FINAL PROJECT REPORT
WTFRC Project #PH-02-240

Title: Development of a molecular assay for screening post-harvest pathogens of apple and pear

Principle Investigators: Peter Sholberg, Karen Bedford and Dan O'Gorman
AAFC-PARC, Summerland, BC

Objectives

1. **The objectives of this study were to develop: a) a PCR assay and b) a diagnostic DNA array for post harvest pathogens of apple and pear** based on the β-tubulin gene that will allow rapid recognition of post-harvest pathogens, simultaneously recognize fungicide resistant strains, and quantitative assessment of pathogens in diverse samples.

2. **In 2002-2003 the objectives were to develop the specific primers and probes** for PCR and DNA array assays through extraction, amplification, sequencing of the β-tubulin gene, alignment of sequences, primer and probe design, and testing of designed primers and probes for cross-reactivity and specificity.

3. **In 2003-2004 the objectives were to conduct direct testing** of diverse samples using the designed assays and to refine the assays to provide quantification of post-harvest pathogens.

Significant Findings

- **The β-tubulin gene was utilized to design PCR detection assays where the ribosomal gene was not useful.**
    - Two multiplex PCR assays for *Penicillium* blue mold have been developed and tested that detect within one day (1) *Penicillium expansum* and other *Penicillium* spp.
    - A multiplex PCR assay for *Botrytis* gray mold has been developed and tested that detects within one day *Botrytis* spp..
    - A PCR assay for *Mucor Piriformis* has been developed and tested that will detect within one day the presence of *M. piriformis*.

- **DNA array probes targeting the β-tubulin gene have been designed and tested, to detect and identify:**
    - *Penicillium expansum* isolates and TBZ resistance**.**
    - *Botrytis* species and TBZ resistance.
    - *Mucor Piriformis*

- **The DNA array was successfully used to distinguish living from dead cells.** This ensures that the DNA detected in a sample is truly representative of the number of viable pathogen cells capable of causing disease.

Methods

DNA extraction and amplification. Fungal cultures grown in liquid culture until biomass was sufficient for DNA extraction were transferred into Bio 101 Fast DNA extraction kit in 2 ml tubes. The extraction protocol supplied with the kit was followed and DNA was eluted in 100 µl volumes and stored at –20ºC until required. DNA was amplified using the polymerase chain reaction (PCR) in 20 µl volumes containing: 0.5-1.0 µl DNA, 1x buffer, 2.0-3.0 µM $MgCl_2$, 1.5-2.0 µM dNTP mix, 0.05-0.4 µM each forward and reverse primers, 1 unit of Taq DNA polymerase and for multiplex PCR, 2.5 units of Ultratherm polymerase. Primer pairs used to amplify the β-tubulin gene were BT2MLev-up4/Bt-Lev-Lo1 and for the ribosomal spacer DNA, the primers were UN18S42/UN28S22 (For Species specific amplification using multiplex reactions please refer to table 1 for the specific primer names). Amplification was performed on a GeneAmp 2700 thermal cycler (Applied Biosystems) with variable cycle conditions depending on primers used. PCR products were run out on a 1.5 % agarose gel (or 2.5% gel for multiplex PCR) stained with ethidium bromide. The gel was visualized using a UV transilluminator and BioPhotonics Gel Print 2000i system linked to a Mitsubishi video copy processor P67UA.

DNA sequencing. The sequence reaction mixture contained 15-20 ng of purified DNA, 1µl of 3.5 µM primer, 3 µl 10 x sequencing buffer and 1 µl of Big Dye terminator mix. Sequencing reactions were carried out on a GeneAmp 2700 thermal cycler set to run for 25 cycles at 95ºC for 30 sec, 50ºC for 15 sec, and 60 ºC for 2.5 min. The same primer pairs used to amplify the DNA in the initial PCR reaction were also used in sequencing reactions using the ABI Big Dye Terminator Kit. Electrophoresis of sequencing extension products was performed on an ABI 377 sequencer. For each species multiple isolates have been sequenced to act as conformation and to check for genetic homogeneity between isolates. Editing of ribosomal and β-tubulin DNA sequences were done using Sequence Navigator software (Applied Biosystems Inc). Miscalled or missed bases were manually corrected using data from a reverse sequence reaction as conformation. Once editing was complete, data were entered into our sequence database. Because of difficulties encountered using sequencing *Mucor piriformis* isolates, ribosomal DNA was cloned following standard protocols. Clones were screened using a PCR method and clones possessing the correct size of insert underwent direct sequencing.

