Project title:	*Trichoderma* green mould – Diagnostic assays for improved disease management
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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

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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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.... Date ……..15th July 2010

Report authorised by:

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.... Date………..15th July 2010

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GROWER SUMMARY

Headline

- A rapid molecular diagnostic test for *Trichoderma aggressivum* identification is now available that can permit early diagnosis of the disease and help mitigate financial losses.

Background and expected deliverables

The fungus *Trichoderma* is comprised of numerous species of which only some cause economic losses to the Mushroom Industry. At the onset of the problem in the mid 1980s, *Trichoderma* (green) compost mould and cap spotting was attributed primarily to *T. harzianum* leading to between 30-100% losses. However, several studies that investigated *Trichoderma* outbreaks in Europe and North America (Fletcher, 1986; Seaby, 1996; Muthumeenashi *et al.* 1998) showed the situation to be much more complex. There are four biotypes, some of which are present in the UK and some of which are economically damaging as summarised below:

Biotype	Current Name	Present in UK	Economically damaging
Th1	*Trichoderma harzianum*	yes	no
Th2	*Trichoderma aggressivum f. europaeum*	yes	yes
Th3	*Trichoderma atroviride*	yes	no
Th4	*Trichoderma aggressivum f. aggressivum*	no	yes

Rapid diagnosis of *T.aggressivum* cannot currently be achieved quickly so this prevents effective disease management. Recent advances in our understanding of *T.aggressivum* molecular biology (Project M 46) may permit the development of species - specific diagnostic tools.

Summary of the project and main conclusions

The project successfully developed extraction methods for *Trichoderma* DNA directly from Phase III compost. Real-time PCR primers and probe for *T.aggressivum* were 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 taxonomy. 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 one 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 4500 propagules per gram of fresh weight and potentially 10 times more dilute (450 propagules/gfw; equivalent to only a few conidial heads of *Trichoderma*). The new assay has been used to successfully test several compost samples from commercial premises for *T.aggressivum,* This will be invaluable to aid the understanding of the pathogen's epidemiology in project M 50.

Financial benefits

A rapid molecular diagnostic test for *Trichoderma aggressivum* identification is now available that can permit early diagnosis of the disease and help mitigate financial losses.

Action points for growers

- Aggressive forms of *Trichoderma* compost mould (*Trichoderma aggressivum*) can cause significant yield losses (in excess of 50%) due primarily to bare areas and cap spotting.

- Early detection and rigorous hygiene measures are key to successful *Trichoderma* management.

- A semi-automated DNA extraction for Phase III compost has been developed permitting rapid diagnoses (<48h) from compost.

- The project has led to the development of the first rapid, cost effective commercial service for compost producers and growers, to screen Phase III compost directly for *T.aggressivum.*

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SCIENCE SECTION

Introduction

The fungus *Trichoderma* is comprised of numerous species of which only some cause economic losses to the Mushroom Industry. At the onset of the problem in the mid 1980s *Trichoderma* (green) compost mould and cap spotting was primarily attributed to *T. harzianum* which could lead to between 30-100% crop losses. However, several studies that investigated *Trichoderma* outbreaks in Europe and North America (Fletcher, 1986; Seaby, 1996; Muthumeenashi *et al*. 1998) showed the situation to be much more complex. Differences in the morphology and pathogenicity of these fungi and more recently molecular analysis has lead to different groups being referred to as initially biotypes of *Trichoderma harzianum* (Th1, Th2, Th3 and Th4) but since 2002 the most damaging strains of *T. harzianum* Th2 and 4 have been raised to species level and should be referred to as *Trichoderma aggressivum* f. *europaeum* and *T.a.*f. *aggressivum* (Samuels *et al*., 2002) as summarised below:

Biotype	**Current Name**	**Present in UK**	**Economically damaging**
Th1	*Trichoderma harzianum*	yes	no
Th2	*Trichoderma aggressivum f. europaeum*	yes	yes
Th3	*Trichoderma atroviride*	yes	no
Th4	*Trichoderma aggressivum f. aggressivum*	no	yes

Many other species of *Trichoderma* have been reported in association with mushroom production with varying degrees of significance with respect to economic damage.

