

**INVESTIGATIONS ON THE DIAGNOSIS, COLONIZATION, AND
EPIDEMIOLOGY OF GRAPEVINES WITH PIERCE'S DISEASE**

A Thesis

by

MANDI ANN VEST

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

December 2004

Major Subject: Plant Pathology

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December 2004

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ABSTRACT

Investigations on the Diagnosis, Colonization, and Epidemiology
of Grapevines with Pierce's Disease. (December 2004)

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Pierce's disease (PD) of grapevines, caused by *Xylella fastidiosa*, is devastating Texas vineyards. Two rapid diagnostic techniques, real-time polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), were compared on the basis of cost, reliability, and their ability to quantify *X. fastidiosa* in diseased tissues. A high correlation was found between the two techniques for measuring bacterial titer *in vitro*. A similar relationship was not detected when applying the methods to diseased tissue. There was a 75% similarity between the techniques when used to diagnose PD in artificially infected grapevines. Where the two methods differed, real-time PCR was more successful in identifying plants known to be infected with the bacterium. In uninoculated grapevines, the two techniques were similar, where the positive rates were 7% and 4% for ELISA and real-time PCR respectively. In a second study, 3 grape cultivars, 'Cynthiana', 'Cabernet Sauvignon', and 'Chardonnay', were inoculated with 2 isolates of *X. fastidiosa* to measure disease development and colonization by the pathogen. The bacteria colonized similar distances from the inoculation point over a 25 week period in all three cultivars. Real-time PCR and ELISA absorbance values suggest that the concentrations of bacteria ranged between 10^4 and 10^6 cells/ml in a 1.27 cm

section of grapevine cane. Concentrations of bacteria didn't vary based on distance from the inoculation point. Marginal leaf-scorch symptoms were seen on 'Cabernet Sauvignon' and 'Chardonnay' grapevines 9 weeks post-inoculation. Leaf-scorch symptoms were not observed on 'Cynthiana'. The vigor of all inoculated grapevines was reduced compared to negative control grapevines the season after initial infection. In a third study, a Texas vineyard planted in Viognier grapevines was surveyed for PD symptoms on 3 separate dates. In October 2003, 45/50 rows had significant aggregation of symptomatic grapevines according to Ordinary Runs Analysis. Aggregation of symptomatic grapevines was found down the row more often than across the row. The rapid rate of disease progress and mortality rate of vines in this vineyard suggest that vine-to-vine spread is occurring and that Viognier vines are highly susceptible to PD.

DEDICATION

God has blessed me so much and carried me always. I am most thankful that He gave me two exceptional parents. I dedicate this work to my parents, Donald and Julie Vest, who have inspired me by their hard work in agriculture and education. They have provided for me the resources to achieve everything I've done to this point. I am so thankful they loved me so much that they sacrifice daily to provide for me. If I do anything in this life that makes a difference, it is because of their hard work and unconditional giving. I am mostly thankful that they taught me to stay rooted in my relationship with God and acknowledge Him in all of my ways. I can do all things through Christ, who is my strength - Philippians 4:13.

ACKNOWLEDGEMENTS

Thank you Father for being my strength and most dependable friend. Shelby, Kristin, and Julie, you made my experience better than I could have ever dreamed. Thanks for laughing with me and at me at times. Thank you for listening and offering help, advice, and a shoulder to lean on.

Erin and Kim, thank you for loving me since we were young and carefree. I am so glad we have grown up together. Casey and Tom, thanks for making me laugh, it always helped. Andy, thank you for inspiring me and encouraging me to walk closer with Him.

Dr. Appel, thank you for giving me the freedom to be myself in this whole process and for the opportunity to learn how this crazy system works. Thanks to my committee for helping whenever I needed it. Jim Kamas, thank you for your hard work and willingness to be the leader of this group. Ed Hellman, Mark Black, and Lisa Morano, thank you for your advice. Thank you USDA for funding this project.

Thanks to Olivier Schill, my crazy French friend who made research fun. Thank you Coy Crain and Julie for sweating with me in the greenhouse and helping with the fun lab work! Thank you Tricia Johnson for being so perseverant on the computer!

Thank you Dad and Mom, I love you! And to the rest of my family, I love you and I am so happy to be a part of the most special family in the world!

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CHAPTER I

INTRODUCTION

Literature Review

Pierce's disease (PD), caused by *Xylella fastidiosa* (82), is considered the single greatest threat to wine grape production in Texas (46,72). The disease has posed a problem in Texas since at least 1990, causing losses of millions of dollars. The risk of PD varies across the state with the High Plains and Trans-Pecos areas being least vulnerable and the central-south Texas regions being the most vulnerable (46). The Hill Country tends to have relatively mild winters, which may contribute to increased risk of PD in central Texas (42,46). Little data has been collected concerning the epidemiology of the disease in Texas, although samples have been collected from symptomatic vines in various vineyards and PD has been regularly confirmed (Mr. James S. Kamas, *personal communication*). Diagnostic methods have included real-time polymerase chain reaction (PCR) (75), enzyme-linked immunosorbent assay (ELISA) (55), plating bacteria from plant tissue (39), Gram-staining and visual microscopic observation (82).

Texas, considered the fifth largest wine producer in the United States, continues to expand its grape production throughout all parts of the state (16,72). A majority of the grape production in the state is located on the Texas High Plains, where disease and pest problems are reduced and soil and weather conditions are conducive to viticulture practices. However, most wineries are found in the Texas Hill Country and around the

This thesis follows the style and format of Plant Disease.

Dallas area, where tourism contributes to their economic viability and PD risk is greatest (10,16).

Presently, Texas grape production is at its highest and is taking its place among the states' most agronomically important crops. As of 2002, 2,900 acres of Texas land is planted in grapevines, and there are at least 46 wineries which produce over one million gallons of wine per year (16). The Texas Wine Marketing Research Institute estimates that production will exceed 2 million gallons in the next 5 years (16). Not only does wine production in Texas continue to contribute to a rich agricultural heritage, it also has a significant impact on the Texas economy. In 2001, the estimated total economic impact of the Texas wine and wine grape industry on the state's economy was \$133 million, 1,800 jobs were provided, \$3 million in direct excise and sales tax were accumulated, and \$10.5 million in indirect and direct tax impacts occurred (16). According to the Texas Agricultural Statistics Service, the year 2002 was difficult for grape growers due to adverse weather, disease, and pests (71). Pierce's disease was one of these adversities and was the focus of this research project.

Pathogen Description and Biology

Pierce's disease was only recently found to be caused by the bacterium *Xylella fastidiosa* (82). The first reported case of PD was described in California by Newton Pierce in 1882 (58). For 80 years after this first discovery, the disease was thought to be caused by a virus because researchers were unable to culture the causal agent (36,38). Later it was found that the causal agent is spread by xylem-feeding leafhoppers

(20,36,43), specifically sharpshooters (22,35,59) such as the blue-green sharpshooter, *Graphocephala atropunctata* (60), or the glassy-winged sharpshooter, *Homaladisca coagulata* (64). These insects feed on various plants that serve as supplemental hosts to *X. fastidiosa* and then vector the bacteria into vineyards (21,68,70). The number of known supplemental hosts is in the hundreds and continues to grow (21,49,68). This multitude of plant species that harbor *X. fastidiosa* probably varies in importance as a source for vector spread. One aspect of the variability depends on whether the bacteria spread systemically within the plant, multiply in high numbers, or persist for long periods (63). After a vector feeds on a vine and transmits *X. fastidiosa* into the xylem, the bacteria inhibits water flow by multiplying and clogging the water-conducting tissues of the vine. Classic PD symptoms include marginal leaf scorch, leaf drop with petiole retention, shriveled grape clusters, and uneven periderm development or green “islands” at the nodal areas (30).

Characterization of the PD bacterium was not possible until it was isolated in 1978 (13,38). In 1987 Wells et al. proposed the name *Xylella fastidiosa* for this group of fastidious, xylem- limited bacteria based on the characterization of 25 phenotypically and genotypically similar strains (82). The strains were isolated from various economically important hosts including grapevine, peach, periwinkle, almond, plum, elm, sycamore, oak, and mulberry. All isolates were single celled, nonmotile, gram negative, aflagellate rods (~0.25 to 1.35 by 0.9 to 3.5 μm). Biochemical assays found similarity among all strains and genetic comparisons indicated at least 85% DNA-DNA

homology (82). Although different strains of *X. fastidiosa* have been classified as a single genus and species, differences remain that are poorly understood (47,63).

Some strains of the bacterium have a wide host range (38), some isolates from one host can multiply and induce symptoms in another (38,47,48,66), while some strains appear to be host specific (66). A strain of *X. fastidiosa* can infect and produce symptoms on mulberry in very cold regions, yet the PD strain spreads most efficiently in the hottest regions of the U.S. and doesn't normally occur in regions that have hard winters (9,61,62). Production of desirable, susceptible grape cultivars has not been successful in Florida due to PD, yet citrus variegated chlorosis (CVC), also caused by *X. fastidiosa*, hasn't occurred after over a hundred years of citrus production in the state. CVC was discovered in Brazil after only 50 years of citrus production, and coffee leaf scorch, another disease caused by *X. fastidiosa*, has been recently described in the same region (15,67). Citrus replaced the coffee industry in Brazil after a period of decline in coffee production; thus it is likely that the citrus strain of *X. fastidiosa* originated from coffee (9). *X. fastidiosa* strain relationships remain vague as the bacterium continues to be found in new hosts.

Diagnostic Tools

PD diagnostic methods need to be sensitive and reliable for research and successful management of the disease. Since *X. fastidiosa* was isolated in 1978, researchers have been developing assays to detect the pathogen in plant tissue more rapidly than culturing, since colonies of the bacterium are not visible for 7 to 10 days on

laboratory media (13,38,52,55,63). Culturing of the bacteria is also difficult due to the fastidious nature of *X. fastidiosa*, slow growth rates, and limited distribution in infected plants (13,37,53). The serology-based enzyme-linked immunosorbent assay (ELISA) has been routinely used since 1976 to detect plant pathogens (81). Polymerase chain reaction (PCR) has been used to amplify pathogen-specific DNA sequences providing a reliable diagnostic tool for plant diseases since 1993 (34). More recently, real-time PCR has been applied to various plant pathogens, speeding up the PCR process by reducing the number of steps and possibilities for human error (74).

These rapid diagnostic tools have their limitations. The ELISA technique employs polyclonal antibodies that reduce the specificity, has been shown to have low sensitivity, and can lead to false positive results (8,27,37,41,68). Grapevines and some other plants have inhibitors that prevent successful detection of plant pathogens with PCR (52). Neither PCR nor ELISA can estimate the viability of *X. fastidiosa* in plant tissue (63). But, ELISA and real-time PCR have been shown to quantify concentrations of *X. fastidiosa* in pure suspensions of bacterial cells (55,75).

Multiplication and Colonization

Several experiments have shown *X. fastidiosa* varies in rates of survival, multiplication, and colonization within hosts (3,18,19,23,24,37,53,65). This variance may be due to the time of year of inoculation (19), the environment (18,19,62), or the type of host plant (23,24,37). *X. fastidiosa* can multiply and move within the xylem of grapevines that are thought to be resistant, tolerant, or susceptible to PD (23,24). Fry et

al. (23) showed that ‘French Colombard’, a susceptible *Vitis vinifera* cultivar, appeared to be a more conducive habitat for the bacterium than ‘Carlos’ and ‘Noble’, both *V. rotundifolia* cultivars native to the southeastern U.S. and thought to be tolerant and resistant to PD, respectively, based on symptom development in the field.

Cultivar selection for grape production in high risk areas for PD can be challenging. This is due, in part, to the fact that mechanisms of resistance, tolerance, and susceptibility are poorly understood (23,24,38,40). For example ‘Cynthiana’ (Norton), *Vitis aestivalis* Michx., appears to have tolerance to PD, but this variety is not widely planted due to limited market potential (46). Popular grape cultivars commonly grown in Texas include *V. vinifera* cultivars ‘Cabernet Sauvignon’ and ‘Chardonnay’ due to high market demand. The former cultivar is considered moderately susceptible and the latter is considered highly susceptible to PD (31). In Texas in 2002, 720 bearing acres were planted in ‘Cabernet Sauvignon’ and 550 bearing acres were planted in ‘Chardonnay’ (71). Behavior of *X. fastidiosa*, with respect to multiplication and colonization in the xylem in these popular cultivars is poorly understood and should be addressed. A cultivar like ‘Cynthiana’, which appears to be resistant to PD, may harbor the bacterium but the vine may be able to tolerate infection by the pathogen. The bacteria may multiply and colonize at different rates in grapevines that vary in susceptibility (40). It would therefore be important to determine the relationship between *X. fastidiosa* colonization and symptom development in grape cultivars commonly utilized in Texas.

