Propagation Success of Grapevines (*Vitis vinifera L.*) Infected with *Xylella fastidiosa*

by

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Abstract

Pierce's disease of grapes, caused by the xylem-limited bacterium *Xylella fastidiosa* Wells, is typically fatal to varieties of *Vitis vinifera* L. The objective of this study was to investigate rooting success of asexually propagated cuttings taken from *X. fastidiosa* infected grapevines and determine if rooted cuttings could survive and produce viable plants for vineyard establishment.

Cuttings were taken January 2008 from dormant *V. vinifera* cv. Merlot and cv. Cabernet Sauvignon grapevines located in the Hill Country and Gulf Coast regions of Central Texas. Prior to our research, symptoms of Pierce's disease were recorded on each grapevine in each vineyard using a Symptomatic Reliability Index.

At the conclusion of six weeks, cuttings were uprooted and evaluated. Rooting percentage, number of roots, root length, root rating, number of shoots, and shoot length, were recorded for each cutting.

Rooting data indicates symptomatic and asymptomatic *X. fastidiosa* infected grapevines have the ability to be propagated asexually through cuttings. To confirm the presence of *X. fastidiosa*, rooted cuttings were tested with Enzyme-Linked ImmunoSorbent Assay and Real-time Polymerase Chain Reaction. Results revealed several asymptomatic and symptomatic cuttings positive for *X. fastidiosa*. This experiment demonstrated grapevine cuttings infected with *X. fastidiosa* can be propagated to produce healthy looking nursery plants that could be sold as clean nursery stock.

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List of Abbreviations

CRD……Completely Randomized Design
cm……..Centimeters
ELISA……....Enzyme Linked ImmunoSorbent Assay
L……...Liters
LSD……..Least Significant Difference
mL……..milliliters
PCR……...Polymerase Chain Reaction
PD……...…Pierce's Disease
QRT……...Quantitative Real-Time
SRI……...…..Symptom Reliability Index
V. vinifera……*Vitis vinifera*
X. fastidiosa……*Xylella fastidiosa*

Chapter I

Introduction and Literature Review

Pierce's disease (PD) is a fatal disease of grapevine (*Vitis spp*. L.) caused by the bacterium *Xylella fastidiosa*. *Xylella fastidiosa* is a gram negative, small, single, rod-shaped, non-flagellated cell approximately 0.5 to 1.0 micrometers in width and 1.0 to 3.5 micrometers in length (Hoch and Burr 2008). *Xylella* moves with a 'twitching' movement typical of type IV pili (Hoch and Burr 2008). *Xylella fastidiosa* is nutritionally fastidious, making it extremely difficult to study its biology (Chen and Civerolo 2008).

Xylella fastidiosa bacterium affects grapevine physiology by limiting movement of water through xylem vessels (Almeida et al. 2005). As the disease progresses, basic plant functions such as transpiration, respiration, and photosynthesis become limited (Hopkins 1989; Purcell 2005). In advanced stages, as grapevines attempt to conserve water by closing stomata, grapevine growth is restricted and the plant dies (Hopkins 1976). Once infected, the length of time required for *X. fastidiosa* to kill plants is cultivar dependent, and varies from a few months to a few years (Goheen and Hopkins 1988).

Pierce's disease was first identified in Southern California in 1884 (Pierce 1892) and in Northern California in 1887 (Hewitt 1970). Pierce's disease was initially called "mysterious disease", "California vine disease", "vine plague", and "Anaheim disease" (Hewitt 1970; Winkler 1974). In 1892, Pierce described a severe grapevine disease that eliminated commercial viticulture within the Los Angeles Basin (Purcell

2005). Aside from the United States, PD has been identified in temperate regions of Central America (Raju et al. 1980), South America (Rossetti et al. 1990; (Li et al. 2003), Taiwan (Leu and Su 1993), and Europe (Berisha et al. 1998).

Symptoms of PD include leaf scorching, premature leaf drop, green islands (areas with green epidermis surrounded by brown periderm), leaf necrosis, and matchsticks (the abscission of leaf blade with the petiole remaining attached to the shoot) (Hewitt 1970). Because of other potential nutrient and water stress symptoms, disease diagnosis based on visual symptoms can be difficult (Hopkins 1989; Thorne et al. 2006). *Xylella fastidiosa* can be verified by DNA testing in vines expressing visual symptoms associated with PD. However, visual symptoms may not appear in infected plants (Wells et al. 1987; Thorne et al. 2006; Hopkins 1985).

Since first identified, PD has been devastating to vineyards. Greenhouse studies concluded *X. fastidiosa* could be transmitted by xylem-feeding insects such as the green sharpshooter (*Draeculacephala minerva*, Ball) (Freitag 1951). Subsequently, other xylem-feeding insect vectors including the blue-green sharpshooter (*Graphocephala atropunctata*, Signoret), and the spittlebug (*Aphrophora saratogensis*, Fitch) have been identified as PD vectors (Newman et al. 2004; Purcell 1974).

Early observational studies tracking movement of PD concluded vineyards in close proximity to riparian habitats (known to host native xylem-feeding vectors) were believed to be areas of high risk for PD (Purcell 1997). In the late 1980's, the introduction of the glassy-winged sharpshooter (*Homalodisca coagulata*, Say) into the

Western United States (transported from infected nursery stock from the Southeastern United States) changed pre-conceived notions about the spread of PD within the United States (Sorensen and Gill 1996). With a greater flying range when compared to other xylem-feeding vectors (Purcell and Saunders 1999), and its ability to travel to areas without water sources (Hopkins and Purcell 2002), the glassy-winged sharpshooter has expanded the range of PD in the United States.

Recent studies have shown PD may be spread by means other than insect vectors. Krell et al. (2007) demonstrated common horticulture tools (pruning shears, etc.) may assist in the spread of *X. fastidiosa* in *V. vinifera*. Krell et al. (2007) was able to successfully transmit *X. fastidiosa* into the xylem of *V. vinifera* cultivars by making direct contact between xylem sap and pruning shear blades. This experiment introduced the possibility of transmitting PD by mechanical methods. Because grapevines are pruned throughout the season to promote fruit quality, open the canopy for harvesting, and train vines for the next growing season (Winkler et al. 1974), transmission of *X. fastidiosa* by cutting equipment has important implications for PD epidemiology

Meyer et al. (2002) investigated the potential for *X. fastidiosa* to be transmitted in grapevines by grafting. Pierce's disease infected grape scions were grafted to healthy non-infected rootstocks. Results provided evidence diseased plant material could be successfully grafted to non-diseased plant material and produce a diseased plant.

High quality plant materials produced by commercial nurseries provide grape growers with plants that contribute to the success of the viticulture industry. To satisfy current demand, these nurseries perform propagation techniques including cuttings, layering, suckering, and grafting (Ohio State University Extension 2008; Buffington 1961; Normann 1991). Of these methods, cuttings and grafting are the most common propagation techniques (Ohio State University 2008).

Dormant propagation wood collected for cuttings is usually collected during winter months (Castagnoli and Miller 2003). When selecting plant material for propagation, there are many factors to consider: trueness of variety, general health of the source plant material, and freedom from disease and insects. Length of cutting will vary depending on variety and internode length (Benz et al. 2006; Guse and Larsen 2001; Jackson 2000). Selected cane cuttings should be between 30 to 46 cm in length, 0.5 to 1.5 cm in width, and have at least three or four nodes (Castagnoli and Miller 2003; Buffington 1961). When a potential cutting is taken from a grapevine, two cuts are made to form the cutting. To quickly identify the distal and proximal ends, the proximal end is cut five centimeters above the upper-most node and is made at a 45°angle. The distal cut is a flat cut perpendicular to the length of the cutting. The distal end of the cutting is placed beneath the soil or rooting media to callus and root (Castagnoli and Miller 2003; Jackson 2000). Once taken, cuttings are often placed into a cool trench about 0.5 meters deep and covered with soil until they are ready to be planted in the nursery (Goode et al. 1982).

To eliminate diseases or pests, fungicides are often applied, or cuttings may be treated in hot water baths (Haviland et al. 2005; Waite et al. 2001). Cuttings should be taken from disease-free nursery material (Jackson 2000; Williams-Woodward 2002), but because plants are not always tested for plant diseases, it is difficult to determine if all plant material sold is free from disease or insects. (Sanderlin and Melanson 2006).

Planting a vineyard is a long-term, expensive investment. Therefore, it is important planted vines are disease and pest-free. It is commonly assumed propagation from diseased or infected plants will result in infected plant material, and potentially diseased plants (Williams-Woodward 2002). In an effort to provide producers with the best available plant material, nurseries offer certified vines. However, because a vine is certified does not guarantee the vine is clean of disease or insect contaminants (Nelson-Kluk et al. 1990). Certified vines are sold as vines that have been propagated from vineyard blocks that were established with clean foundation plant materials. These plants seldom show detectable pathologies. Nurseries also produce and sell non-certified vines. Non-certified vines are not propagated from foundation stock and may not be tested for diseases (Castagnoli and Miller 2003).

The first objective of this study was to investigate rooting success of asexually propagated grape cuttings collected from symptomatic and asymptomatic vines from a vineyard under study for PD epidemiology. The second objective was to determine if rooted cuttings from diseased stock plants could survive and produce viable plants which could potentially be sold as nursery stock for vineyard establishment.

Chapter II

Materials and Methods

Two commercial vineyards located in central Texas were selected as sources for PD infected plant material. Central Texas is a region with a high risk for PD (Kamas et al. 2000; Buzombo et al. 2006). Vineyards were located in the Hill Country and the Gulf Coast grape growing regions of Texas (Figure 1). Both vineyards had previously been assessed for symptoms of PD (Dr. David Appel. 2007. personal communication).

