of project:	Teagasc, Dublin; WHRI, Warwick; FERA, York
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Date project commenced:	1 October 2009
Date project completed (or expected completion date):	31 January 2011

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

Headlines

- Compost pasteurisation conditions of 60°C for 12 hours are needed to eradicate *Trichoderma aggressivum*

- *Trichoderma*-infected compost mixed and diluted into healthy Phase III compost can cause from 5-100% crop reduction due to green mould

- A new molecular detection method for *Trichoderma aggressivum* in Phase III compost has been shown to be very sensitive and reliable

Background and expected deliverables

Trichoderma aggressivum f. europaeum (previously called *T. harzianum* type Th2) is an aggressive compost green mould that predominantly infects Phase II compost at spawning. It was a serious problem in the mid 1980's and 1990's but was largely controlled through improved hygiene at spawning, and fungicide treatment of spawn, when infection risks were high. It has started to appear in bulk Phase III facilities across Europe in recent years despite the fact that hygiene levels are generally considered to be much higher on Phase III facilities compared to smaller, less technologically advanced, facilities. These outbreaks of *T. aggressivum* in bulk Phase III raise the question as to whether or not *T. aggressivum (Th2)* behaves differently in the bulk Phase III system compared to in-situ spawn run systems.

There is no precise information on the conditions (temperature, time, compost moisture, ammonia) that are required to eradicate *Trichoderma aggressivum* (spores and mycelium) during compost pasteurisation. Current recommendations for eradication times, temperatures and ammonia concentrations are approximate. Previous work has shown that *Trichoderma aggressivum* (Th2) can be detected in chicken manure, on Phase II prefilters and in spawning halls. Other work has shown that *Trichoderma aggressivum* (Th4) spores can withstand up to 200 ppm gaseous ammonia and 60°C for 9 hours under laboratory conditions, and 60°C for 10 hours under simulated Phase II conditions. However experiments combining both factors in compost have not been done and combined effects of these factors are likely to be more efficacious than either factor alone. However,

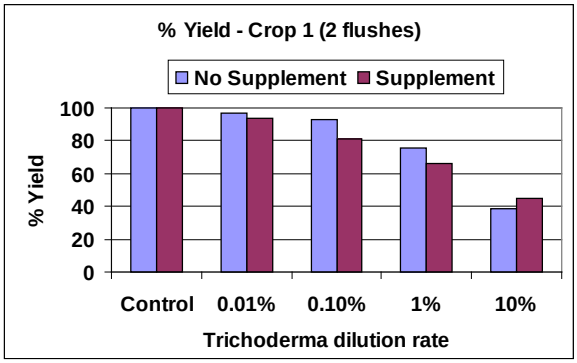
commercial pasteurisation tunnels may have numerous ‘cool zones’ in which *Trichoderma aggressivum* inoculum can survive.

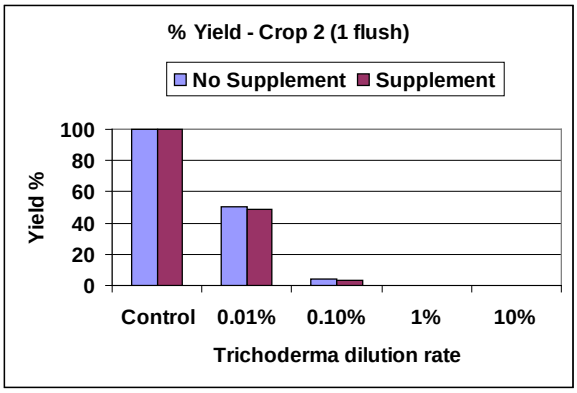
Trichoderma aggressivum (Th2) is known to derive nutrition from the starchy grains used in the manufacture of mushroom spawn. Some level of control was achieved in Canada and the USA when compost spawn and non-grain spawn products were used during spawning rather than standard grain based spawn. Similarly, some facilities are now mixing bulk Phase II with bulk Phase III and results from projects M47 and M49 indicate that this may reduce the incidence of *T. aggressivum* (Th2), providing the bulk Phase III is *Trichoderma*-free. With the increased dependence on bulk Phase III there is a need to determine if non-grain mushroom spawn products offer a control strategy for bulk Phase III systems of production.

A recently completed HDC-funded project (M 48) successfully developed (a) a method to extract *Trichoderma* DNA directly from Phase III compost in conjunction with (b) a molecular test using “real-time TaqMan PCR” technology to detect *T.aggressivum* in Phase III compost. On a small scale, the *T.aggressivum* assay was found to be very sensitive and it was equal to microbiology-based tests but the PCR test result can be achieved within a working day as opposed to the two stage microbiological method that takes at least 8 days. The new assay needs to be verified with samples that are representative of commercial scale production.

Summary of the project and main conclusions

T. aggressivum in Phase III compost

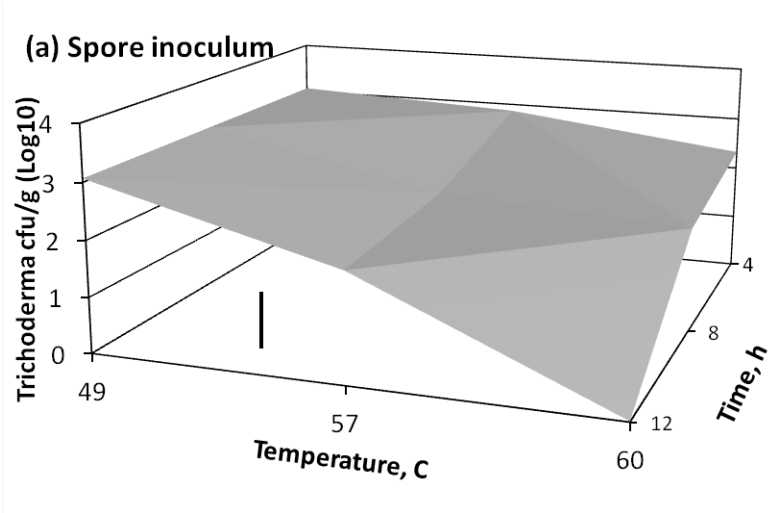




When *Trichoderma* infected compost was mixed and diluted with healthy (uninfected) Phase III compost, there was a significant correlation between the extent of yield loss recorded and how diluted the *Trichoderma*-infected compost was. The more dilute the compost the less the effect on yield loss but a dilution rate of 0.01% caused a 3-6% yield reduction in one experiment and a 50% yield reduction in another. At a 10% dilution rate the yield loss was 40-50% in one crop and 100% in a second crop. Thus the extent of yield loss can be affected by other factors. How heavily colonised the initial *Trichoderma*-infected compost is one factor and how well the infected compost is mixed through the healthy compost is likely to be a second factor. Bulk Phase III compost goes through several mixing stages so it is possible to envisage how a small localised patch of *Trichoderma*-infected compost in a Phase III tunnel could be diluted quite efficiently throughout a sizeable proportion of the compost from the tunnel. Supplementing the Phase III compost as it was being emptied did not have any effect on the severity of the *Trichoderma* which developed although only one supplement was evaluated. *Trichoderma*-infected Phase III compost can infect Phase II compost at spawning.

Trichoderma aggressivum was shown to sporulate sooner and more heavily when it was exposed to the light, as might happen in tray, bag and block spawn-runs in situ, where a grower may visit spawn-running rooms from time to time or rooms may not be totally light-sealed. Thus, *Trichoderma* infections within a Phase III tunnel are likely to be less "visible" although they do contain many spores. In one experiment compost that had been infected with *Trichoderma* and incubated in the light had double the number of *Trichoderma* propagules than compost that was incubated in the dark.

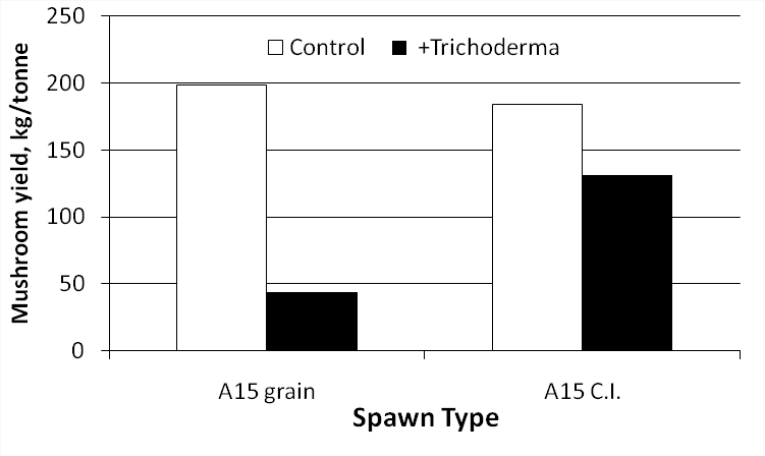
T. aggressivum and pasteurisation conditions



The conditions needed during pasteurisation of Phase I compost to eradicate inoculum of *Trichoderma aggressivum* (Th2) to below a detectable limit were determined to be 60°C for 12 hours. The results showed that both *Trichoderma* spores and *Trichoderma*-infected compost were highly temperature tolerant and survived 57°C for 8 hours. They could also survive in moderately high ammonia concentrations of 300 ppm for several hours. The pasteurisation requirement was not increased for dry (69% moisture) Phase I compost compared with normal (74% moisture) compost. Three types of *Trichoderma* viability testing were used at casing. The detection limit using dilution plating was 10 cfu/g compost. This corresponded with visible *Trichoderma* growth from compost on semi-selective agar, and severe or even complete mushroom yield loss compared with a non-infected control compost.

T. aggressivum and alternative mushroom spawns

The effect of spawning composts with different types of mushroom spawn (grain, Speedy Inoculum™ and commercial casing inoculum (CI) on vulnerability to green mould infection was investigated. Grain spawn and Speedy Inoculum™ were equally susceptible to green mould infection and mushroom yield loss, but casing inoculum was significantly less susceptible.



T. aggressivum and compost analyses

The relationship between compost analysis and susceptibility to green mould infection was investigated. No relationships were found between green mould susceptibility and compost moisture, ash content or pH but compost ammonium nitrogen contents above 0.02% of dry matter increased the likelihood of severe mushroom yield loss resulting from green mould infection.

T. aggressivum detection in Phase III and Phase II compost

Detection of *T. aggressivum* in Phase III compost using the molecular-based "real time Taqman PCR" test was very sensitive and reliable. It was capable of detecting propagules when *Trichoderma*-infected compost was diluted into healthy compost up to a dilution factor of 0.01% (10^{-4}). Microbiological tests (Most Probable Number - MPN and Weed Mould Analysis - WMA) were effective and reliable at detecting *Trichoderma* propagules in Phase III compost when propagule counts were relatively high - 100-1000 propagules per gram fresh weight (gfw). When counts were lower, these methods were variable and false negatives were obtained. This is likely to reflect the difficulty in sampling and subsampling compost that contains a low population of *T. aggressivum* propagules.

Molecular detection of *T. aggressivum* in pasteurised Phase II compost using a real time TaqMan PCR test was inconclusive due to the small number of samples tested.

Microbiological methods were relatively reliable for the detection of *T. aggressivum* in Phase II compost.

Financial benefits

Trichoderma aggressivum can cause yield reductions of up to 100% and although outbreaks can be very sporadic, the impact of an outbreak is very severe. Individual Phase III compost producers in Europe have indicated that compensation to growers for crop loss due to *T. aggressivum* has been in the order of millions of Euro. Thus it makes sense that Phase III compost producers exercise extreme vigilance in the prevention, and early detection of, *T. aggressivum*. This can be achieved through routine testing for *T. aggressivum* and ensuring that Phase II pasteurisation conditions are set at levels known to eradicate *T. aggressivum*, especially in the event of a green mould outbreak.

Action points for growers

Phase I, Phase II

- All pasteurisation tunnels should be examined for cool zones in the compost; tunnel insulation and airflow should be modified accordingly.

- In the event of a green mould outbreak, a pasteurisation temperature of 60°C should be maintained for 10 hours; the effect of a longer (12 hour) pasteurisation on compost quality should also be examined.

- During green mould outbreaks, the use of commercial Casing Inoculum in place of grain spawn should be investigated.

Phase III

- Phase III compost should be monitored for presence of *T. aggressivum* during tunnel emptying. This could be done by placing Petri dishes at strategic locations during emptying and getting any “green mould” cultures identified by FERA or Teagasc. Alternatively, a representative sample of the Phase III compost should be sent to FERA for testing using the sensitive Real Time PCR method.

- Unexpected reductions in yield from bulk Phase III compost should prompt a closer look at the compost for *Trichoderma* green mould. If compost green mould is present, the identity should be confirmed and the Phase III supplier should be notified.

