Project title	Developing an accurate, quantitative and predictive test for Mushroom Virus X.
Project number:	M 51
Project leader:	Dr Kerry Burton, University of Warwick
Report:	Final report, December 2010
Key staff:	Dr Kerry Burton (University of Warwick) Dr Adam Baker (University of Warwick) Dr Dan Eastwood(University of Warwick) Dr Helen Grogan (Teagasc, Kinsealy R & D Centre, Dublin)
Location of project:	University of Warwick
Industry Representative:	Richard Gaze
Date project commenced:	18 January 2010
Date project completed (or expected completion date):	17 October 2010
Key words:	

The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with 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.

Signed on behalf of: Warwick HRI

Signature:……..…….…… Date: ……..……..

Professor Brian Thomas
Acting Director and Head of Department

CONTENTS

GROWER SUMMARY

Headline

- A highly sensitive diagnostic test for Mushroom Virus X has been developed which detects both forms of the disease (brown and pinning disruption symptoms) in spawn-running Phase III compost.

Background and expected deliverables

Mushroom Virus X disease syndrome (known as MVX) causes a number of symptoms, including pinning disruption, crop delay, premature veil opening, various fruitbody abnormalities and brown-coloured mushroom caps. The form of the disease which causes the brown symptom is currently prevalent. However the industry should always be wary of the virus form that causes bare patches (pinning disruption). This form of MVX has been so devastating that some farms have gone out of business as disinfection measures appear to have limited effect.

MVX disease is thought to be spread within the mushroom industry by contaminated compost, with re-infection occurring from contaminated compost, equipment and farm debris. The disease is associated with the presence of double-stranded RNA (ds-RNA) molecules. Identification of the disease is hampered by the current detection method (separation and observation of ds-RNA on gels) which is slow and difficult to interpret. In addition, this current test is so insensitive that a large quantity of material is required (i.e. mushroom fruitbodies) to provide any indication of infection. Using mushrooms for the test is too late in the cropping cycle when the costs of production,, picking and sales commitments have already been incurred.

This project (M51) aimed to develop a detection method which has increased sensitivity to detect the virus. It is based on the technique called Quantitative PCR. PCR is a molecular technique that involves amplification of single-stranded RNA and it is this amplification that allows very low levels of RNA to be detected. Quantitative PCR (also known as ‘real-time’ PCR) is, as the name suggests a quantitative technique and so provides a gauge of the degree of MVX infection.

Summary of the project and main conclusions

Before Quantitative PCR can be adapted into a reliable technique for use for the industry to detect MVX in compost or casing, three key technical questions had to be addressed and solved:

1) Can purified RNA be extracted from compost?

It is relatively easy to extract RNA with high purity from mushroom fruitbodies. It is more difficult to extract RNA from casing material, but this technique has been achieved previously by the scientists undertaking this project. However extracting RNA from compost is a more difficult task, as compost contains large amounts of humic and phenolic compounds which stick to the RNA and interfere with the manipulations as part of the Quantitative PCR methodology.

- A successful method has been designed in this project which extracts purified RNA from compost and without significant RNA degradation.

2) Does mycelium infected with MVX *also* have raised levels of MVX RNA that can be measured by quantitative PCR?

Previous research has shown that MVX-infected mushroom fruitbodies have raised levels of single-stranded RNA (from MVX) compared with non-infected mushrooms.

- This project found that infected **mycelium** in compost and casing also had higher levels of RNA (of MVX) than non-infected compost or casing.

3) Can the new quantitative PCR method detect all forms of MVX?

Recent research suggests that MVX is a syndrome caused by a collection of viruses which may explain the large number of ds-RNA bands and the diverse symptoms associated with MVX including tissue browning and pinning disruption. The key question to answer is: ‘Can quantitative PCR tests be developed that detect all forms of the MVX infections that are commonly found and economically important to the industry’?

- The diagnostic test for MVX developed in this project is based on two MVX sequences. This combined test was able to detect MVX in a range of strains producing the browning symptom and the pinning disruption symptom from spawn-running compost.

This project has therefore developed a detection method for Mushroom Virus X with such an increased sensitivity that it can detect the virus at low levels in **compost prior to cropping**. It can detect the MVX strains that cause browning and pinning disruption. The test will benefit the industry in two ways:

- It will enable detection of early infection when virus levels are low and so give advanced warning to growers.

- It will identify the sources of infection and so enable disease control.

The new diagnostic test is quantitative and indicates the amount of active virus present in a sample. The results indicate that active MVX levels are much higher in the compost than the casing. Therefore compost is a better substrate for testing the presence of the virus. The level of virus detected in compost increases enormously from spawn-run to compost producing mushroom fruitbodies. Virus levels are not affected by the amount of infection introduced by a crop. Even small amounts of infection at spawning leads to approximately the same virus levels during spawn-run and during cropping as high amounts of infection at spawning.

Financial Benefits

Mushroom Virus X is a significant and occasionally devastating disease for the industry. MVX causes financial losses through both yield loss and/or product rejection due to quality issues (brown colouration). The current test for MVX is too insensitive to detect the disease early during mycelial growth in compost and so too late in the cropping cycle to be of much use in real time. The new diagnostic test developed in this project can detect MVX in compost which will give (1) advanced warning to growers of MVX infection and so allow action to be taken to reduce losses due to the disease and (2) the ability to trace the cause of infection which is likely to lead to fewer outbreaks with the knock-on financial benefits. The new test also opens up the possibility of regular compost testing which will give greater

confidence that future crops will be MVX-free

Action Points for Growers

- Growers and compost producers can now use a test to detect MVX and ensure crops and compost are free from infection.

