Project Title:	**Epidemiology of Virus X. Year 3.**
Project Number:	M 39c
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Final Report:	9th September 2004.
Previous Reports:	M 39, M 39a, M 39b
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Date Project Commenced:	1st July 2003
Date Project completed	9th September 2004
Key words:	mushroom, disease, Virus X, dsRNA, crop delay, pin suppression, rollover, symptoms, transmission, bulk handling, Phase III, compost

The results and conclusions in this report are based on an investigation conducted over one year. The conditions under which the experiment was carried out and the results obtained have been reported with detail and accuracy. However because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results especially if they are used as the basis for commercial product recommendations.

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Spores and mycelium containing Mushroom Virus X (MVX) dsRNAs are highly infective with as little as 1g of infected material in a tonne of compost at spawning (0.0001%) being capable of causing significant crop delay and pinning disruption.

Background and Deliverables

This project began in April 1999 to investigate a new viral disease affecting the British mushroom industry. Evidence from around the industry suggests that bulk phase III production has been the most severely affected by Virus X, but non-bulk farms and systems have also been affected. At least four mushroom farms have closed down since the onset of Virus X in 1996, and the quantity of bulk phase III compost being produced has dropped dramatically since then by approx 50%. Three reports have now been produced on the HDC funded research carried out in this area (M 39, M39a, M39b). HDC projects M 39a and b established that infected mycelial fragments and infected compost could readily transmit Virus X dsRNAs, even at low levels of inoculation, but spore transmission experiments failed to prove that spores could transmit dsRNAs into crops due, most likely, to an unsuccessful methodology.

Although some growers have managed to become clear of Virus X, there is strong evidence to indicate that relapses can and do occur on sites which have had the problem before, and which are therefore considered to be virus vulnerable. This continual rumbling around of Virus X suggests that Virus X is capable of operating at very low levels of inoculum, despite what growers perceive to be good viral hygiene measures. The intransigence of Virus X on some bulk Phase III sites has had a devastating effect on the confidence in bulk phase III technology. An initial preliminary experiment at HRI suggested that as little as 0.0001% (1 part in a million) virus x-infected compost in an otherwise healthy compost was sufficient to cause detectable Virus X symptoms in the first flush of the crop.

This project delivers information on the effects of crop contamination with MVX-infected spores and mycelium at different times and under different compost handling treatments.

It also presents information on putative key dsRNA bands associated with the crop delay and pinning disruption symptoms seen in severe outbreaks of the disease. Information is also presented on the effects of continuous crop contamination on dsRNAs in mushrooms.

Summary of Results

Spore infection. Spores from a few MVX infected mushrooms that infect compost in the early days of spawn running are capable of transmitting the MVX symptoms of crop delay and pinning disruption, to new crops. Later infection of a crop may not result in any symptoms but mushrooms will still contain MVX dsRNAs, and will produce MVX infected spores that could build up on the farm.

Rollover crops. There was no further increase in the number of dsRNA bands detected in mushrooms harvested from successive rollover crops, either from compost that was spawn-run in trays, from bulk handled compost in bags, or compost that had been treated with the carbendazim fungicide Bavistin DF.

Low level compost contamination. Extremely small quantities of MVX-infected mycelium (0.0001%) that were incorporated **at spawning** into fresh compost had a dramatic impact on pinning and crop timing and, to a lesser extent, yield. When infection occurred later, when spawn-running was complete then the impact was minimal on this occasion. However, results presented in HDC report M 39b clearly showed that infection at the end of spawn-running or at casing, with specific MVX "strains" could also cause significant crop delay and pinning disruption. MVX dsRNAs were present in harvested mushrooms however, irrespective of whether or not symptoms were expressed. The expression of symptoms in crops following late infections may need some further study in order to try and predict when symptoms are most likely to occur.

MVX strains, symptoms and dsRNA profiles. Only four MVX-infected "strains" (strains 1961, 2687, 1909, and 1283) out of 12 tested, resulted in clear-cut crop delay symptoms when incorporated into freshly spawned compost at spawning (Table 1). DsRNA analysis of the original samples from which the cultures of these four strains were obtained all showed a presence of the dsRNAs H2 & 7 (as a doublet) along with 9 and 15. Mushrooms from compost infected with some of the other "strains" (lacking bands 7, 9 and 15 in the original samples) surprisingly showed the H2 & 7 (as a doublet) along with 9 and 15 in the second flush. It would be interesting to know if the live compost taken at the

end of these non-symptomatic crops, and incorporated into a freshly spawned crop, would result in the associated crop delay and pinning disruption symptoms being expressed.

Table 1. Symptoms and key dsRNAs present in harvested mushrooms from compost infected with different MVX strains at spawning.

Strain	DsRNAs in original sample	DsRNAs in 1st flush mushrooms	DsRNAs in 2nd flush mushrooms	SYMPTOMS
No Symptoms:				
A15				None
A15-1	H2	H2	H2	None
2785	9			None
2643	H2 9	H2 7[1] 8 9	H2 (7 8)[2] 9	None
1911	3f[3] H2 8 9	H2 (7 8) 9	H2 (7 8) 9	None
Ambiguous symptoms:				
2735	H2 9 (4 bb)[4]	H2 (7 8) 9 15 (4 bb)	H2 7 (8) 9 15	Some browns in 1st flush
2637	H2	H2 (7) 8 15	H2 7 (8) 9 15	Yield loss?
2191	H2 9 15	H2 9	H2 9	Yield loss?
2648	3f H2 9	H2 7 (8) 9 15	H2 7 (8) 9 15	2nd flush delay?
2784	3f H2 8 9 15	H2 (7 8) 9 15	H2 7 (8) 9 15	Delay?
Clear symptoms				
1961	**H2 7** 9 **15**	H2 7 (8) 9 15	H2 7 (8) 9 15	**Delay + yield loss**
2687	**H2 7** 8 9 **15**	H2 (7 8) 9 (15)	H2 7 (8) 9 15	**Delay + yield loss**
1909	3f **H2 7** 9 **15**	3s H2 7 8 9 15	3s H2 7 (8) 9 15	**Delay + yield loss**
1283	3s **H2 7** 8 9 **15**	3s H2 7 (8) 9 15	3s H2 7 (8) 9 15	**Delay**

1 H2 7 = two bands H2 and 7 clearly seen as a doublet on an electrophoretic gel.
2 (7 8) = bands very feint
3 "f" or "s" indicates a "feint" or "strong" band on the gel
4 (4 bb) = 4 small dsRNA bands associated with brown mushroom symptom

Infection with a number of "strains" resulted in no symptoms (2785, 2643, 1911), and a few resulted in ambiguous symptoms (2637, 2191, 2648, 2784), which may or may have been due to the small scale nature of the study. Interestingly though, in many cases, there was an increase in the number of dsRNAs detected in mushrooms from compost infected with these strains compared with the original sample (strains 2637, 2648, 2784), with well developed H2 ,& 7 doublets being detected in 2nd flush mushrooms.

Conclusions

- Infection of mushroom compost at spawning with either spores or minute amounts of *Agaricus* material (0.0001%) containing MVX dsRNAs from a symptomatic source, is sufficient to reproduce the symptoms of crop delay and pinning disruption.
- MVX dsRNAs H2, 7, 9 and 15, present in infective material, may be associated with the symptoms of crop delay and pinning disruption.
- Compost carryover form one crop to another will not in itself result in virus, but once a virus outbreak is confirmed , virus infected material could then be carried over, resulting in a build up of virus on the farm.

Commercial Benefits of the project

This project identifies the **very infective nature** of *Agaricus* spores and mycelium that originate from virus-infected crops. It also highlights the susceptibility of freshly spawned compost to infection, from minute quantities of mycelium. Therefore growers should ensure that no live *Agaricus* spores or mycelium are able to enter new crops during the spawning and spawn-running process, **particularly if mushrooms test positive for virus dsRNAs**.

Action Points for Growers

- Protect spawning operations, spawn-running rooms, casing operations and bulk handling operations from infection with mushroom spores or live mycelial fragments.
- Have mushrooms checked on a regular basis to ensure no MVX dsRNA are present at non-symptomatic levels.
- Although carryover of live compost from one crop into a new crop will not in itself cause a build up of virus, it is advisable that compost carry-over does not happen. Once virus is detected in mushrooms it is likely to be present also in the compost so any carryover would lead to a build up of virus levels.
- The very infective nature of MVX-infected mycelium carrying dsRNAs (0.0001% contamination = 1 gram of infected material in a tonne a compost) means that any machinery handling live compost, such as bulk phase III conveyors, should not be used to handle phase II compost.

SCIENCE SECTION

Epidemiology of Virus X. Year 3.

1 Introduction

This project began in April 1999 to investigate a new viral disease affecting the British mushroom industry. Evidence from around the industry suggests that bulk phase III production has been the most severely affected by Virus X, but non-bulk farms and systems have also been affected. At least ten mushroom farms have closed down since the onset of Virus X in 1996, partly as a result of reduced yields and poor quality mushrooms associated with the disease. In addition, the quantity of bulk phase III compost being produced has dropped dramatically since then by approx. 50%. Three reports have now been produced on the HDC funded research carried out in this area (M 39, M39a, M39b). HDC projects M 39a and b established that infected mycelial fragments and infected compost could readily transmit Virus X dsRNAs, even at low levels of inoculation, but spore transmission experiments failed to prove that spores could transmit dsRNAs into crops due, most likely, to an unsuccessful methodology.