Spatial Pattern: Epidemiology

A critical part of epidemiological investigations is identifying the type of disease pattern in a field (50). One important reason for this is that the fate of healthy vines in a vineyard may depend on their spatial relation to those that are already diseased (44). The pattern of diseased vines in a vineyard can suggest whether or not the pathogen is moving from vine-to-vine or from sources external to the vineyard. A random pattern suggests that the pathogen is not spreading from vine-to-vine, and an aggregated pattern suggests the opposite. Statistical analysis of spatial distribution of symptomatic grapevines in a vineyard can lead to understanding of vector x pathogen x host x environment interactions resulting in PD epidemics (50).

Vanderplank (79) proposed that when infected plants are clustered in a field, the pathogen is predominately spreading through adjacent plants. In California before the glassy-winged sharpshooter was introduced, PD incidence was highest on the edge of vineyards along riparian vegetation and decreased with distance from this edge (59). An apparent lack of vine-to-vine spread following initial infections was observed (42). This would be a monocyclic pattern of pathogen spread (7,80). This lack of vine-to-vine spread may have occurred because grapes were either not exposed to repeated infections in summer and/or infections did not persist until the following season (19). A seasonal lack of insect vectors in the field would also explain the monocyclic pattern. Most of the common sharpshooters, such as the blue-green sharpshooter (*Hordnia circellata*) feed on and inoculate younger tissue near the tips of growing vines (60). The bacteria may not

have time to multiply and spread throughout vines before season's end and subsequent annual pruning of the vines removes infected tissues (42). The epidemiology of PD in California has subsequently changed due to the introduction of the glassy-winged sharpshooter. Glassy-winged sharpshooters (GWSS) tend to feed near the base of new shoots and even through the tough bark of branches (64). The differences in feeding behavior may increase numbers of vines having persistent infections until the next season (42) thus making vine-to-vine spread more probable. The disease then becomes polycyclic with a more destructive potential (80). GWSS was first found in the Temecula Valley of California in 1998. Within the next few years spatial patterns of PD in the Temecula Valley vineyards indicated *X. fastidiosa* was spreading within vineyards from vine sources (57). Epidemiological studies have been conducted on PD in California (59,60), but similar analyses have not been done in Texas (42). Observations in Texas indicate that PD may be a polycyclic (7,80) disease (Mr. James S. Kamas, *personal communication*).

Objectives

1. Compare consistency of qualitative results from ELISA and real-time PCR assay methods. Quantitative values for each technique also will be evaluated for their ability to determine concentrations of *X. fastidiosa* in grapevine tissues.
2. Describe colonization of *X. fastidiosa* in grapevine cultivars that were believed to vary in susceptibility to Pierce's disease. Cultivars tested included 'Cynthiana',

‘Cabernet Sauvignon’, and ‘Chardonnay’. Monitor colonization of the bacteria in these vines and PD symptom development over time.

3. Determine spatial pattern of diseased grapevines in a Texas vineyard. Use Ordinary Runs Analysis (28,50) to determine whether PD had an aggregated or random spatial pattern in the vineyard. Monitor disease development over time to determine the rate of PD progress.

CHAPTER II

COMPARISON OF RAPID DIAGNOSTIC TOOLS FOR DETECTING AND QUANTIFYING *XYLELLA FASTIDIOSA* IN GRAPEVINES

Introduction

Enzyme-linked immunosorbent assay (ELISA) is a diagnostic immunoassay used to detect plant pathogens directly in plant tissue (55,81). The ELISA utilizes purified antibodies prepared by injecting a small mammal with an antigen, in this case a component of the plant pathogen *X. fastidiosa*. The antibodies from the animal's blood are extracted, purified, and processed into a serological kit for convenient diagnosis. The technique used most often in diagnosing plant diseases is the sandwich or double antibody technique. This procedure begins with antibody bound to a polystyrene well in a microtiter plate. The sample, consisting of suspect plant tissue homogenized in an extract buffer, is added to the well. Because *X. fastidiosa* colonizes only xylem tissue, plant tissue rich in xylem is selected for testing. If the source antigen, i.e. *X. fastidiosa*, is in the sample it will bind to the antibody. An enzyme conjugate is then added to the well with bound antigen-antibody. A substrate is added to the enzyme conjugate which is bound to the antigen-antibody. If the specific antigen is in the sample being tested, all of the added substances will bind to each other making an immuno-complex. Lastly, a sulfuric stop solution is added. A color change indicates the putative presence of the suspected pathogen (51). Absence of color means the sample was negative or antigen was below detectable concentration. ELISA has been developed for diagnosing PD (39,55) but is sometimes not reliable and may lead to false negatives and false positives.

Concentrations of *X. fastidiosa* must be high (at least 10^4 cfu/ml) for ELISA to give a positive reading (75,76).

After ELISA has been completed, a plate reader can be used to determine absorbance values in each individual well. Putative positives appear as a rusty orange color and negatives are clear. A higher absorbance reading should reflect a higher concentration of *X. fastidiosa*, and one would expect a stepwise decrease in absorbance as the concentration of bacteria in samples decreases.

Polymerase Chain Reaction (PCR) is another method for detecting plant pathogens. Diagnostic PCR is based on constructing millions of copies of specific fragments of pathogen DNA (17,77). The PCR process is highly temperature dependent, heating and cooling is required. During the process, temperature is adjusted to initiate the steps: denature the DNA, hybridize primers to a known sequence (annealing), and extend the complimentary DNA strand on each template strand via Taq polymerase. Primers, chemically synthesized DNA sequences which are complementary to specific sequences of interest, act as initiators to the DNA extension process. Taq polymerase, originally isolated from the thermophilic bacterium *Thermus aquaticus*, incorporates nucleotides into the emerging DNA strand, producing a complementary copy of the DNA template in the region specified by the annealed primers (17,73). After many heat/cool cycles of denaturation, annealing, and polymerization, millions of DNA fragments are synthesized. The PCR product is run on agarose gel stained with ethidium bromide, which aids in visualizing DNA. If bands are seen on the gel and the sample is

not contaminated, further purification allows the sample to be sequenced using special computer software (17,77). This whole process takes 1 or 2 days.

A PCR technique more recently developed is real-time PCR. This procedure is more rapid and easier to carry out (4). Real-time PCR is run in a closed-tube system and requires no post-amplification manipulation for quantification, reducing contamination problems and turn-around times for data analysis (4). During this assay, two *X. fastidiosa*-specific primers define the endpoints of the amplicon (DNA sequence to be synthesized). Once the amplicon is synthesized via polymerase, an oligonucleotide probe hybridizes to the DNA sequence. The probe includes a fluorescent reporter and quencher dye. Polymerase extends the primers until it comes to the attached probe, then reporter dye is released from the probe and read by the Smart Cycler system (Cepheid, Sunnyvale, CA) as fluorescent emissions (4). Results are obtained by measuring the cycle threshold (Ct), the first cycle in which there is significant increase in fluorescence (74). This is a true real-time process because progress can be monitored on a computer screen at any time during the cycle (5). Specific 16S-23S internal transcribed spacer (ITS) primers and probe have been developed for detection of *X. fastidiosa* (75).

Another advantage of real-time PCR is the ability of the process to quantify the pathogen (75). Real-time PCR has been shown to quantify the amount of DNA in the sample being tested by detecting the point during cycling when amplification of a PCR product crosses a fluorescence threshold. The greater the amount of DNA present, the earlier in the PCR process a significant increase in fluorescence is observed (4).

The objective of using both ELISA and real-time PCR as diagnostic techniques in the present study was to compare the consistency of the two techniques in giving qualitative and quantitative results. The results presented show the value of using ELISA and real-time PCR to detect *X. fastidiosa* and reliability of the techniques in quantifying concentration of the bacteria in plant tissue.

Materials and Methods

A description of how grapevines were obtained, potted, arranged in the greenhouse, and inoculated are in the Materials and Methods section of Chapter III of this thesis. ELISA kits, designed to detect several strains of *X. fastidiosa*, were obtained (Agdia, Inc., Elkhart, IN). For real-time PCR, Omnimix HS, a general PCR reaction mix, and reaction tubes were obtained (Cepheid, Sunnyvale, CA). Primers were ordered from the Gene Technologies Laboratory at Texas A&M University (College Station, Texas). Fluorescent probe was obtained (Synthegen, Houston, Texas). The PCR machine used was the SmartCycler[®] (Cepheid, Sunnyvale, California).

One-hundred thirty-five grapevines were inoculated and assayed for the presence of *X. fastidiosa*. To detect the bacteria in grapevines, 2.54 cm pieces of cane were cut from the inoculation point, 15.24 cm distal from the inoculation point, and every 7.62 cm distal from the previous point. Each piece was cut in half, then each half was sliced into 2 mm sections using a razor blade or pruning sheers for tougher tissue, and placed into 1 ml of either sterile succinate-citrate-phosphate buffer modified with ascorbate and 5% polyvinylpyrrolidone (SCPAP) (52) for real-time PCR or ELISA general extraction

buffer (Agdia Inc.). Tools were sterilized between samples by dipping them into 70% ethanol and passing them over a flame. Tubes were stored at 4°C for 48 hours until assayed. ELISA sample tubes containing plant tissue and buffer were vortexed and 100 µl of suspension was pipetted into a precoated well. ELISA was then performed according to product instructions (Agdia Inc.). Real-time PCR sample tubes containing plant tissue and buffer were vortexed and 1 µl of suspension was added to reaction tubes for the assay (75).

After the ELISA reaction was completed, a SPECTRAFluor plate reader and computer software package Magellan (Tecan, Maennedorf, Switzerland) were used to determine absorbance levels in each well. SmartCycler software (Cepheid, Sunnyvale, CA) was used to read real-time PCR results. Initially, a concentration curve was determined for each instrument to calibrate readings with known bacterial concentrations. *X. fastidiosa* was grown on PW medium (39) and a suspension of cells was made and diluted by 1/10 five times. The ELISA absorbance and real-time PCR Ct values of the dilution series were entered into Microsoft Excel and plotted against each other to determine how well their values correlated. ELISA absorbance values and real-time PCR Ct values for direct test on plant tissues were plotted against each other as well.

Results

For real-time PCR, smaller Ct values reflect higher concentrations of template DNA (Table 2.1). Larger ELISA absorbance values indicate higher antigen

concentration. So, the two assays have an inverse relationship (Fig. 2.1). Both ELISA and real-time PCR can give potentially false positive results (75,76). Similar observations were made in our laboratory. Therefore, we established a minimum ELISA absorbance value and a maximum real-time PCR Ct value that could be considered positive for *X. fastidiosa*. A minimum of 10^4 bacterial cells/ml are required for a positive ELISA (75,76). The average ELISA absorbance value for solutions of 10^4 bacterial cells/ml was $A_{492} = 0.174$. The average value for solutions of 10^5 cells/ml was $A_{492} = 0.35$ (Table 2.1). Since ELISA has also been reported to give false positives (76), $A_{492} \geq 0.30$ was considered to be a positive ELISA result. Also, real-time PCR was reported to give a weak positive result, Ct = 37 to 38.5, for *Xanthomonas campestris* (75). Negative controls have elicited late positive results occasionally, after at least 37 cycles. Therefore, only Ct values of 36 or less were considered positive.

Dilution series results for real-time PCR Ct values are shown plotted against ELISA absorbance values in Fig. 2.1 (known cell concentrations are shown at each point on the line). The values for each are shown (Table 2.1). When ELISA absorbance values on \log_{10} scale were plotted against real-time PCR Ct values the result was a linear relationship (Fig. 2.1). We used this relationship to evaluate the efficiency of using real-time PCR and ELISA to quantify concentrations of *X. fastidiosa* in grapevine tissue.