As part of a study investigating the epidemiology of PD within a vineyard, individual vines from both vineyards were rated using a vine Symptomatic Reliability Index (SRI). This portion of the research was performed during the 2004-2007 growing seasons by Dr. David Appel from the Department of Plant Pathology and Microbiology at Texas A&M University. To monitor spread of PD within vineyards, SRI rating maps were created using ArcGIS (Version 9.1: Environmental Systems Research Institute SRI, Redlands, CA). Each vine in the vineyard was assigned a numeric symptom rating value between 1 and 7. Asymptomatic vines showing no symptoms were given a rating of 1. Symptomatic vines expressing significant PD symptoms including dieback were given a rating of 5. Vines missing from the vineyard initially listed on the SRI rating map were given a 6 and vines that were replaced were given a 7 (Table 1). One time each growing season (2004-2007) vines were evaluated for visual PD symptoms.

For the current study, two European *V. vinifera* cultivars, Merlot and Cabernet Sauvignon, were selected. Merlot is a highly vigorous cultivar well adapted to many soil types (Bauerle et al. 2008). Cabernet Sauvignon is adaptable to many soils, but performs best on well-drained, low-fertile soils (Koundouras 2008). Each cultivar is highly susceptible to Pierce's disease (Purcell 2005).

In January 2008, 200 cuttings were collected from both asymptomatic (SRI rating 1) and symptomatic source vines (SRI rating of 3 and 4) from each vineyard. Because of difficulty acquiring healthy green cambium tissue (observed while making cuts on source vine canes), only 130 symptomatic cuttings were collected from the Gulf Coast vineyard. After each cutting was taken, pruning shears were sterilized by spraying with 70% isopropyl alcohol. To record cutting number (1 to 200) and source vine number, each cutting was individually labeled. Source vines were identified by row number and vine number. Spur positions were labeled with corresponding cutting numbers.

Each cutting was a minimum 30.0 cm in length, 1.0 to 2.0 cm in width, and had a minimum of six nodes. A flush cut was made perpendicular to the length of the cutting approximately 2.0 cm below the basal node. A second cut at a 45° angle was performed 2.0 cm above the apical node. No more than four cuttings were collected from a single vine. Only one cutting was taken per cane. Cuttings were taken at the third node, leaving a two-bud spur on the source vine. Cuttings were placed within a sealed plastic bag, and placed on ice within an insulated cooler. Within one day, cuttings were transported to greenhouse facilities in Lubbock, Texas. Temperature

within the cooler was maintained between 1°C and 4°C. To maintain cooler temperature additional ice was provided as needed and cuttings remained inside coolers until insertion into potting media.

Propagation of cuttings was conducted at the Texas Tech Horticulture Greenhouse Complex located in Lubbock, Texas. Six large plastic containers (119.0 x 57.0 x 61.0 cm) were prepared as rooting containers. Rows of drainage holes were drilled in the bottom of each container at approximately 5.0 cm spacing. Rooting containers were filled to a depth of 15.0 cm with Promix 720 (Premier Horticulture, Quakertown, PA.) professional greenhouse potting mixture.

Depending on size of the cutting and internode length, cuttings were prepared for propagation by trimming to four or five nodes. Pruning shears were treated with 70% isopropyl alcohol between each cut. Cuttings were gently inserted into rooting media until the second node from the base was between 2.5 and 4.0 cm below the soil surface. Soil was then watered lightly to settle.

Three containers were used to propagate symptomatic vines and three containers were used to propagate asymptomatic vines. Each container was partitioned into three sections and each section contained four rows. Rows were comprised of five cuttings each of Merlot-Hill Country (M-HC) and Cabernet Sauvignon-Hill Country (CS-HC) vines, and three cuttings each of Merlot-Gulf Coast (M-GC) and Cabernet Sauvignon-Gulf Coast (CS-GC) vines. A total of 48 cuttings were placed in each container (Figure 2). Containers were randomly arranged on greenhouse benches.

Propagation mats (PRO-GRO, Brookfield, WI) maintained soil temperature near 23.8°C (Tom Nemcik (Novavine), personal communications; Hartmann et al. 2002).

Cuttings were watered once each week until roots and shoots formed. Thereafter, cuttings were watered once every four days. Bud break was monitored daily and recorded. After six weeks, all cuttings were extracted from the soil and root growth was evaluated. Collected data included: number of roots, length of longest root, root rating, number of shoots, and length of the largest shoot. Rooting percentage was also calculated. A minimum of one root was required for a cutting to be considered rooted. For each cutting the overall root system was assessed with a rating scale of one to five. A rating of one indicated little or no growth, and five indicated excellent rooting (30 or more roots with significant lateral root growth).

To monitor greenhouse climatic conditions during rooting, a weather station was installed in the greenhouse. A CR23X datalogger (Campbell Scientific, Logan, UT) was used to collect data. Solar radiation was monitored using a pyranometer (model: PY50729, LI-COR Biosciences, Lincoln, NE) placed 0.5 m above the greenhouse bench surface. Type K thermocouples (Omega Engineering Inc., Stamford, CT) were constructed to monitor ambient air and soil temperatures within each propagation container. Soil temperature of each container was monitored using one thermocouple placed near the center of the container 7.5 cm below the surface. Three shielded thermocouples were used to record ambient air temperature inside three of the six rooting containers.

Successfully rooted cuttings were planted into 2.2 L containers containing Promix 720 growing media and placed on greenhouse benches inside the greenhouse Cuttings that did not root or produce shoots were discarded (Figure 3). To monitor presence of potential insect vectors, four yellow sticky traps (15.0 cm x 30.5 cm) (Johnny's Selected Seeds, Winslow, ME) were placed 0.5 meter above potted vines.

Each potted grapevine was watered as needed to maintain moist soil conditions and fertilized once each week for eight weeks with a 20N-4.4P-16.6K water soluble fertilizer (applied 54.0 g/L) (J.R. Peters, Allentown, PA). Subsequently, a 5N-4.4P-20.8K hydrosol hydroponic fertilizer (applied 30.0 g/L) (Verti-gro, Summerfield, FL) was applied once every other week. Applications of Safer Brand Caterpillar Killer with *Bacillus thuringiensis* (BT) (Woodstream Corp, Lititz, PA), Safer insecticidal soap (Woodstream Corp, Lititz, PA), and 70% Neem oil (Green Light Sales Co, San Antonio, TX) were applied at 8.2 g/L every four weeks or as needed to control insect pests. Vines were monitored daily for symptoms of PD.

Presence of *X. fastidiosa* in rooted cuttings was assessed after 16 weeks of growth inside the greenhouse. Enzyme-Linked ImmunoSorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) analytical techniques were used to determine presence of *X. fastidiosa*. Petiole samples were collected from individual vines using pruning shears sterilized after each cut with 70% isopropyl alcohol. Several leaves were taken from each plant and immediately placed into a labeled plastic bag. Bags were put into an insulated cooler with ice for several hours until transfer to the

viticulture laboratory at the Texas Agrilife Research and Extension Center in Lubbock, TX.

Petioles were cut from the leaf blade using a razorblade sterilized with 70% ethyl alcohol. Approximately 0.5 grams of petiole tissue were collected from each vine sample and placed in 5.0 ml of General Extraction Buffer 3 (Agdia, Elkhart, IN), and macerated with a homogenizer (Omni International, Marietta, GA). The homogenizer tip was replaced after each usage. The macerated liquid slurry was transferred into 50 mL conical tubes (BD Biosciences, Franklin Lakes, NJ), and centrifuged for 4 minutes. The supernatant was collected with a 3 mL syringe (BD plastic, Franklin Lakes, NJ) and transferred into two 1.5 mL micro-centrifuge tubes. Tubes were labeled with treatment and sample numbers. This process was repeated for each sample.

ELISA was performed on the second set of samples contained in the 1.5 ml micro-centrifuge tubes using a *X. fastidiosa* DAS ELISA Pathoscreen kit (Agdia, Elkhart, IN). The *X. fastidiosa* specific antibody was raised against surface antigens and directed against one or more cell wall proteins (Carbajal 2004). Using the protocol from Agdia, a modified ELISA protocol was created (Appendix 1).

Results of ELISA were analyzed using the internal spectrometer of a Multiskan EX plate reader (Thermo Scientific, Waltham, MA) and Accent software (Thermo Scientific, Waltham, MA). Results were analyzed using the absorbance mode of the Multiskan at 650 nm. Numerical values greater than 0.3 indicated a positive result.

After ELISA testing, remaining samples within the micro-centrifuge tubes were secured into a sample tray and placed inside a freezer for a period of eight hours. Tubes were then shipped on dry ice to the Department of Plant Pathology and Microbiology at Texas A&M University, College Station, TX. Samples were stored in a freezer maintained at 2°C to 4°C and later tested using Quantitative Real-Time (QRT) PCR.

Samples were tested three days after arrival at the Department of Plant Pathology and Microbiology at Texas A&M University using Dr. David Appel's Lab Protocol for an Applied Biosystems 7300 Real-time PCR Machine (Appendix 2). Each sample was replicated three times. Data was reported in the Applied Biosystems 7300 software.

On September 23, 2008 fresh petiole samples were collected from vineyard source vines that tested negative for both ELISA and PCR and were tested again using a slightly modified protocol (different lab equipment available). Samples were collected within one day, stored on ice, and tested eight hours after collection. Petioles were processed using a mini ballbeater (Biospec Products, Bartlesville, OK) for two minutes. The supernatant was collected using a pipette (Eppendorf, Westbury New York) and tested with QRT-PCR.