Although some growers have managed to become clear of Virus X, there is strong evidence to indicate that relapses can and do occur on sites which have had the problem before, and which are therefore considered to be virus vulnerable. This continual rumbling around of Virus X suggests that Virus X is capable of operating at very low levels of inoculum, despite what growers perceive to be good viral hygiene measures. The intransigence of Virus X on some bulk Phase III sites has had a devastating effect on the confidence in bulk phase III technology. An initial preliminary experiment at HRI suggested that as little as 0.0001% (1 part in a million) virus x-infected compost in an otherwise healthy compost was sufficient to cause detectable Virus X symptoms in the first flush of the crop. Contamination of healthy spawn-run compost with airborne mycelial fragments from an infected compost was also sufficient to produce Virus X-positive mushrooms. The implications of these findings on virus x control are so enormous that this effect needs to be reconfirmed.

Although initial spore transmission work in project M39a was unsuccessful (spores stored for 1-3 months were used rather than fresh ones) there is strong circumstantial evidence to support the idea that Virus x infected spores readily transmit Virus X to healthy crops. A preliminary experiment at HRI also demonstrated that heavy contamination of compost with FRESH spores readily transmitted Virus X to an otherwise healthy crop. It is therefore important to establish if smaller numbers of infected spores can transmit the virus to new crops in order to determine the importance of spore exclusion hygiene in relation to MVX.

HDC Report M39b reported on how the intensity of some dsRNAs, in particular the H2 band, increased following the continuous reinfection of freshly spawned compost with compost from an earlier crop for up to nine crop cycles. As the H2 dsRNA band is known to be closely related to bands 7, 8 and 9 found in MVX infected crops, it would be extremely useful to know if they would eventually be expressed following further continuous reinfections.

The overall aim of this series of projects is to understand the relationship between Virus X infected propagules and the development of mushroom Virus X disease (MVX), in order to develop effective control strategies.

The specific objectives of this project M39 c are as follows:

1. Quantify and qualify some of the parameters required for spore transmission of Virus X into healthy compost
2. Establish the relationship between very low levels of contamination (0.0001%) on the transmission of Virus X into freshly spawned and spawn-run compost
3. Determine if continuous re-infection of freshly spawned compost with compost from an earlier crop leads to additional dsRNAs being detected in mushrooms.
4. Determine the crop symptoms and dsRNA profiles in mushrooms following the infection of freshly spawned compost with different *Agaricus* cultures (MVX "strains") containing a diverse spectrum of dsRNA bands.

2 Spore Transmission Experiment

A preliminary experiment in 2001 (M39?) demonstrated that continuous and heavy infection of spawn-running compost with fresh spores from Virus-X infected mushrooms, transmitted dsRNAs to mushrooms produced in the subsequent crop. In view of the likelihood that most growers would aim to minimise the contamination of spawn-running compost with spores, it was decided that additional information was needed on the effects of lower levels of spore infection of spawn-running compost. An experiment was designed to see if a lighter infection of mushroom spores, applied to compost either at spawning or at the end of spawn-running, was also capable of transmitting MVX dsRNAs.

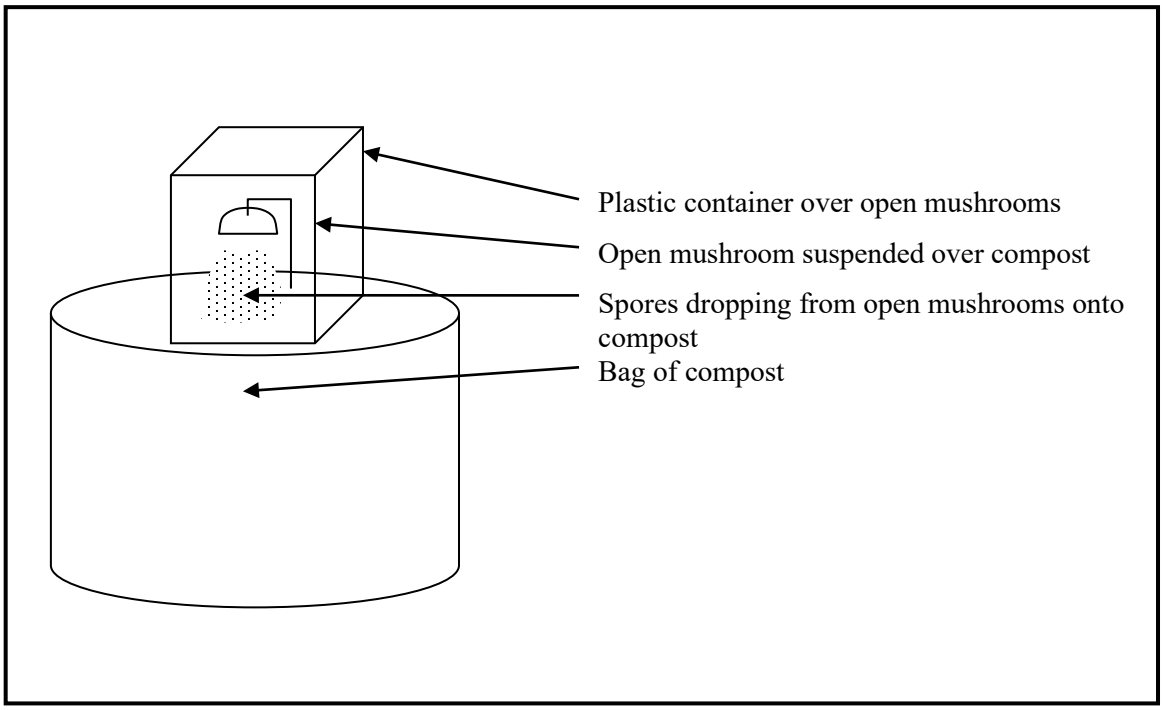
2.1 Materials and Methods

2.1.1 Compost preparation

Heavy gauge polythene bags were filled with 12 kg of spawned compost (HRI compost 10/03; Sylvan A15 spawn) and laid out in a growing room. When all inoculations were completed all bags were cased with a standard commercial casing mix containing casing inoculum (Tunnel Tech English mix).

2.1.2 Inoculation of crop

Fresh mushrooms from a virus–infected crop (strain 1961) were grown and harvested in an adjacent house and used to provide the fresh MVX-infected spores to inoculate bags of compost. Healthy (A15) mushrooms were also harvested from a non-virus infected crop to provide MVX-free spores for the control treatments. Two to four open mushrooms (depending on size) were suspended over the surface of the compost in each bag for a given treatment. The suspended mushrooms were covered with a plastic container to prevent spores escaping into the room and onto other bags (see diagram below). The polythene bag was also pulled up over the containers to prevent the compost from drying out and to provide additional protection against spores escaping into the room. Mushrooms were allowed to drop their spores for 2 to 3 days, after which time they were carefully removed.



Bags of compost were set up as described above for two different mushroom strains, two different inoculation times, and for two different compost handling treatments as follows:

Two strains:	(a)	A15- Control mushrooms
	(b)	MVX strain 1961 mushrooms
Two Inoculation times:	(a)	Just after spawning (Day 3)
	(b)	At the end of spawn-running (3 days before casing)
Two compost handling treatments:	(a)	Spawn-run compost undisturbed prior to casing
	(b)	Spawn run compost bulk handled prior to casing
Replicates:	4 replicates per treatment	
Total number of plots:	2 x 2 x 2 x 4 = 32	

2.1.3 Records and Analyses

Two flushes of mushrooms were harvested picked as closed cups and buttons. No grading was done. Yields were recorded daily and presented as cumulative yield over time.

Mushrooms were sampled and tested for the presence of double-stranded ribonucleic acid (dsRNA) according to the protocol described in Grogan et al. (2003). The mushrooms which were used to provide the spores for the inoculum were also tested for dsRNAs.

2.1.4 Statistical design

Plots were laid out in a cropping chamber using a double blocking system, with each treatment occurring once in each of the 4 columns (rows) running down the length of the house and once in each pair of adjacent rows running left to right across the house. This ensures that all treatments are present in all locations throughout the house to allow for any variations of environment that may occur within the house.

2.2 Results and Discussion

2.2.1 Yield effects

When compost was infected at spawning with MVX-infected spores (strain 1961) there was a one day delay in the onset of both the first and second flushes, in conjunction with the production of heavy pin-sets and lots of small mushrooms. The effect occurred whether or not the compost was bulk handled at the end of the spawn-running period or left undisturbed (Figure 1). However, the effect was not shown by all replicates, suggesting that the effect of spore contamination at spawning can be variable. When compost was infected at the end of the spawn-run with MVX-infected spores (strain 1961) there was no obvious effect on cropping (Figure 2).

These effects due to spore infection are less severe and less consistent than previous experiments using MVX-infected compost (see HDC Report M39b), where crop delay and heavy pin-sets occurred irrespective of the time of infection, and more consistently within replicates.

There were no noticeable effects due to the application of spores from healthy A15 mushrooms to compost either at spawning or at the end of spawn-running. In addition, the total yields from all treatments but one were quite similar, whether the compost had been infected with spores from MVX-infected mushrooms or not, or had been bulk handled

Figure 1. Mushroom Yield following infection of compost early in the spawn-run by spores from either standard A15 mushrooms or mushrooms from an MVX-infected crop (strain 1961) showing crop delay and pinning disruption. LSD = least significant difference at $P = 0.05$.

