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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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Grower Summary

Headlines

- Molecular diagnostic tests can reliably detect specific MVX genetic material in mushrooms and also in spawn-run compost but at very low levels some false negatives may occur
- MVX negative status is characterised by up to nine non-symptomatic dsRNAs
- CSL Plant Clinic, Sand Hutton, York YO41 1LD will provide a virus diagnostic service to the mushroom industry starting sometime in 2007(contact person Tom Nixon).

Background and deliverables

Mushroom Virus X (MVX) disease has been a major disease of the cultivated mushroom in Britain and some European countries since the late 1990's. It has contributed to the closure of a number of farms and compost yards and to increased costs of production through yield and quality reductions. HDC have funded an extensive research programme over several years to look at how the disease is transmitted and controlled (Projects M 39, M 39a, M 39b and M 39c). Results from these works have produced information on MVX disease progression, vulnerable stages of mushroom production and advice to growers on where to focus control measures. Many growers have been able to significantly reduce or eliminate the effects of MVX on their farms as a direct result of the information obtained from this earlier work.

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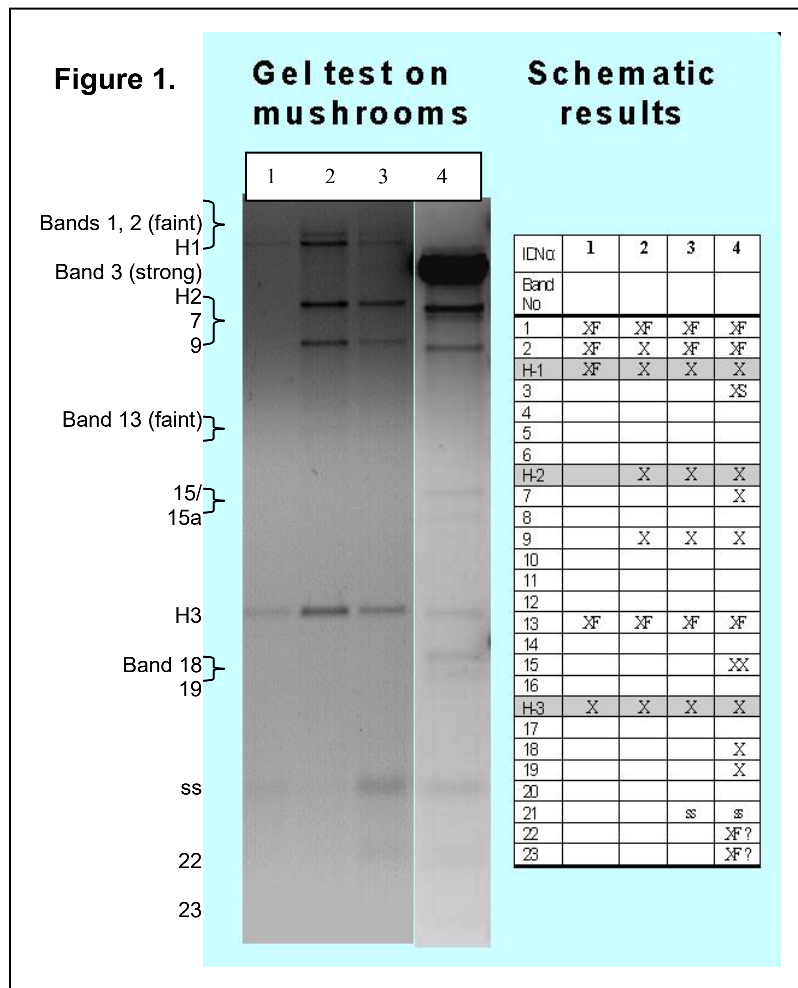
MVX is identified by the presence of many “dsRNA” (double stranded Ribo Nucleic Acid) genetic elements which can be visualised and photographed on an agar gel (Fig.1). An MVX test consists of extracting the dsRNAs from a mushroom sample, purifying them, and then separating them out as bands on an agar gel according to their size. Usually the results are then presented as a schematic and the dsRNA bands numbered from 1 to 23 (Fig 1).



ID No	1	2	3	4
Band No				
1	XF	XF	XF	XF
2	XF	X	XF	XF
H-1	XF	X	X	X
3				XS
4				
5				
6				
H-2		X	X	X
7				X
8				
9		X	X	X
10				
11				
12				
13	XF	XF	XF	XF
14				
15				XX
16				
H-3	X	X	X	X
17				
18				X
19				X
20				
21			ss	ss
22				XF ?
23				XF ?

As MVX decreases in terms of its significance to the British mushroom industry, it has become apparent that whereas a clear “negative” MVX status can be easily ascertained, there are many ambiguous MVX test results. This happens when several dsRNA bands are detected that would not be expected in “healthy” samples. Frequently, these dsRNA bands are present without symptoms and the farm may consider itself to be “MVX-free”. However, it is important to know if the non-symptomatic dsRNAs that can sometimes be detected in samples could eventually give rise to a greater number of MVX-associated dsRNAs and eventually an “MVX-positive” result. There remain several important gaps in our knowledge concerning the recognition of this “MVX-free” status and maintenance of it.

This project had three deliverables: (1) to identify what dsRNA profile constitutes a negative MVX result; (2) to determine if dsRNA numbers and intensity can increase over successive crops if live *Agaricus* material, containing dsRNA, is carried over from crop to crop (reflecting poor virus hygiene measures) and (3) to determine if molecular diagnostic tests for specific MVX dsRNAs (bands 3, 15 and 19) are effective at giving clear positive or negative results that can be confirmed by standard MVX testing procedures.

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Summary of results

Eight *Agaricus* isolates were used to look at what dsRNA profiles could be associated with a "negative" MVX status. Two isolates were from healthy crops (A15-1 and 4599 FY); two were from crops exhibiting brown mushroom symptoms only (4569 SFT & 4583 PS-C); two were historic samples taken at the height of the MVX epidemic (1911 BPG – no symptoms & 1961 WY – severe symptoms); one was from a more recent severe outbreak of MVX (4578 FD) and the final isolate was one produced from a single spore isolate from an MVX infected mushroom that received only the band 3 dsRNA from the parent mushroom (SSI 61). Spawn-run compost derived from each isolate was deliberately introduced into the first of four successive crops at spawning. At the end of the spawn-running period, some spawn-run compost from the previous crop was then introduced into the following crop (to simulate poor virus hygiene) in order to see if the dsRNA profiles or MVX status changed with time.

Crop symptoms and dsRNA profiles.

The results indicate that no significant changes to the dsRNA profile or symptom characteristics occurred over the four crop cycles, contrary to expectation. Those isolates that showed crop delay in the first crop, continued to show similar levels of crop delay in the three subsequently contaminated crops while those crops that were "healthy" and similar to the control, remained "healthy" and similar to the control in all four successive crops (Figure 2). The dsRNA profiles of the "healthy" non-symptomatic crops contained up to nine dsRNAs (bands 1, 2, H1, 5, H2, 9, 12, 13, H3 - see Figure 3) and, to the best of our knowledge at the current time, this type of dsRNA profile represents an MVX "negative" result.

When the very large, strong band 3, present on its own in a single spore isolation from an MVX-infected mushroom (SSI-61), was introduced into an MVX-free crop, there was no effect on the four successive rollover crops. This suggests that band 3 may also be benign but further research is necessary to ensure that it does not have other, as yet unforeseen, effects.