SCIENCE SECTION

Introduction

Trichoderma aggressivum f. europeaum (previously called *T harzianum* type Th2) is an aggressive compost green mould that predominantly infects Phase II compost at spawning. It was a serious problem in the mid 1980's and 1990's but was largely controlled through improved hygiene at spawning and fungicide treatment of spawn when infection risks were high (Seaby, 1996; Grogan & Fletcher, 1993). It has started to appear in bulk Phase III facilities across Europe in recent years (Lemmers, 2010) despite the fact that hygiene levels are generally considered to be much higher than on smaller, less technologically advanced, facilities. These outbreaks of *T. aggressivum* in bulk Phase III raise the question as to whether or not *T. aggressivum (Th2)* behaves differently in the bulk Phase III system compared to in-situ spawn run systems.

There is no precise information on the conditions (temperature, time, compost moisture, ammonia) that are required to eradicate *Trichoderma aggressivum* (spores and mycelium) during compost pasteurisation. Current recommendations for eradication times, temperatures and ammonia concentrations are approximate (Fletcher & Gaze, 2008).

Previous work (Morris et al, 1995a,b) has shown that *Trichoderma aggressivum* (Th2) can be detected in chicken manure, on Phase II prefilters and in spawning halls. Other work has shown that *Trichoderma aggressivum* (Th4) spores can withstand up to 200 ppm gaseous ammonia and 60 °C for 9 hours under laboratory conditions, and 60 °C for 10 hours under simulated Phase II conditions (Morris et al, 2000; Rinker & Alm, 2000). However experiments combining both factors in compost have not been done and combined effects of these factors are likely to be more efficacious than either factor alone. However, commercial pasteurisation tunnels may have numerous 'cool zones' in which *Trichoderma aggressivum* inoculum can survive.

Trichoderma aggressivum (Th2) is known to derive the bulk of its nutrition from the starchy grains used in mushroom spawn (Fletcher, 1997). Some level of *Trichoderma* green mould control was achieved in Canada when a non-grain spawn product was used during spawning rather than standard grain based spawn (Rinker 2000). Similarly some facilities are now mixing bulk Phase II with bulk Phase III and this may reduce the incidence of *T. aggressivum (Th2)*, providing the bulk Phase III is itself *Trichoderma*-free. With the increased dependence on bulk Phase III there is a need to determine if non-grain mushroom spawn products offer a control strategy for bulk Phase III systems of production.

The taxonomy of the genus *Trichoderma* is complex and many *Trichoderma* species are difficult to identity to species level based on microscopic examination of morphological characteristics. Molecular PCR-based techniques have been used to differentiate between the ubiquitous *T. harzianum* (Th1) and *T. aggressivum*, previously known as *T. harzianum* (Th2) and this method is useful for identifying pure cultures of *T. aggressivum* isolated from mushrooms or compost (Chen et al. 1999) but it is not very successful for detecting *T. aggressivum* in compost samples.

A recently completed HDC-funded project M 48 (Lane, 2010) successfully developed a method to extract *Trichoderma* DNA directly from Phase III compost. Real-time PCR primers and probe for *T.aggressivum* were also developed (using the translocation elongation factor gene). It was not possible to develop an assay for *T.harzianum* due to the complexity of this species group. The *T.aggressivum* assay was found to be as sensitive as plating out coupled with a standard PCR assay but importantly could be achieved within a working day as opposed to the existing two stage method taking at least 8 days. The real-time PCR test can be semi-automated to help reduce time and costs and is also semi-quantitative. Levels of *Trichoderma* can be determined to at least a 4,500 propagules per gram fresh weight and potentially 10 fold times more dilute (450 propagules/gfw; equivalent to only a few conidial heads of *Trichoderma*). The new assay has been used to directly test several compost samples from commercial premises for *T. aggressivum* successfully and it will be used as a diagnostic method during the course of this project.

The commercial objectives of this project are therefore:

1. To enhance our understanding of how *Trichoderma aggressivum (Th2)* may behave in bulk Phase III systems of production
2. To identify the eradication conditions for *T. aggressivum (Th2)* during the pasteurisation phase
3. Investigate the potential of non-grain-based mushroom spawn as a control strategy for *T. aggressivm*

To achieve this, the work was broken down into three subsections covering six scientific objectives as follows;

Sub-Section 1: *T. aggressivum* epidemiology in Phase II and Phase III compost

Objective 1. Determine the ability of *T. aggressivum (Th2)* mycelium to infect Phase II compost at spawning.

Objective 2. Characterise the effect of mixing different quantities of *T. aggressivum*-infected Phase III compost into un-infected Phase III compost on the productivity of the compost, with and without supplementation

Sub-Section 2: Compost pasteurisation and agronomy aspects to *T. aggressivum* epidemiology

Objective 3. Determine the eradication conditions for *Trichoderma aggressivum (Th2)* during compost pasteurisation (temperature, time, compost moisture, ammonia) that are required to eradicate *Trichoderma aggressivum (Th2)*.

Objective 4. Test the susceptibility of compost, spawned using non grain-spawn products mixed with Phase II to infection by *T. aggressivum (Th2).*

Sub-Section 3: *T. aggressivum* detection

Objective 5. Confirm that *Trichoderma aggressivum (Th2)* detection methods are effective

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Sub-Section 1: T. aggressivum epidemiology in Phase II and Phase III compost

Objective 1. Determine the ability of *T. aggressivum (Th2)* mycelium (infected compost) to infect Phase II compost at spawning.

Objective 2. Characterise the effect of mixing different quantities of *T. aggressivum*-infected Phase III compost into un-infected Phase III compost on the productivity of the compost, with and without supplementation.

Materials and methods

Inoculum preparation

For Experiment 1 small quantities of inoculum were prepared. Three mushroom spawn grains (Sylvan A15) were coated with *T. aggressivum* spores **(strain FM10)** and placed in the bottom of screw-topped sterile pots (50 mL). Three clean spawn grains were also added. The pots were filled with 30 g of Phase II compost (Kaybeyun Compost Ltd., Monaghan, Ireland). Half the pots were wrapped in tinfoil to exclude light and the remainder were left unwrapped and allowed exposure to laboratory lighting. All pots were incubated at 25 °C for 3 weeks. Two replicates were prepared for each treatment.

For Experiment 2, larger quantities of inoculum were prepared. Pre-spawned (Sylvan A15) Phase II compost was acquired from Kaybeyun compost Ltd., Monaghan, Ireland and used to generate *T. aggressivum* inoculum. One crate (60 x 40 x 30 cm) was filled with 18 kg of compost. Six spawn grains (Sylvan A15) were coated with *T. aggressivum* spores **(strain FM10)** and were placed in the centre of the crate. The crate was spawn run for 17 days under standard conditions. The *Trichoderma*-infected compost was then taken out and mixed by hand to provide a more uniform inoculum. *Trichoderma* inoculum was weighed out in advance prior to incorporation into Phase II or Phase III compost at rates of 10^{-1} to 10^{-6}.

A weed mould analysis (WMA) was done on the compost inoculum to determine the number of *Trichoderma* propagules present per gram fresh weight of the primary inoculum. A 20 g compost sample was blended in 200 mL H_2O, serially diluted and Trichoderma colonies counted. A second, simpler, method based on the Most Probable Number technique was also used. The same WMA serial dilution of compost samples was used. Ten drops of 10 µl were placed on two malt agar Petri dishes (5 per dish) for each dilution.

Positive Trichoderma counts out of 10 were recorded for each dilution and the results checked against MPN tables to give an estimate of the count. Count data was $\log_{10}$ transformed prior to statistical analysis to calculate the mean, standard deviation, variance and co-efficient of variation. Any zero values were give a nominal value of 10 prior to $\log_{10}$ transformation so that a reading of 1 on the Log scale = zero propagules in reality.

Experiment 1. Determining the ability of *T. aggressivum (Th2)* mycelium (infected compost) to infect Phase II compost at spawning.

Fifty grams of *Trichoderma* infected compost was combined from 2 pots of inoculum, prepared in either the presence or absence of light, were added to 450 g of Phase II compost and gently mixed to distribute the infected compost throughout the compost to give a 10^{-1} diluted sample. Once mixed, a 50 g sub sample of the 10^{-1} sample was taken and added to a further 450 g, to give a 10^{-2} diluted sample. This dilution series was continued up to 10^{-6}.

A compost extraction was performed for each compost dilution sample whereby 20 g of compost was soaked for 1 hour in 200 ml water then blended for 1 minute, rested for 5 minutes, blended again for 1 minute then a 5 ml aliquot removed for serial dilution onto OAES medium (Kaufmann et al. 1963). *Trichoderma* recovery was recorded for each dilution level. Colonisation of the Phase II compost by *Trichoderma* and Agaricus was assessed at the end of the spawn-running period.

Experiment 2. Characterisation of the effect of *T. aggressivum*-infected compost added to un-infected Phase III compost on productivity, with and without supplementation.

Crop preparation

Fully spawn-run (Phase III) compost was bulk handled to simulate bulk Phase III conditions and weighed out into 18 kg quantities. Half of the compost was supplemented with Nutrigain supplement at a rate of 0.75% and the remaining half was left unsupplemented. When all experimental treatments were applied, the compost was filled into crates measuring 0.6 x 0.4. x 0.3 m (l x b x d). Crates of compost were cased using Harte peat casing (50 L bag mix) to which casing inoculum had been incorporated at the standard rate.

The casing was also treated with the parasitic nematode product Nemasys at the standard rate.

Crop inoculation

Trichoderma-infected compost-inoculum was added to the spawn-run supplemented and unsupplemented Phase III compost at rates of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} as outlined in Table 1. Control plots were left uninfected.

Table 1. Inoculation rates and quantities

Trichoderma Inoculation rate	% Trichoderma-infected compost added	Phase III compost weight (kg)	Inoculum weight (kg)
0 (Control)	0	18	0
10^{-1}	10%	16.2	1.8
10^{-2}	1%	17.82	0.18
10^{-3}	0.1%	18	0.018
10^{-4}	0.01%	18	0.0018

Once the inoculum was added and mixed into the Phase III compost, a subsample was taken to determine the propagule count following dilution of the inoculum into fresh Phase III compost. A sub- sample was also sent to FERA for analysis by real time PCR.

Trichoderma assessments.

The number of *Trichoderma* propagules/gfw was calculated using sub-samples of the compost-inoculum as well as subsamples of compost dilution treatments taken after the inoculum had been added and mixed through at different rates. Two microbiological methods were used: a Most Probable Number (MPN) test and a weed mould analysis (WMA) test.

The MPN test was based on presence or absence of *Trichoderma* growth in 10 x 10 µl drops of each dilution of a serially diluted suspension prepared from the compost sample. This gave a score of from 0 to 10 for each dilution. The results for the three dilutions which gave scores that ranged from 10 to 0 were selected (e.g. if results for dilutions 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} = 10, 10,10, 10, 7, 2 then the last 3 numbers (10, 7, 2) are selected and used in conjunction with MPN tables to give a MPN value for this sample of 1.33×10^{9}) (Halvorson & Ziegler, 1932). Presence of *T. aggressivum* was tested qualitatively by means of a direct plating test.

The WMA test is described in Sub Section 2 below.

Records.

Mushrooms were harvested from each plot as closed cups over two flushes.

Statistical design and analysis

Each treatment combination was replicated 4 times to give a total of 40 plots (5 infection treatments x 2 supplement treatments x 4 replicates). These plots were positioned in a mushroom growing room according to a random block design. Data were subjected to an analysis of variance (ANOVA) to identify any significant treatment effects or interactions.

The experiment was repeated a second time to confirm results.

Results and discussion

Experiment 1. The ability of *T. aggressivum* (Th2) mycelium to infect Phase II compost at spawning.

Growth of *Trichoderma aggressivum* incubated in the light and the dark.

Trichoderma aggressivum sporulated more heavily when incubated in the light compared to incubation in the dark. This occurred whether or not the culture was on an agar plate (Figure 1) or on compost (Figure 2). None the less significant sporulation still occurred in the dark. These results suggest that the colonisation of compost by *T. aggressivum* in a Phase III tunnel may be less noticeable than if the compost was exposed to light as can happen with in situ spawn-runs in trays, bags and blocks.