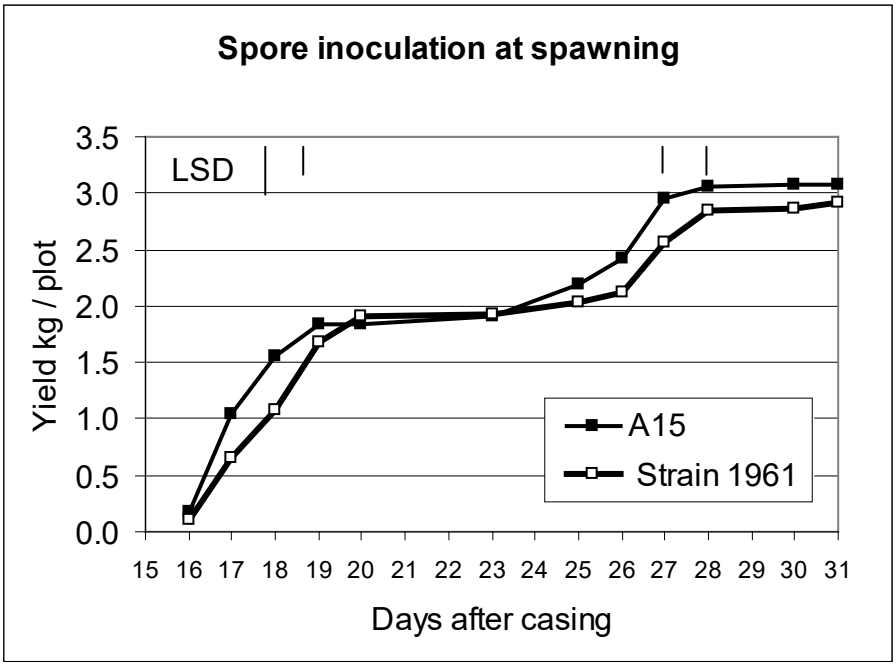
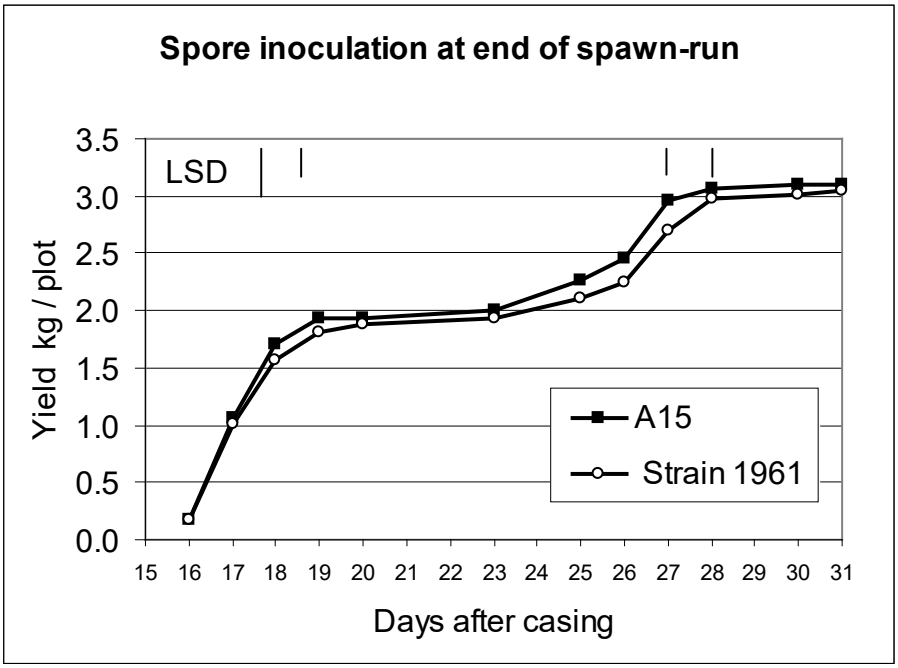


Figure 2. Mushroom Yield following infection of compost towards the end spawn-running by spores from either standard A15 mushrooms or mushrooms from an MV- infected crop (strain 1961) showing crop delay and pinning disruption. LSD = least significant difference at $P = 0.05$.

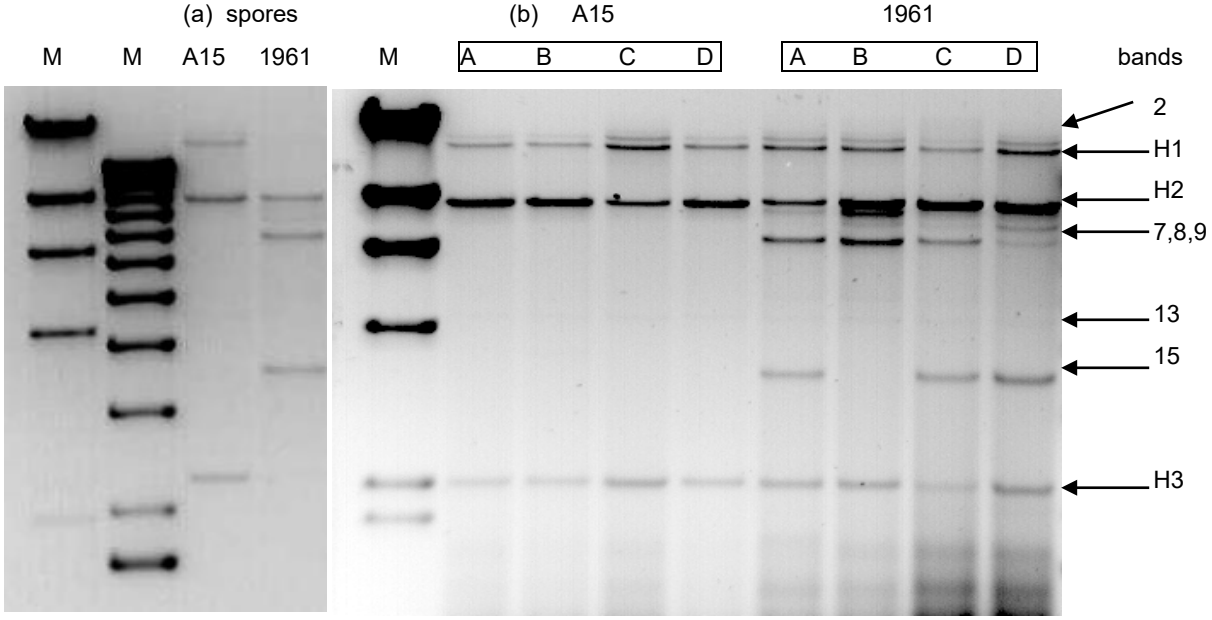


(Figures 1 & 2). These results suggest that infection of spawn-running compost with spores may be less severe than with infected compost. This is in contrast to reported information on La France disease where spores were believed to be the primary infective agent (Schisler et al 1967).

2.2.2 dsRNA profiles

All mushrooms from compost that had been infected with MVX 1961 spores, at either the beginning or end of the spawn-running period, tested positive for the same dsRNAs present in the parent mushrooms from which the spores were derived. The dsRNA profiles were very similar for all treatments, irrespective of whether the spores were applied early or late in the spawn-running process or whether or not the compost had been bulk handled after spawn-running (Figure 3). Although only the compost that was infected at spawning showed crop delay and pinning disruption, the mycelium in the compost from the later infection time, as well as the spores from the mushrooms, are all highly likely to contain MVX dsRNAs and so could be capable of transmitting the disease into other crops. Should that occur at spawning, then symptom expression is also likely to occur. This is an area that should be further explored in order to confirm the infective nature of apparently non-symptomatic, but MVX dsRNA-containing crops.

Figure 3. (a) dsRNA profiles of mushrooms from which spores were derived; (b) dsRNA profile of mushrooms from compost infected with spores from A15 mushrooms and 1961 mushrooms; (A) infected after spawning, spawn-run in-situ; (B) infected after spawning, bulk handled; (C) infected at the end of spawn running, in-situ; (D) infected at the end of spawn running, bulk handled. M = molecular markers.



2.3 Summary

Spores from a few MVX-infected mushrooms are capable of transmitting MVX disease to new crops. The symptoms of crop delay and pinning disruption are more likely to be seen if the infection occurs in the early days of spawn-running. Later infection of a crop may not result in any symptoms but mushrooms will still contain MVX dsRNAs. The presence of MVX dsRNA-infected-material on a farm may therefore go undetected, only becoming apparent when breeches in virus hygiene measures occur that allow the virus to infect the crop at a vulnerable stage or in great quantity.

2.4 Conclusions

- Infection of mushroom compost at spawning with spores from a few mushrooms, containing MVX dsRNAs from a symptomatic source, is sufficient to reproduce the symptoms of crop delay and pinning disruption.

3 Compost Rollover Experiments

Mushroom viruses are carried within the mushroom mycelium and can be transferred into new crops if infected mycelium anastomoses with non-infected mycelium. This could happen when compost from an older crop becomes incorporated into freshly spawned compost of a new crop, such as might happen if trays are not steamed off prior to re-use, or if the same conveyor is used to handle bulk phase II and Phase III compost. The results from the previous Virus X report, M39b, showed that there was an increase in the intensity of the dsRNAs normally found in healthy mushrooms, when compost was continually re-incorporated or "rolled over" into freshly spawned compost for 8 crop cycles. This experiment was continued for a further 10 crop cycles to see if there was any further increase in intensity and diversity of dsRNAs following continued contamination of freshly spawned compost with live compost from a previous crop. In addition, some compost was bulk handled after spawn running, and some was bulk handled and treated with Bavistin DF (active ingredient = carbendazim), to determine if these activities had any influence on the number and intensity of the dsRNAs present.

