FINAL REPORT
WTFRC Project #AH-01-61

Title:	**Development of a comprehensive diagnostic system for apple diseases using a DNA array**
Principal Investigators:	Peter Sholberg, Karen Bedford and Dan O'Gorman AAFC-PARC, Summerland, BC
Co-operator:	André Lévesque AAFC-ECORC, Ottawa, ON

Objectives:

1. **The objective of the project was to develop a DNA array diagnostic system for apple diseases** that includes the major pathogens of apple found in the Pacific Northwest region of North America. The diagnostic system is based on a DNA array of species-specific DNA probes using reverse dot blot hybridization and allows rapid screening and identification of several fungal and bacterial pathogens in one test from various types of sample tissue.

2. **In 2003-2004 the objectives were to refine the array to provide quantitative relative scale assessment of important apple pathogens** useful for predictive monitoring management and storage decisions

Significant Findings:

Year 3

- Fire blight quantitative relative scale assessment correlated well with actual populations present
- Fire blight was successfully monitored in a field experiment with a detection limit of 1-10 cells on flower tissue
- Powdery mildew quantitative relative scale assessment correlated well with actual populations present
- Powdery mildew and Apple scab were successfully monitored in field situations for a second year , hybridized extractions from spore trap samples correlated directly with Apple scab predictive models for ascospore release
- Primers for *Pseudomonas. syringae pv. syringae*, causal agent of bacterial canker of apples and stone fruits have been developed.
- Commercial interest has been expressed in the array

Year 2

- A third generation prototype membrane array, revised after testing of first and second generation prototypes, was developed that incorporates probes for the major pathogens of apple
- Fire blight, Powdery mildew, Apple scab, Gray mold, and Blue mold have been readily detected from plant tissues and environmental samples using probes on the array
- In field testing the array, hybridized extractions from spore trap samples correlated directly with Apple scab and Powdery mildew predictive models for ascospore release

Year 1

- Direct testing of powdery mildew with a PCR assay was made possible with the design of a species-specific primer (PM-UPITS). Environmental samples from a Rotorod® spore sampler were tested successfully for the presence of powdery mildew at very low levels using the primer.
- Genus or species-specific probes ready for testing on a DNA array membrane were designed for powdery mildew, Fire blight, blue mold, grey mold
- Sequencing of collar rot and blossom blast/Bacterial Canker was completed.

Methods:

Year 3 Quantitative relative scale assessment of Fire blight and Powdery Mildew

Fire blight
Dilution series of Erwinia amylovora isolate 1477-1

A 10 fold dilution series of *Erwinia amylovora* cells was simultaneously plated for cell counts and processed for DNA extraction. DNA was extracted using the standard FastPrep protocol for bacterial cell and DNA was eluted in 100µl volumes. Plates were counted after 48 hrs to give an estimation of cell concentration for corresponding DNA extractions. Amplification of DNA was carried out as described under Powdery Mildew below. Amplification products from cell concentrations of 10^6, 10^4,10^2, 10^0 and a zero cell (no template control) were hybridized. Results were compared with treatment samples from day 0 of the screen house experiment.

Screen house experiment

A replicated experiment using potted two year old Jonagold trees was established in a protected screen house. Apple blossoms were inoculated with concentrations of 0, 10^1, 10^3, 10^5, or 10^7 CFU/ml of a mixed cocktail of strains 1477-1(streptomycin sensitive) and 1280 (streptomycin resistant). Three replicate samples of 10 apple blossoms were selected at random from a bulked sample of 6 blossoms from each of 5 trees collected daily beginning the day of inoculation for four days. The replicate samples were suspended in 10 ml of distilled water, sonicated for 3 minutes and plated by traditional dilution plating methods onto a selective medium for Fire blight. The same samples were used for DNA extraction, amplification and hybridization. Individual reps for each treatment were ground separately in Liquid nitrogen. Approximately 0.5 cc of powdered blossom sample was added to 2 ml extraction tubes for processing. The standard FastPrep DNA extraction protocol for plant material was followed with one modification of an additional washing step using 6 M guanidine thiocianate. DNA was eluted in 100 µl volume and kept frozen at -80 C until needed. DNA was amplified in 20 µl reaction volumes using the primers ITS 16 and ITS 23 (McManus and Jones, 1995). The number of strikes that developed on the inoculated trees was recorded on July 13, 2003. Results were compared and analyzsed statistically.