The design of the propagation experiment was a split-plot design with each main plot treatment (symptomatic or asymptomatic) assigned three plastic containers arranged in a completely randomized design (Figure 4). Each container was split into three subplots. Each subplot contained four treatments (cultivar x location). Subplot

treatments were: M-HC, M-GC, CS-HC, and CS-GC. Because of limitations on plant material within each subplot, Hill Country cuttings were replicated five times, while Gulf Coast cuttings were replicated three times. Rooting data were analyzed using the GLIMMIX procedure in SAS (Version 9.1.3; SAS Institute, Cary, NC). All other data were analyzed using the PROC Mixed procedure in SAS.

Cuttings in trial one were propagated January 8, 2008 to February 26. 2008. Successfully rooted cuttings were transplanted February 26, 2008. In trial two, cuttings were propagated March 4, 2008 to April 22, 2008. Cuttings that successfully propagated were transplanted April 22, 2008. All cuttings grew for 16 weeks or until petioles were large enough for DNA analysis using ELISA or QRT-PCR.

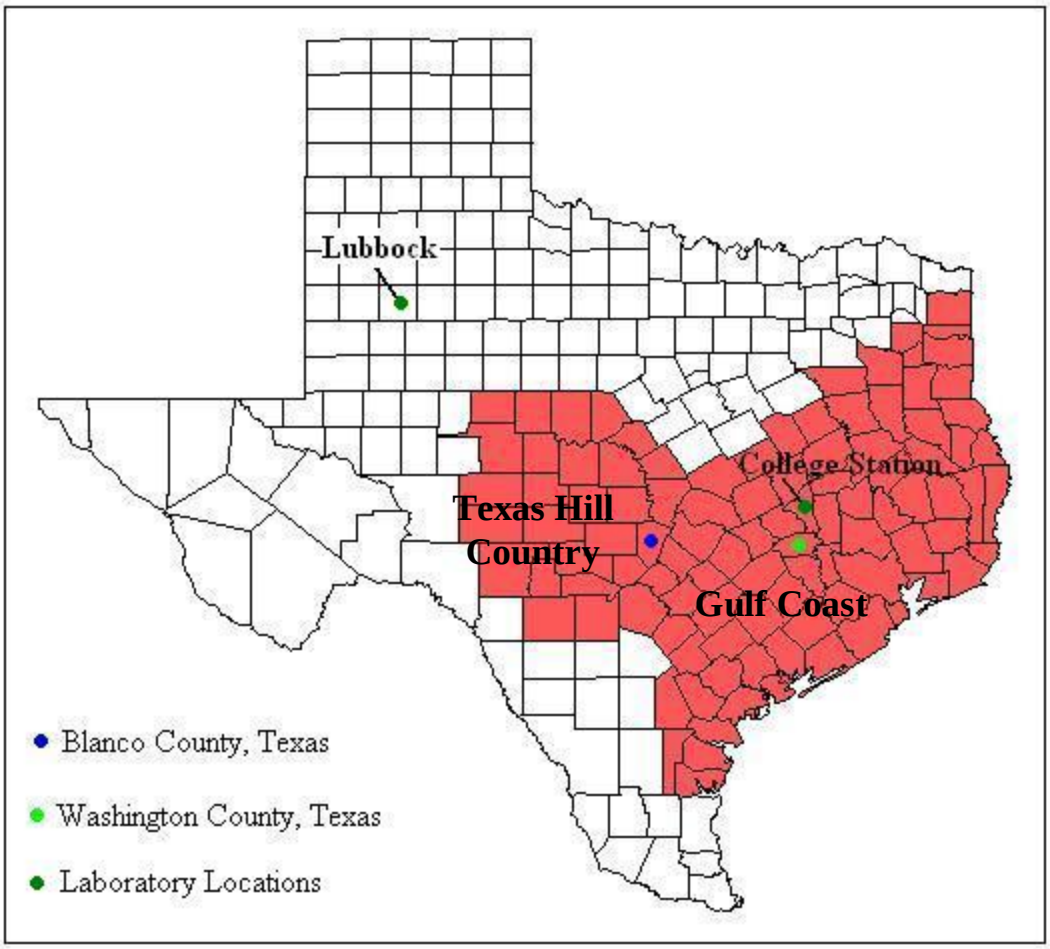

Figure 1. Vineyards chosen were located in the Gulf Coast (Washington County) and the Hill Country (Blanco County) growing regions of Central Texas. The vineyards in the study sites selected were previously evaluated for Pierce's disease epidemiology by Dr. David Appel (Texas A&M University, College Station, TX) from 2004 to 2007. Symptomatic and asymptomatic cuttings were collected from potentially infected vines in the study sites. Cuttings were propagated at the Texas Tech University Greenhouse Complex in Lubbock. Petiole analysis tests were performed in Lubbock at the Texas AgriLife Experimental Research Station and in College Station at Texas A&M University.

Figure 2. Symptomatic and asymptomatic cuttings were collected from potentially infected vines located at two vineyards in Central Texas. Vineyards chosen were located in the Gulf Coast and the Hill Country growing regions of Texas. The vineyards in the study sites selected were previously evaluated for Pierce's disease epidemiology by Dr. David Appel (Texas A&M University, College Station, TX) from 2004 to 2007. Cuttings were separated into two treatments based on symptomology (asymptomatic or symptomatic). Cuttings were then arranged inside plastic containers by cultivar (Merlot or Cabernet Sauvignon) and location (Hill Country or Gulf Coast) in twelve columns and five rows.

Figure 3. Symptomatic and asymptomatic cuttings were collected from potentially infected vines located at two vineyards in Central Texas. Vineyards chosen were located in the Gulf Coast and the Hill Country growing regions of Texas. The vineyards in the study sites selected were previously evaluated for Pierce's disease epidemiology by Dr. David Appel (Texas A&M University, College Station, TX) from 2004 to 2007. Successfully rooted cuttings were planted into 2.2 L pots. Cuttings grew for 16 weeks until petiole samples were tested for *Xylella fastidiosa* using ELISA and QRT-PCR.

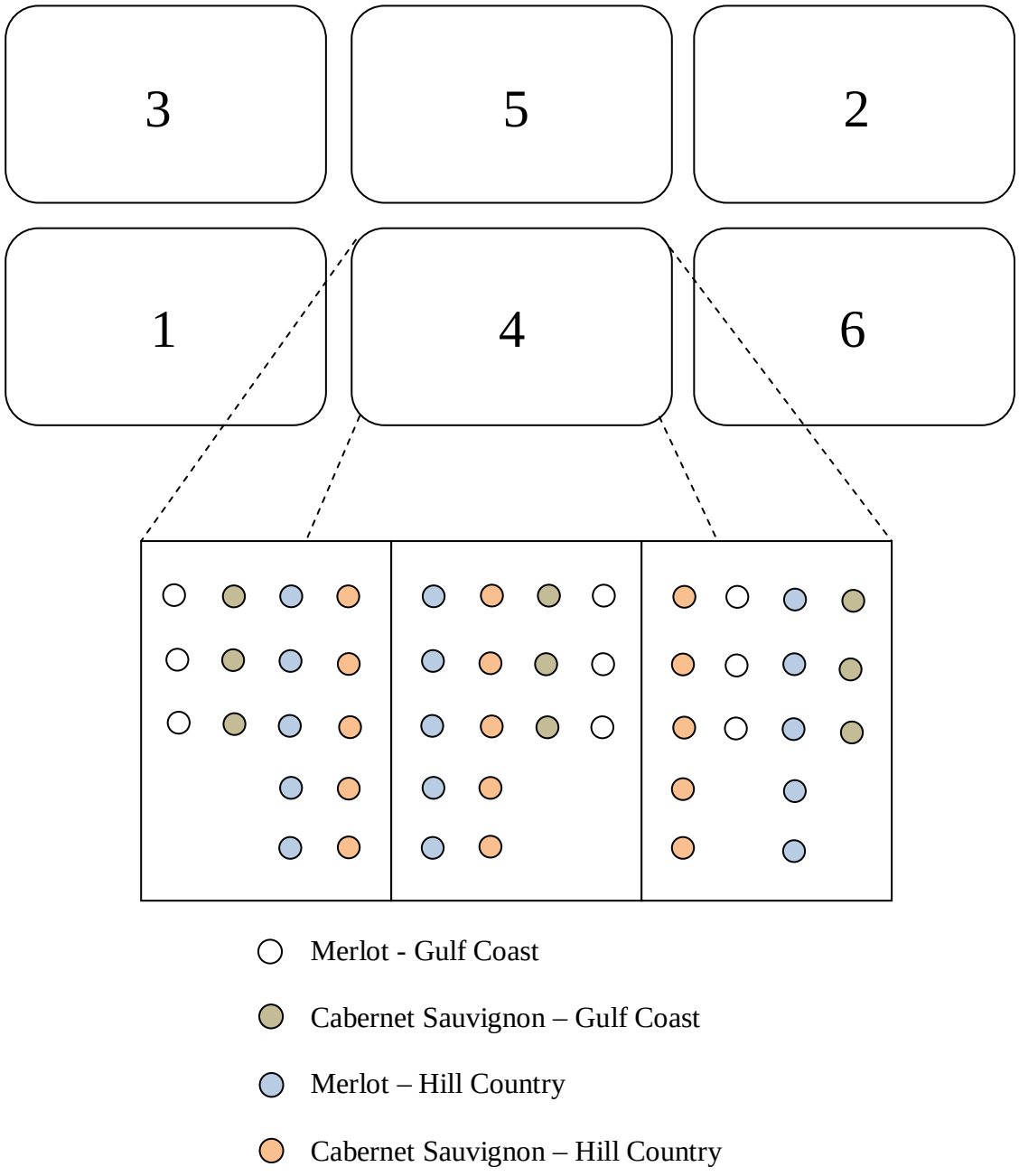

Figure 4. Experimental design. Six rectangle containers were assigned numbers 1 to 6. Three containers were planted with asymptomatic cuttings and three with symptomatic cuttings. Each container was divided into three subplots and each subplot contained combinations of cultivar and location randomly assigned to columns in each division as shown in the figure.