3.1 Materials and Methods

3.1.1 Compost carry-over in trays.

Trays of spawned compost from the Warwick HRI Mushroom Unit were placed in a growing room to spawn-run. At the start of spawn running, a small handful of compost from a previous crop (taken from rollover crop 8, see previous HDC report M39b) was mixed into the compost in the tray. At the end of spawn running, a small handful of compost (about 20 g) was taken from the tray prior to casing, and used to infect another tray of freshly spawned compost. This was done for a total of 11 crops (Rollover 9 to Rollover 19).

3.1.2 Compost carry-over in bulk-handled compost in bags.

Heavy-gauge polythene bags were filled with 20 kg of spawned phase II compost from the Warwick HRI mushroom Unit, and placed in a cropping chamber to spawn run. At the end of the spawn running period the compost was emptied out of the bag onto a sheet of polythene and gently broken up to simulate the bulk handling process associated with the

emptying of phase III tunnels. A small sample of the spawn-run compost (about 20g) was put aside to infect a freshly spawned bag of compost. The compost was then put back into the bags, cased and cropped according to standard growing procedures at Warwick HRI Mushroom Unit. This was done for a total of 9 crops (Rollover 11 to rollover 19).

3.1.3 Bavistin treatment of bulk handled compost.

Spawned phase II compost was treated at spawning with Bavistin DF at a rate of 100 g per tonne (2 g in 100 ml water / 20 kg bag), and again during bulk handling of the spawn-run compost. (There is no label approval for this application but we were aware that Bavistin DF might have been used in this way to control moulds in compost, during the time when MVX was first encountered). In addition to the Bavistin DF treatment, a small quantity of spawn-run compost from a previous crop was also incorporated into the compost at spawning, as described above. These two treatments were done for a total of three crops, Rollover 17 to Rollover 19).

3.2 Results and Discussion

There was no further increase in the number of dsRNA bands detected in mushrooms harvested from successive rollover crops, either from compost in trays (Figure 4), or from bulk handled compost in bags, with or without Bavistin treatment (Figure 5). Bands 2, H1, 13 and H3 occurred in all mushroom samples throughout the rollover crops. Bands 1, 4 and 13 also occurred regularly but were much feinter. Band 4 (13.6 kilo base pairs) was more noticeable in mushrooms from Rollover crops 16, 17 & 18. The titre of some bands following some treatments was occasionally more intense but there did not appear to be any increase in intensity associated with any particular treatment from one crop to the next. Yield data was not recorded from these crops but observations were made as to how healthy the crops looked. By and large most crops performed well, and showed no crop delay or other symptoms associated with MVX, with the exception of Rollover crops 17 and 18. Two of the bags of bulk-handled compost in Rollover 17 failed to crop though the Bavistin-treated bags produced some mushrooms. In Rollover 18, all trays and bags showed central areas of dieback where the compost from Rollover 17 had been incorporated (Figure 6). Mushrooms from this crop however did not contain either La France dsRNAs or any additional MVX dsRNAs (Figure 5) but compost from the bare

Figure 4. DsRNA profiles of mushrooms harvested from a series of rollover (RO) crops in trays. For all gels the left hand lane is a marker lane and the next four lanes are for mushrooms from 4 different Rollover trays.

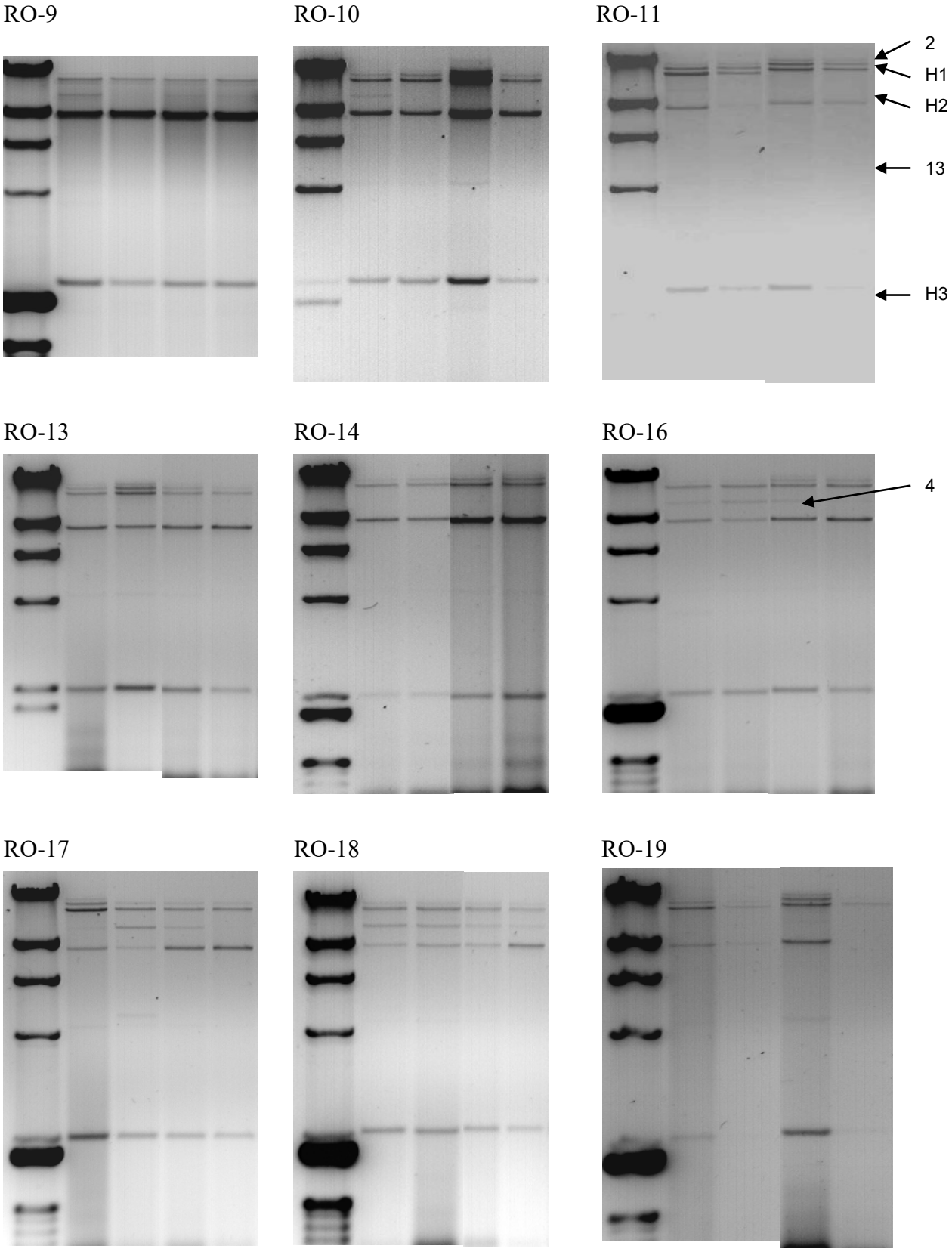
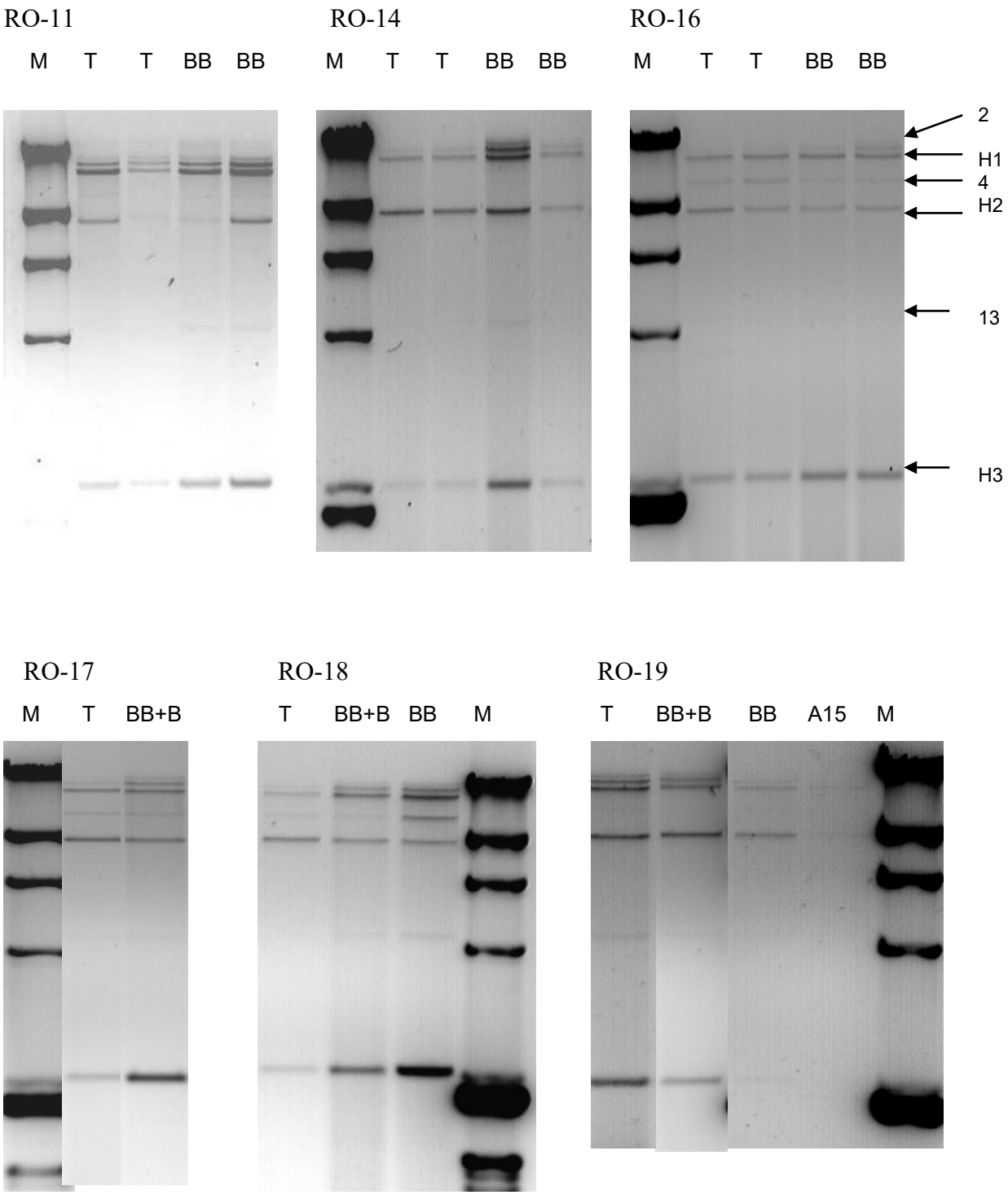


