Final Report for HDC

Project Title	An investigation of the cause and control of a causally unknown mushroom cropping problem (now known as Virus X)
Project Number	M 39
Project Leader	Richard Gaze to May 2000 Helen Grogan from May 2000
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Key Workers	Dr. Bruce Adie Dr. Helen Grogan Mr. Richard Gaze Dr. Mike Challen Dr. Leo Calvo-Bado
Location of project	HRI, Wellesbourne, Warwick, CV35 9EF
Project Co-ordinator	Mr. Andrew Middlebrook (to November 2000) Lords Mushrooms Leeds, Yorkshire, LS12 3PT Mr. Brian Oxley (from November 2000) Monaghan Middlebrook, Langford Farm, Somerset, BS40 5ES
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Key Words	Mushroom, Virus, Virus X, PCR, hygiene, transmission, epidemiology

An Investigation of the Cause and Control of a Causally Unknown Mushroom Cropping Problem (M 39)

CONTENTS

PRACTICAL SECTION FOR GROWERS

Commercial benefits of the project

Within the lifetime of this project three mushroom farms, contributing in the region of 10% of total British mushroom production, have closed down, partly as a result of Virus X disease. In addition, many growers have experienced significant yield reductions due to Virus X. A conservative cost to the industry would be £15 - 20 million/annum. The cost benefit to the industry of developing control measures is likely therefore to be significant.

Background

In autumn 1996 a single farm reported cropping problems for which no satisfactory explanation could be found. The following year several other farms described similar but transitory cropping problems. These were similar to the original site in two major aspects, an inability to pin and no convincing explanation of cause. During late 1997 a second farm, unrelated to the first, began to experience similar symptoms to the original case which continued unabated and unresolved. The conviction that something novel was occurring became difficult to avoid. During 1998 the number of farms similarly affected rose steadily to fifteen. By early 1999, it was impossible to ignore the near certainty that a novel disease was being experienced by a widening sector of the industry.

Examination of mushrooms from a problem British farm by Professor Romaine at Pennsylvania State University, using a modified electrophoretic test, showed novel bands of dsRNA (double stranded ribonucleic acid) distinct from those normally associated with Virus Disease (35nm, La France). A working hypothesis was established that "the novel mushroom disorder being encountered in Britain was caused by an unknown virus or viruses". It was called Virus ‘X’ until it could be more fully characterised by molecular methods.

Objectives

- to validate and extend the analysis of the dsRNA tests carried out by Professor Romaine
- to examine the correlation of dsRNA test results with symptoms on mushroom farms
- to investigate possible transmission mechanisms
- to develop a rapid and practical diagnostic test by producing a specific PCR (polymerase chain reaction) probe
- to investigate the epidemiology of disease spread in order to provide control strategies.

Summary of results

Approaching 400 samples have been analysed in an attempt to confirm the correlation between disease symptoms and dsRNA banding patterns. Twenty-three dsRNA bands have been associated with the Virus X complex, although they never all occur together. The maximum number of bands encountered in any one sample has been 14, and the minimum has been 1 band (Figure 1). Three "hybrid" or "healthy" bands (H1, H2 and H3) are found in mushrooms from infected sites but these are also found in healthy mushrooms from uninfected sites, and are not included in the 23 Virus X bands mentioned above.

Ds RNA bands 3, 9 and 15 were among the most common, and PCR primers have been developed for these bands. These PCR primers will not detect any other bands. However,

they are likely to be much more sensitive than the electrophoretic test, and should detect very low levels of bands 3, 9 and 15, that may not be detected by the electrophoretic test. A fourth PCR primer will detect hybrid band H2, along with bands 7 and 8, all of which are closely related. Current DEFRA-funded research aims to produce additional PCR primers and to determine what primers are necessary to give a reliable alternative to the current electrophoretic test.

The existing PCR primers have been successfully used on mushroom tissue and mycelium from spawn-run compost although further validation using spawn-run compost is necessary. The four PCR primer pairs give positive results when combined together in a single "multiplex" reaction, but it is unrealistic to think that more than four primer pairs could be combined in this way. Further work is needed to optimise and standardise the dsRNA extraction procedure from mushrooms and mycelium.

Evidence was obtained through small scale experiments that suggests that both mushroom spores and mycelium can transmit the virus. A new HDC funded project has begun (M 39a) that aims to clarify the epidemiology and transmission of Virus X by mushroom spores and mycelium.

Following contacts with growers affected by Virus X, evidence has been obtained which strongly suggests that Virus X levels can be reduced by implementing effective virus hygiene measures. This consists of protecting compost from *Agaricus* spores and mycelium at virus-vulnerable stages in the process (such as phase II cool-down, at spawning, during spawn-running, and when bulk handled).

Summary of Virus X symptoms

- **Pinning Disruption.** This manifests itself in various ways. Crops may be delayed by one to several days, with pins slow to develop, or there may be large or small areas of non-productive bed or patches. These can be circular, forming swirls down the length of a shelf, or consist of large swathes of a shelf or tray. Frequently, areas of non-productive bed grade into slow developing pins and then into apparently healthy mushrooms
- **Prematurely opening mushrooms.** This symptom manifests itself as mushrooms that develop apparently normally on the bed and appear to be closed cups. Once picked and turned over, the veil will be broken and they will have prematurely opened, resulting in a loss of quality and downgrading. This symptom often appears only in the second flush
- **Off-coloured or discoloured mushrooms**. On some sites the only symptom to be observed is the presence of off-coloured or brown mushrooms throughout a bed spawned with a white strain. They can be restricted to only a few mushrooms on a bed to consisting of up to 40% of a crop. Frequently, mushrooms appear fairly normal or only slightly greyish or pinkish on the bed, but become progressively more discoloured in storage, again leading to a downgrading of the mushrooms.
- **Loss of yield.** Gradual reductions in yield over time without any overt symptoms have been recorded. This may lead eventually to some of the symptoms above being

encountered. In reality, some pinning delay or disruption may have been present but their cause assigned to some other factor of compost or environment.

- **Malformations.** In cases where symptoms and yield losses are very severe, mushrooms are often malformed and look similar to mushroom malformations associated with virus/La France disease.

Conclusions

- A reliable electrophoretic test has also been developed to determine the profile of novel dsRNA bands in mushrooms. This is a slow and laborious test that permits only 12 samples per week to be analysed.
- Twenty-three novel dsRNA bands have been identified as being associated with Virus X disease in addition to the three "hybrid" bands that occur in healthy mushrooms.
- The correlation between the presence of these bands and novel (virus) disease symptoms has been repeatedly tested and found to be good.
- Both the bands and symptoms have been found to be more numerous and variable than at first anticipated.
- Four PCR primer pairs have been developed which can rapidly detect Virus X dsRNA bands 3, 9, 15 and H2 in mushrooms and spawn-run compost, but the dsRNA extraction procedure needs to be standardised and optimised to give reliable, repeatable, results. When fully operational, it should be capable of processing 40 samples or more per week.
- There is strong evidence to indicate that Virus X is transmitted from crop to crop by both spores and mycelium.
- A great weight of circumstantial evidence consistently indicates that the main point of entry for transmission and accumulation is the spawn running stage. This includes spawning and bulk handling of Phase II onto trays and shelves through to filling fully-run bulk Phase III.
- Virus X outbreaks on some farms have been controlled by implementing effective virus control measures to prevent *Agaricus* spores and mycelium from contaminating compost from Phase II cool-down through to spawning, spawn-running and cropping. One grower has gone from being about 50% down on production, to getting back to normal production levels after ensuring that spawn-running rooms were no longer drawing in unfiltered contaminated fresh air through leaky door seals.
- The nature of the virus complex remains unknown and DEFRA-funded research on the molecular characterisation of the virus complex is currently underway. This should identify which PCR primers are the most useful as a Virus X diagnostic.
- Further information concerning the precise epidemiology of the disease is required to achieve sustainable control and an HDC-funded research project in this area is currently underway (M 39a).

Action Points for Growers

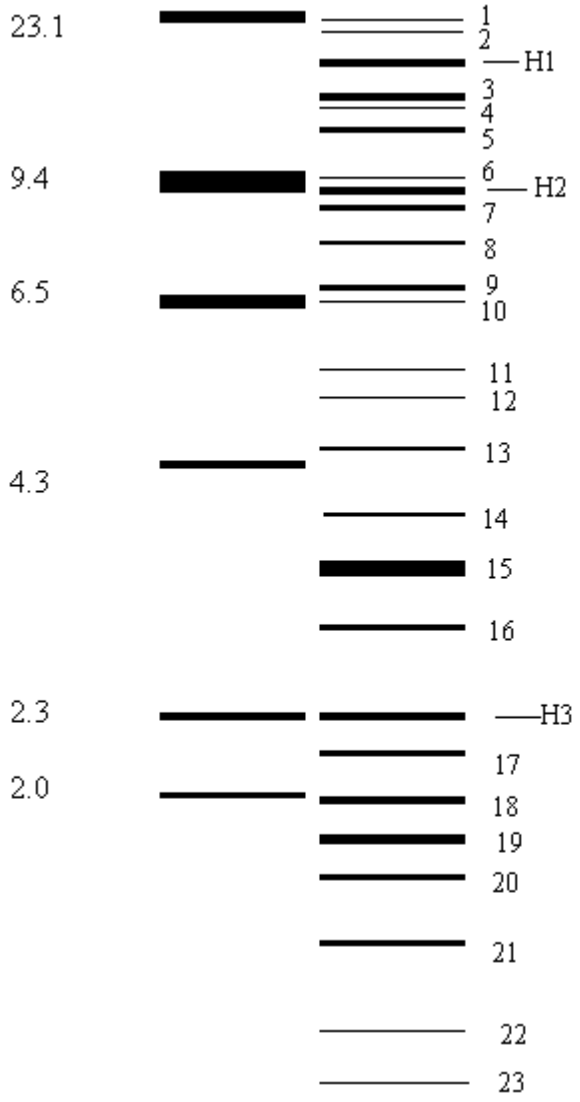
Whilst much still remains to be defined concerning this disease the results of this project clearly indicate the mode and time of virus transfer. Measures to be taken are therefore as follows: -

- Virus control, at its simplest, can be defined as the prevention of repeated and accumulative contamination of new crops by mushroom spores and mycelia from previous, older crops.
- The volume of contaminant and the earliness in the production cycle at which the contamination takes place are both important factors in determining the speed at which virus levels rise.
- The aim of 'virus hygiene' is to progress each crop through the production cycle with no biological contact with previous crops. The 'art' of virus hygiene is the detection of the many, often subtle, routes by which this protection is breached. This detection process is greatly aided by accurate judgement of where the high priority areas lie.
- The following list attempts to identify the vulnerable areas and to offer some indication of their importance. Individual circumstances will alter the emphasis that needs to be placed on each item but the principles remain true across the board. All references to spores and mycelium in this list are exclusively mushroom spores and mycelium.

Process	**Potential Routes of Virus Transmission**	**Vulnerability**
Phase I	• Spores can pass through phase I. If the quantities of spores on site are high AND there is likelihood of cool zones existing in phase II, then contaminated phase I is a potential route of transmission.	Normally Low
Phase II	• Phase II is normally safe until cool down. • Filtration of spores at cool down **MUST** be effective	High
Spawning	• The spawning process itself may offer slight or great opportunity for contamination depending on the system employed. • Tray spawning usually offers only a brief opportunity. • Spawning of bulk phase II onto shelves offers considerably greater opportunity	Medium High
Phase III & spawn running	• Owing to its long duration and early stage in the system, phase III offers a long period for contamination, allowing maximum effect to result from any contamination. **This is probably the most vulnerable area of all.** Some of the many possibilities for contamination include: • Mycelial fragments (or spores) contaminating bulk phase III at spawning/filling • Imperfect filtration of bulk phase III tunnels • Inadequately cleaned bulk phase III tunnels or contaminated trays or shelves. • Spore entry into traditional spawn-running rooms due to inadequate filtration or air recirculation systems pulling in unfiltered fresh air	VERY HIGH

Casing & Case Running	• Traditionally casing and case-running have not been considered to be at risk from virus contamination but, with the advent of high farm spore and mycelial load, and the emergence of Virus X that produces symptoms at a low level, these should now be given more consideration. • A conclusion is undoubtedly to protect casing and case running as well as possible but primarily to reduce the potential level of exposure by reducing the contaminated load on the site. This point is dealt with again under cropping.	Unknown but possibly Medium (High)
Cropping	• Until now, the cropping stage would not have been considered to be at risk from virus contamination. This is now less certain for Virus X. • What is more certain is that cropping open mushrooms now provides an increased source of spores, which have the potential to contaminate the accepted areas of great vulnerability. • The growing of open mushrooms particularly, but also of value-pack mushrooms, has resulted in very high spore loads on many farms. To assist the precautions taken in phase II/III, this spore load **MUST** be reduced, either by picking closed mushrooms or, more practically, by exhaust filtration. Whether recirculation filtration is relevant has yet to be determined.	(High ?) HIGH
Cook-out	• This is undoubtedly very important for other diseases. In some systems the reduction of mycelial debris will be advantageous in the virus context also. For example where bulk phase II or III is filled onto shelves.	Low To High
General	• Virtually all these factors can **appear** to have been dealt with whilst remaining largely ineffectual. • Always doubt the efficacy of any precaution undertaken and if possible challenge it by physical monitoring. • Most importantly "believe" the mushrooms. If they have virus disease, there is a hole, somewhere, in the protection.	High

Figure 1. The dsRNA bands associated with Virus X. Bands H1, H2 and H3 occur regularly in healthy mushrooms and are often referred to as "hybrid" or "healthy" bands. Table on the right shows the results of the analysis of 320 samples



Band Number	No. of samples with specific band (max = 320)	% of samples with specific band
0	2	1
1	26	8
2	**150**	**47**
H1	246	77
3	**82**	**26**
4	5	2
5	18	6
6	15	5
H2	253	79
7	51	16
8	**64**	**20**
9	**190**	**59**
10	4	1
11	3	1
12	28	9
13	**130**	**41**
14	9	3
15	**146**	**46**
16	8	3
H3	318	99
17	5	2
18	44	14
19	46	14
20	12	4
21	15	5
22	6	2
23	5	2

SCIENCE SECTION

1. Introduction

In autumn 1996 a single farm reported cropping problems for which no satisfactory explanation could be found. The following year several other farms described similar but transitory cropping problems (Gaze, 1997b). These were similar to the original site in two major aspects, an inability to pin and no convincing explanation of cause. During late 1997 a second farm, unrelated to the first, began to experience similar symptoms to the original case which continued unabated and unresolved. The conviction that something novel was occurring became difficult to avoid (Gaze, 1998). During 1998 the number of farms similarly affected rose steadily to fifteen. By early 1999, it was impossible to ignore the near certainty that a novel disease was being experienced by a widening sector of the industry (Gaze, 1999a, b and c).

