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Using Real Time PCR and stigma imprinting to expedite the detection of *Erwinia amylovora* on the stigmas of pear and apple flowers for improved timing of fire blight control

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Introduction and objectives

Fire blight is a bacterial disease of pomaceous plants including economically important apple and pear. The disease has the potential to kill full-grown trees in a few weeks and is the most important and feared disease of these crops. The bacteria survive over winter on cankers and are transferred to flowers by splashing rain or insects. Honeybees and flies rapidly disseminate the bacteria from flower to flower. A high percentage of flowers can become colonized within a few days when environmental conditions are conducive for growth and spread. Prompt control measures are essential to prevent widespread destruction by fire blight when the bacteria are present in the flowers.

The bacteria that are placed on the stigma of the flower begin to grow rapidly but do not necessarily infect. This is called the epiphytic phase of bacterial growth. Bacteria on other parts of the plant usually die within a few hours. The epiphytic bacteria grow very rapidly on the flowers and can spread from a few flowers to nearly 100% of the flowers within 1-3 days. Infections usually begin when epiphytic bacteria on the stigmas are washed by rain or dew into the floral cup where they enter the flower and cause infection. Fire blight can be controlled by eliminating the over wintering sources of bacteria and by protecting flowers with properly timed antibacterial sprays.

Growers are not sure when to spray the expensive antibiotic, streptomycin, to control the disease because they don't know when the bacteria are present in the flowers. Environmental models such as Maryblyt or Cougarblight are used to indicate when conditions are favorable and growers use the models to initiate a spray program. However in many cases the bacteria are not present in the flowers because there were no inoculum sources in or near the orchard. As a consequence many sprays are applied with no apparent benefit. Timeliness for spraying is very critical for protecting vulnerable orchards and reducing unneeded sprays

Epiphytic bacteria can be detected on the stigmas of individual flowers by imprinting flowers on selective growth media. This process is simple and inexpensive but it takes up to 3 days to make the determination. We would like to expedite this method because of its utility and simplicity. We propose placing an incubator on site and training a local individual to identify the specific *Erwinia amylovora* colonies. This could potentially reduce the time down to 24 hours instead of 36-48 hours. This timesavings could improve the control of fire blight.

The presence of streptomycin resistant *E. amylovora* in some orchards in Utah county has complicated control of fire blight. Growers are uncertain whether to use streptomycin or Mycoshield because they do not know whether the resistant bacteria are present in their orchard. We plan to use the stigma imprint technique to assess the flowers for the presence of streptomycin resistant bacteria before growers have to spray and before fire blight occurs.

Stigma Imprinting

Individuals provided by Mountainland Packing were trained in the proper method of stigma imprinting. Pear and apple flowers from 8 orchards in Utah County were imprinted by these individuals on CCT selective media beginning at early bloom and continued until most flowering was finished. In most cases 180 flowers were imprinted daily from each orchard. Flowers were taken randomly from within the sampled orchard block with no more than 1 flower taken from individual trees. Plates were incubated at 29C at a temporary laboratory set up at Mountainland Packing in Santaquin. The CCT plates were examined with a microscope at 10X after 24-36 hours incubation by a lay person trained to recognize typical colonies of *Erwinia amylovora*.

Streptomycin resistant *E. amylovora* are known to be present in the Santaquin Pear orchard No. 2. Stigma imprinting was conducted on normal CCT plates and on CCT plates with 100ppm of streptomycin incorporated into the media. The same flower was imprinted first on the normal CCT plate and then on the CCT plus streptomycin plate.

Real Time PCR

Molecular biological techniques and equipment available today might provide for a more rapid detection of epiphytic bacteria than the currently used stigma imprint protocol. The two most viable detection methods readily available on the market are conventional and real-time PCR (polymerase chain reaction). PCR has many applications in science and at its core, relies on similarities or differences in genetic material found in all living organisms. The genetic material, deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), is made up of a series of sequences of 4 compounds (bases). *E. amylovora* contains a circular piece of DNA known as a plasmid, and within this plasmid is a sequence of 944 bases that is unique to *E. amylovora.* PCR uses a series of heating and cooling cycles along with an enzyme and the starting portion of the 944 base sequence (primer) to amplify the full 944 base sequence. This procedure will only amplify the sequence if the full sequence is present bacteria in the unknown sample. With conventional PCR, the product of the procedure is placed in an agarose gel on an electrophoresis apparatus to separate the amplified product from other non target DNA. The final step is to stain the gel with ethidium bromide to view the amplified DNA product using UV light. Real-time PCR (RT-PCR) was chosen because is does not require the use of a thermocycler, equipment to make agarose gels, an electrophoresis apparatus, imaging equipment and does not necessitate the use of the caustic chemical, ethidium bromide. RT-PCR has a thermocycler and the imaging apparatus built-in and non-caustic fluorophores are used in detecting amplified product which eliminates the need for electrophoresis equipment and ethidium bromide. Additionally, portable RT-PCR units are available which provide the option to perform onsite testing to further expedite rapid testing of flowers in orchards. Idaho Technologies (IT), located in the University of Utah research park, offered a ruggedized portable RT-PCR machine (R.A.P.I.D.) on loan for the 2003 apple

and pear bloom period. IT also provided a 2-day advanced training course in the use of their R.A.P.I.D. machine. During the course of the 2-day training, an RT-PCR protocol for *E. amylovora* was optimized resulting in the protocol detailed below:

Cocktail ($MgCl_2$) optimized:
1uM of each AJ primer
2mM each dNTP's
30mM $MgCl_2$
1:3000 SYBR Green
0.8U Taq + TaqStart
template
remainder of reaction cocktail ddH_2O

Thermocycling optimization:

Denaturation:	94ºC for 5 minutes
PCR Cycle (50 cycles):	94ºC for 0 seconds rampdown 20ºC/second
	60ºC for 0 seconds rampdown 20ºC/second
	72ºC for 38 seconds rampdown 20ºC/second
Melt Cycle:	94ºC for 0 seconds rampdown 20ºC/second
	60ºC for 0 seconds rampdown 20ºC/second
	94ºC for 0 seconds rampdown 0.2ºC/second

Amplification of boiled pure culture and live pure culture *E. amylovora* were consistently amplified from concentrations of 10 bacteria/ul. The 944 base pair sequence amplified by the Al Jones primers melted between 85-86ºC.

Once the RT-PCR protocol was optimized in the lab, a stigma collection protocol was designed to fit with the PCR procedure.

Stigma Collection Protocol:
Remove 2-3 day old pear or apple blossom from tree
Imprint stigma set on CCT media
Excise Stigmas and place them in microfuge tube containing 100ul dH_2O
Each microfuge tube contained stigma sets from 9 flowers that corresponded to the 9 stigma imprints from a single CCT plate.

Test runs of the PCR and stigma imprint protocol were completed with crabapple blossoms from trees on the USU campus. The test runs as well as the initial field assays that utilized the above stigma collection protocol did not provide positive PCR results when compared to the standard stigma imprinting procedure on CCT, even when *E. amylovora* was added to the stigma washes. Because of the negative results that were obtained, a series of protocol changes were implemented to troubleshoot the procedure. When working with PCR technology, three primary areas are considered for troubleshooting. First, the proper amount genetic material must be obtained from the sample to provide a positive result. Second,

amplified sequence length and PCR extension timing must be optimized to allow time for extension and annealing of the DNA strands. Third, reaction inhibitors present in the sample (stigma wash) need to be reduced or removed. The following is a list of trouble shooting procedures aimed at optimizing the RT-PCR system for reliable field results.

Both bulk blossom washes and extracted stigmas were tested in all of the following trouble shooting steps

1. Stigma wash was spiked with known concentrations of *E. amylovora* to test for inhibition of the reaction with stigma or floral compounds.
2. 2 and 5 ul of sample added to reaction for both spiked and non-spiked samples to determine if there was an optimum amount of genetic material in the reaction.
3. 2 and 5 ul of sample added to reaction from dilutions of 1:1, 1:10, 1:100 and 1:1000 were done with both spiked and non-spiked samples to determine if the reaction was being inundated with genetic material. Diluting sample material is a common technique to eliminate inhibitors.
4. Samples were spiked and concentrated by centrifugation and the reaction sample was taken from the pellet and the liquid phase and placed in reaction tubes at 2 and 5ul aliquots. This procedure was done to concentrate the bacteria and reduce inhibitors in the PCR reaction.
5. Samples were spiked and either not centrifuged or centrifuged at 1600rpm for 10 minutes in a 3% glycerol solution. Reaction samples were taken from above and below the glycerol gradient and placed in reaction at 2 and 5ul. Additionally, extracts from above and below the glycerol gradient were also diluted 1:100 and 2 and 5ul added to the reactions. These procedures were completed to reduce or eliminate inhibitors and to check for DNA overload.
6. Sixty stigma surfaces were imprinted in the same spot on either a filter paper, a Petri plate containing water agar or in a 1.5ml microfuge tube containing water agar. Each imprinting surface was washed with distilled water and 2 and 5ul of wash was placed into the reaction. This procedure was done to reduce inhibitors entering the reaction and concentrate bacteria in the sample.
7. Stigmatic surfaces were swabbed from 20 flowers with an Idaho Technologies (IT) 123 extraction kit swab. Additionally, bulk blossom washes were subjected to the IT 123 extraction kit. One of the swabs was washed and the wash placed directly into the reaction and another swab was subjected to the IT 123 extraction procedures. Each extraction solution and the wash from the swab had 2 and 5ul placed into separate reactions. The extraction procedure was used to reduce or eliminate inhibitors in the reaction.
8. Blossoms were spiked and washed and the wash was placed directly into the reaction, spun at 13000 rpm for ten minutes with supernatant used as template and treated with a glycerol spin with the upper liquid phase as the template. The annealing time for the PCR reaction was also decreased from 60 sec to 58 seconds.
9. Bulk blossom washes were subjected to the Eppendorf plasmid extraction kit. Each extraction solution had 2 and 5ul placed into separate reactions. The extraction procedure was used to reduce or eliminate inhibitors in the reaction.