Previous HDC projects (e.g. M 1, M 10, M 13, M 14, M 46) have investigated aspects of *Trichoderma* including control and detection. M 46 demonstrated the prevalence of *T.aggressivum* (Th2) as the cause of compost green mould in pre and first flush. It also confirmed the absence of *Trichoderma aggressivum f aggressivum* (Th4).

The project demonstrated the reliability of molecular techniques for identification of *T.aggressivum f. aggressivum* from pure cultures based on the translocation elongation factor (Tef) gene and to a lesser extent the ITS region.

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Aim

To develop a rapid molecular diagnostic test for *Trichcoderma aggressivum* (Th2 & Th4).

Objectives

1. Develop a nucleic acid extraction protocol for Phase III mushroom compost
2. Design a real-time TaqMan PCR assay for *Trichoderma aggressivum* (Th2 & Th4)
3. Evaluate assay testing spiked samples with known pathogen concentration

Materials and methods

Primer Design and Evaluation

TaqMan® assays for *Trichoderma harzianum* and *Trichoderma aggressivum* were designed in Primer Express 3 (Life Technologies, Paisley, UK) and manually checked for specificity using a DNA sequence alignment constructed from all of the available *Trichoderma* sequences in Genbank.

DNA Compost Extraction

Samples of compost (75 g) were added to 1000 ml Nalgene bottles with 20 stainless steel ball bearings (25.4 mm in diameter). 375 ml CTAB soil buffer (120 mM sodium phosphate buffer pH8, 2% cetrimonium bromide, 1.5 M NaCl, 3% Antifoam B) was added to the samples respectively. An internal standard was also added. Nalgene bottles were then placed on a commercial paint shaker for four minutes. A 40 ml subsample was taken and centrifuged at 5000 g for five minutes. 20 ml of the resulting supernatant was added to 2 ml 5M potassium acetate and placed on ice for 10 minutes, followed by a 5 minutes centrifugation at 12 000 g. The supernatant was then added to a clean 50 ml tube containing 15 ml isopropanol plus acid washed silicon dioxide and incubated at room temperature followed by 5 minutes centrifugation at 12 000 g. The supernatant was discarded and DNA was purified from the resulting pellet using a Wizard Magnetic DNA Purification System in conjunction with a Kingfisher ML magnetic particle processor (Thermo Electron Corporation). All DNA samples were eluted into 200 µl Tris-EDTA (TE) buffer and stored at -30°C until required.

Soil extracts were tested using the real-time PCR assay for the internal standard described above and a universal primers for bacteria (J. Woodhall unpublished data). Standard FERA real-time conditions were used except that the master mix consisted of Environmental master mix 2.0 (Applied Biosystems, Warrington, UK) which is known to withstand the inhibition usually associated with environmental extracts. The samples were also tested

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using the nanodrop spectrophotometer (Thermofisher Scientific, Loughborough, UK). Absorbance values at 230, 260 and 280nm were taken to assess DNA quality and quantity.
In order to assess the extraction efficiency of the DNA extraction methods to be tested an internal standard was used. The internal standard would also act as a positive control in routine diagnostics to check extraction quality. A TaqMan assay was complementary to the unique sequence (primer soilF 5'- CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG-3', soilR 5'- GGT GCG TTC GCT GTT AAT GG-3' and a labelled probe (Fam-Tam) 5'-CGACGTCGCATGCTCCCGG-3'). Tests on compost with this assay in the absence of the internal standard were negative indicating no cross-reaction with existing soil nucleic acids.

Production of Trichoderma inoculum

Agaricus bisporus spawn grains (strain Sylvan A15) were added to a heavily sporulating *Trichoderma* culture in a Petri dish and gently shaken to coat the spawn grains with spores. These infected spawn grains were used as primary inoculum to provide *Trichoderma*-infected compost.

***Production of Trichoderma* infected compost.**

Crates (0.6 x 0.4 x 0.3m) were filled with 10 kg of Phase II compost spawned with *A. bisporus (*strain Amycel Delta). Six *Trichoderma*-inoculated spawn grains were placed into a 10 cm deep well in the centre of each crate then covered over with compost. *Trichoderma aggressivum* strain Fm10 and *Trichoderma harzianum* strain Fm2 (from project M 46) were included in this experiment. Control crates with only *A. bisporus* were also included. Three replicate crates were prepared for each treatment. Crates were incubated at 25°C for three weeks after which time the contents of each crate were gently mixed to produce a uniform *Trichoderma*-infected compost mixture which was designated as "undiluted" (10^0) and used for assay validation.

