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CONTENTS

Page

Relevance to growers and practical application

Application 1

Summary 1

Research Report

1. Introduction 3
2. Background 3
3. Materials and Methods 4
   - 3.1 Amplification using the specific primer ThI-int
   - 3.2 Amplification of 18s rRNA gene
   - 3.3 Amplification of *T. harzianum*, Th2-specific fragment
   - 3.4 Restriction enzyme digestion
   - 3.5 Cloning of 460 bp fragment of rDNA into pBluescript
   - 3.6 Ligation
   - 3.7 Transformation
   - 3.8 Plasmid minipreps
   - 3.9 Sequencing

4. Results 8
   - 4.1 Diagnostic PCR for aggressive colonisers
   - 4.2 Diagnostic PCR for *T. harzianum,* Th2 isolates
   - 4.3 Detection of *T. harzianum*, Th2 directly from infested compost

5. Conclusion 12

6. References 14

7. Contract 16

RELEVANCE TO GROWERS AND PRACTICAL APPLICATION

APPLICATION

The objective of Project M10a was to exploit the molecular data generated in Project M10 in order to develop a diagnostic test that would distinguish 'aggressive' and 'non-aggressive' colonising forms of *Trichoderma harzianum.*

SUMMARY

Adoption of good hygiene methods and the careful use of fungicides have reduced the incidence of *Trichoderma* green mould in the GB mushroom industry. However, losses are still estimated to be up to £3-4 million per annum and *Trichoderma* remains a potential threat.

Previous work on Project M10 showed that aggressive colonisation of compost was most frequently due to *Trichoderma harzianum* Th2, one of three biological forms of this species found in the British Isles. DNA-based tests were able to distinguish the aggressive form from the closely related non-aggressive forms. However, the tests developed in Project M10 were not suitable for rapid screening of large numbers of isolates. Results of the current project have led to the development of a sensitive, 2-3 day test for detection of *T. harzianum* Th2. This test is based on a polymerase chain reaction and can be used either directly on extracts made from compost or on cultures of *Trichoderma* isolated by conventional methods.

Horticulture Research International may now be able to offer a diagnostic test based on this procedure through the Mushroom Clinic.

The main findings of this project were:-

1. A diagnostic test, taking 2-3 days to complete has been developed for *T. harzianum* Th2, an aggressive colonising green mould.

2. Aggressive green mould cultures from Canada and the USA (termed Th4) have been analysed using molecular techniques and shown to differ from *T. harzianum* Th2.

3. North American isolates of Th4 are unique to mushroom compost ie they are not found in any other ecological niche.

4. Aggressive green mould cultures from Australia have been analysed using molecular techniques and shown to differ from those found in the British Isles and Northern America.

5. The diagnostic test developed this project could be used to attempt to identify sources of inoculum of *Trichoderma* and to screen compost raw materials.

6. Good hygiene and careful use of fungicides remain the main control methods for green mould.

7. Great care should be taken to avoid spread between continents of aggressive *Trichoderma* strains.

RESEARCH REPORT

1. INTRODUCTION

Trichoderma species are associated with various diseases of mushroom such as cap spotting, green mould and blotch (Beach, 1937, Kligman, 1950, Kneebone & Merek, 1959). In 1985-1986, a green mould epidemic devastated parts of the mushroom industry in the British Isles (Seaby, 1987) and losses ran into several million pounds (Fletcher, 1990). A second Ireland-wide green mould epidemic occurred during 1989-1990 (Doyle, 1991) and in 1993, a smaller but significant outbreak occurred (Doyle, pers. comm.). *Trichoderma harzianum* was recognised as causing the most severe problems in compost. Although the aggressive colonisers were found to be a different biological form (Seaby, 1987, 1989 and Doyle, 1991) there are no reliable characters to identify them. Morphotaxonomically, the aggressive colonisers were not different from the other *T. harzianum* isolates from the compost.

Previous work funded by HDC (Project M10) has successfully used various DNA based techniques to differentiate the aggressive isolates from morphologically similar non-aggressive isolates. The aim of this project was to develop a diagnostic PCR-based test to detect the aggressive colonisers. A diagnostic PCR test was developed to identify aggressive isolates directly and rapidly from mushroom compost.

2. BACKGROUND

The molecular work on *Trichoderma* continued throughout the interim period of the past and the current project (1991-1996) and isolates originating from various countries have been studied. There was no information on aggressive colonisers from countries other than British Isles and North America although green mould has been reported in most mushroom-growing areas of the world. It was also found that *T. harzianum*, Th4 isolates were the aggressive colonisers in North America. The aggressive isolates were found to be exclusive to specific geographic locations.