The symptoms of this new disorder are quite diverse. The most common visible expression appears to be areas of non-productive bed (Plate 1). These areas may vary in size from small patches within a predominantly 'normal' cropping surface to, in extreme cases, being the predominant condition in which mushroom fruit bodies are the exception. In this instance, the symptoms are clumps of mushrooms in an otherwise non-productive bed.

The affected areas themselves consist of either totally unpinned casing, arrested pins or fully formed but late-developing sporophores (mushrooms). The latter symptom, when observed immediately prior to picking, looks like a hollow or depression in an otherwise even mushroom stand.

The shape of patches varies. They may be circular, entirely irregular, or in some cases, particularly on shelves, have a regular pattern along the length of the shelf. They are reported to appear in the first flush and to grow larger in subsequent flushes but also not to grow or not to appear until later flushes. They are always progressive, however, never recovering to produce healthy mushrooms. Commonly distorted or discoloured mushrooms have also been observed.

In some instances the symptomology is quite different. Bare areas of bed are absent; instead the symptoms observed are premature cap opening often associated with an absence of veil and other minor sporophore abnormalities. The appearance of 'brown' mushrooms in a white strain also appears to be associated with this disorder. In some instances all the symptoms described appear together on one site. In all cases some degree of reduced yield occurs. In extreme cases yields may be reduced to almost nil.

The first visual diagnosis of this problem suggested it was Virus Disease (35nm, La France). Samples were subsequently examined by EM, IEM (ISEM) (Barton 1985) and commercial PAGE. However, apart from one positive EM/IEM result, which could not be repeated, no problematical virus levels were detected. The British industry has remained largely free of virus disease for several decades with minor outbreaks being consistently detected by EM and ISEM tests. These tests have proved to be a reliable method of virus detection in the past, providing confidence in the results (Gaze, 1997a). A series of negative results, therefore, initially eliminated virus disease as the cause of the 'new' problem. A similar progression of visual diagnosis of virus, followed by its rejection of it based on standard virus tests, was experienced from all the farms subsequently afflicted by this disorder.

Subsequent to the initial apparent elimination of a viral cause many other potential causes were examined, some logical and many less so. The first case coincided with the adoption of aerated phase I compost. For a short while it was thought that this, for reasons unknown, was the source of the problem. New occurrences of the problem on other sites demolished this hypothesis. Pathogens and compost mould causes were sought but not found. Spawn was incriminated but exonerated as the problem spread and a wide range of spawns became involved. In short, having examined and rejected virus as a possible cause, the disorder remained a mystery until examination at Pennsylvania State University, using a modified electrophoretic test (Wach *et al.,* 1987), showed novel bands of dsRNA distinct from those associated with Virus Disease (35nm, La France).

The present supposition is that the disorder described is caused by a virus or viruses, unknown, which for the moment has been termed Virus 'X'.

The commercial objectives of the project are:

- to validate the electrophoretic test for double stranded viral RNA as carried out by Professor Romaine
- to examine the correlation of test results with symptoms expressed
- to investigate possible routes of viral transmission
- to develop a rapid and practical diagnostic test and
- to investigate the epidemiology of disease spread in order to provide control strategies

It was originally envisaged that the project would largely follow a linear progression. Validation and refinement of the electrophoretic test obtained from Professor Romaine, → correlation of the test with the occurrence of symptoms, → the production and validation of a PCR test, → epidemiological investigations and the production of control strategies.

Early in the project it became apparent that the complexity of the situation was likely to delay production of a PCR. This, together with the extreme urgency of the situation in the field, has necessitated following several lines of approach simultaneously. It has required retention of the more time-consuming and limited electrophoretic test for longer than anticipated.

Due to the nature of the project, the report has been broken down into separate chapters that deal with individual objectives. A general discussion and conclusions are provided at the end of the report.

2. Validation of the Electrophoretic Test

2.1 Introduction

Detection of dsRNA molecules associated with mushroom viruses in the past has been done using a technique known as "poly acrylamide gel electrophoresis" or PAGE. This has been replaced with a modified test in conjunction with agarose gels (Wach *et al*, 1987; Romaine & Schlaghnaufer, 1993). At the start of this project there was a need to ensure that we could repeat the modified page test which originally identified the novel dsRNA bands described by Prof. Peter Romaine from Pennsylvania State University.

2.2 Materials and Methods

Mushroom samples were collected in quantities of 150 grams of fresh material, preferably as closed cups. The samples were frozen on receipt, and then freeze dried prior to use. A 10 gram sample of freeze-dried material was used for each analysis. Samples were obtained from several sites, including sites previously examined by Prof. Peter Romaine. Mushroom samples were then examined at HRI using a slightly modified version of the electrophoretic tests described by Wach et al (1987) and Romaine & Schlaghnaufer (1993). These samples were compared with material known to be infected with Virus (La France) caused by a mixture of virus particles, predominantly 35nm but also containing 25nm and bacilliform particles. The dsRNA banding patterns of the samples were compared.

2.3 Results and Discussion

Figure 1 shows the dsRNA bands detected in three samples: (1) mushrooms from a site with Virus X symptoms, (2) healthy mushrooms from an uninfected site and (3) mushrooms infected with 35nm virus. The banding patterns in all three cases were quite different and were consistent with those found by Prof. Peter Romaine. Figure 1a is a schematic representation of Figure 1 to illustrate the band positions more clearly. Repeated testing also gave the same results, indicating that the test was reliable, and our ability to perform it was confirmed. Early in the project, it was found that the number of bands associated with the new syndrome was greater than the initial work at Pennsylvania State University had indicated. As further sampling was undertaken to test correlation between bands and symptoms this increasing complexity was further endorsed which had implications for PCR development.

Figure 1. dsRNA bands for Virus X, healthy mushrooms and La France (35nm) virus from early studies.

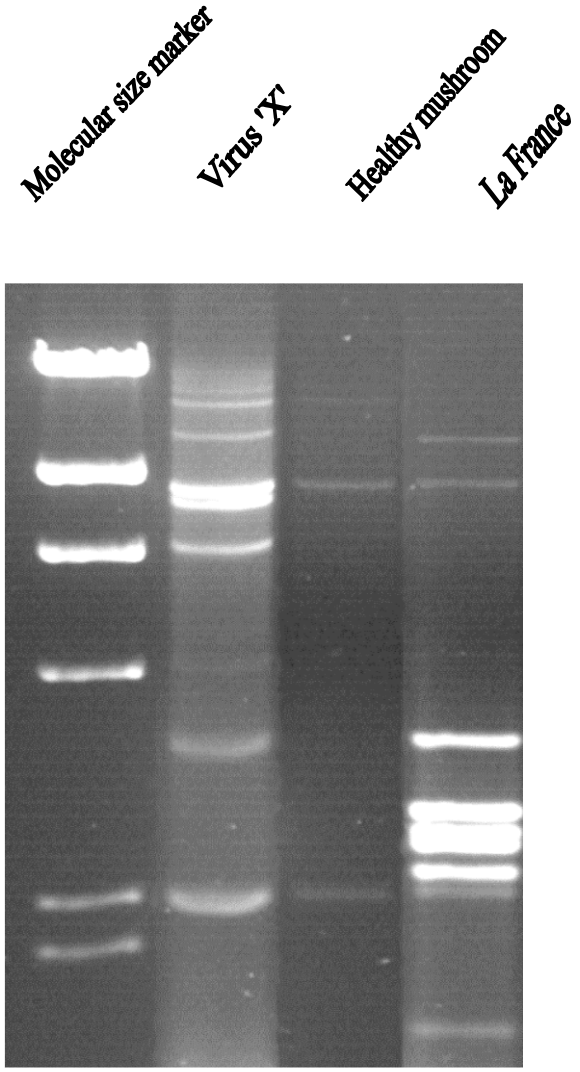
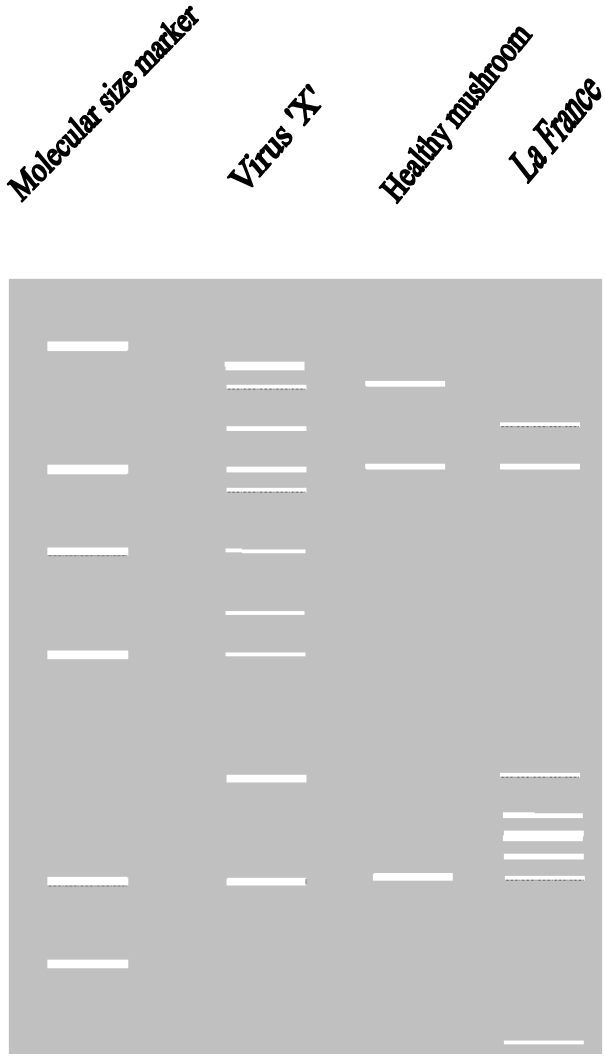


Figure 1a. Schematic representation of the dsRNA bands in Figure 1.



2.4 Conclusions

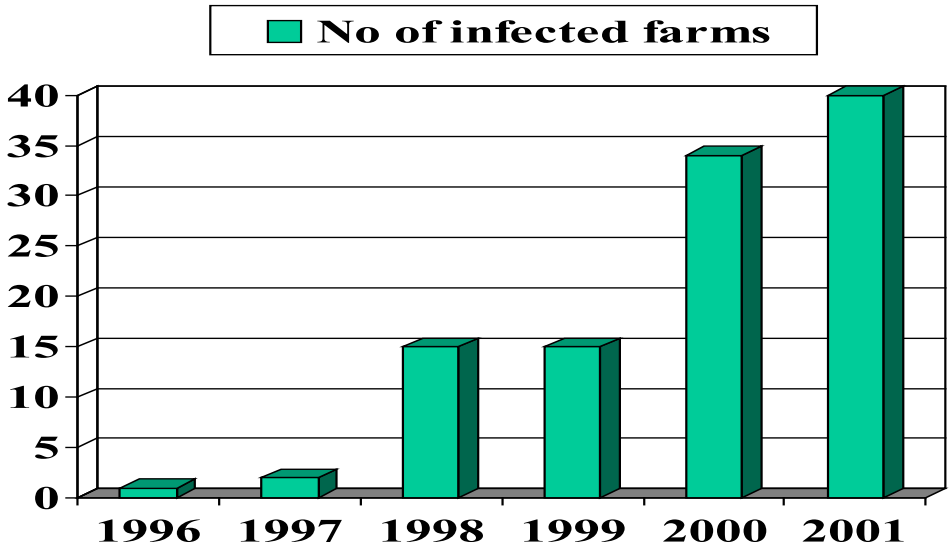
- A reliable electrophoretic test has been developed to indicate the presence of novel dsRNA bands in mushrooms.

3. Correlation between symptoms and dsRNA bands

3.1 Introduction

From 1998 to 2001 there was a steady increase in the number of sites which were experiencing problematical symptoms (Figure 2). Following the initial validation of the test as reliable and repeatable (previous section), the sample-base was widened to include sites with and without symptoms, in order to establish correlation between dsRNA presence and symptoms. This had been anticipated as a finite exercise but it was proved necessary to continue sampling throughout the project when more growers started to report symptoms. During this period it became apparent that the dsRNA banding patterns were more complex than anticipated. Initially, growers were requested to send in samples for dsRNA analysis, as part of this project, but the work to develop the diagnostic PCR test was being compromised by the long slow process of analysing mushrooms from the industry. In October 2000, a Virus X Diagnostic Service was launched by HRI to deal with the high demand for dsRNA tests. The number of samples being received enabled us to test the correlation between the presence of novel dsRNA bands and cropping problems associated with the variety of symptoms being reported.