- When future MVX outbreaks occur, this test can be used as a diagnostic tool to ascertain the source of infection and to test infected equipment and machinery to minimise the risks of re-infection.

- Growers and composters are invited to consider to what extent they may wish to use this test. This information will be necessary for the business case to make the test commercially available. Kerry Burton now of East Malling Research (kerry.burton@emr.ac.uk) would like to make this test available to the industry and to deliver results in a timely fashion. There are financial planning implications however and he requests that growers and composters contact him directly if they are interested.

SCIENCE SECTION

Introduction

Mushroom Virus X disease syndrome (known as MVX) is a serious disease for the mushroom industry which causes a number of symptoms, including pinning disruption, crop delay, premature veil opening, various fruit body abnormalities, and brown-coloured mushroom caps (Grogan *et al.,* 2003). The disease is associated with the presence of double-stranded RNA (ds-RNA) molecules which can be visualised on a gel and known as ds-RNA bands. A set of 26 distinct ds-RNA bands were identified, ranging in size between 0.64 Kbp and 20.2 Kbp. These are inferred to be the causal agents of MVX disease (Grogan *et al*., 2003; Adie *et al*., 2004). As these viruses appear to be little more than double-stranded RNA molecules, the only way to detect them is to use molecular methods.

It is now clear that the collection of MVX ds-RNAs represents a complex of viruses. The 12.75 Kbp band of MVX has been sequenced, and described as an endornavirus, AbEV1 (Maffettone, 2007). Sonnenberg and Lavrijssen (2004) found that sequence analysis of the portion of the 17 Kbp band shared significant similarity with *Cryphonectria parasitica* hypovirus. Most recently it has been hypothesized that the low molecular weight ds-RNAs associated with the symptom of cap browning (2.0, 1.8, 0.8 and 0.6 Kbp in size) are due to the presence of a further distinct virus named as 'Brown Cap Mushroom Virus' (BCMV), which is of the 'partitivirus' class of viruses (Green, 2010).

The current detection method for MVX of visualizing double-stranded RNA bands has shed some light on the disease. However the low sensitivity of this method does not give advanced warnings to growers of compost that is infected, or enable the industry to understand how compost and crops can become infected and what and where the sources of infection are.

A recent Walsh Ph.D. student, Julian Green (working with Kerry Burton, Helen Grogan and Dan Eastwood) has revealed that MVX can be detected in mushroom fruitbodies by a technique known as Quantitative PCR. PCR is a molecular technique that involves amplification and it is this amplification that allows very low levels of an agent to be detected. Quantitative PCR (also known as 'real-time' PCR) is a specific type of PCR which counts the number of PCR cycles before the target RNA reaches a threshold value. This then enables the amount of RNA to be quantified. Green detected very high levels of single-stranded RNA

of MVX origin in mushroom fruitbodies, however we do not know whether this RNA indicating MVX presence can also be detected earlier in the growing cycle e.g. in the compost mycelium.

This project is aimed at developing a highly sensitive detection method for MVX to detect the virus at early infection in the compost when virus levels may be low. A method that is quantitative would allow growers to gauge degree of infection.

There are three questions for the research to answer, these are:

a) Is it possible to extract RNA from compost with sufficient purity and integrity for the Quantitative PCR testing?

   It is relatively easy to extract RNA with high purity from mushroom fruitbodies. It is more difficult to extract RNA from casing material but this technique has been achieved by the scientists undertaking this project. However extracting RNA from compost is a more difficult task as compost contains large amounts of humic and phenolic compounds which stick to the RNA and interfere with the manipulations as part of the Quantitative PCR methodology. The key issue is to develop the technique to extract purified RNA from compost but without significant RNA degradation.

b) High levels of virus single-stranded RNA have been detected in mushroom fruitbodies infected with MVX, from a previous project. The question is whether this is reflected by high levels of single stranded RNA in the mycelium of infected compost and casing?

c) There is increasing evidence that MVX is a collection of different viruses. The question is: Can PCR methods be developed to detect the different forms of MVX that have been found in infected material from industry?

Project Strategy

The project strategy to answer these questions was:

1. The development a method to extract purified RNA (high quality and non-degraded) from mushroom compost.

2. Identify candidate genes for a MVX detection system by screening using whole genome mushroom microarrays. Candidate genes were chosen as those having increased levels as a result of MVX infection in the compost and casing as well as mushroom fruitbodies.

3. Use Quantitative PCR to compare the performance of the candidate genes in detecting MVX from different infected MVX strains of mushrooms collected over a number of years. The genes that are able to detect MVX in all strains and from different conditions being assigned for the diagnostic test.

Materials and methods

While Mushroom Virus X can cause major problems for the mushroom industry, it consists biologically of little more than a collection of double-stranded RNA molecules. This project is aimed at detecting those molecules using molecular methods.

Biological Material

The biological material used in this project was: non-infected and MVX-infected mushroom fruitbodies and mycelium taken from compost, casing and from axenic culture.