On a qualitative basis, results from both assays were fairly consistent. Multiple sections of cane tissue (2.54 cm section at the inoculation point and every third subsequent 2.54 cm section distal from the inoculation point) were assayed for each

Table 2.1. Real-time polymerase chain reaction (PCR) cycle threshold (Ct) and enzyme-linked immunosorbent assay (ELISA) absorbance ($A_{492\text{nm}}$) value means obtained from assaying cell dilutions of *Xylella fastidiosa*. Each assay was performed three times on each dilution. Dilutions were also plated on PW media and colonies were counted.

Bacterial cells/ml*	ELISA absorbance	Real-time PCR Ct
10^2	0.066	36.96
10^3	0.099	33.24
10^4	0.174	31.44
10^5	0.350	28.86
10^6	1.101	24.76
10^7	2.515	21.09

*Suspensions of *Xylella fastidiosa* were plated on PW medium and colonies were counted. Each colony is assumed to be started by a single cell.

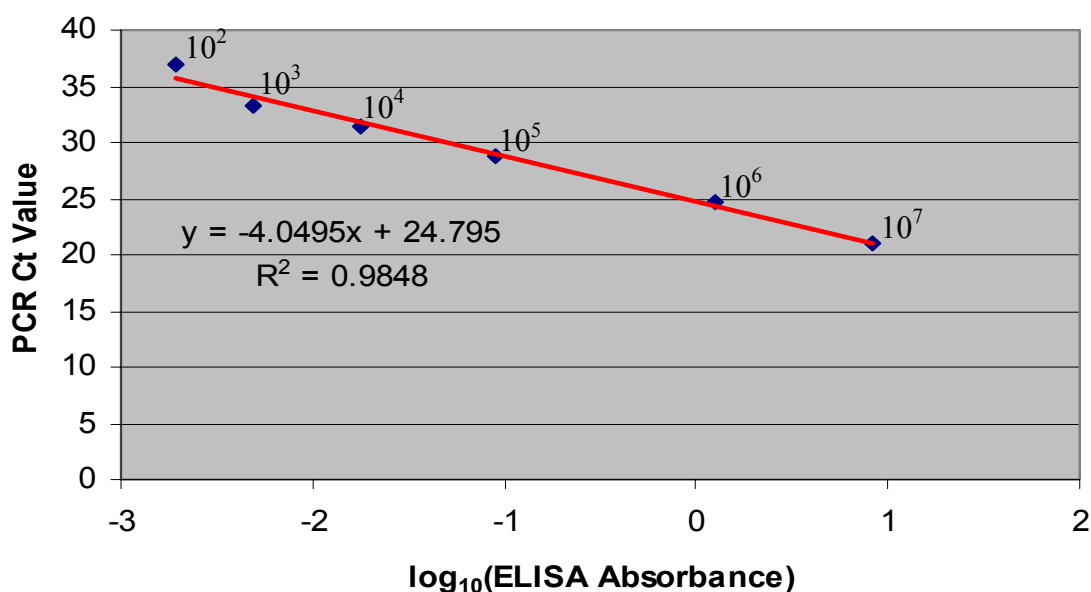


Fig. 2.1. Real-time polymerase chain reaction (PCR) cycle threshold (Ct) values plotted against \log_{10} enzyme-linked immunosorbent assay (ELISA) absorbance values, wavelength 492, observed from a dilution series of *Xylella fastidiosa*. The concentrations of bacterial suspensions are indicated at each point on the line. The correlation between the values is linear when ELISA values are transformed \log_{10} , $R^2 = 0.98$.

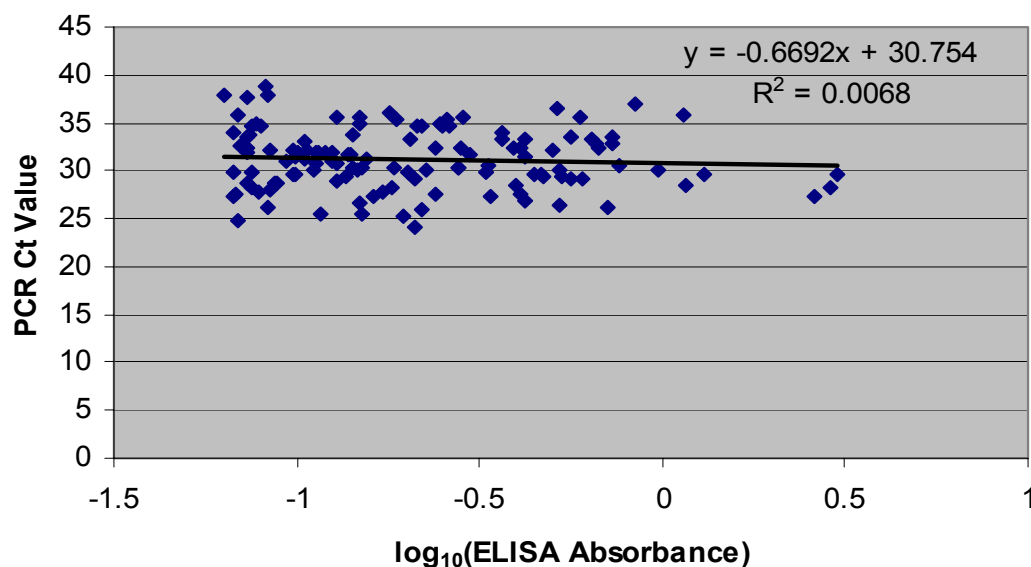


Fig. 2.2. Real-time polymerase chain reaction (PCR) cycle threshold (Ct) values plotted against \log_{10} of enzyme-linked immunosorbent assay (ELISA) absorbance values for *Xylella fastidiosa* found in grapevine tissue. The results for 124 reactions are shown. 2.54 cm of grapevine canes were cut in half, finely chopped, and each half was soaked in 1 ml of ELISA buffer or succinate-citrate-phosphate buffer for 48 hours at 4°C. The tubes were vortexed and the suspension was assayed using ELISA or real-time PCR.

grapevine. A vine was considered positive if at least one 2.54 cm section tested positive. The two assays gave similar results for 102 (76%) of the 135 grapevines. But for 33 grapevines (24%), the two assays gave differing results. Of these 33 grapevines, 25 were considered positive for *X. fastidiosa* by real-time PCR and negative by ELISA. The remaining 8 grapevines were considered positive by ELISA and negative by real-time PCR. Of the 108 known positive grapevines (inoculated with a suspension of *X. fastidiosa*), 42% were considered positive by ELISA and 65% were considered positive by real-time PCR. ELISA absorbance values and real-time PCR Ct values run on similar

plant tissue gave different quantitative results. Real-time PCR Ct values were plotted with \log_{10} ELISA absorbance values for each sample of grapevine cane that was positive with both assays (Fig. 2.2). There was no correlation between \log_{10} ELISA absorbance and real-time PCR Ct values, $R^2 = 0.0068$.

Discussion

Because plating *X. fastidiosa* can be problematic due to time, 7 to 10 days of incubation before colonies appear (13,39), and contamination from other organisms, rapid methods of detecting the bacteria in plant tissue are desirable. PD diagnostic methods need to be sensitive and reliable as well so that researchers can give growers accurate diagnoses. Currently, the only control methods for PD are planting resistant cultivars, exclusion of the pathogen by controlling the vectors, and removal of diseased grapevines and other plants (42,46). Therefore, when growers are told they have PD in their vineyard, sacrifices of plants must be made to prevent further spread. There needs to be a high degree of certainty that a positive diagnostic result from ELISA or real-time PCR means that plant tissue is infected with *X. fastidiosa*. Previous reports have shown that real-time PCR is an effective method of diagnosing plants with a high degree of certainty (74,75). ELISA reports have shown that the assay can detect *X. fastidiosa* when it is highly concentrated in plant tissue (38,39,55).

Most researchers trust PCR over ELISA for giving an accurate PD diagnosis, since PCR targets specific sequences of pathogen DNA. The 16S-23S spacer region was used to design primers and a probe for real-time PCR for several strains of *X. fastidiosa*

(75). This region is commonly used to study prokaryotic diversity because it has series of highly conserved sequences as well as variable sequences, which makes it convenient for PCR primer design (26). The primers and the probe designed previously (75) proved to be effective for real-time PCR in the present investigation. However, the available ELISA test-kit for *X. fastidiosa* diagnostics is not as specific as real-time PCR. This assay employs polyclonal antibodies, which are a mixture of immunoglobulin molecules secreted in the blood of an exposed mammal as a defense against antigens. These molecules each recognize a specific marker or epitope on the surface of the antigen. Polyclonal antibodies are not considered to be as specific as monoclonal antibodies, which are immunoglobulin molecules that only recognize one marker on an antigen. The advantage of using polyclonal antibodies rather than monoclonal is that the chances are higher of getting a positive result when the antigen is present (12). The commercial ELISA kit used in the present study was based on polyclonal antibodies, and it is not specific for the PD strain of *X. fastidiosa*.

A disadvantage of real-time PCR was expense. Each real-time PCR reaction that we performed cost approximately \$8 and each ELISA reaction only cost approximately \$2. The initial cost of buying the real-time PCR machine is quite high, about \$40,000 for a machine that can run 16 reactions at a time. Another disadvantage is that plants can produce PCR inhibitors that prevent successful PCR from plant tissue (52,75). We tried to overcome the inhibitor problem by soaking plant tissue in SCPAP (succinate-citrate-phosphate buffer with 0.02 M sodium ascorbate and 5% insoluble

polyvinylpyrrolidone) to extract *X. fastidiosa* for real-time PCR. This buffer is reported to help bind plant inhibitors that prevent successful PCR (52).

Since real-time PCR is a more sensitive detection method, it is expected that the assay would give more positives than ELISA, assuming these plants were truly infected. Possible explanations for why ELISA showed eight positives not considered positive by real-time PCR are that plant inhibitors prevented successful PCR or the ELISA results were false positives. Each 2.54 cm section of cane was cut in half, and one-half was assayed via ELISA and one-half was assayed via real-time PCR. Not using the same section of cane tissue for each assay may have affected results.

Both assays have been reported to give false positive results occasionally (75,76), and there could be a few reasons for this. Of course, in both reactions there is always a chance that a false positive result was caused by contamination by *X. fastidiosa*. However this is unlikely when proper microbiological techniques are observed and special care is taken to prevent contamination. If a false positive occurs from plant tissue thought to be negative, it could be that the plant tissue was actually infected by *X. fastidiosa*. Real-time PCR might give a false positive if primers start annealing to themselves, making products called primer dimers (17). Or it may be that the probe has degraded causing an increase in fluorescence even though template DNA is absent from the reaction tube. Ordering more of the probe could solve this problem, but the probe is one of the more expensive ingredients in this reaction. Sometimes the protocol and temperatures of the PCR can be adjusted to alleviate the false positive problem. The cycle threshold (Ct) can be adjusted so that level of fluorescence in a reaction tube must

be higher to be considered positive by the SmartCycler system. False positives indicated by ELISA might be caused by the presence of related bacteria which bind to the polyclonal antibodies or by cross contamination of pruning shears and razor blades. It could also be that commercial ELISA kits include faulty chemicals or equipment.

Although qualitative results for the two assays were fairly consistent, quantitative comparisons did not show any correlation (Fig. 2.2). We expected a negative correlation similar to that illustrated in Fig. 2.1. It has been reported that *X. fastidiosa* is not uniformly distributed throughout xylem tissue and colonies tightly aggregate (38,82). The bacterium forms an extracellular matrix that probably helps it stay bound to the xylem (38). Therefore, even homogenizing plant tissue or chopping it very finely does not guarantee that all bacteria will be released into solution. Also, although plant tissue length was measured to keep samples consistent, the amount of xylem tissue is not consistent for all samples. *X. fastidiosa* is confined to the xylem so plant segments with larger amounts of xylem tissue may contain more bacteria. Real-time PCR and ELISA may approximate the bacterial titer in plant tissue, but to use them to quantify bacterial concentrations with any degree of confidence would require further testing. In the future, plant tissue should be weighed so that concentration of bacteria can be compared to the mass of the sample. Also, homogenizing the tissue can increase the amount of bacteria released into suspension. We did not homogenize plant tissue because of the large number of samples we ran. Sterilizing the homogenizer between samples proved to be problematic.