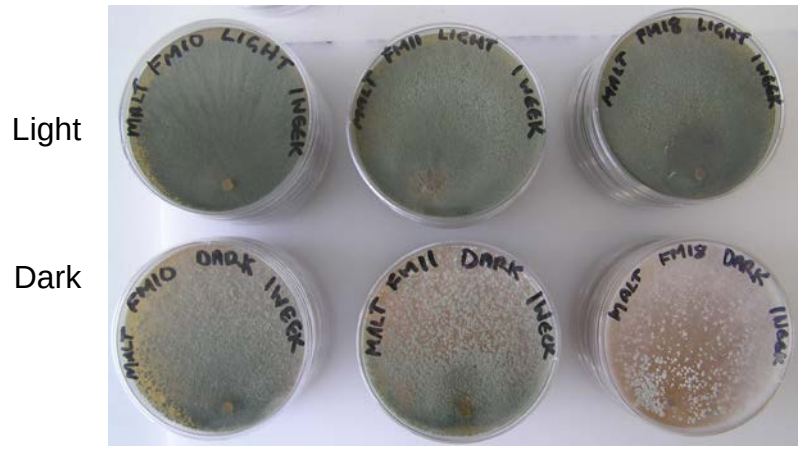


Figure 1. *Trichoderma aggressivum* grown on malt agar and incubated in the light or dark for 7 days

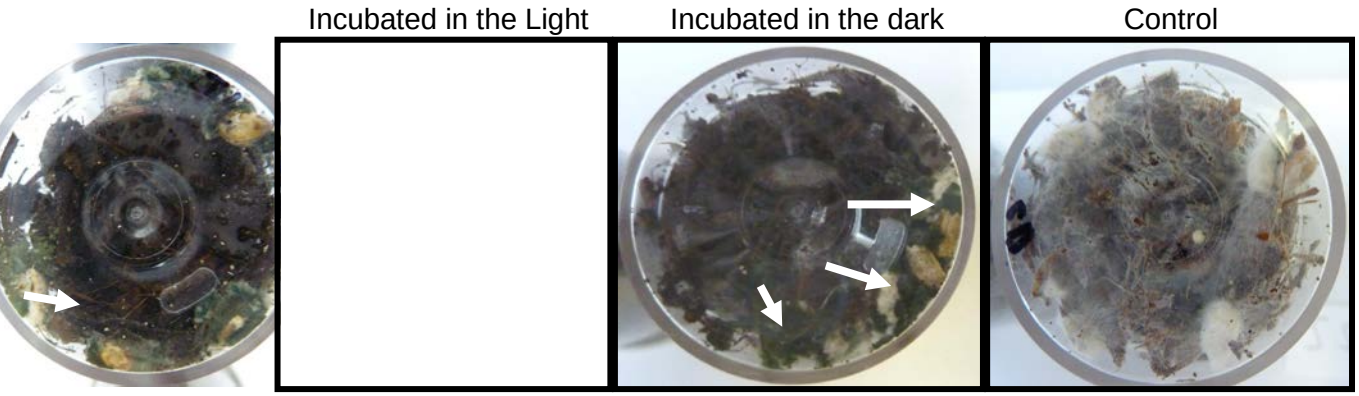


Figure 2. Colonisation of Phase II compost in pots with *Trichoderma aggressivum* after incubation in the light or dark for 3 weeks.

Detection of propagules in Phase II compost.

Trichoderma aggressivum propagules were detected in all Phase II compost samples when infected compost was diluted into it up to a dilution of 1 in 10^6 (1 g in a tonne). From 5 to 10 times fewer propagules occurred when the *Trichoderma*-infected compost had been incubated in the dark (Table 2).

Table 2. Number of *Trichoderma* propagules (colony forming units – cfu's) per gram fresh weight (gfw) detected in Phase II compost following incorporation of *Trichoderma*-infected compost, incubated in either the light or in darkness, at different rates.

	Trichoderma propagules detected cfu's/gfw	
Inoculum Incubated in:	**Light**	**Dark**
Dilution rate: Undiluted	>1 x $10^{9\ \#}$	>0.1 x $10^{9\ \#}$
10^{-1}	>1 x $10^{8\ \#}$	>0.1 x$10^{8\ \#}$
10^{-2}	>1 x $10^{7\ \#}$	>0.1 x$10^{7\ \#}$
10^{-3}	>1 x $10^{6\ \#}$	>0.1 x$10^{6\ \#}$
10^{-4}	>1 x $10^{5\ \#}$	0.1 x10^5
10^{-5}	1 x 10^4	0.14 x10^4
10^{-6}	2.1 x 10^3	0.4 x 10^3

= too many to count

Table 3. Growth of *Trichoderma* and *Agaricus* at the end of spawn-run following inoculation with *Trichoderma*-infected compost at spawning that had been incubated in either the light or dark

	Presence of *Trichoderma* sporulation (T) and *Agaricus* growth (Ag) at the end of spawn run			
Inoculum Incubated in:	**Light**		**Dark**	
Dilution rate : 10^{-1}	T +++	Ag 0	T +++	Ag 0
10^{-2}	T +++	Ag +	T +++	Ag 0
10^{-3}	T +	Ag +++	T +	Ag ++
10^{-4}	T +++	Ag +	T +	Ag ++
10^{-5}	T +	Ag ++	T 0	Ag ++
10^{-6}	T +++	Ag +	T 0	Ag +++

0 = no growth; + poor, ++ = moderate; +++ good.

Trichoderma development during spawn-run following inoculation with *Trichoderma* infected compost at spawning.

The development of *Trichoderma* green mould in the inoculated compost dilution series was variable (Table 3) in response to increasing levels of dilution of *T. aggressivum*. Less *Trichoderma* was visible at the higher dilutions when the inoculum used had been incubated in darkness but *Agaricus* growth was still only moderate. *Trichoderma* sporulation was usually less when *Agaricus* growth was moderate to good. *Agaricus* growth was consistently poor to absent at dilution rates of up to 10^{-2} but it was more variable at higher dilutions rates. These results suggest that *Trichoderma*-infected compost is capable of transmitting *T. aggressivum* and reducing *Agaricus* growth even when present at very low levels of between 10^{-3} to 10^{-6} (0.1% to 0.0001%).

Experiment 2. The effect of *T. aggressivum*-infected compost added to healthy Phase III compost on productivity, with and without supplement.

Trichoderma Inoculum.

The Number of Trichoderma propagules/gfw present in the fully infected inoculum used in the experiments was determined by either the Most Probable Number (MPN) method or Weed Mould Analysis (WMA) method (Table 4). There was an approximate 2-3 fold difference in the propagules/gfw between the two inocula with fewer propagules being present in Crop 1 inoculum compared to Crop 2 inoculum. Crop 1 inoculum looked less well colonised by Trichoderma than crop 2 inoculum. The two methods to determine the propagule numbers gave similar results although the variance was high but this is to be expected with a compost based inoculum.

Table 4. Number of propagules/gfw detected in the *Trichoderma*-infected compost inoculum used in Crops 1 and 2. Statistical analysis was conducted on Log_{10} transformed data.

	Crop 1	Crop 2	
	MPN	**WMA**	**MPN ***
	9.36×10^8	1.9×10^9	1.33×10^9
	4.74×10^8	2.6×10^9	
	13.3×10^8	1.9×10^9	
	3.99×10^8		
	7.92×10^8		
Average count / gfw	7.15×10^8	2.10×10^9	1.33×10^9
(log_{10} value)	(8.854)	(9.293)	
(Variance)	(0.046)	(0.0006)	
(Standard deviation)	(0.214)	(0.025)	

* only one MPN count available for Crop 2 inoculum

Crop Yields

There was a significant reduction in yield for all *Trichoderma* dilution rates in Phase III compost in both Crop 1 and Crop 2 with the effect being much more severe in Crop 2 for the same dilution rates (Figures 3-7). In both crops the effect was less severe, the more dilute the rate of *Trichoderma* inoculum added. There was no significant impact of supplementation on Control yields or on the severity of *Trichoderma*-related crop loss.

When yield was expressed as a % of the control, the yields from the Trichoderma treatments ranged from 38 to 96% in Crop 1 and from 0 to 51% in Crop 2 (only one flush was harvested in Crop 2 due to unforeseen circumstances).

The incorporation of relatively low levels of *Trichoderma*-infected compost (0.01%) into healthy Phase III compost in Crop 2 had a very severe impact on yield compared to Crop 1. The number of *Trichoderma* propagules in the inoculum used in Crop 2 was 2-3 times more than what was in the inoculum for Crop 1 (Table 4) and this may account for some of the difference between the Crops. In addition there was an extra level of mixing introduced in Crop 2, in order to better recreate what happens during Phase III tunnel-emptying and transport and this additional mixing may also have contributed to the differences observed between the two crops.

These results indicate that even a small pocket of *Trichoderma* infected compost in a Phase III tunnel has the potential to affect a much greater proportion of that compost as a result of various opportunities for mixing and diluting the infected compost into the un-infected compost. Under these circumstance the *Trichoderma* infected compost is unlikely to be

"visible" therefore no alert will be raised. Furthermore there is a distinct possibility that growers receiving compost from one area of the tunnel may crop very well while growers receiving compost from a more contaminated batch of compost may experience total yield loss leading to a false conclusion that the compost is not the source. These results suggest that continuous monitoring for the presence of *T. aggressivum* is highly desirable for Phase III producers in order to pick up when low levels of the mould start to occur. Early detection would allow a more rapid response to try and detect where the weakness are that allowed the infection to occur in first place.

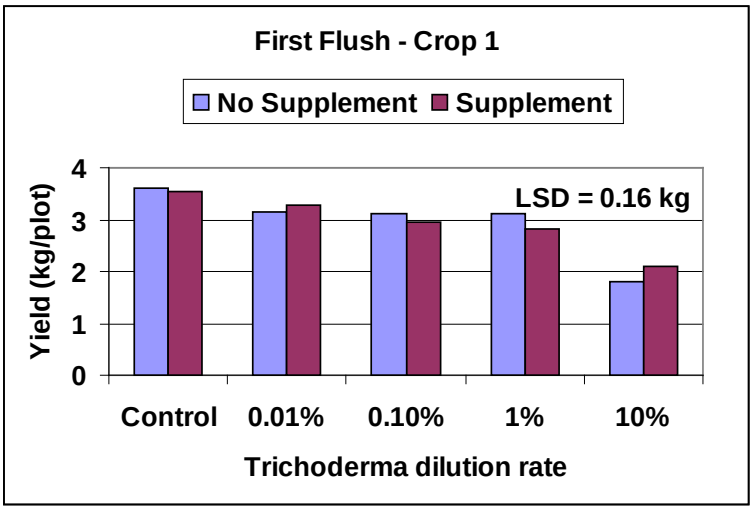


Figure 3. First flush yield in response to the incorporation of *Trichoderma*-infected compost at the end of spawn-run at different rates (Crop 1)

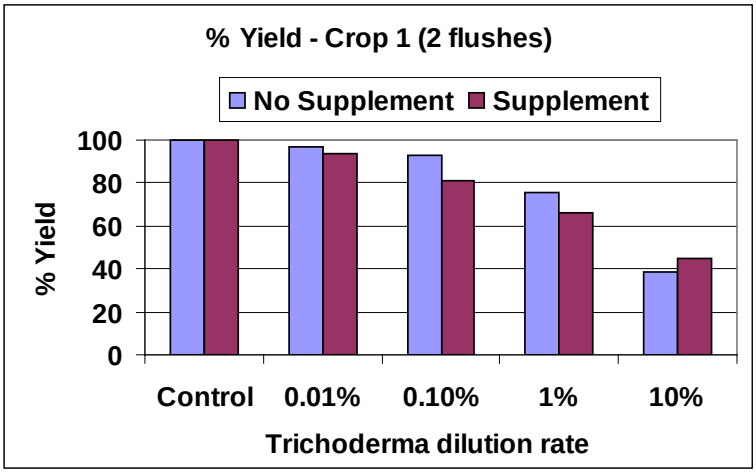


Figure 4. Yield as a % of control over two flushes in response to the incorporation of *Trichoderma*-infected compost at the end of spawn-run at different rates (Crop 1)

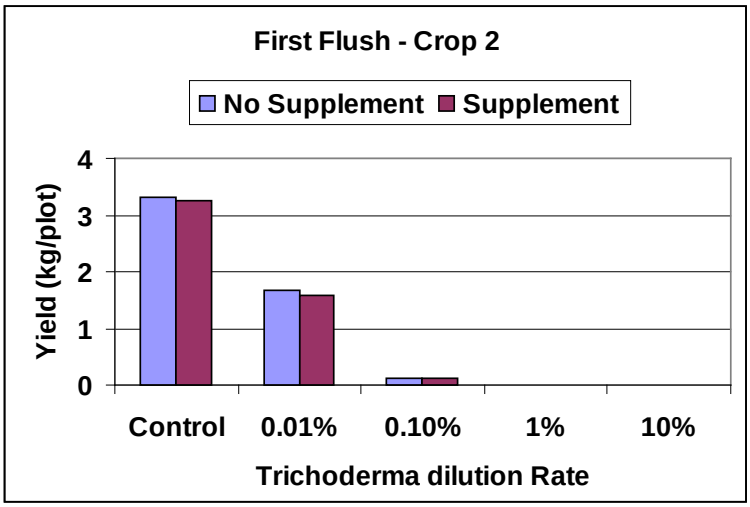


Figure 5. First flush yield in response to the incorporation of *Trichoderma*-infected compost at the end of spawn-run at different rates (Crop 2)

