THE DEVELOPMENT OF NEW TOOLS FOR FIELD AND LABORATORY DIAGNOSIS OF PIERCE'S DISEASE

A Thesis

by

KELLY ASBILL BRYAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2008

Major Subject: Plant Pathology

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Approved by:

Chair of Committee,	David N. Appel
Committee Members,	Carlos F. Gonzalez
	B. Greg Cobb
Head of Department,	Dennis C. Gross

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ABSTRACT

The Development of New Tools for Field and
Laboratory Diagnosis of Pierce's Disease. (May 2008)
Kelly Asbill Bryan, B.S., Texas Tech University
Chair of Advisory Committee: Dr. David N. Appel

Pierce's Disease (PD), caused by *Xylella fastidiosa*, is a devastating bacterial disease of grapevines. One of the few control options is roguing. Roguing depends on precise diagnosis of PD in vines. These experiments were conducted to improve available diagnostic protocols and enhance levels of disease control.

Plots were selected from four different Texas vineyards with a total of four different varieties (Blanc duBois, Cabernet Sauvignon, Chardonnay, and Merlot). An infrared thermometer was used to take temperature measurements of the vines. Samples were taken of each of these vines at the same time and were tested for *X. fastidiosa* by culturing, Enzyme-Linked ImmunoSorbent Assay (ELISA), and Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR).

ELISA found an increase in plant temperature in samples that tested positive for *X. fastidiosa*, but QRT-PCR did not. An infrared thermometer could be used to detect asymptomatic vines, but there are several variables to consider such as grape variety and vineyard location. Grape varieties differed significantly in mean temperatures, as did vineyard locations. PD does not seem to have a pattern in which it spreads, although this could be because of the high level of disease incidence in the chosen vineyards. Both the ELISA and QRT-PCR tests have their own pros and cons for *X. fastidiosa* detection. ELISA takes approximately 6 hours and can be inaccurate in detecting *X. fastidiosa*. QRT-PCR takes 2-3 hours and is a much more sensitive test. A combination of techniques (PrepMan Ultra® and nucleic acid precipitation) can be used to clean QRT-PCR samples when they have degraded and are being affected by inhibitors.

DEDICATION

To Papa – I did it! I will miss you always.

Love,

Kelly

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. David Appel for his help throughout this whole process. I couldn't have done it without your expertise. I would also like to thank my committee members, Dr. Carlos Gonzalez and Dr. Greg Cobb. A special thanks goes out to my lab colleagues, for without their help, this project wouldn't have been possible.

I would also like to express my appreciation towards my family, friends, and most of all to my husband, Casey. You have let me vent, offered me advice, and kept me going when I wanted to quit. I love you all!

TABLE OF CONTENTS

	Page
ABSTRACT	iii
DEDICATION	iv
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF FIGURES	viii
LIST OF TABLES	x
INTRODUCTION	1
MATERIALS AND METHODS	7
Experimental Setup	7
Temperature Measurements	8
Tissue Collection and Processing	8
ELISA Testing	9
QRT-PCR Testing	10
Statistical Analysis	10
RESULTS	11
Vine Temperature Measurements	11
Disease Epidemiology	28
QRT-PCR vs. ELISA	28
QRT-PCR Experimental Procedures	34
DISCUSSION	37
SUMMARY	42
LITERATURE CITED	43
APPENDIX A	45

Page

APPENDIX B 47

APPENDIX C 48

VITA 49

LIST OF FIGURES

FIGURE Page

1 Risk map for PD across the state of Texas 5

2 Plot pattern. 8

3 Dead vine average temperatures (F) throughout a summer day................ 12

4 Mean infrared temperatures for each of the four varieties at all four vineyards for all six time points. 13

5 Mean temperatures of the Texas Hills vineyard varieties. 14

6 Mean temperatures of the Spicewood vineyard varieties........................... 15

7 Mean temperatures of the Palacios vineyard varieties. 16

8 Mean temperatures of the Experimental vineyard varieties. 17

9 Mean infrared temperature for each of the vineyards over the entire summer 18

10 Mean temperature for each of the separate copy numbers in QRT-PCR... 19

11 Blanc duBois sample copy numbers and the corresponding average vine temperatures. 21

12 Cabernet Sauvignon sample copy numbers and the corresponding average vine temperatures. 22

13 Chardonnay sample copy numbers and the corresponding average vine temperatures. 23

14 Merlot sample copy numbers and the corresponding average vine temperatures. 24

15 Mean temperatures for negative, positive, and potential positive ELISA results. 26

FIGURE Page

16 Mean temperature of ELISA results for each variety for the entire summer. 27

17 Number of QRT-PCR negatives and positives for each of the plot locations 29

18 Number of QRT-PCR negatives and positives for each plot location for Blanc duBois at week 33. 30

19 Number of QRT-PCR negatives and positives for each plot location for Cabernet Sauvignon for week 33. 31

20 Number of QRT-PCR negatives and positives for each plot location for Chardonnay at week 33. 32

21 Number of QRT-PCR negatives and positives for each plot location for Merlot at week 33. 33

22 FAM and TAMRA graph lines with inhibitors present from degraded QRT-PCR samples. 35

23 FAM and TAMRA graph lines from a QRT-PCR with no inhibitors due to PrepMan Ultra® and nucleic acid precipitation.......... 36

LIST OF TABLES

TABLE Page

1 Analysis of variance showing no significant difference between the dependent variable of Log of Copy # and the independent variable of Temperature Difference.. 20

2 Analysis of variance for Blanc duBois between the dependent variable of Log of the Copy Number and the independent variables, of Temperature Difference (ambient – vine) and Variety.. 21

3 Analysis of variance for Cabernet Sauvignon between the dependent variable of Log of the Copy Number , and the independent variables, of Temperature Difference (ambient – vine) and Variety 22

4 Analysis of variance for Chardonnay between the dependent variable of Log of the Copy Number, and the independent variables, of Temperature Difference (ambient – vine) and Variety.. 23

5 Analysis of variance for Merlot between the dependent variable of Log of the Copy Number , and the independent variables, of Temperature Difference (ambient – vine) and Variety.. 24

6 Analysis of variance of grapevine temperature measurements against the independent variable of ELISA results 25

7 Comparison of numbers of ELISA and QRT-PCR positives (+). (p+) = potential positive, PPD = possible PD 34

INTRODUCTION

Xylella fastidiosa is a devastating bacterial plant pathogen to many different species of crop plants, but none more so than grapevines. *X. fastidiosa* causes Pierce's disease (PD) of grapes, and is one of the major limiting factors to growing *Vitis vinifera* grapes in the southeastern United States (4). Compared to many other plant pathogens, *X. fastidiosa* is still poorly understood. *Xylella fastidiosa* is a gram negative, fastidious bacterium, or a bacterium that needs specific nutrients *in vitro*. Therefore, *X. fastidiosa* was not cultured until the 1970's (4). Failure to successfully culture the pathogen greatly hampered the expansion of knowledge about *X. fastidiosa* and PD. Today, research scientists are gaining back the ground they lost, but there are still large gaps in our knowledge of this disease. As a result of these knowledge gaps, there are no effective control measures or cures for PD. Also, to add to the lack of knowledge about the bacterium, the disease can be difficult to diagnose (14).

Many of the symptoms associated with PD are the same as those caused by other diseases. Stunting, dieback, leaf scorching, and defoliation can all be found as symptoms in various other diseases or nutrient deficiencies. However, PD does have a few characteristic symptoms. Petiole retention, also known as matchsticking, is one of them. Matchsticks form when the leaf lamina abscises from the petiole. The petiole remains and then dehydrates thus looking like a blackened matchstick (14). One other characteristic symptom is green islands. Green islands occur on the canes of newer growth. As the new growth begins to mature and form periderm, the outer tissue normally turns brown at a relatively uniform rate. In the canes of PD-infected plants, the browning does not occur uniformly. The canes will turn brown near the nodes and remain green in small patches in the intermediate zone (14). These two symptoms, however characteristic they are, are not useful in identification until later in the summer season. This allows several months where a misidentified plant can be a reservoir for the pathogen.

This thesis follows the format of the journal, Plant Disease.

More ways of confidently diagnosing PD is through serological tests or by polymerase chain reactions, but even these can be problematic. ELISAs, or Enzyme-Linked ImmunoSorbent Assays, are relied upon heavily for identification of *X. fastidiosa* and Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR) is becoming more popular as new developments make processing faster (Applied Biosystems). New developments have made QRT-PCR is much more sensitive than ELISA, it can detect one molecule of bacterial DNA (Applied Biosystems, and it can be run in one third of the time. At this point in time, growers are frustrated due to the difficulties in diagnosis and the lack of measures to protect themselves from this devastating disease.

Xylella fastidiosa is vectored by two main insects, *Graphocephala atropunctata* (Signoret), the blue-green sharpshooter (BGSS), and *Homalodisca vetripennis* (Germar), the glassy-winged sharpshooter (GWSS). Both are members of the leafhopper family. The BGSS is native to California, and prior to the introduction of GWSS, it was the primary vector. Now GWSS has surpassed its lesser relative and has devastated the southern grape growing regions of the state. The GWSS is indigenous to the Gulf Coast states and exists throughout much of the Southern United States (7). It is one of the main vectors in Texas. This voracious leafhopper feeds on many different host plants, so many in fact that it would be very difficult to eliminate all of its hosts (7).

The mode of action of *X. fastidiosa* is to aggregate in the xylem of plants and form occlusions. Plant tissue above these occlusions is unable to receive water and therefore dies (1). There are two kinds of xylem tissues, tracheids and vessels. Tracheids are thinner than vessels and overlap each other. Because of their thinness, they translocate a relatively smaller amount of water at a time. Vessels are shorter and wider than tracheids and allow a much greater amount of water to flow up the plant (17). It is in the vessels that *X. fastidiosa* occlusions have been found. These occlusions are not entirely made up of bacteria, in fact, it has been shown that muscadine grapes (*Muscadinia rotundifolia* Michx.) have a defense mechanism that allows them to produce tyloses, gums, and pectins. These substances trap the bacteria and prevent them from spreading systemically (2). Although these vascular plugs help prevent the spread of *X. fastidiosa* throughout the plant,

they also plug the plant's xylem vessels. This causes water stress and leaf symptoms such as leaf scorch (2).

When a plant becomes water-stressed, its temperature can climb. Plants control their temperatures in three different ways. Leaves only absorb about 50% of the total solar energy coming from the sun (17). To dissipate the heat that the leaves accumulate, they can use long-wavelength radiation, sensible heat loss, or latent heat loss (17). Sensible heat loss is basically a product of conduction and convection. If the air temperature is less than the leaf temperature, the air circulation removes heat from the leaf. Latent heat loss occurs by transpiration. As water evaporates from the leaf, it withdraws large amounts of heat. These latter two processes are the most important in the regulation of the leaf's temperature (17). The Bowen ratio describes the relationship between these two processes. The Bowen ratio is sensible heat loss divided by evaporative heat loss (17). When a crop is water-stressed, the stomates partially close which then reduces evaporative cooling (increases Bowen ratio). This decrease in evaporative cooling conserves water, but the plants then have higher leaf temperatures (17). Tu et al. (15) found that when measuring leaf-air temperatures, bean plants infected with *Phaseolus vulgaris* L. and grown under water stress usually had much higher temperatures. To test the correlation between disease incidence and temperature, bean cultivars with tolerance or susceptibility to *Fusarium solani, Rhizoctonia solani,* and *Pythium ultimum* were grown in infested soil (15). The soil was at field capacity to rule out any increase in temperature from water stress. Susceptible cultivars had a significantly higher rate of root rot severity. Temperatures were found to be 2-3 degrees Celsius higher in susceptible plants than tolerant plants (15). They also discovered that the temperature increased with the increasing severity of the disease. In a study by Nilsson et al. (10), the leaf temperature of healthy plants (16-17°C) was considerably lower than in infected plants (23-24°C). In a separate study, differences of 3-5°C were found in the flag leaf temperature of root/vascular diseased plants (10). Jones et al. (6) used infrared thermography to demonstrate that temperature results from the sunny side of a grapevine can be 3°C higher than the shaded side. Jones et al. (6) also recorded the sunny side of a vine as to having a 1.81°C standard deviation, compared to a 0.93°C

standard deviation of the shaded side. Due to the vascular nature of PD, there may be connections between the occlusions and an increase in plant temperature. If so, this could provide a way to identify PD infected plants before the titer of bacteria in a plant becomes high enough to serve as an inoculum reservoir.

The available control measures for PD are expensive, time-consuming, and not altogether effective. The best control for PD is to do preliminary planning. Location of a prospective vineyard should be determined carefully. It has been shown that *X. fastidiosa* resides in a large number of riparian plants and weeds (12). Therefore, it would be wise not to plant a vineyard near rivers or lowlands (12). Especially in areas where PD is common, natural vegetation surrounding the vineyard must be kept to a minimum to reduce the amount of inoculum available to the vectors. A minimum of 150 feet should be maintained as weed-free to serve as a barrier between the vineyard and the surrounding vegetation (7). Also, considerable thought should be given to variety selection. Resistant varieties should be chosen in locations in which PD is known to be endemic. Perry (1976), designed a risk map that showed the varying probability, across the state of Texas, of having PD in vineyards (Fig. 1). This figure is very helpful and should be consulted when planning to establish a vineyard in Texas.