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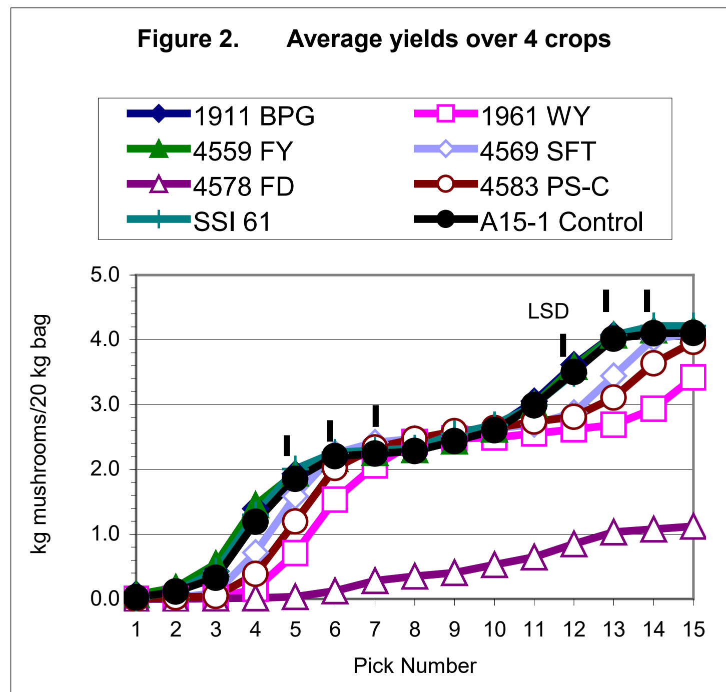
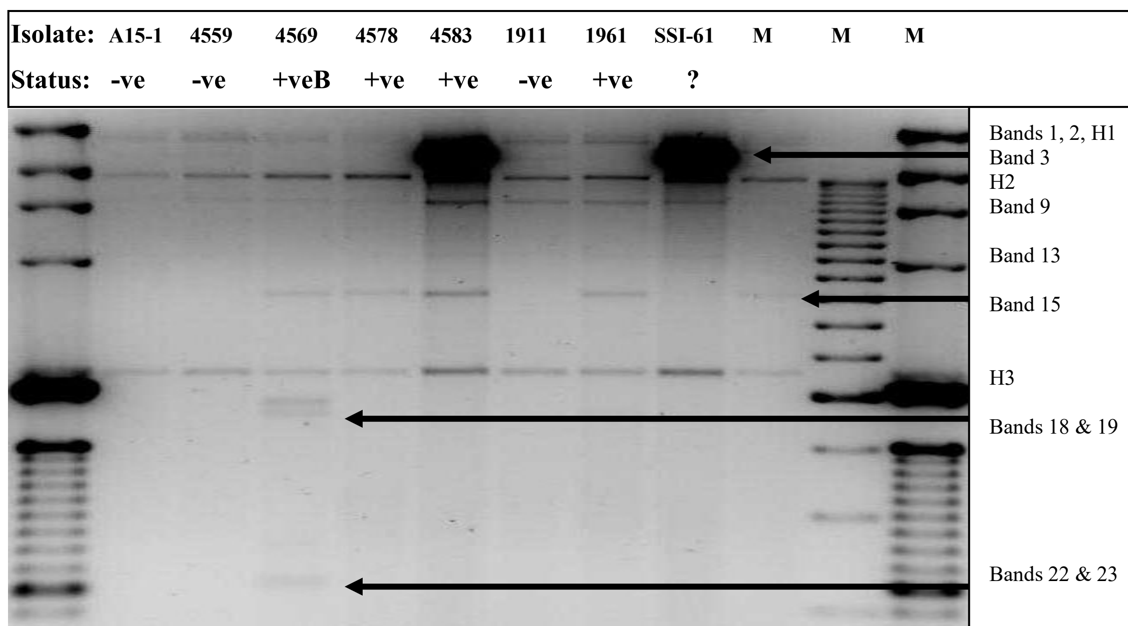


Figure 2. **Average yields over 4 crops**

Figure 3. Positive and negative MVX gel test results for eight mushroom samples (B = brown mushroom symptoms; M = marker lanes).



Molecular tests.

Molecular based rtPCR tests, using crude extracts of either mushrooms or mycelium derived from spawn-run compost, were effective at detecting three specific dsRNAs (bands 3, 15 and 19) and the results consistently agreed with those obtained for the standard MVX gel test done on mushrooms

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subsequently produced from the compost (Table 1). This means that growers can now be confident that molecular diagnostics for these three bands - 3, 15 and 19 - are as good as the standard gel test. However the molecular tests are very specific and only give a positive/negative result for individual bands and provide no information on any other possible bands that may be detected by the standard gel test. Furthermore, false "negative" results from rtPCR tests using crude extracts may occur if the amount of the dsRNA in a sample is very low. An additional "nested" PCR test can be done in such cases, which reduces the number of false negatives. However, both tests can be used to compliment each other. The rtPCR test can be used on the mycelium from spawn run compost and can be done more rapidly and on more samples at a given time. It could be used by bulk phase III producers as a quality assurance test to ensure that none of the MVX bands 3, 15 or 19 is present in fully spawn-run compost. Any positive results would be followed up by a gel test on mushrooms originating from a suspected batch of compost. The standard gel test can only be done on mushrooms and gives a more complete picture of the dsRNAs that may be present in a mushroom sample and this test would be useful for growers who suspect a virus outbreak on their farms.

Table 1. Comparison of PCR results on pure and crude extracts of mushrooms or mycelium with standard gel test results for mushrooms.

	Mushrooms			**Mycelium**
Band 3	Band on gel	PCR Pure	PCR Crude	PCR Crude
A15-1 Cont	-	-	-	-
4559 FY	-	-	-	-
4569 SFT	-	-	-	-
4578 FD	-	-	-	-
4583 PS-C	+	+	+	+
1911 BPG	-	-	-	-
1961 WY	-	-	-	-
SSI 61	+	+	+	+
Band 15	Band on gel	PCR Pure	PCR Crude	PCR Crude
A15-1 Cont	-	-	-	-
4559 FY	-	-	-	-
4569 SFT	+	+	+	+
4578 FD	+	+	+	+
4583 PS-C	+	+	+	+
1911 BPG	-	-	-	-
1961 WY	+	+	+	+
SSI 61	-	-	-	-
Band 19	Band on gel	PCR Pure	PCR Crude	PCR Crude
A15-1 Cont	-	-	-	-
4559 FY	-	-	-	-
4569 SFT	+	+	+	+
4578 FD	-	-	-	-
4583 PS-C	-	-	-	-
1911 BPG	-	-	-	-
1961 WY	-	-	-	-
SSI 61	-	-	-	-

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Conclusions

- dsRNA profiles of mushrooms that are negative for MVX contain up to nine apparently "benign" dsRNAs bands (bands 1, 2, H1, 5, H2, 9, 12, 13, and H3 as listed on MVX gel test results). To the best of our knowledge this profile constitutes "negative" status
- Molecular diagnostic tests for specific bands (3, 15 and 19), in conjunction with a crude extract of dsRNA from mushrooms, were as effective at detecting the presence of these bands in **mushrooms** as the standard gel test.
- Molecular diagnostic tests for specific bands (3, 15 and 19), in conjunction with a crude extract of dsRNA from **mycelium**, were also effective at detecting the presence of these bands in mycelium from spawn-run compost, the presence of which was later confirmed by MVX testing of 1st flush mushrooms. In some cases where infection levels are very low, false negatives may occur. An additional "nested" PCR test can be done in such cases to reduce the number of false negatives. This could be useful when trying to identify potential sources of infection such as debris and dust around farms and compost yards.
- Band 3, in conjunction with the nine "negative" / "benign" dsRNAs, does not appear to have any effect on the crop and to date no obvious symptoms are associated with its presence in mushrooms. Consequently, this "MVX" band 3 dsRNA may also be benign. However, it should not be present in normal healthy mushrooms and its presence should be treated with caution. Its presence reflects the contamination of the crop with virus-carrying material (e.g. mushroom spores, mycelium) and should trigger an increase in virus hygiene measures.
- Over a period of four successive crops, no increase in the number of dsRNA bands was recorded when spawn-run compost from one crop was artificially introduced into the following crop at spawning. This suggests that the initial MVX virus inoculum is derived from outside the farm. Levels on the farm can build up if allowed to do so via breeches in virus hygiene measures allowing it to be transmitted to other crops on the farm.

Commercial benefits of the project

We have obtained clarification on what dsRNA profile constitutes a "negative" result for MVX so growers need not be unduly alarmed by the presence of a range of dsRNAs in

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mushrooms, as illustrated in Figure 3. However, the more dsRNAs that are present, the more it suggests that there is some degree of contamination of crops with live *Agaricus* material and due attention should be paid to the potential virus threat that that poses. Molecular diagnostic tests for three MVX bands (3, 15, 19) that are usually present in various combinations in the most symptomatic crops, have been shown to be effective and consistent on both mushroom samples and mycelium from spawn-run compost, when compared with the standard MVX gel test. This means that both mushrooms and spawn-run compost can be tested now for presence of potentially threatening dsRNAs. All tests should be available from **CSL Plant Clinic, Sand Hutton, York YO41 1LZ** in 2007.