DNA Primer and Array Probe development. Final edited versions of the β-tubulin sequence data for each species were placed into groups. β-tubulin sequences for identical and related species were also down loaded from Genbank and placed into corresponding groups. Sequence data for each group was then aligned in ClustalX software. Alignments were analyzed visually to identify regions exhibiting differences between species. These regions of differences were then marked as possible sites for primer or probe selection. To ensure consistent melting temperatures, and to prevent duplex and hairpin formation, final probe selection and design were carried out using Oligo 6 software (National Biosciences, Hamel, MN). Probe sequences were entered into a Blast search of the Genbank database. The Blast search checks for the occurrence of homologous sequences existing in other species with DNA sequences present in the database.

Testing of Primers and Probes. Designed primers for *Penicillium,* and *Botrytis* were tested alone and in multiplex combinations using various temperature and cycling regimes for the ability to distinguish *Penicillium* and *Botrytis* species. Designed primers for *Mucor piriformis* were tested alone in standard PCR assays using various temperature and cycling regimes for the ability to distinguish the pathogen from other closely related spp. The cultures were detected and identified by characteristic band patterns on stained agarose gels. The DNA array template was constructed by immobilizing the DNA probes on a nylon membrane by a 5'- amino modified spacer arm. The probes were arranged on the membrane in an array pattern, grouping ITS probes (previously developed) and

the β-tubulin speceies specific and TBZ resistance probes together. The constructed arrays were used for testing in hybridization reactions with pure cultures. The cultures were detected and identified by positive hybridization signals, which appear as dark dots on exposed x-ray film. Individual probes on the array were tested for strength of reaction and specificity of reaction. The same set of cultures was used in testing the PCR and DNA array assays. Additional PCR and DNA array hybridization tests were carried out with environmental and commercial samples.

Quantification of pathogen populations. The quantification capabilities of the post harvest DNA array were investigated. A 100 fold dilution of solutions containing spores were simultaneously plated for cell counts and processed for DNA extraction. Plates were counted after 48 hrs to give an estimation of cell concentration for corresponding DNA extractions. Amplification products from concentrations of 10^6, 10^5,10^4, 10^3, 10^2, and 10^1 and zero cells, were used as standards in hybridizations with unknown samples which were also plated out to determine population size per ml. Hybridization results of the DNA concentration standards were used to estimate sample populations for a range of grayscale values (0-255). Grayscale values were then used to estimate cell numbers in inoculated and environmental samples.

Detection of DNA from live verses killed cells. Solutions containing spores of *B. cierea*, *Mucor pirifomris* and *Penicillium expansum* were used in reactions with the DNA array to determine detection of live cell vs. killed spores. Spore from the three pathogens were washed from culture grown on agar plates with sterile water. The spore counts were determined for each pathogen separately using a haemocytometer (*Botrytis* = 8,000 spores/ml, *Mucor* = 78,000 spores/ml and *Penicillium* = 90,000 spores/ml). The spores from all three pathogens were combined and then added to beakers containing 100 ml of 100 ppm free Cl2, and 100 ml of sterile H_20. Three replications of each treatment were carried out. One ml of each replicate was removed after one, 24, 48 and 96 hrs. Samples were used for both plate counts and DNA extraction. The DNA was amplified as described earlier and hybridized with the DNA post harvest array for detection and quantification of the pathogens.