Although these rapid diagnostic methods can help determine if *X. fastidiosa* is in plant tissue, a single method is not 100% accurate. Two or more diagnostic tests are often used when definitive diagnosis is needed. Previous reports on real-time PCR have shown that the technique can be applied early in the season before PD symptoms are showing (74,75). This early diagnosis could allow grape growers to remove infected vines early in the season to prevent further spread of *X. fastidiosa*. However, an intensive sampling of a vineyard would have to be performed to determine which grapevines are infected early in the season, sometimes involving thousands of grapevines. Such intensive sampling of a vineyard would be very expensive and time consuming. Without obvious late season symptoms, the only indication that a vine might have PD is reduced vigor or dieback (83). It is more realistic to wait for PD symptoms, and then test symptomatic grapevines using ELISA, real-time PCR, or culturing (preferably using at least two techniques). After PD has been confirmed, growers should then promptly remove diseased vines as recommended.

CHAPTER III

COLONIZATION OF *XYLELLA FASTIDIOSA* IN THREE GRAPE CULTIVARS

Introduction

Although some grape species appear to be resistant or tolerant to PD, the mechanisms involved are poorly understood (23,24,38,40). Resistant species are those that can exclude or overcome the effect of a pathogen, and tolerant species are those that can sustain the effects of a disease without dying or suffering serious injury (2). *X. fastidiosa* may multiply and colonize at different rates in grapevines that vary in susceptibility (40). ‘Cynthiana’ (Norton), *Vitis aestivalis*, appears to have tolerance to PD (46). ‘Cabernet Sauvignon’, *V. vinifera*, is considered moderately susceptible and ‘Chardonnay’, *V. vinifera*, is considered highly susceptible to PD based on symptom development in the field (31). Behavior of *X. fastidiosa*, with respect to multiplication and colonization in the xylem of ‘Cynthiana’, ‘Cabernet Sauvignon’, and ‘Chardonnay’ has not been investigated. There was a need to determine the relationship between *X. fastidiosa* colonization and symptom development in these grape cultivars.

The objective of this investigation was to describe colonization of *X. fastidiosa* and symptom development in grapevine cultivars that vary in susceptibility to PD. Cultivars tested included ‘Cynthiana’, ‘Cabernet Sauvignon’, and ‘Chardonnay’. Colonization of the bacteria in these vines and PD symptom development will be monitored over time.

Materials and Methods

Plant Materials

Ninety grapevines of ‘Cabernet Sauvignon’, ‘Chardonnay’, and ‘Cynthiana’ were obtained and grown in a greenhouse. The ‘Cabernet Sauvignon’ and ‘Chardonnay’ were obtained from James S. Kamas (Extension Fruit Specialist, Texas Cooperative Extension, Fredericksburg, TX). The original mother plants came from Ge-No’s Nursery in California (8868 Rt. 28 Ave. 9, Madera, CA 93637). ‘Cynthiana’ rooted cuttings were obtained from Double A Vineyards in New York (10277 Christy Road, Fredonia, NY 14063). The grapevines were own-rooted. One year old dormant canes were taken in December 2002 and 30 to 38 cm cuttings were rooted to induce callus. In April 2003, the rooted cuttings were removed from the callus bed. Then cuttings were planted in 3-gallon pots in Sunshine[®] #1 potting mix and placed under drip irrigation in a greenhouse. As the vines grew, new shoots were trained to bamboo poles. Vines were routinely fertilized using Peters[®] 20-20-20 according to product recommendations. Insecticide was sprayed in the greenhouse every two weeks. At the first sign of foliar fungal diseases (sooty mold caused by *Capnodium* spp. and powdery mildew caused by *Uncinula necator*), the fungicide Nova[®] was sprayed subsequently according to product recommendations. From planting until inoculations, vines were pruned on occasion to control growth.

Inoculations

Vines were inoculated in August 6, 2003 with two different isolates of *X. fastidiosa* from symptomatic grapevines in a vineyard near US 290, 10 miles west of Fredericksburg, TX. Petioles from symptomatic vines were surface sterilized and squeezed with forceps to force sap out onto solid Periwinkle (PW) media (14,39). After colonies were visible on media, they were transferred to new PW plates and tested to confirm identity. Real-time polymerase chain reaction (PCR) (75), enzyme-linked immunosorbent assay (ELISA) (55,69), Gram-stain and microscopic visualization (82) were used to verify that the isolates were *X. fastidiosa*. Primers and the probe used in the real-time PCR reaction were derived from the 16S-23S internal transcribed spacer (ITS) region as described in Schaad et al. (75). After isolates were verified and transferred twice onto solid PW, each isolate was aseptically suspended in phosphate buffered saline (PBS) and optical density (OD) readings were taken at A_{600} . Hopkins (39) reports an OD of 0.20 for a suspension of 10^8 cfu per milliliter. Bacterial suspensions were prepared with a slightly higher OD than 0.20 to ensure that an adequate amount of live bacterial cells were present for successful inoculations. The OD's of the two inoculum suspensions at A_{600} were: Isolate 1 = 0.35, Isolate 2 = 0.375. The solutions were dilution plated at concentrations of 10^{-1} - 10^{-5} on solid PW media and later tested with ELISA and real-time PCR to verify isolate identity and concentration. The concentrations according to colony counts on plates were 10^7 cfu/ml.

Table 3.1. Description of nine treatments in greenhouse experiment studying movement of *X. fastidiosa* in ‘Cynthiana’, ‘Cabernet Sauvignon’, and ‘Chardonnay’.

Treatment	No. Plants	Cultivar/ Isolate
T1	35 plants	‘Cynthiana’, inoculated with isolate 1
T2	35 plants	‘Cynthiana’, inoculated with isolate 2
T3	20 plants	‘Cynthiana’, inoculated with PBS, neg. control
T4	35 plants	Cab. sauv., inoculated with isolate 1
T5	35 plants	Cab. sauv., inoculated with isolate 2
T6	20 plants	Cab. sauv., inoculated with PBS, neg. control
T7	35 plants	‘Chardonnay’, inoculated with isolate 1
T8	35 plants	‘Chardonnay’, inoculated with isolate 2
T9	20 plants	‘Chardonnay’, inoculated with PBS, neg. control

T1	T2	T5	T2	T2	T9	T7	T2	T3	T9
T7	T7	T8	T6	T8	T3	T8	T2	T9	T5
T7	T3	T4	T7	T1	T8	T5	T2	T4	T2
T5	T6	T8	T3	T7	T7	T4	T9	T9	T6
T5	T4	T4	T4	T5	T1	T5	T6	T7	T8
T2	T6	T2	T5	T4	T6	T8	T2	T5	T8
T8	T1	T9	T2	T8	T2	T2	T2	T2	T1
T1	T1	T1	T1	T5	T4	T1	T8	T8	T1
T2	T9	T6	T1	T4	T8	T6	T1	T3	T3
T3	T1	T2	T5	T4	T7	T5	T9	T7	T6
T7	T2	T4	T2	T2	T6	T4	T8	T4	T7
T4	T8	T5	T7	T4	T2	T7	T5	T7	T4
T6	T8	T1	T1	T3	T1	T7	T7	T1	T6
T2	T3	T8	T3	T7	T9	T1	T8	T1	T7
T8	T1	T2	T4	T7	T1	T7	T7	T4	T7
T7	T9	T9	T8	T2	T8	T3	T3	T8	T5
T4	T4	T6	T3	T9	T2	T5	T4	T1	T9
T2	T5	T5	T7	T5	T4	T4	T3	T2	T7
T7	T1	T9	T8	T7	T1	T6	T8	T2	T4
T8	T5	T5	T2	T5	T4	T5	T7	T1	T5
T8	T5	T9	T7	T3	T9	T9	T8	T2	T1
T8	T5	T1	T6	T3	T5	T3	T4	T8	T4
T9	T2	T3	T8	T2	T6	T1	T2	T8	T3
T6	T7	T7	T4	T6	T8	T5	T1	T6	T5
T3	T9	T7	T5	T7	T1	T4	T9	T2	T4
T4	T5	T7	T4	T5	T5	T1	T4	T5	T5
T4	T4	T1	T8	T8	T1	T1	T6	T2	T8

Fig. 3.1. Randomized design of treated vines in greenhouse. Descriptions of treatments (T1-T9) are found in Table 1.

The randomized design was based on 5 rows of 54 grapevines each for a total of 270 plants (Fig. 3.1). Each row contained seven plants from treatments 1, 2, 4, 5, 7 and 8 and four plants from treatments 3, 6, and 9, which were the negative controls (Table 3.1).

The grapevines were inoculated on August 6, 2003 as follows. To mark the inoculation point a piece of masking tape was wrapped around a single cane near the base where it emerged from the trunk. To inoculate the plant, a razor blade was used to cut a slit parallel to the stem axis through the periderm and into the xylem of the plant. A syringe with 27 gauge needle was used to insert approximately 20 μ l of inoculum into the slit. This was repeated on the opposite side of the cane to ensure successful inoculation.

Assaying Vines

Four weeks after inoculation, two grapevines from treatments 1, 2, 4, 5, 7, and 8 and one vine from treatments 3, 6, and 9 were randomly picked and assayed for presence of *X. fastidiosa*. The canes which had been previously inoculated were removed from the vines. A 2.54 cm piece of the cane was removed at the inoculation point. A 2.54 cm piece 15.24 cm distal from the inoculation point and then 2.54 cm pieces every subsequent 7.62 cm were removed. Each piece was cut in half, and each half was sliced into 2 mm sections using a razor blade. Then each chopped cane piece was placed into 1 ml of sterile succinate-citrate-phosphate modified with ascorbate and 5% polyvinylpyrrolidone (SCPAP) buffer (52) for real-time PCR or ELISA extraction buffer (Agdia, Inc., Elkhart, IN). Tubes were stored at 4°C for 48 hours until assayed. Sample

tubes were vortexed and then assayed via ELISA and real-time PCR. For real-time PCR, Omnimix HS (a general PCR reaction mix) and reaction tubes were used (Cepheid, Sunnyvale, CA). Primers were ordered from Gene Technologies Laboratory (Texas A&M University, College Station, Texas). Fluorescent probe was obtained (Synthegen, Houston, Texas). Using suspension from the chopped cane tissue in buffer, ELISA was performed according to product instructions and real-time PCR was performed as previously described (75).

After the ELISA reaction was completed, a SPECTRAFluor[®] and computer software package Magellan[®] (Tecan, Maennedorf, Switzerland) were used to determine absorbance levels in each well and results were recorded. Real-time PCR Ct values were recorded from Smartcycler[®] software (Cepheid, Sunnyvale, CA).

Greenhouse conditions reflected ambient temperatures. In the winter, the temperature was set slightly above the freezing point to prevent pipes breaking. Symptom development of the grapevines was recorded on each sampling date. On April 5, 2004 the plants were evaluated based on level of foliation (Fig. 3). The vines were rated based on the number of leaves present: 0 = 20 or more, 1 = between 11 and 20, 2 = fewer than 10 and 3 = dead. Minitab statistical software (Minitab Inc., State College, PA) was used to evaluate these vine vigor ratings. Friedman test (11), for a randomized block design and nonparametric data, was used to evaluate the treatment effect, $\alpha = 0.05$.

Results

The distance *X. fastidiosa* was found from the inoculation point was averaged for the two vines tested for a given treatment on each sampling date. Bacteria apparently survived and flourished in all inoculated grapevine cultivars. There were no significant differences in the distance the bacterium colonized from the inoculation point to the tip of the shoot among the cultivars ‘Cynthiana’, ‘Cabernet Sauvignon’, and ‘Chardonnay’ (Fig. 3.2). No bacteria were found in the control vines during the course of the experiment, so those data are not included. *X. fastidiosa* was detected furthest from the inoculation point in grapevine treatments inoculated with Isolate 1 25-weeks post-inoculation, with the exception of ‘Cabernet Sauvignon’. For grapevine treatments inoculated with Isolate 2, the bacterium was detected furthest from the inoculation point between 17 and 19 weeks post-inoculation.