A) Non-infected or control mushroom fruitbodies and mycelium from colonised casing and colonised compost were collected from A15 crops grown at Warwick HRI under standard conditions on two separate occasions. Axenic mycelial cultures (laboratory cultures) were grown on Compost Extract Medium (Calvo-Baldo, 2000) at Warwick HRI.

B) MVX-infected material

The material used to identify candidate genes by microarray screening are shown below a-d.

a. The culture grown mycelial samples were grown axenically on Compost Extract Medium at Warwick HRI from stock straws (strains 2735, 1283 and 1961). Four replicates samples were used per strain.
b. Four MVX fruitbody samples were collected. Three were collected during visits to infected farms in Ireland (2008); these samples are not therefore assigned a specific

strain but displayed the symptoms of browning. The fourth MVX fruitbody sample was provided by Teagasc (November 2009) and was of strain 2735.

c. All four MVX colonised compost samples were provided by Teagasc (April 2010) and were of strain 4569.
d. Four MVX colonised casing samples were collected. Samples 1 and 2 were provided by Teagasc (November 2009) and were of strains 2735 and 4569 respectively. MVX colonised casing samples 3 and 4 were provided by Teagasc (April 2010) and are both of strain 4569.

The material to compare candidate gene performance with different MVX strains using Quantitative PCR

e. Teagasc grew 5 strains harbouring MVX at different inoculation rates which provided a source of compost and casing samples during September 2010. These strains have been collected from industry infections over a number of years and they produce different symptoms.

The 5 MVX strain numbers are:

Three strains producing 'classic' MVX 'patch' symptom

- 1283
- 1961
- 2735

Two strains producing the 'brown' symptom

- 4549
- 4614

Compost was infected with each MVX strain by two distinct methods which will be referred to as Inoculation rates when displaying the results:

- Low inoculation rate (0.01%): MVX Infected compost added to compost at a rate of 0.01% (i.e. 1 in 10^{-4} dilution) at the time of spawning.
- High inoculation rate (25%): MVX infected spawn added to non-infected spawn at a rate of 25% and then the spawn was used to spawn compost.

Compost was sampled for analysis at two times

- End of spawn run (early)
- During cropping (late)

Casing was sampled during cropping

The experimental layout is shown in Table 1 (first 4 columns).

Table 1. Extraction of RNA from samples for the microarray screening exercise: Description of Samples and the Purity and Concentration of the RNA

Sample name	Material	Strain	MVX Status	Conc	260:280	260:230
Control Casing 1	Casing	A15	Control	580.5	2.15	2.44
Control Casing 2	Casing	A15	Control	657.6	2.07	1.56
Control Casing 3	Casing	A15	Control	159.3	2.16	2.00
Control Casing 4	Casing	A15	Control	396.2	2.06	1.43
MVX Casing 1	Casing	2735	MVX	682.3	2.06	1.91
MVX Casing 2	Casing	4569	MVX	330.1	1.98	2.02
MVX Casing 3	Casing	4569	MVX	324.8	2.11	1.52
MVX Casing 4	Casing	4569	MVX	363.4	2.10	1.57
Control Compost 1	Compost	A15	Control	309.7	2.02	1.54
Control Compost 2	Compost	A15	Control	310.5	2.04	1.70
Control Compost 3	Compost	A15	Control	178.2	1.83	1.10
Control Compost 4	Compost	A15	Control	286.5	1.85	1.22
MVX Compost 1	Compost	4569	MVX	146.0	1.98	1.40
MVX Compost 2	Compost	4569	MVX	116.5	1.95	1.45
MVX Compost 3	Compost	4569	MVX	112.6	1.96	1.25
MVX Compost 4	Compost	4569	MVX	99.0	1.87	1.25
Control Fruitbody 1	Fruitbody	A15	Control	1634.3	2.15	2.20
Control Fruitbody 2	Fruitbody	A15	Control	926.9	2.10	2.26
Control Fruitbody 3	Fruitbody	A15	Control	2461.5	2.14	2.13
Control Fruitbody 4	Fruitbody	A15	Control	1110.2	1.91	1.79
MVX Fruitbody 1	Fruitbody	Brown	MVX	1040.8	2.07	1.72
MVX Fruitbody 2	Fruitbody	Brown	MVX	2002.5	2.14	2.10
MVX Fruitbody 3	Fruitbody	Brown	MVX	960.3	2.08	1.68
MVX Fruitbody 4	Fruitbody	2735	MVX	1020.0	2.05	1.87
Control Culture 1	Culture	A15	Control	981.7	1.99	1.14
Control Culture 2	Culture	A15	Control	131.3	2.04	0.97
Control Culture 3	Culture	A15	Control	703.4	1.99	0.89
Control Culture 4	Culture	A15	Control	1390.2	2.05	1.13
1283 Culture 1	Culture	1283	MVX	293.2	1.98	1.26
1283 Culture 2	Culture	1283	MVX	777.6	2.04	1.45
1283 Culture 3	Culture	1283	MVX	305.8	1.96	1.36
1283 Culture 4	Culture	1283	MVX	789.0	2.03	1.42
1961 Culture 1	Culture	1961	MVX	730.2	2.01	1.40
1961 Culture 2	Culture	1961	MVX	759.0	1.97	0.72
1961 Culture 3	Culture	1961	MVX	680.4	1.94	0.69
1961 Culture 4	Culture	1961	MVX	900.8	1.97	0.71
2735 Culture 1	Culture	2735	MVX	644.3	2.02	1.73
2735 Culture 2	Culture	2735	MVX	991.7	2.08	1.97
2735 Culture 3	Culture	2735	MVX	634.5	2.05	1.84
2735 Culture 4	Culture	2735	MVX	814.2	2.10	1.95

RNA Extraction

RNA was extracted from fruitbody and mycelium grown in pure culture using a standard protocol using Tri-Reagent®.

