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Contents

GROWER SUMMARY

Headlines

- Mushroom Virus X is highly infective. Contamination of mushroom crops with very small amounts of Virus-X-infected *Agaricus* mycelium at any time in the crop cycle, from spawning to casing, will result in the transmission of virus to the crop.
- The development of "brown" or off-coloured mushrooms, one of the symptoms associated with Virus X, is most likely to occur when infection occurs during bulk handling or casing.
- Crop delay and pinning disruption can occur following infection of healthy compost or casing at any time in the crop cycle with either high or low amounts of infective material.

Background and Deliverables

Mushroom Virus X disease is less widespread now than it was in April 2000/2001, partly due to a number of site closures. There have been two or three notable success stories where the disease or presence of virus has been dramatically reduced, if not eradicated. However, in 2002 and 2003 there have also been a number of relapses where Virus X symptoms have re-occurred, correlated with the presence of the viral genetic material "double stranded ribonucleic acid" (dsRNA) being detected in mushrooms. In addition some sites have not yet succeeded in becoming clear of the problem despite major virus-control measures being implemented.

The relapses, and the difficulties of some sites in overcoming Virus X problems, illustrates how the new virus appears to be operating at a different level compared with the La France virus. Bulk handled phase III is intrinsically vulnerable to virus disease due to the production of mycelial fragments capable of forming quick, efficient and continuous virus-loops back to spawning operations. Growing open mushrooms also generates masses of *Agaricus* spores that can infect spawn-running compost. Thus, wherever high spore loads are generated, or wherever bulk operations are carried out, particularly with phase III, an "exclusion-hygiene" approach must be adopted. In the past 10-15 years "exclusion-hygiene" precautions have managed to keep La France virus at bay throughout much of the industry, but such measures have been less effective in protecting crops from Virus X, particularly on sites considered to be "virus vulnerable".

This project delivers information on the how Mushroom Virus X operates within crops to produce a variety of symptoms. We identify factors that affect Virus X symptom expression and establishment in a crop.

Summary of Results and Conclusions

MVX Strains. Nine "strains" or cultures of mushroom Virus X (MVX), with different genetic characteristics (ie different dsRNA banding patterns or profiles), and exhibiting diverse symptoms, were obtained from MVX infected mushrooms from around the country between 2000 and 2002. Mushroom spawn was made for each of the nine MVX strains, and this was used to grow nine

MVX strains of mushrooms to determine how similar or different they were to each other when grown under identical conditions. Three of the strains produced similar dsRNA profiles, characterised by a strong band 3, along with bands H2, 7, 8, & 9 as well as a band 15 (strains 1944, 1283-P, 1940). Three others also produced similar profiles characterised by a clear grouping of H2, 7, 8 & 9 as well as a band 15 (strains 1282, 1545 1961) but they lacked the band 3 of the first grouping. The remaining three strains differed from these two groupings and had different profiles to each other. Strain 2648 had a weak band 3 along with H2, 8 & 9, but no band 7 or 15; strain 2637 had H2, 8 & 9, but no band 3, 7 or 15, while strain 2735 had a weak band 3 along with H2, 8 & 9 but no band 7 or 15, but this strain also had the "brown symptom" bands 18,19, 22 and 23 (see Fig. 1). Crop delay was the only symptom recorded for a wide variety of strains when compost was infected at spawning with 100% MVXinfected material.

Fig. 1. Schematic presentation of dsRNA bands detected in nine MVX strains and A15 controls when grown under identical conditions.

Strain	A15 Control	1282	1545	1944	1961	1283-P	A15 Control	2648	1940	2735	2637
DsRNA Band											
1											
2							Xf	**Xf**		**Xf**	Xf
H-1							Xf	**Xf**		Xf	Xf
3				XS		XS		X	**XS**	**X**	
4											
5											
6											
H-2		X	X	X	X	X		XS	XS	XS	XS
7		**Xf**	**Xf**	**Xf**	Xf	**Xf**		**Xf**	**Xf**		
8		Xf	**Xf**	Xf	**Xf**	**Xf**		**Xf**	**Xf**	**Xf**	**Xf**
9		X	X	**X**	XX	X		X	X	X	**X**
10											
11											
12											
13											
14											**Xf**
15		X	X	X	X	X			**X**		
16											
H-3	X	Xf	X	X	X	X	X	X	XS	X	X
17											
18				Xf	**Xf**					X	
19				Xf	**Xf**					X	
20											
21									**X**		
22										X	
23										X	

XS = strong band; Xf = feint band; XX = two distinct bands at this position; **Bold** = not detected in original sample. H-1, H-2, H-3 = dsRNA bands frequently found in healthy, non-virus affected mushrooms. Bands 18, 19, 22 and 23 are always associated with the "brown mushroom" symptom.

- There are some characteristic groupings of dsRNA bands associated with MVX from diverse sources, with band 3, bands H2, 7, 8 & 9, and band 15 occurring regularly in different combinations.
- Crop delay was the only symptom recorded for a wide variety of strains when compost was infected at spawning with 100% MVX-infected material.

Symptom expression and time of infection. A second experiment, using four selected MVX strains:- 1283-P, 1961, 2735 and 2637, looked at the relationship between symptom expression and time of infection. Off-coloured/brown mushrooms, one of the symptoms reported in industry, was reproduced using strain 2735, which was obtained from a site experiencing this symptom and

which contained the characteristic dsRNA bands associated with browning (bands 18, 19, 21 & 22). However, the expression of the symptom varied depending on the time of infection and quantity of infective material added.

Infection at the end of spawn running

Brown/off-coloured mushrooms occurred consistently, but irregularly, in first and second flushes, when healthy, fully spawn-run compost was infected at a low level (0.01%) during bulk-handling of the spawn-run compost (see Fig. 2).

Infection at casing

Brown/off-coloured mushrooms also occurred consistently when only the casing was infected (Fig. 2).

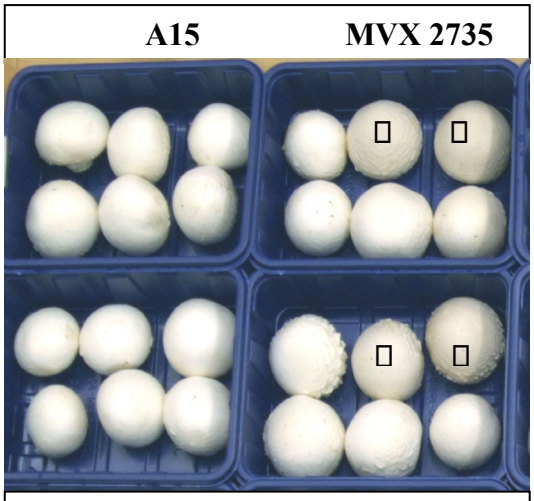


Fig. 2. Brown (*) scaly mushrooms from a crop infected with MVX strain 2735 during bulk-handling (top) or at casing (bottom).

Infection at spawning

When infection occurred at a low level at spawning (0.01%), some brown mushrooms occurred but only in one out of three replicates, and then predominantly in the second flush. Higher levels of infection at spawning (25%-100%) did not produce any brown mushrooms (see table below).

Infection Treatment	A15-1 Control	MVX Strain 2735
100% infected spawn[1]	None	Crop delay
25% infected spawn	None	None
0.01% infected compost[1] at spawning	None	Some brown mushrooms (in 1 out of 3 replicates)
0.01% infected compost at end spawn run	None	Brown mushrooms (all replicates)
1.2g infected compost added to casing at casing	None	Brown mushrooms (all replicates)

1 The term "infected" spawn or compost means spawn or compost that is made using MVX-infected *Agaricus* mycelium (i.e. an MVX "strain"), which contains Virus X dsRNAs.

With two other MVX strains 1283-P and 1961, infection at any time in the crop cycle from spawning to casing, with either high or low levels of infection, caused a 1-2 day delay in the first flush and a 3-4 day delay in the second flush. Both strains resulted in a heavy pin-set, producing masses of small mushrooms compared to the more even pinning in the control. No premature-opening mushrooms were observed, although this had been reported by the farm from which strain 1961 was obtained. These two strains (1283-P and 1961) behaved very similarly in cropping experiments although strain 1961 lacked the strong band 3 present in 1283-P (see Fig. 1). A fourth strain MVX 2637, which had several dsRNA bands but did not have dsRNA bands 3, 7 or 15, showed no symptoms compared to the control.

- The brown mushroom symptom occurs predominantly when there are low levels of infection at bulk handling and casing, and when the characteristic dsRNA bands 18, 19, 21 & 22 are present in the infective mycelium.

- Crop delay and pinning disruption can occur following infection of healthy compost or casing at any time in the crop cycle with either high or low amounts of infective material. This appears to be associated with dsRNA bands H2, 7, 8 & 9 and band 15, with or without a strong band 3.

Repeated continuous contamination (Rollover crops). When live *Agaricus* material was deliberately carried over, again and again, from a previous crop into a new crop, then the titre of the non-symptomatic dsRNAs (H1, H2 and H3), in the mushrooms gradually increased, and a few new dsRNAs also started to occur at feint levels (Fig. 3). After eight consecutive rollover crops, mushrooms contained high titres of H1 and H2, as well as low titres of dsRNAs 2, 12, 13, & 14 although crop timing and yields appeared to be normal. The significance of these "feint" dsRNAs is unclear at the moment. Further rollover crops are planned to see if the dsRNA profiles change further.

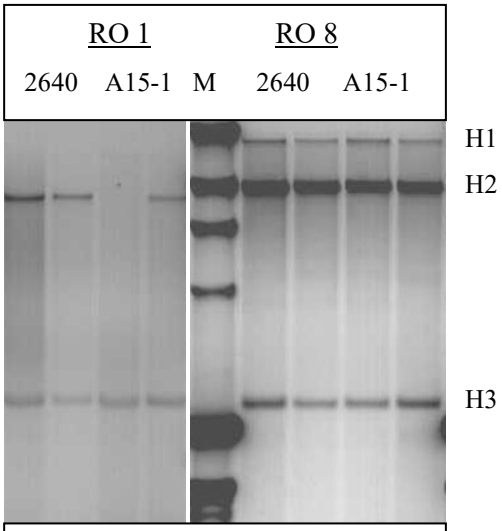


Fig. 3. MVX gel test on mushrooms from Rollover crop 1 (RO1) and rollover crop 8 (RO8).

- Repeated continuous contamination of healthy compost with spawn-run compost or live *Agaricus* material from a previous crops results in an increase in the titre of non-symptomatic dsRNAs H1, H2 and H3.

Dust and debris transmit virus. We know from the above, and previous, experiments that infected *Agaricus* material applied to a crop at casing can result in MVX symptoms and / or dsRNAs being expressed in mushrooms. When mushroom farm dust and debris - collected from a farm with severe MVX problems - was applied to a healthy crop at casing, it resulted in the production of mushrooms with up to ten additional MVX dsRNAs, compared to the controls (Fig. 4). Live *Agaricus* material is likely to be present in the dust fraction on mushroom farms as spores and mycelial fragments on pieces of compost. However we have been unable to isolate any live *Agaricus* cultures from such material due to high background levels of bacteria and mould contamination.

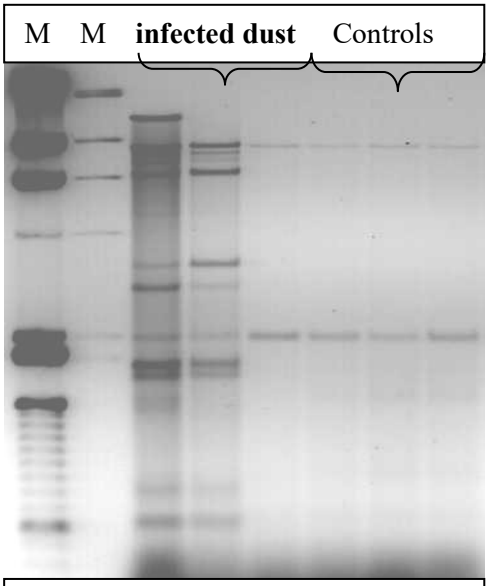


Fig. 4. MVX dsRNA gel for mushrooms harvested from trays treated, at casing, with MVX-infected mushroom-farm dust and debris (M = markers).

