Diagnostic system for apple diseases using a DNA array *(Comparison of Real-time PCR and DNA array technologies).*

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Objectives:

1. Compare the sensitivity, specificity and reproducibility of DNA array, and Real-time PCR detection techniques for Powdery mildew & Fire blight.
2. Evaluate the usefulness of the techniques for monitoring and predictive purposes using fire blight and Powdery mildew predictive models as a reference in comparison to traditional monitoring.
3. Evaluate relative cost effectiveness of the various techniques for commercial development including potential applications to micro-array and microchip technologies.

Methods

Field sampling and DNA extraction

Bee hives were placed in field-20 (apple), field-9 (apple) and field-16 (pear) on (April 18) at PARC Summerland (all have a history of fire blight). Burkhart spore trap tape mounted on a glass slide was smeared with silicon grease and placed at the entrance of each bee hive, to sample the feet of the bees for the presence of fire blight. The samples were collected from the hives and exchanged for new tape daily. The tape was cut into small strips and placed directly in 2ml tube for DNA extraction.

In order to sample mouth part of the bees for the presence of fire blight, plates of sterile 5% sugar water were placed in close proximity to the hives and collected three times a week (Mon. Wed. and Fri.) during bloom.

Blossoms were collected daily from the same apple or pear orchard mentioned above starting in April. Ten fully open blossoms were randomly collected, pooled, placed in a single plastic bag and frozen at -20°C. Each bag of blossoms was ground in liquid nitrogen and three sub-samples of 0.5 g was taken and processed for DNA extraction. Ten leaves were also randomly collected from the same orchards. Samples were pooled, placed in a single plastic bag and frozen at -20°C and processed in a similar fashion to the blossoms.

A weather station was set up at Summerland and data was down loaded weekly. Weather data was analyzed with SpecWare 6.0 software (Spectrum Technologies, Inc., Plainfield, IL), input into the apple/pear IPM software package (Spectrum Technologies, Inc., Plainfield, IL) which contained software designed to forecast infection periods for fire blight.

A spore trap was set up in field 20 at PARC Summerland in early April and I-rods were collected on a daily basis until the end of bloom and weekly there after until the middle of July. Spore trap I-rods were placed in a single 2 ml tube for DNA extraction.

The Fast prep DNA extraction kit (Bio 101 Inc., Vista, CA) was used for DNA extraction from all samples. The DNA was eluted in 100 µl and stored at – 20°C. DNA samples were used in

parallel experiments using Real-time PCR and the apple DNA array hybridization for detection and quantitative analysis of Fire blight and Powdery mildew pathogens.

Real-time PCR: Fire blight and Powdery mildew

Primers for the Real-time PCR test were designed from the pEA29 plasmid sequences. The primers pEA29-F1 and pEA29-F3 were used in conjunction with the reverse primer AJ76 (McManus and Jones). The nested Real-time PCR protocol requires an additional 2.5 hours to complete but would still allow for the detection and quantification analysis to be complete within one working day.

The primer pair PM-ITS / Un28S22 was used in a Real-time PCR assay for the detection of *P. leucotricha,* the apple Powdery mildew pathogen. Similar to fire blight assay, environmental sampling of apple mildew with I-rod spore samplers required a nested PCR for detection with the Real-time PCR analysis.

DNA array: Fire blight and Powdery mildew

Newly designed DNA array membranes have been assembled that include ribosomal DNA and pEA29 plasmid DNA probes which are specific for the Fire blight pathogen. The membrane also includes ribosomal probes for Powdery mildew that were included and tested with the original DNA array prototype. The DNA array analysis requires a 24 to 48 hour window to acquire and interpret the results.

The PCR intended for DNA array analysis, for both fire blight and apple mildew are nested reaction combining universally conserved outer primers, with internally nested species-selective primers. The apple mildew PCR targets the ribosomal DNA which corresponds to the rDNA probes on the array. The fire blight PCR is a multiplex reaction simultaneously amplifying ribosomal DNA and pEA29 plasmid DNA, thereby targeting both DNA regions with specific probes incorporated on the array.

Results and Discussion

Fire blight

Table 1 and Figure 1 shows results for fire blight monitoring using both Real-time PCR and the apple DNA array. The values shown are the average of the three sub-samples taken for each blossom or leaf sample. For the bee hive samples (sticky tape- door mat, and sugar water samples) three separate reactions were conducted from each sample and the values were averaged to give a final quantitative result.

Figure 1 shows the quantification of Fire blight for both detection systems. Trends in detection values were similar but not identical for Real-time and DNA array analysis. But the values never differed more than an order of magnitude from each other for any particular sampling date. This difference however, is likely the result of the different way, or different point in the reaction where, quantification analysis is conducted by Real-time PCR and DNA array Hybridization.