Preparation of Trichoderma-infected compost diluted in Phase III compost for real-time PCR evaluation

Phase III compost was filled into bags (up to 100 g) and *Trichoderma* infected compost, taken from the undiluted 10^0 treatment prepared earlier, was added at the rate of 10, 1, 0.1, 0.01 and 0.001 g (equivalent to 10%, 1%, 0.1%, 0.01% and 0.001 %) giving a dilution range of 10^{-1} to 10^{-5} (Figure 1). Six replicates of each dilution were prepared independently, three of which were sent to FERA for real time PCR diagnostic assay and three which remained at

Figure 1. Quantities of *Trichoderma* - infected compost added to 100 g Phase III

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Teagasc for microbiological and standard PCR assay.

Detection Methods

Compost plate test:

Ten pieces of compost from each replicate were plated out onto malt agar containing 100 mg kg^{-1} Streptomycin and incubated at 25°C for six days, after which they were scored for presence or absence of *Trichoderma*. Selected cultures were tested by PCR to confirm if they were *T. aggressivum* or *Trichoderma* sp.

Compost extract dilution and most probably number (MPN) test:

A compost extract was prepared for each replicate whereby 20 g of compost was soaked for 1 hr in 200 ml sterile water, blended for 1 minute, rested for 5 minutes, blended again for 1 minute then a 5 ml aliquot was removed and retained. Each extract was then serially diluted to 10^{-6} and 10 x 10μl aliquots were dotted onto malt agar + 100 mg kg^{-1} Streptomycin. Plates were incubated at 25°C for three days then scored for presence out of ten for *Trichoderma* growth. The results were analysed using the Most Probable Number method to calculate the number of *Trichoderma* propagules present per gram fresh weight of compost (Halverson and Ziegler, 1932).

Standard PCR detection:

Trichoderma cultures were obtained from the MPN tests and used for PCR testing using primer sequences that could discriminate (a) T*. aggressivum f. europaeum* and (b) the *Trichoderma* genus (Muthumeenakski, pers. comm.). No primers were available to detect *T. harzianum* specifically. DNA was extracted from the plate cultures using a Sigma Genelute Plant Genomic DNA miniprep kit (G2N10). PCR reactions were carried out in an Eppendorf Mastercycler under the following conditions: 94°C for 2 minutes followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 45 seconds and 1 cycle at 72°C for 7 minutes. Reaction Volume was 12.5 µl. Sigma Redtaq Readymix (R2523) was used in the PCR reaction master mix. DNA was stained with ethidium bromide and visualised by UV light in a 1.5% agarose gel.

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Evaluation of the real-time PCR assays

Forty-two samples of Phase III compost were analysed for the presence of *T.harzianum* and *T.aggressivum*. The new DNA compost extraction method was used followed by detection using the newly designed real time PCR assays.

Thermo-cycling conditions as follows:

50 °C for 2 minutes - 1 cycle

95 °C for 10 minutes

95 °C for 15 seconds & 60 °C for 60 seconds (40 cycles)

TaqMan reaction composition:

Reagent	**Volume**	**Final Conc. in 25 µl**
2x Environmental Master Mix	12.5 µl	(1x)
Forward Primer 7.5 µM	1 µl	300 nM
Reverse Primer 7.5 µM	1 µl	300 nM
Probe 5 µM	0.5 µl	100 nM
Water	8 µl	N/A
DNA	2 µl	N/A

Results

Assay evaluation

Specificity

The specificity of both assays was determined against a range of cultures (Table 1). The *T.aggressivum* assay was found to be highly specific and sensitive (Table 2). However, not surprisingly due to the complexity of *T.harzianum* taxonomy, a reliable assay for testing compost for this species could not be developed. This assay however did not cross react with any isolates of *Trichoderma aggressivum* or any other species of *Trichoderma* tested. It failed on one occasion to detect an isolate of *Trichoderma harzianum* although the other five isolates were identified correctly. The main problem was that the sensitivity of the assay was poor in comparison to the *Trichoderma aggressivum* assay. Therefore, although this assay will correctly identify most isolates of *Trichoderma harzianum* it lacks the sensitivity needed for detection of this species at low level in a complex matrix such as compost.