Figure 2. Number of mushroom farms from which novel dsRNA bands were identified from 1996 to 2001.



3.2 Materials and Methods

Mushrooms received for analysis were treated as described in section 2.2. A day-book was kept to record details of conversations with growers, especially what symptoms were being observed.

3.3 Results and Discussion

Symptoms

Farms providing samples invariably described a wide variety of symptoms but over the duration of the project these have been categorised as follows:

- **Pinning Disruption.** This manifested itself in various ways. Crops could be delayed by one to several days, with pins slow to develop, or there could be large or small areas of non-productive bed or patches. These could be circular, forming swirls down the length of a shelf, or consist of large swathes of a shelf or tray. Frequently, areas of non-productive bed would grade into slow developing pins and then into apparently healthy mushrooms (Appendix, Plates 1-3).
- **Prematurely opening mushrooms.** This symptom manifested itself as mushrooms that developed apparently normally on the bed and appeared to be closed cups. Once picked and turned over, they had in fact prematurely opened, resulting in a loss of quality and downgrading (Appendix, Plate 4). This symptom often appeared only in the second flush
- **Off-coloured or discoloured mushrooms**. On some sites the only symptom to be observed was the presence of off-coloured or brown mushrooms throughout a bed spawned with a white strain (Appendix, Plates 5 & 6). They could be restricted to only a few mushrooms on a bed to consisting of up to 40% of a crop. Frequently, mushrooms would appear fairly normal or only slightly greyish or pinkish on the bed, but would become progressively more discoloured in storage, again leading to a downgrading of the mushrooms.
- **Loss of yield.** Gradual reductions in yield over time without any overt symptoms have been recorded. Usually this would lead eventually to some of the symptoms above being encountered. In reality, some pinning delay or disruption may have been present but their cause assigned to some other factor of compost or environment.
- **Malformations.** In cases where symptoms and yield losses were very severe, mushrooms were often malformed and looked similar to mushroom malformations associated with virus/La France disease (Appendix, Plates 7 & 8).

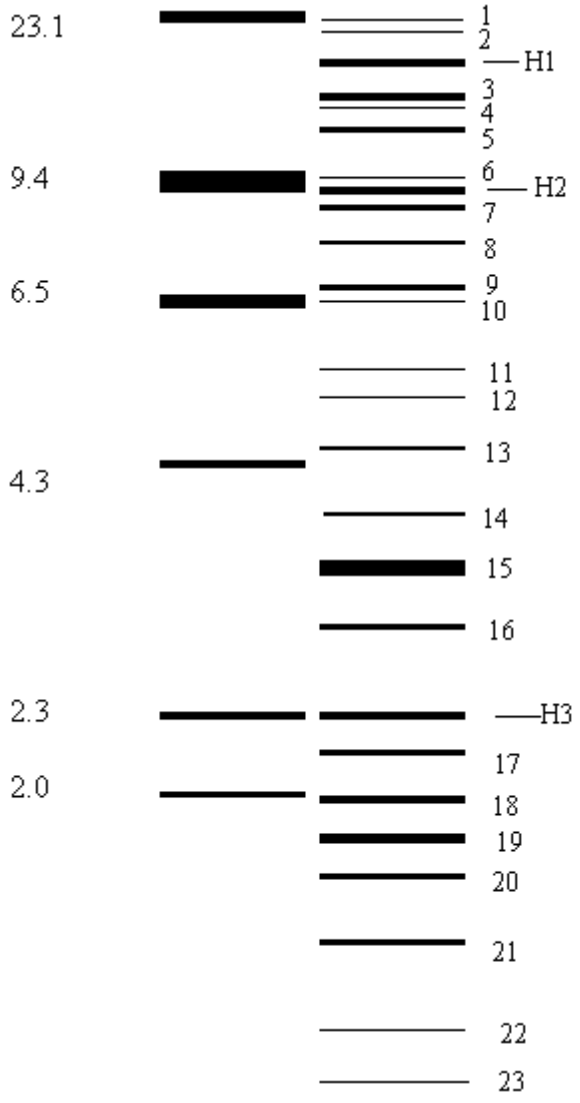
dsRNA banding patterns

During the course of this study a total of 23 different dsRNA bands were recorded and numbered. In addition 3 bands were found, which also occurred in mushrooms from healthy control sites with no history or symptoms of Virus X and these were numbered H1, H2 and H3. After band numbers 1-23 had been established, a few additional bands occurred in a few samples only, which were numbered according to the nearest band above them (bands 0, 8b, 16b and 18b). A schematic of the 23 bands is shown in Figure 3 but it is important to remember that **they do not all occur together.**

The maximum number of novel dsRNA bands recorded in a sample has been 14, in addition to bands H1 to H3 found in healthy mushrooms (14 + [1 to 3] = 17 max in total). The minimum number of novel bands to occur in mushrooms from a site with symptoms is 1, in addition to bands H1 to H3 found in healthy mushrooms (1 + [1 to 3] = 4 max in total). Repeated sampling from any one site indicated that both the number and position of bands could vary slightly, possibly reflecting the amount or type of virus present. The significance of this is not yet clear.

Some bands occurred much more frequently than others. Out of 320 samples tested from October 2000 to May 2001, only 6 of the 23 novel bands occurred in more than 20% of samples. The most common novel bands were bands 2, 3, 8, 9, 13 and 15. All other bands occurred with frequencies ranging from 16 % down to 0.6 % (Figure 3). The H1, H2 and H3 bands occurred in 77, 79 and 99% of samples, respectively.

Figure 3. The dsRNA bands associated with Virus X. Bands H1, H2 and H3 occur regularly in healthy mushrooms and are often referred to as "hybrid" or "healthy" bands.



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15	**146**	**46**
16	8	3
H3	318	99
17	5	2
18	44	14
19	46	14
20	12	4
21	15	5
22	6	2
23	5	2

Correlation of test with symptoms

There was a 100% correlation between the presence of novel dsRNA bands and the presence of one or more of the Virus X mushroom symptoms in the samples tested (Table 1). Control mushrooms from sites without any Virus X symptoms were consistently negative for novel dsRNA bands, but positive for one to three of the H1, H2 and H3 bands (Table 2).

There was no clear association between individual bands and individual symptoms, apart from the discoloration symptom. When Bands 18 and 19, 22 and 23 were all present together in a sample, the major symptom for those samples was that of brown or off-coloured mushrooms (Table 1, farms 3 & 4). However, occasionally "brown" mushroom samples did not produce these bands. Perhaps they were present at undetectable levels and a more sensitive PCR test may pick them up, and there is some evidence emerging from the PCR work that this is indeed the case. Also, if these bands occurred separately rather than as a group of four, then it was unlikely that brown mushrooms was reported as a symptom (Table 1, Farms 5, 7 & 10). Much further analysis is required before the relationship between individual bands and symptoms can be determined.

There appears to be a tendency for the intensity and number of bands to decrease when virus control measures take effect, resulting in a reduction of symptom expression. On those sites where virus control measures have been possible to implement and achieve, usually only band 2 persists after symptoms have disappeared (Tables 1 & 2, farms 2 & 10). What seems likely is that, as with 35nm Virus Disease, the concentration of viral material profoundly influences disease symptom expression. Whether or not one can deduce from these related hypotheses that low concentrations of virus are directly related to low band number is far less certain although band intensity may be correlated with disease level.

DEFRA-funded molecular characterisation work is being carried out at present and the results should help to clarify our understanding of these novel dsRNA molecules. It may also provide information as to whether or not individual bands can be associated with specific symptoms.

3.4 Conclusions

- The correlation between the presence of novel dsRNA bands and novel (virus) disease symptoms has been repeatedly tested and found to be good.
- Both the bands and symptoms have been found to be more numerous and variable than at first anticipated.
- Twenty-three novel dsRNA bands have been identified as being associated with Virus X disease in addition to the three "hybrid" bands that occur in healthy mushrooms.

Table 1. Novel dsRNA banding patterns from 10 different mushroom farms experiencing various symptoms associated with Virus X

Code	Farm 1 1615	Farm 2 1617	Farm 3 1630	Farm 4 1642	Farm 5 1647	Farm 6 1654	Farm 7 1659	Farm 8 1709	Farm 9 1714	Farm 10 1739
dsRNA Band No										
1	X									
2	X	X		X	X				X	X
H-1	X	X	X	X	X	X		X	X	X
3	X		X	X		X	X			X
4									X	
5		X					X			
6	X									
H-2	X	X	X	X	X	X	X	X	X	X
7					X					X
8		X	X	X						
9	X		X	X	X	X		X	X	X
10								X		
11										
12					X		X			X
13	X		X	X	X		X		X	X
14			X							
15	X	X			X	X	X	X		X
16							X			
H-3	X	X	X	X	X	X	X	X	X	X
17										X
18			X	X						X
19			X	X	X		X			
20										X
21		X	X				X			
22			X	X						
23			X	X						
No. of bands (not H1, H2, H3)	7	5	10	9	7	3	8	3	4	10
Symp-toms	various	patches	browns	browns	Early open-ing	patches	various	patches	various	various

Table 2. Novel dsRNA banding patterns from mushroom farms experiencing no symptoms of Virus X (Farms 11-14) or following the implementation of virus control measures (Farms 15, 16, 2 & 10)

Code	Farm 11 1689 HRI	Farm 11 1847 HRI	Farm 12 1661	Farm 13 1743	Farm 14 1839	Farm 15 1679	Farm 16 1626	Farm 2 1958	Farm 10 1959
dsRNA Band No									
1									
2						X		X	X
H-1	X		X		X	X	X	X	X
3									
4									
5									
6									
H-2	X	X		X			X		
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
H-3	X	X	X	X	X	X	X	X	X
17									
18									
19									
20									
21							X		
22									
23									
Symp-toms	None	None	None	None	None	Had browns	Had various	Had patches see Table 1	Had various see Table 1

4. Development of a PCR Test

4.1 Introduction

Advances in molecular biology have meant that molecular tools are increasingly being used to understand disease-biology and to provide molecularly-based diagnostic tests. Polymerase Chain Reaction technology (PCR) is used widely throughout the world in the area of disease detection. It consists of (a) identifying a sequence of genetic material unique to the disease organism of interest, in our case a dsRNA band associated with Virus X disease, (b) designing a DNA (deoxyribonucleic acid) " primer", which can "stick" to that unique piece of genetic material in a crude extract of the (mushroom) tissue suspected of having the disease, (c) adding the appropriate chemicals which will make millions of copies of the unique piece of genetic material of interest which is called the "polymerase chain reaction" or PCR for short. If the unique piece of genetic material was present in the sample then the PCR test will produce many copies and the result will be positive, if there was no matching genetic material then the PCR test produces no unique product and the result is negative.

The current electrophoretic test, which is used to detect the presence of the dsRNA genetic material associated with Virus X disease, is a long and cumbersome process where only a few samples can be dealt with at a time. A PCR test would greatly speed up the detection process and also allow for a greater number of samples to be processed simultaneously. It was decided that a PCR test would be an asset to the detection of Virus X disease in mushrooms as it could also be used on spawn-run compost, a sample type that cannot be tested with the electrophoretic test. However, as the complexity of the Virus X dsRNA banding pattern unfolded during the course of this study, it became apparent that many PCR probes would be necessary as we did not know which dsRNA bands were the most important ones. Work is in progress to identify the key dsRNA bands of interest, and when this is achieved the most appropriate PCR test(s) can be selected.

4.2 Materials and Methods

A schematic representation of the first steps involved in developing a PCR is outlined in Figure 4. Suitable dsRNA was extracted from mushroom virus X infected mushrooms, broadly following the cellulose affinity protocol – method II - described by Romaine & Schlagnhaufer (1993). However, dsRNA was extracted from a relatively large volume of infected mushrooms, as virus titre was lower when compared to that of traditional 35nm virus (La France Isometric Virus – LIV).

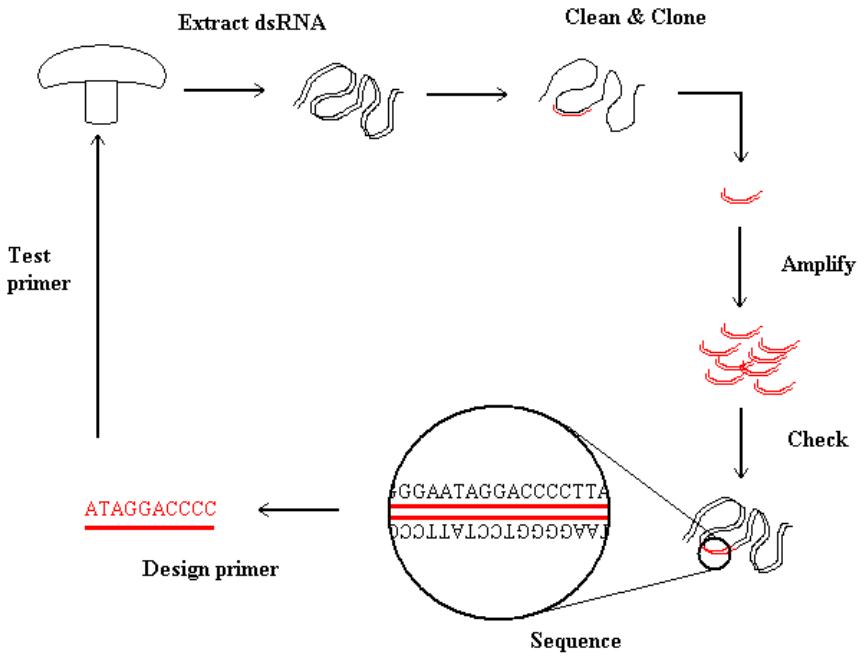

Figure 4. Schematic representation of the steps involved when developing a PCR primer-based diagnostic test.