Results and Discussion

PCR Assay. Two multiplex PCR assays employing both ribosomal internal transcribed spacer (ITS) and β-tubulin primers (Table 1) used in different combinations have been developed to distinguish *Penicillium expansum* from other post-harvest pathogenic *Penicillium* species. Testing of the assays has so far been conducted on known isolates of *Penicillium* spp. obtained from Dr. Peter Sanderson (Washington State), as well as *Penicillium* spp. isolates collected in British Columbia by our laboratory. The *Penicillium expansum* isolates have been sequenced and characterized for resistance or sensitivity to TBZ. Results of testing of the assay were shown in previous reports. Each of the multiplex assays clearly distinguishes *P. expansum* from other *Penicillium* species. Depending on the combination of primers used the multiplex assays can simply distinguish *P. expansum* from other *Penicillium* spp. or it can identify *P. expansum, P. solitum*, and *P. commune*; the multiplex assay used in was unable to amplify *P. aurantiogriseum*. The PCR multiplex system was used to test a larger number of *P. expansum* isolates, and was successful in every case in distinguishing them from the other *Penicillium* spp. tested (reported earlier)

Botrytis cinerea and a second *Botrytis* spp. (*B.tulipea-like*) consistently present in British Columbian apples have also been successfully distinguished using a multiplex PCR assay employing designed primers (Table 1). The PCR primers were also used in amplifications to distinguish *B. cinerea* and the *B.tulipea-like* isolates from nine other *Botrytis* spp. *B. fabae* does however react with the *B.cinerea* primers while the *B. tulipea*-like primers appear to be 100% specific. The PCR assay was also used in trials to help determine the pathogenecity of the two different *Botrytis* species. In inoculations of

apple using one or the other of the *Botrytis* spp., both were shown to cause rot, although lesions caused by *B. cinerea* were generally larger. In inoculation of a mixture of both species, populations of both *B.cinerea* and *B.tulipea-like* were detected and identified by PCR as being present at the margin of the lesions. However, *B.cinerea* was consistently identified by PCR as being in greater numbers, indicating that it is more pathogenic.

The potential exists that a single multiplex PCR could be used for *Penicillium* and *Botrytis* spp. as the bands that appear on the gels for the two genera are distinct. Optimization of multiplex PCR assays becomes more difficult as more primers are used. Further work is needed to explore the possibility of a *Botrytis/Penicillium* multiplex assay.

Previously developed primers for Bull's Eye rot (De Jong et al.) have been used to assay pure cultures of *Pezicula malicortis* and to successfully assay fruit from a tree inoculated with a known isolate of *Pezicula malicortis*. DNA extracted from developing cankers and from lesions appearing on the fruit both tested positive with the specific primers for *Pezicula malicortis.* Isolation of the pathogen from these tissues, on agar plates, was used to confirm the results.

Attemps to design primers for *Mucor piriformis* from the β-tubulin gene were not successful. Therefore primers were designed for this pathogen using ITS sequences from isolates in our collection, isolates received from Dr. Bob Spotts (Oregon State University, Hood River, OR), and one sequence obtained from GenBank which represented a South African isolate. Testing of these primers showed specificity for the pathogen using pure culture (Figure 1).

DNA array. Probes specific for two *Botrytis* spp. (gray mold) and for TBZ resistance in *B. cinerea* and *P. expansum* were designed from β-tubulin gene sequences (Table 1). These and specific ITS probes for *Mucor piriformis*, *Penicillium expansum* and two *Botrytis* spp. were placed on a prototype array membrane for testing. The prototype membrane was tested with the known strains of *P. expansum* and *B. cinerea* resistant or sensitive to TBZ, other *Penicillium and Botyris spp., M. piriformis* and other *Mucor spp*. All of the *Penicillium expansum* isolates reacted with at least two of the three specific ITS probes. TBZ resistant *P. expansum* and *Botrytis* isolates all reacted as expected. The *M. piriformis* probes only reacted with *M. piriformis* isolates. Other *Muco*r and *Penicillium* spp. tested did not react with any of the species specific probes providing confirmation of the specificity of the probes. The various ITS region and β-tubulin gene probes clearly distinguished the two Botrytis spp. when hybridized at 55°C and at 58°C. However, when hybridized at 55°C, the eight other *Botrytis* spp. also showed positive hybridization signals but were distinguished from the two apple pathogens by producing unique hybridization patterns with the various probes. The hybridization temperature of 55°C appears to be optimum for the *Penicillium* and *Mucor* probes and therefore the *Botrytis* probes could still be modified to work more effectively at this temperature.