Fig. 1. Risk map for PD across the state of Texas (Perry 1976).

Unfortunately for vineyard owners, many of the widely used cultivars are susceptible to the disease. *Vitis vinifera* spp. are particularly ravaged by PD; Chardonnay, Sangiovese, Cabernet Sauvignon, and Merlot are just a few examples that are susceptible (on different levels) to PD. Testing is currently done at Texas A&M University to determine which varieties are most suitable for the hot Texas climate and its disease index (G. McEachern, personal communication). So far, the only varieties listed for PD resistance are Blanc duBois, a white wine American hybrid variety, Black Spanish (Lenoir), and Cynthiana (Norton), both red wine American hybrid varieties (3). Black Spanish and Cynthiana are *Vitis labrusca* and *Vitis aestivalis* Michx. respectively (3). Monitoring vector populations is also an important step in controlling PD. This allows the grower to know when the most opportune time for insecticidal spraying. However, there are few insecticides labeled for use on sharpshooters. There are also heavy restrictions on the insecticides used on food intended for human consumption (8). One technique with some promise for control is 'roguing,' or the removal of infected vines. Presumably a diseased asymptomatic plant may have sufficient bacterial titer to serve as an inoculum source. Under those conditions, effective roguing becomes increasingly difficult to accomplish. Improper roguing could lead to the rampant infection and devastation of an

entire vineyard. Current roguing recommendations are made on the basis of the predicted spatial pattern of the disease. In a study by Tubajika et al.(16), the observed spatial patterns of PD led to the conclusion that the distribution of the disease reflected the feeding pattern of the vector. *Xylella fastidiosa* spreads down a row, or vine-to-vine, at a higher frequency than across rows (16). The frequency of vine to vine spread was found to be anywhere between 20-92%, whereas across row spread was found to happen 12-80% of the time (16). This study provides evidence that it may be in the best interest of growers to rogue symptomless plants surrounding an infected plant, as it is probable that they are already infected. To enhance the effectiveness of roguing, it was hypothesized for the present study that one could use an infrared thermometer to detect non-symptomatic vines. If this hypothesis is true, then it would be a simple tool that growers could use to manage PD in their vineyards. The use of infrared thermometry in diseased crops is not a novel idea. There have been several studies that have sought to use this technology and many observed correlations between diseased plants and an increase in temperature (10, 15, 16). The difference in previous studies and this study is that we looked at the plant temperature as a whole, not just the leaf temperatures.

The goal of this project is to better understand the nature of *X. fastidiosa* and be able to develop techniques for management and control of the bacterium. The hypothesis central to this research was that infrared thermometry can be used to diagnose PD-infected plants before symptoms appear. Specific objectives were;

1. To map the spread of PD from known source vines to adjacent vines within and across vineyard rows,
2. To test various existing diagnostic techniques on the mapped vines for their relative abilities to detect the pathogen in symptomatic and symptomless vines,
3. To test a new diagnostic technique, the infrared gun, for utility as a tool in the detection of diseased vines.

MATERIALS AND METHODS

Experimental Setup. To test the objectives of this study, there were several variables to consider. The first was location. Suitable vines were selected in four different vineyards: Texas A&M Experimental Vineyard (ExV) in College Station, Palacios Vineyard (Pal) in Brenham, Spicewood Vineyard (SW) in Spicewood, and Texas Hills Vineyard (TX) in Johnson City. These vineyards were chosen because we were familiar with their layout and varieties. The second important variable was grape variety. Four different grape varieties were chosen from the four vineyards. They were Merlot, Blanc duBois, Chardonnay, and Cabernet Sauvignon. Merlot is considered to be somewhat tolerant to *X. fastidiosa*, while Chardonnay and Cabernet Sauvignon are very susceptible. Blanc duBois is considered to be the best PD-resistant white wine grape in Texas (personal communication, Dr. George Ray McEachern, Horticulture Dept., TAMU, College Station, TX).

Using 2005 vineyard disease survey maps (ArcMAP® summer survey data), disease centers for data collection were chosen. The vineyard disease surveys were plots of the entire vineyards showing each individual vine and its health rating for 2005. In this study, a disease center is defined as a diseased central plant in a plot of 15 surrounding plants. The surrounding vines had been deemed healthy in the 2005 PD surveys. Having healthy vines surrounding the disease centers allowed for observing pathogen spread. The central vines, or disease centers, were selected by two levels of symptom development; incipient and severe. A healthy control plot with asymptomatic vines was also included. The plots, defined as the disease center and the surrounding 14 vines (Fig. 2), were flagged for every variety. There were three plots per variety (healthy control, incipient, and severe) and each of the varieties had plots at two different vineyards (Appendix A).

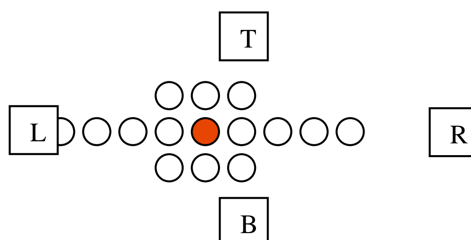

Fig. 2. Plot pattern. Disease center is shown in red and healthy plants in white (T= top, R = right, B = bottom, L = left).

Temperature Measurements. The fifteen chosen plants for each disease center had temperature measurements taken with a Fluke 572 Precision Infrared Thermometer (Everett, WA USA 98206) once a week for six weeks throughout June, July, and August. The emissivity value was set at 0.94. Emissivity is a material's ability to absorb and radiate energy. More specifically it is the ratio of radiation emitted by a surface to radiation emitted by a black body at the same temperature (5). The shaded side of the main trunk, towards the top of the plant, was the locale of the temperature reading in hopes of reducing the amount of influence from the Texas summer sun (2). Also, temperature readings were only taken from 11:00 am-3:00 pm and in good weather conditions. No temperature readings were taken during cloudy or otherwise bad weather days to reduce variability. Three temperature measurements were collected for every vine and then averaged. These data, as well as the overall condition of the vine was recorded manually and then input into an Excel spreadsheet. To serve as a temperature control and to allow for observation of a temperature range, readings were taken from a dead grape vine. The temperatures were taken at specific time intervals (9:00 am, 12:00 pm, 3:00 pm, and 6:00 pm) throughout the day (Fig. 3).

Tissue Collection and Processing. Tissue samples were also collected weekly from each of the fifteen plants for each of the six weeks for a total of 2160 samples. Leaves (8-10), with petioles attached, were taken from each plant and put into a labeled bag. These samples were stored in an ice chest in the field and then were transferred to a 4

C refrigerator at the lab. These samples were processed for culturing on PD3 media, ELISA, and QRT-PCR testing. Culturing was only accomplished on the first time point (Julian week 26). Because of the slow process of preparing samples for culturing, the samples were stored in a refrigerated cold room. This cold room had a malfunction in December 2006 that caused all of the stored samples to rot. Because *X. fastidiosa* is an extremely slow growing bacterium, the media plates were being overrun by secondary organisms before *X. fastidiosa* had a chance to grow. ELISA and QRT-PCR testing were done despite the condition of the degraded samples. For the samples that were processed, 3-5 petioles were selected and surfaced washed in tap water. Using aseptic techniques, the petioles were cut into manageable pieces, approximately 1 inch in length. The petioles were then rinsed in 70% ETOH and transferred to 20% sodium hypochlorite for 4 minutes. After four minutes, the petioles were removed with sterile forceps and rinsed in sterile distilled water. They were then placed into labeled sterile Petri dishes filled with sterile distilled water. The petioles were cut into smaller pieces, approximately 1 cm in length. Using sterile forceps, the petiole pieces were squeezed in the center, allowing sap to exude from both of the cut ends. The cut ends were then lightly touched to the surface of the PD3 media in a prearranged pattern. Plates were wrapped with parafilm and placed into a 28 C incubator. Because of the long incubation time for *X. fastidiosa*, the plates were allowed to incubate for at least 7 days. Most were not removed until 14 days had passed. Plates were checked regularly and results recorded. The PD3 media used was slightly altered in that only ½ the amount of BSA was used (Appendix B).

ELISA Testing. ELISA tests were run using Agdia kits (Agdia, Inc., Elkhart, Indiana, 46514). The Agdia ELISA protocol was followed in this experiment, except for one detail. In the beginning, we used SCPAP Extraction buffer (Appendix C) instead of the General Extraction Buffer (GEB) that Agdia supplies. This buffer substitute was made to avoid disposal requirements of the sodium azide in GEB. However, the yellow tint in the SCPAP buffer interfered with the TECAN SpectraFluor plate reader function forcing a switch back to the suggested GEB. The remaining petioles from the sample bags were surface-sterilized and cut lengthwise and then again horizontally into very small pieces.

Approximately 0.15 grams of the cut petiole tissue was put into each of two labeled 1.5 mL tubes. This allowed enough of each sample to run several ELISAs or QRT-PCRs if necessary. GEB (600μl) was added to the tubes and then they were stored at 4 C overnight. The tubes were removed from the refrigerator and spun at 12,000 rpm for 2 minutes. The supernatant was then drawn out and placed into new labeled 1.5 mL tubes. The remaining tissue and tubes were discarded. The samples were stored in a -20 C freezer until testing. Upon testing, the samples were thawed, while the remaining solutions required for the test were made. All solutions were made according to the Agdia protocol.

QRT-PCR Testing. QRT-PCR tests were run on an Applied Biosystems 7300, using 96-well plates (Applied Biosystems, Foster City, CA, 94404). All plates, reagents, and other QRT-PCR supplies were from Applied Biosytems. Primers came from Schaad et al. (XfR1 and XfF1). The QRT-PCR protocol went through several revisions during the experiment due to inhibitors in the degraded plant tissue and contamination issues. We sought help from our Applied Biosystems representative for the inhibitor problem. It was suggested that using a new product called PrepMan® Ultra (Applied Biosystems, Foster City, CA, 94404) may help in cleaning up the samples. The PrepMan® Ultra protocol was followed except for following: sample amount was increased to 100 ul. A range test was performed to test the PrepMan® Ultra. Samples were diluted 1:10. In the PrepMan® Ultra protocol booklet, there were suggestions on how to further clean up the samples. In this experiment, nucleic acid precipitation was tested to determine if it would have an effect on the inhibitors. To quantify the QRT-PCR data, the copy number of each sample was recorded. This gave us the ability to determine how many bacteria were present in each sample.

Statistical Analysis. All data were analyzed using SPSS 15.0 (SPSS, Inc., Chicago, IL, 60606). Analysis of variance was used to determine if there were significant differences between the dependent variable (temperature) and the independent variables (grape variety, vineyard, QRT-PCR results, ELISA results, and Julian week). Graphs were created to demonstrate how each of the variables was significantly different.

RESULTS

Vine Temperature Measurements. Figure 3 shows the mean vine temperature of a dead vine throughout a summer day in Texas. Temperatures were taken on the east and west sides of the vine. The east side of the vine was warmer in the morning (9:00 am) and the west side of the vine was warmer from noon on throughout the day. Figure 4 shows mean vine temperatures separated according to variety. Vine temperatures increased at a steady rate as the summer progressed. Cabernet Sauvignon had the highest temperatures during most of the summer, followed by Chardonnay, Merlot, and Blanc duBois, respectively. To determine whether there was a difference in vine temperatures of each variety at each separate vineyard, line graphs were created (Figs. 5, 6, 7, and 8). At the Texas Hills vineyard, Merlot and Chardonnay had very similar temperatures over the course of the summer (Fig. 5). There is a dip in the Merlot's temperature due to incomplete data, but these two varieties have a much higher temperature than Cabernet Sauvignon. When looking at the Spicewood vineyard, we see that Cabernet Sauvignon is higher than Chardonnay (Fig. 6). This differs from the Texas Hills results. The Palacios vineyard graph shows a very similar average temperature between Blanc duBois and Merlot (Fig. 7). The dip in temperature in both varieties is due to incomplete temperature data. The Texas A&M Experimental vineyard only had one variety, Blanc duBois (Fig. 8). The mean temperatures for each of the vineyards over the course of the summer were significantly different from each other (Fig. 9). The Experimental Vineyard exhibited the lowest temperature with an average of 90.97 degrees Fahrenheit, followed by Palacios (91.92), Spicewood (96.19), and Texas Hills (96.63).

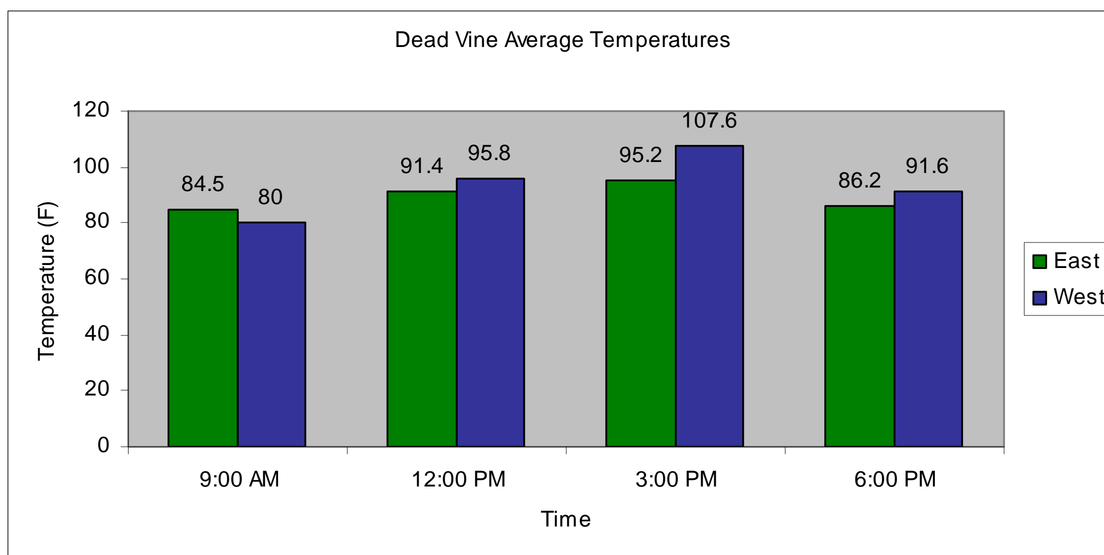

Fig. 3. Dead vine average temperatures (F) throughout a summer day. Temperatures were taken on the east and west side of the vines.