- Dust and debris on mushroom farms that infects casing, can transmit Mushroom Virus X.

Conclusion

These experiments highlight the infective nature of MVX. A variety of symptoms (such as crop delay, brown mushrooms) can be detected by the first flush following a single infection event that can occur any time from spawning to as late as casing. This goes some

way towards explaining some observations on commercial farms at the height of the Virus X outbreaks where bought-in spawn-run blocks from a virus-free source could become severely infected with MVX when grown on a farm with an existing Virus X problem.

Commercial Benefits of the Project

This project highlights the very infective nature of MVX infected material and the need to ensure that there is no opportunity for infected material to contaminate a healthy crop at any stage in the crop cycle. Thus growers can focus on identifying all loopholes in their exclusion hygiene measures in order to become MVX-free. The information in this report should help growers to identify weakness in their system where MVX infection could take place. Having identified weakness, growers can focus their attention and financial spend on improving hygiene measures in the most effective manner.

Action Points for growers

- Assess the potential of your growing practices to produce live *Agaricus* material that can enter the dust fraction of your farm (ie mycelial and compost fragments from uncontained bulk-handled spawn-run compost at filling, spores from open mushrooms, spore and compost debris in central corridors adjacent to cropping rooms, compost debris from non cooked-out crops, poor or no exhaust-air filtration, poor room seals). Rectify any loop-holes.
- Assess the cleanliness of machinery used immediately prior to spawning, casing and filling. Do not allow casing machinery to become contaminated with farm dust and debris before use. Use disinfectants that are known to kill *Agaricus* spores and mycelium.
- Assess the storage conditions of casing materials. Do not allow to become contaminated with any farm dust. Disinfect outer coverings prior to opening if possible.
- Filter exhaust air from growing rooms if possible, especially if growing flats, using the highest grade filter your system allows.
- Disinfect central corridors daily to prevent the build up of live *Agaricus* spores and debris that can enter the dust fraction on the farm, particularly if experiencing an MVX problem. Use disinfectants that are known to kill *Agaricus* spores and mycelium.
- Ensure door and vent seals on spawn-running rooms and tunnels are effective, so that no dust-laden unfiltered air enters rooms during spawn-running.
- **Never** use the same machinery to handle both phase II compost at spawning **and** fully spawn-run compost. The highly infective nature of MVX means that it only takes a very small fragment of live *Agaricus* material to contaminate a healthy crop.

SCIENCE SECTION

Epidemiology of Virus X. Year 2.

1 Introduction

This project began in April 1999 to investigate an expanding problem, which at that time was of unknown cause. A complex of dsRNA molecules was detected in mushrooms from affected sites but not in mushrooms from unaffected sites. This indicated that the problem was almost certainly viral in origin and the condition was termed Virus X disease. It is currently referred to as "Mushroom Virus X" disease or "MVX". Early work at HRI indicated very strongly that MVX can be transmitted both by mushroom spores and mushroom mycelium (Gaze *et al*, 2000; Grogan *et al*, 2003). HDC project M39a (Grogan, 2002a) established that infected mycelial fragments and "infected"[8] compost could readily transmit Virus X dsRNAs into healthy *Agaricus* mycelium, resulting in Virus X infected mushrooms, irrespective of whether the contamination occurred at spawning, during bulk handling of spawn-run compost or at casing. Only one strain of MVX (strain 1283) was used in these initial experiments and the main symptom associated with that strain in those experiments was a time delay in the harvest of both the first and second flushes of up to four days (Grogan, 2002a).

The range of symptoms recorded within the industry, such as premature opening and the occurrence of off-coloured or "brown" mushrooms, was not encountered during these initial experiments. Additional experiments were considered necessary to try to reproduce at least some of these symptoms encountered by mushroom growers. This report (M39b) summarises the results of those experiments, which continue the epidemiology work begun in HDC project M 39a.

This project has three objectives:

[8] The term "infected" compost means compost that contains MVX infected *Agaricus* mycelium (i.e. an MVX "strain") that contains Virus X dsRNAs. "Healthy" compost is compost that is spawned, or spawn-run with *Agaricus* mycelium that free of Virus X dsRNAs.

1. To determine if symptom expression (premature opening, pin suppression, crop delay and brown mushrooms) occurs as a result of dsRNA characteristics or the timing and quantity of infected material that is applied to a crop.
2. To determine if dsRNA bands increase in number and intensity if *Agaricus* mycelium from an earlier crop is continually reintroduced (carried-over) into fresh compost.
3. To detect the presence of *Agaricus* propagules (spores and mycelium) in mushroom farm "dust, debris and air" and to determine their viability and potential to transmit Virus X.

The results for each objective are presented and discussed in individual chapters.

2 General Materials and Methods

2.1 Mushroom crops

All mushroom crops were grown on the HRI Mushroom Unit using a standard wheat straw and chicken manure compost and following standard procedures within the British industry. Phase I compost is produced in non-aerated stacks, which is turned regularly. After about 14 - 19 days on the yard, it is filled into bulk pasteurisation tunnels and subjected to a standard pasteurisation and conditioning regime. The phase II compost is then spawned with a standard commercially available spawn and filled into either wooden trays, holding 50kg, or plastic bags containing up to 20kg. Spawn-running is done in dedicated spawn-running rooms for a period of 17 days, at which time the compost is cased with a commercially bought casing product. Casing inoculum may or may not be added and is specified in each experiment. Cased trays and bags are then moved into temperature controlled cropping rooms and cropped for up to three flushes. Mushrooms are harvested as closed cups to prevent the build-up of MVX-infected spores.

2.2 Virus X cultures

A large collection of Virus X-infected *Agaricus* cultures have been produced at HRI, which were derived from Virus X-infected mushrooms, compost or casing. Corresponding mushroom samples were analysed for the presence of Virus X associated dsRNAs so that the Virus X profile of each culture at the time of sampling is known. During the course of these experiments a selection of "strains" was used to give a broad range of different dsRNA profiles. Details of the cultures are given in Table 1.

2.3 Virus X inoculum preparation

Virus X inoculum was prepared in two ways as follows:

2.3.1 Virus X-infected spawn.

Six plugs of freshly grown Virus X-infected *Agaricus* cultures were used to inoculate jars containing about 150g of sterilised rye grain. Jars were incubated at 25°C for two to four

Table 1. Details of MVX "strains" (*Agaricus* cultures containing MVX dsRNAs) used in experiments and dsRNA bands present in mushrooms from which cultures were taken

***Agaricus* Cultures: MVX-Strain**	**Year isolated**	**Symptoms reported**	**DsRNA Bands present**	
			"Hybrid" bands[8]	**MVX dsRNA bands**
1282	2000	Heavy pinning, delay, clumps, premature opening	H1,H2, H3	5, 8, 9, 12, 13, 15, 19
1283-P	2000	40-80% yield reductions. Patchy crop with large bare areas and crop delay	,H2,H3	3(S)[8], 9, 15,
1545	2000	History of patchy crops, browns, yield reductions, premature opening	H1,H2, H3	9, 15(S),
1940	2001	None, good yields.	H1,H2, H3	2, 6, 9,
1944	2001	Patchy crop, browns, premature opening	H1,H2, H3	2, 3, 8, 15, **[18(S), 19(S), 22(S), 23(S)][8]**
1961	2001	Premature opening	H1,H2, H3	2, 7, 9, 15(S)
2637	2001	None, but previous history of browns	H1,H2, H3	2,
2640	2001	None, but previous history of symptoms	H1,H2, H3	
2648	2001	None, but previous history of browns	H1,H2, H3	3(F)[2], 9, 13
2735	2002	Browns	H1,H2, H3	5, 9, **[18, 19, 22, 23]**
A15-1	2002	None, derived from an A15 mushroom crop with no virus history or dsRNAs	,H3	

[8] These bands also occur in non-symptomatic, otherwise healthy crops

[8] (S) indicates a strong titre for this band; (F) indicates a feint band

[8] Bands 18, 19, 22 and 23 are always associated with the brown mushroom symptom

weeks and shaken weekly to ensure good growth of the Virus X-infected *Agaricus* on the rye grain. When the rye was well colonised the jars of Virus X-infected *Agaricus* were stored in the fridge at about 4°C until needed.

2.3.2 Virus X "infected compost" [1].

Glass screw-capped jars containing about 50g of pre-chopped Phase II compost were sterilised by autoclaving at 120°C for 1 hour on two consecutive days. The compost was then spawned under clean room conditions with Virus X-infected spawn (see 2.3.1). The spawned compost was then incubated at 25°C until fully spawn-run. When needed the spawn-run compost was gently broken up and weighed out into the required quantities under clean room conditions.

2.4 Determination of Virus X dsRNA profiles.

All mushroom samples (150 g fresh weight) for Virus X testing were done via the HRI Mushroom Diagnostic Clinic. Mushroom samples were frozen immediately, and then freeze dried prior to analysis. Total nucleic acids were extracted and purified from 10 g samples of freeze-dried mushrooms. The purified extract was subjected to agarose gel electrophoresis and the resultant gels, depicting dsRNA bands, were photographed and scored for the presence of novel Virus X-related dsRNAs.

[1] The term "infected compost" means compost that contains *Agaricus* mycelium infected with Virus X dsRNAs. "Healthy compost" is compost that is spawned, or spawn-run with *Agaricus* mycelium that is free of Virus X dsRNAs.

3 Symptom Expression Experiments

Two symptom expression experiments were carried out. The first experiment looked at crops of mushrooms grown from a large number of MVX-infected cultures (see Table 1), at a high rate of infection (100%) to see if the symptoms associated with each strain could be reproduced. The second experiment explored the relationship between time of infection, dsRNA profiles and symptom expression for a smaller selection of strains.

3.1 Experiment 1 - Materials and methods

Heavy gauge polythene bags were filled with 12 kg HRI **phase II** compost (batch 05/02) and spawned by hand with spawn made with MVX-infected *Agaricus* cultures (60g-spawn/12kg bag of compost). Two control treatments were included and consisted of:- (a) Commercial Sylvan A15 spawn and (b) home-made spawn A15-1, made from a culture derived from a standard A15 mushroom from a crop with no virus history. Five MVX-strains with different dsRNA profiles and reported symptoms were chosen from Table 1 and are listed below in Table 2. Three replicates were prepared for each treatment. An additional four MVX-strains were included (Table 2) but with just one replicate prepared for each strain.

Table 2. Strains used in Experiment 1 (more details on strains can be found in Table 1).

Experiment 1a Replicated treatments		Experiment 1b Non-replicated treatments	
Spawn strain	Replicates	Spawn strain	Replicates
A15	3	1940	1
A15-1	3	2637	1
1282	3	2648	1
1283-P	3	2735	1
1545	3		
1944	3		
1961	3		

For the replicated component of the experiment, the bags of spawned compost were laid out in a randomised block design consisting of three blocks of 7 bags with one replicate of each

treatment in each block. The non-replicated treatments were placed to one side in the same cropping chamber. All bags were spawn-run for 17 days at 25°C and 95%relative humidity, after which time they were cased with TunnelTech English casing mix, with NO casing inoculum (C.I.) added. Two flushes were harvested and crops were inspected for symptoms. Mushroom samples were taken from each treatment for dsRNA analysis. (Experiment 1 laboratory reference = 6.01).