Marginal leaf-scorch was seen on both ‘Chardonnay’ and ‘Cabernet Sauvignon’ grapevines 9 to 11 weeks post-inoculations. No PD symptoms were ever observed on ‘Cynthiana’ vines. The vines came out of dormancy in March 2004 when leaves started emerging. Variation in foliation levels was observed so vine vigor ratings were developed based on the number of leaves emerging from the grapevines in April 2004, mean ratings shown in Fig. 3.3. All negative control treatments, shown in green, had lower mean disease ratings than inoculated treatments. According to the Friedman’s test, there was a significant treatment effect between the 3 inoculated cultivars, $p\text{-value} < 0.001$. The mean disease ratings for the two inoculated ‘Chardonnay’ treatments were higher than mean disease ratings for the other two inoculated cultivars, or vine vigor was

more reduced in ‘Chardonnay’ than ‘Cynthiana’ and ‘Cabernet Sauvignon’. Mean disease ratings for inoculated ‘Cabernet Sauvignon’ treatments were similar to mean ratings for inoculated ‘Cynthiana’ treatments.

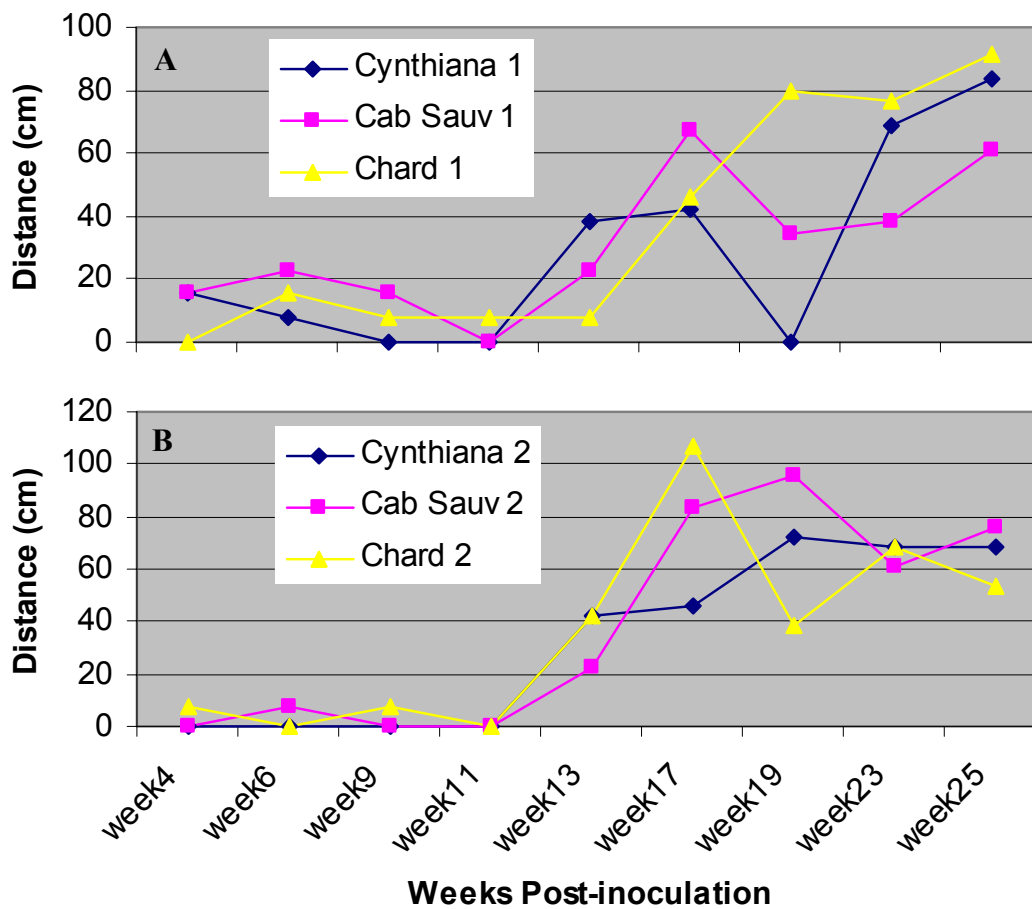


Fig. 3.2. Average distance *Xylella fastidiosa* was found with real-time PCR from the point of inoculation to the tip of the shoot. Graph shows the average distance the bacterium was found for two grapevines of each treatment. **A**, Data from three cultivars inoculated with Isolate 1, and **B**, data from the cultivars inoculated with Isolate 2. Cultivars include ‘Cynthiana’ (*Vitis aestivalis*), ‘Cabernet Sauvignon’ (*V. vinifera*), and ‘Chardonnay’ (*V. vinifera*).

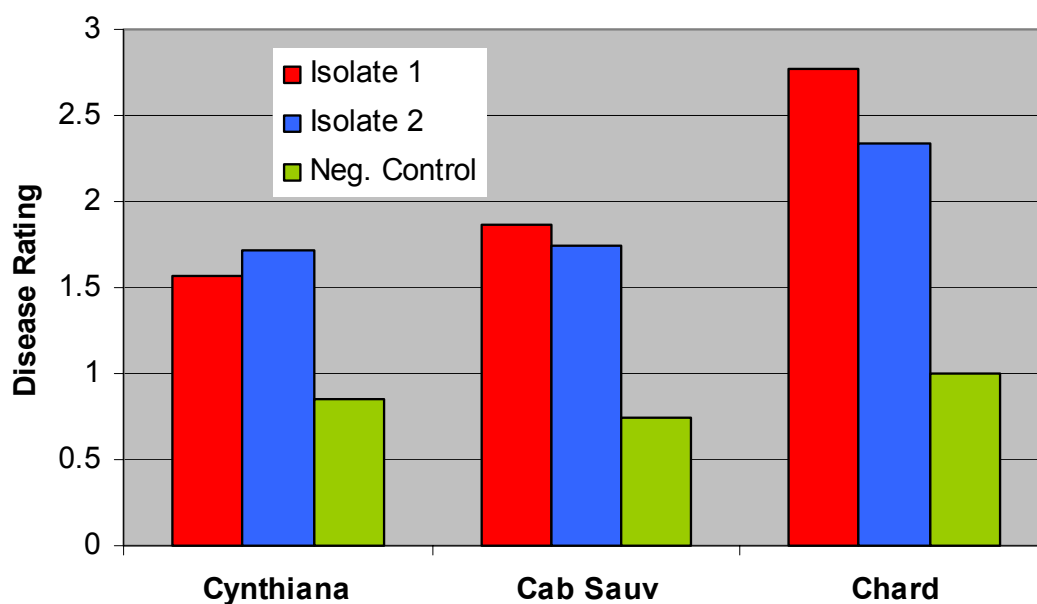


Fig. 3.3. Mean disease ratings for grapevines of three cultivars coming out of dormancy in April 2004. Ratings were based on foliation of the grapevine. 0 = more than 20 leaves emerging, 1 = 11-20 leaves, 2 = 10 or fewer leaves, and 3 = no leaves emerging (dead plant). Grapevines were inoculated with a 10^7 cfu/ml suspension of *Xylella fastidiosa* in August 2003. Green bars indicate vines that were inoculated with phosphate-buffered saline (PBS) only. According to the Friedman test for nonparametric data, there was a treatment effect, p -value < 0.001 .

Discussion

The nature of resistance, tolerance, and susceptibility to PD is a concept that is still poorly defined. Symptom development has been used to describe varying rates of susceptibility in the field (23,24). In this study, symptom development between ‘Cabernet Sauvignon’ and ‘Chardonnay’ was not different. Both cultivars started to develop marginal leaf-burn around the same time, about 9 weeks post-inoculation. But ‘Cynthiana’ never developed any putative PD symptoms. Winkler (83) reported that a

symptom of PD in the spring is delayed foliation. Fig. 3.3 illustrates differences in the level of foliation among various grapevine treatments. The figure indicates that there were differences among negative control grapevine treatments and inoculated grapevine treatments in their mean foliation ratings. Therefore, the infection of ‘Cynthiana’, ‘Cabernet Sauvignon’, and ‘Chardonnay’ by *X. fastidiosa* affected the vigor, or foliation level, of these varieties. The mean disease rating of inoculated ‘Chardonnay’ grapevines was significantly higher than all other treatments, which reflects field observations that ‘Chardonnay’ is highly susceptible to PD (31).

It appears that ‘Cynthiana’, ‘Cabernet Sauvignon’, and ‘Chardonnay’ provide a conducive environment for colonization by *X. fastidiosa*. The rate of movement of the bacterium within the three cultivars was not significantly different. But it may be that ‘Cynthiana’ is the best host of the three cultivars in question, since it provides a conducive environment yet has a high survival rate the following season. ‘Chardonnay’ would be the poorest host for *X. fastidiosa* since it can not survive for very long after infection. So the question to be addressed is what is it about ‘Cynthiana’ that causes it to withstand infection? ‘Cynthiana’, also known as ‘Norton’, is a cultivar native to the United States. Cultivars Norton and ‘Cynthiana’ have nearly identical vegetative features and similar fruit characteristics, although some reports have argued that the two are distinctly different (32,45). Norton was found in 1835 near Richmond, Virginia and introduced by Dr. D.N. Norton (33,54). Parentage of Norton is unknown but is thought to be a natural hybrid of *Vitis aestivalis* and *V. labrusca* L. (25). It has been reported that ‘Cynthiana’ was found in the wild in Arkansas (32,45). This cultivar is known as a

vigorous vine that is relatively cold hardy, able to endure drought and hot weather, and highly resistant to fungal diseases and phylloxera (33,78). *V. aestivalis* grows wild from New England down to Florida and as far west as the Mississippi River (33). It's mechanism of survival with respect to PD may be a matter of co-evolution of the pathogen and the host. In this case, 'Cynthiana' would be tolerant to PD, consisting of different resistance mechanisms not found in susceptible cultivars. The European varieties, in this case 'Cabernet Sauvignon' and 'Chardonnay', were not exposed to the bacterium prior to their arrival in North America. Thus, these varieties could not have built up resistance factors to *X. fastidiosa* as the native cultivars probably have.

Although 'Cynthiana' tolerates PD and has a higher rate of survival, this does not necessarily mean it is wise to plant this cultivar. If growers want to mix popular European varieties with tolerant native varieties in vineyards, they should know they are still running a risk of aiding PD spread by planting vines that can harbor *X. fastidiosa* without showing symptoms. The question remains whether a resistant variety actually exists. With the vast number of hosts for *X. fastidiosa* already identified and the evidence presented here that 'Cynthiana' is a suitable host, it may be that the bacterium can infect and multiply within all grapevine cultivars. Thus all cultivars are potential carriers of *X. fastidiosa* and facilitators of PD epidemics. In other words, there is no evidence to show that any grape cultivar can completely exclude the pathogen or overcome it.

CHAPTER IV

EPIDEMIOLOGY OF PIERCE'S DISEASE IN A TEXAS VINEYARD

Introduction

Spatial pattern may be described as the arrangement of diseased plants relative to each other and to the architecture of the host crop (7,29). The ecology and patterns of spread of *X. fastidiosa* can be studied by observing spatial patterns of PD symptoms in a vineyard. Mapping the incidence of symptoms and monitoring spread over time can lead to key insights into the establishment and subsequent spread of PD (35,59).

Madden et al. (50) proposed 'ordinary runs' as a tool for determining whether infected plants in rows are aggregated or randomly dispersed. A run may exist as an ordered sequence of some two categories, such as diseased or healthy plants in a row of some crop. A run is then defined as a succession of one or more identical categories, which are followed and preceded by a different symbol or no symbol at all (28). In the present case, a run is represented by one or a series of diseased grapevines or healthy grapevines in a trellis. If several adjacent vines in a row showed PD symptoms and several adjacent vines in a row were healthy, the diseased grapevines and healthy grapevines would be considered aggregated. Thus, there would be few runs. If grapevines were infected by external sources with no movement of the pathogen between adjacent vines, one might observe a random mixing of healthy and infected plants and a correspondingly large number of runs. The null hypothesis evaluated in this test of ordinary runs is that the ordered sequence of symptomatic plants is random. The

alternative hypothesis is that the ordered sequence of symptomatic plants is aggregated (50).

Another type of analysis that is beneficial in comparative epidemiology is disease progress evaluated over time. The purpose of this type of analysis is to reveal similarities and differences among epidemics based on disease progress curves. Besides comparing disease progress curves, quantitative comparisons can be made between epidemics using area under the disease progress curve (AUDPC) (6). Because data for only one vineyard are presented in this report, comparisons to other epidemics were limited to previous literature on PD epidemiology (59,83). The information found for the single vineyard in this study will be useful in the future when disease progress data have been monitored and presented for epidemics in other vineyards.