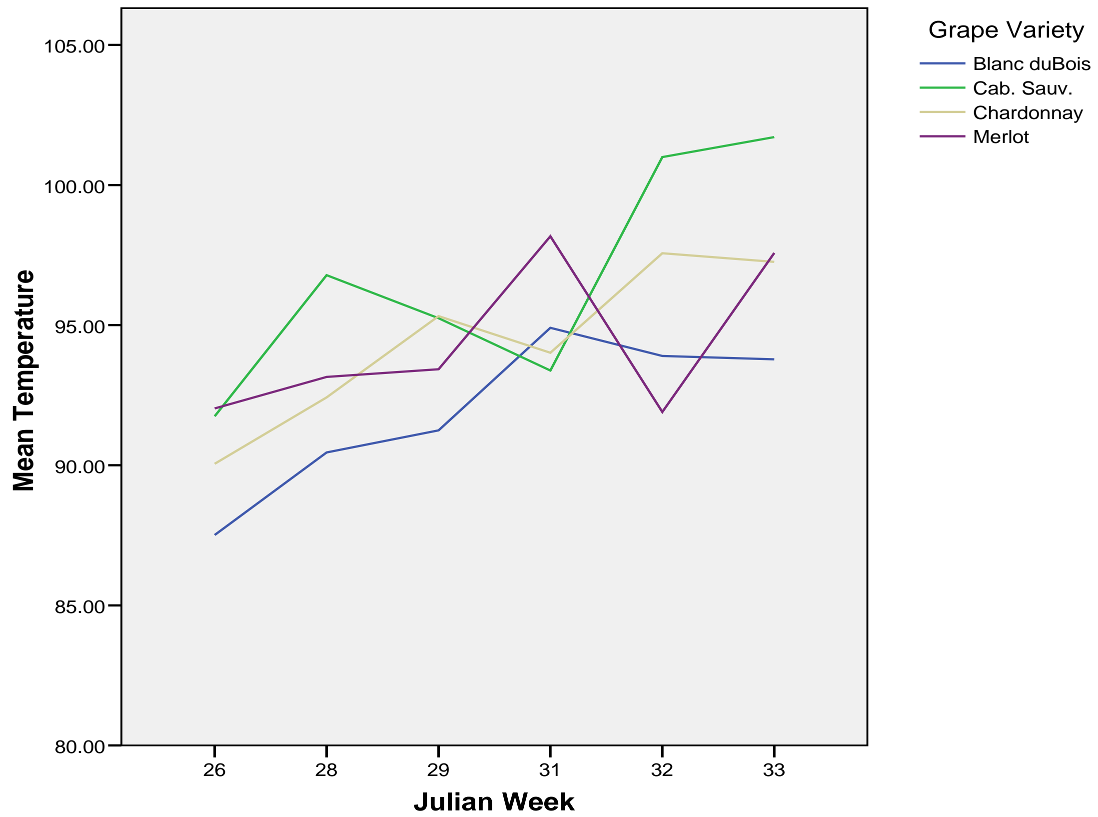

Fig. 4. Mean infrared temperature for each of the four varieties at all four vineyards for all six time points.

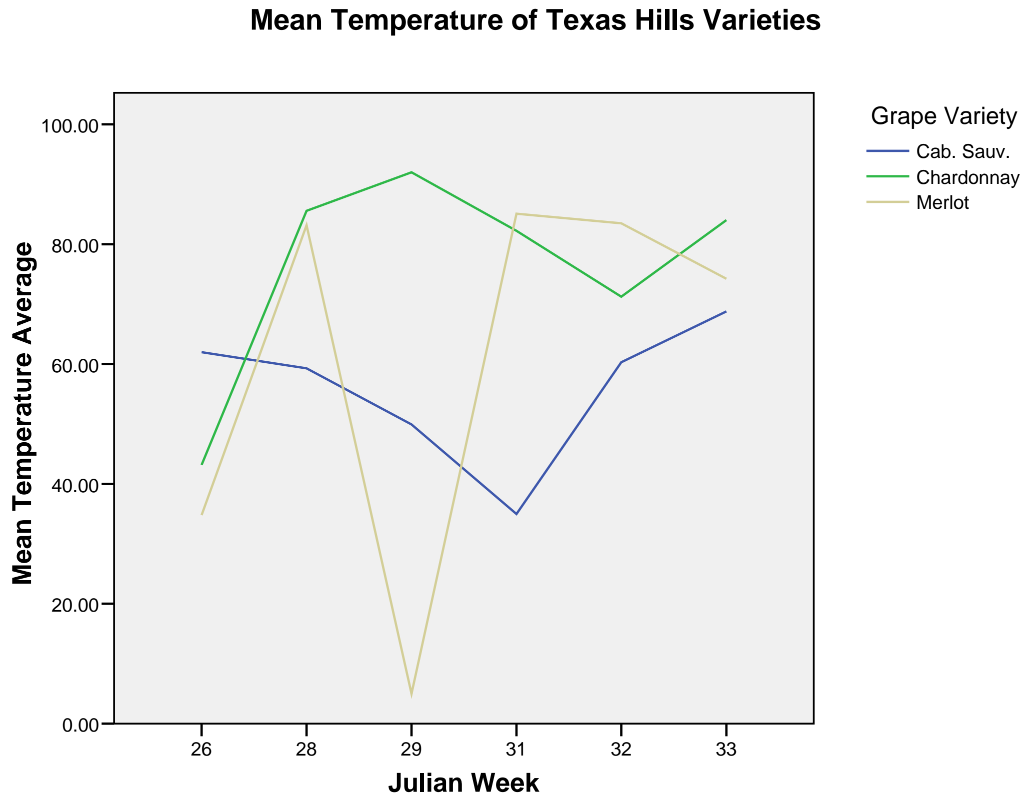

Fig. 5. Mean temperatures of the Texas Hills vineyard varieties. The dip in the Merlot's temperature is because of incomplete data.

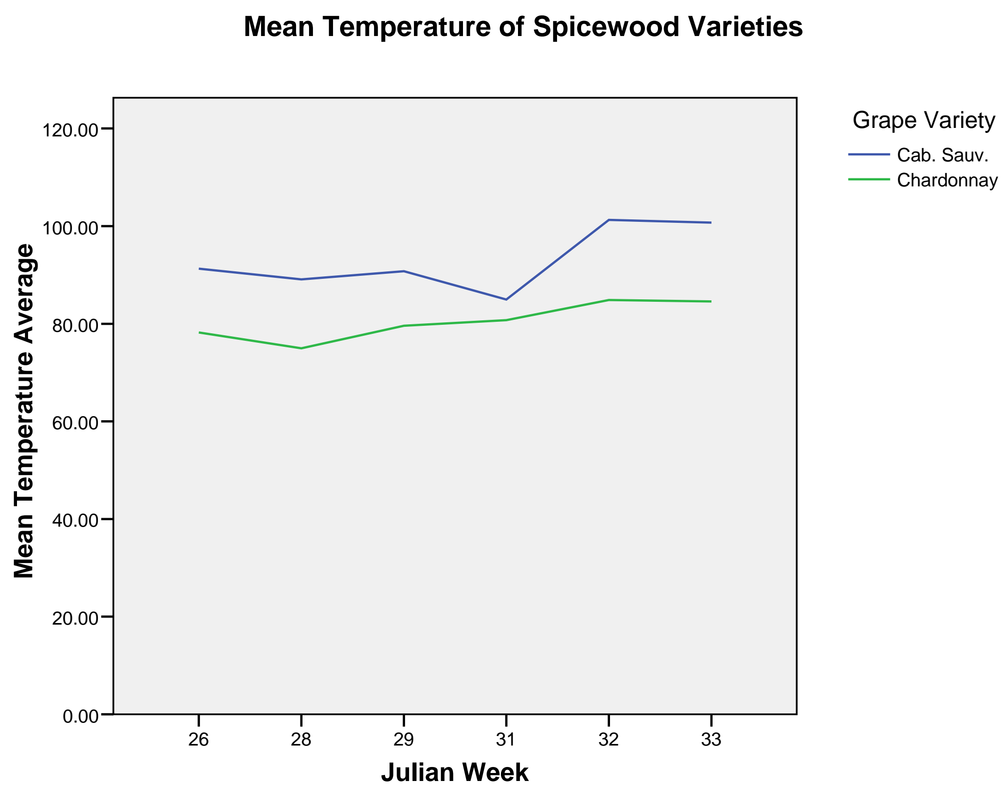

Fig. 6. Mean temperatures of the Spicewood vineyard varieties.

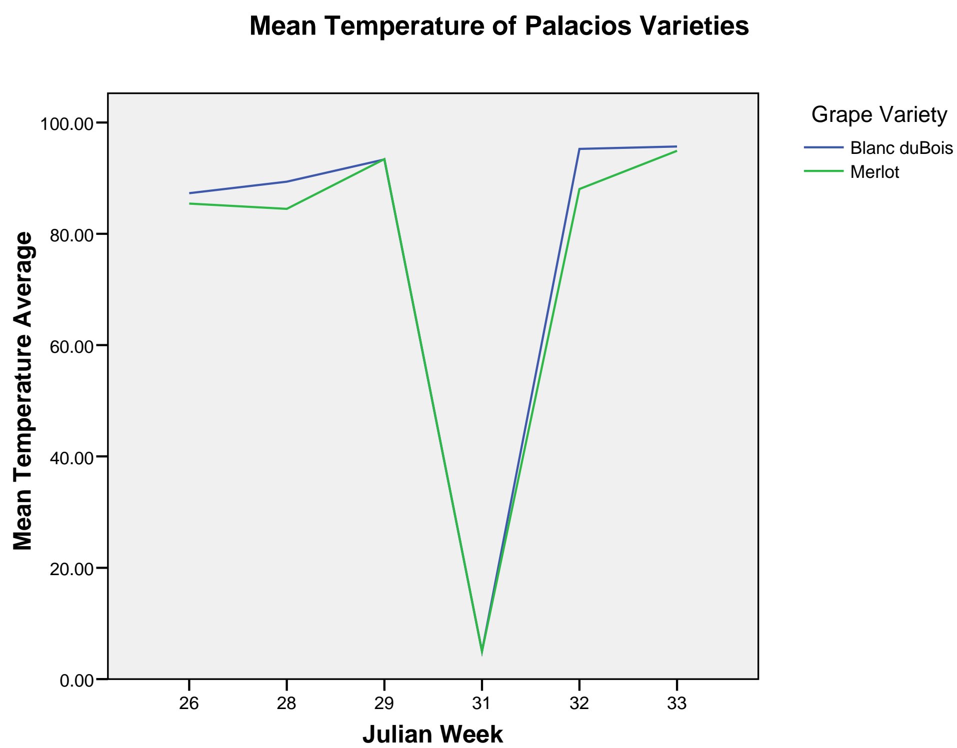

Fig. 7. Mean temperatures of the Palacios vineyard varieties. The dips in both varieties' temperatures are due to incomplete data.

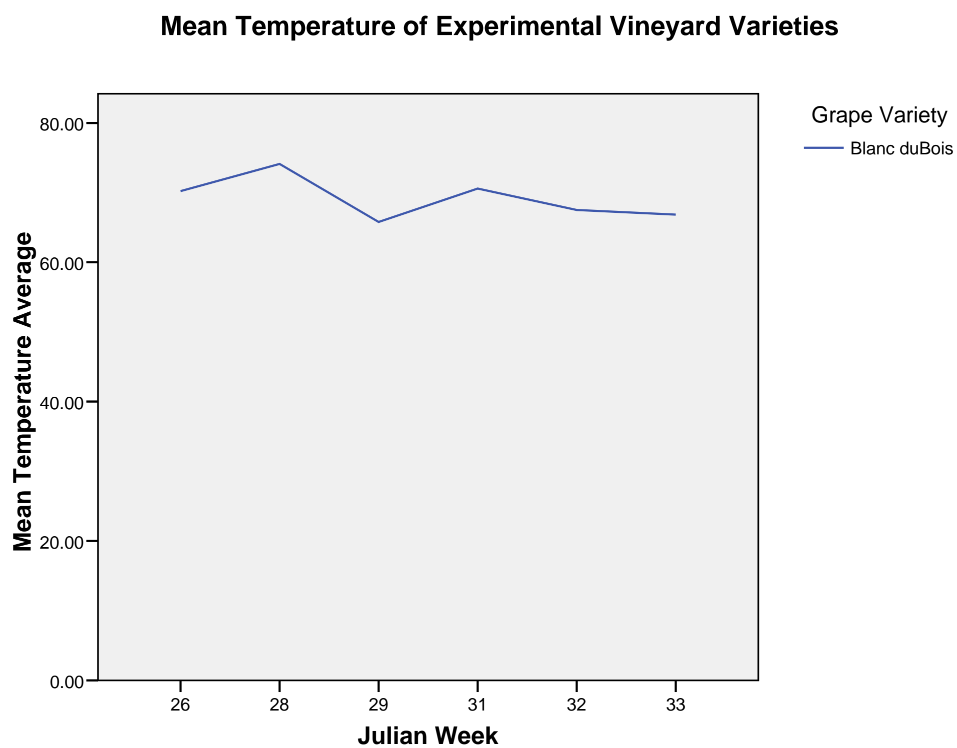

Fig. 8. Mean temperatures of the Experimental vineyard variety.