Chapter III

Results and Discussion

During propagation in trial one, daily minimum air temperatures within each propagation container averaged 18.2°C and daily maximum air temperatures averaged 31.2°C (Figure 5). Average minimum soil temperature was 24.3°C and average maximum soil temperature was 27.2°C (Figure 6). Maximum daily shortwave radiation entering the greenhouse was observed at 785 W m^{-2} (Figure 7). In trial two, daily minimum air temperatures within each propagation container averaged 24.4°C and daily maximum air temperatures averaged 35.7°C (Figure 5). Average minimum soil temperature was 26.2°C and maximum soil temperature was 32.9°C (Figure 6). Maximum daily shortwave radiation was observed at 1027 W m^{-2} (Figure 7).

Rooting success of symptomatic and asymptomatic cuttings taken from vines of *V. vinifera* vines during the first trial (Table 2) and second trial (Table 3) varied. For trial one, rooting percentage was influenced by an interaction between symptomology (symptomatic or asymptomatic) and cultivar (Figure 8). For asymptomatic cuttings, rooting percentage of Merlot cuttings had a greater rooting percentage when compared to cuttings taken from asymptomatic Cabernet Sauvignon vines. However, for symptomatic cuttings, Cabernet Sauvignon cuttings had a greater rooting percentage when compared to Merlot. These results suggest Cabernet Sauvignon vines might have greater field tolerance to PD when compared to Merlot vines. After comparing cultivar sensitivity to PD of twenty-five *V. vinifera* cultivars, Raju and Goheen (1981) suggest Merlot is more susceptible to PD than Cabernet

Sauvignon. Our research indicates under symptomatic conditions, cultivar sensitivity to PD may have a greater influence on rooting of Cabernet Sauvignon cuttings (lower rooting percentage) when compared to Merlot cuttings. However in trial two (Figure 9), rooting of asymptomatic cuttings was three times greater when compared to rooting of symptomatic cuttings. Habermann et al. (2006) reported healthy sweet orange cuttings had a greater rooting percentage when compared to cuttings infected with citrus variegated chlorosis (a disease also caused by *X. fastidiosa*). Low rooting percentage of symptomatic grape cuttings in both trials may have occurred because of the presence of PD in cuttings, or because cuttings taken from source vines were previously weakened by other pathogens, reduced stored carbohydrates, climatic injury, or mechanical injury (Warmund et al. 1985; Winkler et al. 1974).

Vineyard location (Hill Country and Gulf Coast) had an effect on rooting only in trial one (Table 2). In the first trial, cuttings taken from the Hill Country vineyard had 40% greater rooting percentage and produced 78% more roots when compared to those taken from the Gulf Coast vineyard (Table 2 and Figure 10). In trial two, cuttings from the Hill Country vineyard had similar rooting when compared to cuttings taken from the Gulf Coast vineyard (Table 3and Figure 11). Low rooting percentage and root growth of cuttings from the Gulf Coast region may have been influenced by environmental stresses and management practices (disease control, cropping levels, etc.). Howell and Shaulis, (1980) suggest over-cropping weakens grapevine cold hardiness by reducing the amount of carbohydrates available for shoot maturation.

To determine cutting rooting quality, numbers of roots, length of longest root, and root rating were evaluated. In both trials there was no difference between number of roots for each cutting when asymptomatic cuttings where compared to symptomatic cuttings (Tables 2 and 3). Rooting similarities between asymptomatic and symptomatic cuttings may have resulted due to absence of the pathogen or because cuttings surviving propagation did not contain enough *X. fastidiosa* titer levels to affect rooting quality. Krell et al. (2007) suggest non-infected cuttings may not have been exposed to infected cuttings long enough to cause infected *X. fastidiosa* root grafts, as infected cuttings containing *X. fastidiosa* died a few months after planting.

In both trials the number of roots produced by Merlot cuttings, were not different when compared to the number of roots produced by Cabernet Sauvignon cuttings. In trial one the number of roots recorded for Hill Country cuttings were greater when compared to the number of roots recorded for Gulf Coast cuttings (Table 2 and Figure 10). In trial two, Hill Country cuttings produced nearly five times more roots when compared to Gulf Coast cuttings (Table 3 and Figure 11). Hill Country cuttings may have produced more roots when compared to Gulf Coast cuttings due to competition between source vines and native vegetation. Based on observations when both vineyards were visited (no record from vineyard management), the Gulf Coast vineyard appeared to have more native vegetation growing between rows and source vines, potentially limiting growth. Hostetler et al. (2007) suggests excessive weed competition in vineyards can reduce vine health and yield. Cuttings taken from the Hill Country vineyard may have produced more roots when compared to Gulf Coast

cuttings because source vines in the vineyard were experiencing greater competition with other vegetation. Cuttings collected from source vines and source vines themselves were neither tested for nutrient availability before or after the experiment was completed. Although cuttings were not tested for nutrient status, of the 733 cuttings propagated, a little more than one third (243) of all cuttings were able to successfully root and produce vegetative growth suggesting an unknown variable was able to limit propagation.

Length of longest root was influenced by symptomology/cultivar (Figure 8) and symptomology/location (Figure 12) in trial one. Asymptomatic Merlot cuttings were 0.5 greater in length when compared to the longest root from asymptomatic Cabernet Sauvignon cuttings. However, symptomatic Cabernet Sauvignon cuttings were greater in length when compared to symptomatic Merlot cuttings. Length of longest root had similar results to symptomatic rooting percentage. Cuttings may have been influenced by the presence of PD, other pathogens, reduced stored carbohydrates, climatic injury, or mechanical injury.

Length of the longest root was also influenced by an interaction between symptomology and location in the first trial. Asymptomatic cuttings taken from the Hill Country were greater in length when compared to cuttings taken from the Gulf Coast. Symptomatic cuttings taken from the Hill Country were greater in length when compared to cuttings taken from the Gulf Coast. In the second trial, cuttings taken from the Hill Country were greater in length when compared to cuttings taken from

the Gulf Coast (Table 3 and Figure 12). Root ratings in both trials from Hill Country cuttings were greater when compared to root ratings from Gulf Coast cuttings.

Cuttings used in trial two were stored for an additional seven weeks in large storage coolers kept at 1°C to 4°C. This duration of time may have reduced vigor of cuttings and resulted in less root and shoot growth. Alley and Christensen (1970) report 'Thompson seedless' cuttings stored in wood shavings at colder temperatures (0°C to 3°C) had a reduction in number of roots and size of roots when compared to cuttings stored at ambient temperatures (12°C to 19°C) in sand. Treeby and Considine (1982) suggest storing cuttings in refrigeration (4°C) reduces overall stored carbohydrates during the first 36 days of storage. However, in the last 36 days of storage the same cuttings had similar carbohydrate levels of cuttings stored in sand. Similar results found by Warmund et al. (1985) suggest reduced shoot growth may have occurred due to the size of cuttings and late planting date. It appears cuttings stored for an additional seven weeks may have been directly affected by cold temperatures, a result reducing shoot and root growth.

Number of shoots and longest shoot length were recorded for both trials (Table 4 and 5). Longest average shoot length was influenced by the interaction of symptomology, cultivar, and location in trial one (Table 4 and Figure 13). Within asymptomatic cuttings, Merlot cuttings from the Hill country were more than two times greater in shoot length when compared to Merlot cuttings from the Gulf Coast. Similarly, asymptomatic Cabernet Sauvignon cuttings from the Hill Country were almost 50% greater in shoot length when compared to Cabernet Sauvignon cuttings

taken from the Gulf coast. Symptomatic Cabernet Sauvignon cuttings from the Hill Country were almost two times greater in shoot length when compared to Cabernet Sauvignon cuttings from the Gulf Coast. In trial two (Table 5) symptomatic cuttings produced three times more shoots compared to asymptomatic cuttings. Cabernet Sauvignon produced a greater number of shoots when compared to Merlot in Trial two. However in trial two, Cabernet Sauvignon produced three times more shoots when compared to Merlot.

Shoot length measured in trial two, indicates symptomatic and Cabernet Sauvignon cuttings produced greater numbers of shoots when compared to asymptomatic and Merlot cutting shoots respectively (Table 5). Location again had a significant effect; Hill Country shoots were three times larger when compared to Gulf coast shoots.

Rooted cuttings were tested for the presence of *X. fastidiosa* using ELISA and QRT-PCR. All ELISA test results were negative (Table 6). Although it is recognized ELISA results are dependent upon titer levels of the bacterium, these data suggest that all rooted cuttings were *Xylella* free. ELISA test plates included 12 positive and 12 negative *X. fastidiosa* control wells which returned values correctly associated with each control. All ELISA tests were replicated three times and confidence is high with the results. Negative ELISA results may have occurred due to low *X. fastidiosa* titer levels inside petioles at time of sampling. Hill and Purcell (1997) suggest ELISA tests on samples isolated from processed plant tissue can only reliably detect infection loads of 10^5 cells per gram plant tissue or greater.