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Table 1. Specificity of the *T. harzianum* and *T. aggressivum* TaqMan assays (- = Ct 40)

Specificity			
Culture reference	**Name**	***T. harzianum* TaqMan results**	***T. aggressivum* TaqMan results**
2663	*Trichoderma* sp. (not Th1-4)	-	19.78
2664	*Trichoderma aggressivum* f. *eur.*	-	19.78
2665	*Trichoderma aggressivum* f. *eur.*	-	20.99
2666	*Trichoderma aggressivum* f. *eur.*	-	19.92
2667	*Trichoderma virens*	-	-
2668	*Trichoderma viridie*	-	-
2669	*Trichoderma aggressivum* f. *eur.*	-	19.92
2670	*Trichoderma aggressivum* f. *eur.*	-	35.48
2671	*Trichoderma aggressivum* f. *eur.*	-	20.80
2672	*Trichoderma aggressivum* f. *eur.*	-	20.47
2673	*Trichoderma* cf. *atroviridie*	-	-
2674	*Trichoderma aggressivum* f. *eur.*	-	32.98
2675	*Trichoderma harzianum*	30.15	-
2676	*Trichoderma aggressivum* f. *eur.*	-	21.34
2677	*Trichoderma aggressivum* f. *eur.*	-	19.76
2678	*Trichoderma aggressivum* f. *eur.*	-	20.21
2679	*Trichoderma harzianum*	-	-
2680	*Trichoderma aggressivum* f. *eur.*	-	18.68
2681	*Trichoderma harzianum*	25.62	-
2682	*Trichoderma harzianum*	37.91	-
2683	*Trichoderma aggressivum* f. *eur.*	-	21.26
2684	*Trichoderma harzianum*	39.22	-
2685	*Trichoderma aggressivum* f. *eur.*	-	31.05
2686	*Trichoderma harzianum*	34.72	-

Table 2. Sensitivity of the *T. harzianum* and *T. aggressivum* TaqMan assays

Sensitivity		
Concentration	***T. aggressivum* TaqMan**	***T. harzianum* TaqMan assay**
100 ng/ul	23.41	27.02
10 ng/ul	26.58	29.17
1 ng/ul	27.57	30.79
100 pg/ul	33.12	32.61
10 pg/ul	36.56	37.07
1 pg/ul	39.58	-
100 fg/ul	-	-

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The *T.aggressivum* assay was shown to be highly sensitive and detected very low levels of the *T.aggressivum* DNA, however, the *T.harzianum* assay was significantly less sensitive.

Assessing the suitability of the compost extraction protocol

Compost extracts were tested for general bacteria and an internal standard (Table 3) to determine the suitability of the extracts for PCR. The results were comparable to the soil protocol and therefore were evaluated further.

Table 3. Cycle threshold value for the 4 compost samples tested for general bacteria and the internal standard plasmid (Ct values range from 0-40, the lower the value the greater the concentration of DNA present; a Ct value of 40 means no DNA was detected).

Sample	General Bacteria		Internal Standard	
	Ct	**Ct**	**Ct**	**Ct**
1	20.9	17.8	22.6	22.3
2	18.4	17.7	22.6	22.4
3	16.1	15.7	21.5	21.5
4	16.5	16.2	23.1	23.1

The assay was shown to be an excellent method for DNA extraction from Phase III compost.

Trichoderma-infected composted diluted in Phase III compost

Compost plate test

All compost fragments removed from all the *Trichoderma aggressivum*-infected Phase III samples resulted in *Trichoderma* growth (Figure 2). Growth was more rapid and abundant for Phase III compost infected at a high rate of 10% or 1 % (10^{-1}-10^{-2}) but *Trichoderma* growth still occurred at the lowest level of infection of 0.001 % (10^{-5}). *Trichoderma harzianum* was only detected down to 0.1 % (10^{-3}). All the control–uninfected compost was clear of *Trichoderma*.