Recently, the use of PCR technology has been focused in plant pathogen diagnostics (Henson and French, 1993).The variable spacer region in the rDNA repeat unit is an attractive source for generating species-specific PCR primers (Brown *et al.*, 1993; Mills *et al.*, 1992; Moukhamedov *et al.*, 1994; Nazar *et al.*, 1991; Sreenivasaprasad *et al.*, 1994). A primer was designed from the ITS1 sequence of aggressive isolates following the alignment of all *Trichoderma* taxa present in the mushroom compost, in order to amplify a specific and diagnostic fragment. *T. harzianum*, Th2 isolates were also shown to possess a larger ribosomal DNA compared to other taxa. The presence of an intron sequence was identified in the 18S rRNA gene and this was used in conjunction with the ITS1 spacer region to design a set of primers exclusive to *T. harzianum*, Th2 isolates.

3. MATERIALS AND METHODS

3.1 Amplification using the specific primer ThI-int

The specific primer ThI-int (5' primer) was used in combination with a modified universal primer, ITS4-ext (modified from ITS4, White *et al.*, 1990) to amplify a portion of rDNA of aggressive isolates. A 50μl PCR reaction mixture contained 5-10ng of template DNA, 5μl of 10X buffer,1μl of 100μM each of deoxynucleotide triphosphate (dNTP) mix, 1μl each of the primers (20μM stock), 0.5μl(1 unit) of Dynazyme II polymerase and sterile distilled water (up to a total volume of 50μl).The PCR mixtures were subjected to a cycle of 94°C for three minutes and to 30 cycles of one minute at 94°C, 0.30 minute at 66°C and 1 minute at 72°C followed by a final extension time of 5 minutes at 72°C. The sequence of primers are as follows:

ThI-int - CCCCCTCGCGGGTTATTTTTACT

ITS4-ext - TTCTTTTCCTCCGCTTATTGATATGC

3.2 Amplification of 18S rRNA gene

Universal primers NS1 and ITS4 (White *et al.*,1990) amplified a region of rDNA including the 18S, ITS1, 5.8S, and ITS2 from the template DNA, and primers NS1 and NS4 amplified a portion of 18S rDNA alone. The sequence of primers are as follows:

NS1 - GTAGTCATATGCTTGTCTC

NS4 - CTTCCGTCAATTCCTTTAAG

ITS4 - TCCTCCGCTTATTGATAGC

The reaction mixtures contained 1μM of each of primer and amplification occurred during 45 cycles each of 1.5 min at 94°C 2 min at 55°C and 3 min at 72°C.

18S rRNA genes from various *Trichoderma* species were amplified. The amplified DNA was ethanol-precipitated by adding 1 volume of 3M sodium acetate solution. It was redissolved in SDW to give a final concentration of 1 μg/5μl. The DNA was digested using various tetrameric restriction enzymes, namely *Alu* I, *Pal* I, *Rsa* I and *Msp* I and digests were electrophoresed. One fragment ~460 bp from the *Msp* I digest of *T. harzianum*, Th2 isolate (Figure 2b) was gel purified using a Wizard column according to manufacturer's instruction (Promega). It was then cloned into plasmid Bluescript.

3.3 Amplification of *T. harzianum*, Th2- specific fragment

A 50μl PCR reaction mixture contained 5-10ng of template DNA, 5μl of 10X buffer,1μl of 100μM each of deoxynucleotide triphosphate (dNTP) mix, 1μl each of the primers (20μM stock), 0.5μl(1 unit) of Dynazyme II polymerase and sterile distilled water (up to a total volume of 50μl).The PCR mixtures were subjected to a cycle of 94°C for three minutes and to 30 cycles of 1 minute at 94°C, 0.30 minute at 65°C and 1 minute at 72°C followed by a final extension time of 3 minutes at 72°C.

3.4 Restriction Enzyme Digestion

The amplified DNA was quantified by ethidium bromide fluorescence on a UV transilluminator with known quantities of λDNA (Sambrook *et al.*, 1989). DNA (1-2μg) was digested with 10 units of restriction enzyme overnight at 37°C according to manufacturer's instructions (Pharmacia). Digested DNA was electrophoresed in 1.4% agarose gel with suitable molecular weight marker.

3.5 Cloning of 460 bp fragment of rDNA into pBluescript

Preparation and purification of insert DNA.

Primers NS1 and ITS4 were used with total DNA of isolate ThI to amplify a region of nuclear rRNA gene block including 18S, 5.8S, ITS1 and ITS2. The amplified product was digested with restriction enzyme *Msp* I and digestion products electrophoresed on a 1% (w/v) low melting point agarose gel (Sigma) with molecular weight markers. The fragment corresponding to 460 bp was excised, transferred to a microfuge tube, melted in a water bath at 70°C for 2 min and then purified using the Wizard DNA clean up system (Promega) according to the manufacturer's instructions.

3.6 Ligation

Vector ($pBSK^+$, Stratagene) DNA was linearised by digestion with restriction enzyme *Cla* I and purified by the Wizard DNA clean up restriction system (Promega). The purified 480 bp insert DNA was ligated into *Cla* I digested vector DNA using T4 DNA ligase (Pharmacia), 15±1°C, overnight.