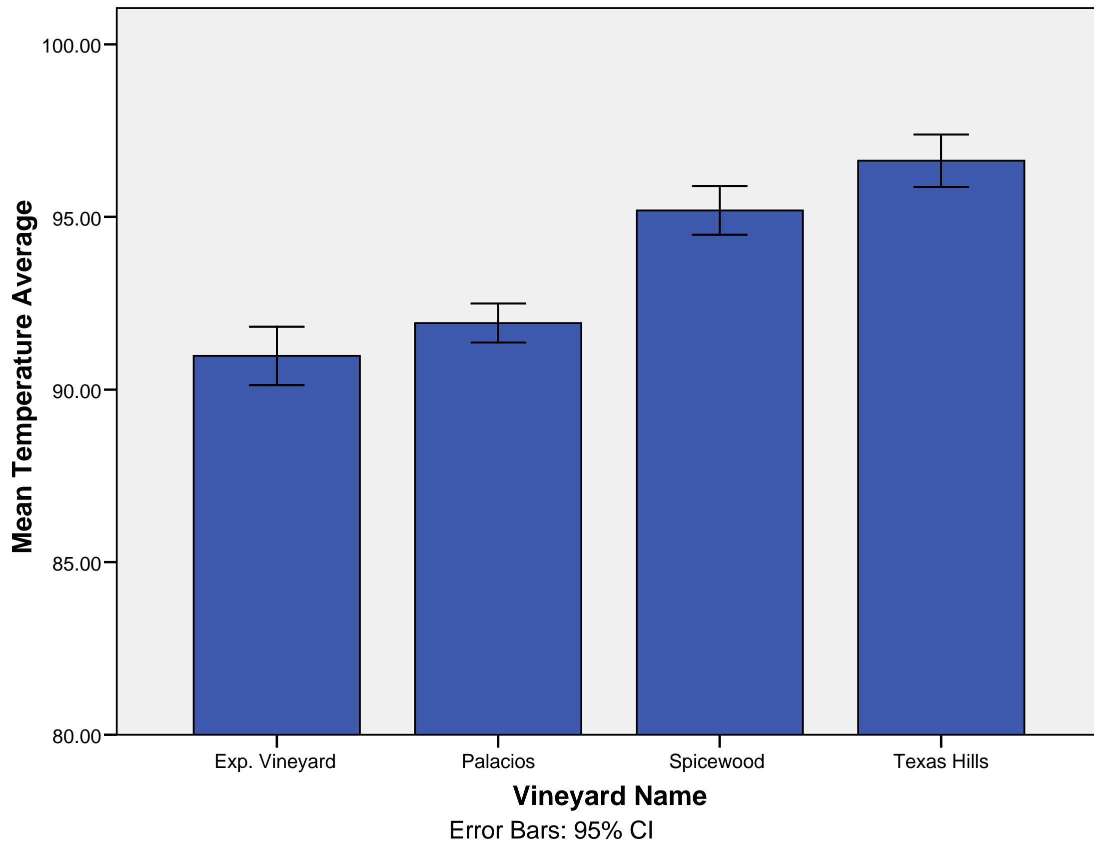

Fig. 9. Mean infrared temperature for each of the vineyards over the entire summer.

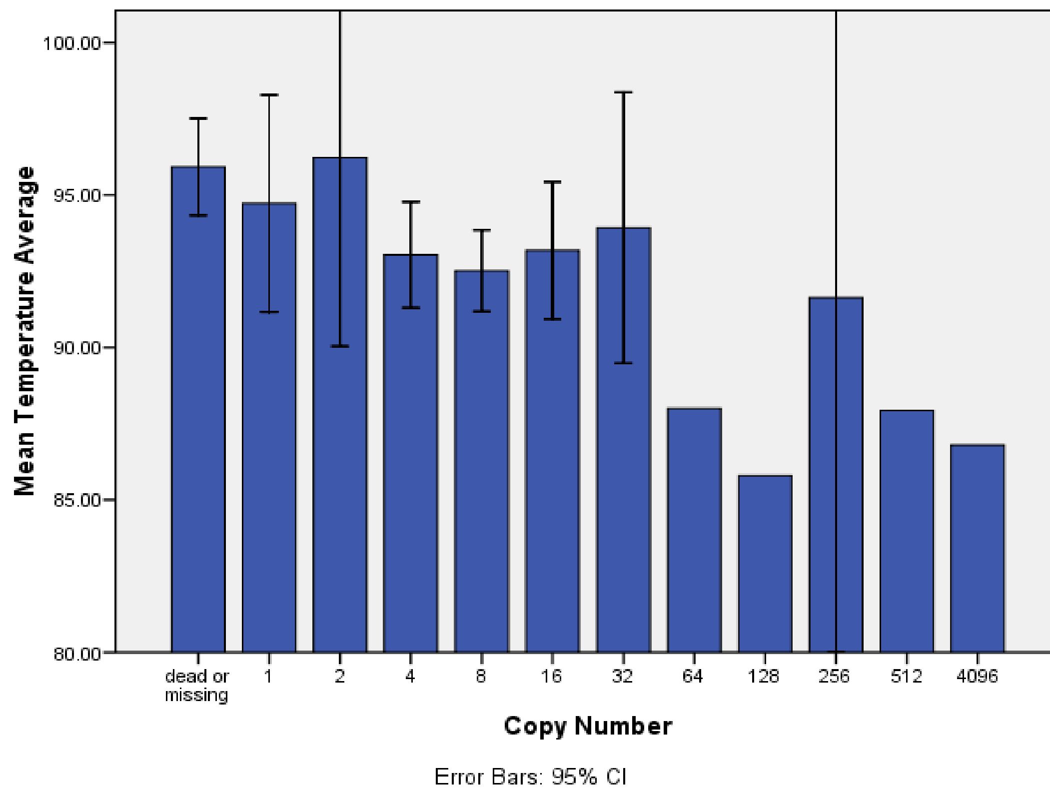

Fig. 10. Mean temperature for each of the separate copy numbers in QRT-PCR.

The average vine temperature for each of the copy numbers was graphed (Fig. 10) and it showed that the trend was for the vine temperature to decrease as the copy number increased. An analysis of variance (ANOVA) was run to determine whether there was an association between vine temperatures and the results of QRT-PCR testing for the presence of *X. fasitidiosa*. The ANOVA was run on the entire data set and used the log of each of the vine QRT-PCR copy numbers and the temperature difference (calculated by ambient temperature minus the vine temperature) (Table 1). The result was that there was not a significant difference between each of the copy numbers and the vine temperature. To determine if grape variety might influence a potential association between copy numbers and vine temperatures, a separate ANOVA was run for each variety (Tables 2, 3, 4, and 5). A significant difference was found in Blanc duBois (p-value = 0.032, Table 2), Cabernet Sauvignon (p-value = 0.037, Table 3), and Chardonnay (p-value = 0.039, Table 4). No significant difference was found in Merlot (p-value = 0.335, Table 5). Graphs were made to visually represent each of the four tests (Figures 11, 12, 13, and 14 respectively).

Table 1. Analysis of variance showing no significant difference between the dependent variable of Log of Copy # and the independent variable of Temperature Difference.

Tests of Between-Subjects Effects

Dependent Variable: Log of Copy #

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2994.171[a]	361	8.294	1.183	.093
Intercept	37.617	1	37.617	5.367	.022
Temp#_Difference	2994.171	361	8.294	1.183	.093
Error	1387.866	198	7.009		
Total	4409.329	560			
Corrected Total	4382.037	559			

a. R Squared = .683 (Adjusted R Squared = .106)

Table 2. Analysis of variance for Blanc duBois between the dependent variable of Log of the Copy Number and the independent variables, of Temperature Difference (ambient – vine) and Variety.

Tests of Between-Subjects Effects

Dependent Variable: Log of the Copy Number (+ .05)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	916.716[a]	111	8.259	1.828	.032
Intercept	9.361	1	9.361	2.071	.161
Temp#_Difference	916.716	111	8.259	1.828	.032
Variety	.000	0	.	.	.
Temp#_Difference * Variety	.000	0	.	.	.
Error	131.048	29	4.519		
Total	1050.785	141			
Corrected Total	1047.764	140			

a. R Squared = .875 (Adjusted R Squared = .396)

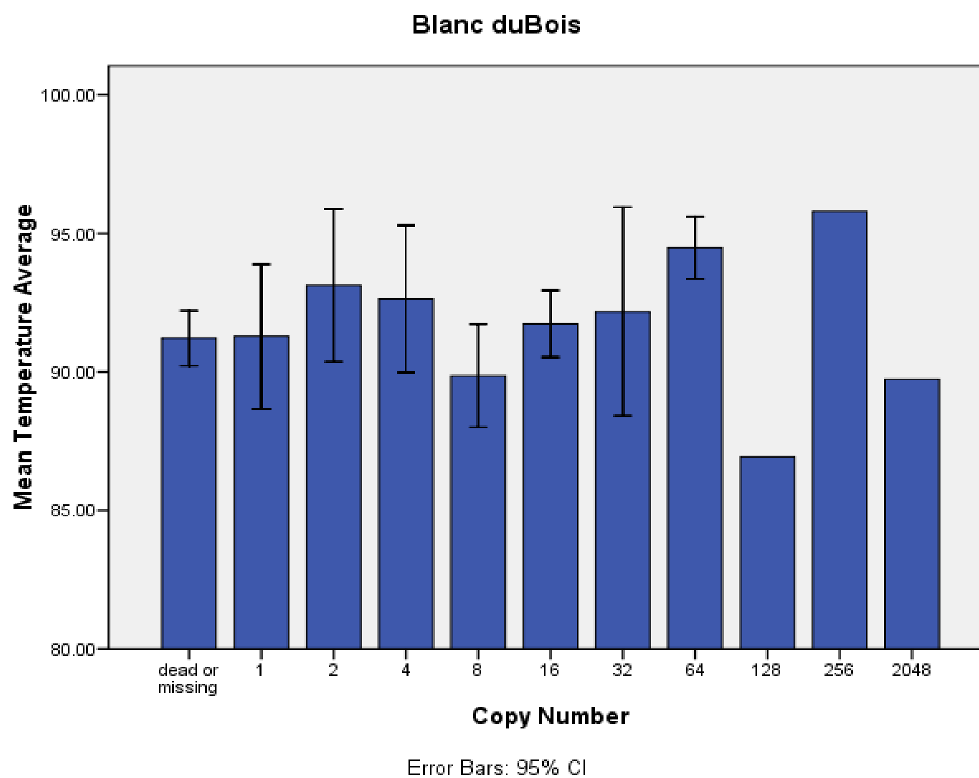

Fig. 11. Blanc duBois sample copy numbers and the corresponding average vine temperatures.

Table 3. Analysis of variance for Cabernet Sauvignon between the dependent variable of Log of the Copy Number , and the independent variables, of Temperature Difference (ambient – vine) and Variety.

Tests of Between-Subjects Effects

Dependent Variable: Log of Copy Number

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1044.782[a]	124	8.426	2.141	.037
Intercept	29.012	1	29.012	7.373	.015
Variety	.000	0	.	.	.
Temp#_Difference	1044.782	124	8.426	2.141	.037
Variety * Temp#_ Difference	.000	0	.	.	.
Error	66.896	17	3.935		
Total	1123.273	142			
Corrected Total	1111.679	141			

a. R Squared = .940 (Adjusted R Squared = .501)

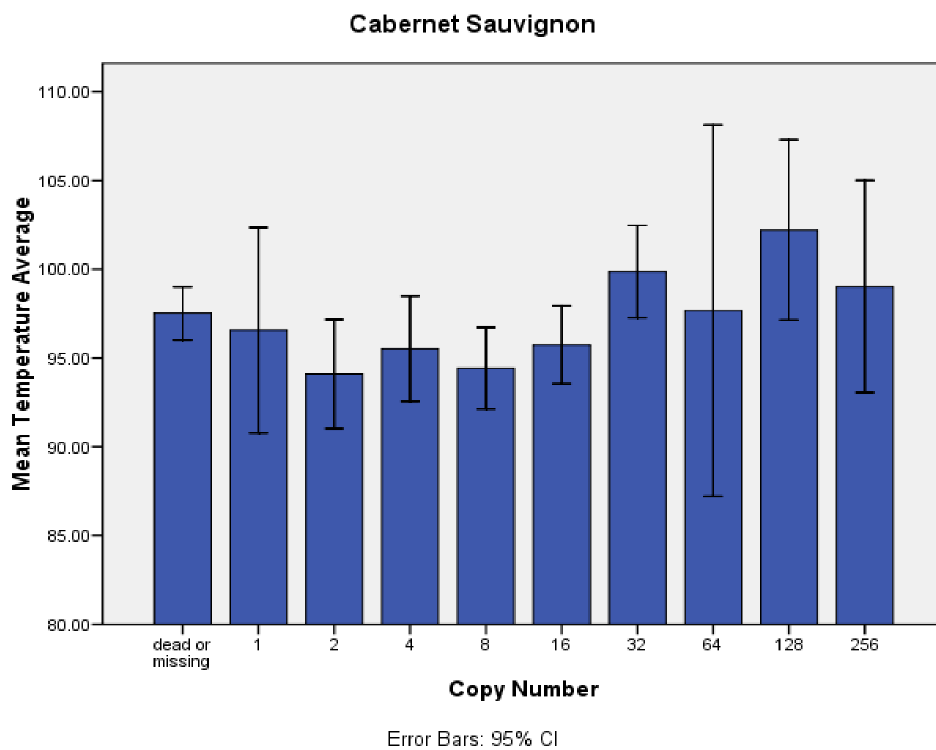

Fig. 12. Cabernet Sauvignon sample copy numbers and the corresponding average vine temperatures.