Quantity of *Trichoderma*-infected compost added to Phase III:

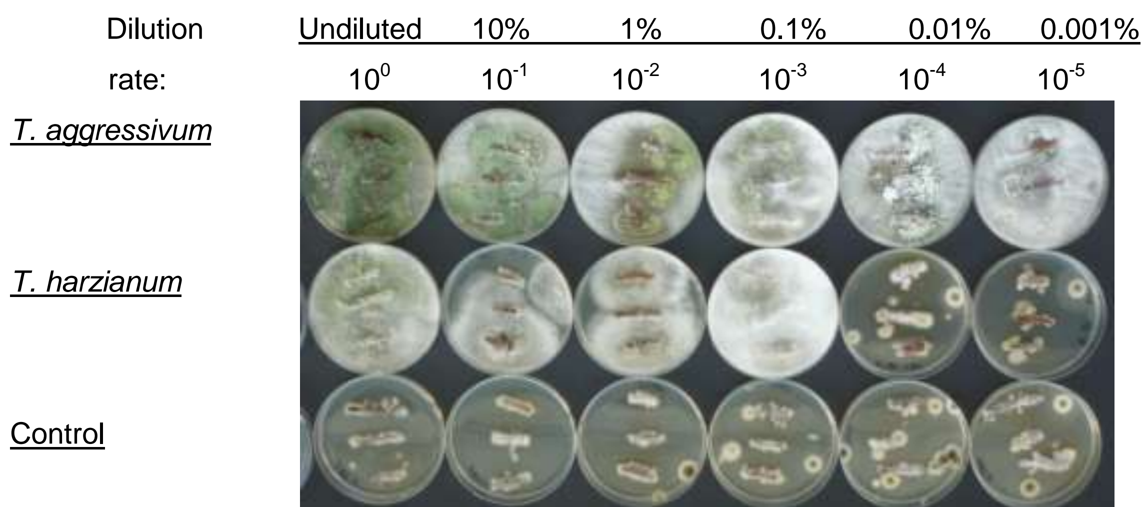

Figure 2. Growth of *Trichoderma* from Phase III compost to which different types and rates of *Trichoderma*-infected compost were added.

Propagule counts (Most Probable Number test)

T. aggressivum propagules were detected in all Phase III compost samples to which *T. aggressivum*-infected compost had been added up to, and including, very low dilution levels of 1 in 10^{-5} or 0.001% in Phase III (Figure 3 and Table 4). The undiluted fully-infected compost contained 2.9 x 10^{9} propagules/gfw according to the MPN test and it is likely that propagules would still be transferred to Phase III compost up to a dilution factor of 10^{6}or 10^{7}.

The number of propagules recovered from the undiluted *T. harzianum* infected compost was significantly lower at 2.2 x 10^{4} propagules/gfw and consequently it was present at lower levels when diluted into Phase III compost (Figure 3 and Table 5). It was only detected up to a dilution rate of 1 in 10^{2} using the MPN test but is likely to have been detectable at a 1 in 10^{3} dilution rate if the test methodology included some additional lower dilutions. The lowest dilutions for the 10^{-3} to 10^{-5} treatments had been omitted in error and hence some propagules may have gone undetected.

PCR tests

Trichoderma cultures obtained from all *Trichoderma aggressivum* compost dilutions were positive for *T. aggressivum f. europaeum* by standard PCR tests. Cultures from selected *T. harzianum* compost dilutions were positive for *Trichoderma* genus Table 6.

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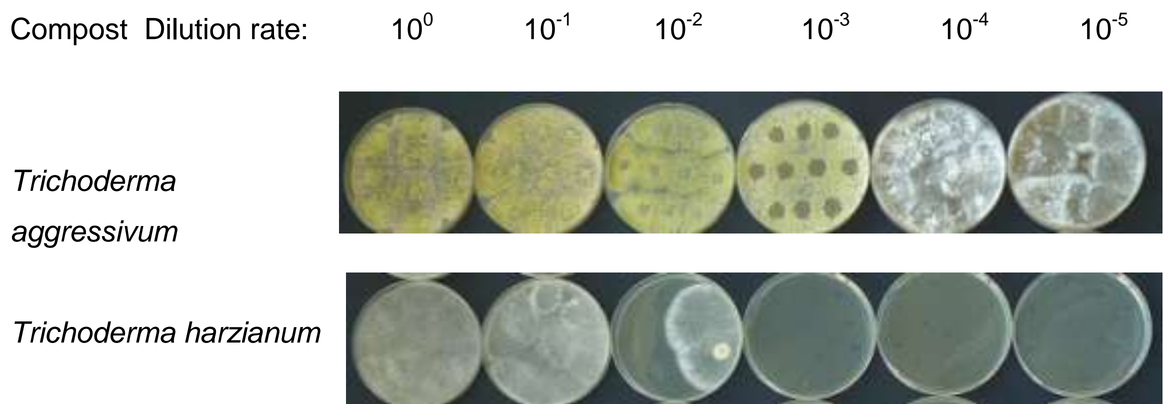