Further testing of samples with QRT-PCR resulted in the identification of four (two asymptomatic and two symptomatic) rooted cuttings positive for *X. fastidiosa*. Canes collected in January were selected at random from source vines previously assessed for PD symptoms. Lack of foliage and petioles during cane collection removed any bias to select possible diseased plant canes. Source vines previously assessed were analyzed as a whole and not by individual canes. Therefore, methods were not taken to determine if canes selected were previously seen with PD symptoms. Higher sensitivity of QRT-PCR compared to ELISA for the detection of *X. fastidiosa* (Krivanek and Walker 2005) may have resulted in positive results where ELISA gave negative results. The ability of QRT-PCR to detect lower concentrations *of X. fastidiosa* due to heightened sensitivity has been reported (Krivanek and Walker 2005; Carbajal et al. 2004). QRT-PCR has been reported to detect infection levels at 10^3cells per gram plant tissue (Carbajal et al. 2004).

Asymptomatic cuttings testing positive for *X. fastidiosa* may have been the result of source vines expressing no symptoms or that selected canes were free of the pathogen. In both vineyards asymptomatic source vines were often located in close proximity to symptomatic vines, sometimes immediately adjacent. After 16 weeks growing in a greenhouse, these four vines expressed no visual symptoms of PD. QRT-PCR results suggest cuttings that exhibit no visual symptoms are able to host *X. fastidiosa*. Of the 99 cuttings which tested negative for *Xylella,* all were symptomless at the time petiole samples were collected for ELISA and QRT-PCR analysis. It may have been possible *X. fastidiosa* population levels had not yet reached an adequate

threshold level to be detected by ELISA or QRT-PCR. Fritschi et al. (2007) observed PD symptoms in Chardonnay and 9621-94 (*Vitis rupestris x V. arizonica*) plants at 113 days post-inoculation, but did not see any symptoms during the first sampling which occurred 34 days after inoculation. Cuttings sampled for ELISA and QRT-PCR were sampled on the 72nd day (Day 245 of the experiment) of vegetative plant growth. On days 166 through 172, cuttings were pruned back to initiate new growth following minor insect damage and leaf scorch caused by extreme greenhouse temperatures. Fritschi et al. (2007) suggests *X. fastidiosa* population levels may require 113 days for symptoms to develop. If cuttings had been grown for a longer time, there may have been a greater number of cuttings with a positive result. No visual symptoms or potential xylem-feeding insects were observed in the greenhouse from day 37 to day 327 on vines that tested positive for *X. fastidiosa*.

Petiole samples were randomly taken from parent vines of cuttings that tested negative in QRT-PCR and were tested for the presence of *Xylella* using QRT-PCR. Thirty-seven source vines sampled in January from the Gulf Coast vineyard were no longer available for sampling in September because they had been removed by vineyard management. Source vines were not tested for *X. fastidiosa* using QRT-PCR prior to cutting collection. At the time cuttings were collected, source vines were dormant and previous knowledge of individual cane status for the pathogen was unknown. Sampled cuttings may have been taken from canes containing no or little *X. fastidiosa*. Mitchell and Bextine (2008) investigated *X. fastidiosa* presence in *V. vinifera* cv. Shiraz. At different tissue positions, ELISA tests confirmed positive and

negative results, indicating *X. fastidiosa* concentrations vary throughout the vine. At the time leaf samples were collected, Gulf Coast vineyard source vines appeared asymptomatic of PD and Hill Country source vines appeared highly symptomatic of PD. However, among the 67 source vines tested from both vineyards, only one tested positive for *X. fastidiosa*. Foliage appearing PD symptomatic from the Hill Country may have been the result of leaves senescing and possibly a potassium deficiency, which explains leaf color and condition in the Hill Country vineyard. Of 66 source vines testing negative for *X. fastidiosa* it might also be possible source vines never contained the pathogen. Source vines were only assessed visually the previous 4 years using a symptom rating scale and only a few vines in each vineyard studied were DNA tested to ensure vines contained *X. fastidiosa*.

Table 1. Symptom Reliability Index (SRI)[1] used to assess Pierce's disease symptoms in commercially grown *Vitis vinifera* L. in Central Texas vineyards.

Rating	Observed Condition of Vitis vinifera L.
1	vines were healthy
2	vines showing early symptoms of PD
3	vines beginning to show advanced symptoms of PD, but no dieback
4	vines beginning to show advanced symptoms of PD including dieback
5	vines were dead
6	vine missing
7	vine had been replaced

[1] Created by Dr. David Appel, Texas A&M University, College Station, Texas.

Table 2. Rooting success of *Vitis vinifera* cuttings during trial one (January 8th to February 19th, 2008) studying the effect of symptomology, cultivar and location on rooting and growth of *V. vinifera* cuttings potentially infected with *Xylella fastidiosa*. Cuttings were grown inside the Texas Tech University Horticulture Greenhouse Complex in Lubbock, Texas.

Treatment	Rooting Percent (%)	Number of Roots	Longest Root (cm)	Rating
Symptomology				
Asymptomatic	61.7a	20.2	7.74	2.4
Symptomatic	47.92b	14.8	4.77	2.0
Cultivar				
Merlot	58.03	20.3	6.85	2.3
Cabernet Sauvignon	53.09	20.0	5.66	2.1
Location				
Hill Country	63.9a[Z]	25.8a	21.10	2.5a
Gulf Coast	45.2b	14.5b	13.87	1.9b
Symptomology x Cultivar				
Asymptomatic Cabernet Sauvignon	53.3b[Y]	-------	6.41b	-------
Asymptomatic Merlot	70.0a	-------	9.06a	-------
Symptomatic Cabernet Sauvignon	52.8b	-------	4.90c	-------
Symptomatic Merlot	43.1c	-------	4.64c	-------
Symptomology x Location				
Asymptomatic Hill Country	-------	-------	9.76a[X]	-------
Asymptomatic Gulf Coast	-------	-------	5.71b	-------
Symptomatic Hill Country	-------	-------	5.38b	-------
Symptomatic Gulf Coast	-------	-------	4.15c	-------

Significance	P>F			
Symptomology	0.0039	0.2263	0.0467	0.1526
Cultivar	0.6851	0.8939	0.0657	0.2207
Location	0.0002	0.0086	0.0008	0.0020
Symptomology x Cultivar	0.0088	0.7890	0.0292	0.5802
Cultivar x Location	0.3111	0.2335	0.9663	0.2295
Symptomology x Location	0.5888	0.1077	0.0338	0.7811
Symptomology x cultivar x Location	0.4176	0.2789	0.9208	0.6323

[Z] Mean separation of Location by LSD P≤ 0.1).
[Y] Mean separation between Symptomology x Cultivar interaction by LSD (P≤ 0.1).
[X]Mean separation between Symptomology x Location interaction by LSD (P≤ 0.1).
LSD= Least Significant Difference.

Table 3. Rooting success of *Vitis vinifera* cuttings during trial two (March 4th to April 15th, 2008) studying the effect of symptomology, cultivar and location on rooting and growth of *V. vinifera* cuttings potentially infected with *Xylella fastidiosa*. Cuttings were grown inside the Texas Tech University Horticulture Greenhouse Complex in Lubbock, Texas.

Treatment	Rooting Percent (%)	Number of Roots	Longest Root (cm)	Rating
Symptomology				
Asymptomatic	33.9a[Z]	1.5	1.1	1.1
Symptomatic	10.6b	3.7	1.7	1.2
Cultivar				
Merlot	11.1b[Y]	2.1	1.0	1.1
Cabernet Sauvignon	33.3a	3.1	1.7	1.2
Location				
Hill Country	24.4	4.2a[X]	2.3a	1.3a
Gulf Coast	20.0	0.9b	0.5b	1.1b

Significance	P>F			
Symptomology	0.0553	0.3145	0.5748	0.3825
Cultivar	0.0679	0.3927	0.1871	0.1616
Location	0.7144	0.0172	0.0039	0.0146
Symptomology x Cultivar	0.1705	0.5443	0.6204	0.3913
Cultivar x Location	0.2727	0.5150	0.8126	0.7604
Symptomology x Location	0.7837	0.1275	0.4078	0.2871
Symptomology x Cultivar x Location	0.2348	0.7517	0.8177	0.8714

[Z] Means separation of Symptomology by LSD ($P \leq 0.1$).
[Y] Means separation of Cultivar by LSD ($P \leq 0.1$).
[X] Means separation of Location by LSD ($P \leq 0.1$).
LSD = Least Significant Difference.

Table 4. Shoot evaluation of *Vitis vinifera* cuttings from trial one (January 8th to February 19th, 2008) studying the effect of symptomology, cultivar and location on rooting and growth of *V. vinifera* cuttings potentially infected with *Xylella fastidiosa*. Cuttings were grown inside the Texas Tech University Horticulture Greenhouse Complex in Lubbock, Texas.

Treatment	Number of Shoots	Longest Shoot (cm)
Symptomology		
Asymptomatic	1.1	4.7
Symptomatic	0.8	3.8
Cultivar		
Merlot	0.9	4.6
Cabernet Sauvignon	1.0	3.9
Location		
Hill Country	1.0	5.2a
Gulf Coast	0.9	3.3b
Symptomology x Cultivar x Location		
Asymptomatic Merlot Hill Country	-------	7.2a[Z]
Asymptomatic Merlot Gulf Coast	-------	3.3d
Asymptomatic Cabernet Sauvignon Hill Country	-------	4.7c
Asymptomatic Cabernet Sauvignon Gulf Coast	-------	3.5d
Symptomatic Merlot Hill Country	-------	3.9d
Symptomatic Merlot Gulf Coast	-------	3.8d
Symptomatic Cabernet Sauvignon Hill Country	-------	5.0b
Symptomatic Cabernet Sauvignon Gulf Coast	-------	2.6e
Significance	P>F	
Symptomology	0.1235	0.4548
Cultivar	0.3790	0.3328
Location	0.3525	0.0099
Symptomology x Cultivar	0.2923	0.3833
Cultivar x Location	0.1112	0.8627
Symptomology x Location	0.7726	0.3006
Symptomology x Cultivar x Location	0.5145	0.0603

[Z] Separation between Symptomology x Cultivar x Location interaction by LSD ($P \leq 0.1$).
LSD = Least Significant Difference.