Action points for growers

- Have mushrooms checked on a regular basis to monitor the presence of viral dsRNAs.
- Have spawn-run Bulk Phase III compost tested regularly for MVX bands 3, 15 & 19 using rtPCR diagnostic tests. Any positive results should trigger an MVX gel test on mushrooms.
- Although carryover of live compost from one crop into a new crop will not in itself cause a build up of virus, it is essential that compost carry-over does not happen as virus outbreaks will occur once virus-infected propagules find their way onto the site. The original source of a virus infection is unknown but reservoirs of infected propagules are bound to be present within the fabric of any farm that has ever had a virus outbreak.
- Protect spawning operations, spawn-running rooms, casing operations and bulk handling operations from infection with mushroom spores or live mycelial fragments.
- The highly infective nature of MVX-infected mycelium carrying dsRNAs means that only a minute amount of infected material is needed to transmit the disease. Consequently any machinery or equipment handling spawn-run compost, such as winches, Bulk Phase III conveyors, etc. should not be used to handle Phase II compost.

SCIENCE SECTION

Mushroom Virus X disease: Recognition of “negative” status

1 Introduction

Mushroom Virus X (MVX) disease has been a major disease of the cultivated mushroom in Britain and some European countries since the late 1990’s. It has

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contributed to the closure of a number of farms and compost yards, and to increased costs of production through yield and quality reductions. HDC have funded an extensive research programme over the past four years to look at how the disease is transmitted and controlled (Projects M 39, M39 a, M39 b and M39 c). Results from these works have produced information on MVX disease progression, vulnerable stages of mushroom production and advice to growers on where to focus control measures. Many growers have been able to significantly reduce or eliminate the effects of MVX on their farms as a direct result of the information obtained from this earlier work.

As MVX decreases in terms of its significance to the British mushroom industry, it has become apparent that whereas a clear "negative" MVX status can be easily ascertained, there are many ambiguous MVX test results, where a small number of putative MVX-associated dsRNA bands are present that would not be expected in "healthy" samples. Frequently, these few putative MVX bands are present without symptoms though there may have been MVX problems on the site in the past. Such farms may be considered "clean" of MVX but there remain several important gaps in our knowledge concerning the recognition of this "clean" status and maintenance of it. We need to establish if the few putative MVX bands that persist on "MVX-clear" sites could give rise, in time, to an outbreak of MVX

It is conjectured that repeated, recycling contamination will eventually cause ambiguous levels of dsRNAs to rise exponentially to the recognised disease levels (as seemed to be the case with La France Isometric Virus -LIV) but this remains unproven. Results from HDC Projects M 39b & M 39c showed that following regular contamination of healthy compost with compost from an earlier crop, for up to 19 cycles, did not result in MVX or La France disease developing, but a range of dsRNAs were none the less detected (Bands 1, 2, H1, 4, 5, H2, 13, H3). The absence of virus symptoms suggests that these dsRNAs may be asymptomatic and benign, and have no relationship with MVX. We need to establish if continuous contamination of healthy compost with other dsRNAs (such as bands 3, 7, 8, 9, 15, 18, 19, 22 & 23) will result in the eventual development of severe symptom expression. HDC Project M 39c began this work, for putative MVX dsRNAs but only for one crop cycle. There was some evidence to suggest that

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there was an increase in the number and intensity of some bands but this work needs to be continued with fewer isolates.

The commercial objective of this work is to provide information on what level of dsRNA in mushrooms constitutes a negative result for MVX, and what level can lead to future development of the disease. This will improve and optimise crop protection strategies.

In the following report we describe the results of experiments where we have challenged a range of isolates, containing different but ambiguous dsRNA banding patterns, with a regime of poor viral hygiene that is continuous and controlled.

The scientific objectives of this project M 39d is as follows:

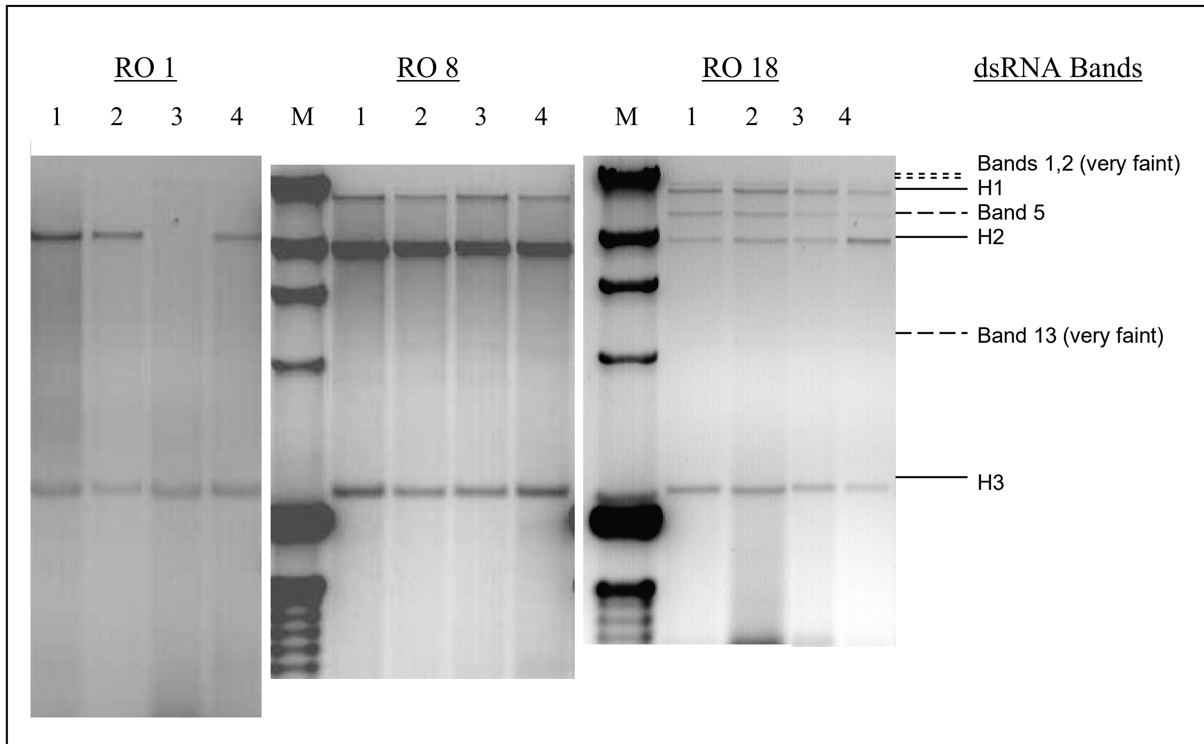
- Conduct rollover experiments for a selected number of isolates with differing dsRNA profiles for a minimum of four crop cycles to determine if dsRNA intensity and number can be increased
- determine what combinations of dsRNAs are associated with an MVX "negative" status in conjunction with an absence of any MVX symptoms
- Evaluate if molecular diagnostic tests for MVX can provide reliable results compared with the standard full extraction & gel test currently used on mushroom samples

2 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, if the same conveyor is used to handle Bulk Phase II and Phase III compost or if Phase III compost propagules contaminate crops or machinery. The results from the previous Virus X reports, M 39b and M 39c, showed that there was an increase in the number and intensity of the dsRNAs normally found in healthy mushrooms (RO1), when compost was continually re-incorporated or "rolled over" into freshly spawned compost for 8, and

19 crop cycles, respectively. The typical dsRNA profile of mushrooms from such "contaminated" rollover crops is shown in Figure 1.

Figure 1. MVX gel test on 4 mushroom samples from Rollover crop 1 (RO1) crop 8 (RO8) and crop 18, showing an increase in number and intensity of dsRNA bands.



These results suggest therefore that bands 1, 2, 5 and 13, as well as bands H1, H2 and H3 are benign or at least not overtly associated with MVX. What they also suggest is that if live (MVX or La France-free) mushroom mycelium does happen to contaminate a fresh crop on a regular basis then there will be a natural build-up of these benign dsRNAs. Thus a dsRNA profile of mushrooms that contains these bands suggests that there has been some contamination of crop with *Agaricus* material either in the form of colonised compost or spores from preceding crops.

A second experiment, reported in M 39c, looked at a range of different MVX strains with various dsRNA band profiles and used the cultures to produce a single crop. That report concluded that complex dsRNA profiles could occur without overt symptoms but that symptoms were more likely to occur if a specific group of dsRNAs (H2, 7, 8, 9 & 15) were present. Bands H2 and 9 are two bands which often occur in non symptomatic crops but band 15 has usually been present in

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severe cases of MVX. Band 3, which occurs as a very strong band, was not always present in severe cases of MVX, and may therefore not play a role in the severity of symptoms. In this project we selected a further eight isolates with various different combinations of dsRNA profiles and conducted rollover experiments with them for four crops to see if there was an increase in the number and intensity of bands with time.