Table 4. Analysis of variance for Chardonnay between the dependent variable of Log of the Copy Number, and the independent variables, of Temperature Difference (ambient – vine) and Variety.

Tests of Between-Subjects Effects

Dependent Variable: Log of the Copy Number (+ .05)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1178.165[a]	122	9.657	1.844	.039
Intercept	9.982	1	9.982	1.906	.180
Temp#_Difference	1178.165	122	9.657	1.844	.039
Variety	.000	0	.	.	.
Temp#_Difference * Variety	.000	0	.	.	.
Error	130.951	25	5.238		
Total	1312.976	148			
Corrected Total	1309.116	147			

a. R Squared = .900 (Adjusted R Squared = .412)

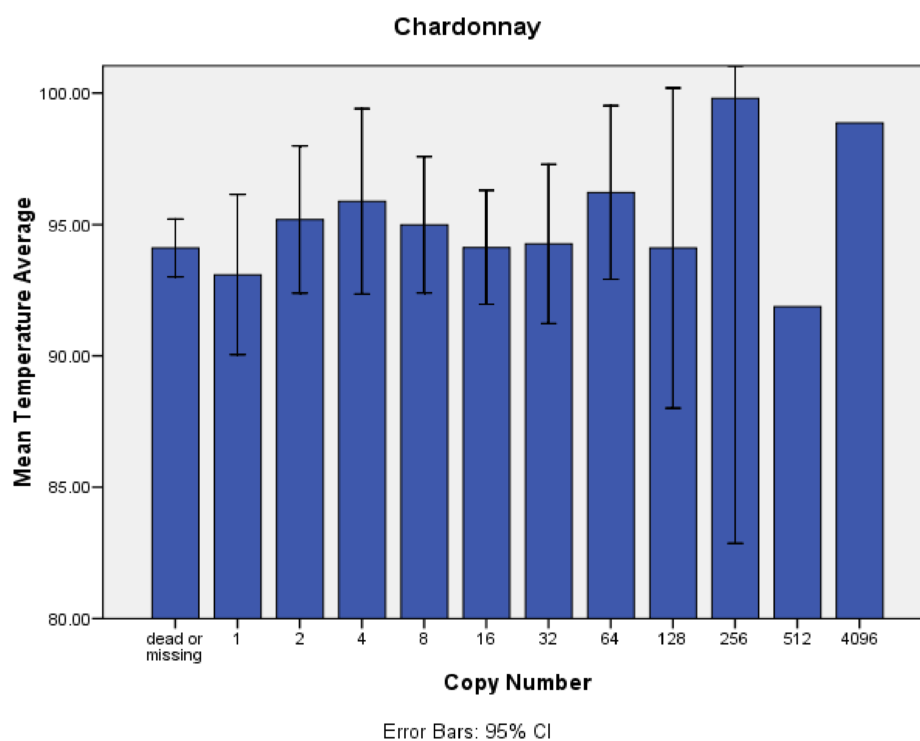

Fig. 13. Chardonnay sample copy numbers and the corresponding average vine temperatures.

Table 5. Analysis of variance for Merlot between the dependent variable of Log of the Copy Number, and the independent variables, of Temperature Difference (ambient – vine) and Variety.

Tests of Between-Subjects Effects

Dependent Variable: Log of the Copy Number (+ .05)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	815.742[a]	112	7.283	1.226	.335
Intercept	5.947	1	5.947	1.001	.332
Temp#_Difference	815.742	112	7.283	1.226	.335
Variety	.000	0	.	.	.
Temp#_Difference * Variety	.000	0	.	.	.
Error	95.058	16	5.941		
Total	922.294	129			
Corrected Total	910.800	128			

a. R Squared = .896 (Adjusted R Squared = .165)

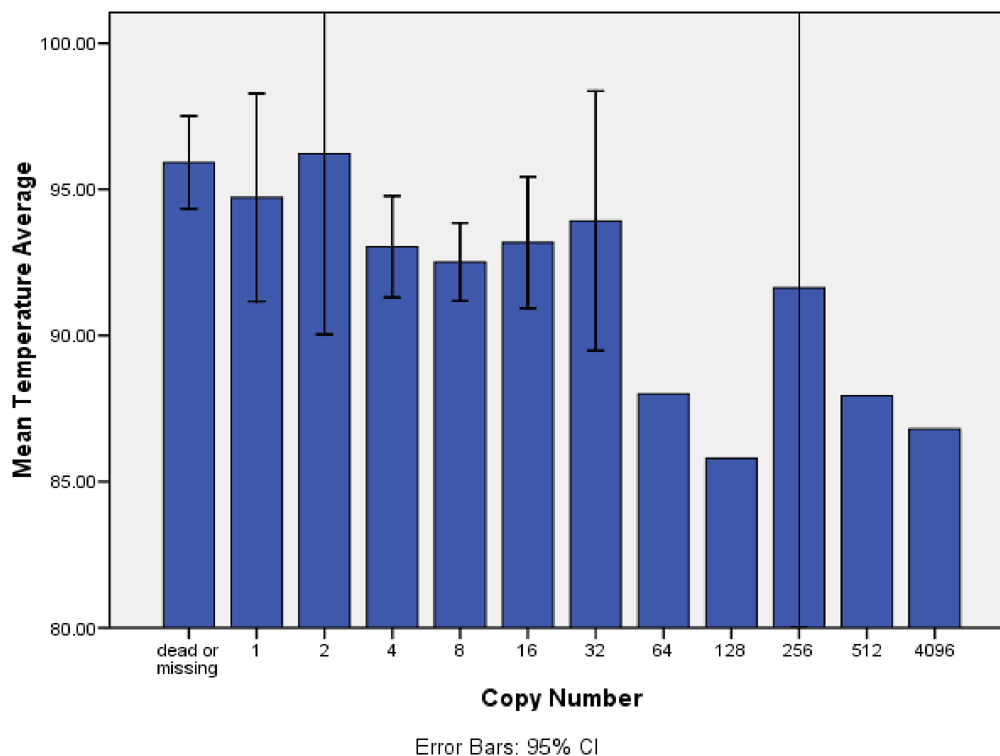

Fig. 14. Merlot sample copy numbers and the corresponding average vine temperatures.

An analysis of variance (ANOVA) was also run to determine whether there was an association between the diagnostic ELISA results and vine temperatures. The variables included the results of the ELISA (positive or negative) and temperature average. The p-values for all tests were significant ($P < .05$) (Table 6). To illustrate the differences between the variables, graphs were created. The ELISA positive vines showed a significantly higher temperature than the ELISA negative vines (Fig. 15). To determine if the individual varieties followed the overall trend, a graph was made (Fig. 16). Chardonnay and Blanc duBois had higher mean temperatures for the ELISA positive vines than the ELISA negative vines. Merlot and Cabernet Sauvignon had higher temperatures for the ELISA negative vines.

Table 6. Analysis of variance of grapevine temperature measurements against the independent variable of ELISA results.

Tests of Between-Subjects Effects

Dependent Variable: Temperature Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	203.912[a]	2	101.956	4.329	.014
Intercept	3662736.586	1	3662736.586	155510.1	.000
ELISA	203.912	2	101.956	4.329	.014
Error	11682.309	496	23.553		
Total	4471905.891	499			
Corrected Total	11886.221	498			

a. R Squared = .017 (Adjusted R Squared = .013)

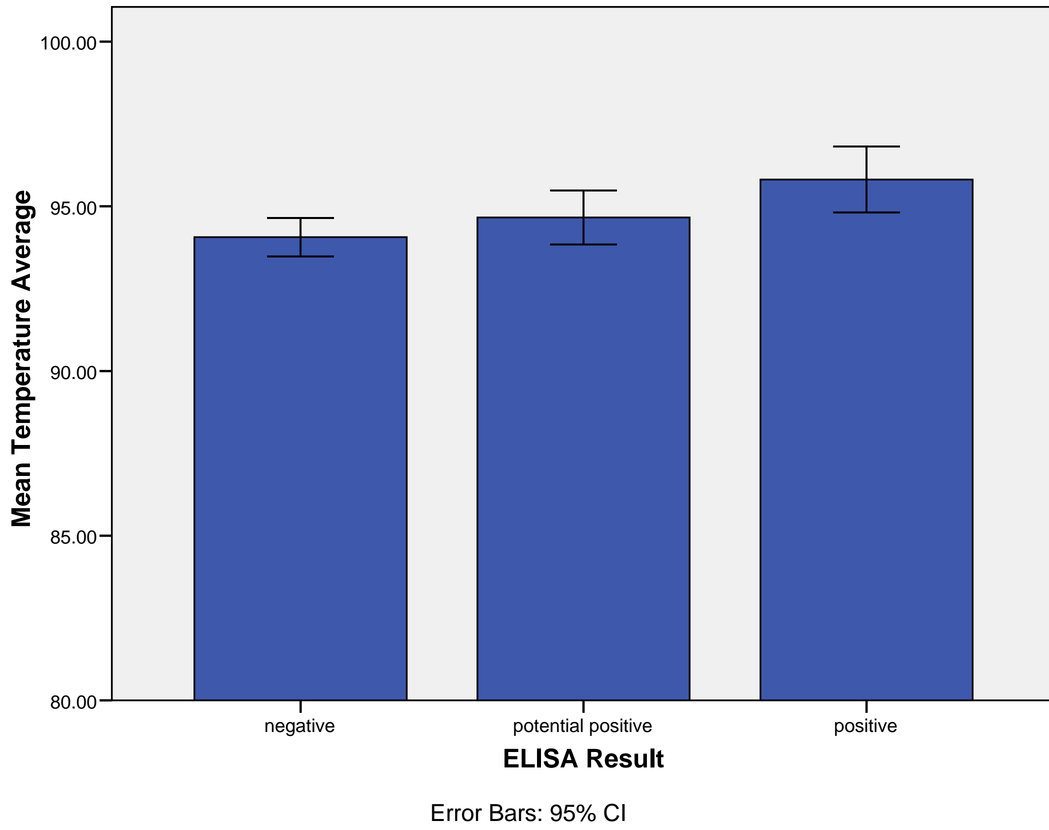

Fig. 15. Mean temperatures for positive, negative, and potential positive ELISA results.

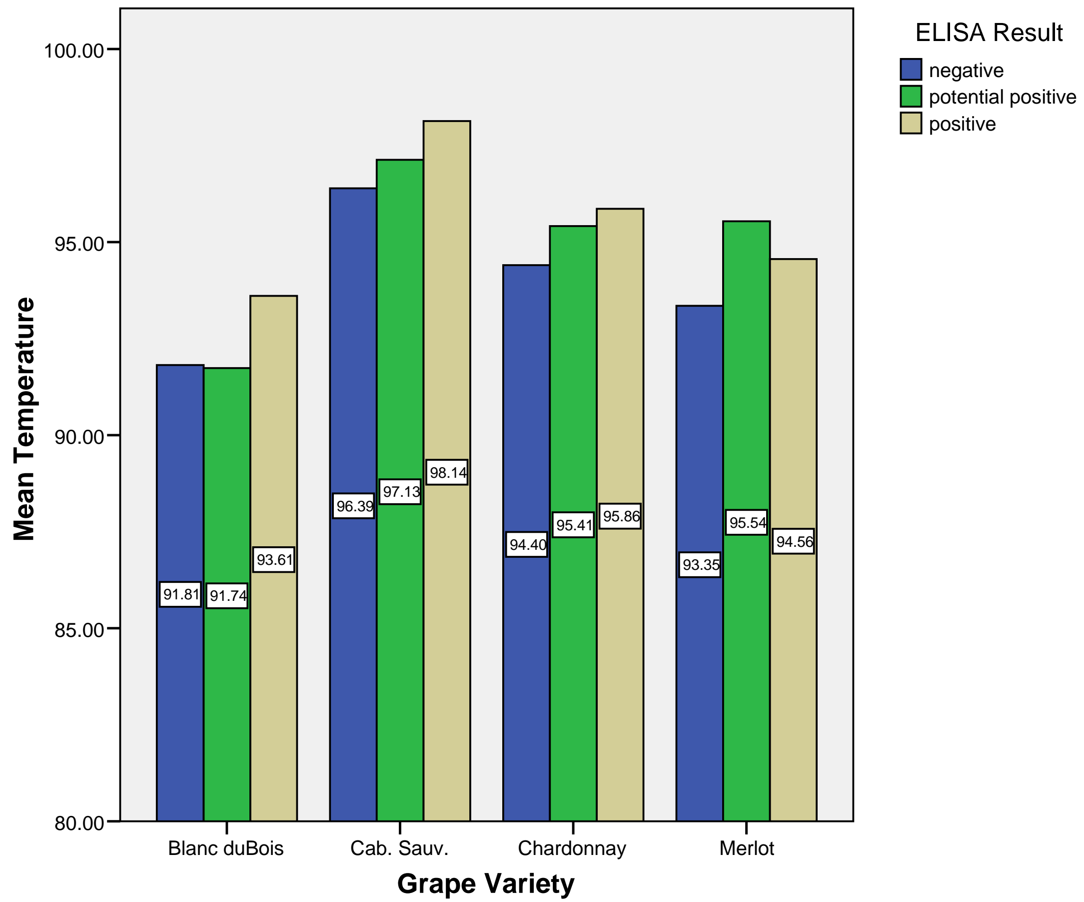

Fig. 16. Mean temperature of ELISA results for each variety for the entire summer.