Figure 5. DsRNA profiles of mushrooms harvested from selected rollover (RO) crops where the compost was spawn-run in situ in trays (T); spawn run in bags then bulk handled (BB); or spawn run in bags, bulk handled and compost treated with Bavistin DF at spawning and during bulk handling (BB+B). A straightforward A15 control sample was included in RO crop 19 for comparison



areas had a high population of moulds (2 x 10^7 propagules per gram fresh weight of compost) which included Scopulariopsis sp (tentative identification) and *Penicillium* sp. Thus the bare areas were probably associated with a compost problem rather than with virus. The last rollover crop, RO 19 cropped well again, showing that the dieback phenomenon was not carried on further.

(Figure 6, picture of crop to scan in.)

3.3 Summary

One conclusion that can be drawn from these data is that Virus X is not present at a low level in normal spawn-run compost in so far as we were not able to trigger the occurrence of MVX dsRNAs by deliberately transferring live compost from one crop to another for up to 19 cycles. Nor did bulk handling the compost in conjunction with compost carry-over for 9 cycles have any effect. Fungicide treatment of the compost with Bavistin DF at both spawning and during bulk handling at the end of spawn-run did not have any effect either though we were only able to look at this treatment for 3 cycles. These results suggest that MVX is not indigenous to mushroom mycelium (or to Sylvan A15 spawn at least). One possibility is that it is present in wild populations of *Agaricus bisporus* which then contaminated commercial crops. Kerrigan (2004) has tested a large number of wild *Agaricus bisporus* from around the world and has frequently found uncharacterised dsRNAs, and he comments that the potential for new viral diseases to emerge is far from negligible. Further DEFRA-funded work is in progress to determine just how similar the dsRNAs in the wild isolates are to those found associated with MVX in commercial mushrooms.

3.4 Conclusions

- Compost carryover form one crop to another will not in itself result in virus, but once a virus outbreak is confirmed, virus infected material could then be carried over, resulting in a build up of virus on the farm.

4 Low Level Compost Contamination

Mushroom farms that produce and handle bulk phase III compost, generate large quantities of macroscopic and microscopic fragments of compost during the tunnel-emptying process. These fragments will undoubtedly be colonised with live *Agaricus* mycelium, and, should they find a route to contaminate fresh phase II compost at spawning, they have the potential to infect the fresh compost with virus, should there be virus present in the live mycelium. Close work with bulk phase III producers at the height of the Virus X epidemic suggested that very low levels of infected mycelium appeared to be capable of contaminating newly spawned tunnels. Experiments were carried out to determine how little infected mycelium was necessary to transmit MVX dsRNAs and symptoms. Very low levels of live MVX-infected compost were introduced into freshly spawned compost at spawning and also at the end of spawn-running. In addition the effect of bulk handling compost was investigated to see if it had an additional effect on the severity of MVX expression.

4.4 Materials and Methods

4.1.1 Compost preparation

Heavy gauge polythene bags were filled with 12 kg of spawned compost (HRI compost 11/03; Sylvan A15 spawn, Experiment 2.23) and laid out in two growing rooms for spawn-running. When all inoculations and treatments were completed all bags were cased with a standard commercial casing mix containing casing inoculum (Tunnel Tech English mix).

4.1.2 Preparation of inoculum

Autoclavable polypropylene pots, containing 150 g of phase II compost were autoclaved for one hour on two consecutive days to produce sterile compost. The sterile compost was inoculated with MVX strain 1283-4c at 1% w/w, and incubated at 25^{O}C until fully spawn run then cold stored until needed. Inoculum (i.e. MVX infected compost) was incorporated into either Phase II or Phase III compost at three different rates and times as follows:

4 Inoculum rates:	1%	10 g inoculum per 1 kg of Phase II
	0.01%	0.1 g inoculum per 1 kg of Phase II
	0.0001%	0.001g inoculum per 1 kg of Phase II (= 1 g per tonne of Phase II)
	None	Control
2 Inoculation times:	At spawning	
	At the end of spawn-running prior to casing.	
2 compost handling treatments:	Compost Bulk-handled after spawn-run	
	Compost undisturbed after spawn-run	

4 replicates per treatment combination

This gives 4 x 2 x 2 x 4 = 64 plots.

Inoculum was placed in the centre of each bag as bags were filled with phase II for all the "At spawning" treatments. For treatments inoculated "At the end of spawn-running", inoculum was placed close to the centre of the bag in bulk handled treatments but was placed close to the surface in non-bulk handled treatments, to reflect what might happen on a mushroom farm.

4.1.3 Records and Analyses

Two flushes of mushrooms were harvested picked as closed cups and buttons. No grading was done. Yields were recorded daily and presented as cumulative yield over time. Mushrooms were sampled and tested for the presence of double-stranded ribonucleic acid (dsRNA) according to the protocol described in Grogan et al. (2003). The mushrooms which were used to provide the spores for the inoculum were also tested for dsRNAs.

4.1.4 Statistical design

Plots were laid out in two cropping chambers with two replicate blocks in each house. Each block was divided into 4 main plots, each with different positions across the width of the house. This arrangement allows for partial estimation of all interactions.

4.2 Results and Discussion

4.2.1 Yield effects

There was no significant effect on cropping patterns due to bulk handling treatments so the data for bulk-handled and non bulk-handled treatments were combined. The most significant effects observed were a 1-2 day crop delay in the first flush, followed by a 4 day delay in the second flush following infection of compost at spawning. The effect was the same irrespective of whether a large (1%) or minute (0.0001%) amount of infective mycelium had been incorporated at spawning (Figure 7). The lowest rate of infection tested (0.0001%) is equivalent to 1 g in a tonne of compost. Trying to exclude this level of infection during bulk handling processes is likely to require an extremely watertight level of exclusion hygiene especially if emptying and filling operations share common corridors and equipment.

In addition to crop delay, compost infected at spawning tended to over pin and produce lots of small mushrooms, making it difficult to pick good quality closed cups. Total yields were a little lower than the controls at between 89 and 95%.

In contrast, when compost was infected at the end of the spawn running period there was little discernable effects. Crop timing was not significantly affected (Figure 8) and mushrooms did not tend to over pin. Total yields were also unaffected ranging from 99 to 105% of the controls. These results differ to those obtained in earlier experiments (See HDC report M 39b) where infection at the end of spawn running or of casing resulted in significant crop delay and pinning disruption for two different MVX strains (1283-P and 1961). Strain 1283-4c, used in the current experiment, was taken from the same source but at a different time to 1283-P, which may account for different effects observed in the different experiments. This is an area that needs to be investigated further.

4.2.2 DsRNA analysis of mushrooms.

All mushrooms from compost that had been infected with MVX 1283-4c mycelium, either at spawning or at the end of the spawn-running period, tested positive for the same dsRNAs present in the parent mushrooms from which the mycelium was obtained. The dsRNA profiles were more intense in mushrooms from compost infected at spawning (Figure 9) whether or not the compost had been bulk handled prior to casing. The dsRNA

Figure 7. Mushroom yield from compost infected with different rates of MVX strain 1283-4c at spawning.