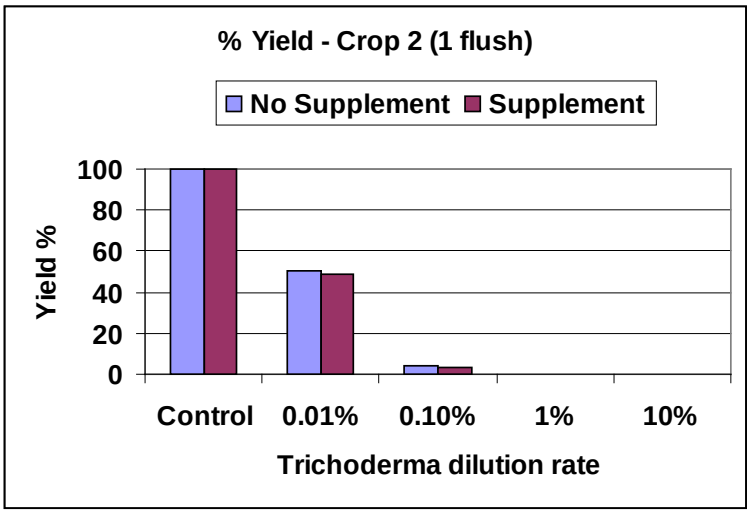
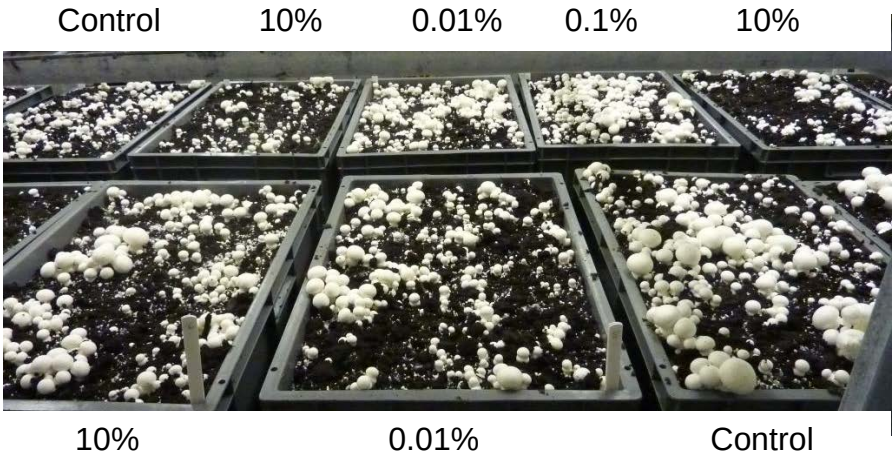


Figure 6. Yield as a % of control over tone flush in response to the incorporation of *Trichoderma*-infected compost at the end of spawn-run at different rates (Crop 2)

First Flush - Crop 1



First Flush - Crop 2

Figure 7. First flush plots for crop 1 and crop 2 with different rates of *Trichoderma* compost inoculum added at the end of spawn-run.

Conclusions

- *Trichoderma aggressivum* appears to sporulate less heavily when infected compost is incubated in total darkness
- *Trichoderma aggressivum* can be detected in compost by microbiological means even when diluted by up to 1 in 10^6

- When *Trichoderma aggressivum* inoculum is in the form of infected compost fragments, it is capable of colonising and sporulating on compost even when very dilute although the effects in the most diluted situations are more variable.
- *Trichoderma* infected compost can cause yield reductions of up to 100% when it is diluted into healthy Phase III compost.
- The more dilute the infection, the less the impact on crop yield but dilution rates of 1 in 10^4 (0.01% or 100 g in a tonne) can, under certain conditions, cause a 50% crop reduction.
- The magnitude of the yield reduction is likely to be influenced by
    - The number of *Trichoderma* propagules on the infected compost
    - The extent of mixing that occurs
    - Compost quality may also affect the severity of symptoms

Sub-Section 2: Compost pasteurisation and agronomy aspects to T. aggressivum epidemiology

Objective 3. Determine the eradication conditions for *Trichoderma aggressivum* (Th2) during compost pasteurisation (temperature, time, compost moisture, ammonia) that are required to eradicate *Trichoderma aggressivum* (Th2).

Objective 4. Test the susceptibility of compost, spawned using non grain-spawn products mixed with Phase II to infection by *T. aggressivum* (Th2).

Materials and methods

Objective 3: Eradication conditions for *Trichoderma aggressivum* (Th2) during compost pasteurisation

Production and inoculation of composts

Phase I composts were prepared in windrows (17 day pre-wet and phase I) as described in Noble et al (1998). The standard compost was prepared from new season wheat straw with broiler poultry manure and gypsum added at 600 kg and 70 kg per tonne of straw respectively. Fresh water was used throughout to produce compost with a phase I moisture content 74±1% (except where stated). Analyses were conducted on samples of the phase I and Phase II composts. Dry matter (DM), N, ammonium (NH^+_4) and ash contents and pH were determined as described in Noble and Gaze (1998).

Except where stated, the *Trichoderma aggressivum* (Th2) isolate 23443B obtained from FERA York, was used. *Trichoderma* spore suspensions containing 1×10^7 spores/mL were prepared by flooding cultures produced on agar plates and exposed to light to encourage sporulation. The suspensions (35 mL) were then sprayed into 10 kg phase I compost contained within plastic bags, which were closed and shaken periodically during spraying to give a concentration of inoculum of 3.5×10^4 spores per gram fresh weight. The same volume of sterile water was used as a control.

Where infected compost inoculum was used, clean Phase II compost was spawned with *Agaricus bisporus* spawn (Sylvan A15) at 0.5% w/w and inoculated with a *Trichoderma* spore suspension in plastic bags as described above. The compost was then incubated at 25°C for 16 days. The bags were then exposed to light for several days until green spores became visible. The compost inoculum therefore contained both *Trichoderma* mycelium and

spores. The infected compost inoculum had a *Trichoderma* propagule count of 10^5 cfu/g and was added at 10% w/w to phase I compost.

Phase II composting was conducted in 'Quickfit' multiadapter flasks immersed in thermostatically controlled water baths, each holding two 10-L flasks (Noble et al, 1997). The prepared Phase I composts (about 3.3 kg samples) were placed on a perforated stainless steel platform within each flask and the flasks immersed in the waterbaths such that the water level was above the level of the enclosed substrate. Each flask was connected to ancillary equipment providing independent aeration of the compost. The oxygen concentration in the substrate was controlled regularly by adjusting the airflow through the compost in each flask within the range 8-16 L kg^{-1} substrate h^{-1} by means of flow meters. The temperature of the substrate in the flasks was monitored with Squirrel multipoint temperature loggers (Grant Instruments Ltd, Cambridge, UK).

For the first 48 hours of the Phase II composting process, the compost temperature was controlled at 49°C to allow a natural succession and gradual build-up of microorganisms. The substrate temperature was then increased to the required pasteurisation temperature for the required time. The temperature was then reduced to 49°C for the remainder of the composting period, which was between five and six days, until the air in the flask was clear of ammonia. A minimum oxygen concentration of 11 (± 1.5)% v/v was maintained in the substrate. The material in each flask was then sampled (2 × 50 g samples per flask) and analysed for viable *Trichoderma* inoculum and spawned with mushroom spawn as described later.

Effect of pasteurisation treatments on *Trichoderma*

1. Pasteurisation temperature and time

The effect of three pasteurisation temperatures (49, 57 and 60°C) × three pasteurisation times (4, 8 and 12 hours) was examined on phase I compost containing either *Trichoderma* spore or infected compost inoculum as described above. Phase I composts without *Trichoderma* inocula were used as control treatments. For each temperature × time × inoculum type treatment, three replicate flasks were prepared.

2. *Trichoderma aggressivum* (Th2) isolates

The following three *Trichoderma aggressivum* (Th2) isolates were used:

(a) 23443B FERA York
(b) T32 Warwick HRI
(c) T40 Warwick HRI

Three replicate flasks of each inoculum treatment, together with an uninoculated control treatment were prepared. Composts were pasteurised at 57°C for 8 hours.

3. Compost moisture content and ammonia concentration

Phase I compost was prepared with the standard moisture content (74% w/w) and also a dry treatment (69% w/w). *Trichoderma aggressivum* (Th2) isolate 23443B spore suspensions containing 1×10^7 spores/mL (35 mL) were then sprayed into 10 kg phase I compost samples contained within plastic bags. The same volume of sterile water was used as a control. The composts were then pasteurised in three replicate flasks for 60°C for 12 hours as described previously.

The ammonia during all of the above pasteurisation treatments was measured with Draeger gas detector tubes.

Cropping procedure

At the end of the flask composting period, compost from each flask (3 kg) was spawned at 0.5% w/w of the fresh weight of compost with *Agaricus bisporus* rye grain spawn (Sylvan A15) and filled into plastic pots, 230 mm diameter x 220 mm depth, covered with paper and spawn-run at 25°C. The pots were positioned on three bench areas or 'blocks'; each block contained a replicate pot of each treatment. After 16 days, the pots were cased with a moist mixture of peat and sugar beet lime (850 g), watered, and again covered with paper. When mushroom mycelium was visible on the surface of the casing, the air temperature was reduced to 18°C, and a relative humidity of 90% and a CO_2 concentration of 0.1% to induce fruiting were maintained. Three flushes of mushrooms were harvested daily over a 17 day period (cap diameter 25-30 mm). The number and weight of mushrooms from each pot was recorded.

***Trichoderma* analysis of composts**

Samples of compost were analysed for *Trichoderma* populations at spawning and at casing. The procedure for analysis is described in Grogan & Harvey (1999). For each compost sample, a 20 g sub-sample was put in a sterile homegeniser bag with 360 mL sterile water. After soaking for 1 hour, the sample was homogenised in a 'Stomacher 400' laboratory blender for 2 × 1 min with a 5 min interval. The resulting compost extract was then serially diluted with sterile water to give a concentration of 1×10^0 to 1×10^5. A 1-mL aliquot of each dilution was pipetted into a series of sterile Petri dishes. For low concentrations, a 3-mL aliquot of diluted compost extract was used. Molten Ohio Agricultural Experimental Station medium, held at 50˚C, was then poured into the dishes, which were then incubated at 25˚C.

The numbers of colonies were recorded after 3, 5 and 7 days, which were then used to calculate the number of colony forming units (cfu) per g fresh weight of compost. Three replicate pieces of compost from the samples were also plated out on malt agar + Streptomycin + Chloramphenol and kept at 25˚C. The growth of *Trichoderma* species was assessed under a binocular microscope after 7 days as follows: 0 no *Trichoderma* present; 1 *Trichoderma* just detectable; 2 moderate *Trichoderma* growth and/or sporulation visible; 3 extensive growth and sporulation of *Trichoderma*.

Materials and methods

Objective 4: Susceptibility of non grain-spawn products to infection by *T. aggressivum* (Th2)

Production and inoculation of composts

Phase II composts were produced at WHRI in bulk tunnels and were also obtained from five commercial sites. The composts (10 kg samples) were inoculated at spawning with 35 mL of suspensions containing 2×10^4 spores/mL of *Trichoderma aggressivum* (Th2) isolate 23443B as described previously. The same volume of sterile water was used as a control. After spawning, the composts were kept in the plastic bags for 16 days and the temperature was maintained at 25°C. The material from each bag was then filled into three replicate plastic pots, each containing 3 kg of spawn-run compost. The mushroom cropping procedure and the *Trichoderma* analysis of compost samples at casing were then similar to those previously described.

Spawning treatments

1. Grain spawning rate

Four spawning rates (0.25, 0.5, 0.85 and 2% w/w) of grain spawn of the strain Sylvan A15 were used with and without spore inoculum of *Trichoderma aggressivum* (Th2) isolate 23443B. The comparison was made using Phase II compost from Warwick HRI and from five commercial composting sites.

2. Spawn strain and type

Lambert Speedy Inoculum™ (strain L901) was compared with grain spawn of strains L901 and A15, with and without inoculum of *Trichoderma aggressivum* (Th2) isolate 23443B. The

comparison was made using Phase II compost from Warwick HRI and from five commercial composting sites.

3. Casing Inoculum

Casing inoculum was compared with grain spawn (both Sylvan strain A15) at a rate of 0.5% w/w, with and without spore inoculum of *Trichoderma aggressivum* (Th2) isolate 23443B. The comparison was made using Phase II compost from Warwick HRI

Effect of compost factors on susceptibility to yield loss from *Trichoderma*

All the composts used in the experiments were analysed for nitrogen, ammonium nitrogen, ash and moisture contents, and pH as described in Noble and Gaze (1998). The effect of different compost analytical factors on yield loss caused by a standard spore inoculum of *Trichoderma aggressivum* (Th2) isolate 23443B was examined.

Results

Eradication conditions for *Trichoderma aggressivum* (Th2) during compost pasteurisation:

1. Pasteurisation temperature and time

Without a definite pasteurisation stage (i.e. compost maintained at 49°C through the Phase II flask composting process) high levels of *Trichoderma* isolate 23443B were detected using both the dilution plating of compost extracts and the plating of compost samples on selective agar (Figures 8 and 9). Without a pasteurisation stage, compost inoculated with *Trichoderma* infected compost resulted in a ×10 higher *Trichoderma* count at casing than compost inoculated with the spore suspension (Figures 8a and 8b). The corresponding plate scores (Figures 9a and 9b) were also higher for the infected compost inoculated treatment than for the spore suspension inoculum. Increasing the pasteurisation temperature from 49 to 60°C and the duration from 4 to 12 hours both progressively reduced the viability of the *Trichoderma* spore and infected compost inocula (Figures 8 and 9). A similar trend was observed when the viability was assessed using a dilution plating of compost extract or by plating of compost on to selective agar (Figures 8 and 9). The spore and infected compost inocula were not reduced to below their detection limits unless the compost was pasteurised for 12 hours at 60°C.