3.7 Transformation

Competent *E. coli* cells were produced by a modified version to that described by Draper *et al.* (1988). The recombinant plasmid DNA was introduced into *E. coli* JM83 competent cells as described by Draper and others (1988). An overnight shake culture of JM83 cells was grown in LB medium (bacto tryptone, 10 g; bacto yeast, 5 g; Nacl, 10 g l^{-1}) at 37°C. An aliquot, 1 ml, was inoculated into 100 ml of prewarmed LB medium in shake culture until the OD_{600} reached 0.5/0.6. The cells were collected by centrifugation at 300 rpm (Mistral, 6L, MSE) for 10 min, resuspended in 50 ml of ice cold solution of 50 mM $CaCl_2$ and incubated for 1 h. The cells were collected by centrifugation at 300 rpm for 10 min at 4°C. The pellet was resuspended in 5 ml of 50mM $CaCl_2$ and 20% v/v glycerol. The aliquots (100μl) were flash frozen in liquid nitrogen and stored at -80°C.

Ampicillin resistance encoded by the plasmid was used as marker to select the transformants. To the ligation mixture 100 µl of competent cells and 10 µl of TCM (Tris-HCl (100 mM pH 7.5), $CaCl_2$ (100 mM), $MgCl_2$ (100mM0 buffer) were added and incubated on ice for 30 min followed by 37°C for 2 min and finally at room temperature for 10 min. Prewarmed LB broth (1 ml) was added to the above, subjected to heat shock at 42°C for 2 min and incubated for 1 h at 37°C. The cells were collected by centrifugation in a microfuge and resuspended in 100µl of LB broth.

Transformed cells with recombinant plasmid were selected for by insertional inactivation of the alpha peptide of β-galatosidase, by plating onto LB agar (Bacto agar No. 1, 0.2% w/v, Oxoid) plates containing ampicillin (100 µg ml^{-1}), X-gal 40 µg ml^{-1} (5-bromo 4-chloro 3-indolyl-β-D galactosidase) and IPTG (5 mM, Isopropylthio-β-D-galactoside) followed by overnight incubation at 37°C.

White colonies containing putative recombinant plasmids were checked by restriction digestion analysis and colony hybridization using the 480 bp fragment as a probe. It was further checked by extracting the plasmid DNA (miniprep) and digestion with *Bam*H I and *Kpn* I enzymes which resulted in an approximately 480 bp length product, visualized on gel.

3.8 Plasmid minipreps

A single white colony was inoculated into 5 ml of LB medium containing ampicillin (100 µg ml^{-1}) and the culture was grown at 37°C overnight in an orbital shaker (200 rpm). The cells were pelleted from a 1.5 ml aliquot by centrifugation in a microfuge (14,000 rpm, 1 min). The pellet was resuspended by vortexing in 100 µl of an ice cold buffer containing 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0), 4 mg ml^{-1} lysozyme (freshly added) and incubated at room temperature for 5 min. To the suspension, 200 µl of a freshly prepared solution of 0.2 M NaOH and 1% SDS was added, the contents were mixed by inversion and then incubated on ice for 5 min. Potassium acetate solution, (5 M, 150 µl, pH 4.8) was added and the tube was vortexed in an inverted position for 10 sec, stored on ice for 5 min and centrifuged in a microfuge for 5 min. The supernatant was transferred to a fresh tube and an equal volume of phenol/chloroform (1:1) added and mixed by vortexing. The contents were

centrifuged in a microfuge for 2 min and to the aqueous phase, 0.1 volume of 3 M sodium acetate pH (5.2) and 2.5 volumes of ice cold ethanol was added. The solution was centrifuged for 5 min and the DNA pellet was washed with 70% ethanol and resuspended in 50 μl sterile distilled water.

3.9 Sequencing

To sequence the insert in pBluescript, approximately 500-800ng of DNA was used. The clones were sequenced using primers M13 forward, T3 and T7 .

Sequencing reactions were performed by cycle sequencing using the Prism Ready reaction dedeoxy terminator cycle sequencing kit (Applied Biosystems) according to manufacturer's instructions.

4. RESULTS

4.1 Diagnostic PCR for aggressive colonisers

The ITS1 sequences of *T. harzianum*, Th1, 2, 3 and 4 were aligned. A region common to *T. harzianum* Th2 and Th4 was chosen for designing a primer specific to these aggressive compost colonisers. The specificity was also checked with multiple sequence alignment with the ITS1 sequences of other *Trichoderma.* The ThI-int primer (23 bp long) in conjunction with ITS4ext primer amplified a ~450 bp fragment from *T. harzianum* Th2 and Th4 isolates alone under specified PCR conditions (mentioned in Materials and Methods) and did not prime amplification from any other *Trichoderma* species (Figure 1a).

The annealing temperature needed to be high to eliminate mismatch priming with *T. harzianum*, Th1 . The application of this PCR was extended to detect *T. harzianum*, Th2 directly from the compost.