Powdery mildew
Methods for extraction and PCR of PM.

I Rods were inoculated with known concentrations of powdery mildew spores. A single leaf was collected from the green house with visible PM symptoms. Spores were washed of with 2 ml of a 0.01% tween solution. A haemacytometer was used to estimate the spore concentration and a 10 fold serial dilution of the spores was carried out. From each dilution, 10 µl from each was spotted

as several drops onto the surface of an I-rod and left to dry in a flow hood. The final concentrations of spores per I-rod were; $2.8x\ 10^4$, $2.8x\ 10^3$, $2.8x\ 10^2$, $2.8x\ 10^1$, $2.8x\ 10^0$ and 0.0. A single I-rod per extraction was used following the standard lab I-rod extraction protocol and DNA was eluted in 100µl volumes. PCR was carried out using the PM-ITS and UN28S22 primers for 37 cycles. Results were used to generate a standard scale for gray scale comparison of concentration.

Testing of the array with field samples of plant tissue (buds, flowers, leaves and fruit) and spore traps

Four rotation impaction spore traps were placed in apple plantings at PARC- Summerland and Agassiz and in an abandoned orchard in Summerland, BC to test for powdery mildew, apple scab and Fire blight. Samplers were set up in April and operated until harvest in September. The spore samplers were set to run 10% of the time, and were sampled daily through bloom and weekly thereafter or weekly throughout the season. Additional samples of leaf and flower tissue were collected for DNA extraction and hybridization from the various fields at regular intervals. Extraction, amplification and hybridization were accomplished as described in Year 2 methods. Apple scab ascospore release from leaves in the abandoned orchard was measured weekly.

Development of probe for Pseudomonas syringae

Published sequences of Pseudomonas syringae were used to design two probes. Probes were tested with known strains of Pseudomonas syringae as described in the Year 2 report.

Results and Discussion

Fire blight

Results of dilution plating with established known concentrations of *Erwinia amylovora* strain 1477-1 and the associated quantitative gray scale hybridization reactions with the array are shown in Table 1 and Figure 1. The dilution plating results of mixed strain inoculum for the screen house experiment and associated quantitative gray scale values are also shown. There is good general agreement between the two sets of data. Concentrations of cells in the order of 10 colony forming units (CFU) per ml of sample were detectable. It must be noted that dilution plating results for one of the screen house inoculation treatments are speculative as an error occurred during the plating. The dilution plating values for the Green treatment should have been approximately 10^5.

Screen house inoculated trees were monitored daily for bacterial populations. Examples of results for the means of the replicate samples of the various treatments are shown in Figure 2. Higher populations were consistently recovered from the trees inoculated with more concentrated inoculum. Consistent with other epidemiological studies of bacterial epiphytes, there was an initial drop in population on the flower surfaces but by day 3 after inoculation populations were increasing. The hybridization results mimicked well the dilution plating results adding confidence that gray scale comparisons can be a consistent indicator of population. .Gray scale hybridization readings were more sensitive than dilution plating. The lower limit for detection with dilution plating was in the realm of 1000 cells per ml as compared to the lower limit of approximately 10 cells per ml for the array. The number of strikes resulting on inoculated trees paralleled the population and gray scale readings (Figure 3). Significant differences were found in the amount of Fire blight that resulted on the inoculated trees (Table 2). There was on occasion considerable variation amongst the replicate samples. Only mean data are shown. It is likely that this is because of the tendency for bacteria on plant surfaces to be log normally

distributed. Consideration of sampling methods and manipulation of the data would have to be explored more fully for commercial use of the array.