10. Bulk blossom washes were treated with TritonX to ensure that all bacterial cells were lysed and the DNA released.

Results

Stigma Imprinting

Individuals trained to conduct the stigma imprinting and the evaluations of CCT plates were competent and proficient at learning the methods and obtaining reliable results. The ability to incubate the plates in Santaquin soon after completing the stigma imprints expedited the reading of the plates so that results were often available within 24 hours. Sherm Thomson or Scott Ockey confirmed the preliminary identification of suspect *Erwinia amylovora* colonies on CCT.

Environmental conditions during bloom were cool with means usually less than 60F during bloom. However despite the low mean temperatures there was a low incidence of flowers colonized by *Erwinia amylovora* in many of the orchards. Fire blight incidence in 2003 was low because of the low temperatures during bloom and consequently the low incidence of colonized flowers.

Streptomycin resistance was detected using the stigma imprints on CCT plus streptomycin plates in the Santaquin pear orchard No. 2 before any fire blight occurred. We estimated that 95% of the *E. amylovora* isolates in the orchard were resistant to streptomycin.

Genola Pear block No.1

The stigma imprints in the pear block No. 1 was very low with 2% or less of the flowers colonized. The mean temperature of 60F was not exceeded during bloom and they were advised that no sprays were necessary. Maryblyt and Cougarblight did not indicate any fire blight risk during bloom. The growers did not spray any bactericides and there was no blossom blight in this pear block.

Santaquin Pear block No. 2

This orchard had numerous overwintering cankers and copious ooze especially in the north end of the block. Stigma imprints revealed 4-13% of the flowers colonized during bloom even though the mean temperature of 60F was never reached during most of the bloom period. Maryblyt and Cougarblight did not indicate any fire blight risk during bloom. It was estimated that there was an average of 8 blossom blight strikes/tree throughout the orchard but some trees had 95% and of the blossom clusters infected which was hundreds of strikes per tree.

Payson Ruby Jon

There were numerous overwintering cankers present in this orchard and many were observed to be oozing during early bloom. A low incidence (1-3%) of positive stigma imprints was detected early in bloom and by mid bloom to late bloom the incidence increased to 20-24% of the flowers colonized. The greatest increase in the incidence of colonized flowers occurred within 2-3 days after the mean temperature exceeded 60F. Maryblyt indicated three

high-risk days and one infection day. Cougarblight predicted high risk on three days because fire blight was present in the orchard in the previous year. The grower was advised to spray on several occasions and the four Mycoshield sprays may have provided some protection. However even with the sprays, the blossom blight incidence was about 5-10 strikes/tree.

Payson Gala
A low incidence of 1-3% of the flowers was colonized during early bloom and the incidence of colonized flowers did not increase even after the mean temperature exceeded 60F. Maryblyt indicated three high-risk days and one infection day. Cougarblight predicted high risk on three days because fire blight was present in the orchard in the previous year. The grower applied Mycoshield four times during high-risk periods. There was an estimate of about 1 strike/20 trees in the block. It was interesting to note that most of the strikes were on the inner side of the two-row trellised apples.

Santaquin Gala No. 1
The incidence of colonized flowers was very low during bloom and only 1-2% was detected on three sampling days. In most cases there were no colonized flowers during the 19 days of sampling. Maryblyt indicated three high-risk days and one infection day. Cougarblight predicted high risk on three days because fire blight was present in the orchard in the previous year. The grower applied Mycoshield two times when environmental conditions appeared favorable for fire blight. It was estimated that only 5-10 blossom blight strikes developed in the orchard.

Santaquin Gala No. 2
The incidence of colonized flowers was only 1-3% during most of the bloom period with the exception of one day with 9% during early bloom. By the time the mean temperature exceeded 60F there were few flowers in the orchard and there was no increase in the incidence of colonized flowers. Maryblyt indicated three high-risk days and one infection day. Cougarblight predicted high risk on three days because fire blight was present in the orchard in the previous year. The grower applied Mycoshield three times when environmental conditions were favorable for fire blight. It was estimated that an average of only 1 blossom blight strike occurred per 20 trees.

Santaquin Gala No. 3
The incidence of colonized flowers was higher in this orchard compared to other Santaquin orchards but still only ranged from 1-6%. Colonized flowers were detected before the mean temperature of 60F was reached and there was no apparent increase in colonized flowers after the mean temperature of 60F was reached. Maryblyt indicated three high-risk days and one infection day. Cougarblight predicted high risk on three days because fire blight was present in the orchard in the previous year. The grower did not spray and there were only 20 strikes in the orchard concentrated in two small areas.

West Mountain Gala
We detected 8% of the flowers colonized during early bloom but samples taken on subsequent days ranged from 1-4%. The mean temperature of 60F was reached late in bloom but there was no obvious increase in the number of colonized flowers. Maryblyt indicated

three high-risk days and one infection day. Cougarblight predicted high risk on three days because fire blight was present in the orchard in the previous year. The grower sprayed Mycoshield three times during bloom when environmental conditions were favorable. Fire blight occurred at an estimated incidence of 1 strike/10 trees.

Real Time PCR

We obtained poor results for all of the standard and trouble shooting PCR reactions. Although the dilutions for the spiked samples were positive in many cases, they were not always repeatable. Positive stigma imprints did not consistently correspond to samples that were positive with the PCR. Table 1 provides detailed trouble shooting procedures and their respective results.

Conclusions

We obtained excellent results with stigma imprinting using apprentice individuals trained for the flower sampling and reading of the plates. Setting up a temporary lab with an incubator at Mountainland Apples expedited the process and made results available usually within 24 hours. This verifies that novice individuals can be trained to do the stigma imprinting and plate reading thus bypassing the need for scientists to collect flowers and read plates. This is a major step in making this technique usable for grower groups or consultants.

The quick turn around time makes stigma imprinting a useful tool to advise growers of the population of the fire blight pathogen present in their orchards. It is a very useful tool to supplement Maryblyt or Cougarblight and add a significant level of confidence to making valuable decisions about the need to spray. Maryblyt and Cougarblight predictions are based on weather and do not take into consideration the amount of inoculum present in an orchard. Therefore Maryblyt might advise growers to spray but a low incidence of colonized flowers might not justify a spray. Alternatively the stigma imprints might show a high incidence of colonized flowers even when Maryblyt or Cougarblight do not show any risk.

These results show that flowers can be colonized with *E. amylovora* before the models predict any risk and before the mean temperature of 60F is reached. The incidence of colonized flowers was usually very low and may not justify any action but at least we know that the bacteria are present and ready to multiply and spread when favorable environmental conditions do occur. We have learned that when inoculum pressure is high because of a high incidence of overwintering cankers that fire blight can occur even when Maryblyt or Cougarblight do not predict any risk. It is also important that we have not seen any fire blight without first detecting *E. amylovora* in flowers using stigma imprints.

We clearly showed in the Santaquin Pear No 2 and Payson Ruby Jon orchards that the presence of large numbers of overwintering cankers resulted in a higher incidence of colonized flowers and consequently a much higher incidence of fire blight. This is true even when conditions were not highly favorable for fire blight. This reinforces the strong recommendation that canker removal is one of the most efficient methods of controlling fire blight. The inadvertent oversight of a few cankers can result in disastrous consequences if conditions are favorable for fire blight. Because of these results we suggest that growers

with orchards with a high incidence of overwintering cankers should probably spray on a calendar basis rather than based on stigma imprints or the predictive models.

These results also show that a low incidence of colonized flowers indicates that blossom blight will probably be minimal. However it should be emphasized that heavy rain, hail or strong winds might create conditions for shoot blight, which is not predicted with stigma imprinting.

Stigma imprinting on plates amended with streptomycin can also determine the incidence of streptomycin resistant *E. amylovora* isolates present in an orchard before fire blight occurs. This enables growers to customize sprays or spray mixtures of streptomycin and Mycoshield to achieve the best control where streptomycin resistance is present.

Streptomycin or Mycoshield sprays do not appear to eliminate or reduce the incidence of colonized flowers. However there is clear evidence that sprays provide some protection against blossom blight. This was apparent in the Payson Gala and Santaquin Gala No. 2. The sprays seemed to protect the outside of the double rows from fire blight but not the inside rows where coverage was not as good.

It might be possible to use the stigma imprinting method to locate overwintering cankers or hot spots in the orchards. Keeping track of where the imprints were taken would enable the grower to return to those trees or areas where the positive samples were taken. This might prove valuable in removing overwintering cankers or spot spraying areas with a high incidence of colonized flowers.

We were able to show that current RT-PCR equipment can be used onsite with relative ease and the need for a technical laboratory is not necessary. The RT-PCR units available on the market have the advantage of being transportable with the only requirement being a source of power.

The results from procedures aimed at releasing bacterial DNA or amending the reaction with DNA provided strong evidence that DNA concentration is not the reason for the negative results. However, the trouble shooting procedures provide solid evidence that the PCR procedure is either being adversely affected by an inhibitor that is preventing reactions even at low concentrations or the 944 base sequence that is being amplified is too long. It is possible that both inhibitors and primer length are both affecting the reaction. Future testing should concentrate on reducing the sequence to a short, but still unique DNA segment and optimizing an extraction procedure aimed at immobilizing the inhibitors present in pear and apple blossoms.

TABLE 1: List of corresponding stigma imprint, blossom wash and PCR results with itemized trouble shooting PCR protocols for each orchard and testing date.