The detection of pathogen cells was low throughout the sampling period and never reached more than 36 cells per sample. The low pathogen population detected by both systems is in line with the very minor amount of blight that was noted in the orchard. The other observation was that the detection levels did not reflect the computer model's predictive pathogen potential. There were similar pathogen numbers detected for both low pathogen potential and for high pathogen potential.

Powdery mildew

Figure 2 shows the combined result for Real-time PCR and DNA array detection and quantification of the Powdery mildew pathogen *P. leucotricha*. Similarly to fire blight the graphical represent of Powdery mildew spores shown in Figure 2 is derived from the average of three separate reactions for each DNA sample extracted from spore traps. The detection values obtained from each detection system are not identical, but both indicate general detection patterns from the orchard spore trap samples. Only on one sample date (May 18/04) do the two detection systems indicate pathogen numbers which differ by more than one order of magnitude.

We attempted to look at how the predictive model for grape Powdery mildew related to the molecular detection of the apple mildew pathogen. This year using Real-time PCR and DNA array as in past several years with the Array, the detection of Powdery mildew occurred several weeks prior to the infection period given by the grape predictive model (data not shown). It is likely that the difference in the epidemiology of the two mildew species does not allow the grape model to be used effectively to predict apple Powdery mildew infections.

Both detection and quantification systems, Real-time PCR and DNA array hybridization are detecting pathogen populations with a nested PCR protocol, down to several cells. Taking the average of triplicate reactions to generate quantification values smoothes out the variability observed with both detection systems. Typically the final population numbers did not vary by greater than one order of magnitude. These differences may be due to the inherent variability of taking values generated at the end of the PCR (DNA array) as apposed to taking more accurate readings of PCR products generated at he beginning of the exponential phase of the reaction (Real-time PCR).

From this study the results from both systems appear comparable. Although for the detection of Fire blight, the speed of which the Real-time PCR system offers for detection and quantification may be a great asset for efficient management of this particular disease. As for other diseases were a rapid (same day) detection system would be required, Real-time PCR may be the technique of choice. For application of disease management in orchards, where several or many different diseases are wanted to be monitored at any given time the DNA array offers a more powerful monitoring tool because numerous samples can be detected within a single reaction tube. However, DNA array analysis is a slightly longer procedure (24-48 hrs).

The use of a DNA (macro) array on nylon membranes as used in this study is likely the best approach to DNA array analysis at this time. The expense of moving to micro chip technology would be inhibitive to any in-house lab or small diagnostic company. The use of micro arrays on glass slide would also increase the lab set up coast as well as sample processing cost. Glass slide micro arrays can cost in the neighbourhood of $100 per slide (Bioscience Corperation; ordering 20 arrays at a time) for a one time use. The nylon membranes used in this study to produce the DNA array are much less expensive to produce (less than $5, see Appendix A) and can be reused numerous times without loss in detection signal strength.

The overall cost of running a DNA array (in house, based on processing 24 sample at one time) would be approximately $12 (Canadian dollars) per samples. Real time PCR would have a slightly higher set up cost for equipment but would only cost approximately $4.88 per reaction (based on home made reaction kits). More information on commercially available Real-time PCR and DNA array services are listed in Appendix A.

Table 1. Summary Table of results comparing Real-time PCR vs. DNA array Hybridization for the detection of *E. amylovora* (fire blight). Also shown are the results of a predictive model that indicates the pathogen potential.

	Model	Positive Nested Q-PCR				Positive Nested PCR/Hybridization			
Date	**Pathogen potential**	**Sample #**	**Sample type**	**Location**	**Estimated # of cells**	**Sample #**	**Sample type**	**Location**	**Estimated # of cells**
Apr 20	V. low	1	DM*	F9	**33**	1	DM*	F9	**20**
Apr 21	V. low	13	DM*	F16	**14**	13	DM*	F16	**20**
Apr 23	V. low	3	DM*	F9	**30**	3	DM*	F9	**2**
Apr 29	V. low	6	DM*	F9	**23**	6	DM*	F9	**20**
Apr 29	V. low	16	DM*	F20	**1**	16	DM*	20	**20**
Apr 30	V. low	39	L**	F20	**7.1**	39	L**	20	**1**
May 4	V. low	31	SW	F20	**20**	31	SW	F20	**5**
May 6	V. low	9	DM*	F9	**7.5**	9	DM*	F9	**2**
May 7	V. low	33	DM*	F20	**1**	33	DM*	F20	**6**
May 18	V. low	44	B**	F20	**≥ 1**	44	B**	F20	**2**
May 20	Mod.	12	DM*	F20	**36**	12	DM*	F20	**2**
May 21	High	45	B**	F20	**19**	45	B**	F20	**1**
May 22	Mod.	34	B**	F20	**≥ 1**	34	B**	F20	**4**
May 23	Mod.	35	B**	F20	**≥ 1**	35	B**	F20	**2**
May 14	V. low	42	B**	F20	**0**	42	B**	F20	**1**
June 4	High	61	L**	F20	**7**	61	L**	F20	**2**
June 24	High	48	B**	F20	**0**	48	B**	F20	**20**
June 24	High	62	L**	F20	**0**	62	L**	F20	**20**