3 Materials and methods

3.1 *Agaricus* isolates

Eight *Agaricus* isolates were selected for testing based on their dsRNA characteristics and details are provided in Table 1. Two isolates were from healthy crops; two were from crops exhibiting brown mushroom symptoms only; two were historic samples taken at the height of the MVX epidemic; one with a faint band 3 and no band 15, the other with a band 15 and no band 3. One isolate was included from a recent severe outbreak of MVX and the final isolate was one produced from a single spore, collected from an MVX-infected mushroom, that contained only the band 3 dsRNA from the parent mushroom.

3.2 Inoculum preparation

For the first rollover experiment (RO1) 1.25 litre polypropylene pots were half filled with Phase II compost which were then autoclaved for 1 hour at 121°C, allowed to rest for 24 hours, then re-autoclaved again to ensure that no viable organisms remained. For each of the *Agaricus* isolates listed in Table 1, two pots of composts were inoculated with eight 5mm plugs; incubated at 25°C for two weeks then refrigerated until needed.

Table 1. *Agaricus* strains used to inoculate healthy crops in rollover experiments. dsRNA bands of interest are underlined.

Strain	Source/code	Crop History	dsRNA Bands in original culture
A15-1 Control	Commercial spawn	Healthy	H1 H3
PY/4559	FY	Healthy	1,2,H1 H2, 9 13 H3
PY/4569	SFT	Brown Mushrooms	1,2,H1 5 H2, 8,9 13 H3, 18,19,23

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PY/4578	FD	Severe crop loss	1,2,H1 5,6,H2, 8,9 13, 15 H3,
PY/4583	PS-C	Brown Mushrooms	3 H2,7, 9 15,15b, H3,18,19
PY/1911	BPG	? Healthy	2,H1, 3f* H2, 8,9 13, H3
PY/ 1961	WY	Severe crop loss	2,H1, H2, 8,9 15 H3
SSI 61	Single spore from MVX strain 1283		3

* band 3 was not seen in any subsequent crops following inoculation with this strain

3.3 Roll-over experiments in bags

Four cropping experiments were conducted one after the other wherein MVX-infected material from either the initial inoculum (rollover crop 1 - RO1) or the previous crop (RO2, RO3, RO4) was introduced into the compost at spawning, to simulate a level of poor crop hygiene, allowing *Agaricus* material to be carried over from an older to a new crop.

Rollover crop 1 (RO1). Heavy duty polythene bags were filled with 20 kg of freshly spawned (Sylvan A15) Phase II compost, and laid out in a growing room. Four replicate bags were prepared for each of the eight cultures listed in Table 1. Each bag was labelled with the appropriate isolate treatment according to the statistical design provided for the experiment. For each of the eight isolates in Table 1, five grams of the spawn-run compost inoculum, described above, was placed in a sterile Petri dish, labelled, and put in a plastic bag to facilitate transport to the mushroom unit. The locations of the relevant bags of spawned compost were identified for each isolate. Wearing disposable rubber gloves, a hole was excavated in the spawned compost to a depth of approx. 15-20 cms. The inoculum from the Petri dish was gently emptied into the hole and covered with the excavated compost – taking care not to disturb the inoculum. The tops of the bags were then folded over loosely to prevent drying out and to facilitate spawn-running. The compost was flattened and compressed with hand pressure in preparation for casing. At the end of the spawn-running period, a small quantity of spawn-run compost was removed from each bag in the RO1 crop to be used as inoculum for all bags in the Rollover 2 crop (RO2). Fresh disposable gloves were used to take

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the samples from each bag. The surface layer of compost was removed from the centre of the bag to reveal the compost below. A small handful (about 20 g) was taken and placed in a sealable polythene bag, labelled with plot position and isolate, brought back to the laboratory and placed in the refrigerator until RO2 compost was spawned. After the samples of compost had been removed from the bags all bags were cased with 7.5-8 litres of casing (Tunnel Tech English with commercial casing inoculum added at standard rate. Two flushes of mushrooms were harvested when mushrooms were at the closed cup stage of development. The crop was then cooked out.

Rollover crop 2 (RO2). Bags of spawned compost (20 kg) were laid out in a new cropping house for the RO2 crop, and labelled with the appropriate isolate treatment according to the statistical design provided for the experiment. The RO1 spawn-run compost sampled earlier was removed from the fridge and introduced into RO2 compost in the same way as described above for RO1. At the end of the spawn-running period, a small quantity of spawn-run compost was removed from each bag in the RO2 crop to be used as inoculum for all bags in the Rollover 3 crop (RO3) as described above.

Rollover crops 3 and 4 (RO3 & RO4). These were conducted in the same manner as described above.

3.4 Experimental design

The layout of each of the four cropping trials was arranged as a Trojan square - this design allows for variation in two dimensions within the cropping house, both across the width and along the length. The design is such that each of the four columns of 8 bags, running along the length of each house, contains a complete replicate of the full set of isolate treatments (Table 1). Similarly, each double row of 8 bags, running across the width of the house, also contains a complete replicate of the set of treatments. There is an additional constraint imposed, such that in each intersection of column and double row, as defined above, no pair of treatments occurs together more than once.

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This design is able to be analysed using the ANOVA algorithm, with the additional advantage that an analysis across all four rollover crops (occasions) can also be produced using ANOVA. Note that the design structure used in the analyses described below was a simplification of the complete design, combining the "between row-column intersection" and "within row-column intersection" as the physical layout of the trial meant that there was no reason to expect the underlying variability to vary between these strata. This simplifies the interpretation of the analysis, as the treatment effect is now only estimated in one error stratum within the design. Note that there are potentially different error estimates associated with comparisons between treatments that do occur together in row-column intersections, and those for treatments that do not.

3.5 Yield and symptom measurements

Yield data from all treatments were plotted as cumulative yield over two flushes to determine if there was any crop delay symptom expressed. Analysis of variance was conducted on yield data to determine (1) if there were any significant effects associated with specific MVX isolates and (2) if there was any increase in the intensity of any effects observed in RO1 in RO2, RO3 & RO4. Crops were monitored for the appearance of other symptoms such as heavy pin sets, poor quality and the presence of brown or off coloured mushrooms.

3.6 Standard MVX dsRNA gel test procedure for mushroom samples

For each MVX isolate treatment, a pooled 150-200g sample of first flush mushrooms was taken for MVX dsRNA analysis consisting of 2-4 mushrooms from each of the four replicate bags. Mushrooms were halved then sliced with half of each mushroom going into a test sample and the second half of each mushroom going into a back-up sample. It was not economically feasible to test every plot individually. The sliced mushrooms were frozen, then freeze-dried and ground to a powder. A purified total dsRNA extract was obtained and run on an electrophoresis gel to separate out the different dsRNAs (according to protocols described in Grogan *et al.* 2003). DsRNA bands were recorded for each MVX isolate for each rollover crop. On some occasions dsRNA analysis was conducted

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on 2nd flush mushrooms also. Two micro litres (μl) of the purified extract was retained for rtPCR testing (see below).

3.7 RtPCR molecular test procedures for mushroom samples

In addition to full dsRNA extraction from mushrooms and visualisation of them on a gel, mushroom samples were also tested for the presence of specific dsRNAs (Bands 3, 15 and 19), using a reverse transcriptase polymerase chain reaction (rtPCR) molecular diagnostic method. RtPCR was conducted on 2 μl of the purified extract obtained for the dsRNA gel test. In addition, RtPCR was conducted on a crude extract of dsRNA. This was obtained using a Tri-Reagent extraction method on 50 mg of the same ground mushroom sample used in the purified extraction process (see http://www.mrcgene.com/tri.htm). RtPCR results were scored as positive or negative for each of the three bands in question (Bands 3, 15 and 19). Control tests included rtPCR detection of *Agaricus* ribosomal RNA in all mushroom samples, and rtPCR detection of each of the three bands in an archive MVX sample as a positive control.