Despite the cellulose CF11 extraction technique favouring dsRNA, the extraction of mushroom DNA and ssRNA is unavoidable. These unwanted materials were enzymatically degraded using DNase and S1 nuclease (Promega) prior to cloning. This minimised the cloning, or 'copying', of non-dsRNA template and thereby favoured the cloning of viral dsRNA. In turn, a 'Qiagen' treatment (Qiaquick PCR Purification kit – cat. no. 28104) was used to remove the added enzymes as well as other impurities, including salts, from the dsRNA sample.

Because technologies such as PCR and sequencing require DNA rather than dsRNA the "Superscript™ Choice System" (Life Technologies) was used in conjunction with random hexameric primers to clone, or 'copy', lengths of the virus genome. These cloned fragments of viral dsRNA are called complementary DNA (cDNA). To this end the manufacturer's cloning kit instructions were followed.

Having 'copied' small sections of the virus dsRNA, these clones or cDNA fragments required amplification. Bacterial cells (*Eshericha coli* DH5α) were used for this purpose. Copied sections of the virus genome (cDNA's) were first blunt end ligated (EcoRV) into plasmids (pbluescript II KS+), which in turn were transferred, by the heat shock technique, into the

competent bacterial cells. Differential screening in the presence of X-Gal and Ampicillin (final concentration = 50mg/litre) allowed recombinant cells to be distinguished from non-recombinants. As the recombinant cells grew and multiplied they also replicated the inserted plasmid with its cloned section of cDNA. Many more fragments of cloned dsRNA (cDNA's) were therefore harvested from the bacteria than were inserted.

At this stage in the protocol it was desirable to confirm that the cDNA originated from the virus i.e. it was necessary to establish that the amplified cDNA originated from the dsRNA and not from any contaminating material. PCR primers will only be effective if the material on which they are based is specific to the virus dsRNA.

The origin of cDNA fragments was confirmed as viral dsRNA by Northern hybridisation. This involved the hybridisation of DIG-11-dUTP (Boehringer) labelled cDNA fragments to their matching dsRNA band that was denatured, transferred and immobilised on a nylon membrane (positively charged) after running in a 0.7% (w/v) agarose gel. Denaturing, transfer and immobilisation of dsRNA elements was carried out using established protocols (Cole *et al*., 1998). Following immunological (Anti-DIG-AP, Fab fragments - Boehringer) and chemiluminesence (CDP-star - Boehringer) procedures, visualisation of the immobilised matching fragment was made possible by exposing light sensitive film to the nylon membrane. Once the origin of the cDNA was established they were sequenced as a pre-requisite to designing a specific primer for the PCR test.

Sequencing reactions were prepared using 200–500 ng of purified plasmid containing the cDNA insert or 30-90ng of a purified PCR product. Cycle – sequencing reactions were performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems, Perkin – Elmer Corp.). The cycling conditions were as follows:

96°C - 2 mins **x 1 cycle**

95°C – 30 secs
55°C – 10 secs
60°C – 4 mins
} **x 30 cycles**

4°C – Hold

Sequencing gels were run at HRI using Applied Biosystems ABI 377 apparatus.

Assembly of dsRNA sequences, design of PCR primers and analysis of sequence data was carried out using commercial software at HRI or via the European Bioinformatics Institute (EBI).

Primer validation

Primer validation took several forms. Initially primer pairs were assessed individually using purified dsRNA. The suitability of the PCR test to work on non-purified dsRNA extracted from mushroom material, mycelium – taken from spawn run compost, and spores was then established. Total nucleic acid, which included viral dsRNA, was extracted from tissue by physical disruption of the cells using 0.1mm glass beads and then cleaned using first 'Chelex' (3% w/v – Biorad) and then 'Qiaquick PCR Purification columns' (Qiagen). Validation then moved to using combinations of primer pairs that allowed several primers to synthesise cDNA from viral dsRNA prior to individual Polymerase Chain Reaction (PCR) tests. Finally, optimisation of the PCR test was carried out.

Thermoscript™ RT-PCR System (Life Technologies) was used to produce cDNA to dsRNA material before Dynazyme (Finnzymes) was used for the PCR amplification. Visualisation of PCR products was by way of ethidium bromide stained agarose gels (1% - w/v) and UV trans-illumination. Appropriately sized marker lanes were also included on each gel to facilitate PCR product size determination.

4.3 Results and Discussion

Several dsRNA bands have now been cloned resulting in the production of many cDNA fragments. These have been catalogued and stored. Many have been sized (Figure 5) and some progressed to DIG probe production (Figure 6). Several of these cDNA fragments have also been sequenced.

Northern analysis suggests that some of the different dsRNA bands may be 'related'. That is to say, some DIG probes developed to specific dsRNA molecules (bands) were observed to hybridise to other bands in addition to themselves (Figure 7).

Sequence information generated to the cDNA fragments allowed the designing of primers specific to that sequence (Figure 8).

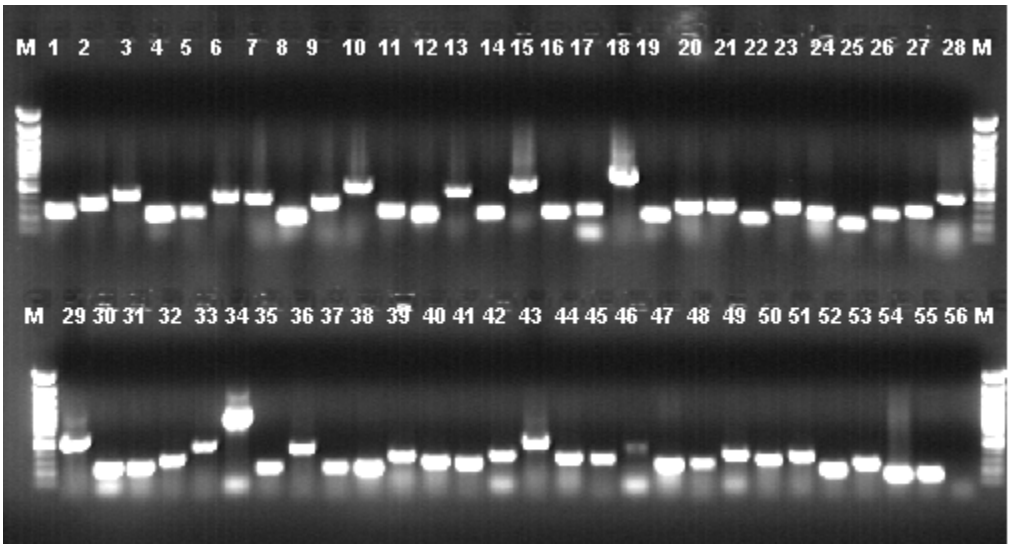

Figure 5. Analysis of DNA products (lanes 1-56) generated from bands H2 and 15 by PCR amplification of the plasmid (pbluescript II KS +) multiple cloning site. Variation in fragment size reflects the use of random hexameric primers and efficacy of cloning. 100 base pair markers (lanes M) are included as standards.

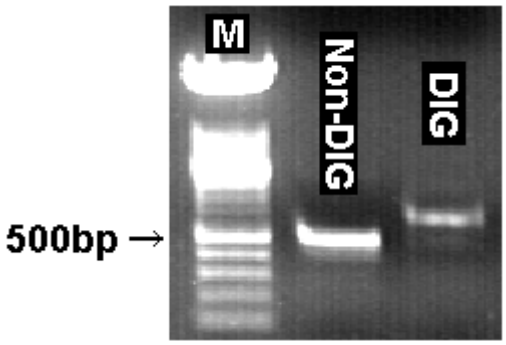

Figure 6. Visualisation of DIG labelled probe for Northern analysis in 1% (w/v) agarose gel. M = Marker lane (100bp); Non-DIG = Polymerase Chain Reaction amplicon of Band 3 (clone 198); DIG = DIG 11 dUTP labelled Polymerase Chain Reaction amplicon of Band 3 (clone 198). Note that DIG 11 dUTP labelling reduces mobility.

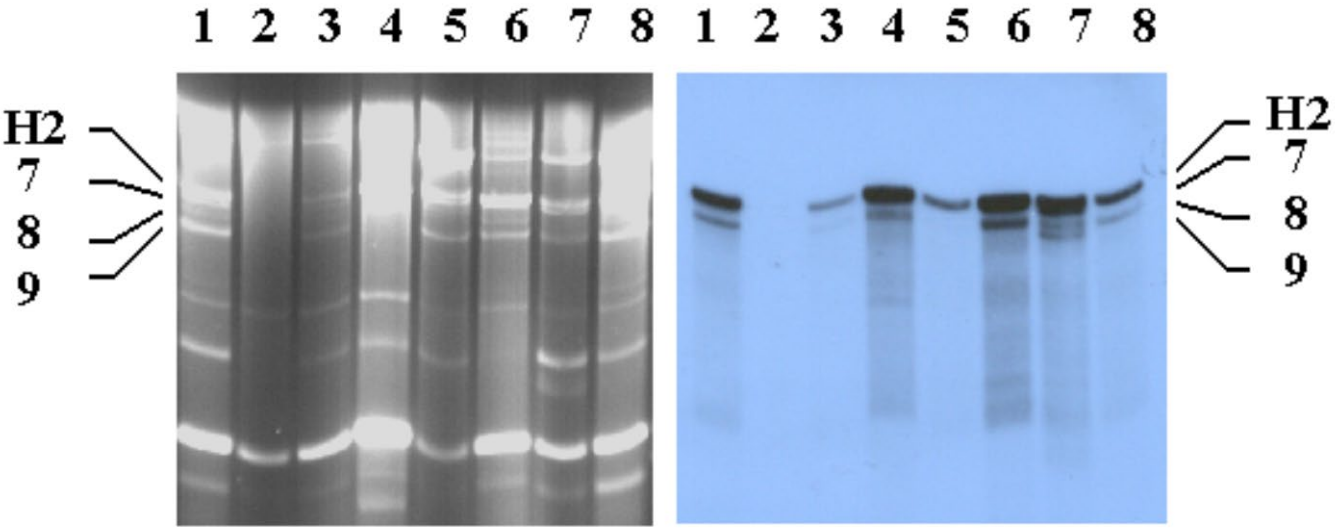

Figure 7. Northern analysis of dsRNA associated with Mushroom Virus X and a DIG probe made from band H2 (clone 102). Dig probe has hybridised ('stuck') to H2 as expected but also dsRNA bands 7, 8, and possibly 9 (lane 7). Original gel (left) indicates the presence of many dsRNA bands with no homology to the probe.

Plasmid sequence — Band H2 clone sequence

GGGCTGCAGGAATTCGATGGAACGACAAGCTGAGATGCATACTGA
TGACCAACCTTTTCCCGTTGCTAGCTTTGGAGCACGAAACGAGCC
CTTATCCAGAGTATGTGGATTGGAGGATTGTCCGCAAATTGGATCA
ACCAATACTCATCTACACATACCAGAGGGAGACATGGGAGGCAA
GTATGAACAACCCTATGACCCCACTATGACTTTGTTGTCAAGAGTT
GAAGATGAGGACGGCACAGTCGTGTGGAATGGCGGTGTTGTTGA
GAGAAAGGGCAATTACAGAGTTCAGGCTTTCTACCCTTACAATAT
ACTGTTCCCAATTACGTAGCTGAGAGAGAAGAGGTGATGATGGA
ATTCCTAGGCTTTTCAGAGAGCAACCTAGAACCAGGCCGATCAG
GTTGAATACTTCAACAAGATGTTTCAGTACGCCAAAACT TACCTT
CGGACAAGCGAGAGAATCAATGGGGAAAACTTCGCCAGTTCGCA
GATTTGCATTCCTTGTACTTCCCTGTCGGTGATCAAGAGTGGCTCG
TGAAGGTGATCAAGCTTATCGATACCG

Band H2 — Plasmid sequence

Figure 8. Band H2 (clone 102) sequence, showing primer sites (underlined) and PCR amplified region (between underlined sections). Small lengths of the flanking plasmid sequence have also been presented. Total cDNA fragment = 532 base pairs; PCR amplified region = 240 base pairs.

Having designed primer pairs it was first necessary to establish that they amplified the desired product (Figure 9). This was done using the best template possible; purified dsRNA.