The post harvest DNA array was also used to detect and quantify pathogens from several environmental and commercial samples (Table 2). An experiment was set up to investigate the detection and quantification of live vs. killed cells, because the detection of DNA by itself does not indicate the viability of the pathogen and its ability to cause disease. Detection of DNA from dead cells would lead to an overestimate of the true pathogen population. The results from this experiment showed detection values of live cells using the DNA array were in general agreement with both spore counts estimated with a haemocytometer, or from total plate counts of viable cells for all three pathogens (Figure 3; Table 3). No Cl_2 treated cells were detected with the DNA array after 48 hours (Table 3).

Table 1. Primers and probes designed for PCR assays and DNA array tests for post-harvest pathogens of apple and pear.

Post-harvest	Target species	Primer or Probe	Name
Blue mold	*Penicillium expansum*	ß-tubulin primer	Pex-bt-f
		ß-tubulin primer	Pex-bt-r
		TBZ sensitive probe	Pex-bt-H3-
		TBZ resistance probe	Pex-bt-H1-
		TBZ resistance probe	Pex-bt-H1-
		rDNA probe	PE-H2c
		rDNA probe	PE-H3
Blue mold	*Penicillium* other	ß-tubulin primer	Pen-other-bt-f
		ß-tubulin primer	Pen-other-bt-r
Blue mold	*P. solitum*	ß-tubulin primer	Psol-bt-f 1
		ß-tubulin primer	Psol-bt-f 2
		ß-tubulin primer	Psol-bt-r
Blue mold	*P. commune*	ß-tubulin primer	Pcom-bt-r
Blue mold	*P. aurantiogriseum*	ß-tubulin primer	Paur-bt-f
		ß-tubulin primer	Paur-bt-r 1
		ß-tubulin primer	Paur-bt-r 2
Grey mold	*Botrytis cinerea*	ß-tubulin primer	Bcin-bt-f
		ß-tubulin primer	Bcin-bt-r 1
		ß-tubulin primer	Bcin-bt-r 2
		rDNA probe	BC-H2d
		rDNA probe	BC-H3d
		ß-tubulin primer	Bcin-133-H3
		TBZ sensitive probe	Bot-95-H1
		TBZ resistance probe	Bot-95-H2
Grey mold	*Botrytis* spp. from	ß-tubulin primer	Bstok-bt-f
		ß-tubulin primer	Bstok-bt-r 1
		ß-tubulin primer	Bstok-bt-r 2
		rDNA probe	BT-H1d
		rDNA probe	BT-H2d
		ß-tubulin probe	Bstck-144-H4
Mucor rot	*Mucor Piriformis*	rDNA probe	Mpir-ITS-
		rDNA probe	Mpir-ITS-
		rDNA primer	Mpir-ITS-36f
		rDNA primer	Mpir-ITS-108f
		rDNA primer	Mpir-ITS-506r
		rDNA primer	Mpir-ITS-585r

Table 2. Summary of amplification and hybridization results using primers and probes designed for the detection of two *Botrytis* spp. (*B. cinerea, B. tulipae*-like) *Penicillium expansum* and *Mucor piriformis*.

Sample	PCR results		Hybridization results	
	Universal primers	Species-specific primers	Species specific probes	TBZ resistance probes
Pure culture samples				
Penicillium expansum (R)	pos	pos	pos	pos
P. expansum (S)	pos	pos	pos	pos
P. commune	pos	pos	-	-
P. solitum	pos	pos	-	-
P. aurantiogrisium	pos	-	-	-
Botrytis cinerea (R)	pos	pos	pos	pos
B. cinerea (S)	pos	pos	pos	-
B.tulipea-like	pos	pos	pos	Not tested
B. fabea	pos	pos	*pos	*pos
B. streptothrix	pos	pos	*pos	*pos
B. porri	pos	-	*pos	*pos
B. squamosa	pos	-	*pos	*pos
B. aclada	pos	-	*pos	*pos
B. tulipea	pos	-	*pos	*pos
B. crystollina	pos	-	-	-
Botrytis sp.	pos	-	-	-
Mucor pyriformis	pos	pos	pos	NA
M. plumbeus	pos	-	-	-
M. hiemalius	pos	-	-	-
M. rouxii	pos	-	-	-
Environmental samples				
Orchard litter	pos	pos	pos	-
Apple tissue	pos	pos	Not tested	Not tested
DPA (dump tank)	pos	pos	pos	pos
Dump tank water	pos	Not tested	pos	-
Flume water	pos	Not tested	pos	-

* pos; samples gave positive hybridization signals, but are distinguishable from either of the two *Botrytis* apple pathogens by generating unique hybridization patterns.