Date/orchard	Stigma Imprint result (%)	Bulk Plate streak result (CFU/ml)	PCR type and Result	PCR protocol troubleshooting
22 April 2003				
McMullen Pear	0	0	Negative	Standard Protocol
Stratton Pear	4	0	Bulk / Negative	Standard Protocol
24 April 2003				
McMullen Pear	0	1	Negative	Standard Protocol
Stratton Pear	0	8	Plate #4 Positive	Standard Protocol
North farm gala	0	0	Bulk / Negative	Standard Protocol
Allred Ruby Jon	0	0	Negative	Standard Protocol
Allred Gala	0	0	Bulk / Negative	Standard Protocol
Charlie Gala	0	0	Bulk / Negative	Standard Protocol
28-29 April 2003				
McMullen Pear	2	Positive TNTC[a]	Negative	Standard Protocol
Stratton Pear	7	Postive TNTC	Negative	Standard Protocol
Allred Ruby Jon	0	0	Negative	Standard Protocol
West Mtn. Gala	0	0	Negative	Standard Protocol
West Mtn. Cameo	NA	0	Bulk / Negative	Standard Protocol
North Farm Gala	0	0	Negative	Standard Protocol
Allred Gala	2	100	Bulk / Negative	Standard Protocol
Charlie Gala	0	0	Bulk / Negative	Standard Protocol
South Ridge Pear	NA	Unk	Bulk / Negative	Standard Protocol
30 April 2003				
McMullen Pear	0	Unk	Bulk / Negative	5ul of template in reaction tube
Stratton Pear	12	Positive TNTC	Bulk / Negative	5ul of template in reaction tube
Allred Ruby Jon	0	Positive TNTC	Bulk / Negative	5ul of template in reaction tube
West Mtn Gala	0	0	Bulk / Positive	5ul of template in reaction tube
North Farm Gala	0	0	Bulk / Negative	5ul of template in reaction tube
Allred Gala	0	0	Bulk / Negative	5ul of template in reaction tube
Charlie Gala	0	500	Bulk / Negative	5ul of template in reaction tube
1 May 2003			Bulk / Negative	5ul of template in reaction tube
McMullen Pear	0	0	Bulk / Negative	5ul of template in reaction tube
Stratton Pear	11	Positive TNTC	Bulk / Negative	5ul of template in reaction tube
Allred Ruby Jon	1	0	Bulk / Negative	5ul of template in reaction tube
Valley View	0	0	Bulk / Negative	5ul of template in reaction tube

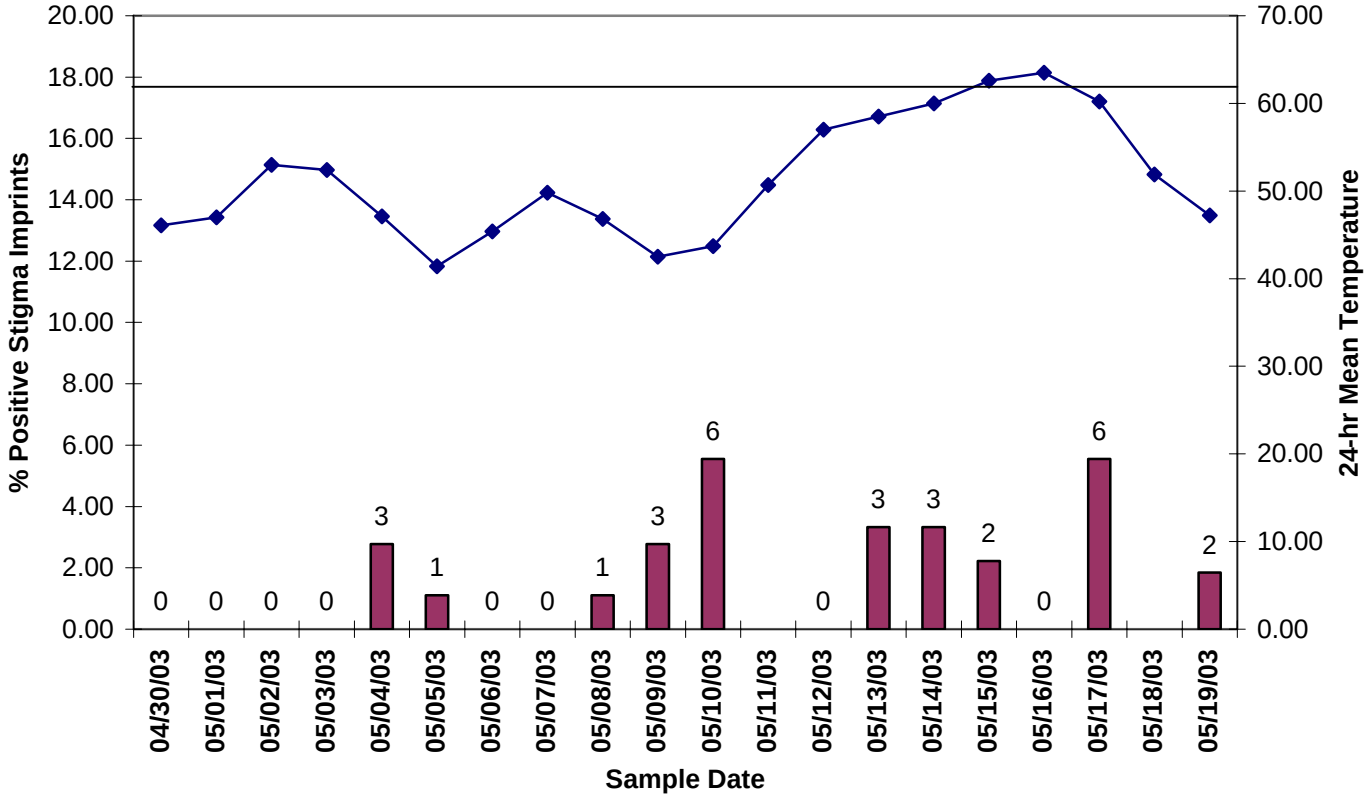
North Farm Gala	0	0	Bulk / Negative	5ul of template in reaction tube
Allred Gala	2	0	Bulk / Negative	5ul of template in reaction tube
Charlie Gala	0	0	Bulk / Negative	5ul of template in reaction tube
2 May 2003				
McMullen	1	Positive TNTC	Bulk / Negative	5ul of template in reaction tube
Allred Gala	3	1500	Bulk / Negative	5ul of template in reaction tube
Valley View	0	0	Bulk / Negative	5ul of template in reaction tube
Charlie Gala	0	0	Bulk / Negative	5ul of template in reaction tube
North Farm 1-10	0	0	Bulk / Negative	5ul of template in reaction tube
North Farm 11-15	0	0	Bulk / Negative	5ul of template in reaction tube
North Farm 16-20	0	0	Bulk / Negative	5ul of template in reaction tube
West Mtn. South	0	0	Bulk / Negative	5ul of template in reaction tube
West Mtn. Canker	0	Positive TNTC	Bulk / Negative	5ul of template in reaction tube
West Mtn. North	0	0	Bulk / Negative	5ul of template in reaction tube
West Mtn. Center	0	0	Bulk / Negative	5ul of template in reaction tube
Allred Ruby Jon	0	6	Middle spiked samples from wash and supernatant were positive with both 2 and 5ul template.	Spiked samples with Ea1, non-spiked samples, 100x and 1000x dilution of each sample type, 2 and 5ul of template for each sample type. Aliquots of samples taken from non-centrifuged blossom wash, spun down pellet and supernatant for tests.
Stratton Pear	13	Positive TNTC	South spiked samples from supernatant and 1000x dilution with both 2 and 5ul template were positive, also, spiked wash with 5ul template was positive.	Spiked samples with Ea1, non-spiked samples, 1000x dilution of each sample type, 2 and 5ul of template for each sample type. Aliquots of samples taken from non-centrifuged blossom wash, spun down pellet and supernatant for tests.
6 May 2003				
Stratton Pear	NA	Positive TNTC	All samples were negative for Ea including positive control. May have been due to primer concentration.	Ea alone, Stratton blossom wash, Spiked Stratton blossom wash. Each treatment was diluted at 0, 100x, with and without a 1600rpm 3% glycerol spin. Additional run was completed with each of the previous treatments but both supernatant and pellets were used as template.

9 May 2003				
Stratton Pear	NA	Positive TNTC	All samples tested were negative for Ea.	Stigmatic surfaces imprinted into microfuge tubes containing water agar, imprints were also done on filter paper and water agar Petri plates. All blossom washes were were spiked with Ea and run without extraction and with the Idaho Technologies 123 extraction kit. Additionally, stigmatic surfaces were swabbed with the IT 123 extraction kit swabs and then subjected to the 123 extraction procedure.
13 May 2003				
Charlie Gala and Stratton Pear	0 NA	0 Positive TNTC	All reactions were negative.	Blossoms were washed and the wash was placed directly into the reaction, spun at 13000 rpm for ten minutes with supernatant used as template and treated with a glycerol spin. The annealing time for the PCR reaction was also decreased from 60 sec to 58 seconds.
14 May 2003				
Charlie Gala, Thomson apple, Crabapple and Stratton pear	0 0 NA NA	NA[b] NA NA NA	Stratton pear at the $1x10^7$ CFU/ml spiked concentration was positive.	All samples were spiked with known concentrations of *E. amylovora* and their wash run against equivalent pure bacterial concentrations.
16 May 2003				
Charlie gala, Allred Ruby Jon and Valley View gala	0 23 0	NA NA NA	All reactions were negative for Ea.	Blossom washes for these orchards were selected based on their stigma imprint results. Each blossom wash had 2 and 5ul added to a reaction.
28 May 2003				
Allred gala, Allred Ruby Jon and Charlie gala	NA NA NA	NA NA NA	All reactions were negative for Ea.	Blossom washes had the Eppendorf plasmid extraction kit performed on them. Separate blossom wash aliquots were treated with 3% TritonX to break open all bacterial cells to release their DNA. Each blossom wash had 2 and 5ul added to a reaction.

[a]TNTC=To numerous to count.