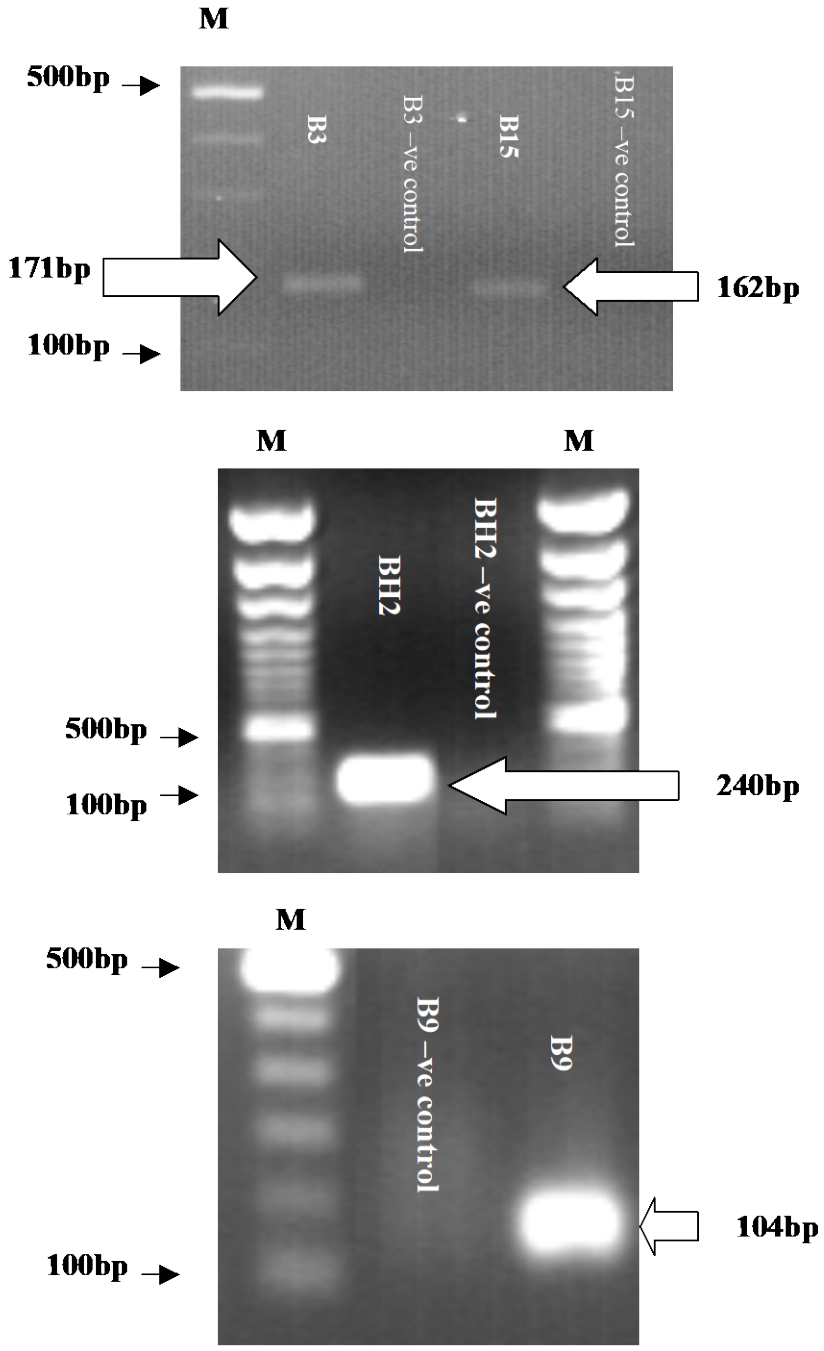

Figure 9. Validation of primers designed to MVX dsRNA bands 3 (B3) and 15 (B15) (top gel), H2 (BH2) (middle gel), and 9 (B9) (bottom gel). PCR product size indicated. Negative control (-ve) and marker lanes (M) (100bp ladder) included on each gel.

Primer pairs were also shown to produce the correct sized PCR products when used with the more crude 'Chelex' preparation of total nucleic acid from mushroom tissue (Figure 10).

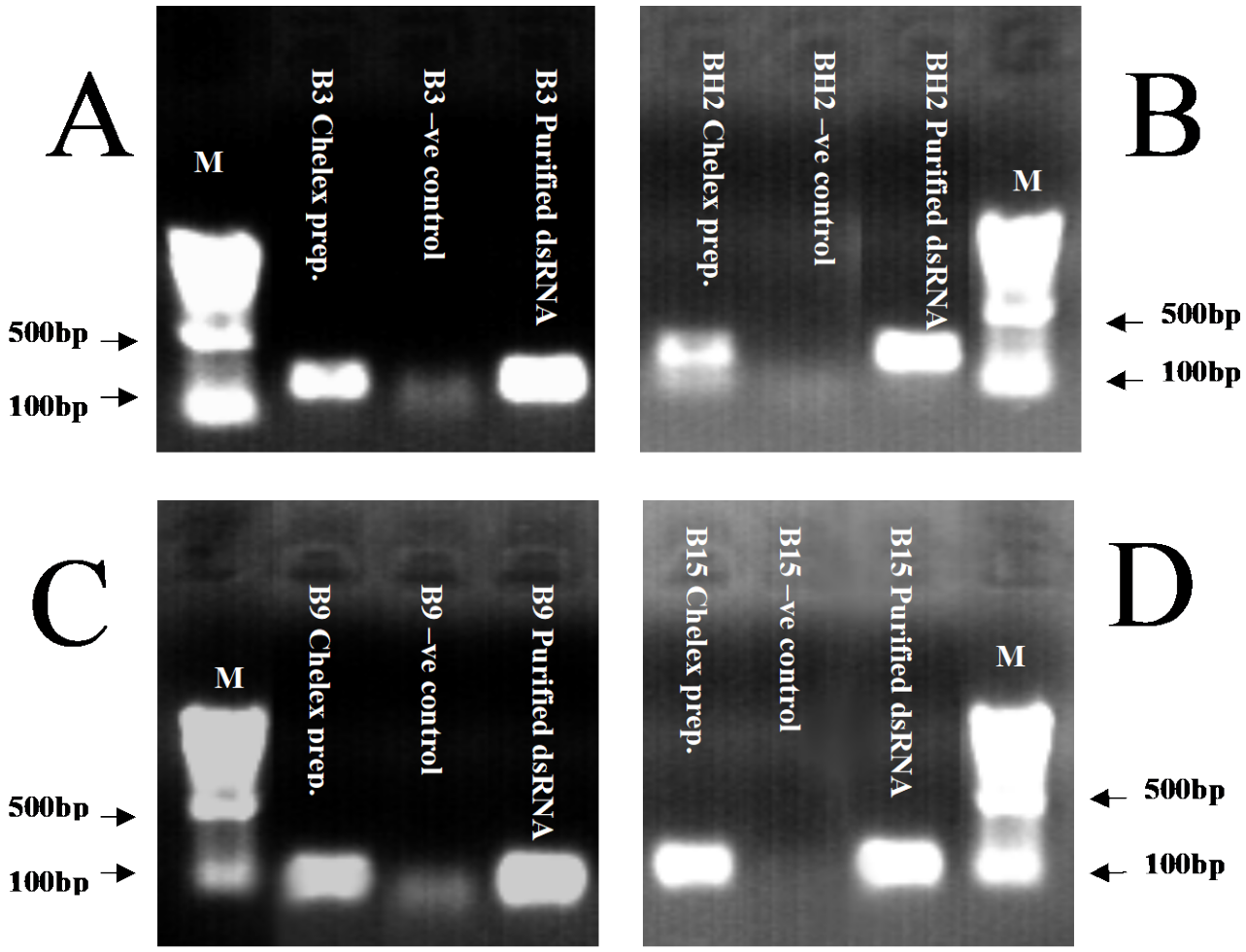

Figure 10. PCR products produced by four different primer pairs to crude 'Chelex' prepared nucleic acid. A = Band 3 primer pair; B = Band H2 primer pair; C = Band 9 primer pair; D = Band 15 primer pair. Also shown on each gel are positive control (purified dsRNA), negative control (-ve control), and 100 base pair ladder marker (M).

Moreover, the crude dsRNA preparation technique allowed the rapid detection of dsRNA molecules not only from Virus X infected mushrooms, but also from germinated spores (Figure 11) and spawn-run compost (Myc 2, Figure 12), something that is not possible using the existing 'total dsRNA extraction technique'. Spawn run compost from a control crop that did not contain dsRNA bands 3 and 15 (Myc 1) did not test positive.

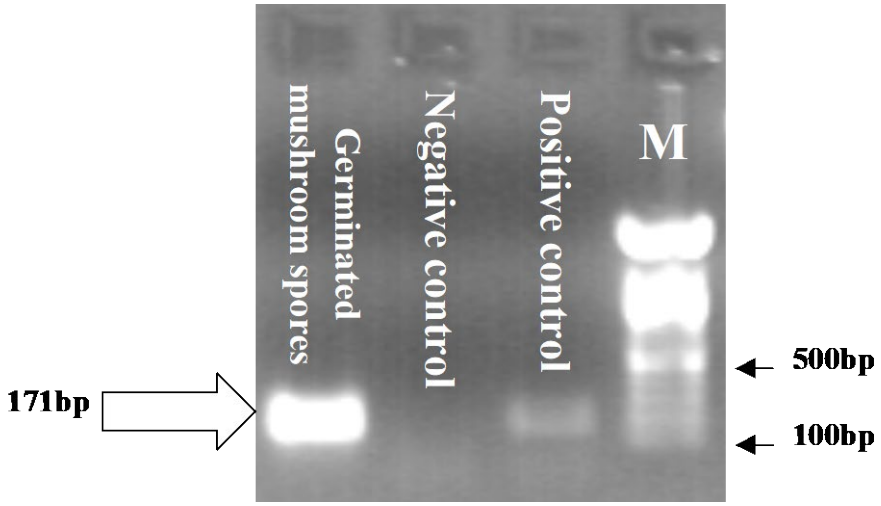

Figure 11. Detection of MVX band 3 in germinated mushroom spores (from a Virus X infected mushroom) using PCR on crude 'Chelex' prep dsRNA. Marker (M) (100 base pair ladder), negative and positive control lanes also shown.

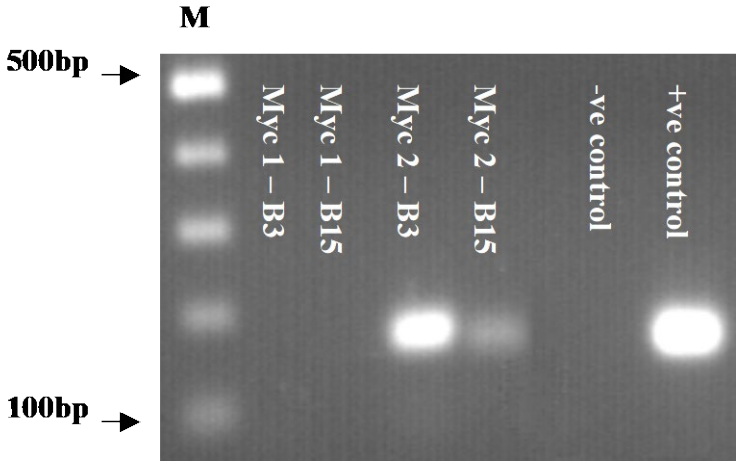

Figure 12. PCR test of crude 'Chelex' prepared nucleic acid taken from two different samples of spawn run compost (Myc 1- Control; Myc 2, Virus X infected) using primer pairs specific to MVX band 3 and band 15. Marker lane (M)(100bp) and positive and negative control lanes also included.

Having demonstrated that the primer pairs amplified the correct products individually it was shown that amplification also occurred when primer pairs were combined during the cDNA synthesis stage, prior to PCR. Combining primer pairs at this stage allowed a single Reverse Transcription (RT) reaction to synthesise cDNA to several bands. This multiplex RT reaction

was then divided between separate PCR reactions to establish specific dsRNA bands presence or absence. Combining several primers in one RT reaction in the above manner saved both time and money and therefore would be desirable for the final PCR test. Initially two primer pairs (B3 and B15) were combined during the cDNA synthesis stage (RT) (Figure 13). Successful amplification with two primer pairs from crude 'Chelex' prepared nucleic acid lead to the use of four and then six primer pairs in combination during the RT stage of the amplification process.

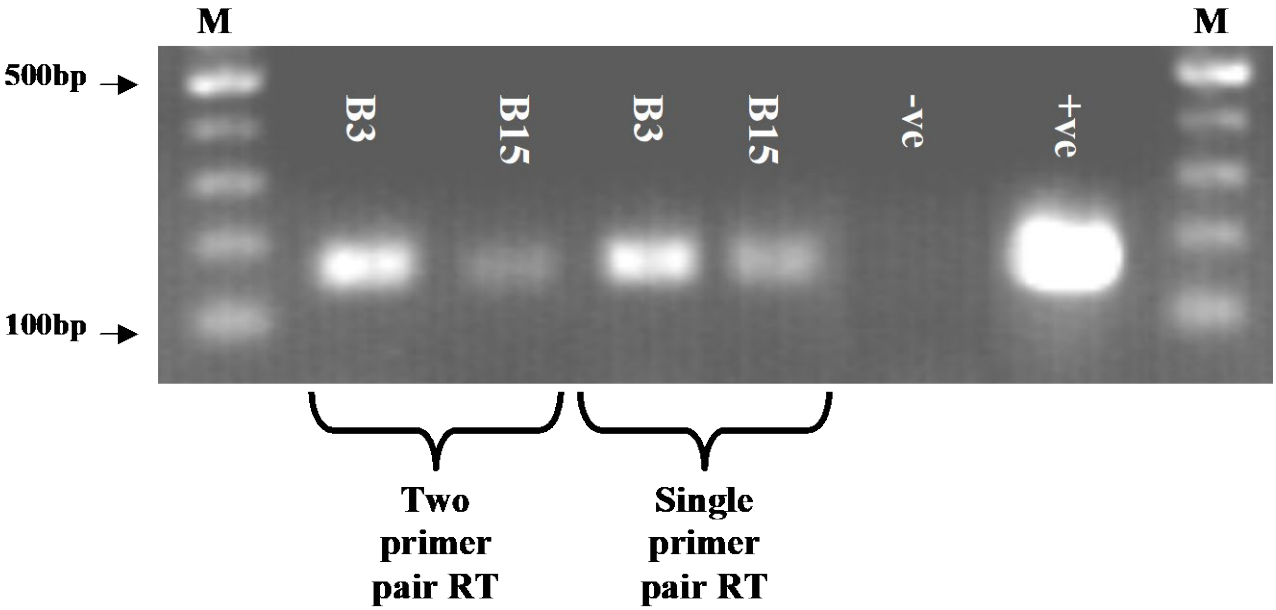

Figure 13. Combination of two primer pairs within the same RT reaction followed by individual PCR tests for the detection of specific bands from crude 'Chelex' prepared nucleic acid. Marker (M) and positive and negative controls also shown.