Materials and Methods

A section of a vineyard in the Texas Hill country was chosen because it was known to have PD and also because of it was recently planted in February 2000. The PD epidemic was increasing in this section of the vineyard when we started making observations. The vineyard is located between Fredericksburg and Stonewall, Texas on Highway 290. The only cultivar planted in this section is Viognier, *Vitis vinifera*, a European white wine grape variety. The section consists of 50 rows of 54 vines, with 1.22 m between vines and 12.80 m between posts. The vineyard was surveyed in July 2003 when symptoms were starting to show and then in October 2003 when symptoms were more advanced. A survey was conducted in May 2004, prior to the seasonal

development of symptoms of marginal leaf scorch and leaf drop with petiole retention (38). Vines were rated in May 2004 based only on health and mortality.

The survey consisted of walking along the rows and rating each vine. In July and October 2003 ratings were based on symptoms of PD and a numeric value was given to describe the stage of disease: 1 = healthy, 2 = incipient symptoms (marginal leaf scorch), 3 = advanced symptoms (marginal leaf scorch, leaf drop with petiole retention, uneven periderm development), 4 = dead, 5 = removed, 6 = radically pruned. In May 2004, only four ratings were observed: 1 = healthy, 2 = dieback, 3 = dead, 4 = removed. Survey results were recorded in ArcMap (ESRI, Redlands, CA), a GIS software tool that helps to visualize geospatial data. Then ordinary runs analysis was conducted to determine whether diseased vines were aggregated or randomly dispersed within and across rows.

The formula for ordinary runs to determine the expected number (E) of runs (U) on a given row is as follows:

$$E(U) = 1 + 2m(N-m)/N$$

where m = number of infected plants in a row, and N = total number of plants in row. If there is aggregation, the observed number of runs will be less than the expected $E(U)$.

The standard deviation of the number of runs s_u is found by:

$$s_u = (2m(N - m)[2m(N - m) - N]/[N^2(N - 1)])^{1/2},$$

and the standardized number of runs Z_u is given by:

$$Z_u = [U + 0.5 - E(U)]/s_u.$$

A row of vines is considered to have aggregation of symptomatic and healthy plants if the value of the standardized number of runs is a large negative number, if $(-Z_u)$ is greater than 1.64 ($P = 0.05$) (50).

For the May 2004 survey, we were interested in disease progress since the first survey in July 2003. May data was entered into ArcMap, and then numbers of vines that had regressed or improved in health were analyzed. A simple analysis of the mortality rate of vines over time in this vineyard was done. The increase or decrease in amount of disease incidence in a plant population over time is defined by the rate of change with time. Here we used $y =$ disease incidence, and describe the epidemic in terms of dy/dt , where $t =$ time. This formula, dy/dt , represents the absolute rate of change for PD incidence (6).

After the survey data was entered into ArcMap, it was observed that there were fewer symptomatic vines at one end of the vineyard. This could be an indication of the possible location of inoculum reservoirs outside of the vineyard. Thus, numbers of symptomatic vines per row were graphed to illustrate spatial trends of disease development.

Results

The results for ordinary runs analysis for the July and October surveys are shown (Table 4.1). In July 2003, 9 out of 50 rows showed significant aggregation of symptomatic vines within rows and 1 out of 54 showed aggregation across rows. In October 2003, 45 out of 50 rows showed significant aggregation within the rows and 11

out of 54 showed aggregation across rows. Aggregation was more likely within rows than across rows.

Table 4.1. Ordinary runs analysis for Pierce's disease symptomatic grapevines in a vineyard in the Texas Hill Country near Fredericksburg.

Survey Date	Direction ^a	
	Within Rows	Across Rows
July '03	9/50	1/54
October '03	45/50	11/54
May '04 ^b		

^a Values correspond to the number of rows with significant aggregation ($-Z_U \geq 1.64$) relative to the total number of rows tested.

^b Data was collected in May based on the health of vines, no putative PD symptoms were observed. Vines were either healthy, had dieback, were dead, or had been removed.

Table 4.2. State of the vines considered healthy in July in the Viognier block of a Texas vineyard, according to assessments taken in October 2003 and May 2004. In July 2003, 2146 vines were considered healthy. The numbers as well as the proportions considered healthy, symptomatic, dead, or removed in October and May are shown.

Vine Ratings	Assessment Date			
	October '03		May '04	
Healthy	1264 ^a	0.58 ^b	1670 ^a	0.78 ^b
Foliar symptoms	714	0.33	-	-
Dieback	128	0.06	282	0.13
Dead	40	0.02	180	0.08
Removed	0	0	14	0.007

^a Actual number of vines.

^b Proportion of vines out of 2146 which were healthy in July '03.

Next, disease progress over time was analyzed for the three survey dates; vines rated as healthy in July 2003 changed in October 2003 and May 2004 (Table 4.2). Thirty-three percent of vines that were healthy in July had marginal leaf scorch in October. But 78% of vines that were healthy in July were healthy in May. The other 22% of vines healthy in July had dieback, were dead, or had been removed in May. Foliar symptoms do not show until June or July in Texas, so no foliar symptoms of PD were seen in May.

Perhaps a more dramatic interpretation is numbers of dead vines at each survey date is illustrated (Fig. 4.1). Only 43 vines were dead in July, but in May ten times as many vines were dead. At this rate, this vineyard could be decimated in less than a year.

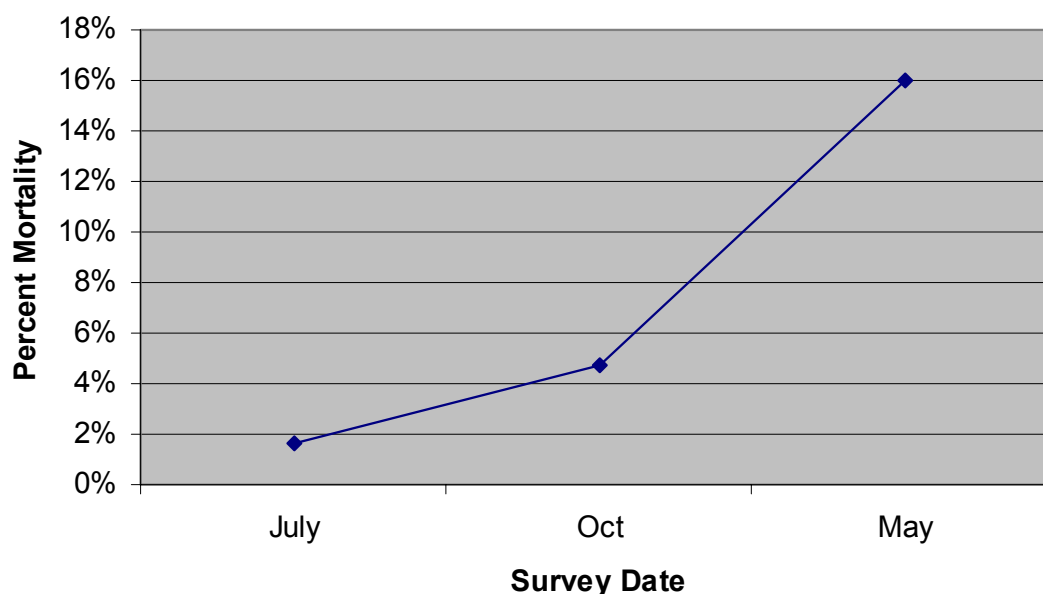


Fig. 4.1. Mortality rate of vines in the Viognier block of a Texas vineyard from July 2003 to May 2004. The block originally contained 2700 grapevines. In July, 43 were dead, in October 128 were dead, and in May 431 grapevines were dead.

Disease incidence on each survey date is illustrated (Fig. 4.2). The vines were planted in February 2000, therefore disease incidence is reported as 0 on this date. Survey data was not collected until July 2003, so information on symptom development between 2000 and 2003 was not available. In July 2003, 19% of the vines in this vineyard block were showing PD symptoms. In October, 48% of the vines showed symptoms, and 22% showed symptoms in May 2004. There are too few points to define a incidence curve with any suitable degree of confidence (56).

PD symptoms decreased with distance from the east end to the west end of the vineyard block and also decreased from the north side to the south side of the block (Figs. 4.3A and 4.3B). On average fewer than 10 vines per row were symptomatic in rows 41 through 50, the west end of the plot, and on average about 15 vines per row were showing symptoms on the east end of the block (Fig. 4.3A). Also, the north end of the block differs from the south end in numbers of symptomatic vines. The average number of symptomatic vines is about 13 on the north end and about 6 on the south end (Fig. 4.3B). Maps of this vineyard block and vines were color coded based on the disease ratings on each survey date (Figs. 4.4A – 4.4C). These figures illustrate where PD infections have actually occurred in this vineyard.

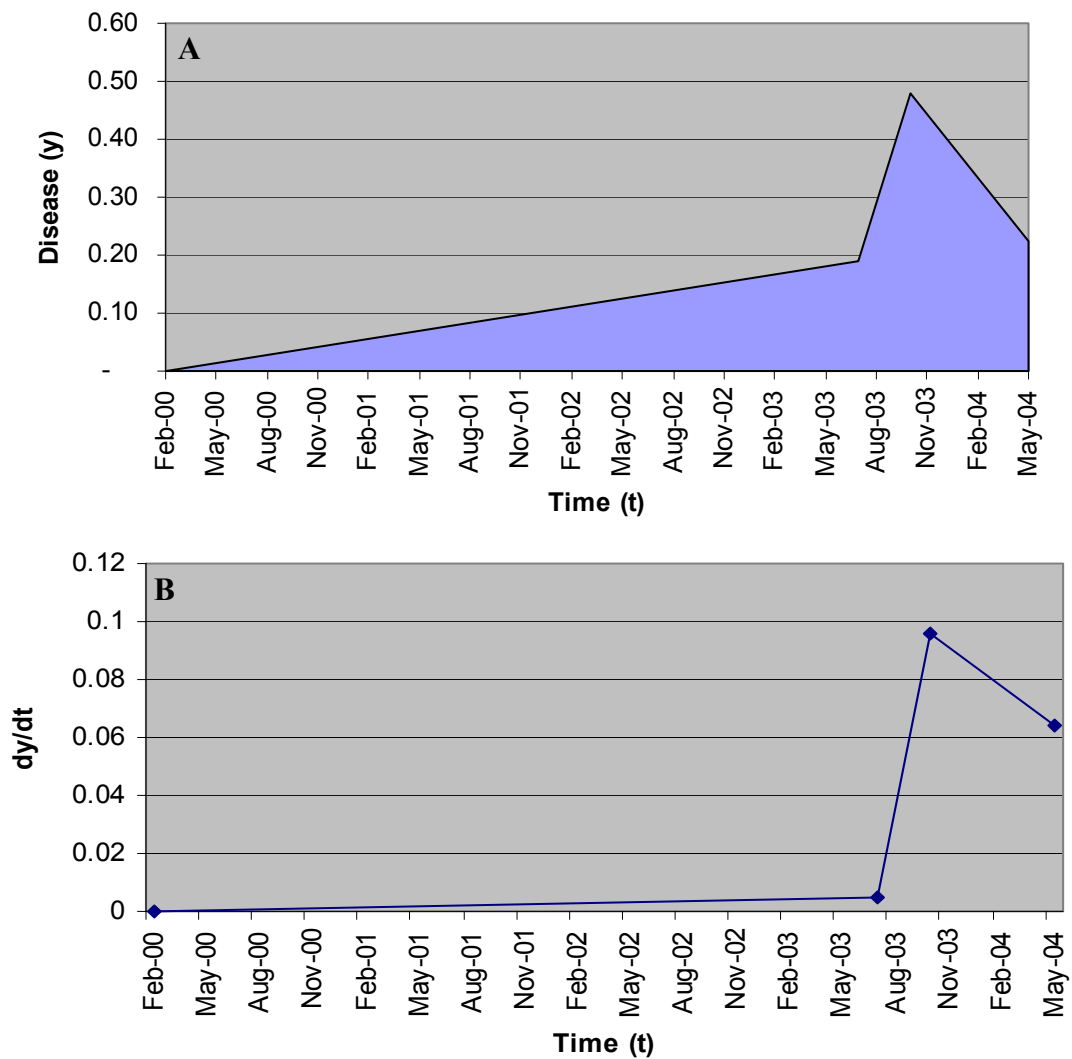


Fig. 4.2. Pierce's disease incidence in the Viognier block of a Texas vineyard on four different dates. The vines were planted in February 2000, so disease incidence is reported as 0.00 for this date. **A**, Disease incidence was 0.19 in July 2003, 0.48 in October, and 0.22 in May 2004. **B**, The absolute rate of change of Pierce's disease incidence versus time.