Initial attempts to extract RNA from colonised compost using a method based on the FastRNA Pro Soil-Direct kit (MP Biochemicals) failed to adequately separate RNA from the humic acids present in the compost. This was evident as the extracts were brown in colour and was reflected by low absorbance ratios (Table 2).

Table 2 The 260:230 ratio for RNA extracted from four samples of compost by modified and original protocol. The ratio is much greater using the modified protocol indicating improved purity.

	Original Protocol	**Modified Protocol**
Sample 1	0.27	1.22
Sample 2	0.12	1.45
Sample 3	0.40	1.40
Sample 4	0.37	1.40

A number of additional steps were added to the protocol in order to enhance the purification of RNA from the humic acids. This procedure is based on the FastRNA Pro Soil-Direct kit with the following modifications:

- Freeze drying and milling compost samples at -80° C
- Addition of reducing agent into lysing buffer.
- Removal of phenolics and humic acids by size exclusion column
- Clean up and concentration of RNA sample using the RNeasy Minelute cleanup kit.

The final protocol gave consistently purer samples, as indicated visually in Figure 1 and in the ratio of absorbance at 260nm and 230nm detailed in Table 2. This modified method was used to extract high quality RNA from all subsequent compost and casing samples for this study.

Following extraction, RNA concentration, purity and integrity was assessed using the Nanodrop 1000 (Thermo Scientific) and the Agilent 2100 Bioanalyzer.

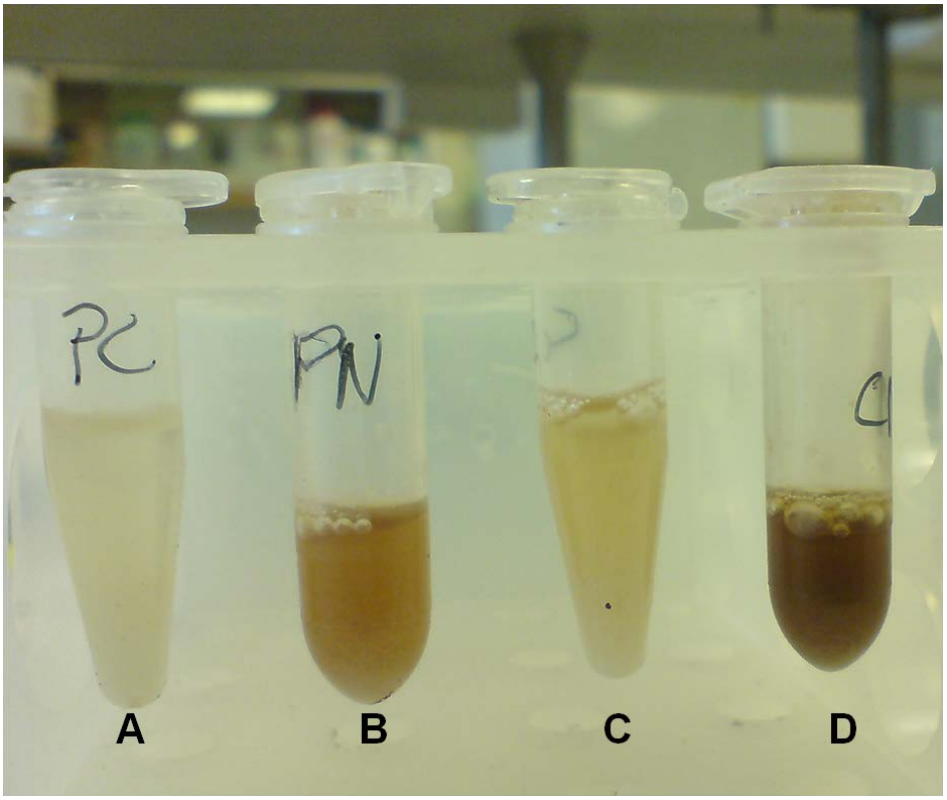

Figure 1 RNA Extracts from colonised compost showing the removal of phenolic and humic substances as indicated by the reduction in brown coloration. Samples A and B are extracts from compost during spawn-run, and C and D are extracts from compost producing mushrooms. Samples A and C were extracted using the modified method. Samples B and D were extracted using the original method.

Use of Microarrays for Screening

The purpose of this part of the project is to screen all the known MVX sequences and genes and all of the mushroom genes to identify the best candidate genes that can be used to indicate the presence of MVX in compost, casing and mushroom fruitbodies. To do this screening exercise we used the latest cutting-edge genomic technologies of *microarrays*.