**** DM** (door mat) = siliconized plastic tape placed at the entrance to bee hives and ***S** (sugar water) sampled daily for DNA extraction.*

***** B** (blossoms); and L (leaves), randomly sampled from the orchard on a daily basis and used in DNA extractions.*

Figure 1. Graphical representation of results shown in Table 1 comparing Real-time PCR vs. DNA array Hybridization for the detection of *E. amylovora* (fire blight). Also shown are the results of a predictive model that indicates the pathogen potential.

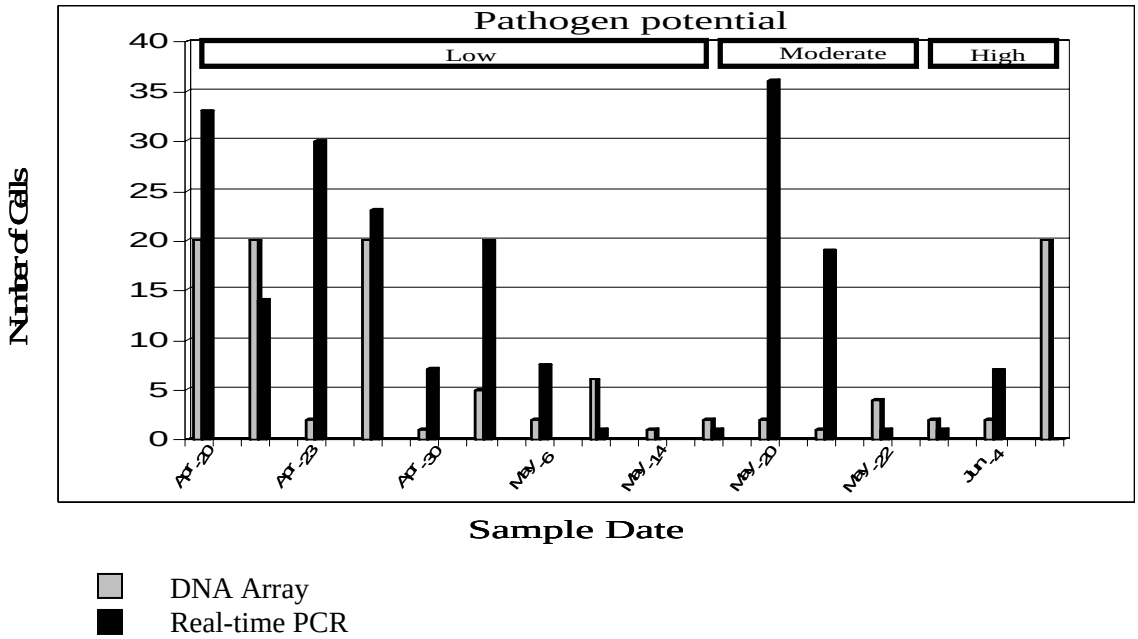
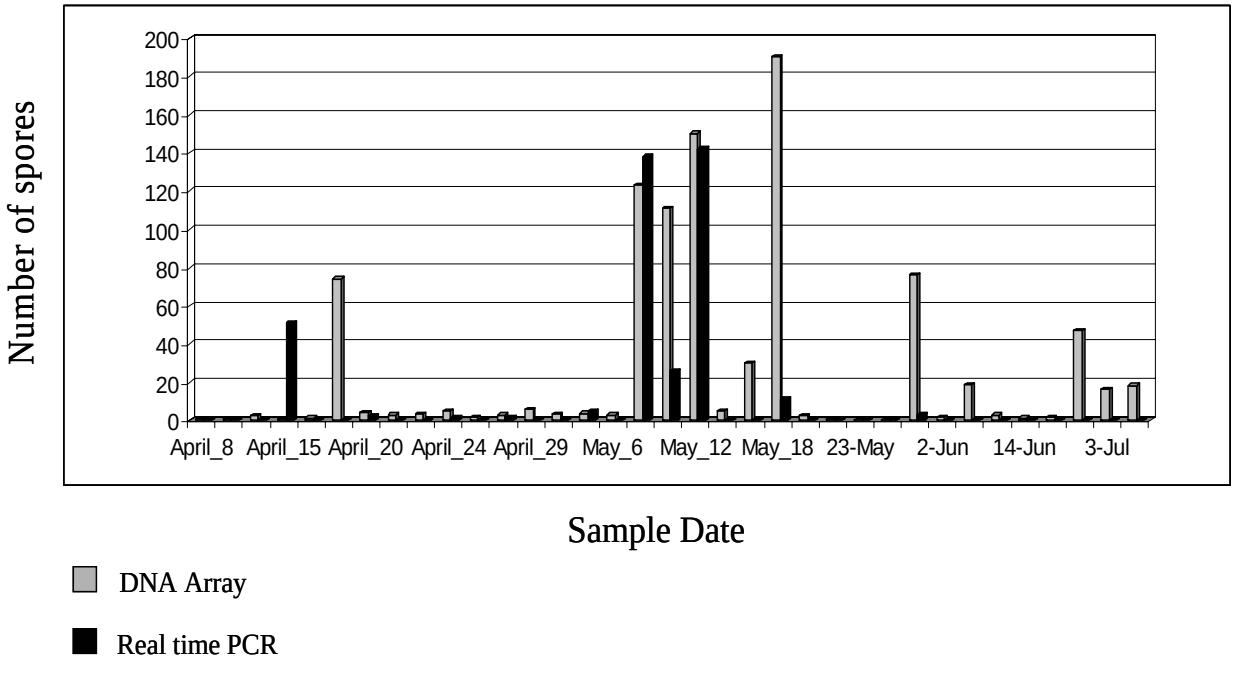