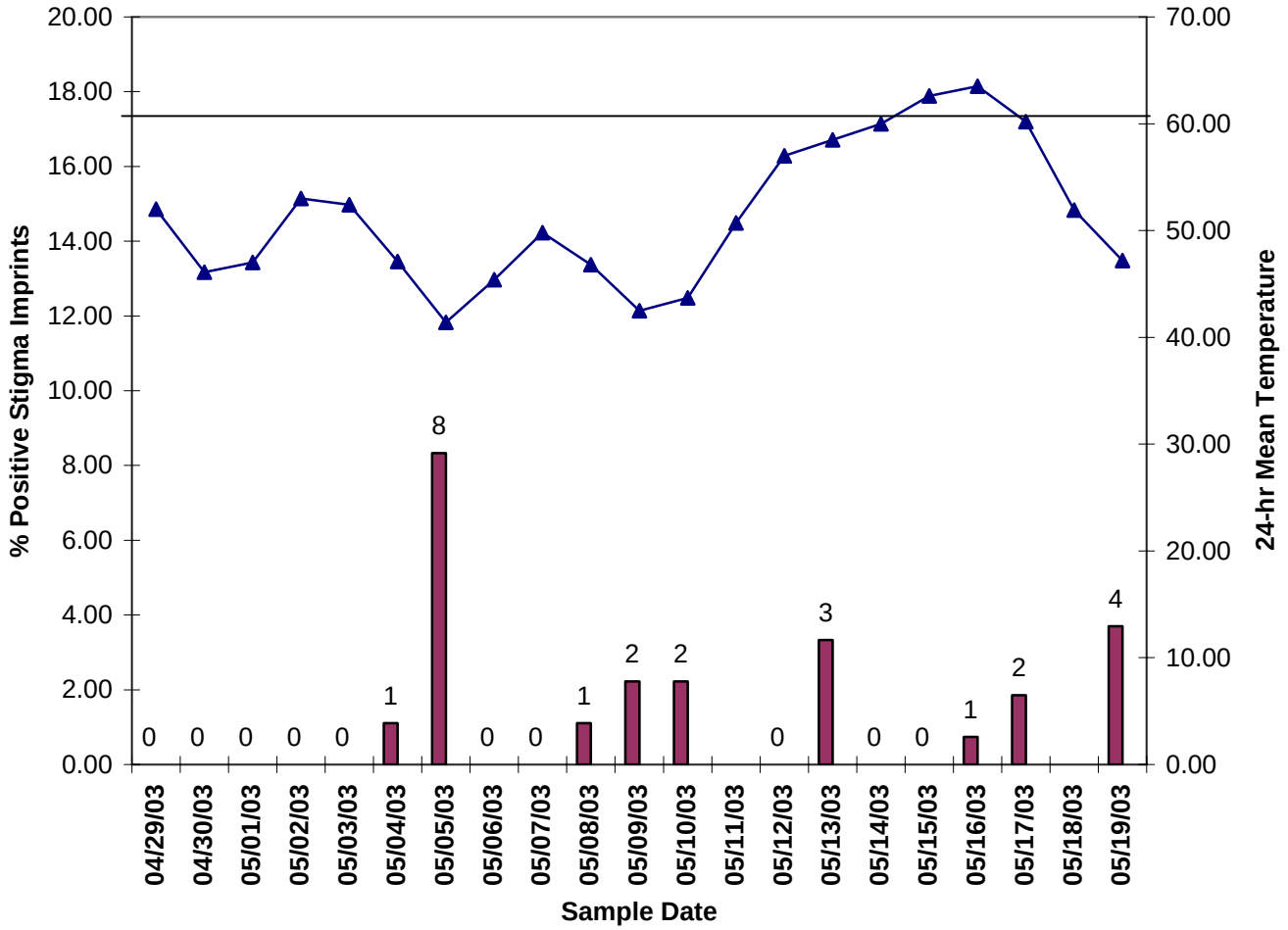
[b]NA=Not Applicable since the stimga imprints or blossom washes were not done on those days.

2003 Stigma Imprint Data for Santaquin #3 Gala



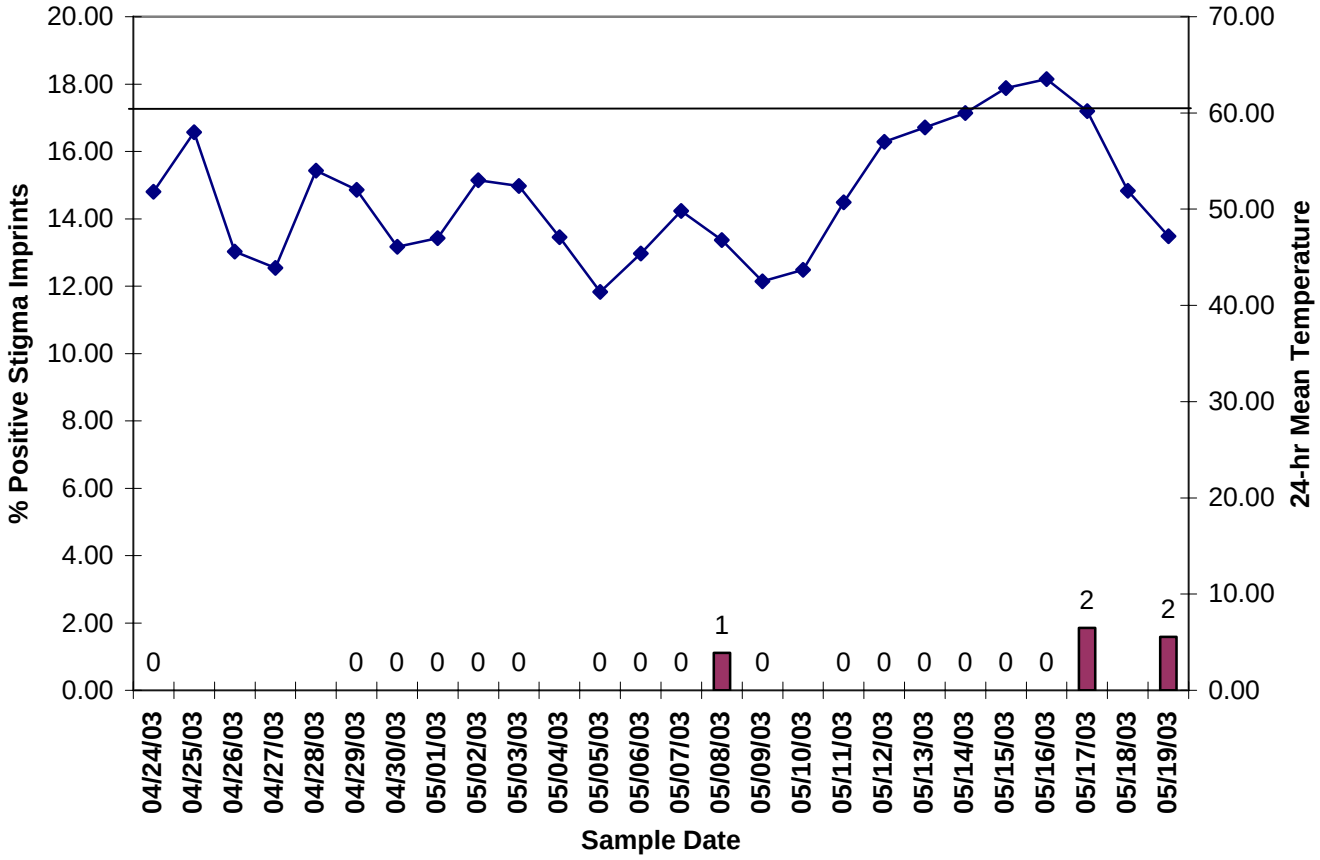
4/30—Bulk Blossom wash 1ml=no Ea growth on CCT
5/1-- Bulk Blossom wash 1ml=no Ea growth on CCT
5/2-- Bulk Blossom wash 1ml=no Ea growth on CCT
5/4-- Strep CCT test on the 5 positive imprints=1/5 resistant.
5/19--Strep CCT test on the 1 positiveimprints=1/1 resistant.

2003 Stigma Imprint Data for West Mountain Gala



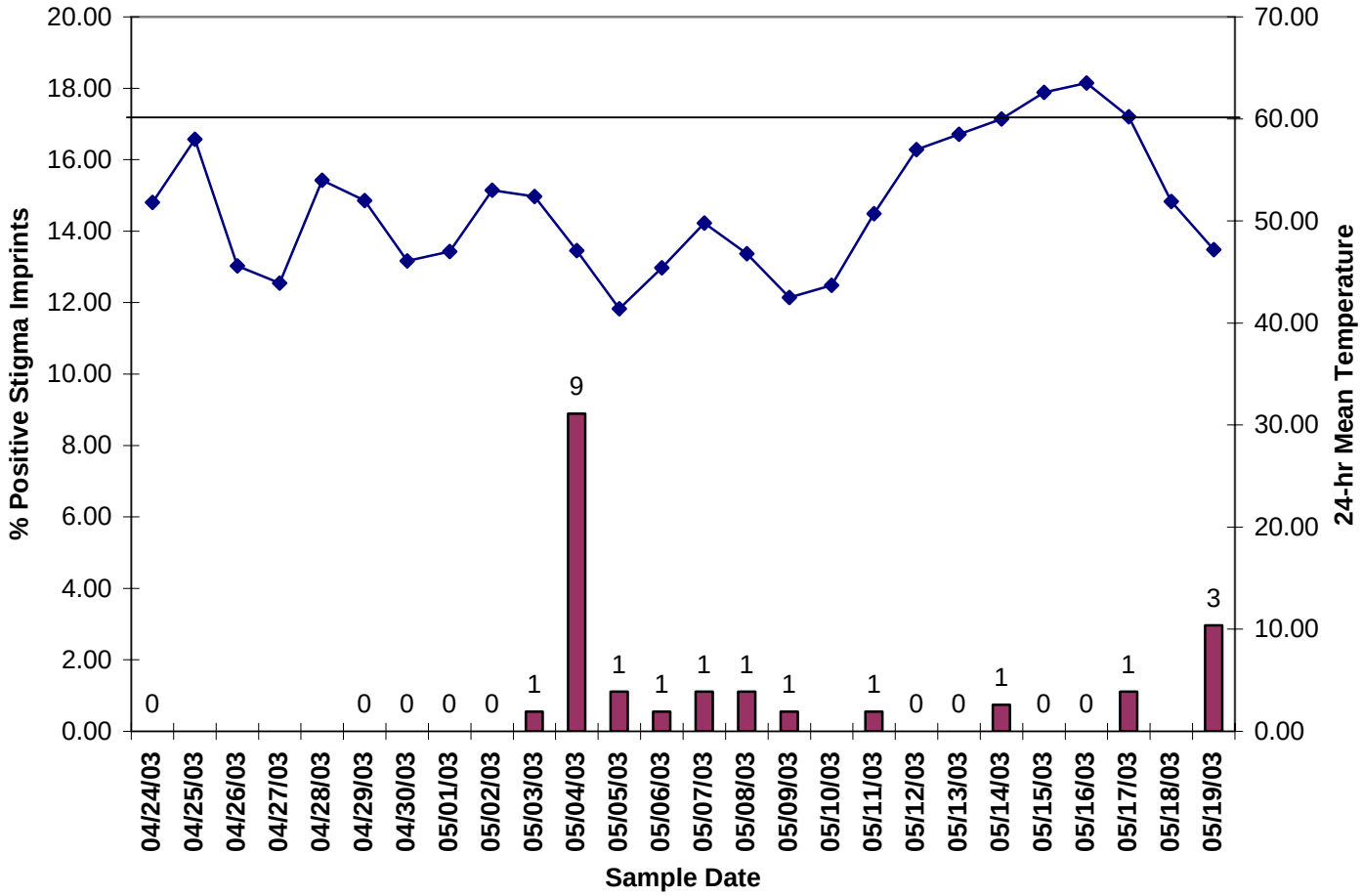
4/29—Bulk blossom wash 1ml= no Ea growth on CCT
4/30—Bulk blossom wash 1ml on each CCT and CCT/strep=no growth on either
5/1-- Bulk blossom wash 1ml on each CCT and CCT/strep=no growth on either
5/2-- Samples collected near canker, south, center and north sections of orchard
near canker=negative for stigma imprints; 1ml bulk blossom wash=TNTC on CCT
north sample=negative for stigma imprints; 1ml bulk blossom wash=0 on CCT
center sample= negative for stigma imprints; 1ml bulk blossom wash=0 on CCT
south sample= negative for stigma imprints; 1ml bulk blossom wash=0 on CCT
5/5-- Strep CCT test on the 15 positiveimprints=0/15 resistant
5/19—Strep CCT test on the 2 positive imprints=0/2 resistant.