A multiplex of four primer pairs was also tested during the cDNA synthesis stage followed by individual PCRs and this was also shown to generate reliable amplicons (Figure 14).

A multiplex of six primer pairs was also tested but was found to be unreliable. Whilst amplification was satisfactory when purified dsRNA was used as the template, amplification was sometimes poor when the more crude 'Chelex' preparation was used as the template dsRNA (Figure 15). For example, an electrophoretic test showed that sample 1 (Figure 15) possessed all six bands being tested for in the multiplex of 6 PCR primers, but the PCR products for bands 19 and 23 were very weak. Similarly, an electrophoretic test showed that sample 2 (Figure 15) contained three bands of the six that should be detected by the multiplex of 6 primers (3, H2, and 9) but bands H2 and 9 were not detected.

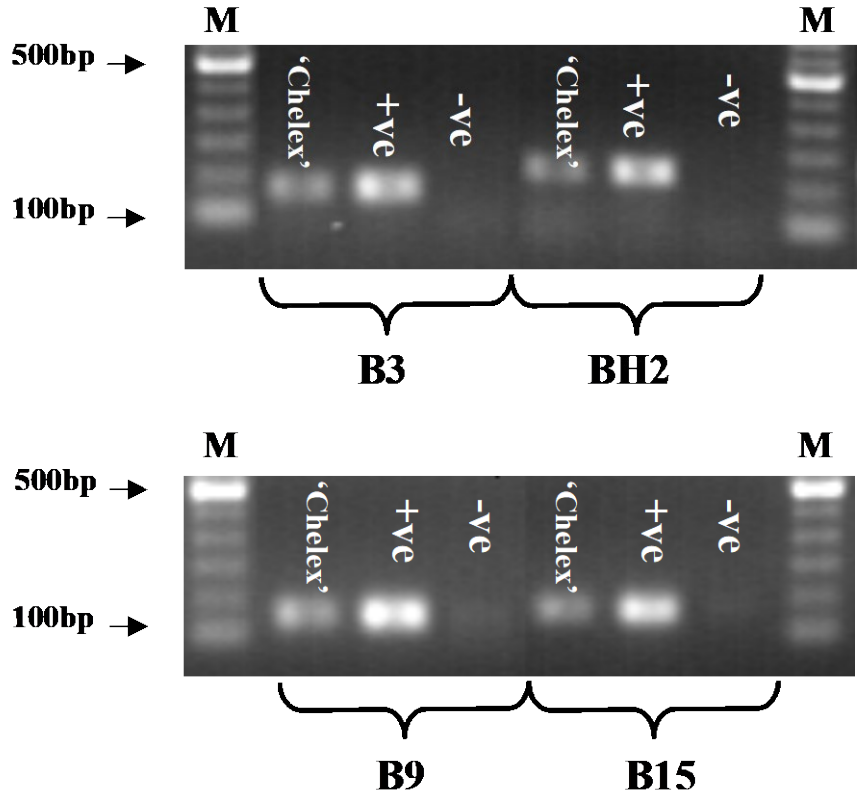

Figure 14. Combination of four primer pairs within the same RT reaction followed by individual PCR tests for the detection of specific bands from crude 'Chelex' prepared nucleic acid. Positive and negative control reactions are included as are marker lanes (M) (100bp).

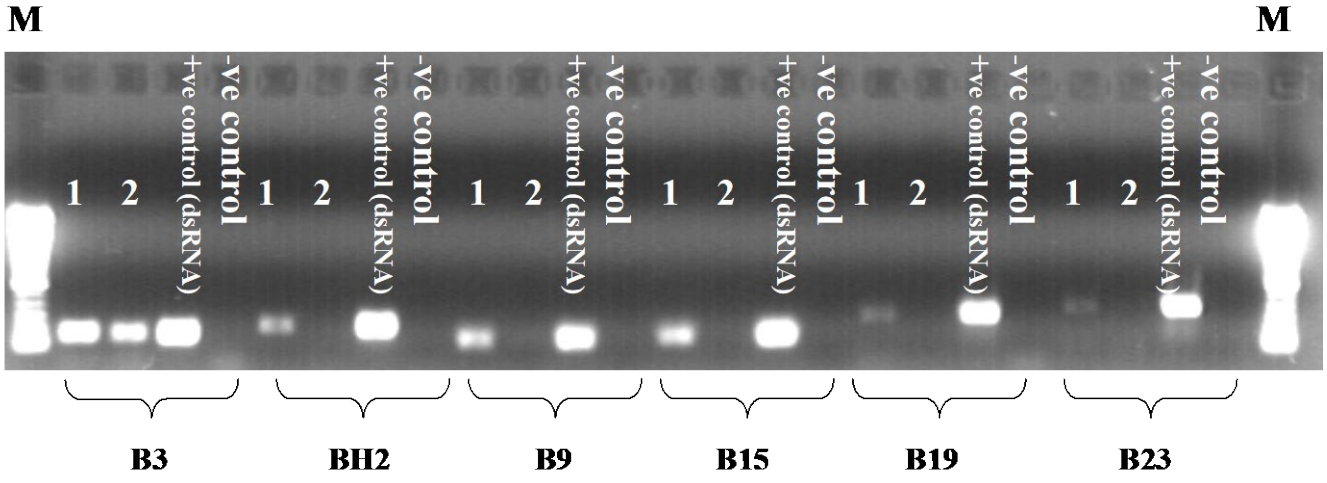

Figure 15. Combination of six primer pairs within the same RT reaction followed by individual PCR tests for the detection of specific bands from crude 'Chelex' prepared nucleic acid. Electrophoretic tests showed that sample 1 contained all six bands, while sample 2 contained bands 3, H2 and 9. Positive (purified dsRNA) and negative control reactions are included as are marker lanes (M) (100bp).

Repeat testing of multiple samples, like the use of six primer pairs in a single RT reaction, has generated variable results. Initial findings suggested that dsRNA molecules, when present, were distributed relatively evenly between cap, stipe and gill tissue and little difference existed between mushrooms from the same sample (Figure 16). However, further testing suggested that variation might exist between mushrooms from the same sample (Figure 17).

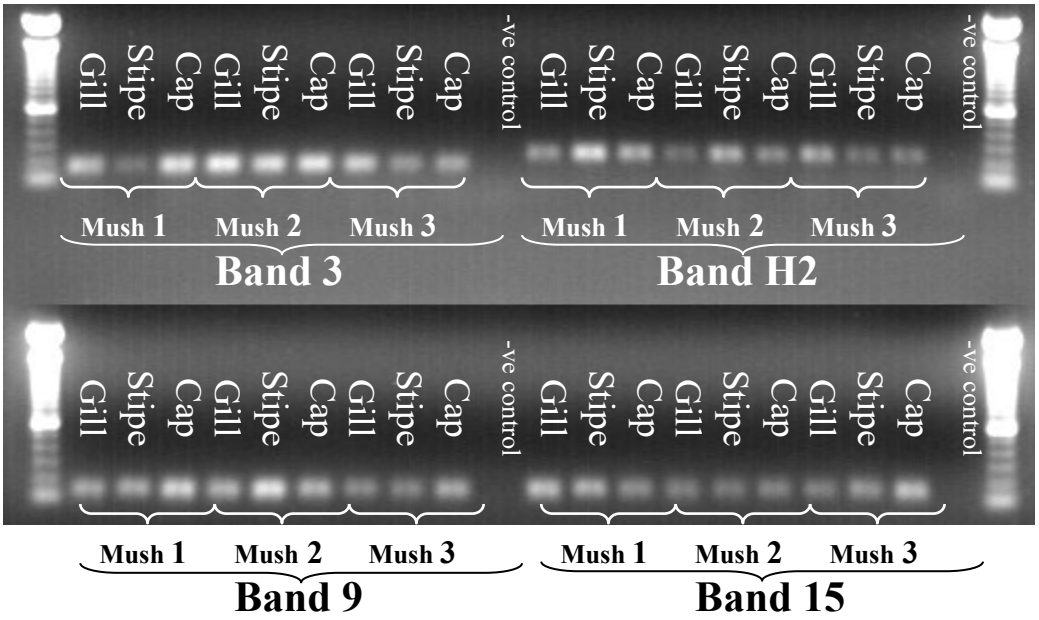

Figure 16. RT – PCR testing for four different dsRNA molecules (Bands 3, H2, 9, and 15) within the cap, stipe and gill tissue of three different mushrooms sampled from the same location at the same time. All tests used crude 'Chelex' prepared dsRNA as template. Negative controls (-ve) and marker lanes (M) (100bp) are also indicated.

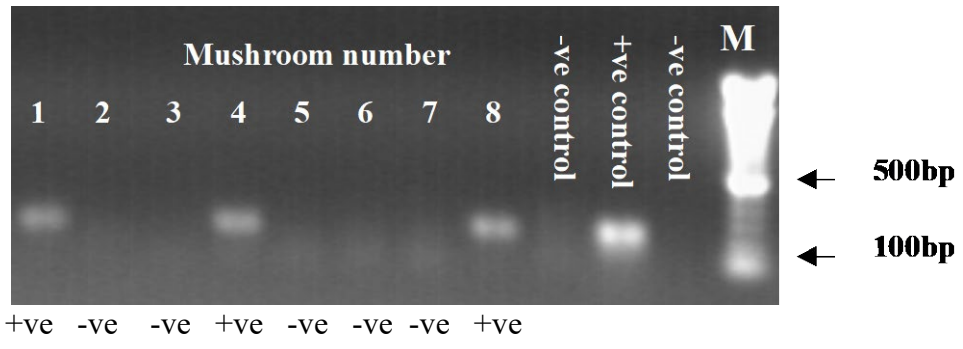

Figure 17. RT – PCR testing for MVX dsRNA H2 from eight different mushrooms sampled from the same location at the same time. All tests used crude 'Chelex' prepared dsRNA as template. Negative (-ve) and positive (+ve) controls and marker lanes (M) (100bp) are also indicated.

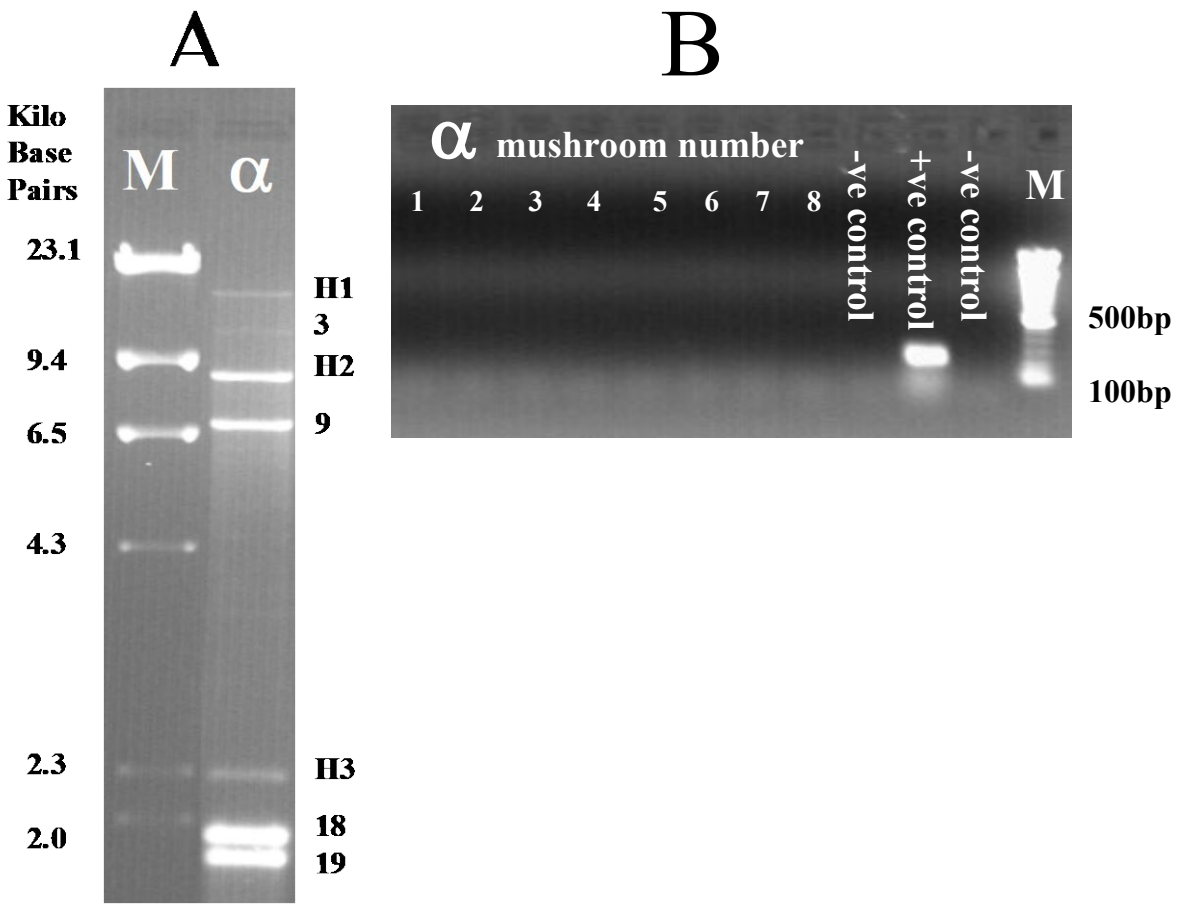

Figure 18 a & b. Comparison between the gel electrophoretic test results (A) showing band H2 and RT – PCR test results (B) indicating no band H2 amplicon. Both tests used sample α mushrooms. RT – PCR testing done on crude 'Chelex' prepared dsRNA. Marker lanes and negative and positive controls are included where appropriate.