4.2 Diagnostic PCR for *T. harzianum*, Th2 isolates

The primers NS1 and ITS4 amplified a fragment of approximately 2.4 kb from different species of *Trichoderma* but a ~3.0 kb fragment from *Trichoderma harzianum,* Th2 (Figure 2a). A product was amplified from at least ten isolates of *T. harzianum,* Th2 (Th2 Shiitake, Th2(01), Th3(06), T13JF, TD3, T9JF, Th2A KPNT, D01001 and ThI), twelve isolates of *T. harzianum*, Th1 (Th1M, T28JF, 13UCD, TD15, LK, Rinker2, Rinker3, Th1USA, Th1-3USA LETray, 59USA and 93-1USA), thirteen isolates of the *T. viride* complex (Th3b, A006022, T11JF, Th3McG, TvSH18, DTvir, A043004, PC1150, IMI282744, 5A, 23USA, 47USA and 92USA), six isolates of *T. harzianum,* Th4 (AF, BE, BQ, Rinker1, RM10casing and RM10manure), three isolates of *T. longibrachiatum* (LESpawn, T25JF and IMI232088), two isolates each of *T. citrinoviride* (T5JF and T27JF) and *T. hamatum* (T1JF and Th3USA) and one isolate each of *T. polysporum* (IMI206039), *T. piluliferum* (IMI185209), *T.saturnisporum* (IMI146852), *T. reesei* (ICMP6526), *T. longibrachiatum* (TISSP), *T. hamatum* (IMI204016) and *T. aureoviride* (IMI138258). Digestion with *Rsa* I resulted in one fragment for all whereas digestion with *Alu* I, *Pal* I and *Msp* I resulted in many fragments. Generally many fragments were shared for all isolates revealing the conserved nature of the rDNA genes. A fragment approximately 460 bp in size was generated with *Msp* I digestion (Figure 2b), only from *T. harzianum*, Th2 isolates. This fragment from isolate ThI was cloned into plasmid Bluescript. The Southern blot containing *Msp* I digests of a representative isolate of *T. polysporum, T. piluliferum, T. harzianum,* Th1 (T28JF), *T. harzianum*, Th2 (Th2Shiitake), *T. saturnisporum, T. reesei, T. citrinoviride* (T27JF), *T. longibrachiatum* (T25JF), *T. viride* (DTvir), *T. koningii* (IMI282744), *T. harzianum,* Th3 (Th3b), *T. hamatum* and *T. aureoviride* was probed with the cloned fragment. Many higher molecular weight fragments from all taxa hybridized to the cloned fragment (result not shown). Therefore, the attempt to use this fragment as a probe was not successful. The additional length observed in the 18S rRNA gene with *T. harzianum*, Th2 isolates and the cross-reaction of the cloned fragment with other taxa could be explained by the presence of intron sequence (s) dispersed with conserved sequences and the cloned fragment not being the intron sequence or exclusive to *T. harzianum,* Th2 isolates. To confirm this, sequence analysis of the cloned fragment was carried out.

Eight clones were sequenced and shown to be identical. A region of 439 nucleotides was determined (Figure 3). The comparison of conserved sequence elements P, Q, R and S (Cech, 1988) confirmed the presence of group I intron in 18S rRNA gene of *T. harzianum,* Th2 isolates. The cloned fragment actually contained Q, R and S sequences. The intron sequence covered the first 236 bp of the clone and the rest of the sequence showed absolute homology to the 18S rRNA of published sequences.

Many fungi are known to possess introns in the 18S rRNA gene, at the 3' end for e.g. *Ustilago maydis* (De Wachter *et al.,* 1992), *Phialophora americana* and *Cenococcum geophilum* (Rogers *et al.,* 1993) and *Protomyces inouyei* (Nishida *et al.,* 1993). Amplification with primers NS1 and NS4, ITS1 and ITS4 and NS1 and ITS2 primers revealed the presence of the additional length at the 3' end of 18S rRNA gene of *T. harzianum,* Th2 isolates (result not shown) and it was further confirmed by sequence analysis.

The intron sequence ends at 1177 bp on the alignment of several 18S rRNA sequences from 13 fungal species (Morales *et al.*, 1995). The group I intron sequence length varied from 340 bp to 411 bp in various fungal species (Nishida *et al.*, 1993). The cloned fragment contained only a part of group I intron sequence. Future work utilized the intron sequence to design a specific primer to *T. harzianum,* Th2 isolates.

Two primers, one from the intron sequence, 21 bp in length (5'TAACAACACGCCTGCTTAAGA3') and the other, from ITS1 region, 23 bp in length, (5'GAGAAGGCTCAGATAGTAAAAAT3'), of *T. harzianum*, Th2 isolates, were designed. These primers amplified ~820 bp fragment from all 52 isolates of *T. harzianum*, Th2 and did not prime amplification from any other *Trichoderma* species (Figure 1b).

Subsequently, a method to detect *T. harzianum*, Th2 directly from the compost was developed.

4.3 Detection of *T. harzianum*, Th2 directly from the infested compost

The detection test can be carried out in two different ways, either directly on the compost or

on *Trichoderma* colonies grown out of the compost.