Figure 3. Growth of *Trichoderma* in one MPN plate series for *T. aggressivum* and *T. harzianum* for each compost dilution rate

Table 4. Mean number (MPN test) of *T. aggressivum* propagules detected in Phase III compost to which *Trichoderma* infected compost has been added and diluted

Grams of *T. aggressivum* - infected compost added to 100 g of Phase III	Undiluted	10g	1g	0.1g	0.01g	0.001g
Dilution rate	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Trichoderma propagules detected /g.f.w: Rep 1	7.4 x10^{9}	1.7 x10^{8}	3.9 x 10^{6}	5.7 x10^{4}	3.9 x10^{3}	1.4 x10^{3}
Rep 2	0.6 x10^{9}	2.7 x10^{8}	2.0 x 10^{6}	6.9 x10^{4}	7.8 x10	10 x10^{3}
Rep 3	0.7 x10^{9}	1.7 x10^{8}	1.5 x10^{6}	4.3 x10^{4}	5.1 x10^{3}	1.8 x10^{3}
Mean:	**2.9 x 10^{9}**	**2.0 x10^{8}**	**2.5 x 10^{6}**	**5.6 x10^{4}**	**5.6 x10^{3}**	**4.5 x10^{3}**
Standard deviation:	**0.63**	**0.61**	**1.30**	**1.31**	**2.17**	**4.97**

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Table 5. Mean number of *T. harzianum* propagules detected in Phase III compost to which *Trichoderma*-infected compost had been added and diluted

Grams of *T. harzianum*-infected compost added to 100 g of Phase III	Undiluted	10g	1g	0.1g	0.01g	0.001g
Dilution rate	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Trichoderma propagules detected /g.f.w.: Rep 1	2.3 $x10^4$	2.4 $x10^3$	11 x 10^2	#	#	#
Rep 2	1.7 $x10^4$	1.9 $x10^3$	7.3 x 10^2	#	#	#
Rep 3	2.5 $x10^4$	1.8 $x10^3$	7.3 $x10^2$	#	#	#
Mean:	**2.2 x 10^4**	**2.0 $x10^3$**	**8.6 x 10^2**	**#**	**#**	**#**
Standard deviation:	**0.38**	**0.33**	**2.12**			

\# propagules were not detected with the methodology used

Table 6. Comparison of methods to detect *Trichoderma* spp. in Phase III mushroom compost.

***Trichoderma* species**	**Compost dilution**	**(1) Compost plate test**	**(2) MPN Test**	**Propagules gfw**	**PCR test on culture from (1) or (2)**	**Real time PCR**
T. aggressivum	10^0	+	+	2.9 x 10^9	+	+
	10^{-1}	+	+	2.0 $x10^8$	+	+
	10^{-2}	+	+	2.5 x 10^6	+	+
	10^{-3}	+	+	5.6 $x10^4$	+	+
	10^{-4}	+	+	5.6 $x10^3$	+	+
	10^{-5}	+	+	4.5 $x10^3$	+	+
T. harzianum	10^0	+	+	2.2 x 10^4	+ #	-
	10^{-1}	+	+	2.0 $x10^3$	+ #	-
	10^{-2}	+	+	8.6 x 10^2	nt	-
	10^{-3}	+	-	Undetected	nt	-
	10^{-4}	-	-	Undetected		-
	10^{-5}	-	-	Undetected		-

+ /- = positive/negative result; nt = not tested, # No *T. harzianum* specific primers available, test was positive for *Trichoderma* genus and negative for *T. aggressivum.*