Table 1. Quantitative Gray scale values generated with known concentrations of bacterial inoculum

Dilution plating: 1477-1		Screen house inoculum: 1477-1 and 1280*		
Concentration	Gray scale value	Concentration		Gray scale value
2 X 10^6	88.5	Blue	10^7	94
2 X 10^4	47	Green	$>10^3$	47
2 X 10^2	27	Pink	10^2	39
2 X 10^0	25	Yellow	10^0	11
Control 0	0	White	0	3

* 1477-1 was used as a standard streptomycin susceptible strain; 1280 was used as a standard streptomycin resistant strain

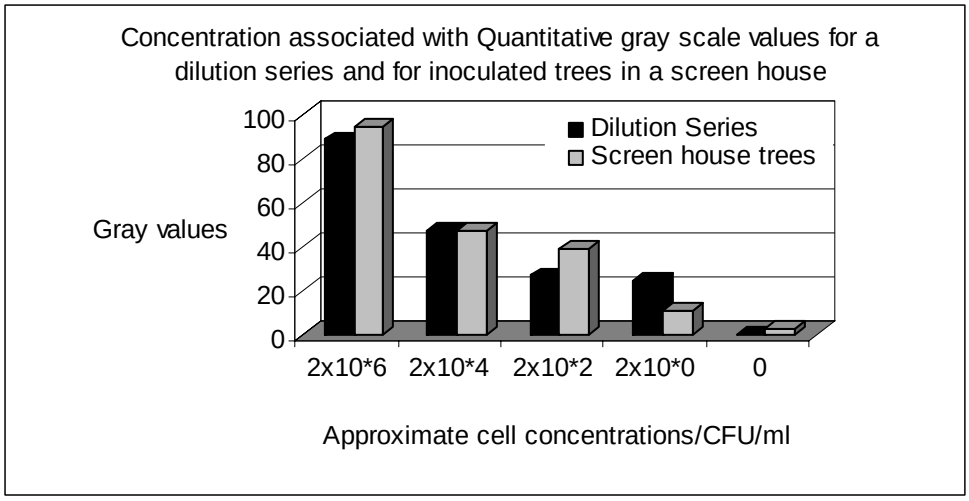

Figure. 1 Comparison of known concentrations of Fire blight bacterial cells and quantitative gray scale assessment for a laboratory dilution series and field inoculated trees

Table 2. Mean number of Fire blight strikes occurring on potted Jonagold trees inoculated with various concentrations of Fire blight inoculum

Treatment	Inoculum concentration CFU/ml	Mean Number of strikes per tree*
White	10^7	0.8 a
Yellow	10^5	2.2 ab
Pink	10^3	3.6 bc
Green	10^1	5.4 cd
Blue	0	7.4 d

* mean of 5 trees. Values analyzed with SAS General Linear Means LSD Means procedure. P=0.05. Numbers followed by different letters are significantly different.