Compost was freeze-dried and ground into a fine powder using a coffee-blender. The powder (100mg) was transferred to a 2ml Eppendorf tube and thoroughly mixed with 1ml of extraction buffer (250mM $NaCl_2$ 200mM Tris-HCl pH7.5, 1% SDS and 1mM EDTA). It was incubated at 37°C for 30 minutes. To this tube, 800µl phenol/chloroform (1:1) was added and the suspension was mixed thoroughly. The tube was centrifuged for 30 minutes at 14000 rpm in a microcentrifuge. The aqueous (upper) phase was purified twice by passing through a chelex-100(BioRad) column. Chelex resin was suspended in sterile distilled water to give a final concentration of 20%(w/v) solution. The slurry was used to pack a 5ml syringe to make a column. Sterile distilled water was used as elution solution and each time 2.5 ml of solution was eluted from the column. After passing through a second column, 1.25 ml of isopropanol was added to the eluent and this was then centrifuged for 10 minutes at 6000rpm in a table-top centrifuge. The supernatant was discarded and the pellet was washed once with 80% ethanol. The pellet was dried under vacuum and dissolved in 100µl of sterile distilled water. Fifty µl of this solution was then purified through Qiagen's QIAquick PCR purification column according to the manufacturer's instruction. The final volume of the extract was 50µl and this was diluted 20 to 50 times accordingly for PCR. In the PCR reaction mixture, 5µl of this solution was used.

Following the alternative method, the weed mould analysis of the compost was done (see Appendix I). The detection test was carried out on the *Trichoderma* components cultured on OSEA plates. A tiny part of the fungal colony was removed using a 1µl inoculation loop and added to a microfuge tube containing 50µl of sterile distilled water. The contents were snap-freezed using liquid Nitrogen and crushed using micro-pestle while thawing. The freezing/crushing cycle was repeated twice. The contents were boiled for 10 min and cooled on ice rapidly.The mixture was passed through Qiagen's Qiaquick column following the manufacturers' protocol and DNA was dissolved in 50µl of sterile distilled water. 5 µl of this solution or an appropriate dilution was taken for PCR. The latter method helps in determining the percentage of problematic organisms per gram fresh weight of compost.

A ~820 bp fragment was amplified from 1 mg of *T. harzianum*, Th2 isolate powder when

mixed with 100mg of compost powder (Figure 1b). In addition the presence of an aggressive isolate was also detected using this method directly from compost thought to contain *Trichoderma* sent for analysis to the Mushroom Clinic, HRI. The entire process can be completed within 2 days.

5. CONCLUSION

Molecular analyses of isolates of *T. harzianum* found in commercial mushroom houses and compost yards in the British Isles have enabled the unambiguous differentiation of aggressive compost colonizer *T. harzianum* Th2 from Th1 and Th3. *T. harzianum* Th2 unlike Th1 and Th3, has not been isolated from niches other than mushroom compost (Muthumeenakshi *et al.,* 1994 and Muthumeenakshi, 1996). Hence when serious compost infestation by *T. harzianum* occurred in North America it was speculated that *T. harzianum* Th2 may be responsible and that inadvertently it had been introduced from the British Isles. Comparison of rDNA and mtDNA RFLPs and the nucleotide sequences of the spacer region of rDNA together with some distinct differences in morphological characteristics, however, clearly showed that these two micro-organisms are not the same and that they represent two distinct taxa of *T. harzianum*. Based on these data, we propose that North American isolates should be designated *T. harzianum* Th4.

Within the limits of isolates that were analysed *T. harzianum* Th2 and Th4 were not found anywhere else other than mushroom compost and were restricted to their respective geographic locations. The relatively recent appearance of *T. harzianum* Th2 and Th4 which are genetically distinct from Th1 and Th3, in two geographically distant locations could suggest that: (1) they form otherwise insignificant components of the *T. harzianum* group of fungi which have flourished on introduction to the artificial environment created by the composted components in which mushrooms are grown; (2) that aggressive isolates have originated from a non-aggressive close relative as a result of recent speciation in an isolated genetically homogeneous sub-population (Brasier, 1987).

DNA sequence analysis of regions of the rDNA gene block of *T. harzianum* Th2 and Th4 has highlighted differences between these isolates and non-aggressive isolates. These differences

have been used to develop a diagnostic test based on the polymerase chain reaction amplification of specific DNA fragments.

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Contract between HRI (hereinafter called the "Contractor") and the Horticultural Development Council (hereinafter called the "Council") for a research/development project.

1. TITLE OF PROJECT

Contract No: M10a
Contract date: 1.3.95

DEVELOPMENT OF A RAPID DIAGNOSTIC TEST FOR COLONISING FORMS OF *TRICHODERMA*

2. BACKGROUND AND COMMERCIAL OBJECTIVE

Trichoderma species affect mushrooms in various ways producing cap spotting and also colonising the compost and interfering with mushroom mycelial growth. Recently *T. harzianum* has been found to be associated with the compost problem. Different forms of *T. harzianum* have been recognised by Seaby (1985, 1987) and Doyle (1991). Forms of *T. harzianum* are indistinguishable morphologically but differ in their ability to grow in mushroom compost. Differentiating between the "aggressive and non-aggressive" form requires precise laboratory work and takes about a week.