Table 5. Shoot evaluation of *Vitis vinifera* cuttings from trial two (March 4th to April 15th, 2008) studying the effect of symptomology, cultivar and location on rooting and growth of *V. vinifera* cuttings potentially infected with *Xylella fastidiosa*. Cuttings were grown inside the Texas Tech University Horticulture Greenhouse Complex in Lubbock, Texas.

Treatment	Number of Shoots	Longest Shoot (cm)
Symptomology		
Asymptomatic	0.1b	0.6
Symptomatic	0.3a[Z]	1.2
Cultivar		
Merlot	0.1b	0.6
Cabernet Sauvignon	0.3a[Y]	1.1
Location		
Hill Country	0.3a[X]	1.4a
Gulf Coast	0.1b	0.4b

Significance	P>F	
Symptomology	0.0310	0.3825
Cultivar	0.0310	0.1616
Location	0.0227	0.0146
Symptomology x Cultivar	0.2254	0.3913
Cultivar x Location	0.1757	0.7604
Symptomology x Location	0.2254	0.2871
Symptomology x Cultivar x Location	0.8714	0.8714

[Z] Means separation of Symptomology by LSD ($P \leq 0.1$).
[Y] Means separation of Cultivar by LSD ($P \leq 0.1$).
[X] Means separation of Location by LSD ($P \leq 0.1$).
LSD = Least Significant Difference.

Table 6. Results of ELISA and Real-time PCR conducted from successfully propagated cuttings and samples from parent vines taken from two vineyards located in Central Texas to detect the presence of *Xylella fastidiosa*.

	Number of Vines	Reps.	Number of Samples	Source Vine Result
Samples taken from rooted plants in greenhouse:				
Enzyme-linked Immunosorbent Assay (ELISA)	103	3	309	103 - Negative
Real-time Polymerase Chain Reaction (Real-time PCR)	103	3	309	99 - Negative 4 - Positive
Samples collected from parent vines of negatively tested cuttings:				
Real-time Polymerase Chain Reaction (Real-time PCR)	67	3	201	66 - Negative 1 - Positive

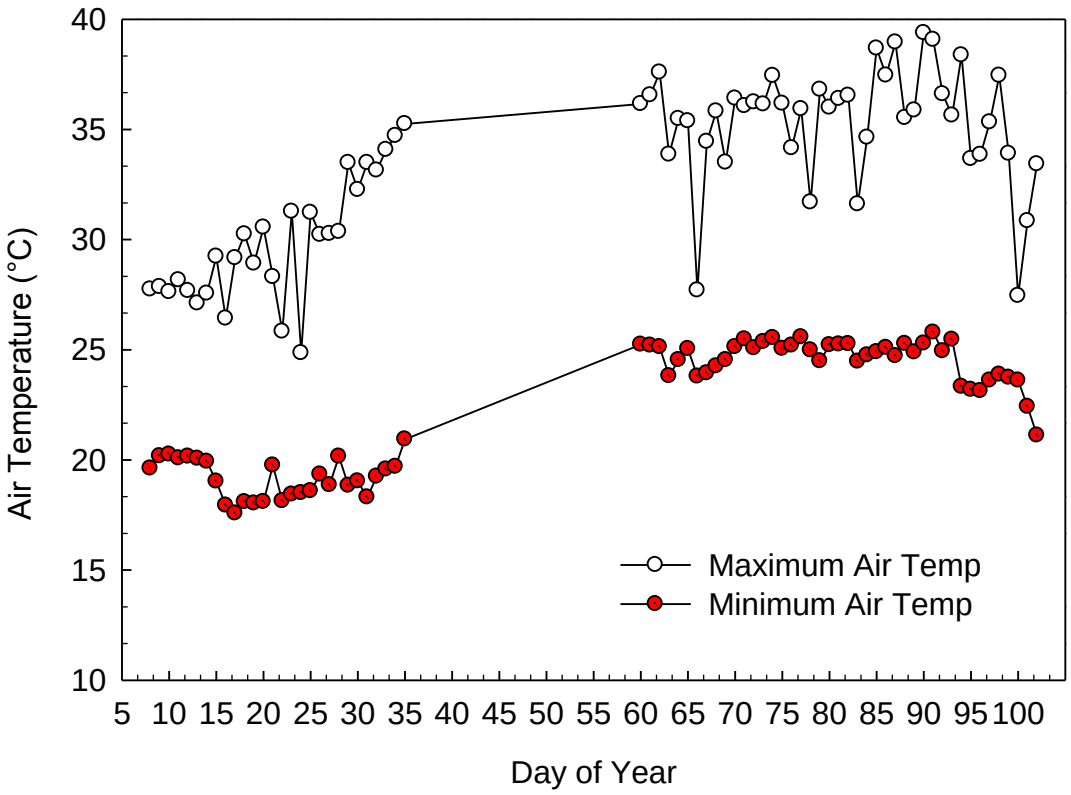

Figure 5. Mean minimum and maximum ambient air temperatures recorded 0.5 m above propagation bench used to propagate *Vitis vinifera* cuttings.

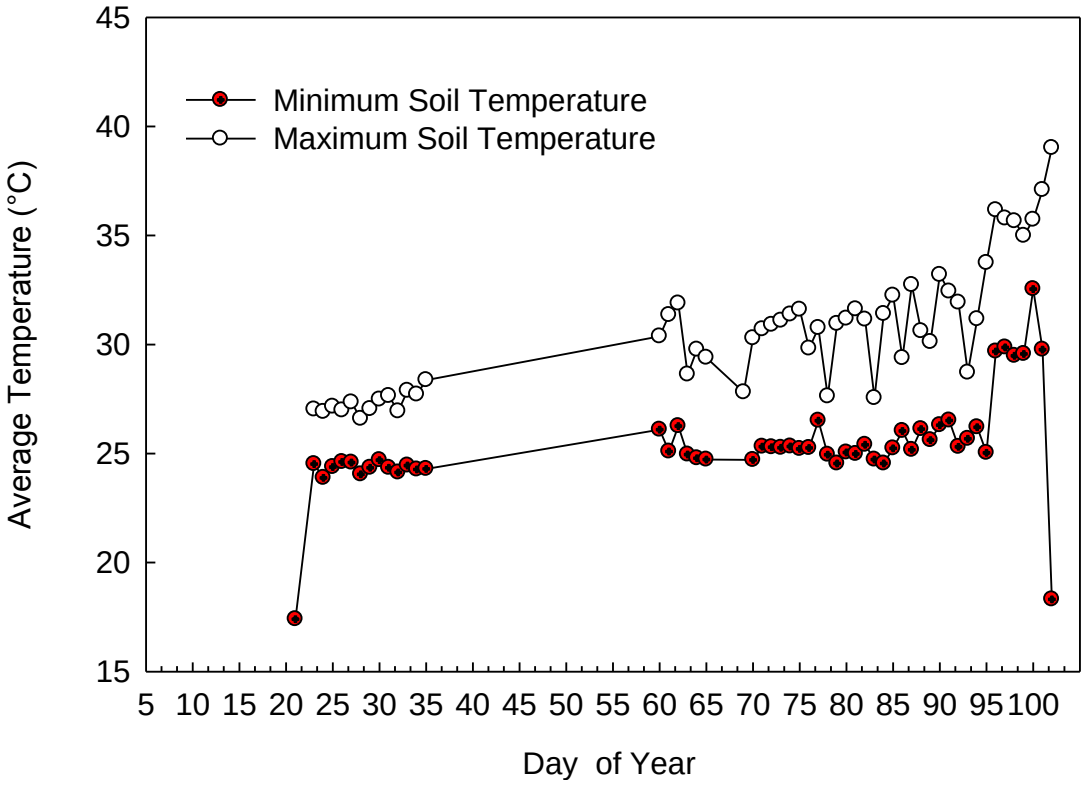

Figure 6. Average minimum and maximum soil temperatures recorded 7.5 cm below soil surface of *Vitis vinifera* cuttings.