Mushroom yield from the clean Phase II composts were 187 ± 11 g/kg and were not significantly affected by the pasteurisation treatments in the absence of *Trichoderma*

inoculum. Without a definite pasteurisation stage, the *Trichoderma* spore inoculum resulted in more than 60% yield loss compared with the clean control compost (Figure 10a). No mushrooms were harvested (i.e. 100% yield loss) when no pasteurisation stage was used on phase I compost inoculated with infected compost inoculum (Figure 10b). The mushroom yield loss resulting from spore and infected compost *Trichoderma* inocula became progressively smaller as the pasteurisation temperature and time were increased.

The results in Figures 8-10 indicate that the eradication conditions for the spore inoculum and infected compost inoculum are similar (60°C for 12 hours), in spite of the infected compost inoculum producing a ×10 higher *Trichoderma* count in unpasteurised compost than the spore suspension inoculum. Figures 8-10 also show that the detection limit for *Trichoderma aggressivum* (Th2) in compost based on dilution plating, growth from compost on semi-selective agar, or mushroom yield loss were similar.

2. *Trichoderma aggressivum* (Th2) isolates

Isolates T32 and T40 were not detectable after a pasteurisation of 57°C for 8 hours. There was no significant mushroom yield loss resulting from a spore inoculum in spawned compost. However, green spores were visible on the casing one of the three pots inoculated with T32 at the end of the third flush.

3. Compost moisture content and ammonia concentration

No viable *Trichoderma* inoculum was detected in the 'normal' or 'dry' composts following pasteurisation at 60°C for 12 hours. Mushroom yields from composts inoculated with *Trichoderma aggressivum* (Th2) 23443B and control composts were not significantly different following this pasteurisation treatment. There was also no significant difference in mushroom yield between 'normal' and 'dry' composts (mean mushroom yield 163 ± 16 g/kg spawned substrate).

Ammonia concentrations during all the pasteurisation treatments ranged from 0 to 500 ppm (Figure 11). This was as a result of both the natural variability of the phase I composts used, and the pasteurisation treatments, with a tendency for the highest temperature (60°C) to produce higher ammonia concentrations than the lowest temperature (49°C). Viability of *Trichoderma* inoculum (spores and infected compost) was reduced by increasing ammonia concentration during pasteurisation (Figure 11). However, both types of *Trichoderma* inocula were able to survive 300 ppm ammonia; no *Trichoderma* survival was detected in a spore inoculated compost exposed to 500 ppm ammonia although only one compost sample reached this concentration.

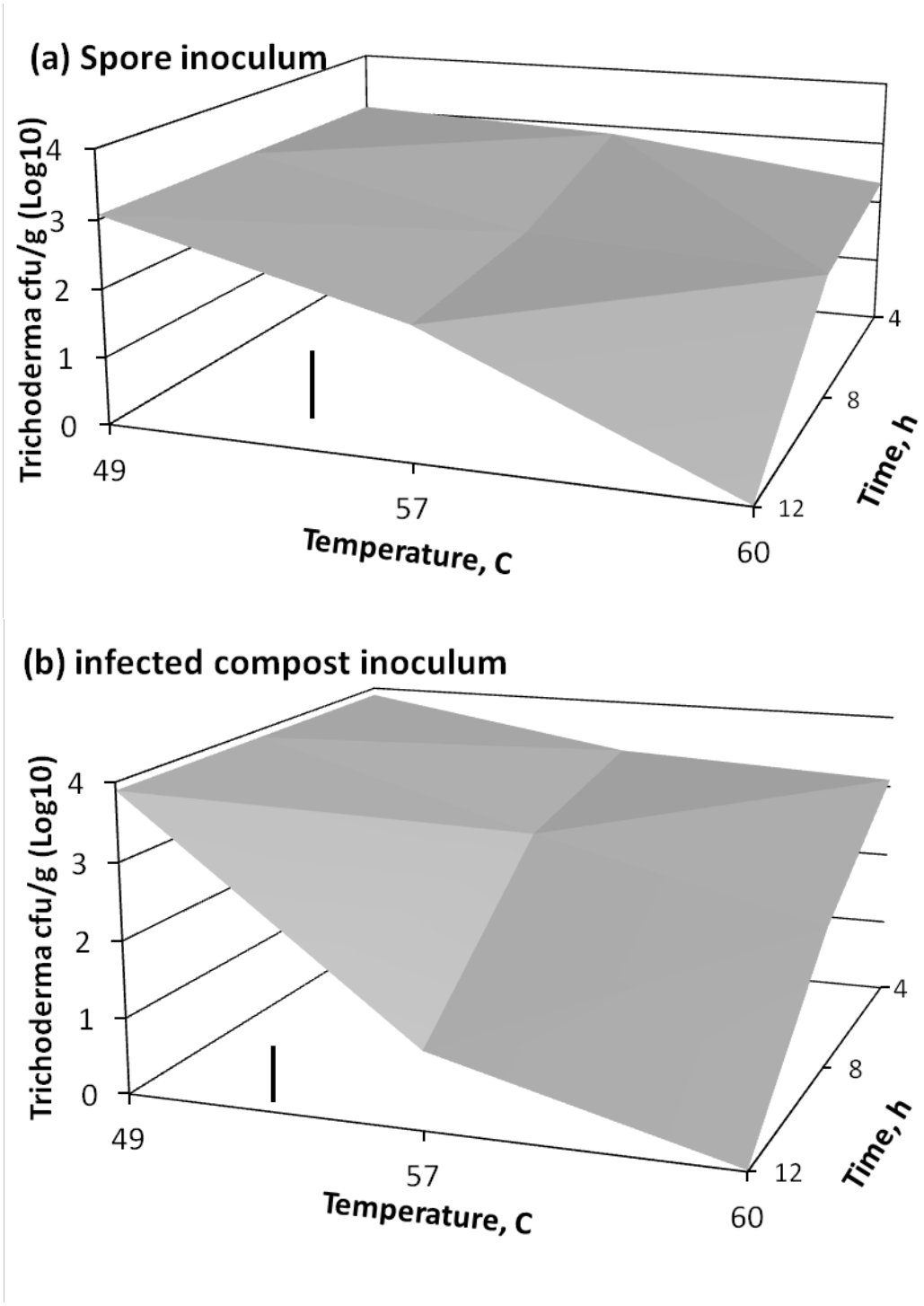


Figure 8. Effect of pasteurisation temperature and time on *Trichoderma aggressivum* (Th2) 23443B in compost using (a) spore and (b) infected compost inocula. Viability assessed at casing by dilution plating of compost extract. Each point is the mean of three replicate composting flasks and two compost samples per flask. Bar indicates LSD between temperature and time treatments ($P = 0.05$).

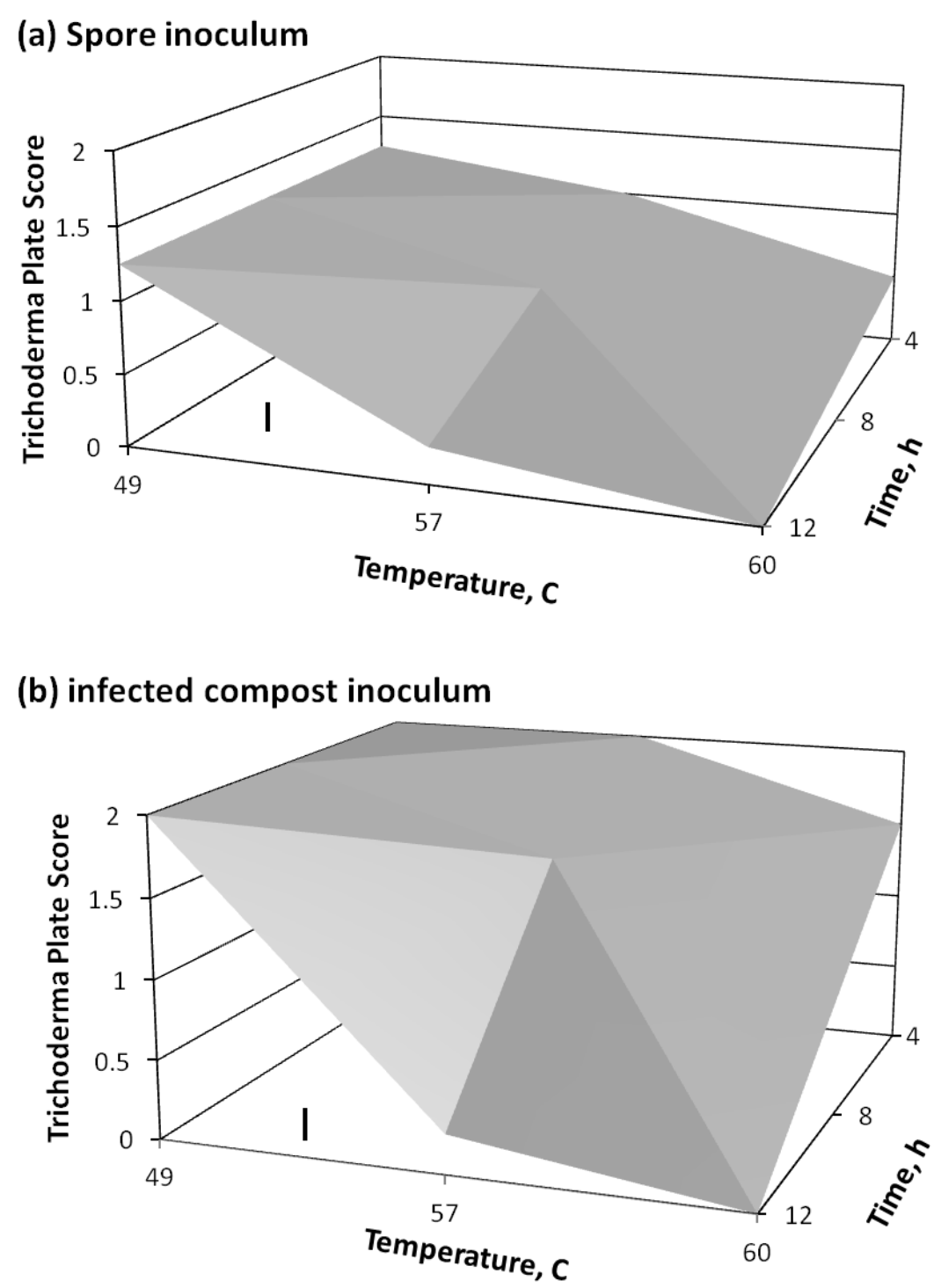


Figure 9. Effect of pasteurisation temperature and time on *Trichoderma aggressivum* (Th2) 23443B in compost using (a) spore and (b) infected compost inocula. Viability assessed at casing by plating of compost on selective agar and scoring for *Trichoderma* growth. Each point is the mean of three replicate composting flasks and two compost samples per flask. Bar indicates LSD between temperature and time treatments (P = 0.05).