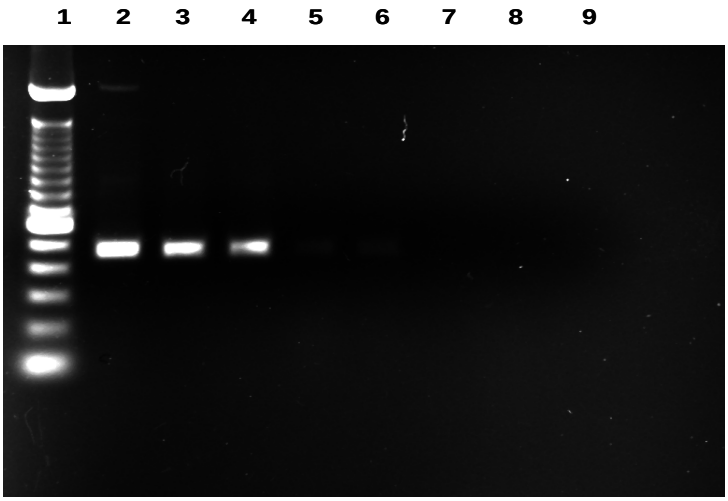

Figure 1. Species specific PCR for *Mucor piriformis* targeting unique sites in the rDNA spacer region. The image shows a species-specific band of approx 500 bp. generated from *M. piriformis* DNA. **Lane 1**, is a 100bp ladder; **lane 2**, *M. piriformis* (57); **lane 3**, *M. piriformis* (563); **lane 4**, *M. piriformis* (1979); **lane 5**, *M. plumbius* (85); **lane 6**, *M. hiemalis* (87); **lane 7**, *M. rouxii* (1985); **lane 8**, *Mucor sp*. (1979); **lane 9**, negative control.

Post Harvest Array Template

	rDNA - ITS					B-tubulin		
	A	B	C	D	E	F	G	H
1	UN-H1-up	BC-H2d	BT-H1d	PE-H2c	MPir-183H1	Bot-95-H1 **gag**	Bcin-133-H3	Pex-84-H3 **gag**
2	Bot-all-H1b	BC-H3d	BT-H2d	PE-H3	MPir-414-H1	Bot-95-H2 **gcg**	Bstck-144-H4	Pex-84-H1 **gcg**
3								Pex-84-H2 **gtg**

Post Harvest Array Membrane

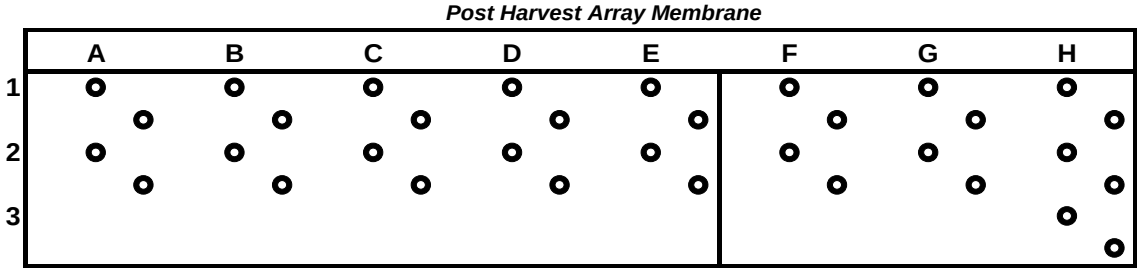

Figure 2. Template of the post harvest DNA array membrane. The template shows the arrangement of probes designed to simultaneously detect *P. expansum* and TBZ resistance; *Botrytis cineria*, *B. tulipae*-like sp. and *Botrytis* Genus TBZ resistance; and *Mucor piriformis*. The probes target both ribosomal ITS DNA and the ß-tubulin gene sequences. The ß -tubulin probes detecting TBZ resistance includes codon 198. These probes not only detect two point mutations, which lead to resistance but also identify *P. expansum*. For *Botrytis,* the TBZ resistance probes are genus specific. The ß -tubulin probe for *Botrytis* species identification are located in different region which does not include codon 198. The probes for *Botrytis cinerea* also reacts with *B. fabae*. The ribosomal probes for *Botrytis* can distinguish between the two apple pathogens but are not completely species specific