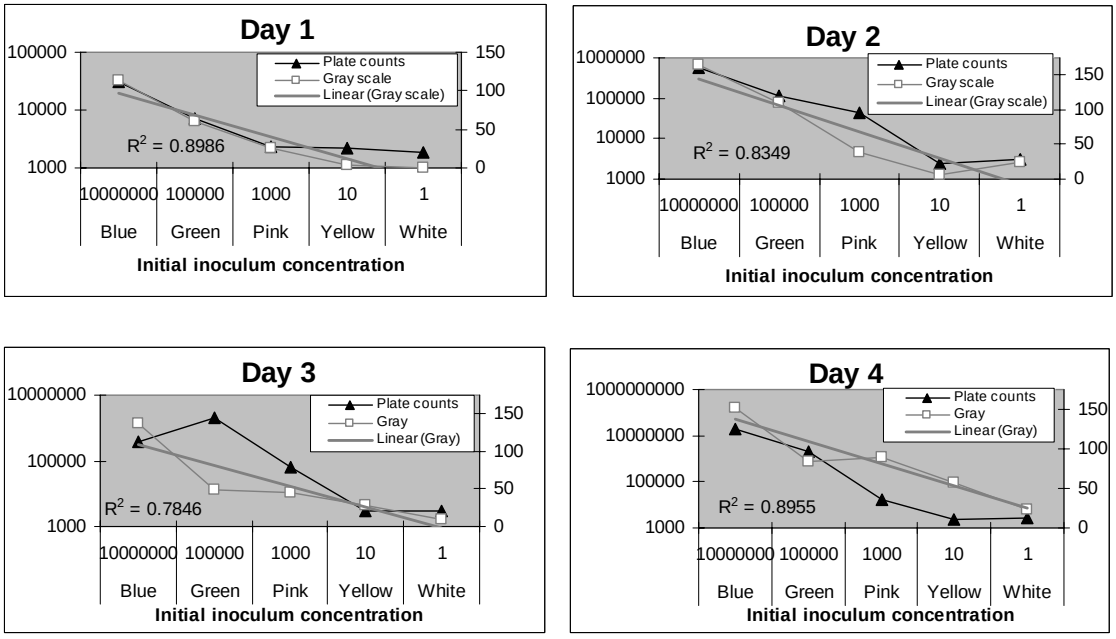

Figure 2. Comparison of means of dilution plate counts and gray scale values generated for daily samples of blossoms from inoculated Jonagold trees one to four days after inoculation. Trend lines fitted to the gray scale data had approximate R^2 values between .8 and .9.

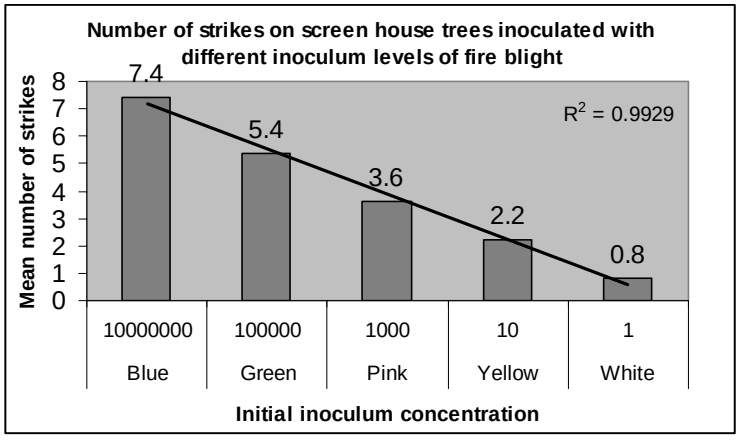

Figure 3. Mean number of Fire blight strikes that developed on screen house Jonagold trees inoculated with different concentrations of fire blight inoculum.

Powdery mildew

Lower detection limits for powdery mildew spores on I-rods were approximately 100 spores (Figure 4). This value is higher than that for Fire blight but there are no negative epidemiological implications from the field work conducted. Field monitoring of powdery mildew with I-Rod traps in several locations was very successful. Results from two locations are shown in Figure 5. In each case powdery mildew was detected before the powdery mildew predictive model registered an infection period. In the case of the Garnet Valley location irrigation was inadvertently left on for an extended period before bloom. The leaf wetness sensor was above the

irrigation so infection periods may have occurred that were not detected by the instrumentation. The relevance of the grape powdery mildew model predictive model to apple powdery mildew is questionable. Mildew spores were present as early as April 18 at the Garnet Valley abandoned orchard location, and again on April 28 and 29, 2003. The trees were at 50% green tip on April 30, 2003. In Field 20, results were similar; conidia were present early as well, no infection periods were recorded until much later. Positive hybridization results were recorded consistently through the bloom period, and again in mid June and for the rest of the growing season (data not shown).

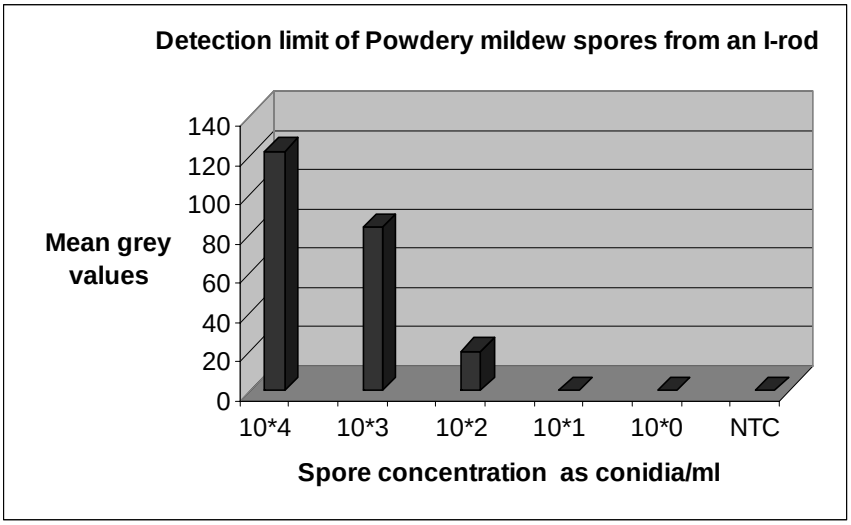

Figure 4. Comparison of gray scale values for known concentrations of powdery mildew spores showing lower limit of detection at approximately 100 spores per I-rod.