3.2 Experiment 1 - Results

3.2.1 Crop delay

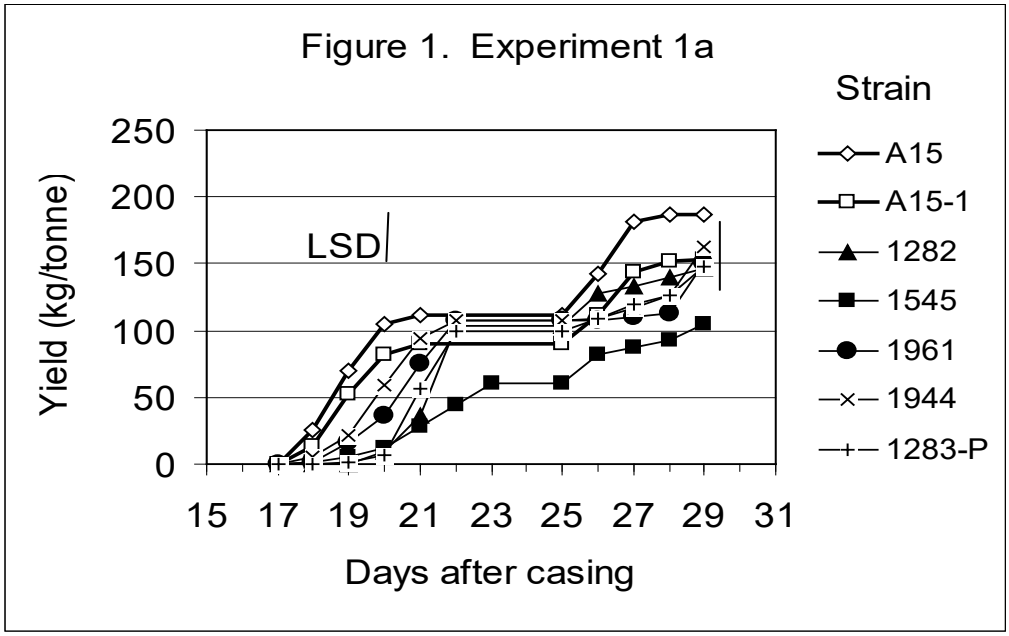
All MVX strains in experiment 1a, and 3 out of 4 MVX strains in Experiment 1b showed a delay in the timing of both first and second flush. A delay of from 1 to 3 days to reach 50% of the first flush yield was recorded for all MVX strains compared to either of the two controls A15 and A15-1 (Table 3). Only strain 2637 showed no delay in crop timing. This was the strain with the least number of extra dsRNA bands (Table 1). The timing of A15-1 strain, using home-made spawn, was not significantly different to the commercial A15 strain (although yields were lower).

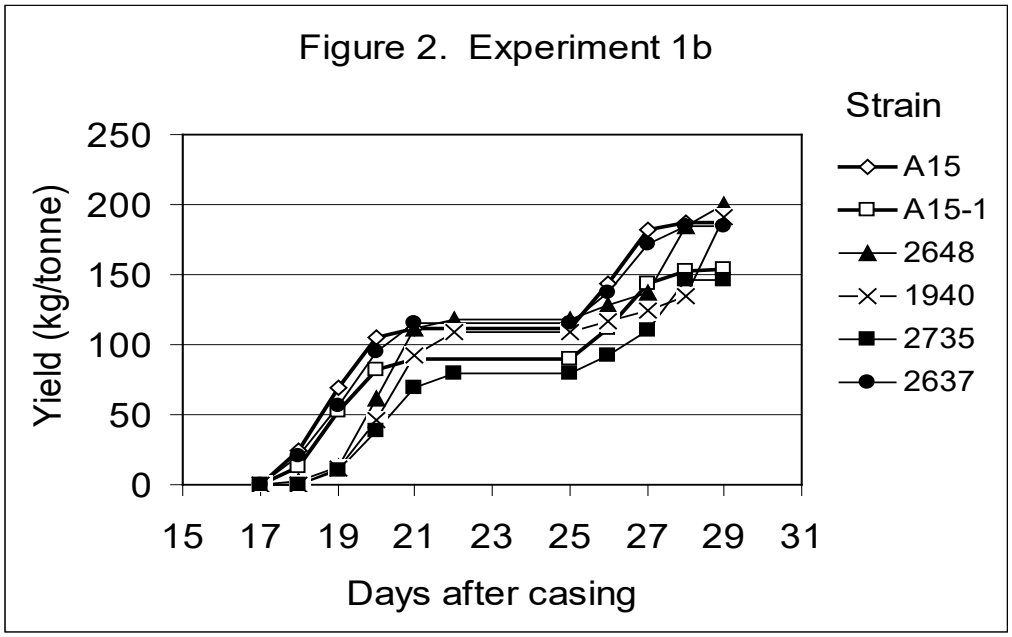
Table 3. Number of days after casing to harvest 50% of first flush. LSD = Least Significant Difference at $P = 0.05\%$.

Experiment 1a		Experiment 1b (Non-replicated treatments)	
Spawn strain	No. of days to 50% of 1st flush yield	Spawn strain	No. of days to 50% of 1st flush yield (approx.)
A15	**18.7**	**A15**	**18.7**
A15-1	**18.8**	**A15-1**	**18.8**
1282	21.2	1940	20.3
1283-P	20.9	2637	19.0
1545	21.4	2648	20.0
1944	19.8	2735	20.0
1961	20.4		
LSD (0.05%)	0.70	LSD	(not appropriate)

3.2.2 Yield effects

The yield from laboratory-made A15-1 spawn was well below that of the commercial Sylvan A15 spawn. This is likely to reflect the smaller laboratory-based process used, coupled with the fact that the *Agaricus* culture used to make the spawn has originated from a mushroom rather than a bone fide mother culture. In Experiment 1a the total yields over two flushes for all the MVX strains, using laboratory-made spawn, were not significantly different to the A15-1 control, although strain 1545 yielded well below A15-1 (Figure 1). In the non-replicated experiment 1b, strains 1940, 2648 and 2637 yielded better than strain A15-1 (Figure 2).





3.2.3 Other symptoms

There were no obvious symptoms expressed by any strain other than crop delay. No premature opening mushrooms occurred or no mushrooms displayed off-colour or browning symptoms.

3.2.4 dsRNA profiles

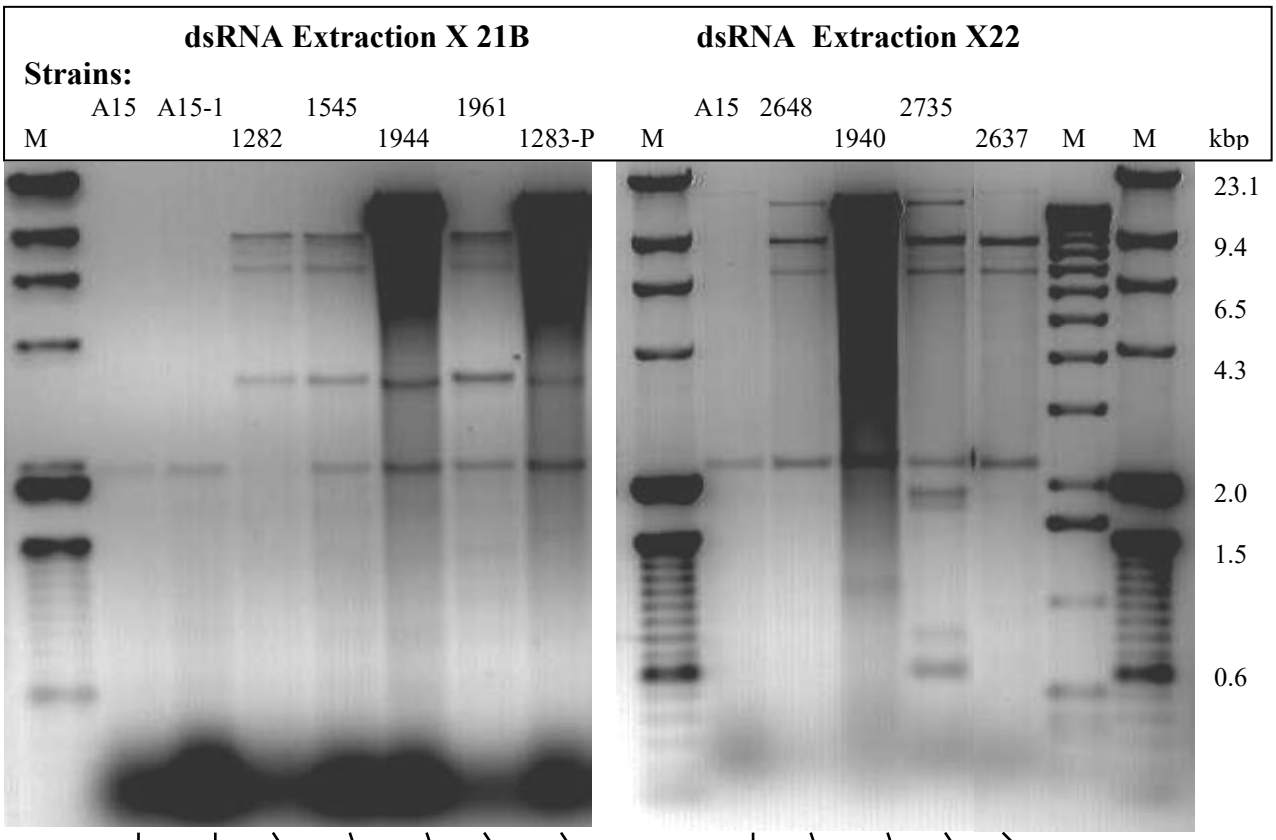
The A15 and A15-1 controls remained predominantly free of extra dsRNAs, containing bands H1 and H3, depending on the extraction. A band 2 was found in an A15 sample, but this band is occasionally found in non-symptomatic samples from sites with no history of Virus disease.

The dsRNA profiles of mushrooms from the experiment were somewhat different to those of the original sample of mushrooms from which the culture was taken (compare Figure 3 with table 1). This was particularly the case for bands that are usually at a low titre and feint such as bands 2, 4, 5, 6, 7, 8, 12, 13, and 19. However some bands which were strong in the original sample were not strong in the experiment (e.g. bands 18, 19, 22 & 23 in strain 1944). In addition, some samples from the experiment had several additional bands to the original sample (e.g. strain 1940 and 2637). It is particularly interesting to see that strain 2637, which only had a feint band 2 (plus H1, H2, H3) when originally sampled, produced mushrooms with additional bands 8, 9 and 14 when that culture was used to make spawn. The detection of band 3 in strains 1940 and 2735 is also interesting as this band was not detected in the original samples (Table 1), and is one of the bands which can occur at a high titre on MVX-positive samples (e.g. 1944, 1283-P).

3.3 Experiment 1 - Discussion

The most interesting observation from this experiment is how similar the dsRNA profiles of some MVX strains were when they were all grown under identical conditions. For example, strains 1282, 1545 and 1961all had a distinct grouping of H2, 7, 8 & 9 along with a band 15 while strains 1944, 1283-P and 1940 had a very strong band 3, the H2, 7, 8 and 9 grouping and a band 15. Strains 2648 and 2735 were similar in that they had a modest band 3, the H1, (7), 8, and 9 grouping but no band 15. However, those strains having similar dsRNA profiles did not crop in a similar manner, e.g. compare crop timing

Figure 3. DsRNA profiles of mushrooms harvested from crops grown with spawn made from *Agaricus* cultures obtained from various sources. M = marker lanes, marker band sizes in kilo base pairs (k.b.p.). Bottom schematic represents the dsRNA bands scored from the gels above, as some bands may be very feint.



Band	A15	A15-1	1282	1545	1944	1961	1283-P
1							
2					+	+	
H-1							
3					XS		XS
4							
5			+				
6							
H-2			X	X	X	X	X
7			**Xf**	**Xf**	**Xf**	Xf	**Xf**
8			Xf	**Xf**	Xf	**Xf**	**Xf**
9			X	X	**X**	XX	X
10							
11							
12			+				
13			+				
14							
15			X	X	X	X	X
16							
H-3	X	X	Xf	X	X	X	X
17							
18					Xf	**Xf**	
19			+		Xf	**Xf**	
20							
21							
22					+		
23					+		

Band	A15	2648	1940	2735	2637
1					
2	Xf	**Xf**	+	**Xf**	Xf
H-1	Xf	**Xf**		Xf	Xf
3		X	**XS**	**X**	
4					
5				+	
6			+		
H-2		XS	XS	XS	XS
7		**Xf**	**Xf**		
8		**Xf**	**Xf**	**Xf**	**Xf**
9		X	X	X	**X**
10					
11					
12					
13		+			
14					**Xf**
15			**X**		
16					
H-3	X	X	XS	X	X
17					
18				X	
19				X	
20					
21			**X**		
22				X	
23				X	

XS = strong band; Xf = feint band; XX = two distinct bands at this position, XX two distinct bands at this position; **Bold** = not detected in original sample, + = detected in original but not in mushrooms from the experiment.

and yield data for MVX 1944, and 1283-P, or 1282, 1545 and 1961 (Figures 1 & 3). The remaining strain 2637, which was the only one to crop normally, had no band 3, an H2, 8 and 9, and no band 15.