2003 Stigma Imprint Data for Santaquin #1 Gala



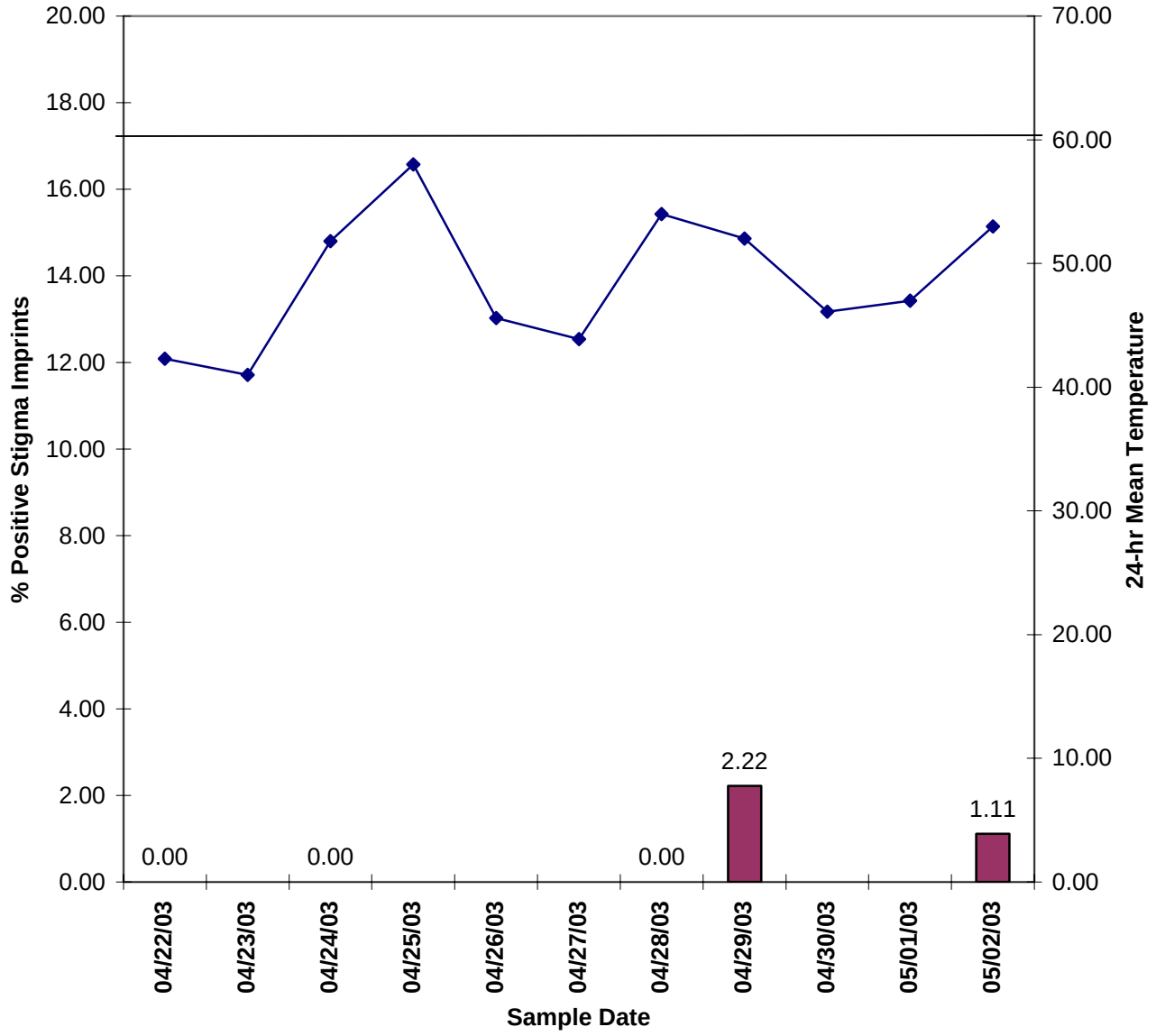
4/24—Bulk blossom wash 1ml= no Ea growth on CCT
4/29—Bulk blossom wash 1ml= no Ea growth on CCT
4/30—Bulk blossom wash 1ml= ~500 cfu on CCT; no Ea growth on CCT/strep
5/1-- Bulk blossom wash 1ml= no Ea growth on either CCT or CCT/strep
5/2-- Bulk blossom wash 1m= no Ea growth on CCT
5/19-- Strep CCT test on the 1 positive imprints=0/1 resistant.

2003 Stigma Imprint Data for Santaquin #2 Gala



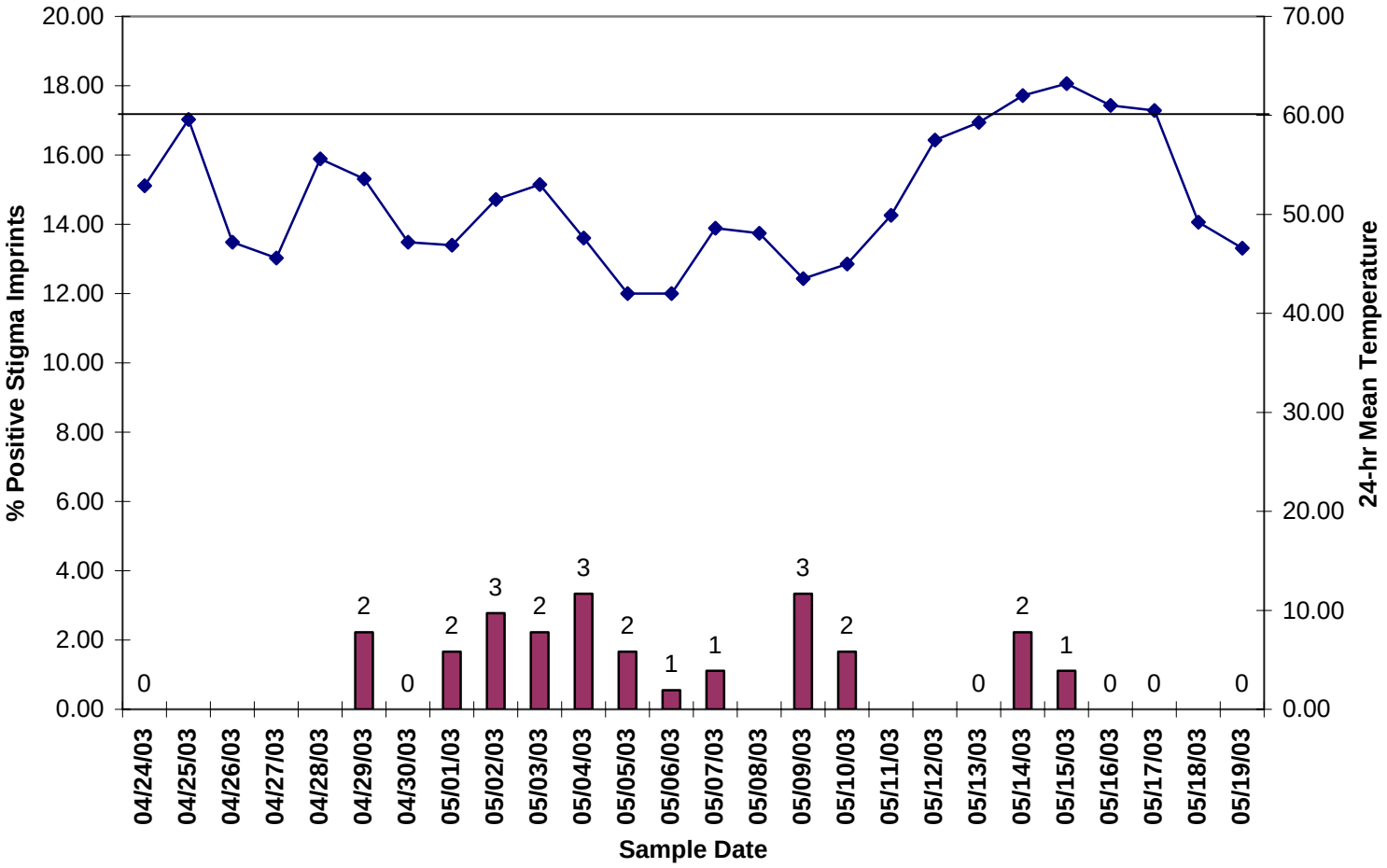
4/24—Bulk blossom wash 1ml= no Ea growth on CCT
4/29—Bulk blossom wash 1ml= no Ea growth on CCT
4/30—Bulk blossom wash 1ml= no Ea growth on either CCT or CCT/strep
5/1-- Bulk blossom wash 1m= no Ea growth on CCT
5/2-- Samples collected in the south, center and north sections of orchard
north sample=negative for stigma imprints; 1ml bulk blossom wash=0 on CCT
center sample= negative for stigma imprints; 1ml bulk blossom wash=0 on CCT
south sample= negative for stigma imprints; 1ml bulk blossom wash=0 on CCT
5/4-- Strep CCT test on the 16 positive imprints=10/16 resistant.
5/19-- Strep CCT test on the 4 positive imprints=4/4 resistant.