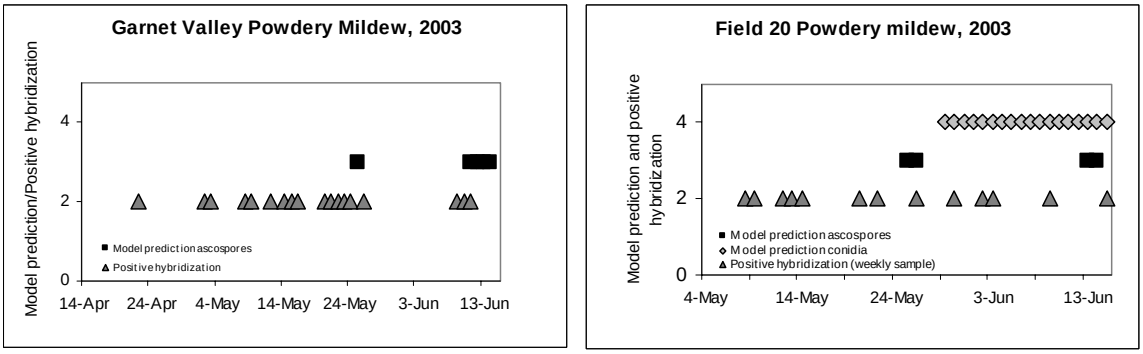

Figure 5. Comparison of powdery mildew monitoring results in an abandoned orchard, left, and in a field location, right, at PARC-Summerland. I-Rods were sampled daily through bloom and weekly thereafter.

Apple scab

A second year of results from monitoring apple scab in an abandoned orchard is shown in Figure 6. The monitoring equipment and spore trap were established April 14, 2003 at green tip. Data are shown through June 15, 2003. Model predictions of infection periods are shown as diamonds on the graph. Each positive hybridization for apple scab is shown as an X, and the numbers of mature ascospores sampled from leaves are shown as triangles. A curve representing the predicted percentage of mature ascospores is shown as a series of squares. The first infection

period predictions by the disease forecasting models were on April 24 and April 25, 2003 as were the first positive hybridizations. These results are encouraging for specificity and sensitivity of the array probes as the models predicted only 8% of the ascospores would be mature on these dates, and the number of mature ascospores counted from sampled leaves was fairly low. A large number of positive hybridizations occurred between April 26 and May 4 and May 5 but no infection periods were recorded. A possible explanation for this is that the irrigation was inadvertently left on in the field so leaves on the orchard floor were receiving constant wetting but the leaf wetness sensors providing disease forecasting data were not. Peak ascospore counts occurred at full bloom, May 14, about a week before a second round of infection periods and positive hybridizations beginning May 20 and May 21, 2003.