Previous work funded by HDC (Project M10) has successfully used DNA-based techniques to differentiate *T. harzianum* strains.

Further work is still required to develop a rapid (1-2 day) test.

This funding will help establish molecular diagnostic techniques within HRI and may assist in attracting further funding from overseas to create a global centre for *Trichoderma* testing.

3. POTENTIAL FINANCIAL BENEFIT TO THE INDUSTRY

Trichoderma continues to be a serious threat to mushroom production. A reliable diagnostic procedure would be of direct benefit to producers and would indirectly benefit the industry by underpinning research on control of *Trichoderma* (see 5. below).

Losses due to *Trichoderma* have been estimated at approximately £3M per annum.

4. SCIENTIFIC/TECHNICAL TARGET OF THE WORK

New techniques enable very precise identification of fungal strains. These utilise certain biochemical reactions and the ultimate test (PCR test) is very specific and quick.

HDC-funded research has resulted in differentiation of *Trichoderma* isolates using RFLP analysis. Although very reliable and accurate, this test takes at least one week to complete and is labour intensive. The aim of this project will be to develop a PCR test which could be completed in 1-2 days. This test is required by the industry for confirmation of identification of aggressive isolates.

5. CLOSELY RELATED WORK

HRI is currently involved with a project investigating the efficacy of carbendazim as a control agent for *Trichoderma*. A project proposal has been submitted to MAFF for the biological control of *Trichoderma*. Both of these projects require accurate identification of isolates used in experiments.

6. DESCRIPTION OF THE WORK

Previous work has established the level of molecular variation within and between aggressive and non-aggressive isolates of *Trichoderma*. This information will now be used to develop a PCR-based test that will identify aggressive isolates from the UK and also aggressive isolates from the USA/Canada and Australia (all of which vary from UK isolates). Rapid movement of isolates has occurred within the British Isles and it is likely that aggressive isolates will move between continents. It is therefore important to develop tests that will identify 'exotic' isolates.

Milestones

1. Establish molecular diagnostic techniques at HRI

2. Synthesize oligonucleotide primers for PCR detection of *Trichoderma*

3. Assess reliability and sensitivity of PCR tests

The programme of work will include transfer of expertise in molecular diagnostic techniques to HRI staff using isolates collected during HDC funded project M10. Experiments conducted in project M10 have provided basic information on the DNA sequence of conserved and semi-conserved regions of a range of *Trichoderma* isolates. Analysis of these data will provide the sequence required for the development of an isolate specific molecular test. This is likely to be an oligonucleotide (probably about 20 bases long) for either a polymerase chain reaction of ligation chain reaction test. This laboratory test will then be developed further to cope with samples taken directly from compost. The sensitivity of a compost-based test will be assessed by comparison with culture DNA-based tests.

7. COMMENCEMENT DATE, DURATION AND REPORTING DATES

Start date 01.04;95; duration 8 months.

The experimental work will be completed by October 1995 and the final report will be produced by the end of November.

8. STAFF RESPONSIBILITIES

Project Leader: Dr Peter Mills
Other staff: To be appointed
Project co-ordinator: George Pointing

Figure 1

a)

Diagnostic PCR+ for aggressive isolates of *T. harzianum* Th2 and Th4

Lane No.	*Trichoderma* species
1-5†	*T. harzianum* Th2
6-9	*T. harzianum* Th1
10-13	*T. harzianum* Th3
14	*T. citrinoviride*
15	*T. longibrachiatum* Th2
16	*T. hamatum* Th3
18-22†	*T. harzianum* Th4

† Amplification of 450 bp fragment from aggressive isolates of *T. harzianum*.
+ Primers used
ThI int - CCCCCTCGCGGGTTATTTTACT
ITS4 ext - TTCTTTTCCTCCGCTTATTGATATGC

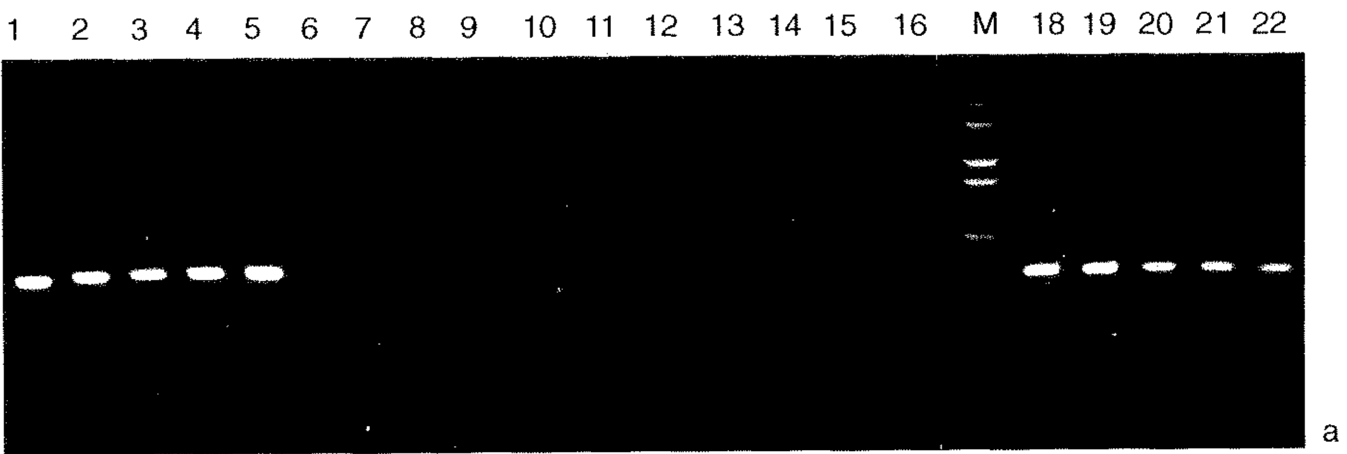
b)