Another interesting observation is that some dsRNAs were "lost". For example, the intense "brown-bands" 18, 19, 22 & 23, which were in the original mushrooms from which strain 1944 was obtained (Table 1 & Figure 3), were very feint in the mushrooms harvested from the experiment. Similarly, some dsRNAs were "gained", i.e. band 3 and 15 in MVX 1940, and band 8, 9 and 14 in MVX 2637. Such differences in dsRNA profiles between the original mushroom samples and subsequent mushroom samples (derived from experiments using cultures obtained from that original sample) are likely to be the result of several factors. The culture from which the MVX inoculum is made for a given stain is usually derived from a single mushroom and there may be different dsRNAs in individual mushrooms, or the titre of a given dsRNA may be lower than the limit of detection. Similarly, the dsRNA extraction procedure can be inefficient so that dsRNAs present at a low titre may get washed away or "lost" in the extraction and detection process. This may explain why quite a few dsRNAs were detected at a feint level in the experiment but not in the original mushroom samples which provided the culture. It does not explain however how the very strong "brown bands" which were present in the original sample of 1944, were only faintly detected in the experiment, as one would have expected them to have had ample opportunity to proliferate in the mycelium during spawn-running and cropping.

The only symptoms observed during this experiment was that of crop delay and, to some extent, yield reduction. No prematurely-opening mushrooms or off-coloured/brown mushrooms were detected, despite the fact that some of the cultures used originated from crops expressing such symptoms. The expression of the various symptoms that have been associated with MVX must therefore be influenced by other factors. The conditions for infecting crops on commercial units will be different to the controlled infections reported here. On mushroom farms there is a high possibility that any infection will occur unevenly, giving rise to a possible gradation of dsRNA titre, which may affect symptom expression. There is also the possibility of multiple infection events occurring throughout the life of the crop. Alternatively, the time at which a crop is infected may be significant or the quantity of virus-infected material that gets into a healthy crop may be important. Some of these factors will be explored in the following experiment.

3.4 Experiment 2 - Materials and Methods

Four MVX strains were selected for further study (Table 4), using lower infection rates than in Experiment 1 above, to explore the relationship between time of infection, quantity of infective material, dsRNA profile and the symptom expression. A15-1 was included as a control as all compost used in Experiment 2 was initially spawned with commercial strain Sylvan A15. Four different infection treatments were included for each strain used and these are summarised in Table 5.

Heavy gauge polythene bags were filled with 12 kg HRI **phase II** compost (batch 06/03), which had been spawned with the commercial strain Sylvan A15 at 0.5%. Three replicate bags were prepared for each MVX strain and for each of the infection treatments, but four replicates were prepared for the A15-1 controls. Those bags that were intended to be infected at spawning were infected at this time. All bags were then positioned in two cropping houses in a "supplemented balanced incomplete block design" for a control strain and four test strains in four blocks each of four units. There are four blocks, each of which includes the control strain (A15-1) plus three of the four test strains, i.e. each of the four test strains is omitted from just one of the blocks. Within this structure, the application times/rates are applied as in a split-plot design to four bags within each strain.

All bags were spawn-run for 17 days at 25°C and 95% relative humidity. At this time the spawn-run compost was physically broken up and shaken within each bag, to simulate the bulk-handling that occurs with bulk phase III compost. Those bags intended to be infected at the end of spawn-run were infected at this time. All bags were then cased with 8 litres of TunnelTech English casing mix, with casing inoculum (C.I.) added. Those bags intended to be infected at casing were infected at this time. The crop was watered and aired following standard procedures at HRI and two flushes were harvested. First and second flush mushroom samples were taken from each treatment for dsRNA analysis. Six mushrooms were selected from each treatment and assessed for quality. They were then cold-stored at 4°C for 7 days and assessed again. (Experiment 2 laboratory reference = 6.03).

Table 4. Details of the MVX strains, and the A15-1 strain used in Experiment 2.

***Agaricus* Cultures: MVX-Strain**	**Symptoms reported**	**DsRNA Bands present**	
		"Hybrid" bands[8]	**MVX dsRNA bands**
1283-P	40-80% yield reductions large bare areas crop delay	H2, H3	3(S)[8], 9, 15,
1961	Premature opening	H1, H2, H3	2, 7, 9, 15(S)
2637	None, but previous history of browns	H1, H2, H3	2
2735	Browns	H1, H2, H3	5, 9, **[18, 19, 22, 23][8]**
A15-1	None	H3	

Table 5. Infection treatments used in Experiment 2.

Infection treatment	**and timing**	**Details**
25% spawn	At spawning	25% of the commercial spawn required to spawn the compost was replaced with home-made spawn, made using cultures of the strains being tested (see section 2.3.1)
0.01 % infected compost	At spawning	1.2 g of infected compost, prepared as described in section 2.3.2, was added to 12 kg bags of spawned compost just after spawning.
0.01 % infected compost	At end of spawn-run	1.2 g of infected compost, prepared as described in section 2.3.2, was added to 12 kg bags of fully spawn-run compost at the end of spawn-run. The compost was broken up and shaken within the bag to simulate the bulk handling process.
1.2 g infected compost	At casing	1.2 g of infected compost, prepared as described in section 2.3.2, was added to the 8 litres of casing required for each 12 kg bag of fully spawn-run compost.

[8] These bands also occur in non-symptomatic, otherwise healthy crops

[8] (S) indicates a strong titre for this band

[8] Bands 18, 19, 22 and 23 are always associated with the brown mushroom symptom

3.5 Experiment 2 - Results

There were significant differences between the four MVX strains used and the A15-1 control with respect to crop timing and yield, with MVX strains showing more variability than the controls (Figure 4). There were also significant differences in the responses of individual MVX strains to the time at which crop-infection with MVX material took place.

3.5.1 Crop timing

All the A15-1 controls, which had been "infected" with healthy *Agaricus* material instead of MVX material, cropped very uniformly, with no differences in crop timing or yield occurring as a result of the addition of the extra *Agaricus* material (Figure 4, Tables 6 & 7). However, infection of healthy compost with small amounts of MVX strains 1283-P and 1961 at spawning, or at the end of spawn-run, or infection of casing, caused a 1-2 day delay in the onset of the first flush, and a 2-4 delay in the second flush (Figure 4, Tables 6 & 7). No significant delay in crop timing was observed when MVX strains 2637 or 2735 were used. This is noteworthy given that crop delay has not been a problem for several growers, particularly when the main problem is off-coloured or brown mushrooms.

3.5.2 Crop yield

There were no significant differences in the yields from any of the four control treatments (Figure 4) with the average yield at the end of two flushes being 236 kg/tonne. Yields from infection treatments for MVX 1283-P and 1961 were generally well below the control at the natural end of the second flush (day 27) and some were still significantly lower than the controls by day 31 when the crop was terminated. Yields from some of the infection treatments for MVX 2637 or MVX 2735 were not significantly different to the controls statistically, but there was a trend towards slightly lower yields compared with the control.

Figure 4. Cumulative yield of mushrooms over two flushes from A15-spawned compost infected with four MVX strains at different times during the crop cycle. Controls were "infected" with *Agaricus* inoculum derived from a non-virus infected source (A15-1). LSD = least significant difference at P = 0.05% for day 21 (end of first flush in the control), day 28 (end of second flush in the control) and day 31 (total yield at end of experiment). Average results for the control treatment (A15-1) included with results for each MVX strain for comparison

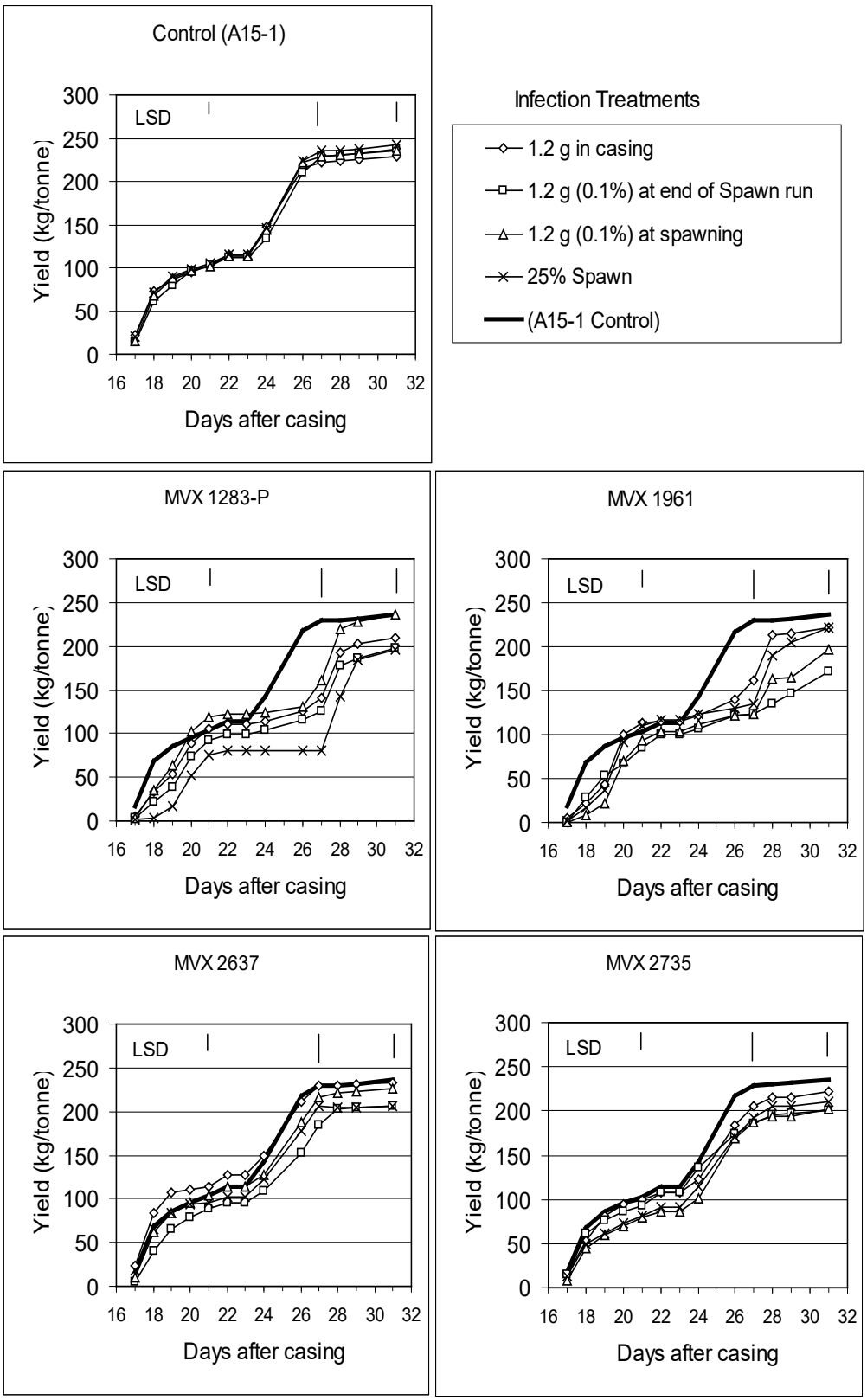


Table 6. Number of days after casing to harvest 25% of total yield. LSD = Least Significant Difference at *P* = 0.05%; d.f. = degrees of freedom.

Infection treatment	**Strains**						**L.S.D. [1] (13 d.f.)**
	A15-1 Control	**MVX 1283-P**	**MVX 1961**	**MVX 2637**	**MVX 2735**		
25% spawn	17.9	20.0	19.4	18.0	18.5		0.98 (13 d.f.)
0.01% at spawning	17.9	18.8	19.7	18.1	18.6		0.98 (13 d.f.)
0.01% at end of spawn run	18.2	19.1	18.5	18.6	17.9		0.98 (13 d.f.)
1.2g/bag at casing	17.8	18.8	19.1	17.5	18.2		0.98 (13 d.f.)
L.S.D.[2] (27 d.f.)	0.64	0.74	0.74	0.74	0.74		
Average over all treatments	17.9	19.2	19.2	18.0	18.3		0.82 (5 d.f.)