2003 Stigma Imprint Data for Genola Pear



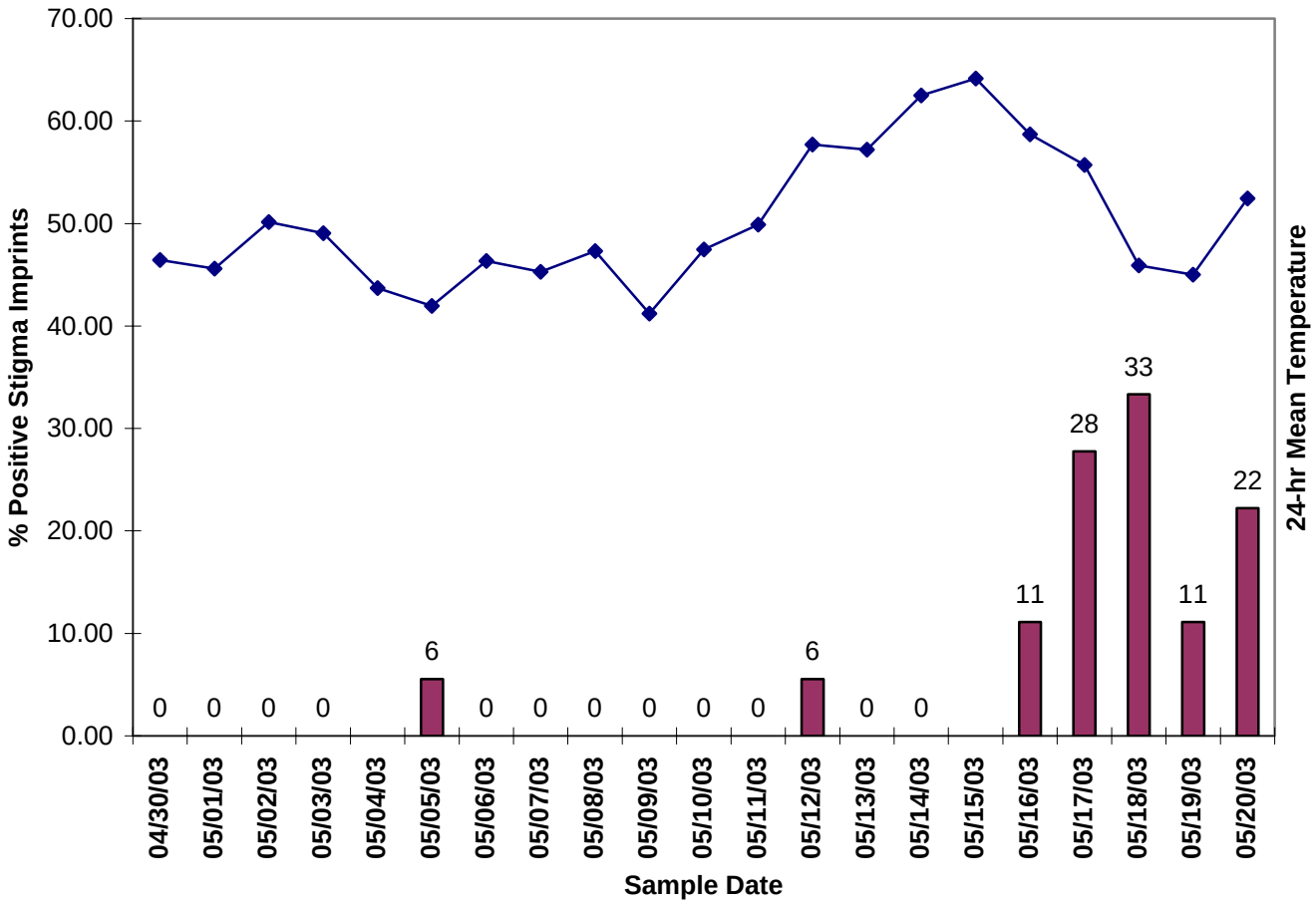
4/22—Bulk blossom wash 1ml= no Ea growth on CCT
4/24—Bulk blossom wash 1ml= 1 cfu Ea on CCT
4/29—Bulk blossom wash 1ml= TNTC cfu Ea on CCT
5/1-- Bulk blossom wash 1ml= no Ea growth on either CCT or CCT/strep
5/2-- Single Positive imprints on row 3 and row 26; Bulk blossom wash 1ml= TNTC cfu Ea on CCT

2003 Stigma Imprint Data for Payson Gala



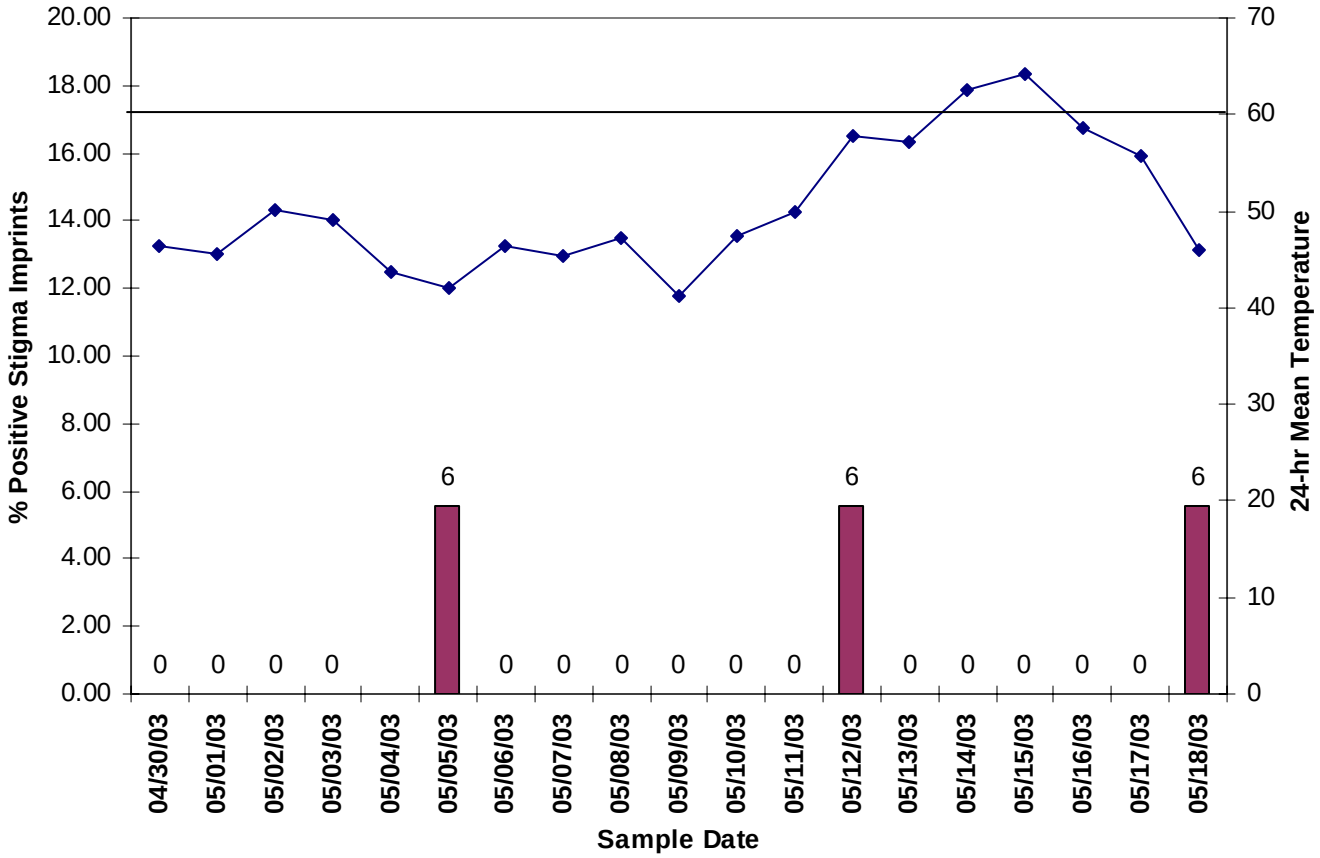
4/24—Bulk blossom wash 1ml= no Ea growth on CCT
4/29—Bulk blossom wash 1ml= ~100cfu Ea on CCT
4/30—Bulk blossom wash 1ml= no Ea growth on either CCT or CCT/strep
5/1—Bulk blossom wash 1ml= no Ea growth on CCT
5/2—Bull blossom wash 1ml= ~1500cfu Ea on CCT
5/4--- Strep CCT test on the 6 positive imprints=2/6 resistant.