Diagnostic PCR+ for aggressive isolates of *T. harzianum*.

Lane No.	*Trichoderma spp.*
1-12†	*T. harzianum* Th2
13	*T. harzianum* Th1
14+	*T. harzianum* Th2
15	*T. harzianum* Th3
16	*T. harzianum* Th4
17+	Compost extraction infested with *T. harzianum* Th2
18	Compost extraction
19	-ve control
M	Marker

† Amplification of 820 bp fragment from aggressive isolates of *T. harzianum* Th2 only
+ Primers used:
ThI 18S int - 5'TAACAACACGCCTGCTTAAGA3'
ThI int Rev - 5'GAGAAGGCTCAGATAGTAAAAAT3'

Lane M refers to DNA molecular weight VI (Boehringer Mannheim); the sizes of DNA fragments in base pairs are: 2176/1766/1230/1033/653/517/453/394/298/234/220/154


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M 18 19 20 21 22
a

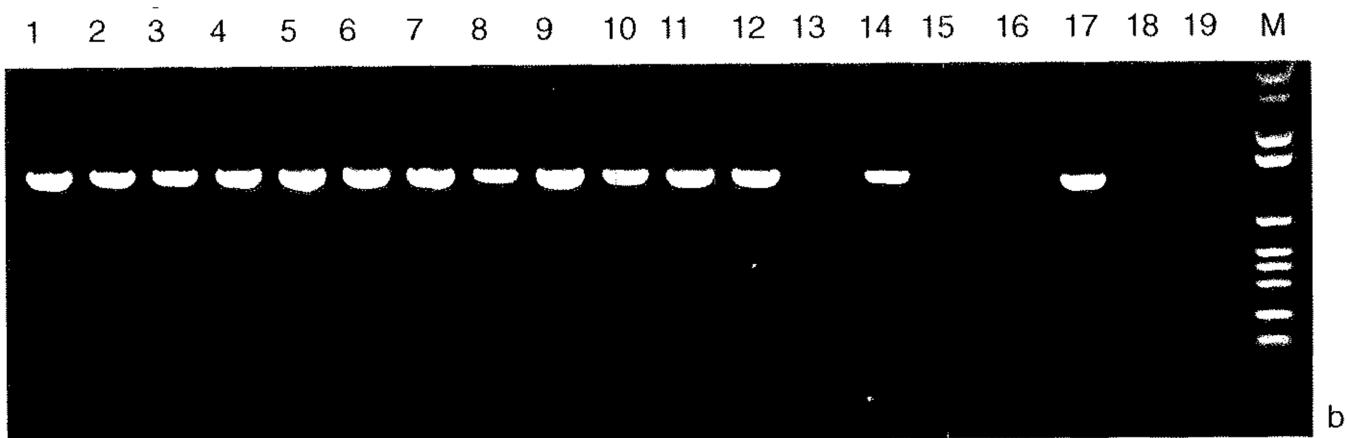

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 M
b

Figure 2

(a)

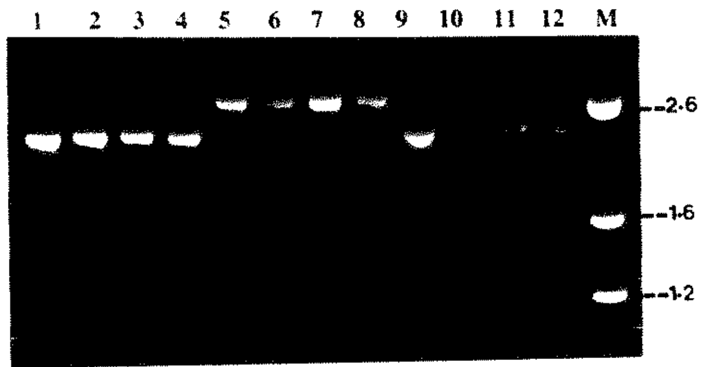
PCR amplified fragment of rDNA of *Trichoderma harzianum* Th1, 2 and 3 isolates with primers NS1 and ITS4 (M, digested pGEM used as molecular size markers).