Figure 1. Apple mildew detection and quantification from I-rod spore samplers using Real-time PCR and the DNA array analysis.



Appendix A

Table A1. Estimated cost of in house processing of DNA array for 24 samples

FastPrep			**per sample**		**24 sample**
FP tubes & reagents			4.75		114.00
2 ml tubes	(3x0.0543)		0.16		3.84
tips	(18x0.022)		0.40		9.60
		Total	**5.31**	**Total/24**	**127.44**
PCR			**per sample**		**24 sample**
0.5 ml tubes	(1x0.065)		0.065		1.56
tips	(10x0.022)		0.22		5.28
Taq	(1x0.454)		0.45		10.80
oligos	(2x0.0126)		0.025		0.60
		Total	**0.76**	**Total**	**18.24**
Hybridization			**per sample**		**24 samples**
membrane	(2.5x10 cm)		0.068		1.62
tips	(5x0.022)		0.11		2.64
tubes	(1x0.065)		0.065		1.56
5'-oligo	(52x0.005)		0.26		6.24
anti-Dig	(3x0.88)		2.64		2.64
CDP*	(10x0.185)		0.31		0.31
		Total	**3.45**	**Total**	**15.01**

Total cost of materials to run 24 samples equals $160.69
plus labour of 8 hours (hands on) @ ~ $150.00 = $310.69 **or $12.94 per sample**
(Does not include cost of general laboratory buffers)

Table A2. Estimated cost of in house processing of Real-time PCR for 24 samples

Real time PCR (home made reaction buffer)					
			per sample		**24 sample**
0.5 ml tubes	(1x0.065)		0.065		1.56
CYBR Green	(1x0.25)		0.25		6.0
tips	(10x0.022)		0.22		5.28
Taq	(1x0.454)		0.45		10.80
oligos	(2x0.0126)		0.025		0.60
		Total	**1.01**	**Total**	**24.24**

Total cost of materials to run 24 samples equals $24.24
plus labour of 5 hours (hands on) @ ~ $93.00 = $305.94 **or $4.88 per sample given**

Table A3. Examples of institute or corporation pricing for manufacturing of DNA arrays or DNA array processing and analysis

Corparation or Institute	Manufacture – Price/array	Array processing/array
Bioscience Corperation	$125	-
BIoDeffencse	$150	$300
Celonex	$50	$100
UW- Madison GE center	$305	-
Uof Arizona (GA&TCF)	$17	$116
UVGE	€240	€150

Table A4. Examples of institute or corporation pricing for PCR or Real-time PCR analysis

Corparation or Institute	PCR	Real Time PCR
Neogen Ltd	$79 (1-9 samples)	-
Pro DNA Diagnostics	$ 9.40	$13.25
U of AlbanyCFG Services	$15	$30
U of MAssachusetts	-	$6 (24 sample min)