2003 Stigma Imprint Data for Thomson Apple



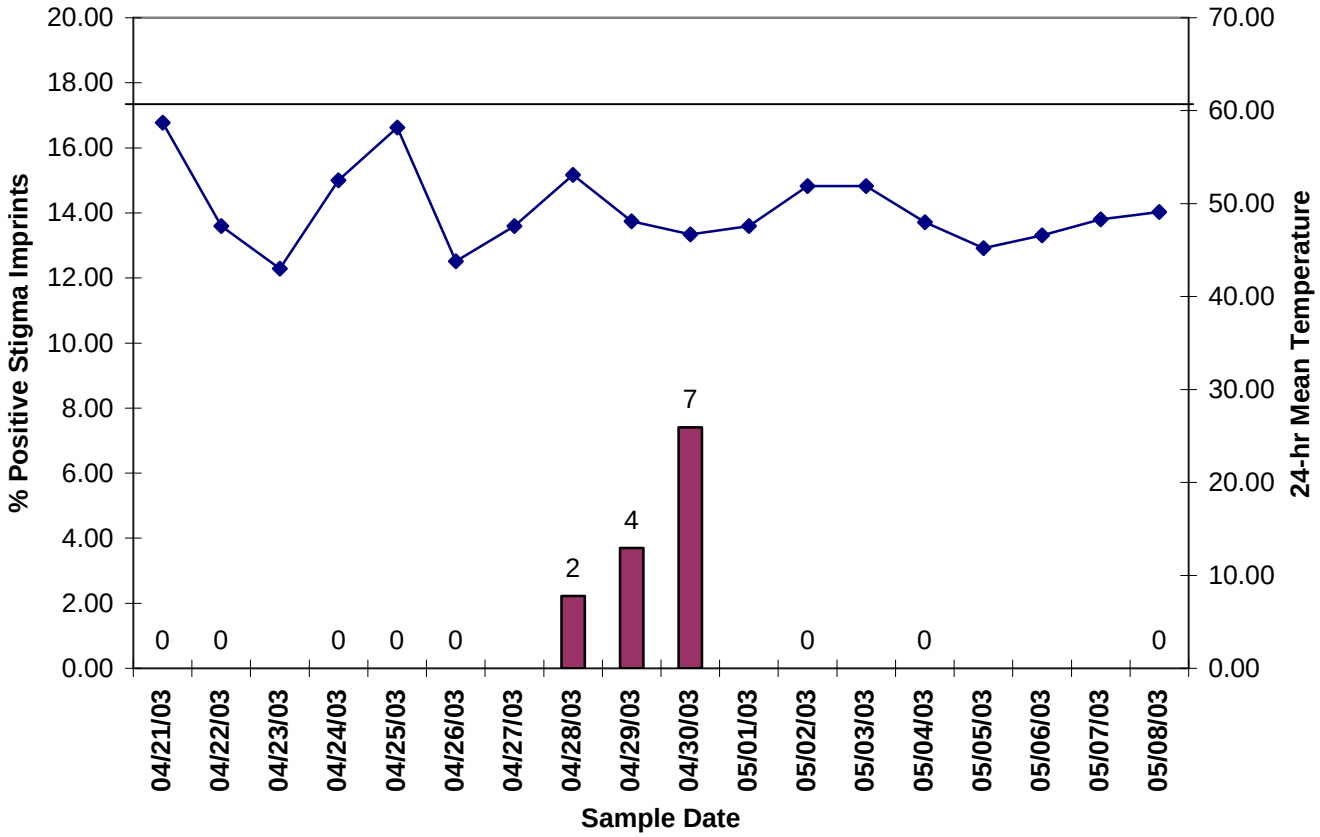
5/5—Strep CCT test on the 1 positive imprints=0/1 resistant.
5/18—Strep CCT test on the 6 positive imprints=0/6 resistant.
5/19—Strep CCT test on the 2 positive imprints=0/2 resistant; samples taken after frost
5/20—Strep CCT test on the 4 positive imprints=0/4 resistant.

2003 Stigma Imprint Data for Thomson Pear

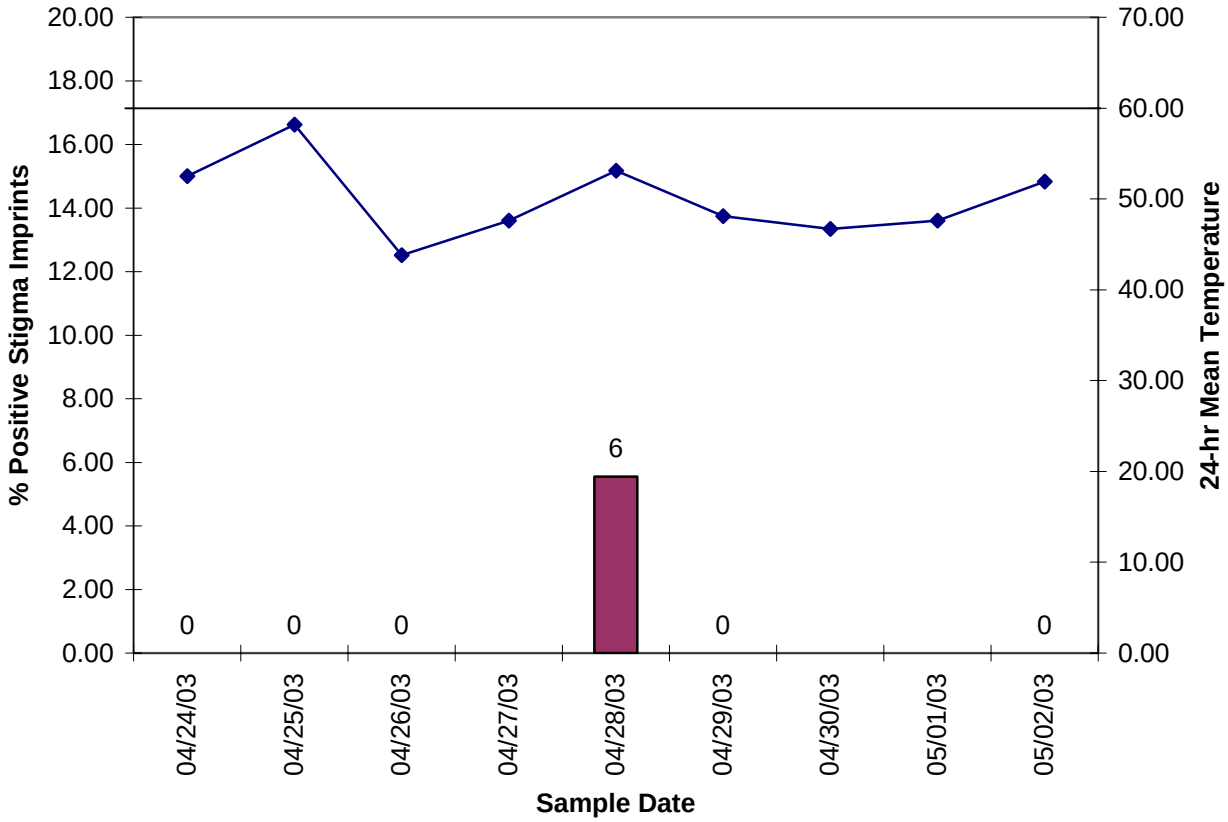


5/5—Strep CCT test on the 1 positive imprints=0/1 resistant.
5/18—Strep CCT test on the 1 positive imprints=0/1 resistant.

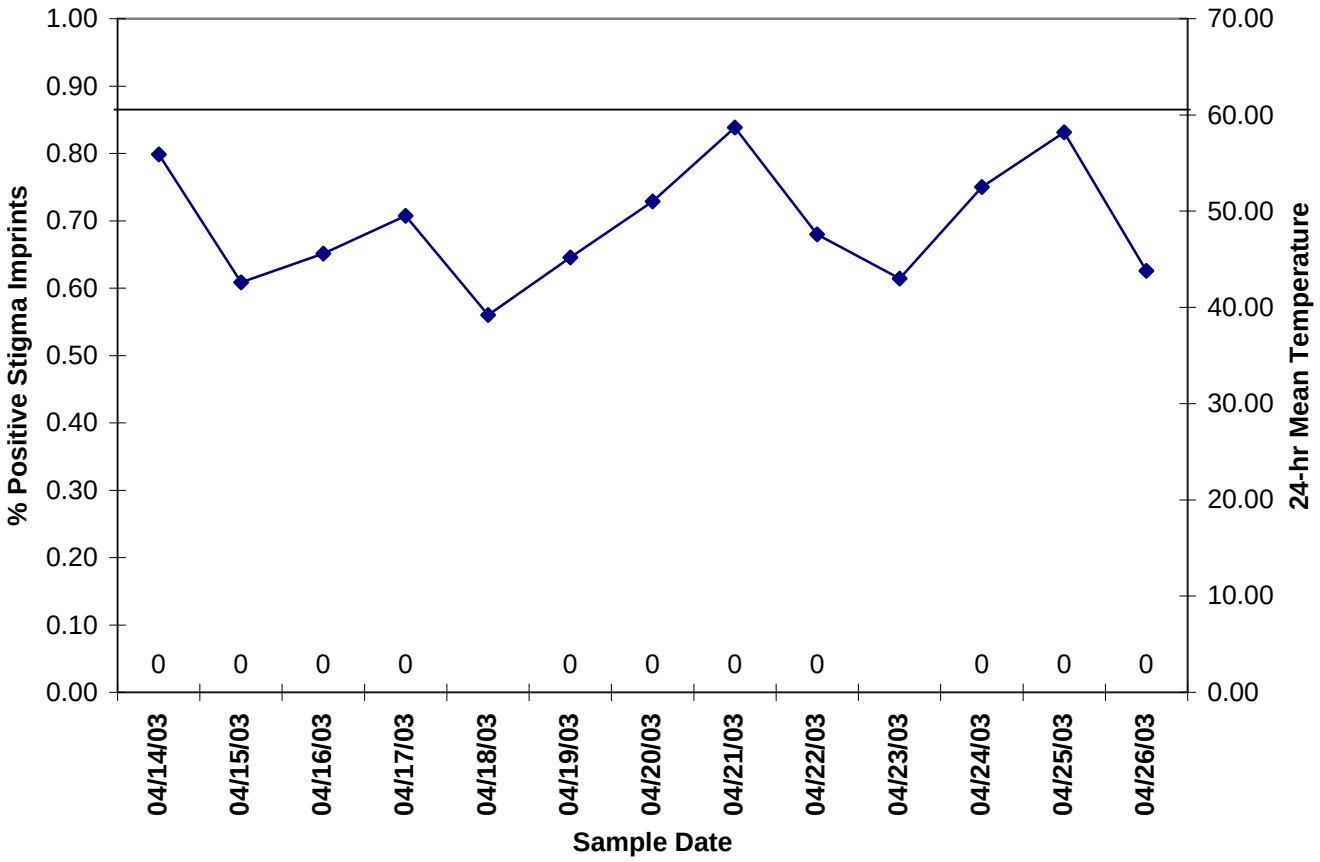
2003 Stigma Imprint Data for Kaysville Gala Apple