3.8 RtPCR molecular test procedures for spawn-run compost samples

RtPCR tests were conducted on the spawn run compost samples that were used as inoculum for each rollover crop for two reasons. One was to verify which of the three dsRNAs 3, 15 and 19 were present in the inoculum and the second was to obtain information to validate the use of rtPCR diagnostics for spawn-run compost. RtPCR is difficult to do when samples contain a high loading of complex organic molecules such as the humic substances that are present in mushroom compost, and which can inhibit some of the chemical reactions taking place. To increase the likelihood of the rtPCR tests working effectively, spawn run compost was incubated on nutrient agar plates for 5 days and the *Agaricus* mycelium that grew out from the compost was scraped off, freeze-dried and ground, thereby minimising the amount of compost material present in the rtPCR reaction tubes. The freeze-dried mycelium was then used to generate a crude extract of whatever

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dsRNAs were present in spawn-run compost, using the Tri reagent method. RtPCR was then conducted as described above.

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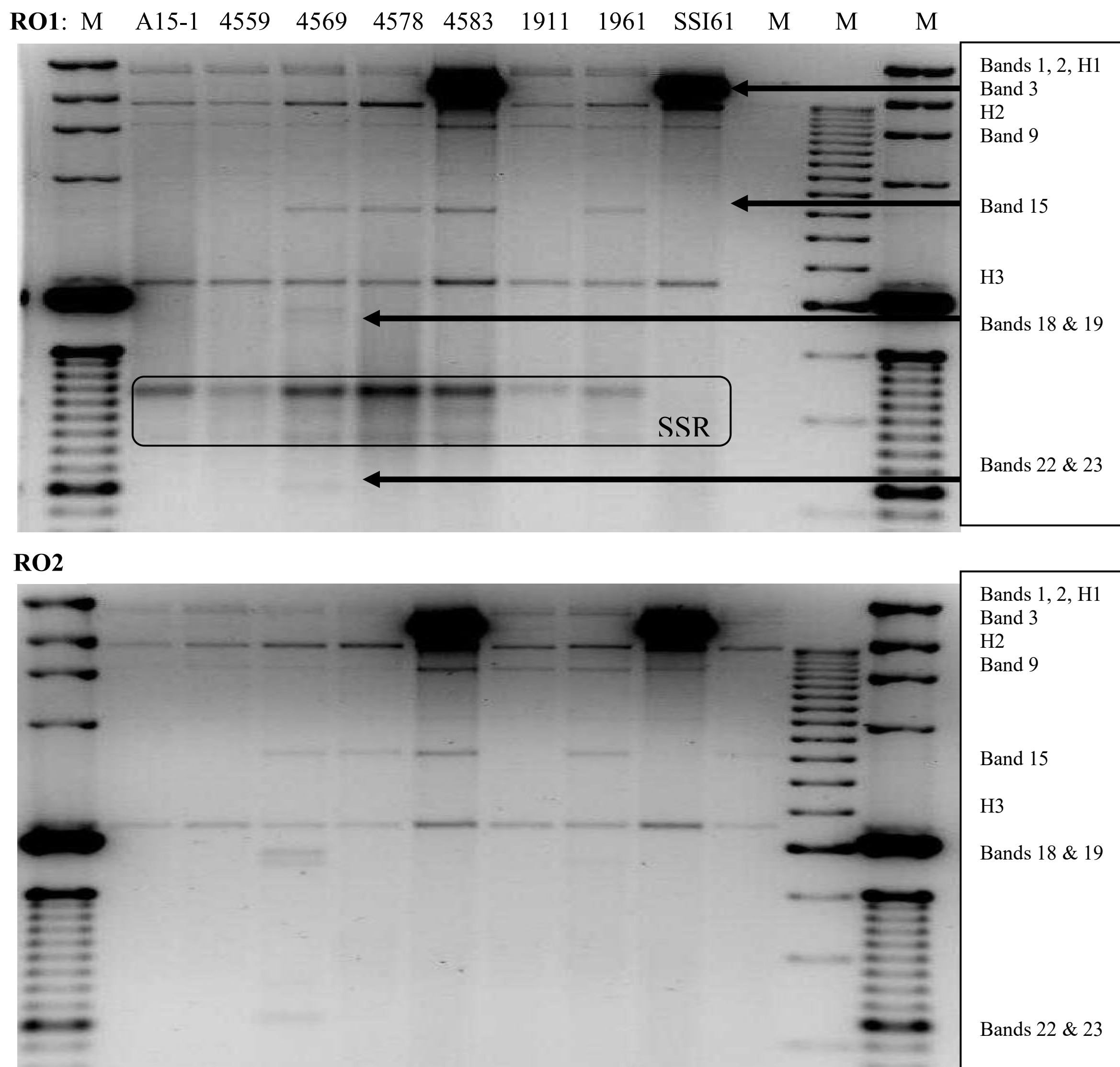
4 Results and discussion

For each of the individual isolates tested there was virtually no further increase in the number of dsRNA bands detected in mushrooms harvested from successive rollover crops (Figure 2). DsRNA profiles for each isolate remained more or less the same from RO1 to RO4. Of the eight isolates tested, four consistently showed significant crop delay relative to the control in each of the four rollover crops (4569 SFT, 4578 FD, 4583 PS-C and 1961 WY) and three isolates (1911 BPG, 4559 FY and SSI 61) showed no significant crop delay relative to the control (A15-1) in each of the four rollover crops. The data for all four rollover crops have been combined to produce Figure 3.

Figure 2. DsRNA profiles of eight 1st Flush mushroom samples harvested from



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rollover crops RO1, RO2, RO3 & RO4. M = marker lanes; SSR = single stranded RNA sometimes detected in fresh mushroom samples.

Figure 2. continued. DsRNA profiles of eight 1st Flush mushroom samples harvested from rollover crops RO1, RO2, RO3 & RO4. M = marker lanes; SSR = single stranded RNA sometimes detected in fresh mushroom samples.