and will detect other *Botrytis* spp. The ribosomal DNA probes are species specific for both *P. expansum* and *M. piriformis*.

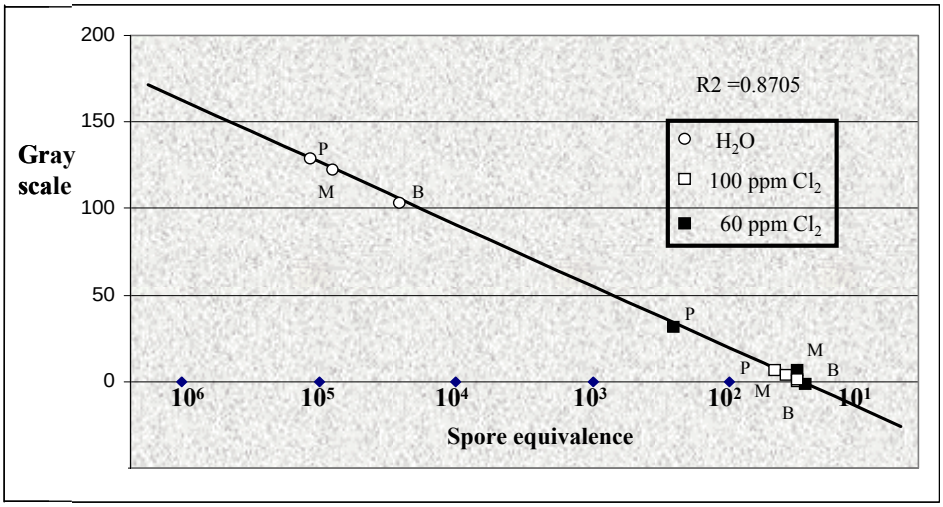

Figure 3. Quantification of live vs. killed cells. Samples shown were taken at 1 hour after inoculating 100 ppm and 60 ppm C l_2 solutions. Standard curve was generated with gray values (0-255) obtained from positive hybridization reactions of known standards and was used to estimate spore concentrations in each sample. Values for each pathogen are displayed separately (P = *Penicillium*; M = *Mucor*; and B= *Botrytis*) for H2O, 100 ppm and 60 ppm free Cl_2 treatments.

Table 3. Comparison of plate counts and DNA array detection of live vs. killed cells.

Sampling times	H_2O		100 ppm free Cl_2		60 ppm free Cl_2	
	DNA array*	**Plate count***	**DNA array***	**Plate count***	**DNA array***	**Plate count***
1 hr	$2.3x10^5$	$2.0x10^5$	$9x10^1$	$1.7x10^1$	$3.5x10^2$	$6.7x10^1$
24 hrs	$2.1x10^5$	$1.5x10^5$	$5x10^1$	$3.0x10^1$	$1.2x10^2$	0
48 hrs	$1.7x10^5$	$1.7x10^5$	0	0	not tested	not tested
96 hrs	$1.7x10^5$	$1.7x10^5$	0	$3.0x10^1$	not tested	not tested

*Values given for DNA array are spore equivalence derived from DNA extracted from a known concentration of spores.
** For plate count method values were determined by dilution plating and are given in cfu/ml.

Budget
Project total: $37,500

Year	2002-2003	2003-2004
Salary and Benefits	$15,000	$17,500
Materials and Supplies	2,500	2,500*
Travel		
TOTAL	$17,500$^{\tau}$	**$20,000**

$^{\tau}$ Matching funding from the Agriculture and Agri-Food Canada Matching Investment Initiative was granted in June 2003.