Microarray Design

This project used the world's first whole genome microarrays of the mushroom *Agaricus bisporus* which were custom designed for this project and manufactured by Agilent Technologies. These are based on the 60,000 probe format, each probe is a 60-mer oligonucleotide (oilgo). The recent annotation of the *Agaricus bisporus* genome (in May 2010) identified 10,438 proposed genes which were used to design up to 5 oligo probes per

gene by the software e-array. Technically speaking the correct term for these proposed genes is Open Reading Frame (or ORF) which refers to a region of the DNA which is inferred to be a gene but not proven to be a gene. Each oligo was intended to be from a different part of the gene. These accounted for about 52,000 oligos on each array. In addition, further probes were designed for the array taken from the version 3 arrays which in turn originated from Warwick projects (including the previous MVX projects) and the EMBL data-base (total 2,197 replicated 3 times) these probes can be identified by the prefix ABP_. Finally additional probes were added to the microarrays to act as controls and for spike-ins.

Microarray processing

The microarray screening examined 4 different developmental/environmental states of the mushroom (i.e. fruitbodies, mycelium in axenic culture, compost and casing) and 2 infection states (MVX infected and non-infected). Four independent replicate RNA samples were examined as biological replicates. The total number of microarrays used was 4 X 4 X 2 =32.

RNA samples were processed using the Quick Amp Labeling Kit, one-color (Agilent) according to the manufacturer's instructions. Once labelled, the samples were hybridised to the microarray slides overnight and scanned using Agilent's High-Resolution C Scanner. Analysis of the image was carried out using the Feature Extraction Software (Agilent), which creates a project, assigns grid, links intensity to probe ID and carries out quality control metrics. This project was then imported in the Genespring software (Agilent) where normalisation was carried out and statistical analysis was undertaken.

Statistical Analysis of Microarray data

Samples were grouped by type (fruitbody, colonised compost, colonised casing and culture grown mycelia) and MVX status (control (A15) versus MVX strain). Differences were identified by 2 way ANOVA with Benjamini and Hochberg False Discovery Rate (Benjamini, and Hochberg, 1995) correction for multiple testing. Those probes identified as showing a statistically significant difference in expression, between control and MVX samples, of greater than 100 fold were selected for further analysis.

Quantitative PCR

RNA was extracted from 20 infected compost samples and10 infected casing samples from the 5 MVX infected strains as indicated in the *Materials and Methods* (*Biological Materials*

section)and Table 4. RNA was also extracted from non-infected casing and compost colonised with strain A15. RNA samples were DNase treated (RQ1 RNase free DNase, Promega) according to manufacturer's instructions. DNase treated samples then underwent reverse transcription using Superscript II (Invitrogen) reverse transcriptase according to manufacturer's instructions producing cDNA samples ready for PCR analysis.

Primers were designed, using Primer Express (Applied Biosystems), to specifically amplify sections of each of the identified bands based on their previously established sequence. These primers were then optimised using Sybr Green for detection and the ABI 7900HT thermocycler (Applied Biosystems). Standard curves were run, which were assessed for linearity and efficiency to ensure accurate quantification.

Samples were run in triplicate (three technical replicates for each biological sample) and primers for the ribosomal RNA 18S were also run as an endogenous control. Gene expression data is corrected for 18S level and is given relative to control samples.

Results

1) To development of a method to extract RNA (high quality and non-degraded) from compost.

When the four modifications were applied to the RNA extraction method, major improvements were observed in the purity of the RNA. Phenolic and humic contamination were reduced as evidenced by the reduction in the brown colour of the extract (Figure 1) and the increase in the 260/230 and 260/280 ratios (Table 2). The RNA was shown to be of good integrity with little evidence of RNA degradation as the Bioanalyzer traces show clearly-defined peaks for the two ribosomal RNAs (18S and 28S) with a low baseline (see Figure 2).