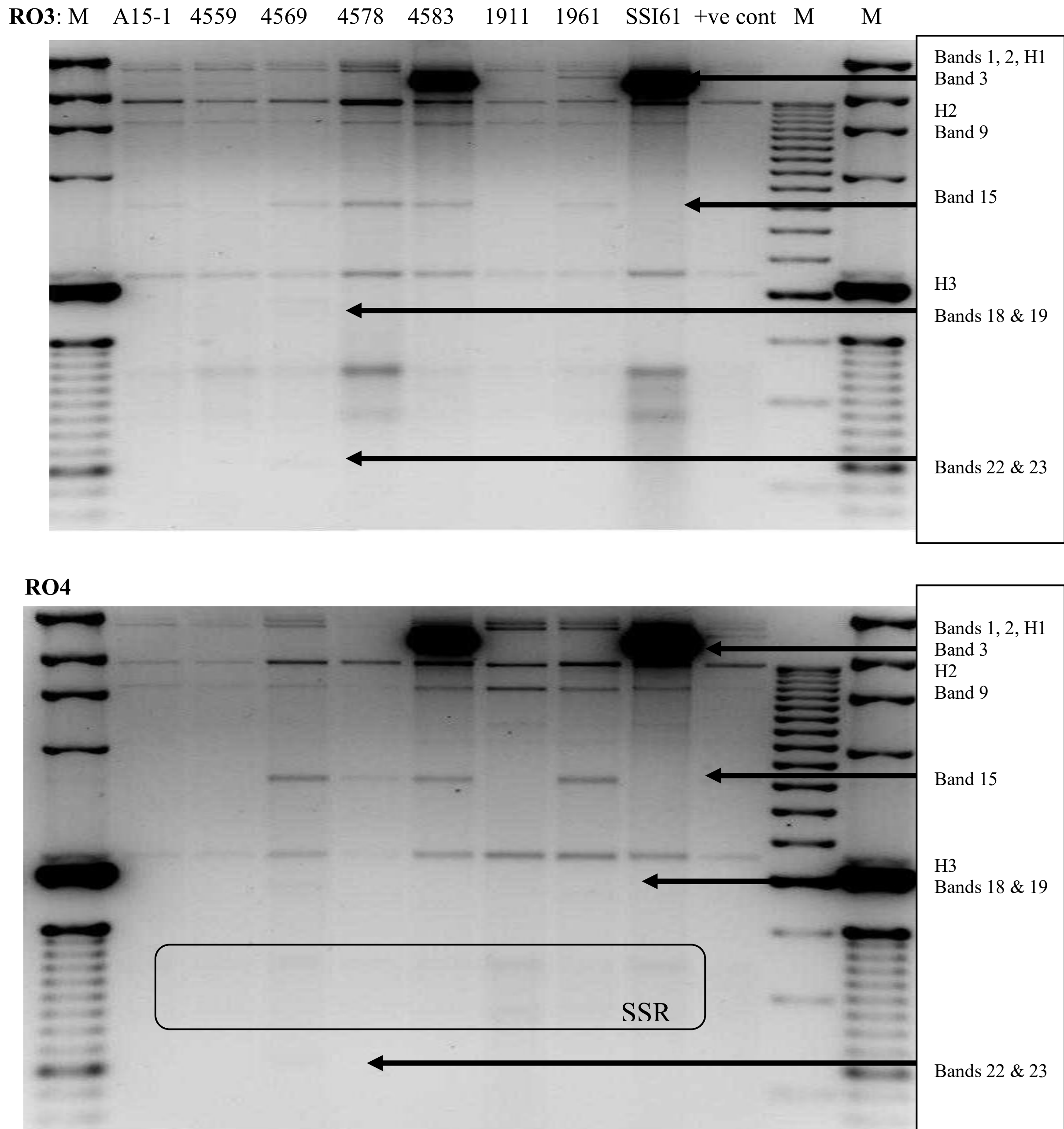

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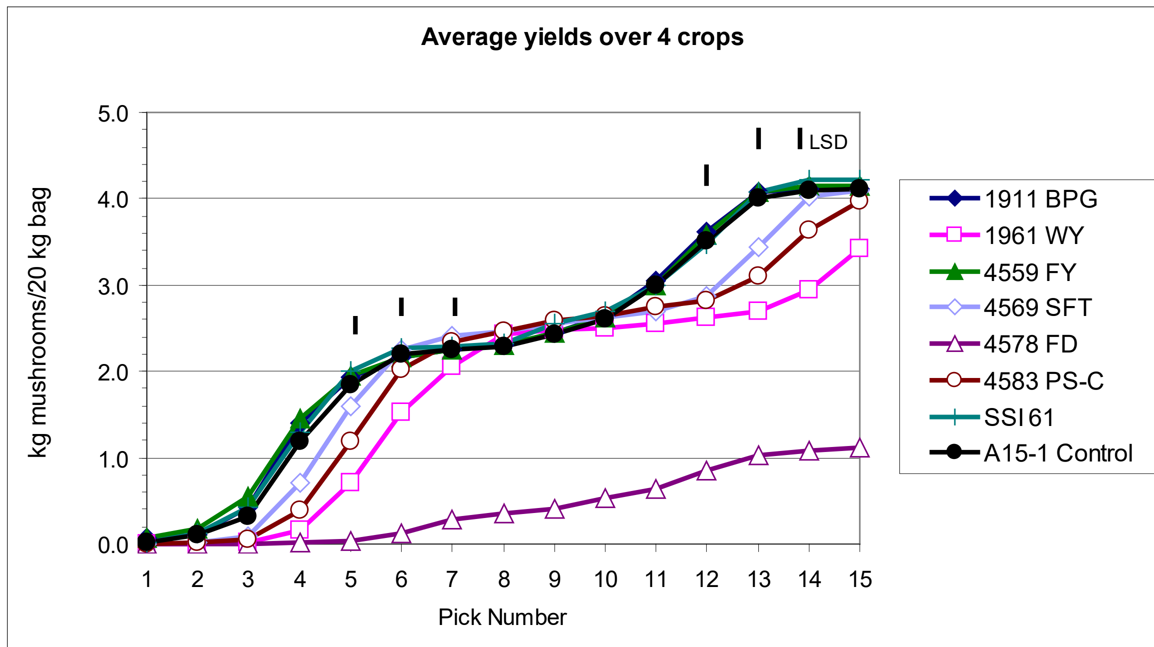
Figure 3. Yield of mushrooms harvested following the introduction of different *Agaricus* isolates into freshly spawned compost for each of eight isolates including a control A15-1. Yields for four successive rollover crops were amalgamated for each isolate as trends were the same in all crops. LSD = least significant difference at *P*< 0.05; 72 degrees of freedom.



4.1 “Negative” status

Bands 1, 2, H1, H2, 9, 13 and H3 occurred in all mushroom samples including the Control A15-1 throughout the rollover crops. A weak band 5 occurred in four samples including the control while a weak band 12 occurred in all samples in RO 2 and RO3, in all samples except the control in RO1, and all samples except the control and 4578 in RO4. These nine bands: 1, 2, H1, 5, H2, 9, 12, 13 and H3; are believed to be background dsRNAs present in normal crops where some *Agaricus* material may contaminate crops from one crop cycle to another, via mushroom spores or spawn-run compost fragments. This profile would therefore constitute a “negative” for MVX. If freshly spawned compost is kept free of any contaminating material the dsRNA profile may contain as few bands as 1, 2, H1 and H3. The additional bands appear to occur when *Agaricus* material such as spores or mycelium from an older crop infiltrate a new crop.

4.2 Band 3

Two isolates, 4583 PS-C and SSI 61, had a strong band 3 in the original starting culture. This band continued to be transmitted to all four rollover crops via the

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incorporation of spawn-run compost from one crop to another. Incorporation of isolate 4583 PS-C resulted in consistent crop delay while incorporation of isolate SSI 61 did not (Figure 3). This would suggest that band 3 in itself does not have an impact on *Agaricus* production. However, band 3 would not be considered to be normal background and its presence in a mushroom sample should trigger a review of virus hygiene measures. Band 3 has not been detected on its own in commercial mushroom samples however the fact that we were able to obtain an *Agaricus* culture with just Band 3 derived from a single spore from an MVX-infected mushroom containing a wide range of other dsRNAs, suggests that it could also happen in a commercial crop.

A faint band 3 was detected on one occasion in RO 3, in mushrooms from compost inoculated with 1961 WY, which did not originally have this band. It was not detected again in mushrooms from the following crop, RO4. The one off nature of this occurrence suggests that it may have been as a result of contamination of one of the plots during RO2 as the molecular rtPCR test on the inoculum used for RO3 eventually gave a weak positive when tested with a more sensitive test than the standard test (see below).

4.4 Band 15

Four isolates, 4569 SFT, 4578 FD, 4583 PS-C and 1961 WY, all had a band 15 in the original starting culture and this band continued to be transmitted to all four rollover crops via the incorporation of spawn-run compost from one crop to another. Incorporation of these four isolates into freshly spawned compost resulted in varying degrees of crop delay (Figure 3). To date band 15 is the only dsRNA band that appears to be correlated with crop delay although the magnitude of the delay can be quite variable from mild to severe. Any MVX isolate that has shown crop delay and pinning disruption in experimental crops has had the band 15 dsRNA. However we do not have band 15 dsRNA in a single spore to test the hypothesis that is categorically correlated to crop delay symptoms. This is an area for future work. It would be useful to prove categorically if band 15 on its own is consistently associated with crop delay and pinning disruption symptoms as this would mean that a diagnostic test for band 15 would be critical to determine the potential risk to a mushroom crop. The evidence at the moment is circumstantial

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so it is still prudent to monitor for the presence of band 15 until such time as we determine otherwise.

A band 15 was detected on one occasion in the Control A15-1 mushrooms from RO3 but was not detected again in mushrooms from the following RO4 crop. The one-off nature of this occurrence suggests that it may have been as a result of contamination of one of the plots during RO3. The molecular test results (see below) confirm it was not detected in the mycelium-inoculum added at spawning using the standard molecular rtPCR test so one or more of the control plots may have been contaminated at a later stage, probably casing. No crop delay was recorded for the A15-1 control treatment. In HDC reports M 39b and M 39c we demonstrated how infection of spawn-run compost at casing with either mushroom spores or mycelium containing band 15 did not always result in crop delay but the mushrooms from such late infected crops always contained band 15. This suggests that in our RO3 rollover experiment the control treatment somehow became infected at casing with band 15-containing material (probably some spawn-run compost fragments from an adjacent band15-containing treatment). A more sensitive test of the mycelium inoculum would help to further determine if band 15 was detectable in the inoculum or not (see below)

4.5 Bands 18, 19, 22 & 23 - the “brown bands”

One isolate, 4569 SFT, had the four low molecular weight “brown” bands 18, 19, 22 & 23 in the original culture, which were transmitted to all four rollover crops via the incorporation of spawn-run compost from one crop to another. However no brown mushrooms were detected in any of the crops. This might reflect the fact that brown mushroom symptom expression is complex. Previous research has shown that the symptom can be erratically expressed if a low level of infected mycelium is incorporated at spawning, (as in this trial), but consistent expression of the symptom requires that the infected mycelium is introduced into the crop around casing time (M 39b).