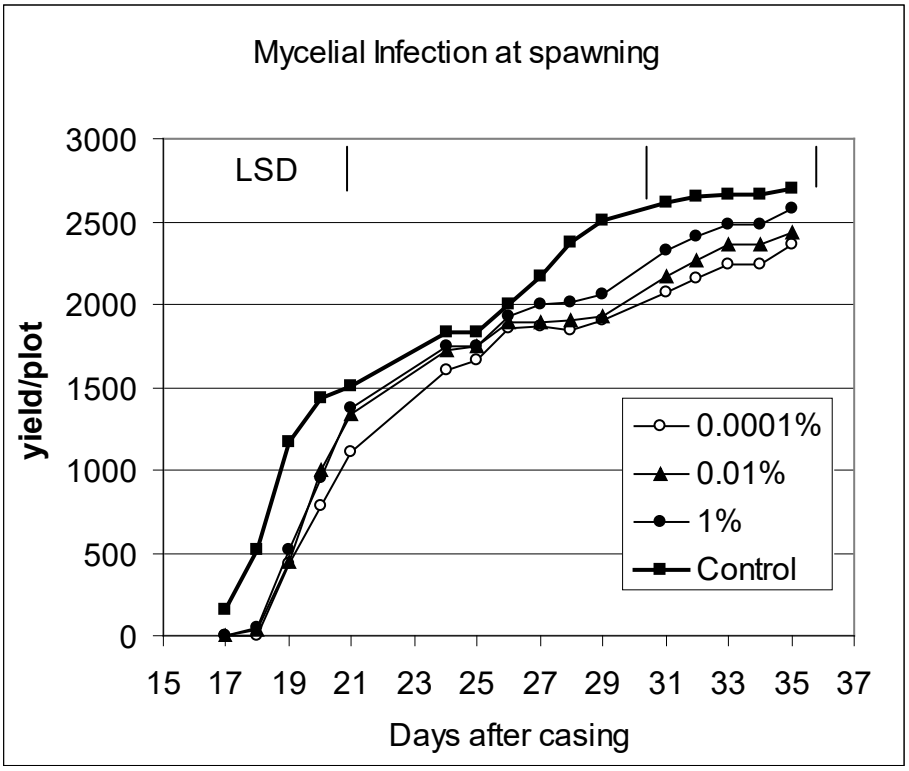
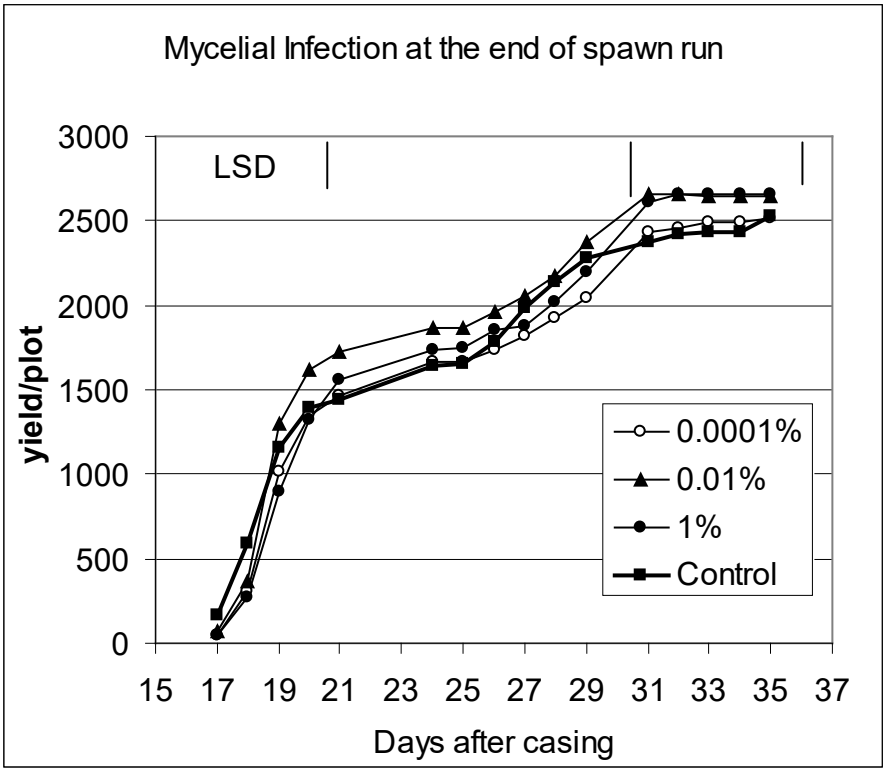


Figure 8. Mushroom yield from compost infected with different rates of MVX strain 1283-4c at the end of spawn running.



profiles in mushrooms from compost infected at the end of spawn run showed more variation however (Figure 10). When compost was bulk handled at the end of spawn-running, and then inoculated in the centre of the bag, the dsRNA profiles of mushrooms was very similar, irrespective of how much inoculum had been added, suggesting that even the very small amount of inoculum added successfully transmitted dsRNAs into the re-anastomosing mycelium following bulk handling. When the inoculum was placed close to the surface of fully spawn-run, but not subsequently bulk handled, compost the mushrooms showed an increase in dsRNA intensity in line with the amount of inoculum added, suggesting that transmission of the dsRNAs from the larger amounts of inoculum that are close to the compost–casing interface, are transmitted more rapidly upwards into mushrooms however it is difficult to fully understand if there are different mechanisms operating which influence the intensity of dsRNAs in mushrooms following contamination of the compost at different times and levels.

4.3 Summary

Extremely small quantities of MVX 1283-infected mycelium (0.0001%) that were incorporated **at spawning** into fresh compost were capable of having a dramatic impact on pinning and crop timing and, to a lesser extent yield. When infection occurred later after spawn-running was complete then the impact was minimal on this occasion, even with relatively large quantities of infected material. This is in contrast to previous experiments where significant crop delay occurred following late infections at the end of spawn-run and in casing. The intensity of the dsRNA profiles in the harvested mushrooms however did not have any relationship with the intensity of symptoms expressed.

Figure 9. MVX dsRNAs in mushrooms from compost inoculated **at spawning** at different rates (C = control, 1 = 0.0001%; 2 = 0.01% and 3 = 1%) and either left Undisturbed or Bulk handled at the end of the spawn-run. M = marker lanes.

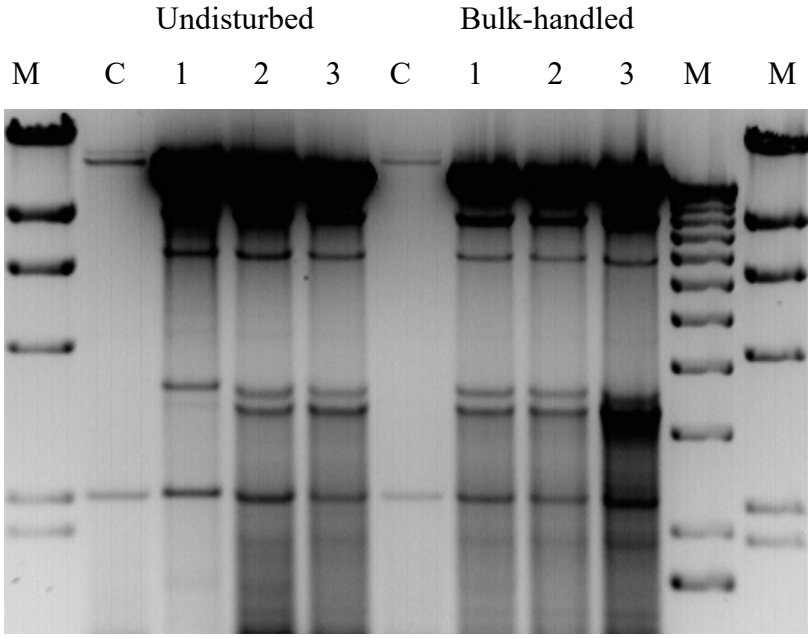
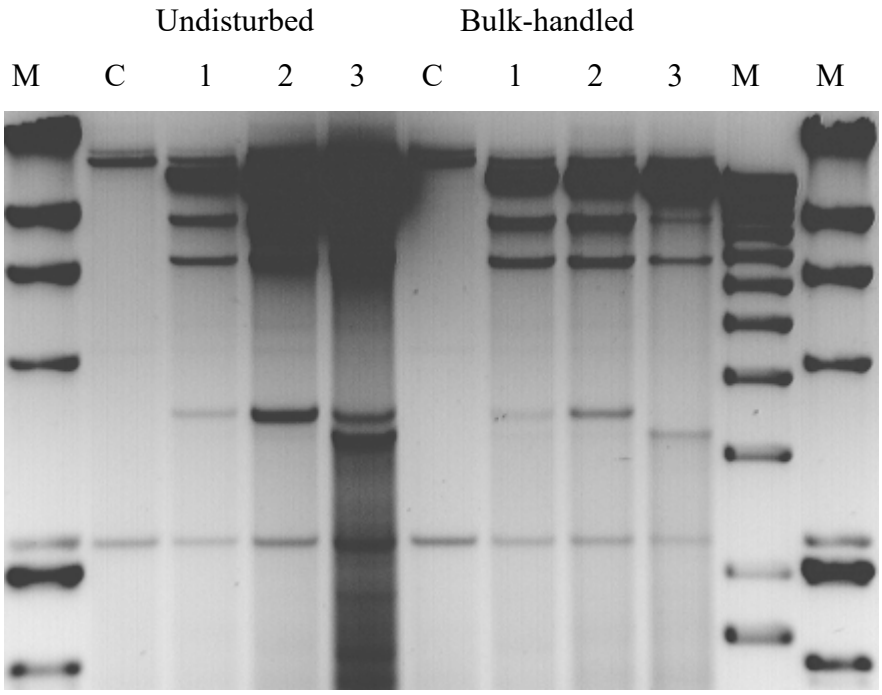


Figure 10. MVX dsRNAs in mushrooms from compost inoculated **at the end of spawn running spawning** at different rates (C = control, 1 = 0.0001%; 2 = 0.01% and 3 = 1%) and either left Undisturbed or Bulk handled at the end of the spawn-run. M = marker lanes.



4.4 Conclusions

- An extremely small quantity of “patch type” MVX infected material at spawning is all that is needed to cause pinning disruption and crop delay. Exclusion-hygiene measures at spawning therefore need to be of a very high standard.
- MVX-positive mushrooms can occur in the absence of any overt symptoms. The presence of MVX-infected material on a farm should be monitored to remind growers of the need for extra vigilance to prevent contamination of younger crops with MVX positive material.