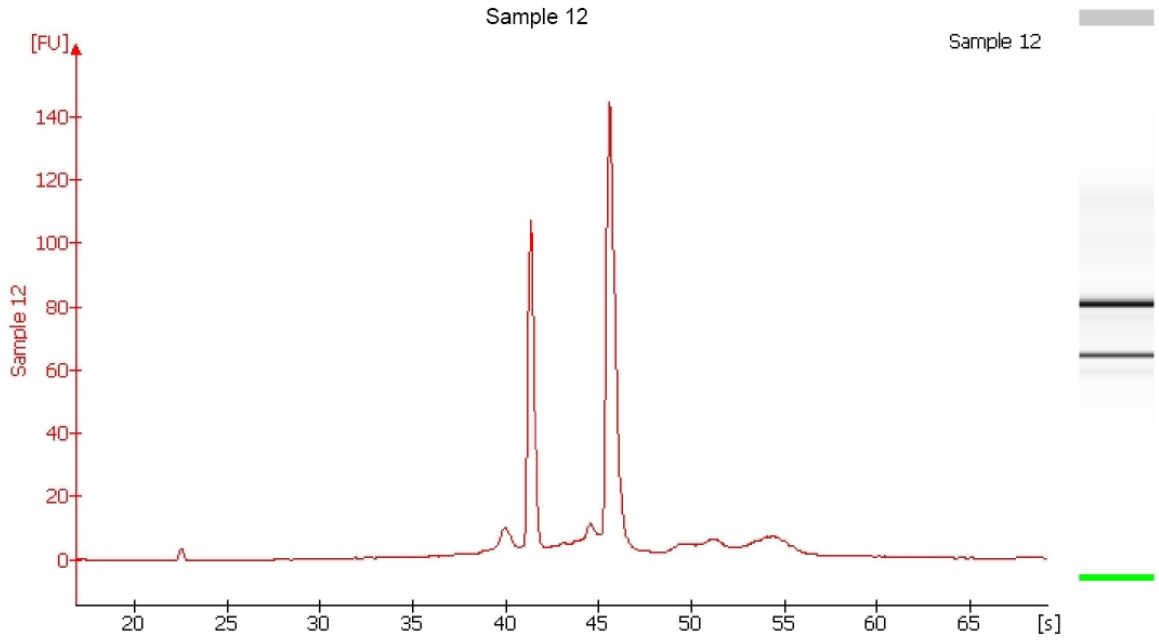

Figure 2 A representative Bioanalyzer trace of extracted RNA. The low base-line and well-defined peaks indicate a low level of RNA degradation.

2) Identify candidate genes for a MVX detection system by screening whole genome mushroom microarrays.

The aim of this component of the project is to identify specific RNA sequences and genes which can indicate the presence of MVX in infected compost and casing, and then which genes show the highest and most consistent increase upon infection to be chosen as the candidate genes for MVX detection. The microarray experiment examined gene expression of 55,000 probes for each of the 32 samples. A total of 1.76 million items of data were collected by this experiment which were analysed and processed using specialist software called GeneSpring. As the final purpose of the project was to develop a detection method, the data processing was focussed to identify probes which show large changes upon infection by MVX.

The number of probes showing at least 100-fold difference between control (non-infected) and MVX infected samples were:

- 97 probes in fruitbody samples
- 102 probes In mycelium of colonised casing
- 88 probes in mycelium of colonised compost
- 31 probes in mycelium grown in axenic (laboratory) culture

All but three of the identified probes related to non-*Agaricus* genome transcripts i.e. probably of MVX origin. The 3 genes of *Agaricus* origin were identified from the compost samples.

The consistency of these probes across sample types was assessed and it was found that no probe with a 100-fold difference featured in all 4 sample types. Specifically there was no cross over between the probes identified in the mycelium of colonised compost and mycelium grown in axenic culture. The axenic cultures were the least important for the detection of the disease commercially, so the focus of attention was moved to looking at the probes which show large changes in mushroom fruitbodies, and the mycelium of compost and casing. 74 probes were found to have changes by 100-fold difference or more in fruitbodies, mycelium in compost and mycelium in casing (see Figure 3).

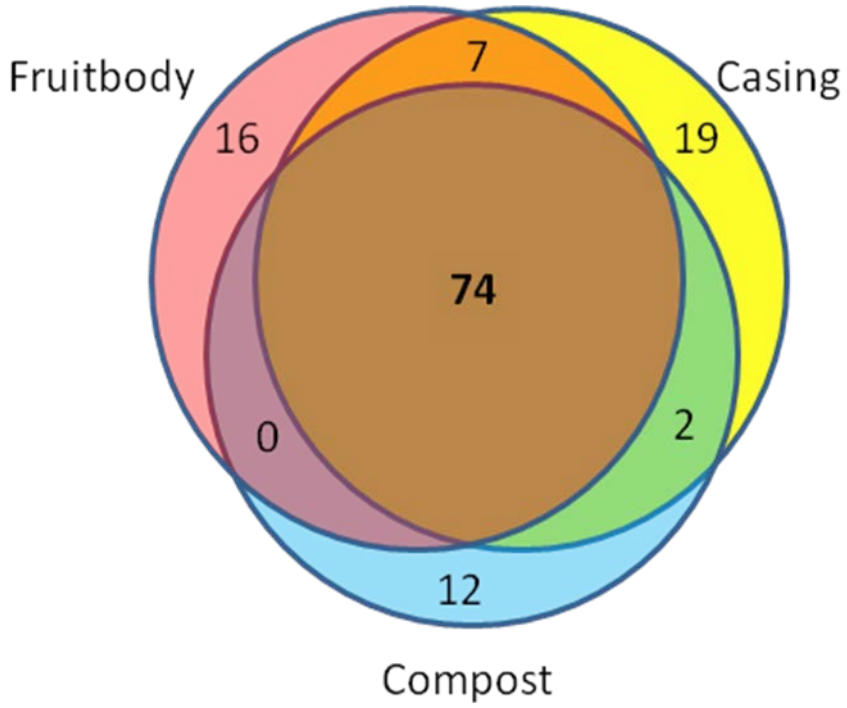

Figure 3 A Venn diagram showing the number of probes which increase (by a factor of 100 fold or greater) due to MVX infection in the mushroom fruitbodies, and mycelium in colonised casing and colonised compost samples.

The probes were then selected that showed the most consistent and significant up-regulation and the best 'spread' of detection across the range of MVX types examined. These probes were then related to the greater length of sequence or band from which the probes was originally designed. The selected probes related to bands 18, 19, 20, 22+, 23, the Low Molecular Weight Bands and band 9 (see Table 3). These probes were then used for developing the quantitative PCR test.