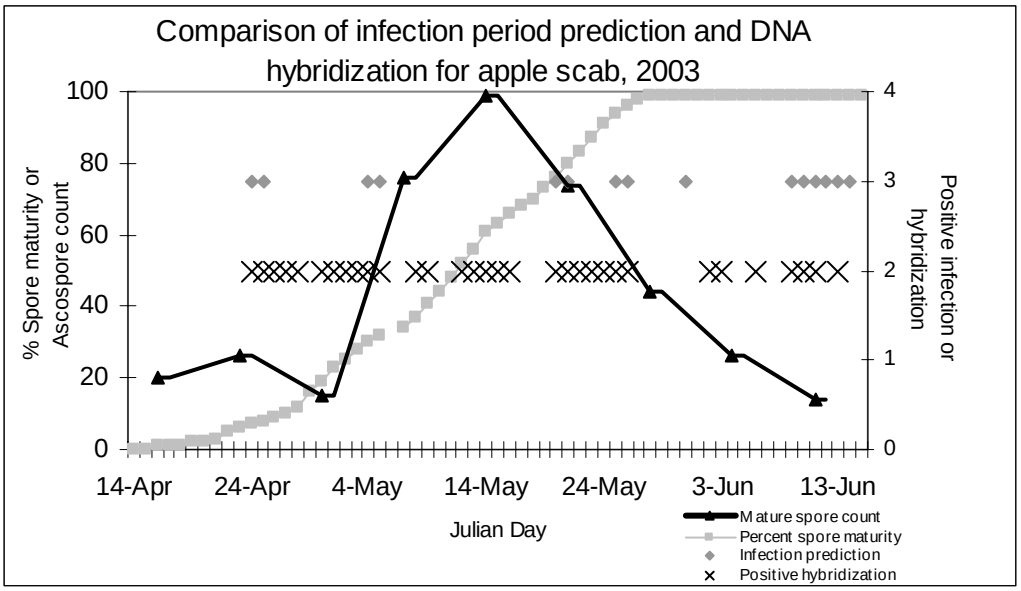

Figure 6. Comparison of ascospore numbers, predicted infection periods, and positive hybridization results for apple scab from an abandoned orchard location.

Development of probe for Pseudomonas syringae

The primers (B1 and B2) used for to amplify P.syringae pv. syringae target the syrB gene. The hybridization probe I made for this gene is a 20 mer and is nested between B1 and B2 primers. The primers have been tested only with P. syringae DNA and work; more extensive testing with different *P. syringae* isolates has not yet been conducted.

Summary

In the course of this project an array system that recognizes apple diseases in laboratory and field situations has been developed. The array can be used for bacterial and fungal diseases alike. It has shown application in field monitoring for apple scab, fire blight and powdery mildew over two years. Quantification using a comparative gray scale system appears feasible for powdery mildew and fire blight. Lower detection limits are currently 100 spores for powdery mildew and 10 cells for Fire blight. Preliminary discussions with Agdia and Wilbur-Ellis have been conducted regarding possible commercialization of the array.

Budget

Title: **Development of a comprehensive diagnostic system for apple diseases using a DNA array**

Principal Investigators: Peter Sholberg

Project total: $97,500

Year	**2001-2002**	**2002-2003**	**2003-2004**
Salary	25,000	25,000	25,000
Materials and Supplies	5,000	5,000	5,000[1]
Travel		2,500	5,000[2]
TOTAL	30,000	$32,500	35,000[3]

[1]supplies includes such items as petri dishes, agarose, pipet tips, tubes, storage boxes, chemical reagents, extraction kits etc.

[2] travel for sampling

[3]funds matched by the Matching Investment Initiative of Agriculture and Agri-Food Canada

References:

McManus, P.S. and Jones, A.L., 1995. Genetic fingerprinting of Erwinia amylovora strains isolated from tree-fruit crops and Rubus spp. Phytopathology 85, 1547-1553.

M. Scortichin, U. Marchesi, M.T. Dettori and M.P. Rossi. 2003. Genetic diversity, presence of the *syrB* gene, host preference and virulence of *Pseudomonas syringae pv. syringae* strains from woody and herbaceous host plants. Plant Pathology 52, 277-286.

Publications:

Sholberg, P.L., Bedford, K.E., Probert, S., and O'Gorman, D. 2001. Characterization of Botrytis cinerea from apple for pathogenicity, fungicide resistance and vegetative compatibility (Abstr.). Phytopathology 91:S81.

D. O'Gorman, P.L. Sholberg, K. Bedford, and C.A. Lévesque, 2002. Development of a comprehensive diagnostic system for apple disease using a DNA array (Abstr.) Can J. Plant Path. 24:393

P.L. Sholberg, D.T. O'Gorman and K. Bedford. DNA evidence that apple and pear powdery mildew are caused by *Podosphaera leucotricha* (Ell. &Ev.) Salm. (Submitted). Can. J. Plant Path.

D. O'Gorman, K.E. Bedford, P.L. Sholberg, S.C. Stokes, P. Haag and C.A. Lévesque, 2003. Design and testing of a comprehensive DNA array diagnostic system for apple diseases (Abstr.) American Phytopathological Society 2003 annual general meeting.