Disease Epidemiology. To determine if *X. fastidiosa* spreads down a row or across a row, the vines were graphed according to their plot arrangement and QRT-PCR values (Fig. 17). Overall, there did not appear to be any pattern in the locations of QRT-PCR positives. Many *X. fastidiosa* positive vines were found down and across the rows from the disease center. When the varieties were separated to compare the number of QRT-PCR positives per plot location, they were all different (Figs. 18, 19, 20, and 21). There were not more QRT-PCR positives down a row (right or left) than across or vice versa. It appeared that the disease spread randomly without pattern. For instance, when looking at Blanc duBois (Fig. 18), it appears that there was more disease spread across a row (bottom) than down a row. When looking at Chardonnay (Fig. 20), there were more positives being shown down a row (left) than across.

QRT-PCR vs. ELISA. Because of a refrigerator problem, the samples rotted, and although we were able to obtain a few positive cultures, it was decided that no culturing data would be used in this project. The only diagnostic techniques that were tested were ELISA and QRT-PCR. A table was made to compare the ELISA and QRT-PCR results (Table 7). There were 94 ELISA positives, 380 QRT-PCR positives, and a total of 47 samples that were both ELISA and QRT-PCR positive. When comparing ELISA potential positives to QRT-PCR positives, 92 samples yielded positive results by both methods. There were 222 samples that were ELISA negative when the QRT-PCR was positive. ELISA detected *X. fastidiosa* in 38% of the symptomatic vines, while QRT-PCR detected *X. fastidiosa* in 97% of the symptomatic vines (Table 7). Out of 84 symptomatic vines, 46% came up as ELISA potential positive. Another contrast can be made by observing that QRT-PCR detected *X. fastidiosa* in 296 asymptomatic vines, the ELISA tests only found 10 samples that were positive for *X. fastidiosa* that were asymptomatic.

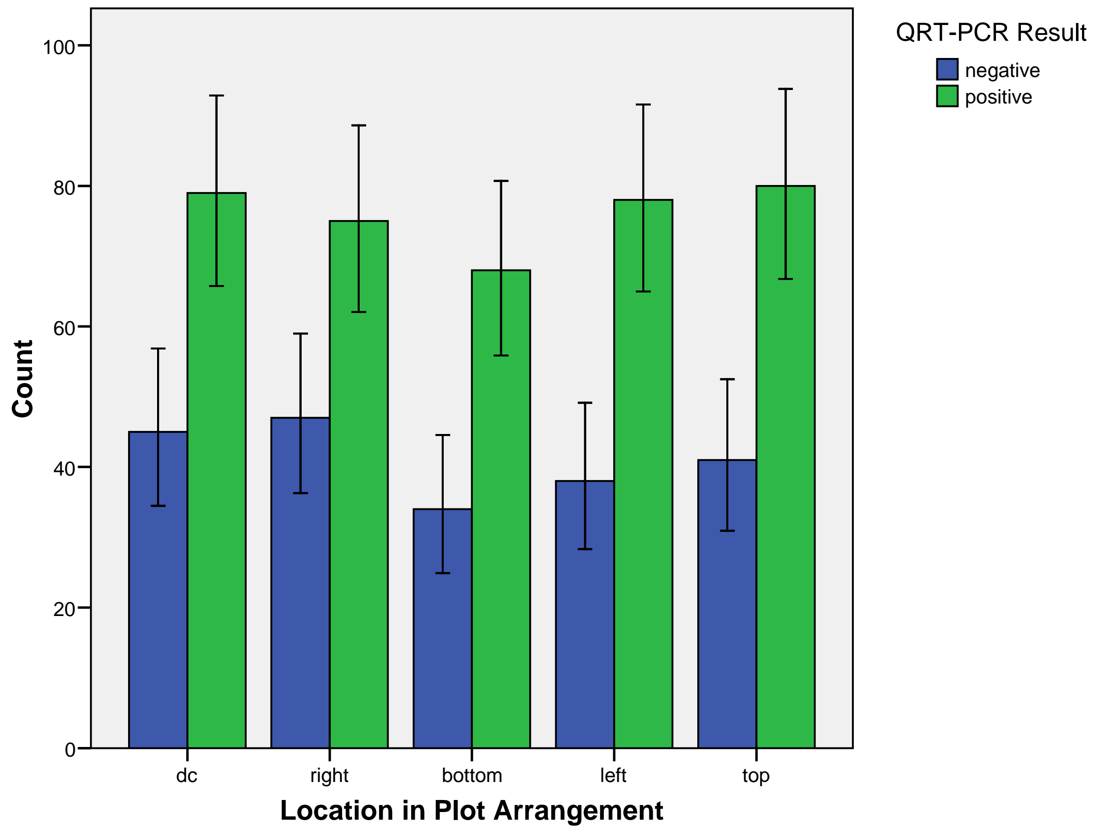

Fig. 17. Number of QRT-PCR negatives and positives for each of the plot locations. Dc is the disease center, right refers to the vine to the right of the dc, bottom refers to the following vineyard row, left refers to the vine to the left of the dc, and top refers to the preceding vine row.

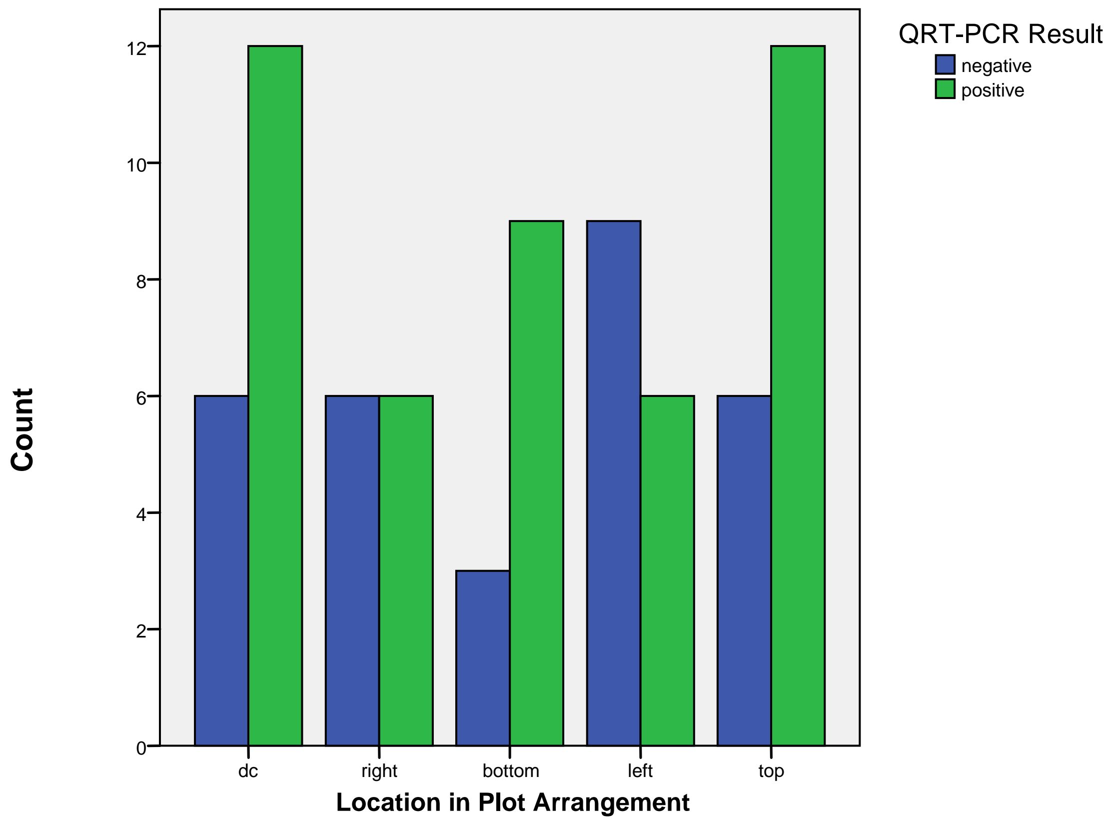

Fig. 18. Number of QRT-PCR negatives and positives for each plot location for Blanc duBois at week 33. Dc is the disease center, right refers to the vine to the right of the dc, bottom refers to the following vineyard row, left refers to the vine to the left of the dc, and top refers to the preceding vine row.

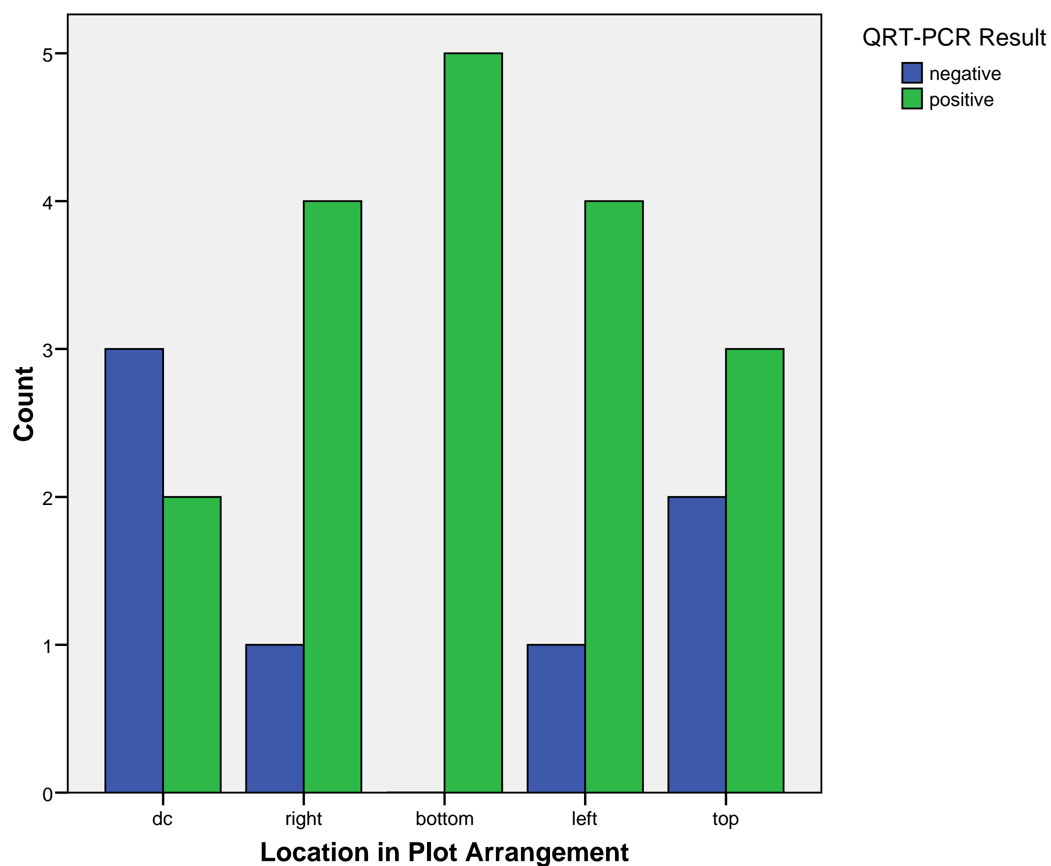

Fig. 19. Number of QRT-PCR negatives and positives for each plot location for Cabernet Sauvignon for week 33. Dc is the disease center, right refers to the vine to the right of the dc, bottom refers to the following vineyard row, left refers to the vine to the left of the dc, and top refers to the preceding vine row.