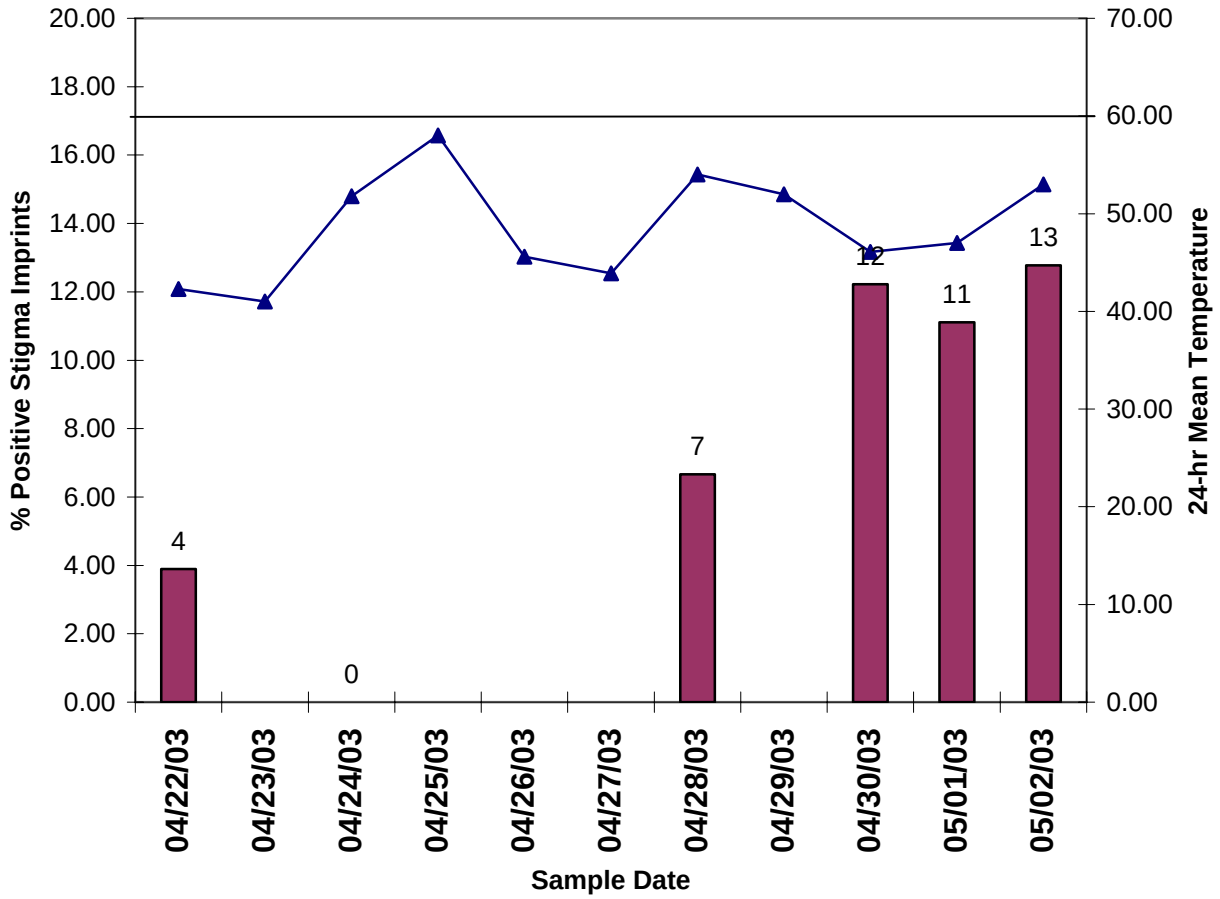
2003 Stigma Imprint Data for Kaysville IPM Apple



2003 Stimga Imprint Data for Kaysville Pear



2003 Stigma Imprint Data for Pear block #2



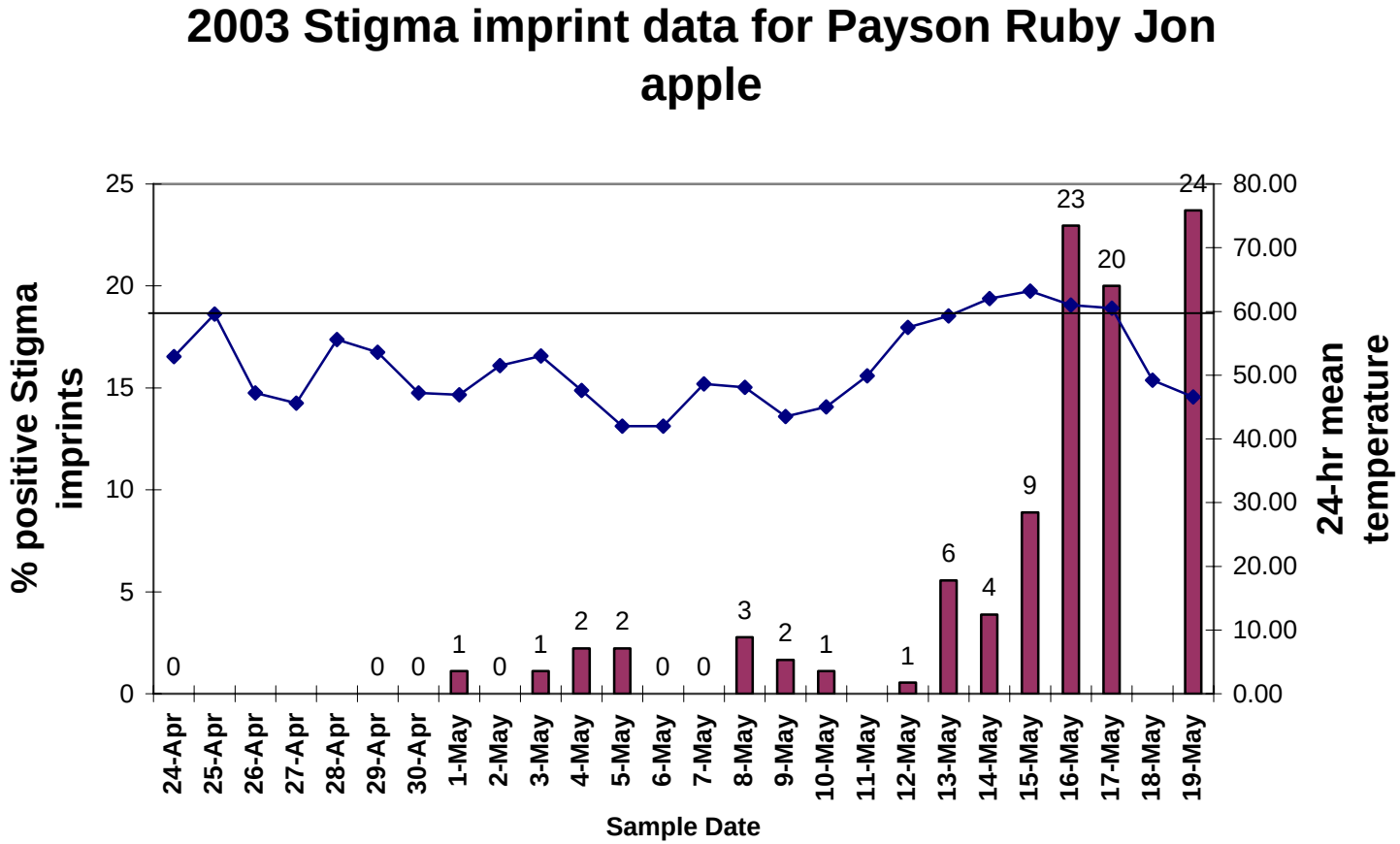
4/22—Bulk blossom wash 1ml=no Ea growth on CCT
4/24—Bulk blossom wash 1ml= 8cfu Ea on CCT
4/30—Bulk blossom wash 1ml= TNTC cfu Ea on both CCT and CCT/strep
5/1—Bulk blossom wash 1ml= TNTC cfu Ea on CCT plate
5/2-- Samples collected in the south, center and north sections of orchard and imprints done on corresponding squares on Strep plates.

north sample=All positive stigma imprints on CCT had corresponding positive imprints on CCT/strep plates-plate 15 had 5 CCT positives and 4 CCT/strep positives; 1ml bulk blossom wash=TNTC on CCT and CCT/strep

center sample= All positive stigma imprints on CCT had corresponding positive imprints on CCT/strep plates-plate 10 had 1 CCT positives and 0 CCT/strep positives; 1ml bulk blossom wash=~1500 on CCT and CCT/strep

south sample= One positive stigma imprint on CCT -plate 3 had 1 CCT positive and 0 CCT/strep positives; 1ml bulk blossom wash=8 cfu Ea on CCT and 4 cfu on CCT/strep

5/1-5—Blossom Blight symptoms found in orchard near severely cankered tree.



4/24—Bulk blossom wash 1ml= no Ea growth on CCT
4/29—Bulk blossom wash 1m= no Ea growth on CCT
4/30—Bulk blossom wash 1ml= TNTC cfu Ea on CCT and TNTC cfu Ea on CCT/strep
5/1—Bulk blossom wash 1ml= no Ea growth on CCT
5/2-- Samples collected near canker, south, center and north sections of orchard
near canker=negative for stigma imprints; 1ml bulk blossom wash=no Ea growth on

CCT or CCT/strep
north sample=negative for stigma imprints; 1ml bulk blossom wash=6 cfu Ea on CCT and no Ea growth on CCT/strep
center sample= negative for stigma imprints; 1ml bulk blossom wash=0 on CCT and CCT/strep
south sample= negative for stigma imprints; 1ml bulk blossom wash=0 on CCT and CCT/strep

5/19— Strep CCT test on the 32 positive imprints=25/32 resistant