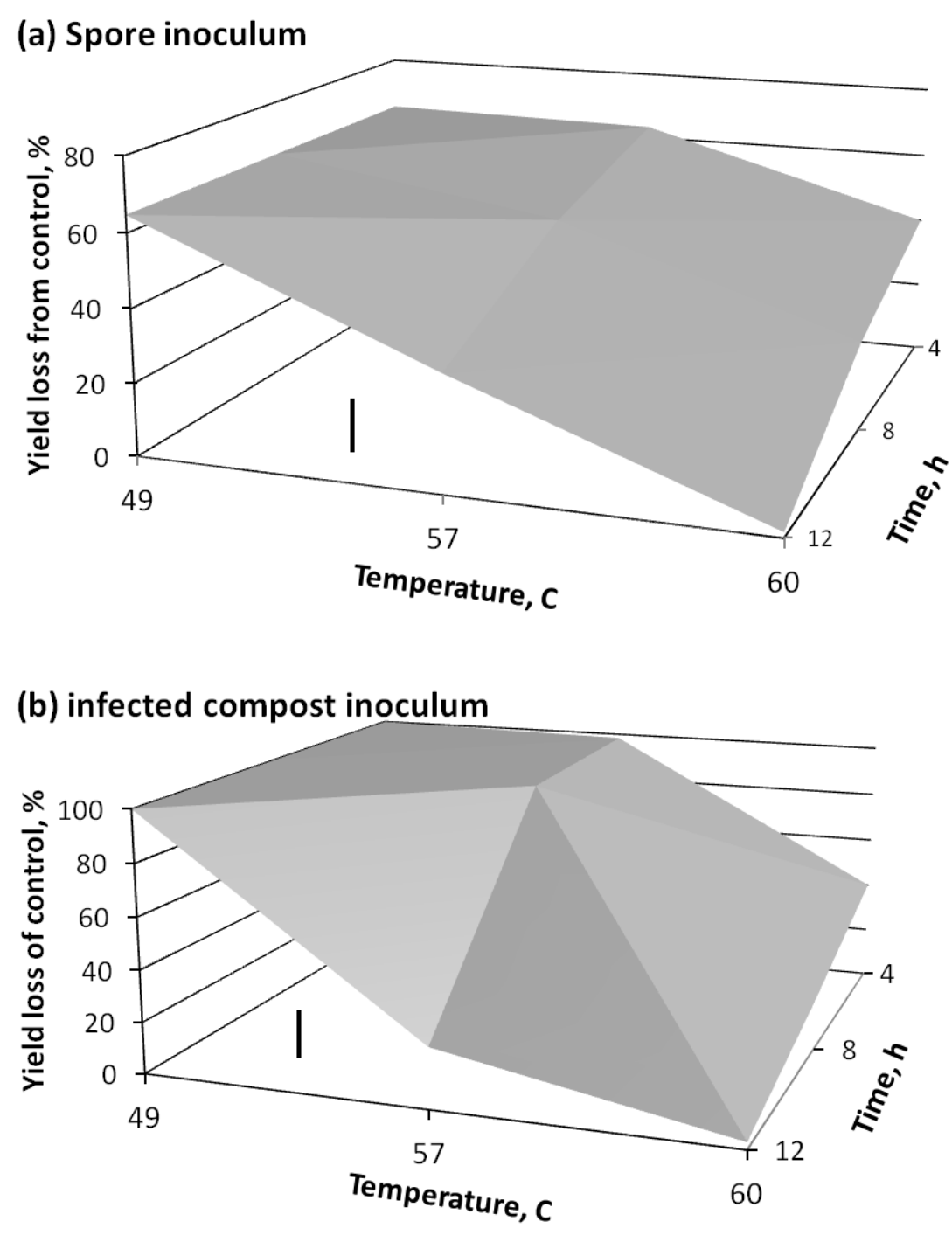


Figure 10. Effect of (a) spore and (b) infected compost inocula of *Trichoderma aggressivum* (Th2) 23443B on mushroom yield loss following different pasteurisation times and temperatures. Percentage yield loss compared with mushroom yield from clean Phase II compost. Each point is the mean of three replicate composting flasks and culture pots. Bar indicates LSD between temperature and time treatments (P = 0.05).

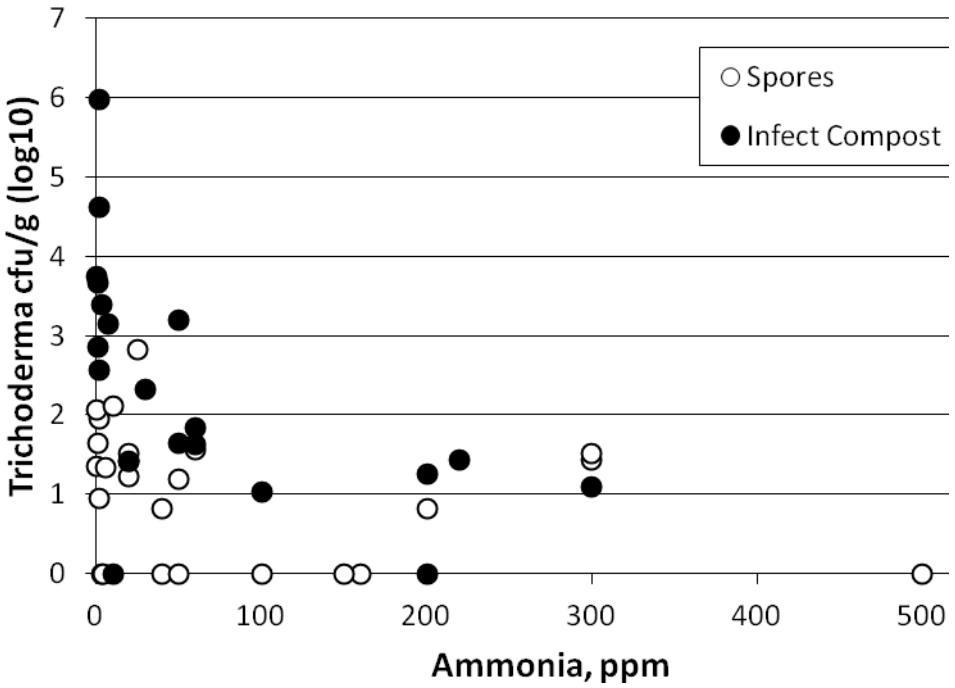


Figure 11. Effect of ammonia concentration during pasteurisation on *Trichoderma aggressivum* (Th2) 23443B in compost using (a) spore and (b) infected compost inocula. Viability assessed at casing by dilution plating of compost extract. Each point is the mean of two *Trichoderma* counts taken on two compost samples.

Susceptibility of non grain-spawn products to infection by *T. aggressivum* (Th2)

1. Grain spawning rate

Spawning rate had no effect on the yield of mushrooms from clean Phase II composts (Figure 12).The *Trichoderma* inoculum had a severe negative on mushroom yield, with the yield loss being greatest at the lowest spawning rate.

2. Spawn strain and type

The *Trichoderma* inoculum again had a severe negative effect on mushroom yield (Figure 13). There was no significant difference in mushroom yield between A15 or L901 grain spawn or Speedy Inoculum™ L901, either in the presence or absence of *Trichoderma* inoculum.

3. Casing inoculum

Mushroom yield from clean phase II compost was not significantly affected by the use of A15 grain spawn or caccing inoculum (Figure 14). The *Trichoderma* spore inoculum reduced mushroom yield in both composts although the yield loss due to green mould was reduced by caccing inoculum compared with grain spawn.

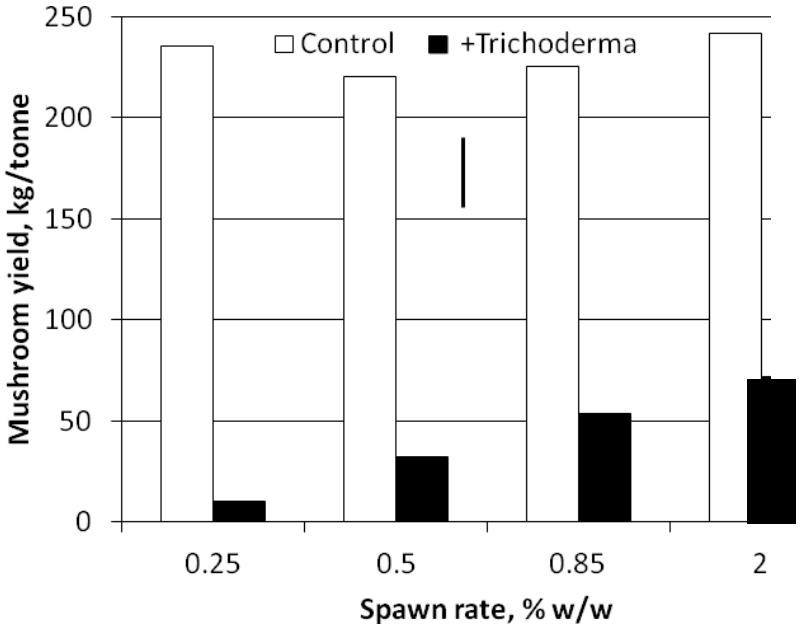


Figure 12. Effect of grain spawn rate and *Trichoderma aggressivum* (Th2) 23443B spore inoculum on mushroom yield from Phase II compost. Each point is the mean of six replicate composts and three pots per compost. Bar indicates LSD between spawn rate treatments (*P* = 0.05).

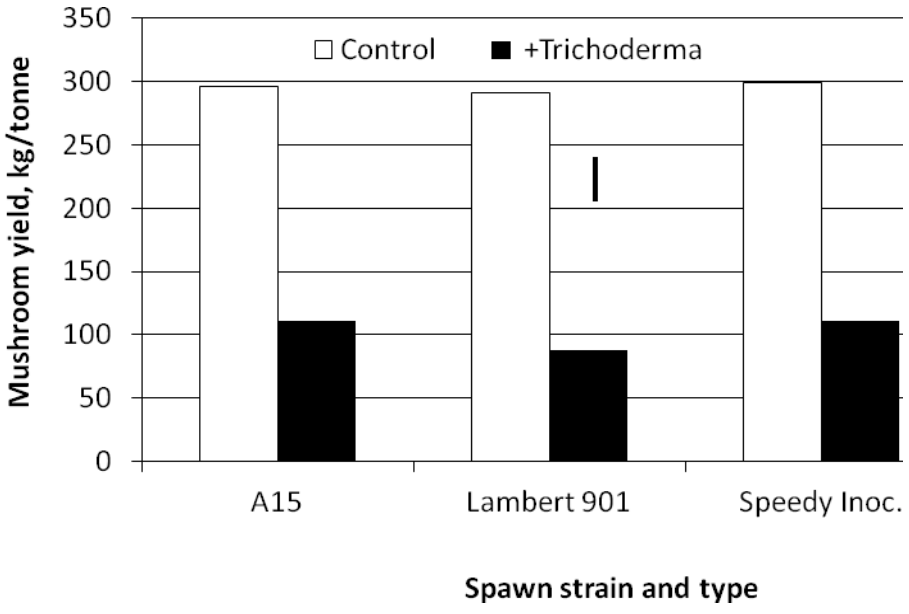


Figure 13. Effect of spawn strain and type and *Trichoderma aggressivum* (Th2) 23443B spore inoculum on mushroom yield from Phase II compost. Each point is the mean of six replicate composts and three pots per compost. Bar indicates LSD between spawn treatments (*P* = 0.05).

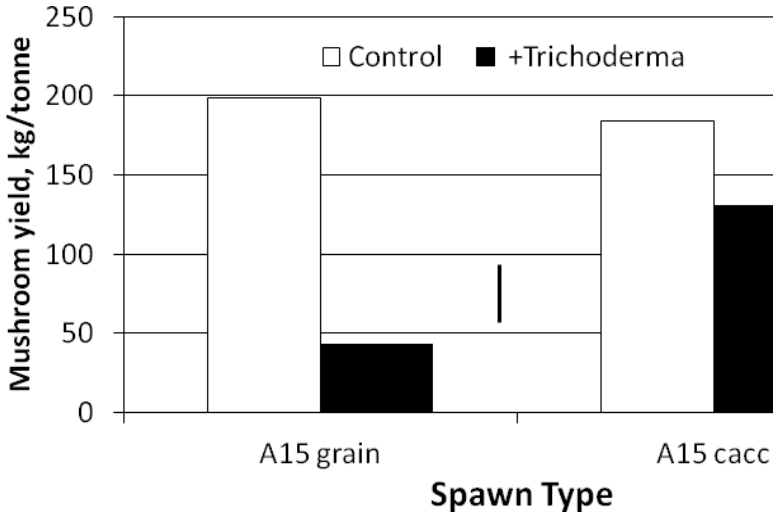


Figure 14. Effect of spawn type at 0.5% w/w and *Trichoderma aggressivum* (Th2) 23443B spore inoculum on mushroom yield from Phase II compost. Each point is the mean of three replicate pots. Bar indicates LSD between spawn treatments (*P* = 0.05).

4. Effect of compost factors on susceptibility to yield loss from *Trichoderma*

There were no relationships between compost moisture, ash content or pH and the mushroom yield loss resulting from a *Trichoderma* spore inoculum applied to grain spawned composts. The only infected composts that produced a mushroom yield that was at least 50% of the uninfected composts were those with an ammonium nitrogen content of 0.02% of dry matter or less. All composts that had a higher ammonium nitrogen content produced less than 25% of the control mushroom yield when *Trichoderma* inoculum was added (Figure 15). Similar trends were observed for total nitrogen content of composts, and nitrogen and ammonium nitrogen contents expressed as a proportion of organic matter.

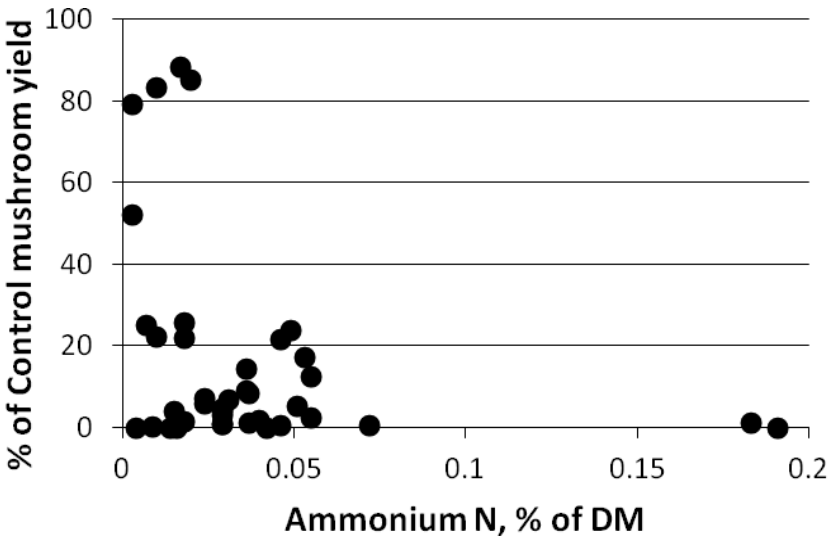


Figure 15. Relationship between compost ammonium nitrogen content and percentage mushroom yield loss due to a spore inoculum of *Trichoderma aggressivum* (Th2) 23443B. Each point is the mean yield from three replicate pots.