Additionally, testing with RT - PCR has not always given results that concur with those from the electrophoretic test. For example, sample α (Figure 18a & b) that had previously tested positive for bands H2 and 9 when using the electrophoretic test (Figure 18a) did not test positive for bands H2 when eight different mushrooms were tested with the RT-PCR (Figure 18b).

4.4 General Discussion

Despite very encouraging results from the PCR tests, not only on mushroom material but also on spores and spawn run compost, there is still a need for further validation of the test. The above results have shown that there is variation in the results and further optimisation and standardisation is necessary before it can be relied upon as a stand-alone test. The now tried-and-tested total dsRNA electrophoretic test still provides extremely useful information and will continue to be used until such time as the PCR test is considered reliable and informative. The continued validation process of the PCR test will ultimately allow a much greater degree of confidence in the results generated from it. (It is anticipated that a working PCR test will be available in June 2002).

Both the RT and PCR stages have been shown to be reproducible with purified dsRNA. The extraction of purified dsRNA is a long slow process that limits the numbers of samples that can be processed at any one time. The next challenge is to demonstrate that the PCR test will consistently give the same results when used in conjunction with a crude extract of dsRNA, which can be done rapidly.

The optimisation and standardisation of the crude dsRNA extraction procedure upon which the PCR test is conducted is crucial to the development of a fast, effective and reliable PCR test. Variations in the crude extract of template material can be magnified downstream. Thus, it is possible that small variations in suitability of template material might give rise to hugely variable results, to the point that one extraction might test positive and another, from exactly the same material, test negative. Some work has already been done in this area and the results suggest that certain features need to be incorporated into the extraction protocol. In addition it has become apparent that the crude extract needs to be made from more than one mushroom in order to minimise the likelihood of sampling a "negative" mushroom from within a largely "positive" crop. Preliminary studies to optimise and standardise the PCR test are encouraging, however work is ongoing to thoroughly optimise the extraction protocol whilst maintaining it’s simplicity.

Currently, the relative importance of any individual band in relation to specific disease symptoms is unclear. More complete characterisation of these bands will hopefully allow

only the bands of importance to be tested for by PCR. For example, if the presence of band 10 is found not to affect the mushroom it need not be included within the test.

The Dept of the Environment, Farming and Rural Affairs (DEFRA, formerly MAFF) has provided funds to continue with the refinement of the PCR test. In addition they have funded work to characterise the virus or viruses that make up Virus X. This should enhance our understanding of how the virus or virus complex is operating. The work to date has highlighted the fact that it is a much more complex situation compared with 35nm Virus disease, and this may have implications on our ability to control or contain it.

4.5 Conclusions

- Four PCR probes have been developed which can rapidly detect Virus X dsRNA bands 3, 9, 15 and H2 in mushrooms and spawn-run compost, but the dsRNA extraction procedure needs to be standardised and optimised to give reliable, repeatable, results.

5 Transmission Experiments

5.1 Introduction

While the PCR test was in progress a number of small transmission experiments were carried out in order to determine if the virus was transmitted by the same routes as traditional 35nm virus (La France disease), that is via *Agaricus* spores and mycelium. It is not possible to get a purified extract of mushroom virus, with which to do transmission experiments, as can be done with plant viruses. Consequently epidemiological studies on mushroom viruses must use material that contains the virus material, namely spores and mycelium from an infected crop. Epidemiological work on La France disease indicated that when 10 - 100 *Agaricus* spores from virus-infected mushrooms were added to compost during spawn-run, virus symptoms developed in the subsequent crop (Schisler *et al,* 1967). This group also produced *Agaricus* cultures derived from infected mushrooms, and used them to make spawn. When this infected material was mixed with healthy spawn, and used to spawn compost, the subsequent crop went on to develop severe virus symptoms. It is highly likely that Virus X is also transmitted via spores and mycelium from infected sources however it is important to establish this fact scientifically. The following experiment was carried out to provide this information. Detailed epidemiological information concerning the amount of infected material required to (a) transmit the disease, and (b) to produce symptoms is not within the scope of this current project and will be carried out in a subsequent project.

5.2 Materials and Methods

Mushrooms and compost were collected from a tray-farm experiencing "patches" of unproductive bed along with a sample of healthy mushrooms from a source with no history of "patches" (HRI). Pure *Agaricus* cultures were made onto nutrient agar from the following:-

1. Cap of a healthy mushroom from an uninfected source (HRI)
2. Cap of a mushroom close to a patch
3. Stipe of a mushroom close to a patch
4. Spores from a mushroom close to a patch
5. Spores from a mushroom in an area with no patches
6. Mycelium derived from compost beneath a patch with no mushrooms.

DsRNA analysis was carried on the original mushroom samples, as well as the cultures derived from the mushrooms, spores and compost using the validated electrophoretic test described in section 2. Spawn was then produced from these cultures using commercially prepared rye grain. The spawns were inoculated into standard HRI formula III compost and

grown on for testing. Samples of mushrooms were taken at first and third flushes and analysed for the presence of dsRNA bands.

5.3 Results and Discussion

Viral dsRNA was detected in four of the six mycelial cultures used to make spawn. No novel dsRNA was detected in the control culture derived from a healthy mushroom from an uninfected crop (Table 3, Treatment 1). No dsRNA was detected in a spore-culture derived from apparently healthy mushrooms taken from an area where no patches occurred (Treatment 4). The dsRNA banding patterns of the remaining cultures were similar with two to three novel bands being present (see Treatments 2 and 6 in Table 3).

The viral dsRNA passed from pure mycelial cultures, derived from positive-testing cap and stipe tissue, into spawn, and went on to produce positive-testing mushrooms in the 1st and 3rd flushes (Tables 3 & 4, Treatments 2 and 3). Similarly the viral dsRNA passed through spores from a positive-testing mushroom (Table 4, Treatment 5) to produce positive-testing mushrooms in the 1st and 3rd flushes. The viral dsRNA was also found to be transmitted through spores from a negative-testing mushroom from a heavily infected crop (Table 4, Treatment 4). When spawn was made from a mycelial culture obtained from compost under a "patch", the mushrooms that were subsequently produced, gave "intensely positive" results (Tables 3 & 4, Treatment 6). This treatment also cropped very poorly. Only the control treatment (Table 3 & 4, Treatment 1) produced negative-testing mushrooms in the 1st flush. However, by the third flush, the control treatment also tested positive (Table 3).

The results of this unreplicated trial show that the viral dsRNA was able to pass through mushroom spores to subsequently produce infected mushrooms. Remarkably this appeared to be the case not only from spores derived from a positive testing mushroom but also from a negatively testing one from a heavily infected crop. Therefore it is important to note that a negative result does not mean total absence of the viral dsRNA, but rather a non-detectable level.

The mushrooms derived from spawn that was made from "patch" mycelium (Treatment 6), tested intensely positive. The plausible explanation for this is that the ultimate symptom of a total absence of mushrooms in "patches" is an expression of very high levels of virus. The

broad conclusion based on this limited, but quite convincing, evidence is that Virus X is transmissible via both spores and mycelium.

Table 3. The dsRNA patterns for three of the six treatments in Table 4, at three stages; (a) culture used to make spawn, (b) 1st flush mushrooms and (c) 3rd flush mushrooms.

	Source of culture used to make spawn:								
	Control healthy mushroom cap **Treatment 1**			**Virus X-positive mushroom cap** **Treatment 2**			**"patch mycelium"** from compost beneath a patch **Treatment 6**		
Band No	(a) Culture	(b) 1st Fl.	(c) 3rd Fl.	(a) Culture	(b) 1st Fl.	(c) 3rd Fl.	(a) Culture	(b) 1st Fl.	(c) 3rd Fl.
1									
2									
H1		X	X	X	X	X	X	Xs*	Xs
3			X	X	X	X	X	Xs	Xs
4					X				
5									
6									
H2			X	X	X	X	X	Xs	Xs
7			X			X		Xs	Xs
8			X					Xs	Xs
9			X	X	X	X	X	Xs	Xs
10									
11									
12								X	
13								X	
14									
15			X				X	Xs	Xs
16									
H3	X	X	X	X	X	X	X	X	X
17									
18									
19									
20									
21									
22									
23									

* Xs = very strong band

Table 4. Cropping experiment using spawn made from mycelium from various sources.

Treatment	Source of mycelial culture used to make spawn:	ds RNA test results	
		1st Flush	3rd Flush
1	Virus-X positive mushroom (Cap)	positive	positive
2	**Control**, healthy mushroom (Cap)	**negative**	positive
3	Virus-X positive mushroom (Stipe)	positive	positive
4	Spores from **negative-testing** mushroom from a heavily infected crop	positive	positive
5	Spores from a positive-testing mushroom from a heavily infected crop (same crop as in 4)	positive	positive
6	Mycelium isolated from compost under a patch	Intensely positive	Intensely positive

The control plot (Treatment 1), spawned with material derived from a healthy mushroom duly tested negative at the first flush. At the third, however, it tested positive. This was so surprising that the test was repeated, with the same result. Sampling procedures were examined for fault but only two explanations seem in anyway plausible. Either the control plot had not been sampled and another plot had been sampled twice (in error), or the plot had become infected during the course of the trial. This latter option is physically feasible as the second, unsampled flush, had got out of control and produced many open mushrooms. This piece of evidence, suggesting transmission during cropping, remains circumstantial. The balance of probability would suggest that within crop transmission had occurred and there is some evidence from within the industry also which suggests that within-crop transmission can take place.

This experiment needs to be repeated to test the hypothesis that Virus X can be transmitted within a crop, from a 1st flush to a third flush, and, if proven, this data will have serious implications in the eventual control measures proposed.

5.4 Conclusions

- There is strong evidence to indicate that Virus X is transmitted from crop to crop by both spores and mycelium.

6 Epidemiology

6.1 Introduction

It is important to know how diseases spread within a crop in order to determine the best control measures to implement. This is especially true for new diseases where there is no previous experience to draw on. However, a new virus disease of mushrooms may well respond to the recognised control measures used to combat 35nm Virus disease (La France). Thus, once the new disease symptoms being encountered in Britain were attributed to a new virus, affected growers were advised to implement the virus-control measures used against 35nm virus, until more detailed information could be obtained on the epidemiology of the new virus. During the course of this project some work was done to test this hypothesis, that standard virus control measures would reduce the incidence of the new virus.

6.2 Materials and Methods

Investigations have taken place both on infected sites and at the HRI mushroom unit. The latter being employed to grow compost to fruiting in isolation in order to try to pinpoint the time of infection or transmission. Compost has been sampled:

- straight from a Phase II tunnel
- after spawning
- after spawn-run

6.3 Results and Discussion

A considerable weight of evidence has been accumulated, all of it unreplicated and much of it circumstantial. However, the conclusion that emerges is consistent and convincing, due to the sheer volume of evidence. It would appear that the primary entry point for mycelial or spore transfer, and thus viral transfer and accumulation, is broadly the same for Virus X as that found in 35nm Virus Disease, namely the spawn-running period. That is the period between spawning and casing, including filling of shelves with either bulk phase II or III compost.

Test results on mushrooms from infected sites and mushrooms from the same compost removed and spawn-run away from the infected site, strongly support this conclusion.

Some farms have overcome Virus X on their farms. This has been achieved by implementing efficient virus control measures that minimise the contamination of compost by *Agaricus* spores and mycelium from Phase II cool-down, through spawnimg, spawn-running, and cropping.

A more detailed epidemiology project (M 39a) has now begun at HRI to quantify the amount of infected spores and mycelium needed to transmit Virus X, and to determine the vulnerability of the different stages in the production cycle from spawning through to cropping.

6.4 Conclusions

- A great weight of circumstantial evidence consistently indicates that the main point of entry for transmission and accumulation is the spawn running stage. This includes spawning and bulk handling of Phase II onto trays and shelves through to filling fully-run bulk Phase III.
- Virus X outbreaks on some farms have been controlled by implementing effective virus control measures to prevent *Agaricus* spores and mycelium from contaminating compost from Phase II cool-down through to spawning, spawn-running and cropping.

7 Miscellaneous

Attempts have been made to purify, or concentrate, the viral dsRNA, which occurs in infected material at low levels. This has been largely unsuccessful. A few virus particles have been observed but evidence connecting them to the new disease syndrome is poor. The only point of interest being at present that the viral particles observed appeared to be the unusual size of approximately 30nm.

Attempts are being currently made to produce material of higher viral concentration. One surprising phenomenon that appears to be occurring simultaneously with the presence of Virus X is the total absence of 35nm particles.

8 General Results and Discussion

At the beginning of the project a small amount of speculative evidence suggested that this disease was viral in cause. Unreplicated and sometimes quite crude experiments carried out on British farms and in the USA indicated that the problem was mycelially transmitted. One quite controlled trial in the USA gave quite strong evidence that a small amount of spawn derived from infected material could transmit the infection when mixed with a large quantity of healthy spawn.

The project so far has established that the electrophoretic test is repeatable and reliable and more importantly that there seems to be a virtually total correlation between the appearance of novel bands and disease symptoms.

As the project has progressed and disease incidence has increased the novel banding has been shown to be both variable and more extensive than first thought. The significance of this has yet to be established. Despite the increasing complexity, the correlation of test results and symptoms remains good. In parallel to these observations the symptoms themselves appear to be more variable than at first perceived.

Evidence has accumulated indicating that the virus is both spore and mycelially transmitted.

Approaching 400 samples have been analysed in an attempt to establish the symptom-result correlation and the incidence of the disease. Most importantly of all, the transmission patterns on some commercial premises have been examined, and a reduction in Virus X levels have been recorded where effective protection (from contamination by spores and mycelial fragments) could be given to phase II cool-down, spawning, and spawn-running,..