Table 3 Summary table of selected probes which demonstrate presence of MVX from Microarray Screen. Eight Probes were selected and their expression is shown in Mushroom Fruitbodies, Mycelium in Compost, Casing and Culture. Expression is presented as fold change relative to non-infected control samples and therefore increased expression due to MVX are indicated by figures greater than 1.

Probe Name	Band	Mushroom Fruit Bodies	Mycelium in Compost	Mycelium in Casing	Mycelium in Pure Culture		
					1283	1961	2735
UNC:b9c7t7.abi	9	860	0.9	12.8	94.5	1.1	50.4
UNC:19-14_T7.abi	19	3999	3804	3013	2.1	3.1	0.9
UNC:B23C82T3.ABI	23	2413	1016	12493	0.9	2.6	0.3
ABP_2008_JG_BH_2	22+	3306	2209	1842	1.6	1.3	0.9
ABP_2120_JG_BW_1	18	4281	2287	1157	1.6	2.1	1.0
ABP_2122_JG_BW_2	LMW	1487	750	14100	0.7	2.7	0.3
ABP_2125_JG_BW_2	19	3141	1553	470	1.4	1.8	0.9
ABP_2135_JG_BW_1	20	2912	412	669	1.3	1.4	0.9

3) Use of Quantitative PCR to compare the performance of the candidate genes in detecting MVX from a range of infected mushroom strains collected over a number of years.

The candidate genes identified by the microarray work were hybridised to the microarray by a 60 nucleotide region of the gene. The next step in preparation for the Quantitative PCR work is to design optimal primer pairs based on the full sequence known for each band or gene. Using these primer pairs PCR reactions were carried out of the RNA extracted from the samples of the 5 strains harbouring MVX and the control non-infected mushroom culture. The Quantitative PCR results are given in Table 4 which shows the ratio of gene expression of infected material compared with the non-infected control material i.e. the fold change, if the value is greater than one, then this indicates up-regulation upon infection.

Table 4 The detection of five MVX strains by 8 different primer sets by quantitative PCR of RNA extracted from compost and casing. The results are given as fold change i.e. the ratio of PCR data from infected compared with non-infected material. Early and late refer to the time the material (compost or casing) was sampled; early compost was during spawn run and late compost and casing was during cropping. High and low refer to rate of inoculation (see *Materials and Methods*).

Strain	**Early Late**	**Material**	**High Low**	Band 9	Band 19	Band 23	Band 22+	Band 18	LMW Bands	Band 19 JG	Band 20
				Expression Level Relative to Control Samples							
1283	Early	Compost	Low	5226	44	61	25	1498	107	4540	24
1283	Early	Compost	High	4373	2	2	1	65	2	69	0
1283	Late	Compost	Low	422748	501	1	1	0	0	0	0
1283	Late	Compost	High	487015	487	1	1	0	0	0	1
1283	Late	Casing	Low	181	1	2	1	10	2	59	1
1283	Late	Casing	High	67	4	3	2	17	5	449	5
1961	Early	Compost	Low	0	1	2	1	1	3	58	1
1961	Early	Compost	High	6	2	1	1	5	1	76	1
1961	Late	Compost	Low	716	2253	3	1	1	2	1	2
1961	Late	Compost	High	414	557	2	2	0	1	0	2
1961	Late	Casing	Low	0	0	0	1	2	1	25	0
1961	Late	Casing	High	0	0	0	0	1	0	45	0
2735	Early	Compost	Low	1009	4	4	3	88	6	269	2
2735	Early	Compost	High	1162	1	1	1	27	1	56	1
2735	Late	Compost	Low	165098	179	0	1	0	0	0	1
2735	Late	Compost	High	180953	733	2	1	1	1	1	1
2735	Late	Casing	Low	391	9	7	4	70	9	436	7
2735	Late	Casing	High	82	5	5	3	31	10	239	5
4569	Early	Compost	Low	3781	43533	52187	28860	855758	86387	2546853	20651
4569	Early	Compost	High	9327	114196	111845	16328	2545364	214745	6892898	66755
4569	Late	Compost	Low	311576	151590	198	214	88	67	73	219
4569	Late	Compost	High	729949	589142	757	471	238	416	221	957
4569	Late	Casing	Low	759	1230	1276	1097	7845	1317	69021	894
4569	Late	Casing	High	948	13871	9084	3922	89844	12103	647222	12565
4614	Early	Compost	Low	761	2	1	1	3	1	2	0
4614	Early	Compost	High	2098	1	1	1	30	1	185	1
4614	Late	Compost	Low	228597	241	0	1	0	0	0	0
4614	Late	Compost	High	308930	272	0	0	0	0	0	0
4614	Late	Casing	Low	226	2	2	2	31	2	55	1
4614	Late	Casing	High	206	1	0	1	0	0	0	0

The primer sets based on Band 9 gives strong signals for all strains harbouring MVX except strain 1961 (when sampled from compost inoculated early or from casing). Fortunately the primer sets based on Band 19JG produce strong signals (25 to 76 fold) for the samples when the Band 9 primers produce weak signals.

Based on these results Band 9 can be seen to give consistently raised expression levels in MVX harbouring strains across the sample types analysed See Table 5. If only one test was to be performed then this primer set would be recommended. However, we recommend two PCR tests to include the primer sets Band 9 and Band 19JG. Band 19JG also shows high levels of expression and complements Band 9. For samples that were not easily detected by the Band 9 primer set, Band 19JG showed good detection, this is especially the case for strain 1961 (see Tables 5 and 6).