5. Detection of *Trichoderma* inoculum

The detection limit for *Trichoderma* cfu in compost using the dilution plating of compost extract method was about 10 cfu/g compost (i.e. 1 $\log_{10}$ unit). This was better than for the plating of compost pieces on semi-selective agar where several negative plate results were obtained from samples than gave a positive result for the dilution plating method. To examine the relationship between the *Trichoderma* population detected at casing, following spore inoculation of compost at spawning, and the subsequent mushroom yield loss, data from projects M 47 and M 50 were combined (Figure 16). This shows that there was severe or even complete mushroom yield loss at the *Trichoderma* population detection limit of 10 cfu per g compost. However, there was no relationship between the detected *Trichoderma* population at casing and the mushroom yield loss. Almost all composts that had a *Trichoderma* count above the detection threshold at casing suffered a mushroom yield loss of about 80% or greater. The exceptions were the five composts in Figure 8 that had a low ammonium nitrogen content.

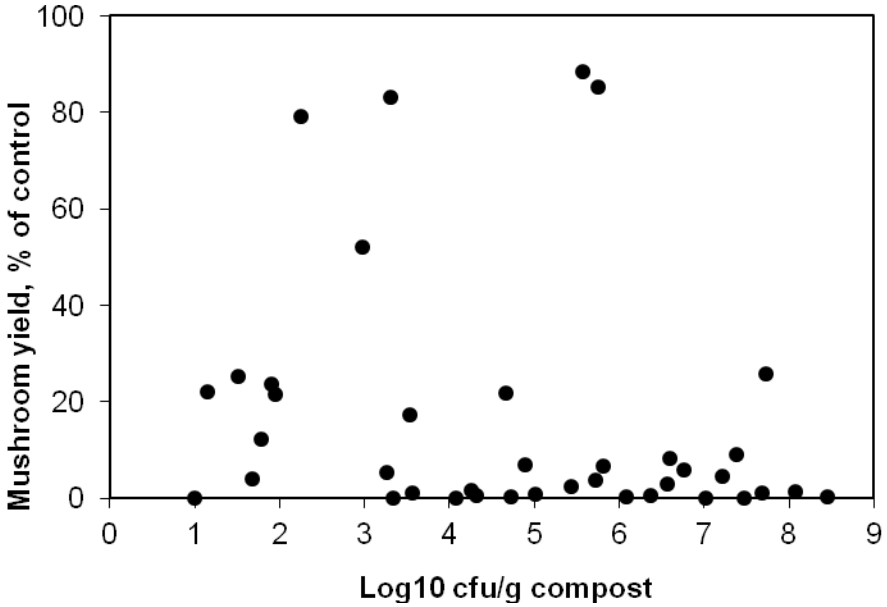


Figure 16. Relationship between *Trichoderma* population in compost at casing and percentage crop loss due to a spore inoculum of *Trichoderma aggressivum* (Th2) 23443B applied at spawning. Each point is the mean yield from two replicate compost samples and three replicate cropping pots.

Discussion

Beyer et al. (2000) found that *Trichoderma aggressivum* (Th4) spores and inoculated corncobs and rye grains failed to survive a 2-h pasteurisation at 60-62°C. By contrast, the results of this project have confirmed other results (Morris et al., 2000c; Rinker & Alm, 2000) that demonstrated that *Trichoderma aggressivum* (Th2 and Th4) is extremely temperature tolerant and can survive in ammonia concentrations of more than 200 ppm for several hours. However, unlike previous work which was conducted in the laboratory and in simulated pasteurisation conditions, this work was conducted in Phase I compost in pasteurisation flasks. The results of this work have shown that for a high inoculum of *Trichoderma aggressivum* (Th2), either as a spore suspension or as infected compost, a compost pasteurisation temperature of 60°C needs to be maintained for 12 hours to reduce the inoculum to below a detectable level. It is possible that a high ammonia concentration (500 ppm) may reduce this temperature-time requirement but this requires further investigation. Commercial Phase II composts typically have ammonia concentrations of 300-700 ppm during pasteurisation, although levels may be higher or lower than this range. It is not clear whether the effect of ammonia concentration on the viability of *Trichoderma* is independent of the pasteurisation treatment since increasing compost temperature between 49 and 60°C resulted in a higher ammonia concentration. This work also highlights the

need to find out where the coolest zones, and possibly the lowest ammonia concentrations, in a pasteurisation tunnel are, since these will be the most vulnerable for survival of *Trichoderma* inoculum.

For several plant pathogens, there is a greater survival risk in dry composts than in moist or wet composts, although this usually only applies to green waste composts with a moisture content less than about 50% w/w (Noble et al, 2009). No increased survival risk of *Trichoderma aggressivum* (Th2) was found in drier (69% moisture) Phase I compost than in wetter compost.

Rinker and Alm (2000) and Beyer et al. (2000) reported that non-grain spawn products including Speedy Inoculum™ reduced the susceptibility of composts to green mould infection, although the growth of *Trichoderma* was similar on casing spawn (caccing inoculum or CI) as on grain spawn. A previous HDC project (M 47) showed that caccing inoculum used at a high rate (10% w/w) was less susceptible to green mould infection than grain spawn used at a normal (0.5% w/w) rate, although the materials were not compared at the same rate. The results here showed that the caccing inoculum provided better protection against green mould infection than grain spawn or the Speedy Inoculum™ product used at the same rate (0.5% w/w). The results of HDC Project M 47, as well as those of Rinker and Alm (2000) and Beyer et al. (2000) all showed that spawn-run compost mushroom inoculum was less susceptible to green mould infection than grain spawn when added to Phase II compost. It is therefore possible that a combination of caccing inoculum followed by Phase II/Phase III mixing could be used during a *Trichoderma* outbreak.

This work has demonstrated that green mould infection and mushroom yield loss following a standard *Trichoderma* spore inoculum varies in different composts. This confirms earlier observations by Grogan and Gaze (1995). No relationship between compost moisture and susceptibility to green mould was found; Beyer et al. (2000) also found that both 'normal' and 'wet' composts were susceptible to green mould although disease symptoms were worse in the wet composts. This work has shown that at increasing levels of compost ammonium nitrogen, there is a greater effect of *Trichoderma* on mushroom yield. However, even at low compost ammonium nitrogen contents, the growth of *Trichoderma* was not inhibited. Work by Gerrits (1988) indicated that the optimum compost ammonium nitrogen content at filling of Phase I for subsequent mushroom yield was around 0.4% of dry matter (equivalent to about 0.06% of dry matter at spawning). However, he showed that the effect on mushroom yield of ammonium nitrogen content at spawning between 0.02 and 0.12% of dry matter was fairly small, and significant mushroom yield loss only occurred if the

ammonium nitrogen content exceeded the latter value. This was in the absence of a *Trichoderma* infection. This project has shown that ammonium nitrogen is not the only compost factor influencing mushroom yield loss due to green mould since some composts with very low ammonium nitrogen levels also had severe mushroom yield losses. It would therefore be worth comparing the analysis and green mould susceptibility of composts with low ammonium nitrogen levels. Beyer et al. (2007) indicated that certain organic acids in composts may encourage green mould infection.

The detection limit for *Trichoderma* (Th2) cfu in compost using the dilution plating of compost extract method was about 10 cfu/g compost (i.e. 1 $\log_{10}$ unit). Severe or even complete mushroom yield loss due to *Trichoderma* was recorded at this detection limit. This indicates that *Trichoderma* (Th2) may be able to reduce mushroom yields when present at population levels in compost that are undetectable using the sampling and detection methodology used in this project. Results of Grogan et al. (1996) and Beyer et al. (2000) indicate that there is a minimum spore inoculum level at spawning, equivalent to about 100 spores per kg compost, below which no detectable mushroom yield loss occurs. The lack of a relationship between the detected *Trichoderma* population at casing and subsequent mushroom yield loss confirms earlier results of Grogan and Gaze (1995) who found that the *Trichoderma* count after cropping did not relate to the mushroom yield loss due to *Trichoderma* in different composts. This may be due to *Trichoderma* and *Agaricus* being able to grow together in certain composts (e.g. those with a low ammonium nitrogen content). However, it may also be due to sampling variability within the compost. The detection methodology for *Trichoderma* involves sampling only a small part of the entire compost mass, whereas the mushroom yield loss reflects the level of *Trichoderma* infection in the entire compost.

Conclusions

- The *Trichoderma aggressivum* (Th2) isolate 23443B was capable of causing severe or even complete mushroom crop loss when present in compost at levels that were at 0the detection limit of the sampling and dilution plating methodology used (about 10 cfu per g compost).
- A compost pasteurisation temperature of 60°C needed to be maintained for 12 hours to reduce spore and infected compost inocula of *Trichoderma aggressivum* (Th2) isolate 23443B to below a detectable limit.

- The above pasteurisation requirement was not increased in dry (69% moisture) Phase I compost.
- Both types of inoculum of *Trichoderma aggressivum* (Th2) 23443B (spores and infected compost) survived an ammonia concentration of 300 ppm during pasteurisation.
- Two other *Trichoderma* isolates, T32 and T40, did not survive a pasteurisation treatment of 57°C for 8 hours.
- There was no difference between grain spawn and Speedy Inoculum^{TM} in susceptibility to green mould infection but yield loss was significantly reduced by the use of caccing inoculum.
- There was no difference in susceptibility to green mould infection between spawn strains Sylvan A15 and Lambert 901.
- Increasing spawn rate from 0.25 to 2% w/w slightly reduced the effect of green mould infection on mushroom yield loss.
- There were no relationships between compost moisture, ash content or pH and the yield loss resulting from a *Trichoderma* spore inoculum.
- The only grain-spawned infected composts that produced a mushroom yield that was at least 50% of the uninfected composts were those with an ammonium nitrogen content of 0.02% of dry matter or less; however, not all composts with a low ammonium nitrogen content were resistant to green mould infection.
- The detection limit for *Trichoderma* using dilution plating (about 10 cfu per g compost) was better than for the plating of compost pieces on semi-selective agar where several negative plate results were obtained from samples than gave a positive result for the dilution plating method.

Sub-Section 3: T. aggressivum detection

Objective 5. Confirm that *Trichoderma aggressivum (Th2)* detection methods are effective

Materials and methods

Compost samples

Forty samples of spawn run compost were obtained from Experiment 2 (above) just after they had been infected at the end of the spawn run with different rates of *Trichoderma* infected compost from 10^{-1} to 10^{-4}. Half the samples had received supplement and half were unsupplemented. Four additional samples of fully infected compost (10^{0}) from four separate plots were included (samples A, B, C and D). Sample A was used to provide the compost inoculum for the Experiment 2 (Table 4). In addition, four samples of pasteurised Phase II compost, inoculated with a spore suspension prior to pasteurisation and subsequently pasteurised for 8 hours at 57 °C, were obtained for testing from the pasteurisation experiments described above.

Real time PCR

A rapid real time PCR protocol for the detection of *Trichoderma aggressivum* in spawn-run compost samples was developed and described in project report M48 (Lane, 2010). Nucleic acid was extracted from all the compost samples using the protocol developed in M48 and then tested for presence of *T. aggressivum* by real time PCR.

***Trichoderma* assessments.**

The number of *Trichoderma* propagules/gfw was calculated for the majority of samples using one or both of two microbiological methods. In addition all samples were tested using the molecular method. Propagule counts and results were compared where possible. The microbiological methods consisted of Most Probable Number (MPN) test and a weed mould analysis test (WMA) as described above.

Results and discussion

Detection of *T. aggressivum* in spawn-run compost

The fully infected compost (10^{0}) gave a consistent CT value of 23 while the WMA and MPN tests gave average counts of 2.1×10^{9} and 1.5×10^{9}, respectively, (Table 5). The WMA and

MPN counts were not significantly different from each other and the means from both methods had large variances, though within each replicate, the results from the two methods were similar in magnitude. The differences between the replicates is likely to be a reflection of the heterogeneity of the sample and sampling

As expected the control samples, (supplemented and unsupplemented), were negative (CT = 40; WMA and MPN = 0) (Table 6). The real time TaqMan PCR assay detected *Trichoderma aggressivum* in all replicates of all dilutions from 10% to 0.01% (10^{-1} to 10^{-4}). Giving CT values in the range of 22 to 36. The CT values for the most dilute samples (0.01% - 10^{-4}) was between 34and 36. In future tests the dilution series could be extended further to 10^{-5} or more to acquire information on the detection limit of this assay.
There was no significant differences in detection between the supplemented and unsupplemented samples suggesting that supplementation did not aggravate *Trichoderma aggressivum* under the conditions of this experiment.