Lane No	Isolate Code	*Trichoderma spp.*
1	Th1M	*T. harzianum* Th1
2	T28JF	
3	UCD13	
4	LK	
5	ThI	*T. harzianum* Th2
6	KPNT	
7	D010011	
8	Th2Shiitake	
9	Th3(b)	*T. harzianum* Th3
10	A006022	
11	T11JF	
12	Th3McG	

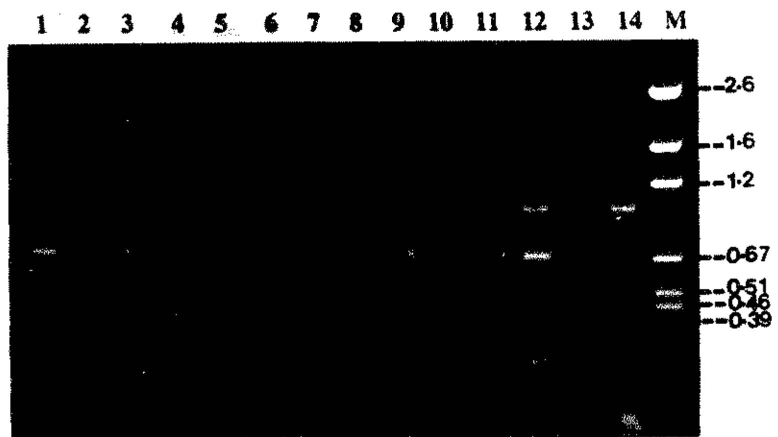
(b)

PCR amplified fragment of rDNA with primers NS1 and ITS4 for isolates of *Trichoderma* species. digested with *Msp* I. Arrow indicates ~460bp fragment specific for *T. harzianum* Th2 isolates only (M, digested pGEM used a molecular size markers).

Lane No.	*Trichoderma spp.*
1	*T. polysporum*
2	*T. piluliferum*
3	*T. harzianum* Th1
4	*T. harzianum* Th2
5	*T. saturnisporum*
6	*T. reesei*
7	*T. citrinoviride*
8	*T. longibrachiatum* Th1
9	*T. longibrachiatum* Th2
10	*T. viride*
11	*T. koningii*
12	*T. harzianum* Th3
13	*T. hamatum* Th1
14	*T. aureoviride*



a



b

ThI - 18S int clone 439 bp

1 GCGCGCGAAAGGCGTCGGTGGCCAGGTTAATAGCCTCGGGTACGGGAAAAACGAGGGCAG

61 ATGCACAATGGACAATCCGCAGCCAAGCCTCTAAGTGCCCGAGACGTATCGAGGAGGTTC
Q

121 AGAGACTTGACGGGGGTGGGTAGGCTCGCTG†**TAACAACACGCCTGCTTAAGA**TAAAGTCC
R S

↓
181 GTCCCTCGGTGAAAGCCGAAGGGGTTGCTTCTTGCAAAAGAGGGCCCAGTAAAACGGGAG

241 CCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACACAATGAGGATT

301 GACAGATTGAGAGCTCTTTCTTGATTTTGTGGGTGGTGGTGCATGCCCGTTCTTAGTTGG

361 TGGAGTGATTTGTCTGCTTAATTGCGATAACGAACGAGACCTTAACCTGCTAAATAGCCC

421 GTATTGCTTTGGCATACGC

Figure 3 **Sequence of cloned fragment from 18S rRNA gene of isolate ThI**

Q, R, and S refer to conserved sequence elements of group I introns; the arrow indicates the end of intron sequence and the beginning of partial sequence of 18S rRNA gene.
†The shadowed region indicates the primer

APPENDIX I

Weed Mould Analysis of Compost
(adapted from method for analysis of silage)

This method gives quantitative and qualitative results. *Trichoderma* colonies can be subjected to diagnostic test for isolate determination.

Compost (20g) is placed in stomacher bag and 360 mls of sdw are added. Sample is allowed to soak for 1 hour then put into the stomacher machine for 1 minute. The sample is allowed to rest for 5 mins then re-stomached for 1 min. One ml of this compost solution is put into 9 mls of sdw and shaken well to give a 10 fold dilution. This is serially diluted further to 10^5. One ml of each dilution is placed in a Petri dish and molten OSEA agar is poured into the dish and swirled around to mix. When set, the Petri dishes are incubated at 25°C for up to two weeks. Most weed mould species grow within a few days. The mean number of colonies is calculated and multiplied by a factor of 18 in addition to the dilution factor to give a figure of cfu's per gram fresh weight.

Ohio State Experimental Agar (OSEA) medium

Glucose	5.0g
Yeast Extract	2.0g
Sodium nitrate	1.0g
Glucose	5.0g
Yeast Extract	2.0g
Sodium nitrate	1.0g
Magnesium sulphate	0.5g
Sodium proprionate	1.0g
Ox-bile desiccated	1.0g
Distilled water	1.0L
Agar Technical no. 3	3.0g/200ml
	6.0g/400ml

Autoclave for 15min at 121°C.