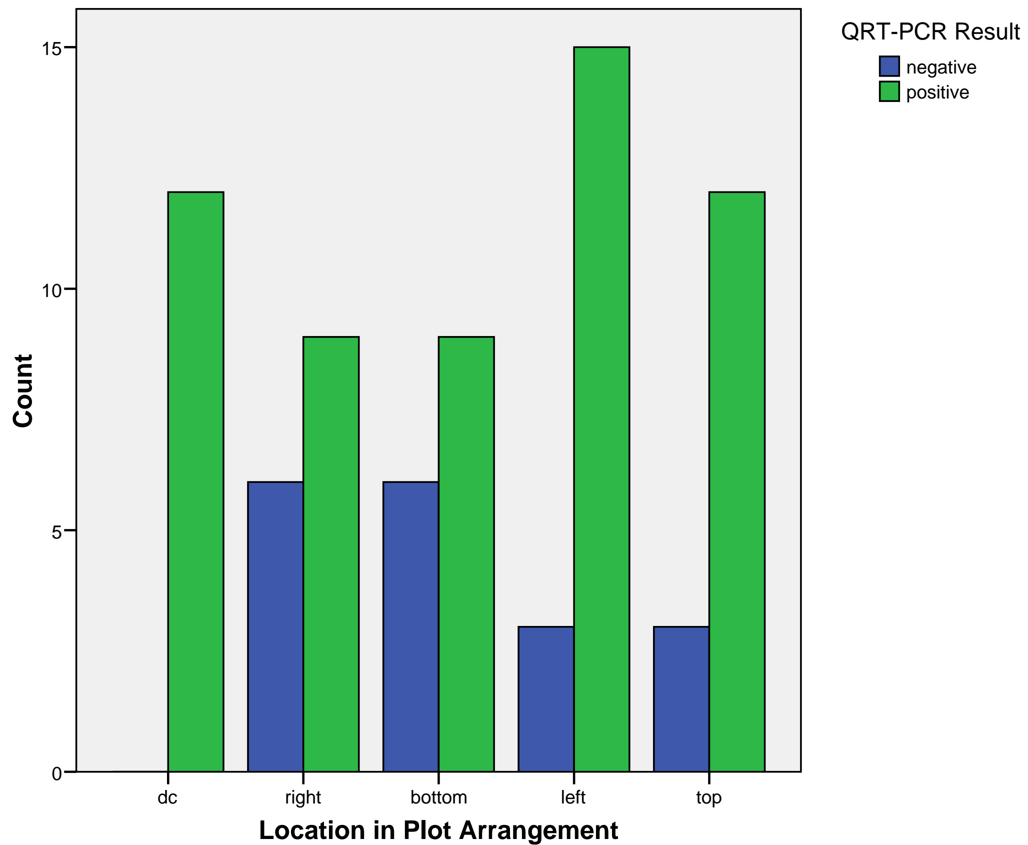

Fig. 20. Number of QRT-PCR negatives and positives for each plot location for Chardonnay at week 33. Dc is the disease center, right refers to the vine to the right of the dc, bottom refers to the following vineyard row, left refers to the vine to the left of the dc, and top refers to the preceding vine row.

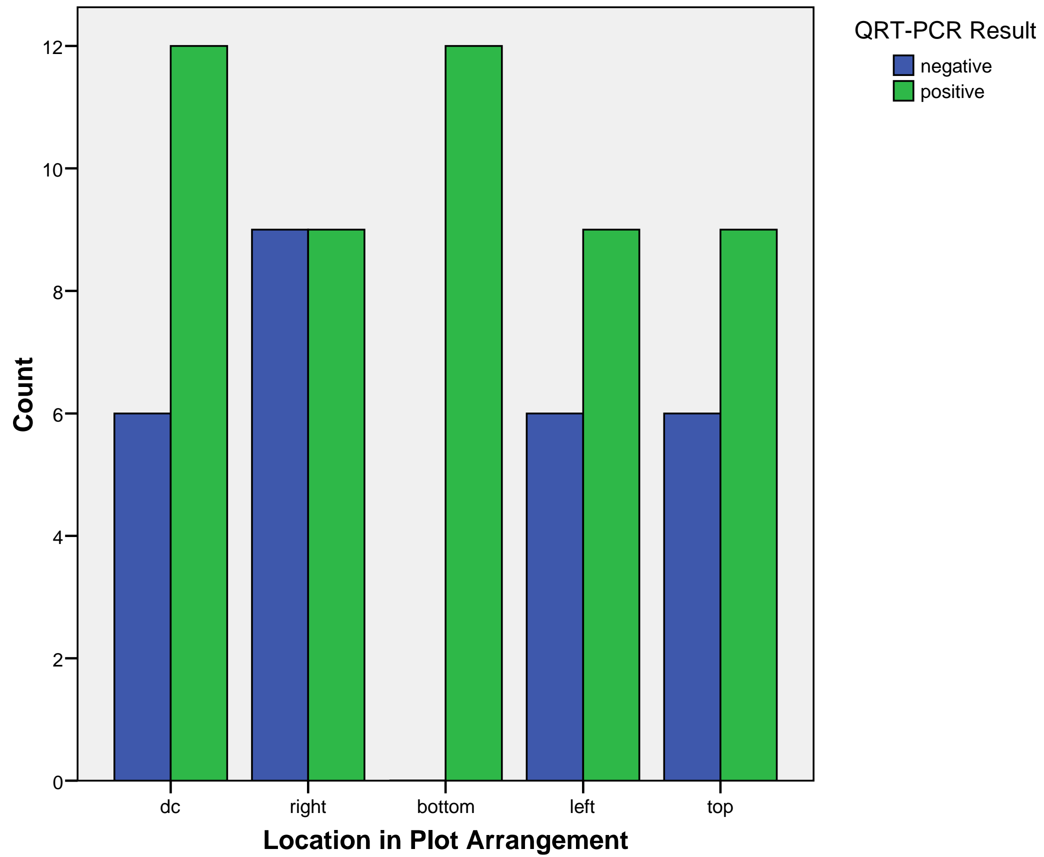

Fig. 21. Number of QRT-PCR negatives and positives for each plot location for Merlot at week 33. Dc is the disease center, right refers to the vine to the right of the dc, bottom refers to the following vineyard row, left refers to the vine to the left of the dc, and top refers to the preceding vine row.

Table 7. Comparison of numbers of ELISA and QRT-PCR positives (+). (p+) = potential positive, PPD = possible PD.

ELISA vs. QRT-PCR	Totals / Counts
Total ELISA (+)	94
Total QRT-PCR (+)	380
Total ELISA (+) and QRT-PCR (+)	47
Total ELISA (p+) and QRT-PCR (+)	92
Total ELISA (-) and QRT-PCR (+)	222
Total QRT-PCR (+) and PPD	82
Total ELISA (+) and PPD	32
Total ELISA (p+) and PPD	39
Total ELISA (+), QRT-PCR (+) and PPD	18

QRT-PCR Experimental Procedures. It was discovered that there were inhibitors present in our QRT-PCR samples (Fig. 22). When looking at Figure 18, it can be seen that both the FAM and TAMRA lines go up as the cycles progress. This indicates that there is a problem. As the FAM signal goes up, the TAMRA should go down because the FAM is being cleaved from the quencher, thus causing the FAM to fluoresce. To solve our problem with these inhibitors, we tested various ways to clean up the samples. PrepMan Ultra® and Nucleic Acid Precipitation were the two methods chosen for experimentation. In a PrepMan Ultra® range test, it was determined that although it eliminated many of the inhibitors, it also brought the amount of *X. fastidiosa* down too low. Nucleic acid precipitation was then used in combination with the PrepMan Ultra® so that the *X. fastidiosa* concentration would not have to be diluted. After nucleic acid precipitation was performed, the samples showed no signs of inhibitors (Fig. 23). It was concluded that the precipitation did indeed take care of the remaining inhibitors and the samples did not require dilution.

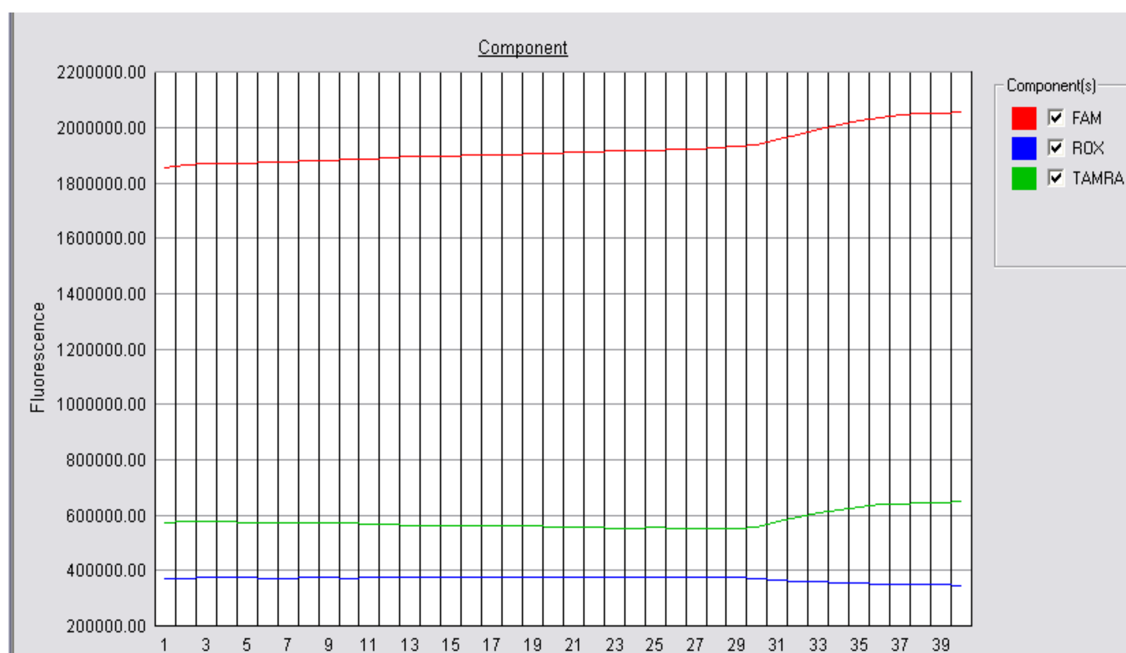

Fig. 22. FAM and TAMRA graph lines with inhibitors present from degraded QRT-PCR samples.

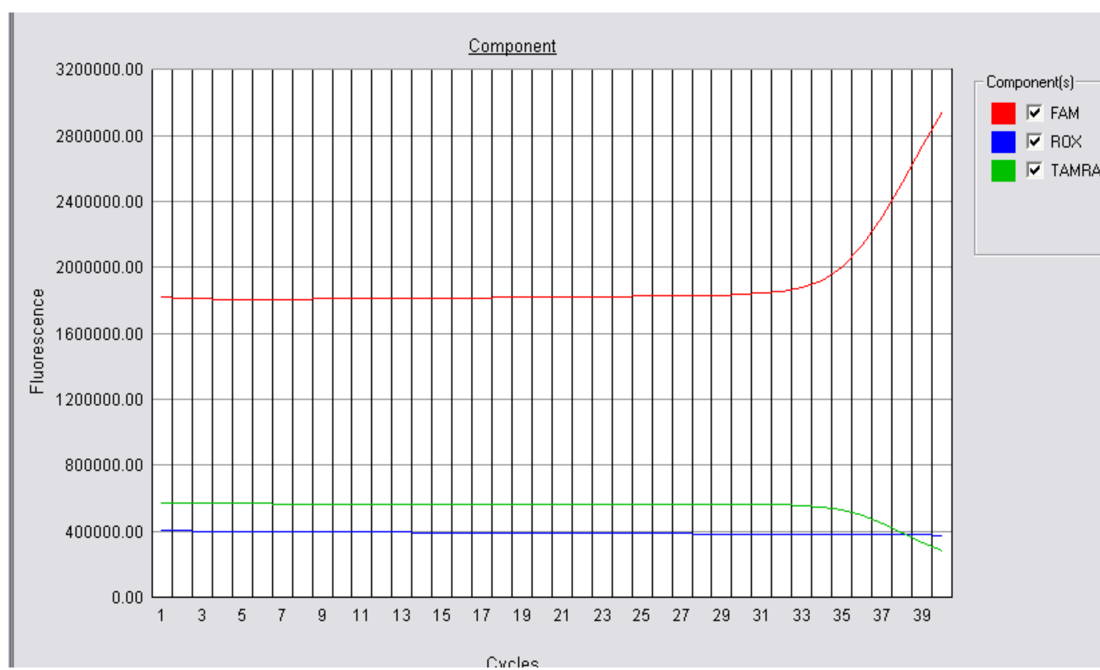

Fig. 23. FAM and TAMRA graph lines from a QRT-PCR with no inhibitors due to PrepMan Ultra® and nucleic acid precipitation.