Therefore the results of this project indicate that combination of two Quantitative PCR tests based on primers for Band 9 and Band 19JG represents a diagnostic test that can detect a wide range of MVX infections in compost or casing at low levels of infection. The Quantitative PCR data for band 9 and band 19JG are shown in Tables 5 and 6 respectively.

The authors wish to express a point of caution in interpreting the data shown in Tables 4-6. The increase or fold-change in gene expression due to MVX infection for some primer set-sample combinations is in the region of thousands to hundreds of thousands. It is tempting to see lower increases, of say 25 fold, as a weak signal. However this is still a strong signal indicating MVX infection, the larger results are simply very, very strong.

Table 5 Fold Change compared to control samples for primer set to Band 9

		Early compost		**Compost during cropping**		**Casing during cropping**	
	Inoculation level →	low	high	low	High	low	High
STRAIN							
1283		5,200	4,500	423,000	487,000	181	67
1961		0.4	5	716	414	0	0.5
2735		1,000	1160	165,000	181,000	390	82
4569		3,800	9,300	312,000	730,000	760	950
4614		760	2,100	229,000	309,000	226	206

Table 6 Fold Change compared to control samples for primer set to Band 19JG

		Early compost		Compost during cropping		Casing during cropping	
	Inoculation level →	low	high	low	High	low	High
STRAIN							
1283		4,540	69	0	0	59	449
1961		58	76	1.5	0.4	25	45
2735		269	56	0	0.7	436	239
4569		2,500,00	6,900,00	73	221	69,000	647,000
4614		2.3	185	0.4	0	55	0.3

Discussion

The use of a combined test involving two quantitative PCR reactions based on different primer pairs has been shown to be successful in detecting all of the MVX forms that were investigated. Examination of Table 4 appears to show an inverse relationship between the quantitative PCR results using the band 9 primer and the Band 19JG primer, i.e. a decline in one (from early to late compost) is accompanied by an increase in the other and vice versa. This suggests that the mycelium has a limited capacity to support both viruses, and one predominates at the expense of the other.

The project has also contributed to the understanding of the biology of MVX disease. Prior to this project, fully quantitative data were not available to examine the effects of infection levels and the rates of MVX proliferation. There is now quantitative evidence that very low levels of infection at the time of spawning results in massive increases in measurable MVX in the mycelium of the compost from spawn-run to cropping. This applies for 4 of the strains tested. This strongly infers that any amount of infection, no matter how small, is likely to lead to serious disease development.

The amount of measurable MVX in the casing was generally much smaller than the compost. Therefore casing does not represent a good substrate for testing MVX. The lower amounts of MVX in the mycelium of casing may be because this substrate is non-nutritious

and that casing mycelium has a different morphology to compost mycelium with the majority of the mycelium in strands.

Conclusions

The control of MVX disease can now be enhanced:

- A new diagnostic technique has been developed that can detect both forms of Mushroom Virus X (browning symptom and pinning disruption symptom) at low levels in Phase III compost.
- This test provides advanced warning to growers and can be used to identify the sources of infection.
- Growers and composters now have the opportunity to encourage the commercial availability of this test by informing HDC and/or Kerry Burton.
- The quantitative results of this project also show that
    - small amounts of early infection at the time of spawning leads to massive increases in infection in the compost during spawn-run and cropping
    - The virus levels are low in the casing. Therefore compost is the better substrate for testing for MVX. It is also interesting that MVX does not thrive in the mycelium of casing.

Knowledge and Technology Transfer

The interim results of this project have been discussed with the HDC Mushroom Panel on 15th June and 2nd November 2010, and the full results were presented on 8th March 2011. Kerry Burton intends to present the results to an International Mushroom Conference in Arcachon, France in October 2011. The Mushroom Panel discussed how to establish if growers would use the potential diagnostic service, suggestions were made to advertise in the Mushroom press and discuss at the International Mushroom Conference.

Glossary

- ds-RNA – Double-stranded RNA. The genes of MVX exist as ds-RNA
- Gene – A region of natural DNA which codes for a protein
- Microarray – A technique for examining gene expression in huge numbers based on oligonucleotides ‘printed’ on microscope slides

- MVX - Mushroom Virus X, a collection of individual double-stranded RNA viruses
- Oligo or oligonucleotide – a small region of DNA synthesized with a specific sequence
- ORF or Open Reading Frame – This term refers to a presumed gene but not proven to be a gene
- PCR – Polymerase Chain Reaction, this technique is used to amplify specific sequences of DNA (see also Quantitative PCR)
- Primer pairs – primer pairs are used to amplify specific regions of DNA by PCR
- Quantitative PCR – A technique based on PCR for which the measurements are taken for each round of cycles, quantity is evaluated when the level of an amplified gene exceeds a threshold level
- RNA – Ribonucleic acid, this can be the form of gene sequence that directly code for proteins and is inbetween DNA and proteins. However for some viruses their genetic code is carried on RNA, and can be either single-stranded or double-stranded.
- Sequence – the sequence of a gene is the order of bases which together code for RNA or proteins

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