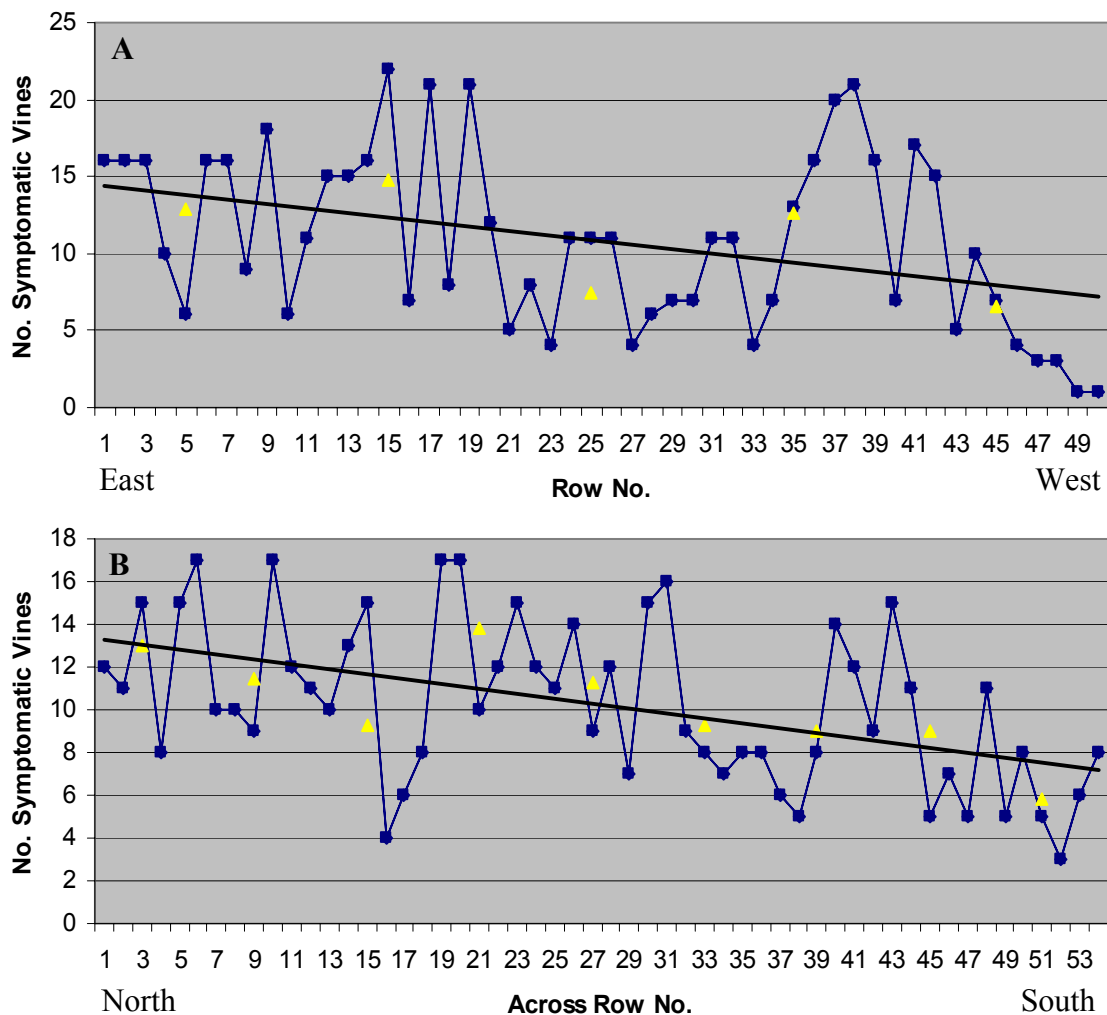


Fig. 4.3. Numbers of symptomatic vines in each row in the Viognier block of a Texas vineyard in July 2003. Yellow triangles refer to the average number of symptomatic vines per row. The black line is the best fit linear trend line for the averages. The number of symptomatic grapevines decreases on the west side of the vineyard block. **A**, There were a total of 54 vines per row. Row 1 was on the east side of this block and row 50 was on the west side. **B**, There was a total of 50 vines per across row. Row 1 was on the north side of this block and row 54 was on the south side.

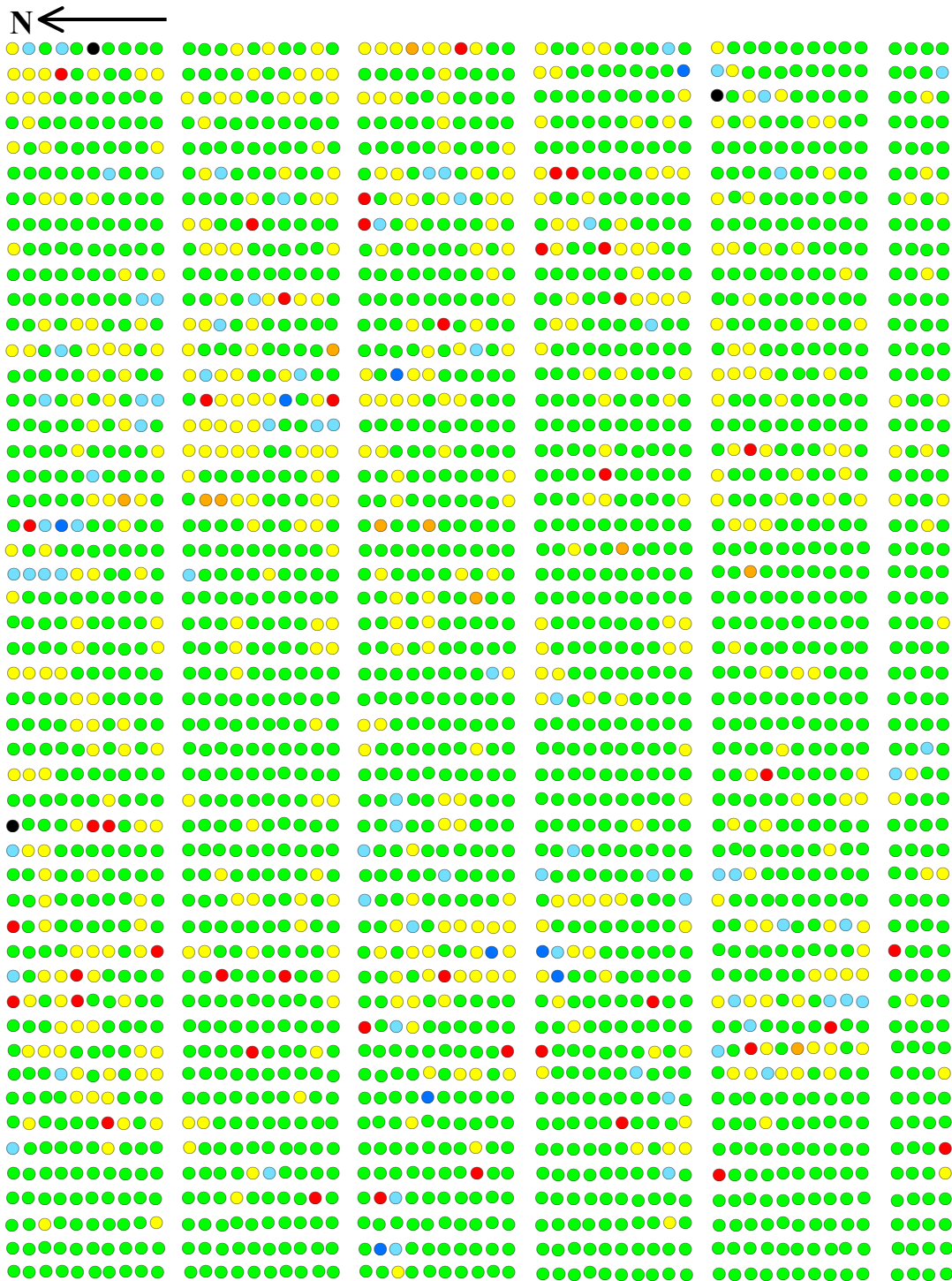


Fig. 4.4A. Map of the Viognier block of a Texas vineyard showing disease ratings of 2700 grapevines observed in July 2003. Green = healthy, Yellow = incipient symptoms, Orange = advanced symptoms, Red = dead, Black = removed, Blue = radically pruned.

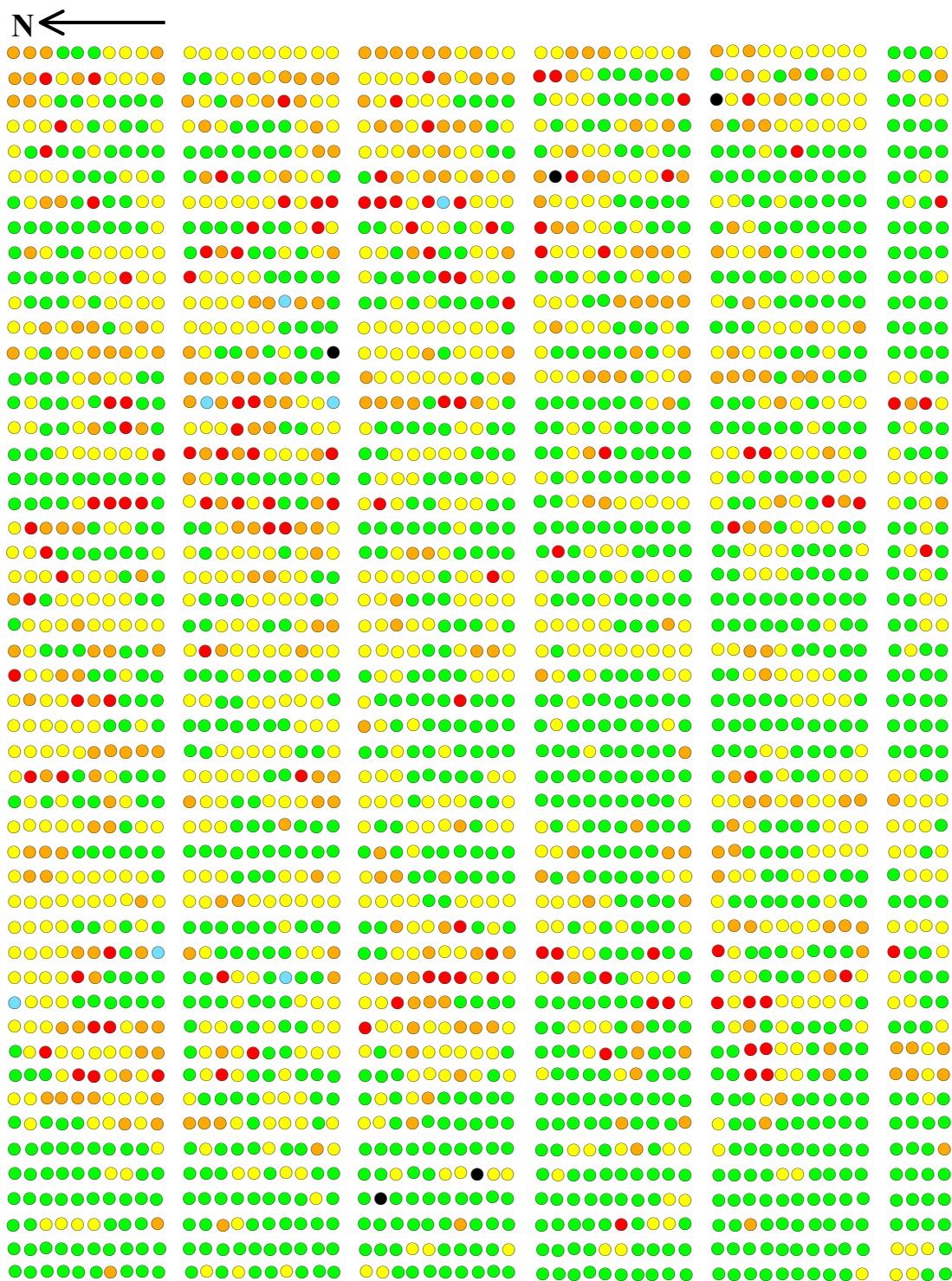


Fig. 4.4B. Map of the Viognier block of a Texas vineyard showing disease ratings of 2700 grapevines observed in October 2003. Green = healthy, Yellow = foliar symptoms, Orange = dieback, Red = dead, Black = removed, Blue = radically pruned.