5 MVX Strains, Symptoms and dsRNA profiles

Previous experiments using MVX strains originating from diverse sources and with diverse symptoms were reported on in HDC report M 39b. Results from that work indicated that there were at least two distinct patterns of MVX symptoms which were associated with distinct dsRNA profiles. Crop delay and pinning disruption (associated with bare areas, patches and non-productive areas) were one group of symptoms, while the presence of brown or off coloured mushrooms in an otherwise normal crop were the second type of symptom. It was apparent that the dsRNA profiles of MVX strains that produced similar symptoms, were different suggesting that some of the dsRNAs present in affected crops may not be contributing to the disease symptomology. It was decided to take a selection of MVX strains with different combinations of dsRNA bands and to compare the symptomolgy, in order to ascertain, if possible, which dsRNAs were correlated with symptom expression. Strains were chosen that had combinations of bands 3, H2, 7, 8 & 9, and band 15 as these were invariable found in samples from the most severely affected sites in Britain. Band 3 was not always present in samples from severely affected sites, whereas bands H2 7, 8, 9 and 15 usually were, so our hypothesis was to test if any strains containing H2, 7, 8, 9 and 15 would be capable of reproducing severe symptoms under controlled experimental conditions. Since the crop delay and patching symptoms, tentatively associated with these dsRNAs, developed consistently following inoculation at spawning (see chapters 2 and 4), it was decided to look at a wider range of strains at one single inoculation time rather than a smaller number of strains at two inoculation times.

5.1 Materials and Methods

5.1.1 Strain selection.

Table 1 outlines the MVX strains selected for this experiment and the dsRNAs that were detected in the original samples. Isolates were grouped into

- Those containing combinations of bands H2, 7, 8 & 9 **only**
- Those containing bands H2, 7, 8 & 9 **AND** band 15
- Those containing bands 3 **AND** H2, 7, 8 & 9 but **NO** band 15
- Those containing bands 3 **AND** H2, 7, 8 & 9 **AND** band 15

Strain MVX 2735, which produces the brown mushroom symptom, was also included which had bands H2 & 9 and the 4 small dsRNAs correlated with browning. Two controls

were included consisting of (1) commercial A15 and (2) A15-1 inoculum made in the same way as the MVX strain inoculum. Crops from A15-1 inoculum usually contain band H2, which tends not to be present in A15 control crops. All strains contained bands other than those listed above, but they are not being considered in this experiment.

Table 1. MVX mushroom samples with different dsRNA profiles, from which *Agaricus* cultures were taken for use in MVX experiments.

Strain/ (sample number)	Symptoms associated with sample (where known)	Putative key dsRNA bands present in original samples	Other dsRNA bands present
A15			1,2,H1, 13, H3
A15-1	Reduced yield ?	H2	1,2,H1, 13, H3
MVX 2637		H2,	2, H1, H3
MVX 2785		9	H1, 12, 13, H3, 18, 20
MVX 2643		H2 9	H3
MVX 2735	Brown mushrooms	H2. 9 (4bb)*	H1, 5, H3
MVX 2191		H2 9 15	H3
MVX 2648	Some variability	3 (feint), H2, 9	H1, 13, H3
MVX 1911	Some variability	3 (feint), H2, 8, 9	2, H1, 13, H3
MVX 2784		3 (feint), H2, 8, 9 15	2, H1, 12,13, H3
MVX 1961	Delay, reduced yields	H2, 7, 9 15	2, H1, H3
MVX 2687	Delay, reduced yields	H2, 7, 8, 9 15	H1 16, H3, 18, 20
MVX 1909	Delay & swirls	3 (feint), H2, 7, 9 15	H1, 13, 16, H3, 18
MVX 1283	Delay, bare patches,	3 (strong), H2, 7, 8, 9 15	H3

* (4 bb) = four small dsRNAs associated with the brown mushroom symptom

5.1.1 Compost preparation

Heavy gauge plastic bags were filled with 3.5 kg of phase II compost (HRI compost 01/04, Experiment 6.04) and spawned with 20 g of Sylvan A15 spawn (0.6%). The bags of compost were placed into 26 cm diameter pots. Once the compost was inoculated, all pots were cased with commercial casing (Tunnel Tech English) containing commercial casing inoculum (C.I.).

5.1.2 Compost Inoculation

MVX inoculum was prepared for each strain listed in Table 1, as described in section 4.1.2. (i.e. sterilised compost was inoculated with spawn made from each of the cultures in Table 1 to provide MVX-infected compost to inoculate the pots of healthy compost prepared above). When ready for use, the MVX-infected inoculum was added to the freshly spawned Phase II compost at a rate of 0.01% (0.35 g per 3.5 kg compost). Inoculum was placed in the centre of each bag of compost. Three replicates were prepared for each treatment.

5.1.3 Records and analyses

Two flushes of mushrooms were harvested picked as closed cups and buttons. No grading was done. Yields were recorded daily and presented as cumulative yield over time. Mushrooms were sampled and tested for the presence of double-stranded ribonucleic acid (dsRNA) according to the protocol described in Grogan et al. (2003).

5.1.4 Statistical design

Plots were laid out in a cropping chamber on aluminium racking at three levels, in a randomised block design. Each level constituted a block, and one replicate of each treatment was randomly assigned to a position within each block. Yield data were analysed using ANOVA (analysis of variance).

5.2 Results and Discussion

5.2.1 dsRNA analysis of mushrooms and symptoms

Table 2 shows the results of dsRNA analysis of 1st and 2nd flush mushrooms taken from crops infected with different MVX strains at spawning, in comparison with the dsRNA profiles of the original strains. The gel image of the dsRNAs for 1st and 2nd flush mushrooms are shown in Figures 11 and 12. The control treatment A15-1 showed no change in the dsRNA profile compared with the original samples, and A15 showed no dsRNAs apart from those normally present in all mushrooms (H1 and H3) but which are not being considered in this experiment.

Only four strains resulted in clear-cut crop delay symptoms (1961, 2687, 1909, and 1283). DsRNA analysis of the original samples from which cultures of these strains were obtained all showed a presence of the dsRNAs H2, 7 (as a doublet) along with 9 and 15, and

sometimes 8, although band 8 is usually very feint. Mushrooms from these treatments also produced very similar profiles to the infecting strain with the exception of strain 1909, which went on to produce a very strong band 3 in mushrooms whereas in the original sample the band 3 signal was feint.

A number of treatments resulted in no symptoms (2785, 2643, 1911), and a few resulted in ambiguous symptoms (2637, 2191, 2648, 2784), which may or may have been due to the

Table 2. Symptoms and key dsRNAs present in harvested mushrooms from compost infected with different MVX strains at spawning.

Strain	DsRNAs in original sample	DsRNAs in 1st flush mushrooms	DsRNAs in 2nd flush mushrooms	SYMPTOMS
No Symptoms:				
A15				None
A15-1	H2	H2	H2	None
2785	9			None
2643	H2 9	H2 7[1] 8 9	H2 (7 8)[2] 9	None
1911	3f[3] H2 8 9	H2 (7 8) 9	H2 (7 8) 9	None
Ambiguous symptoms:				
2735	H2 9 (4 bb)[4]	H2 (7 8) 9 15 (4 bb)	H2 7 (8) 9 15	Some browns in 1st flush
2637	H2	H2 (7) 8 15	H2 7 (8) 9 15	Yield loss?
2191	H2 9 15	H2 9	H2 9	Yield loss?
2648	3f H2 9	H2 7 (8) 9 15	H2 7 (8) 9 15	2nd flush delay?
2784	3f H2 8 9 15	H2 (7 8) 9 15	H2 7 (8) 9 15	Delay?
Clear symptoms				
1961	**H2 7 9 15**	H2 7 (8) 9 15	H2 7 (8) 9 15	**Delay + yield loss**
2687	**H2 7** 8 9 **15**	H2 (7 8) 9 (15)	H2 7 (8) 9 15	**Delay + yield loss**
1909	3f **H2 7 9 15**	3s H2 7 8 9 15	3s H2 7 (8) 9 15	**Delay + yield loss**
1283	3s **H2 7** 8 9 **15**	3s H2 7 (8) 9 15	3s H2 7 (8) 9 15	**Delay**

1 H2 7 = two bands H2 and 7 clearly seen as a doublet on an electrophoretic gel.
2 (7 8) = bands very feint
3 "f" or "s" indicates a "feint" or "strong" band on the gel
4 (4 bb) = 4 small dsRNA bands associated with brown mushroom symptom

small scale nature of the study. Interestingly though in many cases there was an increase in the number of dsRNAs detected compared with the original sample (strains 2637, 2648, 2784) with well developed H2, & 7 doublets being detected in 2nd flush mushrooms.

Band 3 was detected at a feint level in several of the original samples (1911, 2648, 2784, 1909, but only went on to be expressed in mushrooms in one of these treatments:- 1909. This sample originated from a site that had severe MVX problems and has since closed down whereas the other samples originated from sites that did not suffer as badly (to the best of our knowledge).