Evaluation of real-time PCR assays using artificially inoculated compost samples

Table 7. Cycles threshold values for the artificially inoculated compost samples

Sample Number	**Strain/Dilution**	***T.aggressivum* TaqMan**	***T.harzianum* TaqMan**
1	Negative control	40	40
2	Negative control	40	40
3	Negative control	40	40
4	Negative control	40	40
5	Negative control	40	40
6	Negative control	40	40
13	Th1 10^0	40	40
14	Th1 10^0	40	40
15	Th1 10^0	40	40
19	Th1 10^{-1}	40	40
20	Th1 10^{-1}	40	40
21	Th1 10^{-1}	40	40
25	Th1 10^{-2}	40	40
26	Th1 10^{-2}	40	40
27	Th1 10^{-2}	40	40
31	Th1 10^{-3}	40	40
32	Th1 10^{-3}	40	40
33	Th1 10^{-3}	40	40
37	Th1 10^{-4}	40	40
38	Th1 10^{-4}	40	40
39	Th1 10^{-4}	40	40
43	Th1 10^{-5}	40	40
44	Th1 10^{-5}	40	40
45	Th1 10^{-5}	40	40
49	Th2 10^0	21.45	40
50	Th2 10^0	21.71	40
51	Th2 10^0	24.39	40
55	Th2 10^{-1}	24.04	40
56	Th2 10^{-1}	-	40
57	Th2 10^{-1}	29.95	40
61	Th2 10^{-2}	27.07	40
62	Th2 10^{-2}	27.04	40
63	Th2 10^{-2}	30.98	40
67	Th2 10^{-3}	30.84	40
68	Th2 10^{-3}	34.14	40
69	Th2 10^{-3}	33.60	40
73	Th2 10^{-4}	34.51	40
74	Th2 10^{-4}	34.95	40
75	Th2 10^{-4}	34.52	40
79	Th2 10^{-5}	34.55	40
80	Th2 10^{-5}	37.46	40
81	Th2 10^{-5}	37.75	40

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Phase III compost spiked with Th1 or Th2, over a dilution range of 10^{0} - 10^{-5} with three replicate treatments. The *T.aggressivum* assay showed excellent specificity and sensitivity, detecting dilutions of 10^{-5}. However, the *T. harzianum* assay failed to detect the pathogen.

Figure 4 demonstrates the potential of the assay to detect a further tenfold dilution.