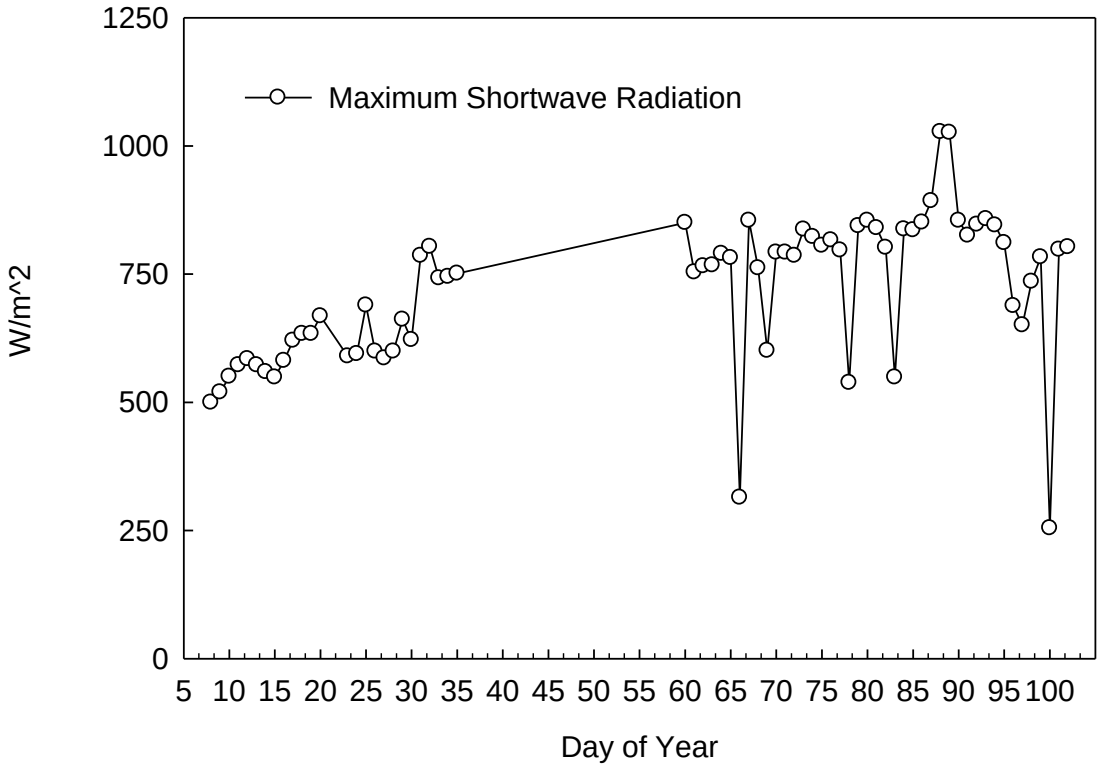

Figure 7. Daily maximum shortwave radiation measured inside greenhouse used to propagate *Vitis vinifera* cuttings.

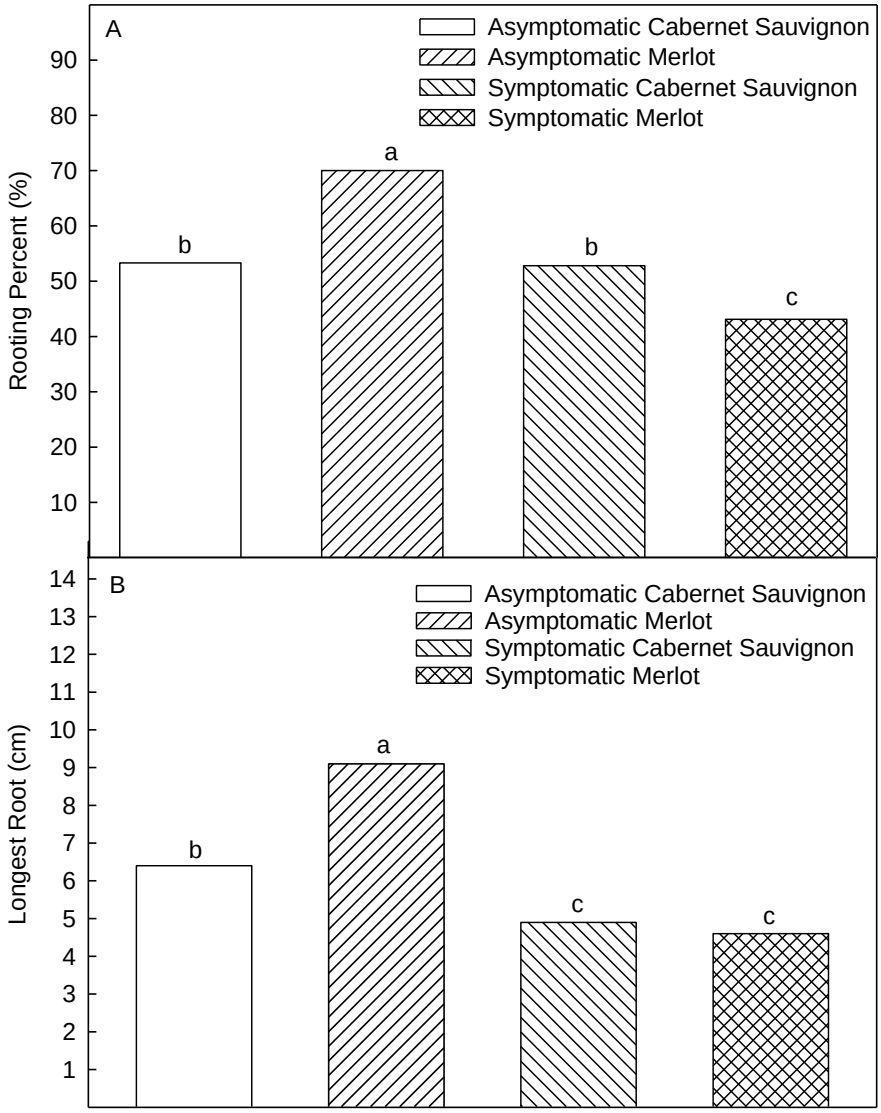

Figure 8. Symptomatic and asymptomatic cuttings were collected from potentially infected vines previously evaluated for Pierce's disease epidemiology by Dr. David Appel (Texas A&M University, College Station, TX) from 2004 to 2007. Rooting percentage was influenced by an interaction between symptomology (symptomatic or asymptomatic) and cultivar for (A) rooting percentage and (B) longest root in trial one. Rooted asymptomatic Merlot cuttings had a greater rooting percentage and greater longest root length when compared to cuttings taken from asymptomatic Cabernet Sauvignon vines. However, symptomatic Cabernet Sauvignon cuttings had a greater rooting percentage and showed no difference in longest root length when compared to Merlot cuttings.

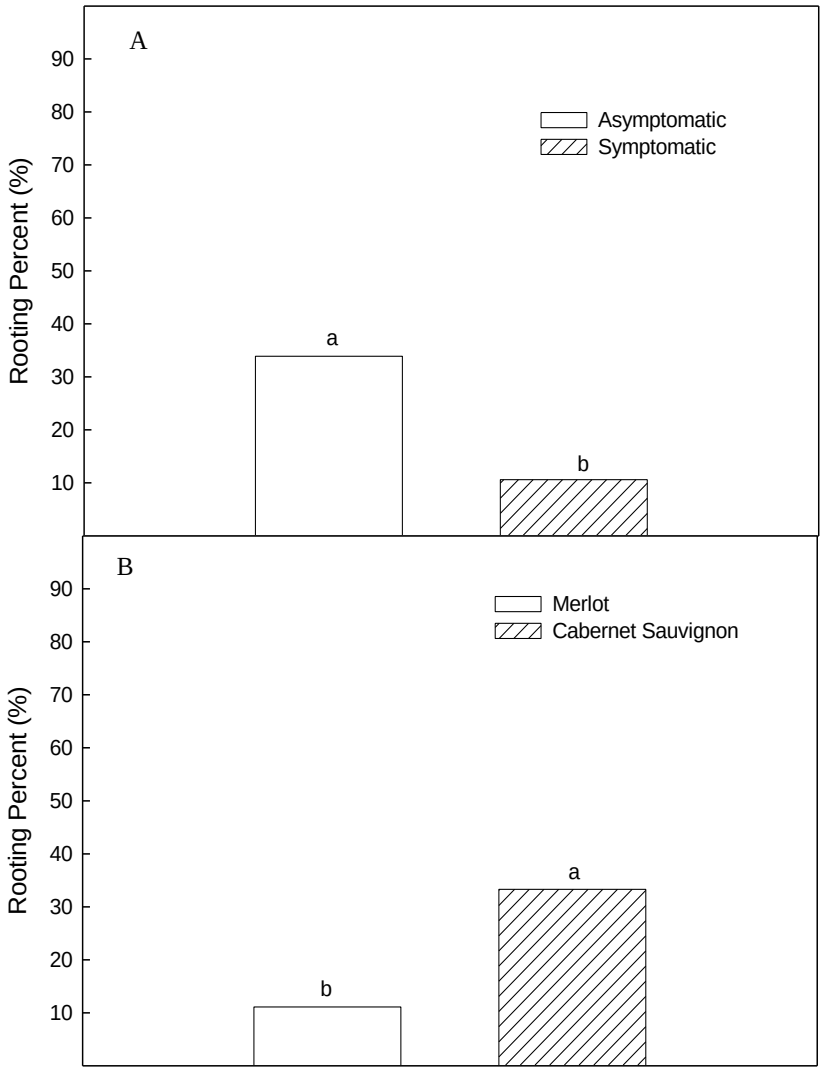

Figure 9. Symptomatic and asymptomatic cuttings were collected from potentially infected vines previously evaluated for Pierce's disease epidemiology by Dr. David Appel (Texas A&M University, College Station, TX) from 2004 to 2007. In trial two, (A) asymptomatic cuttings had a greater rooting percentage when compared to symptomatic cuttings. Also in trial two, (B) Cabernet Sauvignon cuttings had a greater rooting percentage when compared to Merlot cuttings.