One of the *Agaricus* cultures selected for these experiments, 4569 SFT, was obtained from a mushroom sample originating from a crop showing the brown mushroom symptoms and which had tested positive for bands 18 and 19 in the

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original MVX gel test. However, throughout the course of the rollover crops no bands 18 & 19 were detected either by gel test or by molecular PCR tests. The "loss" of the brown bands from this culture may be due to sub culturing the isolate. Alternatively, more powerful "nested" PCR tests may be needed to detect its presence in the pure extract. It would be interesting to know if this culture would be capable of causing brown mushroom symptoms if it was incorporated into a crop around casing time: - the infection time that leads to the most consistent expression of the brown mushroom symptoms.

A faint band 19, associated with the brown mushroom symptom, was detected in the 2nd flush mushrooms of RO1 for isolate 1961 WY, and was consistently detected at a faint level in 1st flush mushrooms in RO2, RO3 and RO4. No brown mushrooms were detected in any of the crops. The band 19 for 1961 WY is just visible in the RO2 gel reproduced in Figure 2, but the bands - although present - were too faint to be visible on the reproduced gel images for RO3 and RO4. The band 19 appears to occur without any of the other associated bands 18, 22 or 23. The molecular test results (see below) confirm it was present in the mushrooms but not detected in the inoculum, however the molecular test on compost is not as sensitive as the molecular test on mushrooms. Band 19 may have been in *Agaricus* mycelium in the inoculum but at a level that was not detected with the test used. The consistent occurrence of band 19 in all four rollover crops suggest it is a true result, and that it has been transmitted via the inoculum in each rollover crop. More sensitive molecular tests were conducted on the inoculum which also gave a negative result raising the question of whether or not rtPCR can detect low level concentrations in spawn-run compost and how compost should be sampled for such tests. Further work also needs to be done to clarify the significance of the occurrence of one brown band (19) in isolation in terms of its potential to produce brown mushroom symptoms if given the right conditions.

4.6 Molecular diagnostic tests.

In addition to the full extraction and gel electrophoretic test conducted on mushroom samples from the rollover crops (Figure 2), additional molecular tests were conducted to detect specific dsRNA bands. RtPCR tests were used to detect

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dsRNA bands 3, 15 and 19 in the following samples: - (A) the purified dsRNA extract obtained for the gel electrophoretic test; (B) a crude extract of dsRNA from the same sample of mushrooms and (C) a crude extract from *Agaricus* mycelium present on the spawn-run compost used as inoculum in each of the rollover crops. The results are presented in Table 2.

In all cases where there was a band 3 or 15 was identified on a gel, the rtPCR test on the same purified extract was also positive, as would be expected. RtPCR tests on a crude mushroom extract of these samples were also positive, giving confidence that the simpler test agrees with the more laborious test. On one occasion a weak band 3 was detected in the mushrooms of 1961 WY in RO3 using the standard gel test. This was confirmed with rtPCR on the crude extract of the mushrooms. An RtPCR test on the crude extract from the inoculum-mycelium, using the same method, was negative, suggesting perhaps that the infective material was not in the inoculum but was introduced into the crop at a later stage, possibly casing. However, a more powerful "nested" PCR technique was successful at detecting the band 3 in the inoculum-mycelium. Thus, negative results with PCR must be interpreted with caution as false negatives are possible when crude extracts of the material are used. Similarly for Band 19, where this was clearly identified on a gel, the rtPCR tests on both the purified and crude extracts were positive. For one sample however (1961 WY), band 19 was detected only as a very faint band, initially in 2nd flush mushrooms (RO1), then in 1st flush mushrooms (RO2, RO3 & RO4). RtPCR tests on the purified extracts were positive but on the crude extracts they were negative, indicating that diagnostic tests using crude extracts may give false negative results. Again, when "nested" PCR tests were done on these samples for band 19 they were positive, highlighting the possibility of false negatives with PCR techniques when using crude extracts.

4.7 Molecular diagnostic tests on spawn-run compost samples.

One of the most interesting aspects of this project was to see if molecular diagnostic tests could be used to detect specific dsRNAs in spawn-run compost as an indicator of the potential for MVX dsRNAs to then be present in the mushroom

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crop from that compost. The standard rtPCR tests on all the spawn-run compost inoculum used for all the strains in all the rollover crops were positive for any dsRNA bands that were known to be present in the original cultures (Table 2) with the exception of band 19 in 4583 PS-C as discussed above. This result gives confidence that molecular diagnostic tests on spawn run compost could be a useful tool to detect the potential for MVX infection with regard to the specific bands tested, namely bands 3, 15 and 19.

The PCR diagnostic test however has highlighted some interesting results in a few cases where a faint band occurred in all crops RO1 to RO4 (band 19 1961 WY) or where a treatment was contaminated as a once off event (band 3 in 1961 WY RO3; band 15 in A15-1 RO3).

For one of the MVX isolates used -1961 WY - a band 19 was consistently detected in the mushrooms in all four rollover crops. Band 19 was not detected in the original culture although bands 18 and 19 have been detected in mushrooms from some experimental crops, using this isolate -1961 - (see M39b and M39 c). Given that band 19 was consistently detected in the mushrooms, as a faint band on a gel, by PCR on the pure extract, and by nested PCR on the crude extract it would be reassuring to be able to detect it in the compost inoculum as it is reasonable to assume that this band is being transmitted with the mycelium inoculum. However, neither the straightforward PCR test nor the more sensitive nested rtPCR tests were able to detect the band 19 in the inoculum mycelium used for any of the RO crops. This potential “false negative” may be explained by a number of factors:

- The concentration of the band 19 dsRNA may not have reached a high enough level in the mycelium of the spawn-run compost compared to the level in the mushrooms produced 17 days later
- The sample size may not have been big enough to provide sufficient template dsRNA for the PCR to work
- Band 19 dsRNA levels may possibly be much higher in mushrooms than in mycelium due to the fact that they are associated with a mushroom symptom rather than a mycelium symptom.

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A nested PCR test on the mycelium inoculum for 1961 WY RO3 gave a positive result for band 3 where the simple PCR test on the same sample was negative. (We were unable to conduct a nested PCR on the mycelium inoculum for A15-1 RO3 for band 15 to clarify if band 15 might also be detected by the more sensitive test). Thus nested PCR tests are more sensitive and would eliminate a number of false positives. However there would be a cost implication if nested PCR tests were conducted as standard as it requires two PCR tests to be conducted instead of one.

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Table 2. RtPCR diagnostic test results for dsRNA Bands 3, 15 and 19 on (A) purified full extract of 1st Flush mushrooms; (B) crude extract from the same mushroom sample; (C) crude extract of mycelium in spawn-run compost used as inoculum. Presence/absence of the band on the electrophoretic gel using the purified full extract is in the first column for each rollover crop for comparison. F = faint band on gel; (+F2) = faint positive on 2nd flush mushrooms; X = positive for band not normally associated with this isolate; (N+; N-) = positive/negative by nested PCR technique; ? = unconfirmed result.

Band 3	RO1				RO2				RO3				RO4			
	band on gel	(A) PCR full ext	(B) PCR mush	(C) PCR myc-Inoc	band on gel	(A) PCR full ext	(B) PCR mush	(C) PCR myc-Inoc	band on gel	(A) PCR full ext	(B) PCR mush	(C) PCR myc-Inoc	band on gel	(A) PCR full ext	(B) PCR mush	(C) PCR myc-Inoc
A15-1 Cont	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4559 FY	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4569 SFT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4578 FD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4583 PS-C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1911 BPG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1961 WY	-	-	-	-	-	-	-	-	**xF**	**x**	**x**	- **(N+)**	-	-	-	-
SSI 61	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Band 15	RO1				RO2				RO3				RO4			
	band on gel	(A) PCR full ext	(B) PCR mush	(C) PCR myc-Inoc	band on gel	(A) PCR full ext	(B) PCR mush	(C) PCR myc-Inoc	band on gel	(A) PCR full ext	(B) PCR mush	(C) PCR myc-Inoc	band on gel	(A) PCR full ext	(B) PCR mush	(C) PCR myc-Inoc
A15-1 Cont	-	-	-	-	-	-	-	-	**x**	**x**	**x**	-	-	-	-	-
4559 FY	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4569 SFT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4578 FD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4583 PS-C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1911 BPG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1961 WY	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SSI 61	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Band 19	RO1				RO2				RO3				RO4			
	band on gel	(A) PCR full ext	(B) PCR mush	(C) PCR myc-Inoc	band on gel	(A) PCR full ext	(B) PCR mush	(C) PCR myc-Inoc	band on gel	(A) PCR full ext	(B) PCR mush	(C) PCR myc-Inoc	band on gel	(A) PCR full ext	(B) PCR mush	(C) PCR myc-Inoc
A15-1 Cont	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4559 FY	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4569 SFT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4578 FD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4583 PS-C	-	-	-	-	-	-	-	**?**	-	-	-	-	-	-	-	-
1911 BPG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1961 WY	- (+F2)	-	-	-	+F	+	- **(N+)**	- **(N-)**	+F	+	- **(N+)**	- **(N-)**	+F	+	- **(N+)**	- **(N-)**