The real time TaqMan PCR assay was more successful at detecting *T. aggressivum* in the more dilute samples containing 0.1% or less of *Trichoderma* infected compost. The MPN test gave a number of zero counts for these sample (Table 6), making the MPN method less reliable for the detection of *T. aggressivum* when it is present in very low quantities. Nonetheless these levels of infection caused serious yield reductions (See Figure 5 – repeated below).

The Co-efficient of variation is a statistic that can be used to compare the variation in data based on different units of measurement. The lower the co-efficient of variation the less variable the data, irrespective of the magnitude or units of the means being compared. At the 10% dilution rate, the co-efficient of variation was generally low for both the molecular detection method (CT value) and the MPN method (no data for WMA for this rate) for detecting *Trichoderma* propagules (Table 6), however when the dilution rates were1% to 0.01% the co-efficient of variation was consistently lower for the molecular detection method (CT value) compared to either the MPN or WMA methods. Both the MPN and WMA analysis gave several “zero” counts for *T. aggressivum* at dilution rates of 0.1% and 0.01% (although some modification of the testing procedure may improve detection rates). Thus the molecular method would appear to be the most consistent and most reliable detection method – especially when the concentration of *Trichoderma aggressivum* propagules is very low**.** This suggests that this technique would be ideally suited to routine testing of bulk Phase III compost in order to pick up low levels of *T. aggressivum* in a Phase III tunnel that might not impact on crops until the infection became more established.

Table 5. Comparison of three methods to quantify *T. aggressivum* in Phase III compost

Dilution	Sample ID	CT	Propagule count (MPN)	Propagule count (WMA)
10^0	A	23	1.3×10^9	2.1×10^9
	B	23	0.35×10^9	0.38×10^9
	C	23	0.47×10^9	0.46×10^9
	D	23	23×10^9	53×10^9
Average		23	1.5×10^9	2.1×10^9
($\log_{10}$ value)			(9.176)	(9.323)
Variance			(0.686)	(0.985)
(Co-efficient of variation)		0	(0.09)	(0.11)
			n.s. *P* = 0.83	

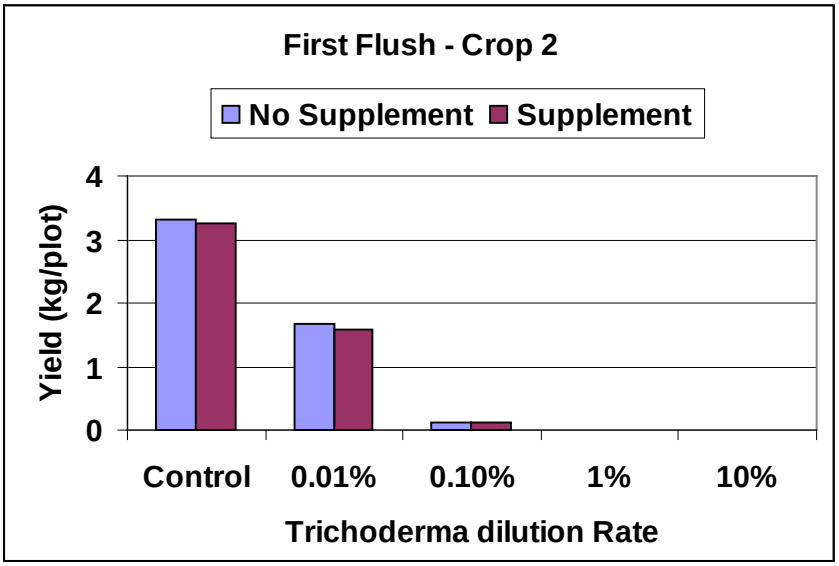


Figure 5. First flush yield in response to the incorporation of *Trichoderma*-infected compost at the end of spawn-run at different rates (Crop 2)

Table 6. Cycle threshold (CT), MPN and WMA estimates of *T.aggressivum* populations in spawn run compost samples infected with *T. aggressivum* compost at different dilutions. MPN and WMA values are Log10 (cfu's/gfw). S = supplemented; US = unsupplemented.

	CT			**MPN**			**WMA**		
Treatment	CT value	**Mean**	Co-efficient of variation	MPN (Log10)	**Mean (Log10)**	Co-efficient of variation	**WMA** (Log10)	**Mean (Log10)**	Co-efficient of variation
10% - S	26	**25**	0.03	6.233	**6.16**	0.04			
	25			6.114					
	25			5.851					
	24			6.439					
10% - US	22	**23**	0.05	6.233	**6.24**	0.02			
	24			6.233					
	24			6.114					
	22			6.380					
1% - S	29	**28.75**	0.02	5.734	**5.19**	0.2	4.114	**4.104**	0.15
	29			6.233			4.877		
	29			3.892			3.409		
	28			4.898			4.014		
1% - US	26	**25**	0.03	4.690	**4.41**	0.1	6.467	**4.558**	0.41
	24			3.833			2.000		
	25			4.770			5.129		
	25			4.364			4.637		
0.1% - S	30	**29.25**	0.05	4.029	**2.42**	0.68			
	30			1.000					
	30			3.653					
	27			1.000*					
0.1% - US	30	**29.25**	0.02	1.000*	**1***	n.r.			
	29			1.000*					
	29			1.000*					
	29			1.000*					
0.01% - S	35	**35.25**	0.01	2.100	**1.55**	0.41	0.523	**0.75**	0.39
	35			1.000*			1.000*		
	35			2.100			0.477		
	36			1.000*			1.000		
0.01% - US	34	**34.5**	0.02	1.000*	**1.43**	0.35	1.000*	**1***	n.r.
	34			1.833			1.000*		
	35			1.892			1.000*		
	35			1.000*			1.000*		
Control - S	40	**40**	0	1.000*	**1***	n.r.			
	40			1.000*					
	40			1.000*					
	40			1.000*					
Control - US	40	**40**	0	1.000*	**1***	n.r.			
	40			1.000*					
	40			1.000*					
	40			1.000*					

* Zero values were give a nominal value of 10 prior to $\log_{10}$ transformation so that a reading of 1 on the Log scale = Zero propagules. N.r. = not relevant

Detection of *T. aggressivum* in pasteurised compost

No *T. aggressivum* was detected in the four pasteurised compost samples that had been infected using the molecular method. *T. aggressivum* was detected at a low level in one sample only by WMA and only this sample resulted in crop loss – inferring that the *Trichoderma* propagules in the other samples had been effectively killed during the pasteurisation regime. In this case it would appear that the WMA method was more reliable than the molecular method at detecting *T. aggressivum* propagules. However as only a small number of samples were tested it is difficult to draw any firm conclusions. The nature of pasteurised Phase II compost is also quite different to Phase III compost and thus the molecular test may not be optimised for this type of sample. Further testing would be required to determine the suitability or otherwise of the molecular test for detecting *T. aggressivum* in Phase II compost.

Table 7. Detection of *Trichoderma aggressivum* in pasteurised compost inoculated prior to pasteurisation

***Trichoderma* strain added**	**CT value**	**WMA**	**Crop symptoms**
isolate 23433B	>40 (= not detected)	11 cfu/g compost (Log10 = 1.041)	52% Crop loss
isolate 23433B	>40 (= not detected)	Not detected	None
isolate T32	>40 (= not detected)	Not detected	None
isolate T40	>40 (= not detected)	Not detected	None

Conclusions

- Molecular detection of *T. aggressivum* in Phase III compost using a real time Taqman PCR test is very sensitive and reliable; it is capable of detecting propagules when *Trichoderma*-infected compost is diluted into healthy compost up to a dilution factor of 0.01% (10^{-4}).
- Most Probable Number (MPN) and Weed Mould Analysis (WMA) tests were effective and reliable at detecting *Trichoderma* propagules in Phase III compost when propagule counts were relatively high 100-1000 cfu's/gfw (Log10 values = 2-3). When counts were lower these methods were variable and false negatives were obtained. This is likely to reflect the difficulty in sampling and subsampling a compost sample that contains a low population of *T. aggressivum* propagules.

- Molecular detection of *T. aggressivum* in pasteurised Phase II compost using a real time TaqMan PCR test was inconclusive due to the small number of samples tested.

Summary of conclusions

Sub-Section 1: *T. aggressivum* epidemiology in Phase II and Phase III compost

- *Trichoderma aggressivum* appears to sporulate less heavily when infected compost is incubated in total darkness
- *Trichoderma aggressivum* can be detected in compost by microbiological means even when diluted by up to 1 in 10^6
- When *Trichoderma aggressivum* inoculum is in the form of infected compost fragments, it is capable of colonising and sporulating on compost even when very dilute although the effects in the most diluted situations are more variable.
- *Trichoderma* infected compost can cause yield reductions of up to 100% when it is diluted into healthy Phase III compost.
- The more dilute the infection, the less the impact on crop yield but dilution rates of 1 in 10^4 (0.01% or 100 g in a tonne) can, under certain conditions, cause a 50% crop reduction.
- The magnitude of the yield reduction is likely to be influenced by
    - The number of *Trichoderma* propagules on the infected compost
    - The extent of mixing that occurs
    - Compost quality may also affect the severity of symptoms

Sub-Section 2: Compost pasteurisation and agronomy aspects to *T. aggressivum* epidemiology

- The *Trichoderma aggressivum* (Th2) isolate 23443B was capable of causing severe or even complete mushroom crop loss when present in compost at levels that were at 0the detection limit of the sampling and dilution plating methodology used (about 10 cfu per g compost).
- A compost pasteurisation temperature of 60°C needed to be maintained for 12 hours to reduce spore and infected compost inocula of *Trichoderma aggressivum* (Th2) isolate 23443B to below a detectable limit.
- The above pasteurisation requirement was not increased in dry (69% moisture) Phase I compost.

- Both types of inoculum of *Trichoderma aggressivum* (Th2) 23443B (spores and infected compost) survived an ammonia concentration of 300 ppm during pasteurisation.
- Two other *Trichoderma* isolates, T32 and T40, did not survive a pasteurisation treatment of 57°C for 8 hours.
- There was no difference between grain spawn and Speedy Inoculum^{TM} in susceptibility to green mould infection but yield loss was significantly reduced by the use of caccing inoculum.
- There was no difference in susceptibility to green mould infection between spawn strains Sylvan A15 and Lambert 901.
- Increasing spawn rate from 0.25 to 2% w/w slightly reduced the effect of green mould infection on mushroom yield loss.
- There were no relationships between compost moisture, ash content or pH and the yield loss resulting from a *Trichoderma* spore inoculum.
- The only grain-spawned infected composts that produced a mushroom yield that was at least 50% of the uninfected composts were those with an ammonium nitrogen content of 0.02% of dry matter or less; however, not all composts with a low ammonium nitrogen content were resistant to green mould infection.
- The detection limit for *Trichoderma* using dilution plating (about 10 cfu per g compost) was better than for the plating of compost pieces on semi-selective agar where several negative plate results were obtained from samples than gave a positive result for the dilution plating method.

Sub-Section 3: *T. aggressivum* detection

- Molecular detection of *T. aggressivum* in Phase III compost using a real time TaqMan PCR test is very sensitive and reliable; it is capable of detecting propagules when *Trichoderma*-infected compost is diluted into healthy compost up to a dilution factor of 0.01% (10^{-4}).
- Most Probable Number (MPN) and Weed Mould Analysis (WMA) tests were effective and reliable at detecting *Trichoderma* propagules in Phase III compost when propagule counts were relatively high 100-1000 cfu's/gfw (Log10 values = 2-3). When counts were lower these methods were variable and false negatives were obtained. This is likely to reflect the difficulty in sampling and subsampling a compost sample that contains a low population of *T. aggressivum* propagules.

- Molecular detection of *T. aggressivum* in pasteurised Phase II compost using a real time TaqMan PCR test was inconclusive due to the small number of samples tested.

Knowledge and technology transfer (to date)

HDC New Projects, Measures against green mould, HDC News 156, September 2009. p11.

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Recommendations for future work

1. The effect of high ammonia concentrations (500 ppm) on *Trichoderma aggressivum* (Th2) should be investigated; this could be achieved by adding urea to the Phase I compost. The independent effects of ammonia and compost temperature on eradication of *Trichoderma aggressivum* (Th2) should be determined.
2. The effect of extended pasteurisation periods (10 hours and longer) on compost mushroom cropping performance should be examined.
3. The temperature-time profiles of commercial Phase II pasteurisation tunnels should be investigated, in particular, the presence of cool zones in the compost. Variations in ammonia concentration (if any) should also be monitored.
4. The effect of using commercial casing inoculum in place of grain spawn on the susceptibility of compost to green mould requires further investigation, particularly in combination with Phase II and Phase III blending.
5. The analysis and green mould susceptibility of composts with low ammonium nitrogen levels should be investigated in order to develop green mould tolerant composts.
6. Further work is required on the minimum spore inoculum needed to produce a detectable mushroom yield loss in different Phase II and III composts, and how this relates to subsequent *Trichoderma* counts at casing and after cropping.

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