DISCUSSION

One purpose of this study was to investigate whether there are opportunities to improve current diagnostic protocols for Pierce's Disease. These protocols include the use of ELISA and QRT-PCR. Each of these techniques has advantages and disadvantages for diagnosing Pierce's disease. For instance, ELISA is less expensive, but it takes the longest amount of time and is not as precise as QRT-PCR. QRT-PCR can detect one bacterium and takes half the amount of time; however, it can be expensive. There have been several studies comparing the ELISA and QRT-PCR techniques, resulting in mixed results. Some studies, such as those by Sherald et al. (13) and Nomé et al. (11), showed that ELISAs could be relatively accurate in detecting *X. fastidiosa*, while other studies show that ELISAs are not very reliable in detecting all infected samples and that QRT-PCR is the better choice (9)(16). In 1980, Nomé et al. tested ELISAs to see if they could detect *X. fastidiosa* in plant tissue. They found that the ELISA tests correctly identified *X. fastidiosa* in 11 of 12 symptomatic almond plants (11). It was also found that ELISA confirmed that all 15 of known infected grape vines were positive for the bacterium (11). In the study by Sherald et al. (13), they evaluated the use of a rapid ELISA test kit for detection of *X. fastidiosa* in landscape trees. ELISAs were able to detect *X. fastidiosa* in all of the asymptomatic elms (*Ulmus Americana*) and sycamores (*Platanus occidentalis*) that had showed severe symptoms the previous year (13). The ELISA tests were also able to detect 17 of 18 diseased trees (12 before symptoms appeared and 5 after symptoms appeared) (13). While these studies seem to conclude that ELISA assays are indeed a suitable test for the detection of *X. fastidiosa*, it is important to point out that they were not perfect. In both studies, there were discrepancies where the assay did not detect *X. fastidiosa* in a known infected and symptomatic plant. Although Sherald et al. (13) indicated that their ELISAs detected *X. fastidiosa* in asymptomatic plants, it is important to remember that the asymptomatic trees had severe PD symptoms the previous year. This indicates that these trees already had a large population of the bacterium. ELISA assays need much larger titers to determine whether or not the sample is positive than QRT-PCR, which

theoretically can detect 1 bacterium in a sample (Applied Biosystems). Tubajika et al. (16), while analyzing the spatial patterns of PD, found that only 85% of their symptomatic vines came up as ELISA positive. Another study, by Minsavage et al. (9), compared PCR and ELISA and found that ELISA could detect 3 x 10^4 bacteria per milliliter and PCR was almost 100-fold more sensitive by detecting 3 x 10^2 bacteria per milliliter. The implication of this large sensitivity gap is that QRT-PCR would be much more reliable in detecting *X. fastidiosa* in plant tissue before the plant becomes symptomatic, possibly before the titer is high enough to be labeled as an inoculum source.

When comparing ELISA testing to QRT-PCR in the present study, it was not unexpected to see a large amount of QRT-PCR positives and fewer ELISA positives. ELISA, while not as consistent (Table 4), provided a better basis for the temperature conclusions because it was only able to detect vines that had a high titer of *X. fastidiosa*. Presumably those vines were actually being affected by the high amount of bacteria residing in their vascular system. There were disadvantages for both techniques. ELISA testing was time consuming, the accuracy varied, and they could not detect small amounts of *X. fastidiosa*. QRT-PCR had the tendency to have contamination issues and was affected by inhibitors.

Because of the problems with degraded samples, new techniques had to be tried to get clean QRT-PCR results. To get rid of the inhibitors, it was suggested to try PrepMan Ultra®. This succeeded, but at the cost of a lower *X. fastidiosa* concentration. To keep the concentration high, more of the sample was used and nucleic acid precipitation was tried to further clean it up. This combination of techniques worked. This experimentation provided new insight into sample degradation problems. It is now known that heavily degraded samples can be cleaned enough to provide clear results for QRT-PCR.

Another purpose to the study was to determine whether vine temperatures might be a useful tool for assessing the health status of vines at risk to infection by *X. fastidiosa*. The temperature gun did detect differences among the mean temperatures of vineyards throughout the summer. This was expected as each vineyard was at a different location throughout south and central Texas. Also, there were differences among the different

grape varieties. These differences are more difficult to explain. Because each of these 4 varieties has a slightly different tolerance level to PD, it could be speculated that the temperature difference has something to do with this. It was hypothesized in this study that the most susceptible, Chardonnay, would have the highest mean vine temperature. This was not the case; in fact, the highest was Cabernet Sauvignon, a moderately tolerant variety. The reasons for this anomaly are unknown. When dividing the varieties up by vineyard (location), we saw different results. At Texas Hills, the two highest temperatures were from Chardonnay and Merlot (Fig. 5), with Cabernet Sauvignon showing the lowest average temperature. At Spicewood vineyard, it was Cabernet Sauvignon that had the highest temperature and Chardonnay that had the lowest (Fig. 6). Palacios vineyard varieties, Blanc duBois and Merlot, had very similar temperatures (Fig. 7). The Experimental vineyard only had one variety, Blanc duBois (Fig. 8) Although we observed many temperature differences between varieties and vineyards, and are not sure why, it does seem to indicate that perhaps there is not one single solution to solve this problem. An infrared gun cannot be used effectively if all varieties have different healthy and diseased plant temperatures. Perhaps it will possible with more research into plant diseases and how they affect plant temperatures, for us to come up with varietal 'equations' that allow us to determine if a plant is diseased by looking at outside air temperature versus the plant temperature based on each variety.

One way to examine the utility of vine temperatures for diagnosis is to determine how they relate to the diagnostic results of the ELISA and QRT-PCR. Mean temperatures for ELISA positive and negative vines differed than those of QRT-PCR positive and negative vines. According to the QRT-PCR vine temperature ANOVA (Table 1), vines that were infected with *X. fastidiosa* had no significant difference between the different copy numbers of each sample. According to a study by Tu et al. (15), diseased plants are supposed to have an increase in temperature, we did not see this in the QRT-PCR samples. To further test these results, an ANOVA was run using the ELISA variable in place of the QRT-PCR variable. This ANOVA showed significant differences between the variables of Temperature Average and ELISA results (Table 6). When ELISA results were graphed

against Mean Temperature, the resulting graph contradicted the QRT-PCR results: ELISA negative vine temperatures were significantly lower than the ELISA positive vine temperatures. To explain these contradicting results, it could be concluded that because of the high precision of QRT-PCR, many of the positives that were shown had low concentrations of the bacterium and these low concentrations would probably not be affecting the plant, thus not causing an increase in temperature. However, when we graphed the average temperature against the separate copy numbers, the trend was completely opposite of our hypothesis (Fig. 9). The average temperature decreased as the copy number increased. To better understand this phenomenon, graphs were made of each variety and its copy numbers (Fig. 11, 12, 13, and 14). The graphs of Blanc duBois, Chardonnay, and Cabernet Sauvignon did not have an observable trend. Merlot had a decreasing trend, which did not coincide with our hypothesis. The implications of this study are unclear in that there are contradicting results in the significance of temperature difference between positive and negative ELISA and QRT-PCR samples and in that when there is an observable trend, it does not proceed as was hypothesized. More research will need to be conducted to better explain the phenomenon that occurred in this study. As for the use of an infrared thermometer, if the mean temperature for each of the varieties (of the QRT-PCR negatives) for all six weeks was used as a base temperature, it's possible to use the infrared thermometer gun to detect whether or not an asymptomatic plant is diseased. Although, at the moment it does seem possible to utilize this new development, more extensive research would need to be done on vine temperatures before committing to vine roguing by way of infrared gun measurements.

The plot arrangement hypothesis that PD spreads down a row faster than across cannot be confirmed by the results of this study. All data that were graphed did not indicate any patterns. Each variety had positives at different locations. This would indicate that one cannot expect healthy vines next to a diseased vine to have a greater probability of being infected. However, according to previous work done by Vest (18), patterns can be detected under some circumstances. It is possible that this pattern was not

seen in this project's target vineyards because of a very high disease incidence. It can be hypothesized that the high disease incidence is overwhelming any visible pattern.

The setup of this study provided many opportunities to gather general data about *X. fastidiosa* and its effects on grape varieties and temperature. Although assumed, we have proof that vine temperatures increase during the day as well as over the summer. Grape varieties also have differing temperatures. Cabernet Sauvignon had the highest mean temperature overall the course of this study. This was surprising in the fact that it's not considered to be the most susceptible. One would have expected Chardonnay to have a higher temperature, considering that *X. fastidiosa* infected vines have a higher temperature than healthy ones. One thing we took into consideration was that there was a statistically significant difference between vineyard temperatures. We divided the variety temperatures into the separate vineyard locations, and found the results were different (Fig. 5, 6, 7, and 8). This means that there are some location effects on vine temperatures. Texas Hills vineyard had the highest mean temperature and it was significantly higher than Spicewood vineyard. These two vineyards are not very far apart geographically speaking, so why does it have a higher temperature? If different locations affect vine temperatures, then this would be something to keep in mind when measuring vines temperatures for roguing purposes.

Further research needs to be done before an infrared thermometer can be used effectively in detecting *X. fastidiosa* infected grapevines. There are too many factors affecting the vine temperatures. Location of the vineyard would need to be taken into consideration, as well as grape variety, since significant differences were found between both. Contradicting results from ELISA and QRT-PCR regarding positive and negative vine temperatures also calls for more research. Study of disease epidemiology would need to be conducted in less heavily infected vineyards so that we can determine if there is a disease spread pattern. One valuable contribution to diagnostics was that we were able to design a technique that cleans up QRT-PCR samples that were heavily degraded and had inhibitors present.

SUMMARY

We have demonstrated that there are contradictory results regarding a significant temperature difference between *X. fastidiosa*-infected vines and healthy vines. ELISA testing stated that there was a significant temperature difference between vines, but QRT-PCR results showed that there was no significance. Further and more extensive research would need to be done before infrared thermometer gun measurements could be used as a basis for early detection and roguing of asymptomatic plants.

While we were not able to get conclusive evidence on culturing, the differences between ELISA and QRT-PCR were not enough to warrant exclusive use of one or the other. Both tests served their purpose and there were pros and cons for each. QRT-PCR may have been the faster of the two tests, but unless the data is quantified, the temperature data will be skewed.

The spread of *X. fastidiosa* did not have a specific pattern. According to the data the disease did not spread down a row more than across a row and vice versa. However, there is some evidence that because the targeted vineyards had a high disease incidence, the spread pattern was washed out.

QRT-PCR samples that are heavily degraded and full of inhibitors can be cleaned up using a combination of techniques. PrepMan Ultra® and nucleic acid precipitation will clean up a sample enough to run a QRT-PCR test on it.

Vine temperatures increased as the summer progressed and each grape variety had a different mean temperature. Cabernet Sauvignon had the highest followed by Chardonnay, Merlot, and Blanc duBois. However, when divided up by vineyard locations, the varieties had varying temperatures. At Texas Hills the highest average vine temperature belonged to Merlot and Chardonnay, while at Spicewood the Cabernet Sauvignon had the highest temperature.

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APPENDIX A

Outline of Plots, Locations, Varieties, and Health Status of Disease Centers

I. Location : Texas Hills Vineyard

A. Variety : Chardonnay

Disease Centers: Health Status and Row Location

1. Severe – 64.14.6 (Dead)
2. Incipient – 58.16.4
3. Healthy – 56.15.9

B. Variety : Cabernet Sauvignon

Disease Centers: Health Status and Row Location

1. Severe – 20.11.8
2. Incipient – 18.4.3
3. Healthy – 17.4.4 (New Planting)

C. Variety : Merlot

Disease Centers: Health Status and Row Location

1. Severe – 31.12.5
2. Incipient – 33.10.6
3. Healthy – 32.2.5

II. Location : Spicewood Vineyard

A. Variety : Chardonnay

Disease Centers: Health Status and Row Location

1. Severe – 62.1.9 (New Planting)
2. Incipient – 63.8.1
3. Healthy – 63.5.2

B. Variety : Cabernet Sauvignon

Disease Centers: Health Status and Row Location

1. Severe – 81.5.4
2. Incipient – 82.6.5
3. Healthy – 81.4.1

III. Location : Palacios Vineyard

A. Variety : Blanc duBois

Disease Centers : Health Status and Row Location

1. Severe – 6.3.7
2. Incipient – 4.6.3
3. Healthy – 6.5.2

B. Variety : Merlot

Disease Centers : Health Status and Row Location

1. Severe – 17.10.2
2. Incipient – 14.3.4
3. Healthy – 15.8.2

IV. Location : Texas A&M Experimental Vineyard

A. Variety : Blanc duBois

Disease Centers : Health Status and Row Location

1. Severe – 2.10
2. Incipient – 3.3
3. Healthy – 3.10

APPENDIX B

PD3 Medium with 1/2 BSA

	600 mL	700 mL	1 L
Citric acid	.6 g	.7 g	1 g
Succinate	.6 g	.7 g	1 g
MgSO4 * 7 H2O	.6 g	.7 g	1 g
KH2PO4	.6 g	.7 g	1 g
K2HPO4	.9 g	1.05 g	1.5 g
Soytone	1.2 g	1.4 g	2 g
Potato Starch	1.2 g	1.4 g	2 g
Tryptone	2.4 g	2.8 g	4 g
0.1% Hemin Chloride	6 mL	7 mL	10 mL
Gelrite	5.4 g	6.3 g	9 g
BSA	1.8 g	2.1 g	3 g

Hemin Chloride Stock Solution (0.1%)

Hemin Chloride	100 mg
NaOH (.05 M)	100 mL

APPENDIX C

SCPAP Extraction Buffer Recipe

	1 Liter	500 mL	250 mL	125 mL
Succinic Acid	1 g	.5 g	.25 g	.125 g
Citric Acid	1 g	.5 g	.25 g	.125 g
K_2HPO_4	1.5 g	.75 g	.38 g	.19 g
KH_2PO_4	1 g	.5 g	.25 g	.125 g
Sodium Ascorbate	3.96 g	1.98 g	.99 g	.495 g
Polyvinylpyrrolidone	50 g	25 g	12.5 g	6.25 g
dH_2O	942 mL	471 mL	236 mL	118 mL

VITA

Name: Kelly Asbill Bryan

Address: Department of Plant Pathology & Microbiology
120 Peterson Building
2132 TAMU
College Station, TX 77843-2132

Email Address: kelly.a.bryan@gmail.com

Education: B.S., Agronomy, Texas Tech University, 2005
M.S., Plant Pathology, Texas A&M University, 2008