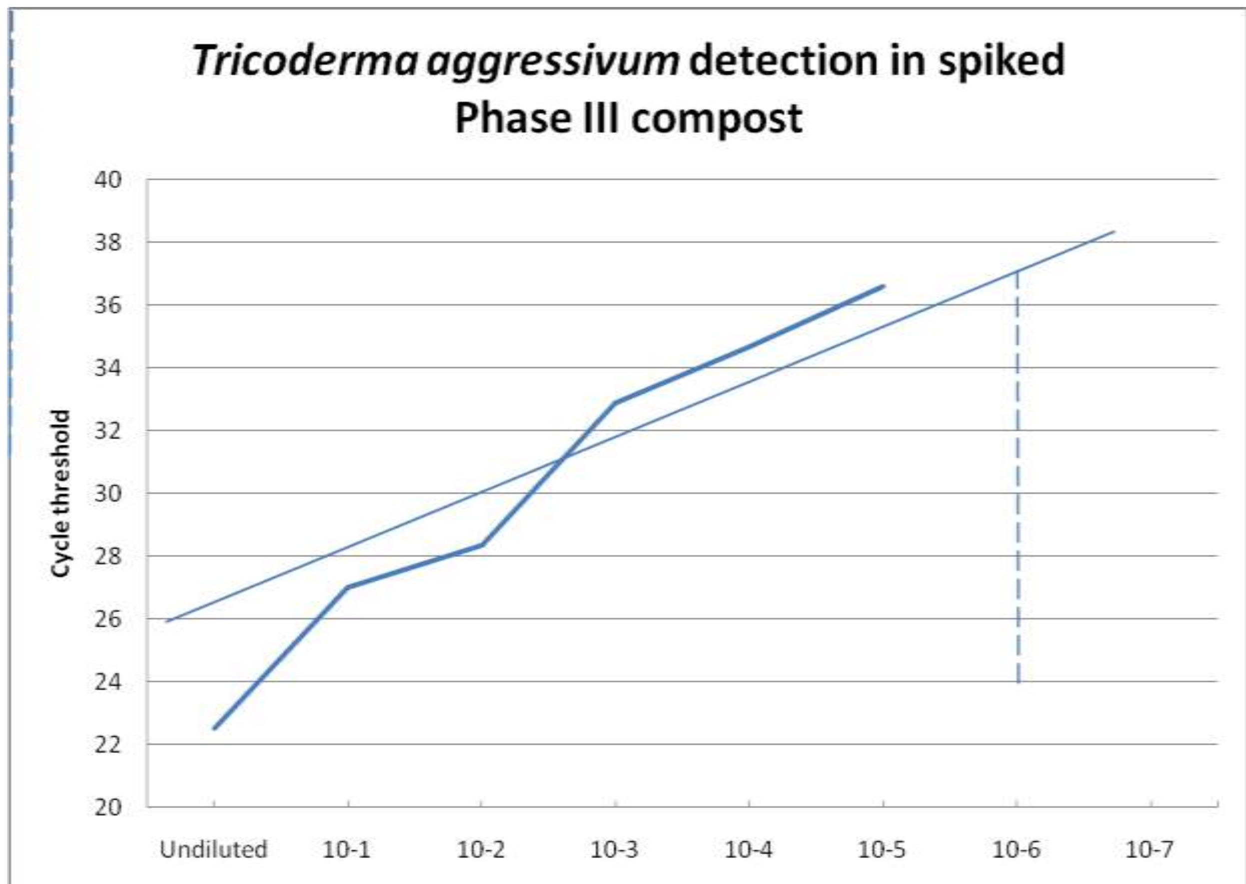

Figure 4. Detection of *Trichoderma aggressivum* in spiked Phase III compost

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Discussion

A new rapid, specific and sensitive diagnostic assay for improved disease management of *Trichoderma aggressivum* has been developed. The *T.aggressivum* real-time TaqMan PCR assay was scrutinised against 24 different *Trichoderma* cultures (Table 1); all *T.aggressivum* cultures were detected with no evidence of any cross-reactivity highlighting the specificity of the assay. The assay was also shown to be highly sensitive detecting at a least 10 pg/ul DNA. However, designing a reliable assay for detecting *T.harzianum* (Th1) was not possible due to the taxonomic complexity of the species. Although this assay did not cross react with any other species including *Trichoderma aggressivum* it did not consistently identify all isolates of *Trichoderma harzianum* and was significantly less sensitive.

To investigate the suitability of the DNA Phase III compost extraction method, samples were spiked with plasmid DNA. The resulting extracts were then tested using general bacteria primers and internal standard primers. The results were equivalent to an existing soil protocol and therefore the method was suitable for further evaluation. Phase III compost samples were provided for assay which were spiked with different dilutions of *T.harzianum* or *T.aggressivum.* For the real-time PCR assays, as expected none of the *T.harzianum* spiked samples were detected which supports previous concerns over the assays reliability. However the Th2 assay proved to be sensitive to detect *T.aggressivum* to a dilution of 10^{-5} that contained approximately 4500 propagules per gram fresh weight of compost. Based on the sensitivity assay in vitro it is predicted the assay could detect a least a further tenfold dilution (450 propagules/gfw) equivalent to several conidial heads of Trichoderma. The assay will be used extensively in a further project M 50 to help investigate the epidemiology of Trichoderma compost green mould. Further work to evaluate the specificity and sensitivity will be necessary before publication.

Traditional microbiological methods (culturing) coupled with standard PCR gave comparable results to the new real-time PCR assay although the new assay was much faster (1-2 days as compared to 7-8 days). The new extraction method is semi automated permitting sixteen samples to be processed at once. It is possible to have a diagnosis within 24-48 hours and compared to the conventional methods (that take at least 8 days) is a vast and significant improvement to permit rapid decision-making. Since the development of the extraction protocol and TaqMan assay several samples from commercial premises have been tested successfully proving the robustness of this assay. There is now a rapid and cost effective service that enables growers to screen their compost for *T.aggressivum.*

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Technology transfer

- Interim reports to HDC mushroom panel
- HDC news-short news item on project initiation
- HDC news-feature article (in press)
- Final and interim reports to HDC mushroom panel
- Visit to TunnelTech North

Glossary

Biotype	Current Name	Present in UK	Economically Damaging
Th1	*Trichoderma harzianum*	yes	no
Th2	*Trichoderma aggressivum f. europaeum*	yes	yes
Th3	*Trichoderma atroviride*	yes	no
Th4	*Trichoderma aggressivum f. aggressivum*	no	yes

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