It was surprising to see additional dsRNAs develop in 1st and 2nd flush mushrooms when they were lacking in the original strain, especially the H2 & 7 doublet , and band 15 (treatments 2643, 1911, 2735, 2637, 2648, 2784) , particularly when they were present without overt symptoms. It would be interesting now to take cultures from these mushrooms containing the extra bands, or compost from the end of crop, to see if infection studies or rollover crops would eventually give rise to the crop delay symptoms associated with their presence in strains 1961, 2687, 1909, and 1283. Rollover crops with A15-1 alone has failed to increase the variety of dsRNAs in mushrooms, apart from increasing the intensity of the H2 band, which suggests the need for some other factor to be present.

5.3 Summary

Only four MVX-infected "strains" (strains 1961, 2687, 1909, and 1283) out of 12 tested, resulted in clear-cut crop delay symptoms when incorporated into freshly spawned compost at spawning. DsRNA analysis of the original samples from which the cultures of these four strains were obtained all showed a presence of the dsRNAs H2 & 7 (as a doublet) along with 9 and 15. Mushrooms from compost infected with some of the other "strains" (lacking bands 7, 9 and 15 in the original samples) surprisingly showed the H2 & 7 (as a doublet) along with 9 and 15 in the second flush, although no obvious symptoms occurred. It would be interesting to know if the live compost taken at the end of these non-symptomatic crops, and incorporated into a freshly spawned crop, would result in crop delay and pinning disruption symptoms.

5.4 Conclusions

- Complex dsRNA profiles in mushrooms may occur without any overt symptoms in the crop
- There is some evidence to suggest that the crop delay symptom is more likely to occur if a specific group of dsRNAs (H2, 7, 9 & 15) are present in infective material which enters the crop cycle at spawning time.

Figure 11. dsRNA profiles of 1st flush mushrooms from compost infected at spawning with different MVX strains (see Tables 1 & 2). M = marker lane. Arrows indicate location of some key bands

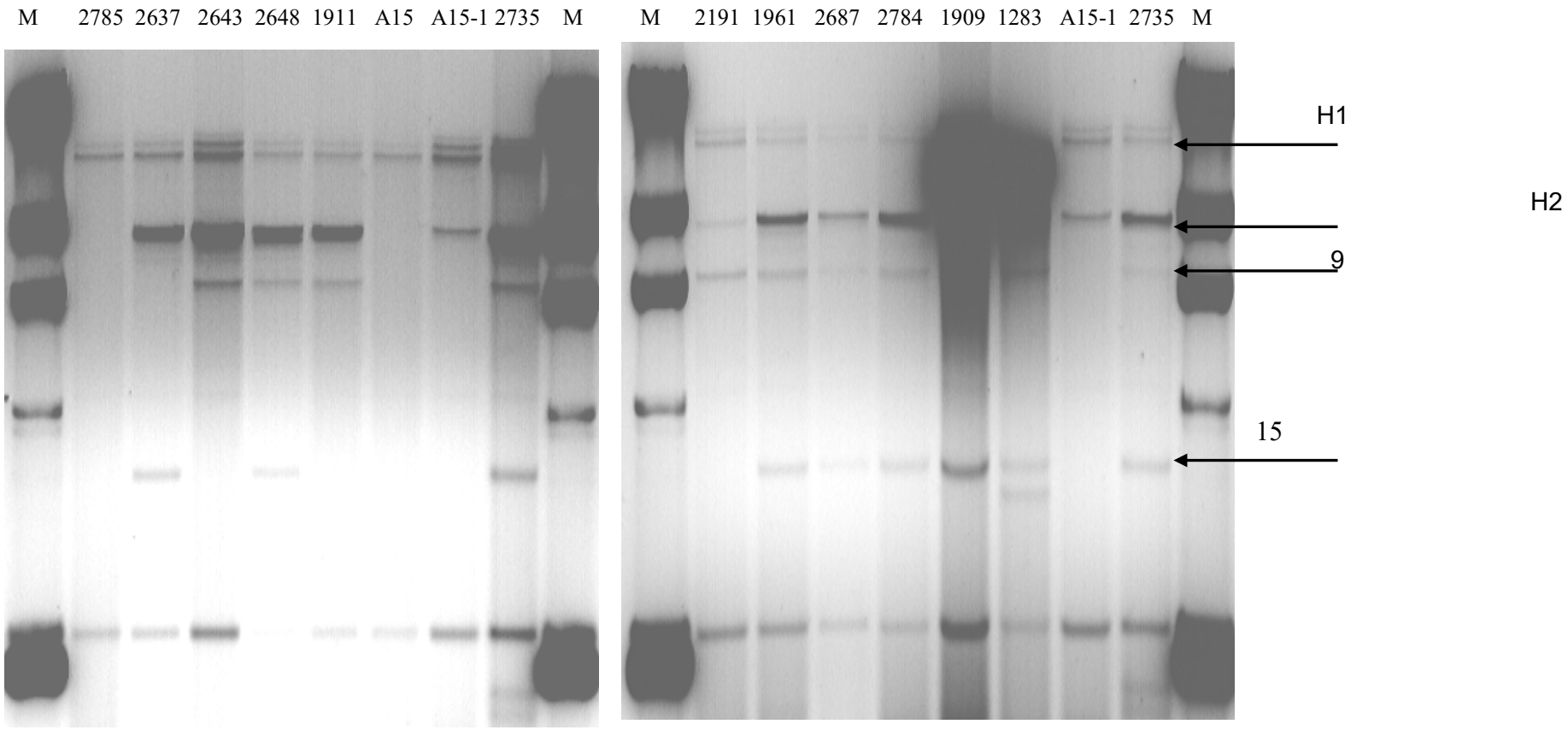
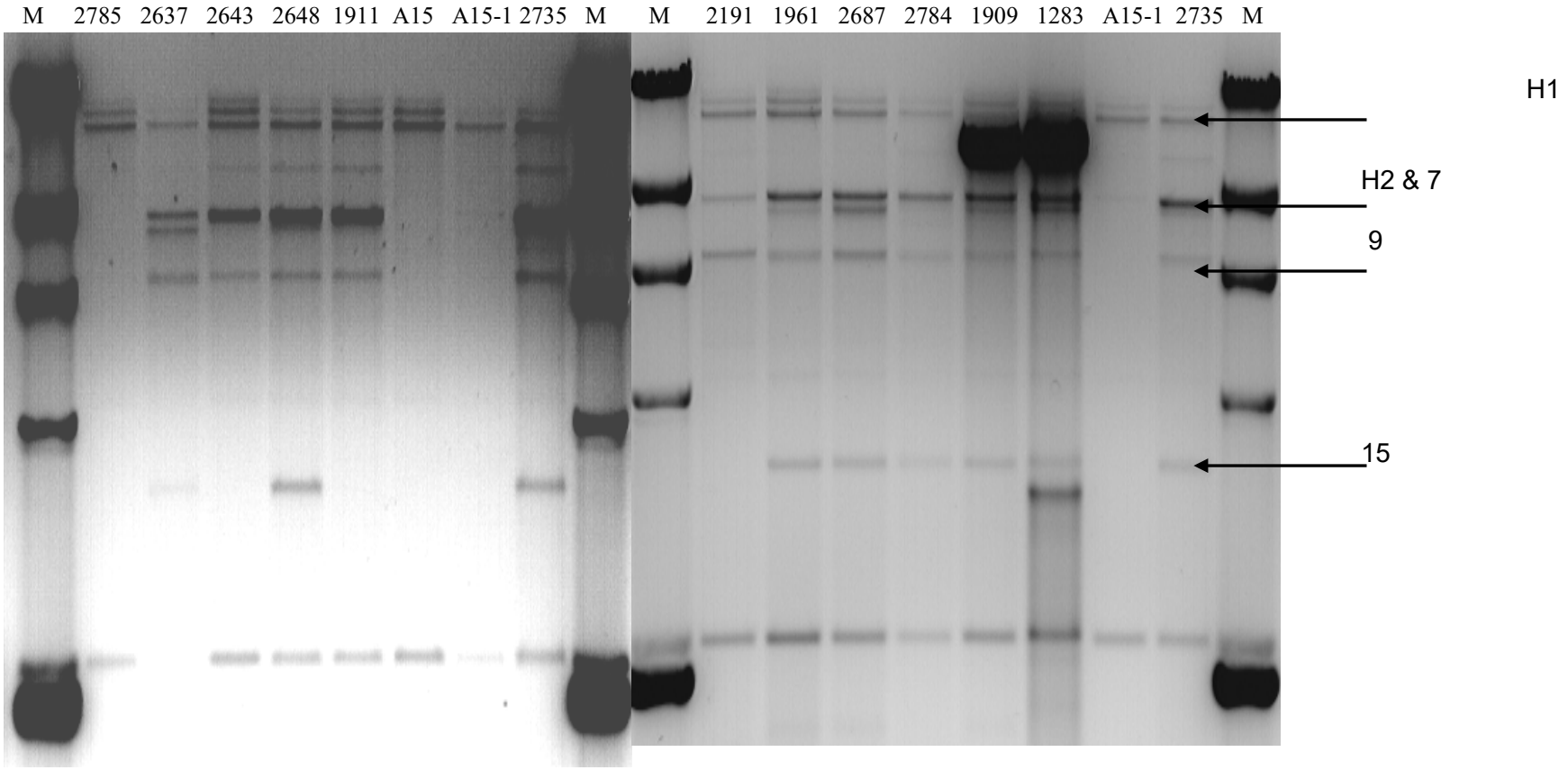


Figure 12. dsRNA profiles of 2nd flush mushrooms from compost infected at spawning with different MVX strains (see Tables 1 & 2) . M = marker lane. Note H2 & 7 doublet for many samples.



6 References

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