1 LSD's for comparison with the Control
2 LSD's for comparisons within individual strains

Table 7. Number of days after casing to harvest 75% of total yield. LSD = Least Significant Difference at *P* = 0.05%; d.f. = degrees of freedom.

	Strains					
Infection treatment	A15-1 Control	MVX 1283-P	MVX 1961	MVX 2637	MVX 2735	L.S.D.[1]
25% spawn	24.9	28.4	27.5	25.1	25.5	0.96 (21 df)
0.01% at spawning	24.8	27.2	28.3	25.5	25.5	0.96 (21 df)
0.01% at end of spawn run	25.2	27.4	27.2	26.1	24.7	0.96 (21 df)
1.2g/bag at casing	24.7	27.1	26.8	24.8	25.4	0.96 (21 df)
L.S.D.[2] (27 d.f.)	0.77	0.89	0.89	0.89	0.89	
Average over all treatments	24.9	27.5	27.4	25.3	25.3	0.72 (5 d.f.)

1 LSD's for comparison with the Control
2 LSD's for comparisons within individual strains

3.5.3 Symptoms and quality

Different symptoms were observed during the course of this experiment depending on which MVX strain was used and at what time the MVX infection took place (Table 8). The control mushrooms were of good size and quality throughout the crop. All MVX 1283-P and MVX 1961 treatments generally produced heavy pin-sets with mushrooms consequently being very small and not capable of growing into good quality closed cups (Figure 5 and 6). Mushrooms from MVX 2637 treatments were of good quality, irrespective of any infection treatment (Figure 5, Table 8). The late infection treatments for MVX 2735, (i.e. those at the end of spawn-run and at casing) consistently produced a number of pale brown mushrooms, often with a sticky, scaly cap texture. Early infection at spawning with a 25% infected material did not produce any off-coloured mushrooms on the bed however, early infection with a low infection rate (0.01% infected compost at spawning) resulted in one plot producing 50 off-coloured mushrooms on the bed, predominately in the second flush (Figure 7, Table 9). Mushrooms from another plot, which produced no off-coloured mushrooms on the bed, became off-coloured during storage at 4°C with the off-coloration sometimes mottled rather than uniform in appearance.

Table 8. Symptoms occurring in crops treated at various times with different MVX strains.

	Strains				
Infection treatment	A15-1 Control	MVX 1283-P	MVX 1961	MVX 2637	MVX 2735
25% spawn	None	Small mushrooms	Small mushrooms	None	None
1.2 g at spawning 0.01%	None	Small mushrooms	Small mushrooms	None	Some brown mushrooms
0.01% at end of spawn run	None	Small mushrooms	Small mushrooms	None	Brown mushrooms
1.2g/bag at casing	None	Small mushrooms	Small mushrooms	None	Brown mushrooms

Figure 5. Mushrooms sampled from all treatments and stored for 7 days at 4°C. Treatments:- A = 25% infected spawn at spawning; B = 1.2 g (0.1%) infected compost at spawning; C = 1.2 g (0.01%) infected compost at end of spawn-run; D = 1.2 g infected compost into casing. * = mushrooms which were off-coloured prior to storage; + = mushrooms which became off coloured during storage. Note off coloured mushrooms in most treatments for MVX 2735 and small mushrooms for MVX 1961 and 1283-P.

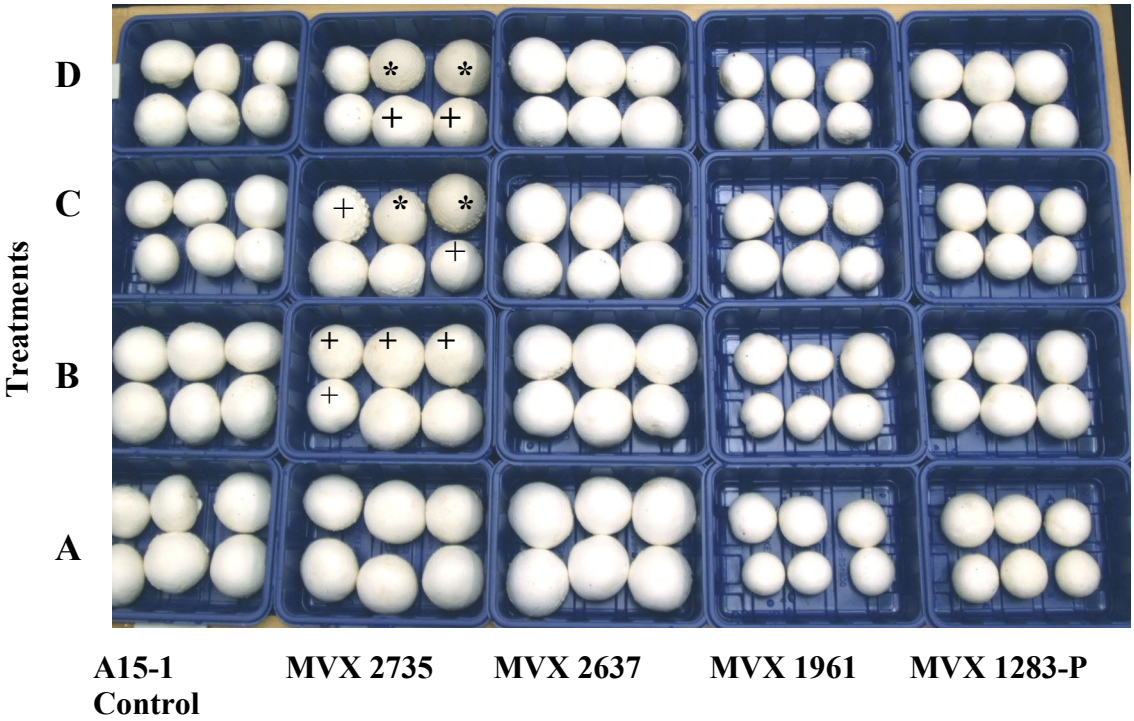


Figure 6. Plots for A15-1 (left) and 1961 (right) during the first flush. Note fewer, better quality mushrooms on the control plot compared with the dense pin-set and crowded small mushrooms on the 1961 plot.

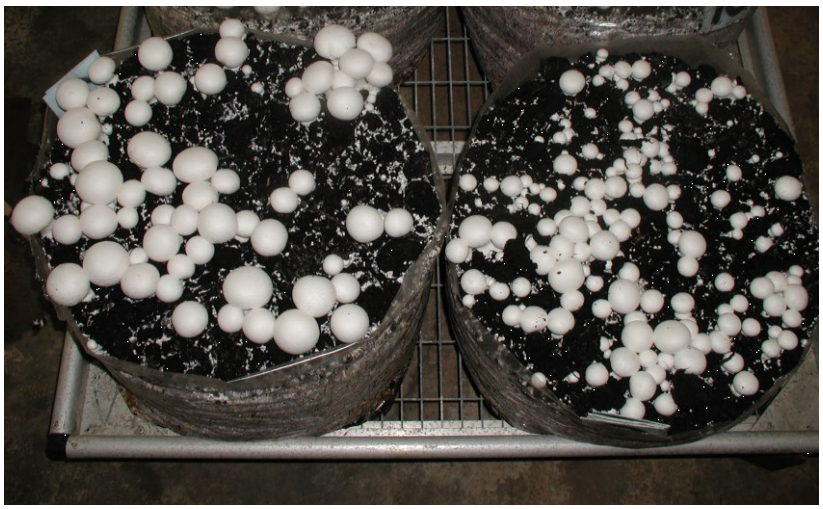


Figure 7. Average number of brown or off-coloured mushrooms harvested from 12kg bags of compost infected at different times with MVX 2735.