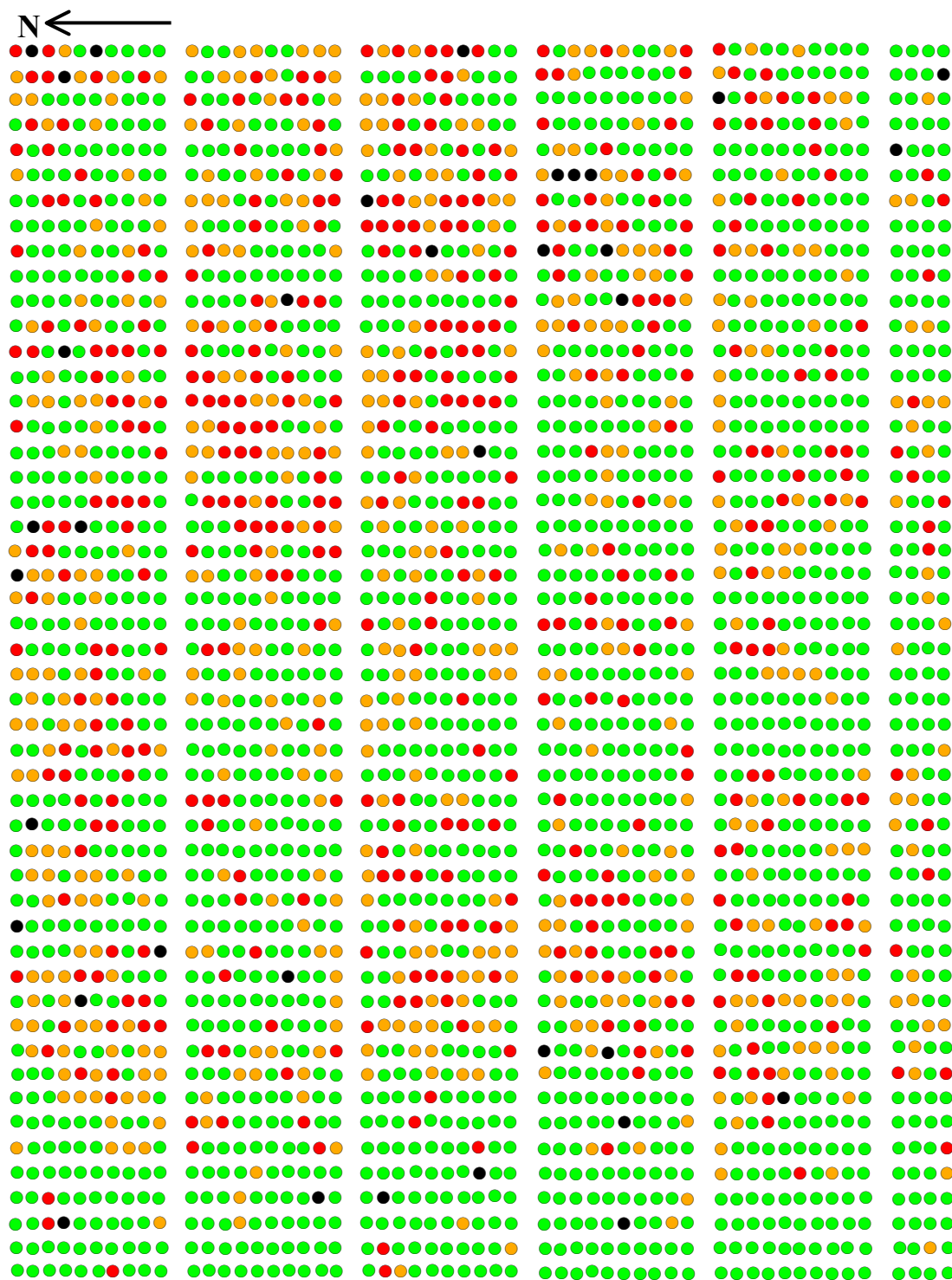


Fig. 4.4C. Map of the Viognier block of a Texas vineyard showing disease ratings of 2700 grapevines observed in May 2004. Green = healthy, Orange = dieback, Red = dead, Black = removed.

Discussion

Vanderplank (80) compared rate of disease spread to interest accrued on money. When a principle amount of money is invested, the interest accrued on that principle is directly related to the original amount. In the same way, the rate of increase or decrease in disease incidence can be related to the initial amount of inoculum present in a field of diseased plants. PD of grapevine has previously been described as a simple-interest type disease (6,7,80) suggesting that newly infected grapevines do not serve as inoculum sources for direct infection of adjacent healthy vine. This has also been called a monocyclic type of disease with only one cycle of infective inoculum produced per growing season (6). Actual data for PD epidemics in Texas have not been previously reported to determine whether PD is a monocyclic or polycyclic disease. A polycyclic disease is one in which the pathogen multiplies through several generations during a season in the course of the epidemic, similar to compound interest accrued on money (80). Once a plant becomes infected with a pathogen, it becomes an inoculum source for further spread.

An explanation for the severity of PD in the Gulf Coastal Plains states is that it is a compound interest or polycyclic type of disease, and vine-to-vine spread is occurring readily (1,42). Infection later in the season could involve supplemental hosts as well as infected grapevines, leading to exponential spread of PD. The Gulf Coast climate differs dramatically from California in rainfall and more importantly for PD, Gulf Coast states have warmer nights and a longer growing season (42).

A 1949 report on PD epidemics in southern California says that in the beginning diseased vines were irregularly scattered, then diseased vines were more aggregated, and finally large numbers of diseased vines were seen on the edges of vineyards adjacent to alfalfa fields and pastures (59,83). Spatial distribution and disease progress data were not recorded for the first few years after the vineyard we studied was planted. We think that initial infections occurred in an irregular and perhaps random fashion in the northwest portion of this vineyard block. But 3 years later in the epidemic, it appears vine-to-vine spread is occurring in the vineyard. Clustering of diseased vines in this vineyard suggests infected vines contribute to spreading *X. fastidiosa* to adjacent vines in a row at greater rates than across rows (Table 4.1). Also, disease progress (Table 4.2, Figs. 4.1,4.2) indicates the rate of disease spread increased. This could mean that previously infected vines were serving as inoculum sources for further spread, which is typical of a polycyclic disease (6).

Clustering of diseased vines as well as polycyclic disease progress suggests several things about an epidemic. First, the epidemic is in the advanced stages and control of PD is unlikely at this point. Second, infected vines are probably serving as inoculum sources. Vines adjacent to infected vines have a higher probability of contracting PD (7). Also, an advanced PD epidemic in this area can occur in less than 3 years. Therefore, the first few years after planting a vineyard in this area are critical. Exclusion of the pathogen is a crucial factor in PD control. So growers should administer insecticide to prevent sharpshooter infestations, remove potential inoculum

reservoirs from the perimeter and within the vineyard, and remove diseased vines as soon as PD is confirmed (42,46).

The spatial distribution of infected vines may indicate where the primary inoculum reservoir is outside of this vineyard. Previous data from California showed steep decreases in PD symptoms with distance from sharpshooter and inoculum source areas (59). Purcell (59) observed spatial patterns of PD that were associated with distance from natural riparian vegetation. The vineyard block in the current study is bordered on each side by various cultivated or riparian vegetation. On the northern perimeter of the block is an older vineyard block, planted in Sauvignon Blanc. On the east side is a cultivated field, usually planted in oats or rotated with watermelon. This field is adjacent to riparian vegetation, including many tree species, weeds, and grasses growing along a small seasonal creek. On the south side are a driveway and a field usually plowed or planted in wheat, corn, or sorghum. Another vineyard block and a weedy pasture are on the west side, and it is planted in Syrah and Petite Verdot.

The numbers of infected vines per row changes from one side of the vineyard to the other (Fig. 4.3). On average more vines were infected on the east side than on the west side (Fig. 4.3A). Rows on the north side had more infected vines than rows on the south side (Fig. 4.3B). The northeast side of this vineyard block could be where sharpshooters with *X. fastidiosa* are more often bringing it into the vineyard. The field and riparian vegetation on the east side could be a source area. If the primary source area is the vineyard on the north side, this gives more evidence that vine-to-vine spread plays a significant role in PD epidemics in this environment.

In comparing this epidemic to data taken in California prior to the introduction of the glassy-winged sharpshooter, one major point is apparent: the rate of disease development in Texas is much more rapid than that seen in California. Although, it could be that Vignier, the only cultivar planted in this block, is highly susceptible to PD, causing rapid disease development. Purcell (59) reported that secondary spread of PD is probably not substantial, based on data collected in California in the early 1970s. And in an experiment conducted in California from 1941 to 1946, it was observed that diseased vines were not the important source of pathogen spread (83). Experiments were also performed to test the effects roguing had on disease dispersal, and it was shown that roguing had no effect on the spread of PD (83). Data presented in the current study gives evidence for earlier theories that PD in Texas is a polycyclic type of disease, vine-to-vine spread is a significant part of the epidemic (1,42).

CHAPTER V

SUMMARY AND CONCLUSIONS

Pierce's disease is a huge problem for Texas grape growers, as illustrated by the vineyard surveys presented in Chapter IV. PD in a vineyard can mean great loss for growers. Management strategies include planting resistant cultivars (which usually have little market value), exclusion of the pathogen by controlling the vector with insecticide, and removal of diseased grapevines and other potential hosts. All control measures require economic loss. Therefore it is important to accurately diagnose PD, to know which cultivars are resistant, and to understand patterns of pathogen dispersal in a vineyard.

One problem is that the growers don't know how to accurately diagnose their grapevines. Typically growers send in symptomatic plant tissue to a diagnostic lab and pay a fee. Diagnostic labs may be limited by the availability of time and resources for accurately diagnosing a plant disease like PD. Should a grower be given results based on real-time PCR or ELISA, he/she should understand the degree of certainty that comes with the result. Results of evaluating the two diagnostic methods show that real-time PCR is more sensitive than ELISA for diagnosing PD. Of the known positive grapevines that were tested, 40% tested positive by ELISA and 65% tested positive by real-time PCR. But real-time PCR is more expensive and initial cost may prevent some researchers from employing this technique. At this point, ELISA is still the more accessible and cheaper tool for diagnosing PD. Isolating *X. fastidiosa* from plant tissue may be the most definitive method for detecting the bacterium, but this method is not

ideal for rapidly diagnosing PD because of slow growth and contamination problems. Using all three of the above mentioned methods would increase the level of certainty for diagnosing PD, but this would require more time and money.

Planting resistant cultivars could ensure that a vineyard is PD free. But many of the cultivars considered resistant are not economically desirable. Also, it could be that truly resistant cultivars don't exist. 'Cynthiana', a cultivar native to the United States, can tolerate infection by *X. fastidiosa* without showing PD symptoms. It is possible that native cultivars thought to be resistant, can also serve as hosts to the bacterium. Resistance factors still need to be determined in order to define resistance with respect to PD.

PD epidemics vary with respect to rate of disease spread, aggregation of diseased vines, and spatial distribution of diseased vines. The differences are due to vectors present in and around vineyards and environmental conditions. In Southern California, after the glassy-winged sharpshooter was introduced, epidemic rates increased and patterns of diseased vines changed from random to aggregated. Southern California also has mild winters and hot summers which appear to correlate with serious PD epidemics. Northern California vineyards still have a non-aggregated spatial pattern of diseased vines along the edge adjacent to water sources and riparian habitats. The Texas vineyard survey results indicate a spatial pattern similar to that now seen in Southern California. Diseased grapevines were aggregated down the rows and rate of disease spread was rapid. This polycyclic type of disease spread suggests a few things about managing PD in this area. First, there are probably many supplemental hosts of *X. fastidiosa* in and

around this vineyard. Growers should remove potential hosts and plant grapevines far from riparian areas. Second, once vines become infected, they will likely become inoculum sources for further spread to adjacent vines. Vineyard managers should promptly remove diseased vines to prevent further spread. Also, sharpshooters are likely spreading *X. fastidiosa* down the row more often than across the row. This movement pattern of the sharpshooters may suggest how growers should administer insecticides.

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