9 Overall Conclusions

- A reliable electrophoretic test has been developed to indicate the presence of novel dsRNA bands in mushrooms that are associated with Virus X disease. This is a slow and laborious test
- Twenty-three novel dsRNA bands have been identified as being associated with Virus X disease in addition to the three "hybrid" bands that occur in healthy mushrooms.
- The correlation between the presence of these bands and novel (virus) disease symptoms has been repeatedly tested and found to be good.
- Both the bands and symptoms have been found to be more numerous and variable than at first anticipated.
- Four PCR probes have been developed which can rapidly detect Virus X dsRNA bands 3, 9, 15 and H2 in mushrooms and spawn-run compost, but the dsRNA extraction procedure needs to be standardised and optimised to give reliable, repeatable, results.
- There is strong evidence to indicate that Virus X is transmitted from crop to crop by both spores and mycelium.
- A great weight of circumstantial evidence consistently indicates that the main point of entry for transmission and accumulation is the spawn running stage. This includes spawning and bulk handling of Phase II onto trays and shelves through to filling fully-run bulk Phase III.
- Virus X outbreaks on some farms have been controlled by implementing effective virus control measures to prevent *Agaricus* spores and mycelium from contaminating compost from Phase II cool-down through to spawning, spawn-running and cropping.
- The nature of the virus complex remains unknown and DEFRA-funded research on the molecular characterisation of the virus complex is currently underway. This should identify which PCR probes are the most useful ones as a Virus X diagnostic.
- Further information concerning the precise epidemiology of the disease is required to achieve sustainable control and an HDC-funded research project in this area is currently underway (M 39a).

10 References

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11 Appendix

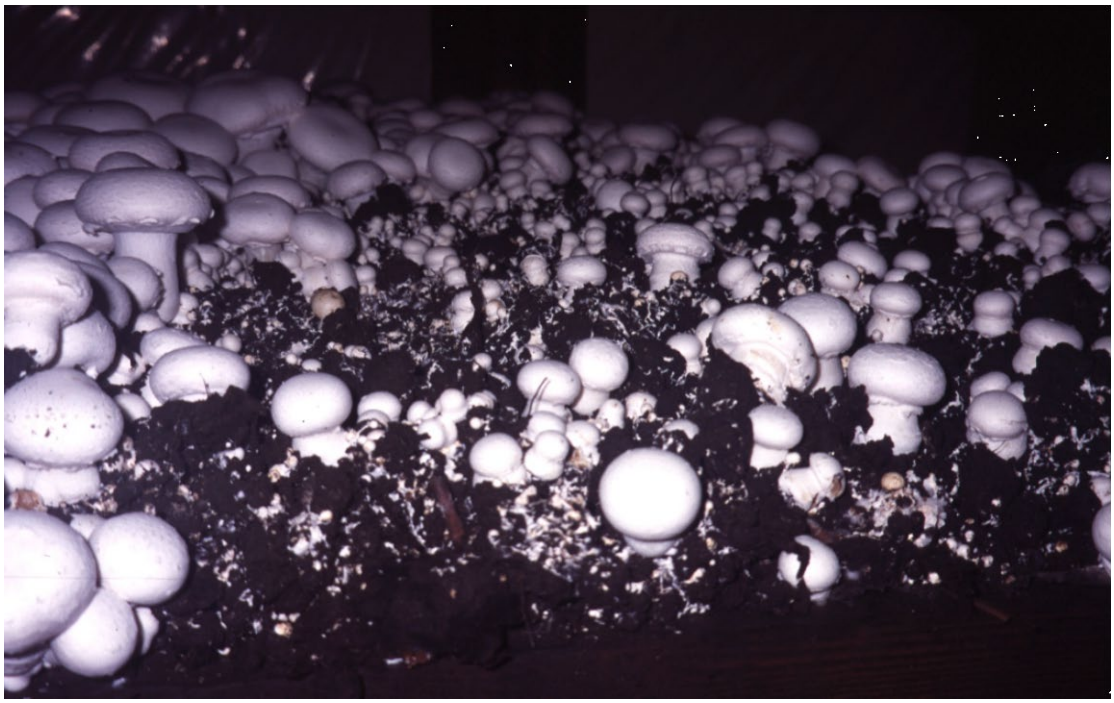

Plate 1. Bed showing area on the right where pinning has been delayed (with the pins relatively small) compared to an area on the left where mushrooms pinned normally.

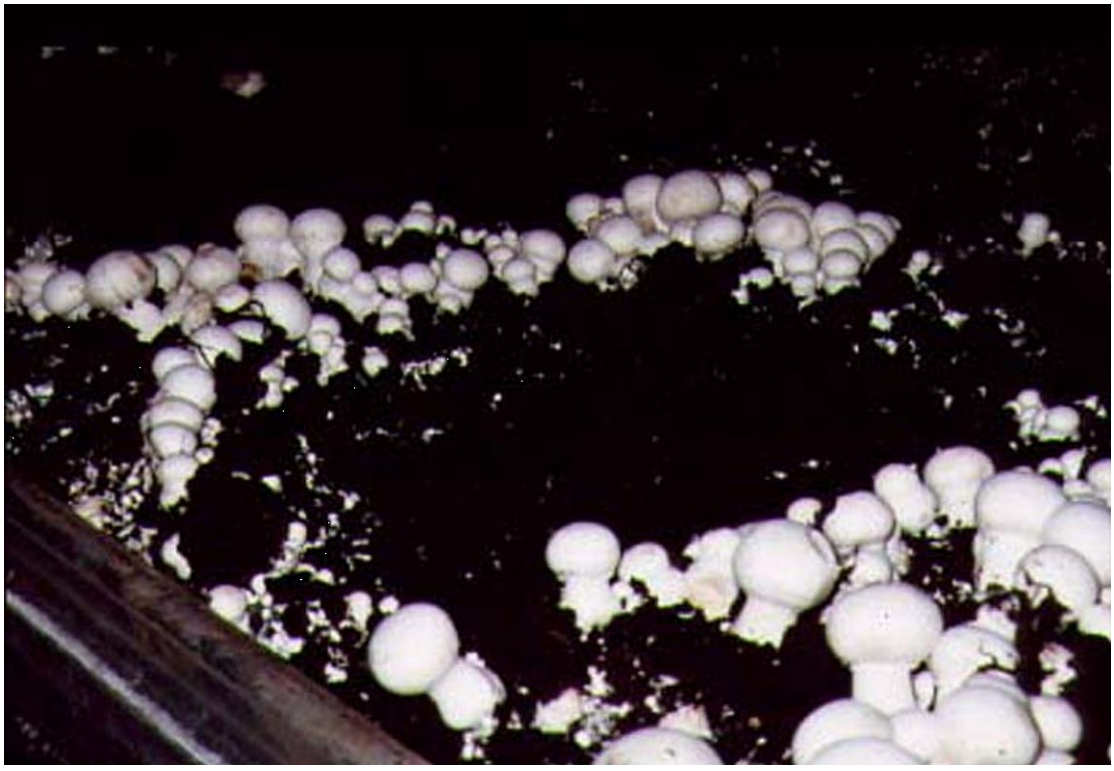

Plate 2. Bed showing bare patches where no mushrooms pinned up.

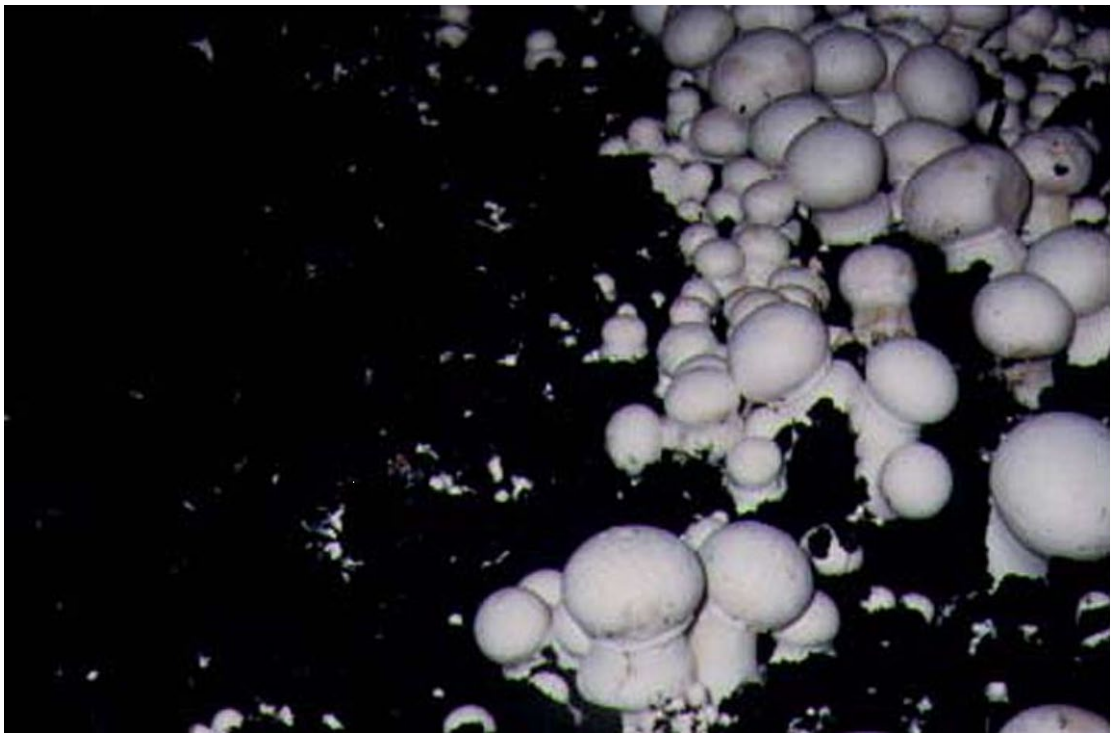

Plate 3. Bare area where no pins have developed on the left grading into normal mushrooms on the right

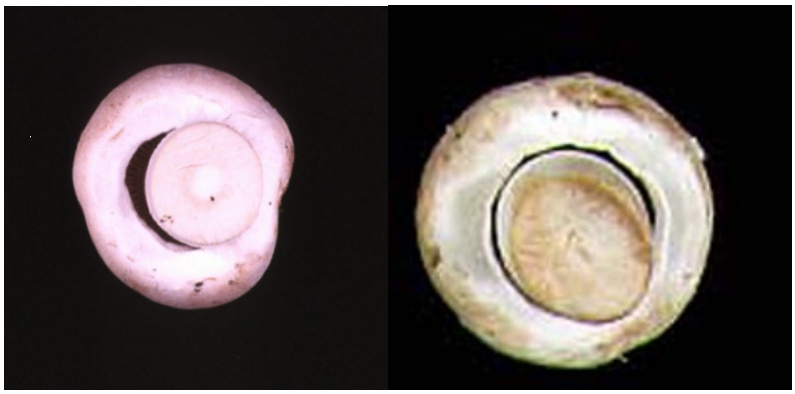

Plate 4. Prematurely opening mushrooms.

Plate 5. Discoloured "brown" mushroom on right compared with a normal mushroom from the same crop.

Plate 6. Off-coloured "brown" mushrooms in the middle of an otherwise normal-looking crop.

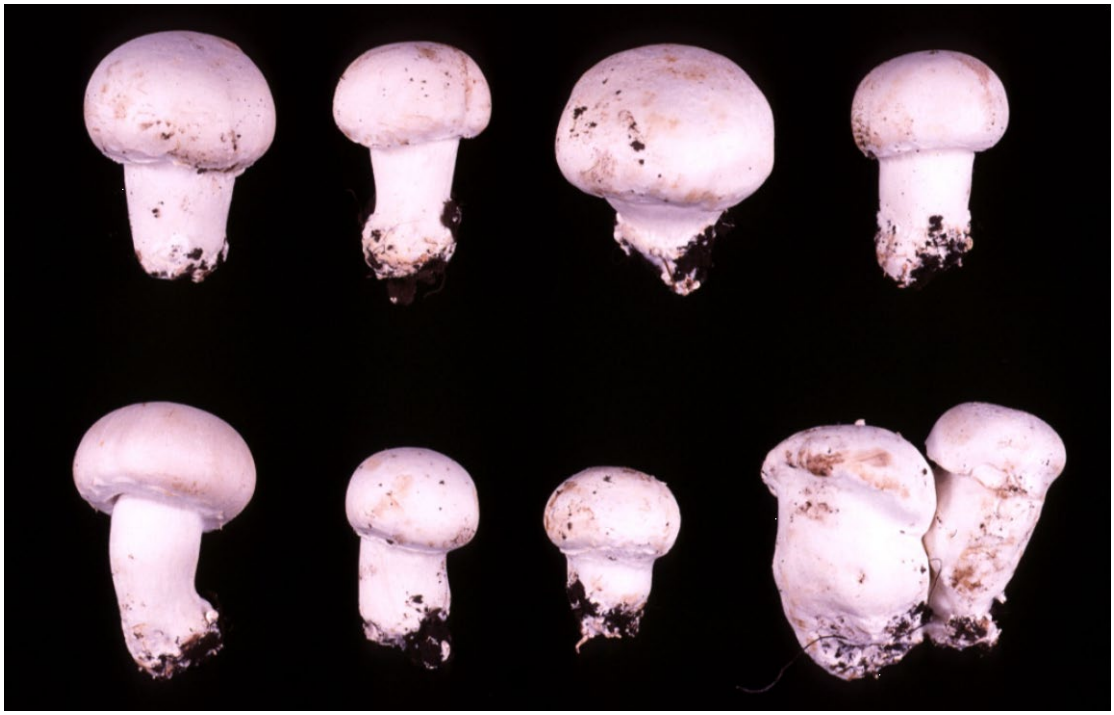

Plate 7 (above) and 8 (below). Malformed mushrooms from heavily infected crops.