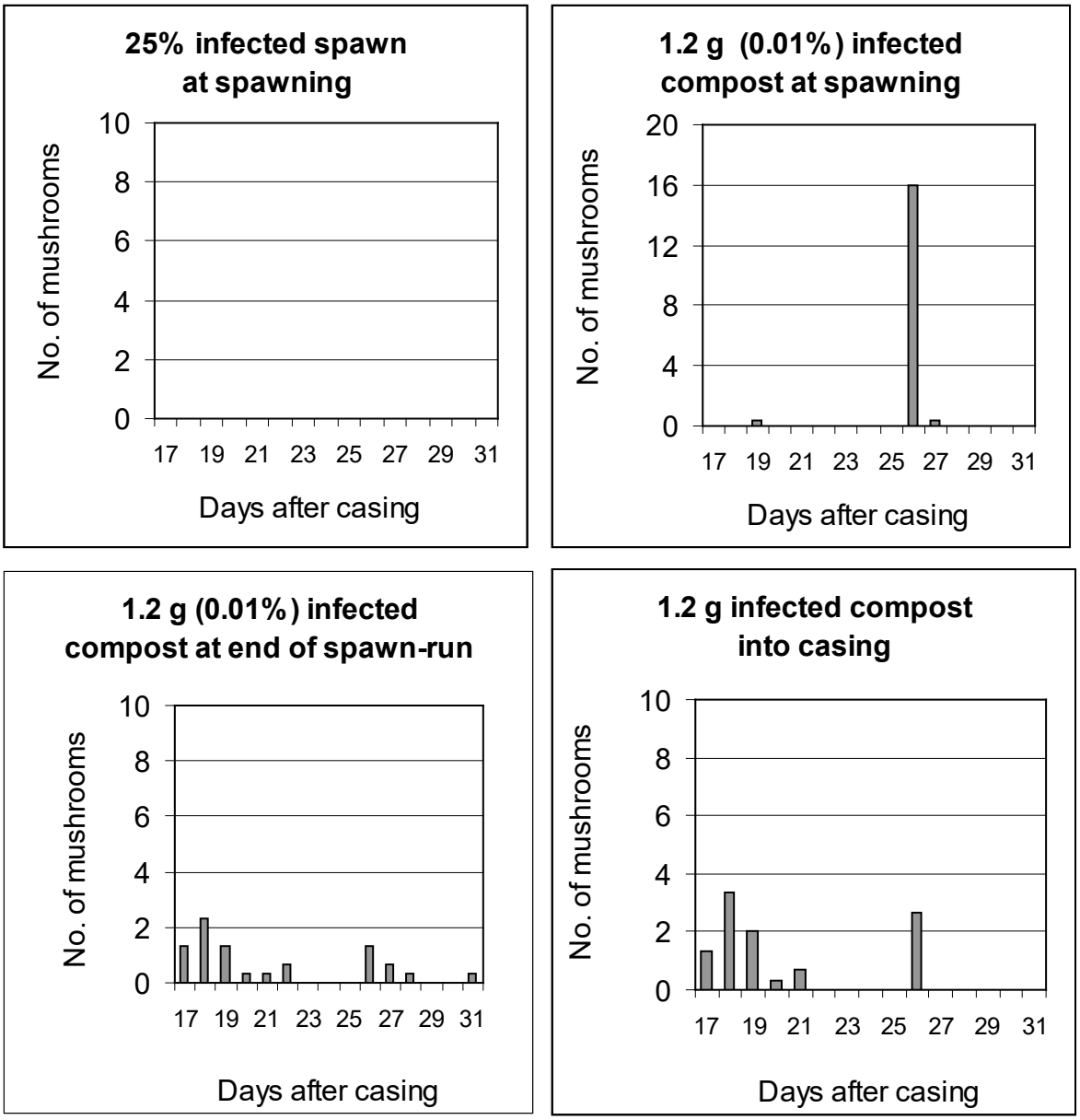


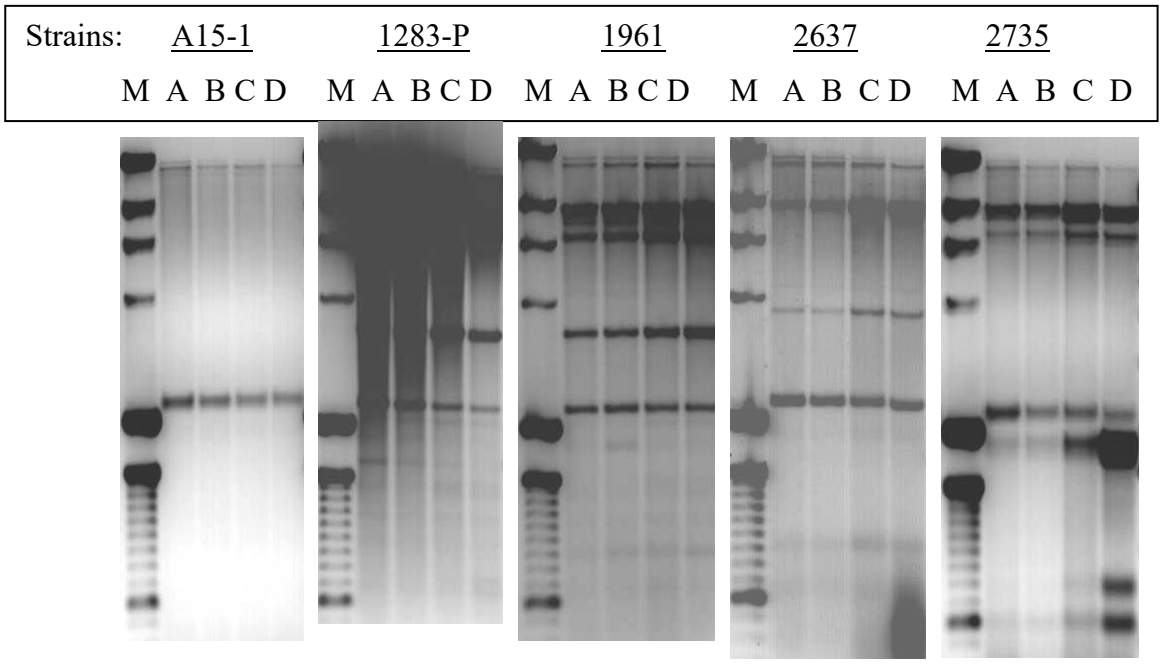
Table 9. Number of brown mushrooms harvested calculated per tonne of compost

Virus Infection type and time			
25% infected spawn at spawning	0.01% infected compost at spawning	0.01% infected compost at end of spwawn run	0.15% infected compost into casing (1.2 g / 8 litres)
0	**1,389**	**750**	**861**

3.5.4 DsRNA profiles

Mushrooms from all the control treatments were predominantly clear, containing the asymptomatic bands H1 and H3, as well as feint bands 1,2, and 13 (Figure 8).(At this point in time we do not know the significance of these feint dsRNA bands that occur in mushrooms from healthy crops with no virus history). Mushrooms from all 1283-P treatments had identical profiles, but the intensity of the bands decreased with the quantity of inoculum used and with the time of infection, with the latest infection time giving rise to mushrooms with the weakest profile (Figure 8). Mushrooms from all 1961 treatments had identical profiles but on this occasion the intensity of the bands was strongest for the latest infection times, i.e. at the end of spawn-run and at casing (Figure 8). Mushrooms from all 2637 treatments had identical profiles, and again the intensity of the bands was strongest for the latest infection times, i.e. at the end of spawn-run and at casing (Figure 8). Mushrooms from all 2735 treatments also had identical profiles , and again the intensity of the bands was strongest for the latest infection times, i.e. at the end of spawn-run and at casing, particularly for the four small dsRNAs associated with the off-coloured brown-mushroom symptom (Figure 8).

Figure 8. Ds RNA profiles of mushrooms harvested from crops treated with different strains of MVX material, at different quantities and at different times. M = marker lane; A = 25% at spawning; B = 0.01% at spawning; C = 0.01% at end of spawn-run (during bulk handling); D = 1.2 g infected compost added to casing (see text for further details).



3.6 Experiment 2 - Discussion

For the first time in an experimental crop we have reproduced the brown mushroom symptom which has been recorded by growers around the UK at various times in the past number of years. The symptom occurred most consistently when a small quantity of infective material was incorporated into the casing of a healthy crop or into healthy spawn-run compost during bulk handling. Only a small proportion of the crop produced the symptom and there was considerable variability in symptom expression between the different replicates, with some plots producing the symptom predominantly in the first flush with others produced the symptom mostly in the second flush. When infection with a small quantity of inoculum (0.01%) occurred earlier, at spawning, some brown mushrooms were also produced but in only one replicate out of three, and the bulk of them were in the second flush, and with a high proportion of mushrooms developing the symptom. No brown mushrooms occurred when a higher level of infection occurred at spawning (25% spawn), confirming the results of Experiment 1 with 100% spawn. Correlated with this expression of the symptom was a very strong titre of dsRNA in mushrooms from crops infected late in the crop cycle compared to those infected earlier. Some mushrooms, from plots that produced no brown symptoms on the bed, became off-coloured/light brown when cold stored for a week.

No premature opening symptoms occurred with MVX strain 1961. However, infection of compost or casing with this strain consistently caused crop delay followed by over-pinning, leading to too many mushrooms on the bed, and thereby reducing the quality and size of the mushrooms being picked. It is both surprising, and from a cropping perspective, worrying, that there was very little difference in the effects due to large or small quantities of infected material incorporated at spawning through to a small quantity of infected material incorporated into the casing.

Crop delay (which can give the impression of bare areas) was recorded for all treatments with 1283-P but total yields were not dramatically lower than the controls. Again, as with 1961, there was very little difference in the effects due to either large or small quantities of infected material incorporated at spawning through to a small quantity of infected material incorporated into the casing.

Both MVX strains 1283-P and 1961 had similar effects on mushroom crops, irrespective of the time at which crops were infected, yet these two strains had different dsRNA profiles,

with the most noticeable difference being the lack of an intense band 3 in 1961. What they have in common is the H2, 7,8, & 9 grouping along with a band 15. MVX strains 2735 and 2637 did not have a band 15, and infection of crops with these strains at less than 100% (see experiment 1) did not produce any crop delay. Further experiments, with additional strains having the H2, 7, 8, & 9 dsRNA grouping along with a band 15, are needed to determine if this combination of bands are responsible for crop delay symptoms.

In three out of the four MVX strains tested, the dsRNA profile of mushrooms from crops infected at the end of spawn running or at casing, were stronger than for mushrooms from crops infected at spawning. This was particularly noticeable for the strain that gave the off-coloured/brown mushroom symptom. The difference in the titre of the dsRNA depending on infection time suggests that some interaction is taking place between the dsRNAs and *Agaricus* within the mycelium, which is influencing the amount of viral replication or expression that is taking place. An understanding of the dynamics of dsRNA replication, expression and persistence within Agaricus mycelium and fruitbodies may shed light on the factors necessary for the expression of the premature opening symptom, which has not yet been reproduced in experiments.

The dsRNAs extracted from the control mushrooms included low titre detection of bands 1, 2, and 13 in addition to the bands H1 and H3, normally associated with "asymptomatic" healthy crops. These feint bands are occasionally recorded in mushrooms from virus-free crops and crops with no virus history, as well as in crops from farms that have "recovered" from MVX. They may be associated with, or be similar to, the "asymptomatic" bands H1 and H3 but their relevance to the MVX story is not known at this time. DEFRA funded research on the molecular characterisation of MVX dsRNAs , currently in progress should help to clarify the relationship between all the dsRNAs found in *Agaricus* samples.

3.7 Conclusions

- Several different "strains" of Mushroom Virus X were all capable of causing significant crop delay when a high level of infection (100%) occurred at spawning, but no other symptoms were produced.
- Infection of crops with several different "strains" of Mushroom Virus X, when grown under identical conditions, produced mushrooms with remarkably similar dsRNA profiles, with at least two distinct profile types being recognisable.
- Symptom expression with a "brown-mushroom" MVX strain (2735) was related to time of infection. The off-coloured/brown mushroom symptom occurred most consistently when a small quantity of infected material was incorporated into the crop at casing or during bulk handling, though contamination of compost at spawning, also with a small quantity occasionally produced significant numbers of brown mushrooms in the second flush.
- Infection at the end of spawn-run or at casing produced mushrooms with a higher titre of dsRNA in three out of four MVX strains tested.
- For two MVX strains from sites with severe problems (1283-P and 1961), infection of a crop with a small amount of material at casing appeared to be equally effective in causing crop delay as infection at spawning with a similar or greater quantity.
- One strain (2637), which contained dsRNA bands 1, 2, 5, 13 & 14 but lacked key dsRNAs believed to be important in Virus X cropping problems (bands 3, 7, 8, 9, 15) caused no crop delay and produced good quality mushrooms. Yields and cropping patterns for all infection treatments were similar to, but more variable than the control.

4 Compost Rollover Experiments.

Mushroom viruses are carried within the mushroom mycelium and can be transferred into new crops as a result of an infected hypha anastomosing with a non-infected hypha. Thus, if spawn-run compost from a virus- infected crop is somehow carried-over into a new crop, then there will be an opportunity for mushroom viruses to spread and develop in the new crop. Compost carry-over in trays from uncooked-out or poorly cooked-out virus-infected crops was implicated in the spread of La France virus into the next crop grown in them (Hollings & Stone, 1971). This lead to better cooking-out procedures for mushrooms crops when the crop was terminated. Mycelial carry-over however is also an important route of infection for bulk spawn-running tunnels where huge quantities of live *Agaricus* material are handled in close proximity to new crops, offering regular and abundant opportunities to infect new crops if there are breeches in the exclusion-hygiene measures needed for handling bulk phase III.

An experiment was designed to simulate continuous mycelial carry-over from crop to crop using a small quantity of live spawn-run compost from one crop to infect the freshly spawned compost of another.

4.1 Materials and methods

Two strains were included in the rollover experiments, an A15 control culture (A15-1) taken from a A15 mushroom crop with no virus history, and Strain 2640 (see Table 1). The mushrooms from which Strain 2640 was obtained only contained dsRNA bands H1, H2 & H3, but the they came from a farm which had a rumbling presence of MVX symptoms, and which has had mushroom samples before and after sample 2640 that were positive for MVX dsRNAs. Strain 2640 was chosen for the rollover experiment as it was considered that, due to its history, it may have MVX dsRNAs present at undetectable levels. If this was the case then they may increase in titre if given the opportunity to do so by purposefully rolling over compost from one crop to another. Eight rollover crops were done in all.

For each rollover crop, four 50 kg trays of phase II compost were spawned with Commercial A15 spawn. Two trays were placed in each of two separate chambers for spawn-running. For the first crop (Rollover Crop 1) one lot of two trays received an additional 2.5 g (1%) of home-made A15-1 spawn, and the second lot of two trays received 2.5 g (1%) of spawn made

from Strain 2640. The A15-1 trays were spawn-run in a separate chamber to the 2640 trays to prevent any cross contamination. Spawn-running was usually done for 17 days, but occasionally it was longer if freshly spawned phase II compost was unavailable. At the end of the spawn-running period a 5-10 g sample of the spawn-run compost was taken from each tray and used to infect two new trays of freshly spawned compost. The spawn-run compost was gently mixed into the new trays, which were then covered with paper, and allowed to spawn-run for 17 days. At all times A15-1 treatments were kept separate from 2640 treatments during spawn-running, and when transferring spawn-run compost into new trays (rollover events).

Some cecids were present in Rollover crop 6 so we decided to take new A15-1 and Strain 2640 cultures from mushrooms harvested from Rollover crop 6, and to make more spawn. The freshly spawned trays of Phase II compost for Rollover Crop 7a were then infected with 5g (2%) of the new spawn per tray, instead of spawn-run compost from the previous cecid-contaminated crop. Spawn-run compost was once again transferred into Rollover crop 8.

Five of the rollover crops were cased and cropped to produce mushrooms for MVX dsRNA testing. Four of the eight crops were taken through and cropped for two or three flushes to record yield and to observe any potential symptoms emerging due to the rollover events. On these occasions, the spawn-run trays were cased with a standard commercial casing mix with **no** casing inoculum added. Once cased, the four trays were moved into a clean chamber for cropping.