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SSI 61	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

5 Conclusions

- dsRNA profiles of mushrooms that are negative for MVX symptoms may contain a variety of apparently benign dsRNAs which include bands 1, 2, H1, 5, 9, H2, 12, 13, and H3 as listed on MVX gel test results (Appendix 1). To the best of our knowledge this profile constitutes "negative" status.
- Molecular diagnostic tests for specific bands (3, 15 and 19), in conjunction with a crude extract of dsRNA, were as effective at detecting the presence of these bands in **mushrooms** as the standard gel test. Although the gel test gives a more complete picture of dsRNAs present in a mushroom sample, rtPCR molecular diagnostic tests could be used to check for the presence of specific dsRNAs in single mushrooms. RtPCR tests are also very useful for tracking the movement of dsRNAs in experimental situations.
- Molecular diagnostic tests for specific bands (3, 15 and 19), in conjunction with a crude extract of dsRNA, were also effective at detecting the presence of these bands in **mycelium** from spawn-run compost, the presence of which was later confirmed by MVX testing of 1st flush mushrooms. Although false negatives are always possible, an additional more sensitive "nested PCR" test can eliminate some false negatives but further work is necessary to determine how accurate molecular diagnostics can be in predicting the presence of MVX dsRNAs in non-mushroom samples (e.g. spawn-run compost, dust and debris).
- Over a period of four successive crops, no increase in the number of dsRNA bands was recorded when spawn-run compost from one crop was artificially introduced into the following crop at spawning suggesting that the critical factor in MVX outbreaks is the initial contamination of the site or farm by MVX-infected material followed by continual breeches in virus hygiene measures on the site.

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6 References

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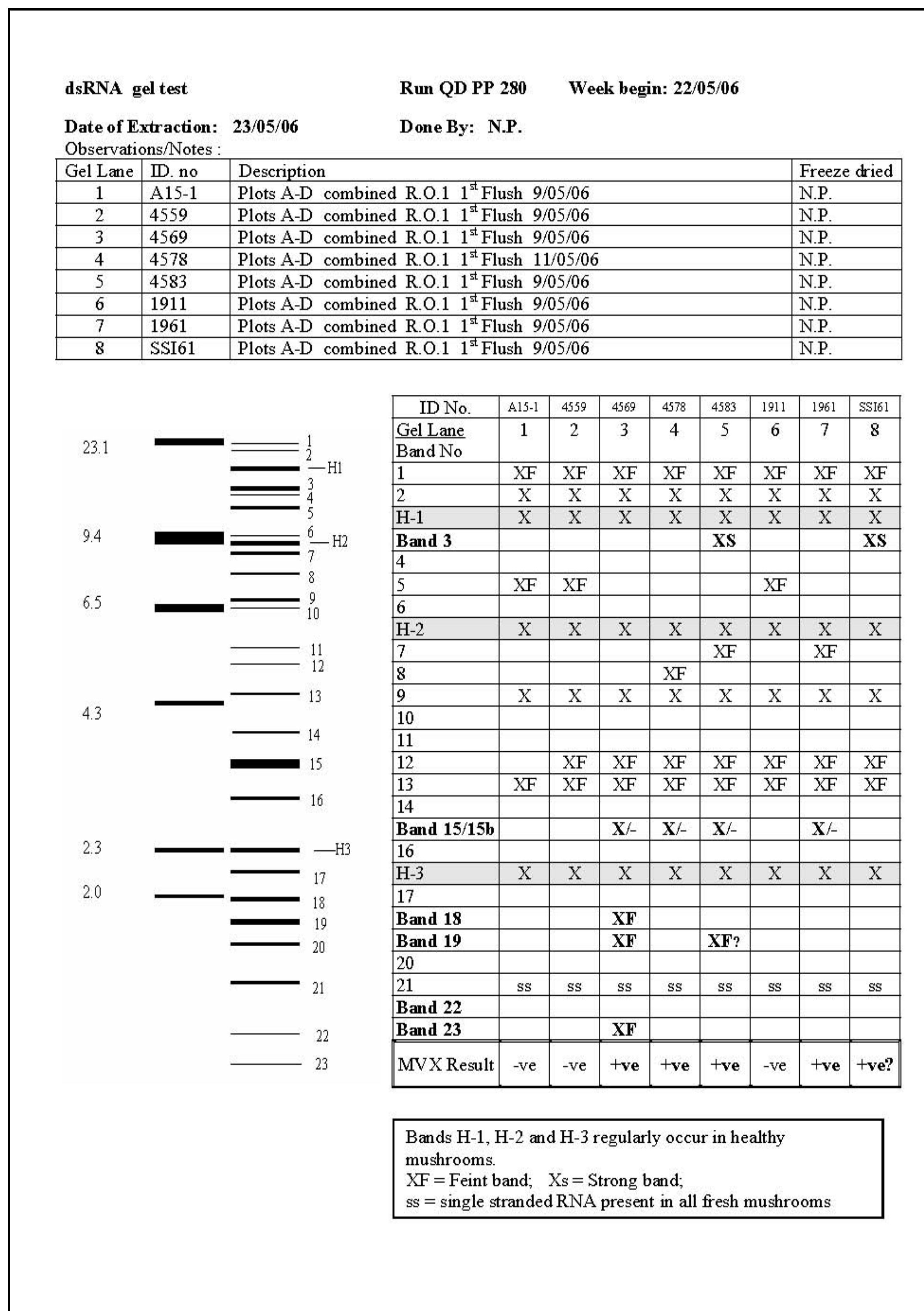
7 Appendix 1

MVX gel test schematic result:



dsRNA gel test **Run QD PP 280** **Week begin: 22/05/06**

Date of Extraction: 23/05/06 **Done By: N.P.**

Observations/Notes :

Gel Lane	ID. no	Description	Freeze dried
1	A15-1	Plots A-D combined R.O.1 1st Flush 9/05/06	N.P.
2	4559	Plots A-D combined R.O.1 1st Flush 9/05/06	N.P.
3	4569	Plots A-D combined R.O.1 1st Flush 9/05/06	N.P.
4	4578	Plots A-D combined R.O.1 1st Flush 11/05/06	N.P.
5	4583	Plots A-D combined R.O.1 1st Flush 9/05/06	N.P.
6	1911	Plots A-D combined R.O.1 1st Flush 9/05/06	N.P.
7	1961	Plots A-D combined R.O.1 1st Flush 9/05/06	N.P.
8	SSI61	Plots A-D combined R.O.1 1st Flush 9/05/06	N.P.

ID No.	A15-1	4559	4569	4578	4583	1911	1961	SSI61
Gel Lane Band No	1	2	3	4	5	6	7	8
1	XF	XF	XF	XF	XF	XF	XF	XF
2	X	X	X	X	X	X	X	X
H-1	X	X	X	X	X	X	X	X
Band 3					**XS**			**XS**
4								
5	XF	XF				XF		
6								
H-2	X	X	X	X	X	X	X	X
7					XF		XF	
8				XF				
9	X	X	X	X	X	X	X	X
10								
11								
12		XF	XF	XF	XF	XF	XF	XF
13	XF	XF	XF	XF	XF	XF	XF	XF
14								
Band 15/15b			**X/-**	**X/-**	**X/-**		**X/-**	
16								
H-3	X	X	X	X	X	X	X	X
17								
Band 18			**XF**					
Band 19			**XF**		**XF?**			
20								
21	ss	ss	ss	ss	ss	ss	ss	ss
Band 22								
Band 23			**XF**					
MVX Result	-ve	-ve	**+ve**	**+ve**	**+ve**	-ve	**+ve**	**+ve?**

Bands H-1, H-2 and H-3 regularly occur in healthy mushrooms.
XF = Feint band; Xs = Strong band;
ss = single stranded RNA present in all fresh mushrooms