4.2 Results

4.2.1 dsRNA profiles

The dsRNA profiles of A15-1 and Strain 2640 mushrooms from Rollover crops (RO) 1, 3, 6, 7a and 8 are presented in Figures 9, 10 and 11. The A15-1 profiles in RO 1 mushrooms had only a feint H1 band, and a H2 band in only one of the two samples tested. An additional dsRNA band "2" was present in mushrooms from RO 3 onwards. By RO 6 the H2 band had become quite strong and a few feint bands were detected around the band 19/22/23 area. Additional bands occurred in mushrooms from RO 7a, when spawn from new cultures replaced the compost as the carry-over material. New bands included bands 5, 13 and 14. In RO 8 the H2 continued to be very strong and a new band, 12 appeared in one sample. None

of the bands 3, 7, 8, 9 or 15, which are frequently found in MVX-positive samples, were detected.

Apart from RO 1 and RO 3, the dsRNA profiles for mushrooms from the Strain 2640-infected crops were almost identical to those for the A15-1 control crops (Figures 9, 10, 11), suggesting that the starting culture of 2640 was not much different to the A15-1 culture used in the control.

Figure 9. Photographs of electrophoretic gels showing dsRNA bands present in mushroom samples from selected rollover crops continually re-infected with live material of either A15-1 or Strain 2640 (two replicates each). Individual rollover crops had received either additional hand-made spawn (RO 1 and RO 7a) or additional spawn-run compost from the previous crop (RO 3, RO 6 and RO 8) added to the freshly spawned compost at spawning. M = marker lane.

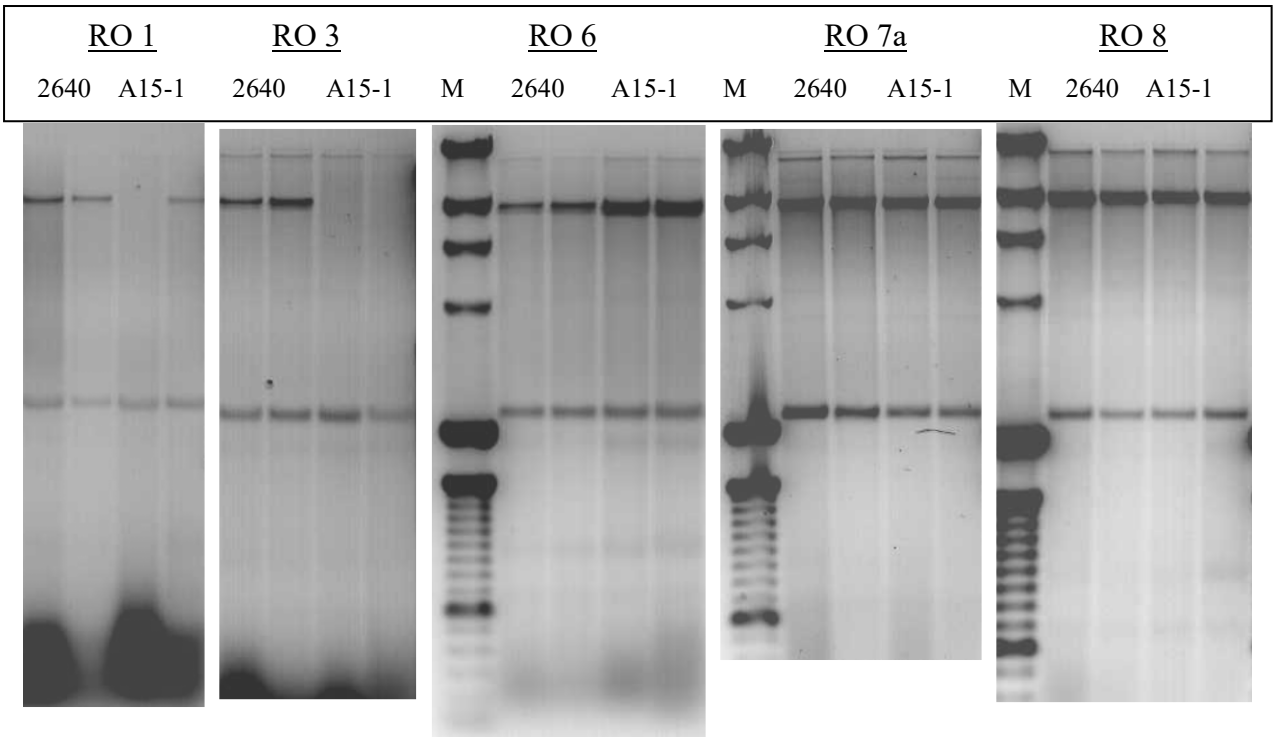
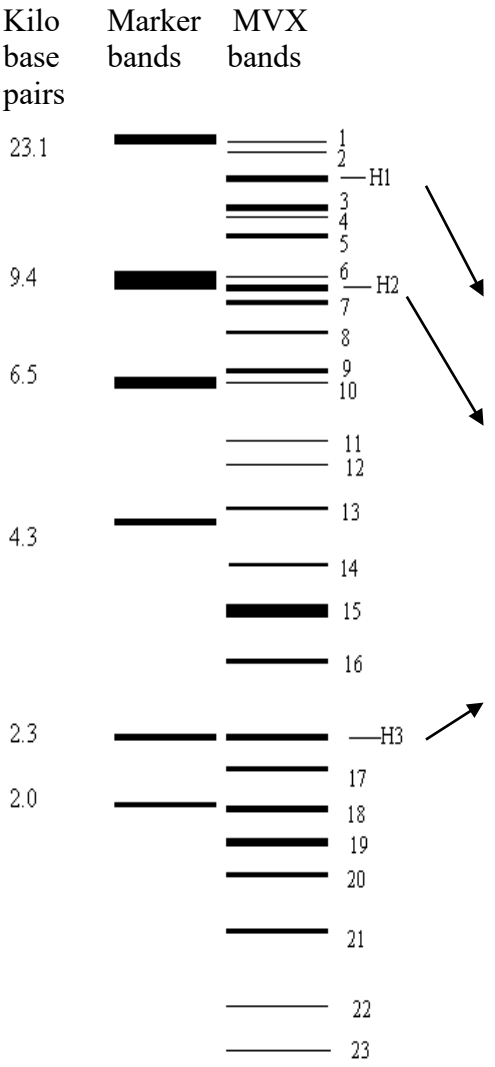


Figure 10. Schematic of A15-1 dsRNA profiles for mushrooms from 5 different rollover crops.



Rollover	RO.1	RO.1		RO.3	RO.3		RO.6	RO.6		RO.7a	RO.7a		RO.8	RO.8
Strain:	A15-1	A15-1		A15-1	A15-1		A15-1	A15-1		A15-1	A15-1		A15-1	A15-1
Band No.														
1														
2				X	XF		X	X		X	X		X	X
H-1	XF	XF		X	X		X	X		X	X		X	X
3														
4														
5										XF	X			
6														
H-2		X					XS	XS		XS	XS		XS	XS
7														
8														
9														
10														
11														
12													XF	
13										XF	XF		XF	XF
14										XF	XF			XF
15														
16														
H-3	X	X		X	X		X	X		XS	XS		X	X
17														
18														
19							XF	XF					XBF	XBF
20														
21														
22							XBF	XBF					XBF	XBF
23							XBF	XBF					XBF	XBF

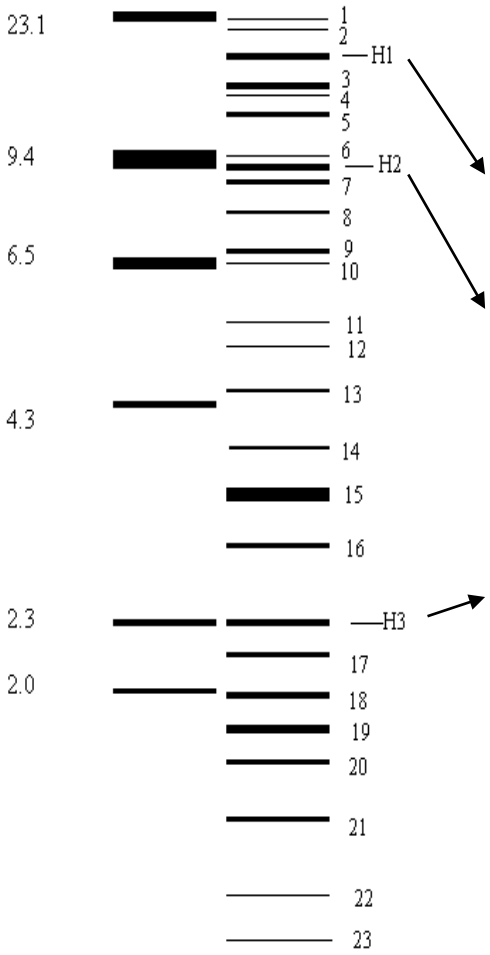
XF = feint band;
XS = strong band
XBF = feint band adjacent to this position

Figure 11. Schematic of Strain 2640 dsRNA profiles for mushrooms from 5 different rollover crops.

Kilo base pairs

Marker bands

MVX bands



Rollover crop:	RO.1	RO.1		RO.3	RO.3		RO.6	RO.6		RO.7a	RO.7a		RO.8	RO.8

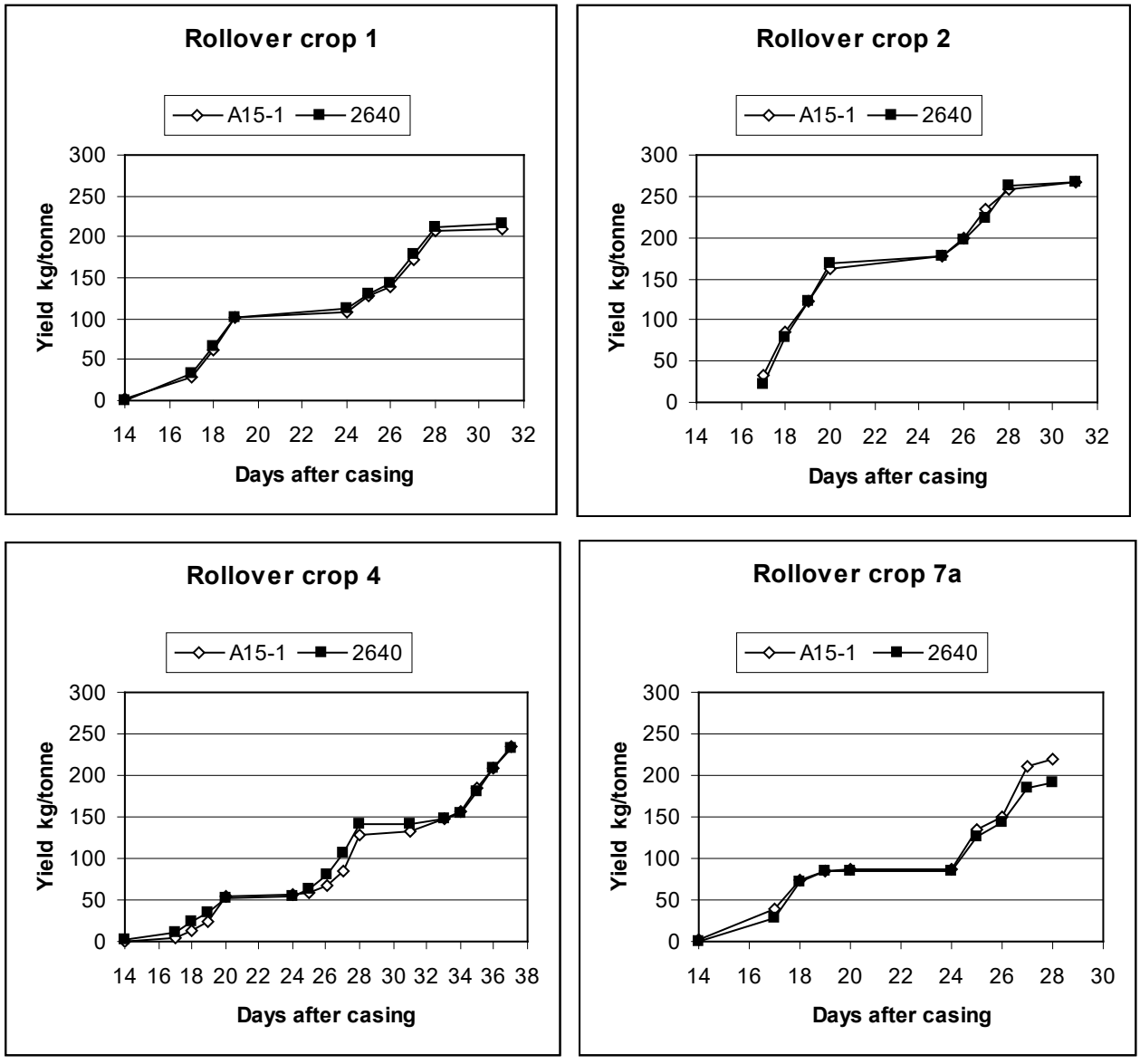
Strain:	2640	2640		2640	2640		2640	2640		2640	2640		2640	2640
Band No.														
1														
2				X	X		X	X		X	X		X	X
H-1	XF	XF		X	X		X	X		X	X		X	X
3														
4														
5										XF	XF			
6														
H-2	X	X		X	X		XS	XS		XS	XS		XS	XS
7														
8														
9														
10														
11														
12														
13										XF	XF		XF	XF
14										XF	XF		XF	XF
15														
16														
H-3	X	X		X	X		X	X		X	X		X	X
17														
18														
19										XF			XBF	XBF
20														
21														
22							XBF	XBF					XBF	XBF
23							XBF	XBF					XBF	XBF

XF = feint band
XS = strong band;
XBF = feint band adjacent to this position

4.2.2 Yields and Symptoms

There were no differences in the yields or crop timing between the A15-1 control treatment or the Strain 2640 treatment in RO crops 1, 2, 4 or 7a (Figure 12). All crops looked healthy. Occasionally mushrooms pinned unevenly or deeply but this is likely to be due to the fact that no casing inoculum was used.

Figure 12. Yields from four sequential crops that had either additional hand-made spawn (RO 1 and RO 7a) or additional spawn-run compost from the previous crop (RO 2 and RO 4) added to the freshly spawned compost at spawning.



4.3 Discussion

After eight rollover events, where live *Agaricus* material was continuously carried over into each new subsequent crop, the titre of dsRNAs in the eight crop had increased in both number and intensity compared with the first crop. There were no major differences between two different starting strains, an A15-1 control strain grown from a mushroom crop with no MVX history, and a potentially MVX-positive strain from a mushroom crop, not exhibiting any signs of virus at the time it was sampled, but which was from a farm with a rumbling low level presence of MVX. The dramatic increase in the titre of the apparently asymptomatic dsRNA "H2" is interesting, as is the presence of the additional bands 2, 5, 12, 13, and 14. While these bands are not among the bands most frequently associated with MVX problems (3, 7, 8, 9, and 15), their presence is unsettling. They may be associated with low levels of 25 nm virus particles and samples of mushrooms from RO 9 will be tested for their presence. There were no discernible effects on crop yields or timing, with two flushes being cleared by day 28 after casing for all crops, and yields being in the region expected for the composts being used. Further rollover crops are planned to see if any further dsRNA bands develop.

4.4 Conclusions

- The titre of the asymptomatic "hybrid band" H2 has increased significantly in harvested mushrooms after eight consecutive (rollover) crops, each infected with some live *Agaricus* material from the previous crop.
- Several minor dsRNAs (bands 2, 5, 12, 13 & 14) have been detected in mushrooms from the eighth rollover crop but crop timing and yields appear normal.
- There were no differences between two sources of initial *Agaricus* material.

5 Detection of *Agaricus* propagules in mushroom farm dust and debris

A small experiment carried out in 2001 showed that mushroom farm dust and debris was capable of transmitting MVX dsRNAs to a healthy crop, if it was used to contaminate the casing of a healthy crop (Grogan, 2002b). Due to the "dirty" nature of this material it has been extremely difficult to extract clean *Agaricus* cultures with which to carry out controlled scientific experiments. Such *Agaricus* cultures derived from farm dust and debris, in conjunction with controlled experiments, could prove that mushroom farm dust and debris is a potent source of MVX infectious material. DEFRA funded work at HRI (Mills *et al.*, 2002) has led to the development of an *Agaricus* specific medium which supports the growth of *Agaricus* but not that of bacteria or several common mould species. Although still in need of further development, it may be of some benefit in isolating *Agaricus* cultures from mushroom farm dust and debris.

5.1 Materials and methods

5.1.1 Sources of dust and debris

Dust and debris were collected from three locations on a farm that was experiencing severe MVX problems as indicated below.

- Dust and debris collected from central corridor with cropping rooms on either side
- Dust and debris collected from a cleaned and disinfected winch
- Dust and debris collected form the beams of fumigated phase III spawning/emptying hall

Dust and debris were also collected from two locations on the MVX-free mushroom unit at HRI.

- Dust collected from central corridor with cropping rooms on either side
- Dust collected from outdoors behind cropping houses

5.1.2 Obtaining pure Agaricus cultures

All samples of the mushroom farm dust and debris obtained from the two locations listed above were tested for the growth of Agaricus, thereby confirming their potential as sources of MVX. Serial dilutions of the samples were prepared and were plated out on DEFRA *Agaricus* Selective medium (Mills *et al.* 2002). Small particles of debris were

also plated out directly onto *Agaricus* Selective medium. All Petri dishes were examined for *Agaricus* growth at weekly intervals.

A second experiment was also set up to try and obtain live *Agaricus* cultures from the debris. Sub-samples of debris were placed into small screw-capped glass jars containing phase II compost so that any *Agaricus* material contained within the sample would have the opportunity to colonise the compost. No spawn was added so that any *Agaricus* growth that occurred could only have come from the sample. Jars were incubated at 25°C and checked for growth at weekly intervals over three months.

5.1.3 MTIST technology.

Samples of air were taken at another site with MVX problems and were treated with the *Agaricus* specific antibody and fluorescent stain (Mills *et al,* 2002). Air samples were also taken using the Anderson sampler technology (Grogan, 2002a) in conjunction with the DEFRA selective medium to determine the concentration of viable Agaricus propagules in the air, for comparison with the MTIST method.

5.1.4 Inoculation of mushroom crop with farm dust and debris

The dust and debris samples, collected from the farms and locations listed above, were used to infect the casing of freshly cased trays of spawn-run compost. An uninfected control was also included. Trays were cropped and observed for symptoms. First and third flush mushrooms from all six treatments were harvested and analysed for dsRNA.

5.2 Results

5.2.1 Isolating pure Agaricus cultures.

No pure cultures were obtained as a result of serial dilution or plating out onto DEFRA *Agaricus* specific medium. This medium was not specific enough and several moulds grew which prevented the observation of any *Agaricus.* The medium needs further development before it can be termed *Agaricus* specific. No *Agaricus* grew when phase II compost was inoculated with dust and debris obtained from mushroom farms. Alternative methodologies need to be explored to try and confirm the presence of live *Agaricus* material in dust and debris.

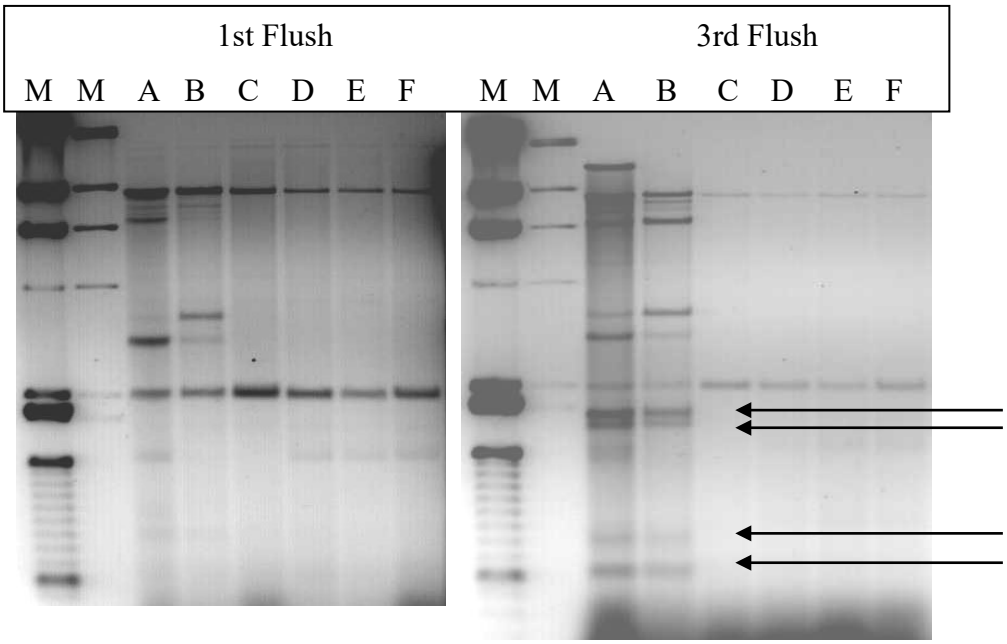
5.2.2 MTIST technology

The MTIST *Agaricus* antiserum detected *Agaricus* spores at several times during a week long trial on a commercial mushroom farm, however the failure of the *Agaricus* selective medium to detect any viable *Agaricus* propagules at the same time means the results have to be interpreted cautiously. At this point in time it is impossible to say whether or not the *Agaricus* specific antibody is detecting "live" or "dead" *Agaricus* spores and mycelium. Further work is needed to validate the antibody before the MTIST test can be used as a diagnostic tool.

5.2.3 Crops infected with mushroom-farm dust and debris.

First and third flush mushrooms from trays infected with the dust and debris from two out of three locations on an MVX infected site contained abundant dsRNAs, while mushrooms from trays infected with dust and debris from an MVX-free site contained a few very feint minor bands. Mushrooms from a control, uninfected tray were also clear of MVX dsRNAs (Figure 13).

Figure 13. MVX gels for 1st flush and 3rd flush mushrooms from trays treated with mushroom-farm dust and debris from various sources: A,B,C from an MVX infected farm; D,E from a virus free farm, F = non infected control. M = marker lanes. Arrows highlight bands associated with the off-coloured/brown mushroom symptom.



The intensity of the bands increased between first and third flush, and the bands associated with the off-coloured brown mushroom symptom also occurred only in third flush mushrooms, though no brown mushrooms were observed in the crop (Figure 13).

5.3 Discussion

It was disappointing not to be able to isolate live *Agaricus* cultures from the dust and debris that resulted in the presence of a large number of MVX dsRNAs in mushrooms, grown from a crop that was contaminated with the same material. It is widely accepted (Lemke,1979; Lecoq *et al*, 1979) that fungal viruses are transmitted through anastomosis between infected mycelium, or germinated infected-spores and healthy mycelium. Therefore the fact that the dust and debris from an infected farm, applied to a healthy crop, and resulting in MVX dsRNAs in subsequent mushrooms implies that there was some live *Agaricus* material in the dust that grew and anastomosed with the healthy mycelium, thereby transmitting the dsRNAs into a healthy crop.

It is not difficult to imagine how live *Agaricus* spores and mycelium can persist in the dust fraction of a farm. Mushrooms are continuously shedding huge quantities of spores, even when "opens" are not being grown intentionally. Spawn-run compost can be spilt on the cropping house floor and/or outside during deliveries and filling of Bulk phase III. Any material that escapes cooking-out or disinfection can potentially harbour *Agaricus* spores and/or "live" compost debris. The circumstantial evidence is that live *Agaricus* material persists on farms.

"MTIST" technology, using *Agaricus* specific antibodies, is currently being developed with DEFRA funding which may be able to identify live *Agaricus* but some method of distinguishing "live" *Agaricus* from "dead" *Agaricus* has to be found, as there will always be a plenty of dead *Agaricus* spores and mycelium present on a mushroom farm.

5.4 Conclusions

- Dust and debris from around mushroom farms is capable of infecting otherwise healthy crops with MVX dsRNAs after the crop has been cased
- All precautions should be taken to minimise the raising of mushroom-farm dust during spawning and casing operations

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