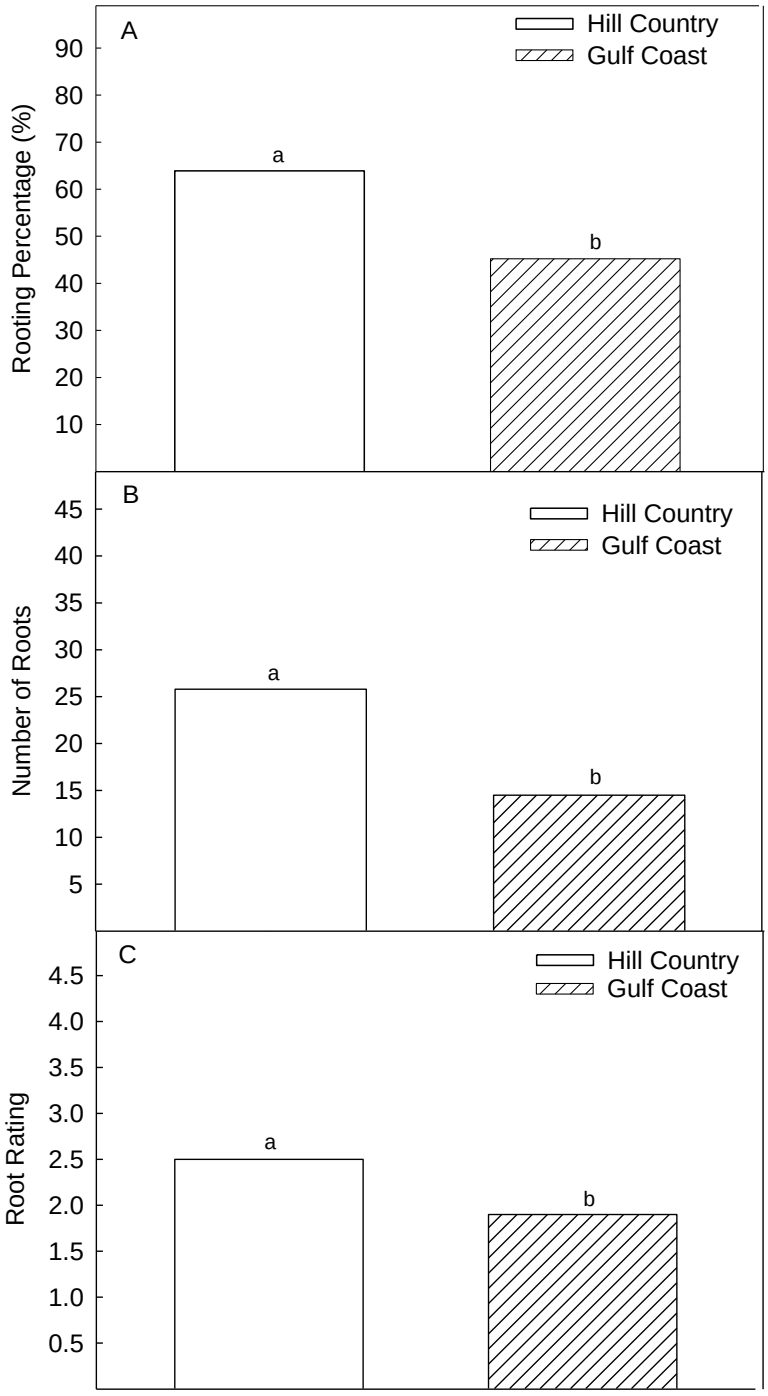

Figure 10. Symptomatic and asymptomatic cuttings were collected from potentially infected vines previously evaluated for Pierce's disease epidemiology by Dr. David Appel (Texas A&M University, College Station, TX) from 2004 to 2007. For trial one, Hill Country vineyard cuttings had greater (A) rooting percentage, (B) number of roots, (C) root rating when compared to cuttings collected from the Gulf Coast vineyard.

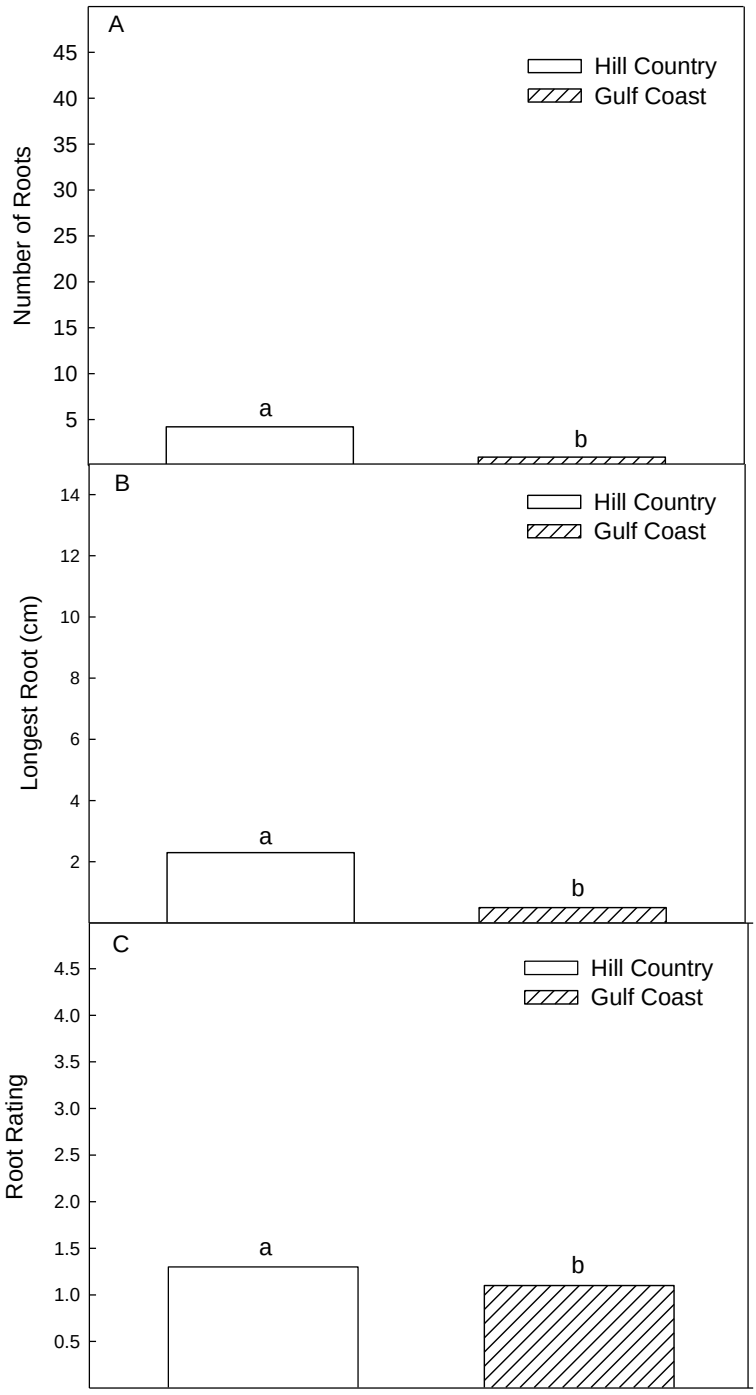

Figure 11. Symptomatic and asymptomatic cuttings were collected from potentially infected vines previously evaluated for Pierce's disease epidemiology by Dr. David Appel (Texas A&M University, College Station, TX) from 2004 to 2007. In trial two, the Hill Country vineyard has a greater (A) number of roots, (B) longest root, and (C) root rating.

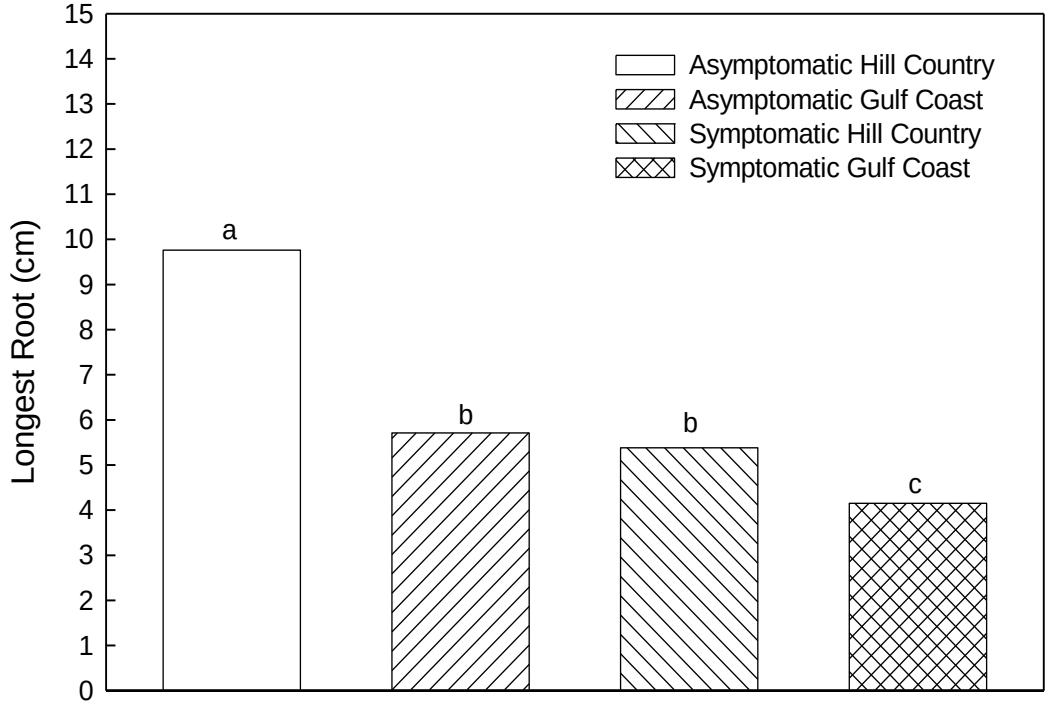

Figure 12. Symptomatic and asymptomatic cuttings were collected from potentially infected vines previously evaluated for Pierce's disease epidemiology by Dr. David Appel (Texas A&M University, College Station, TX) from 2004 to 2007. Longest root length was influenced by an interaction between symptomology (symptomatic or asymptomatic) and location in trial one. Rooted asymptomatic Hill Country cuttings had a greater longest root length when compared to asymptomatic Gulf Coast, symptomatic Hill Country, and symptomatic Gulf Coast cuttings.

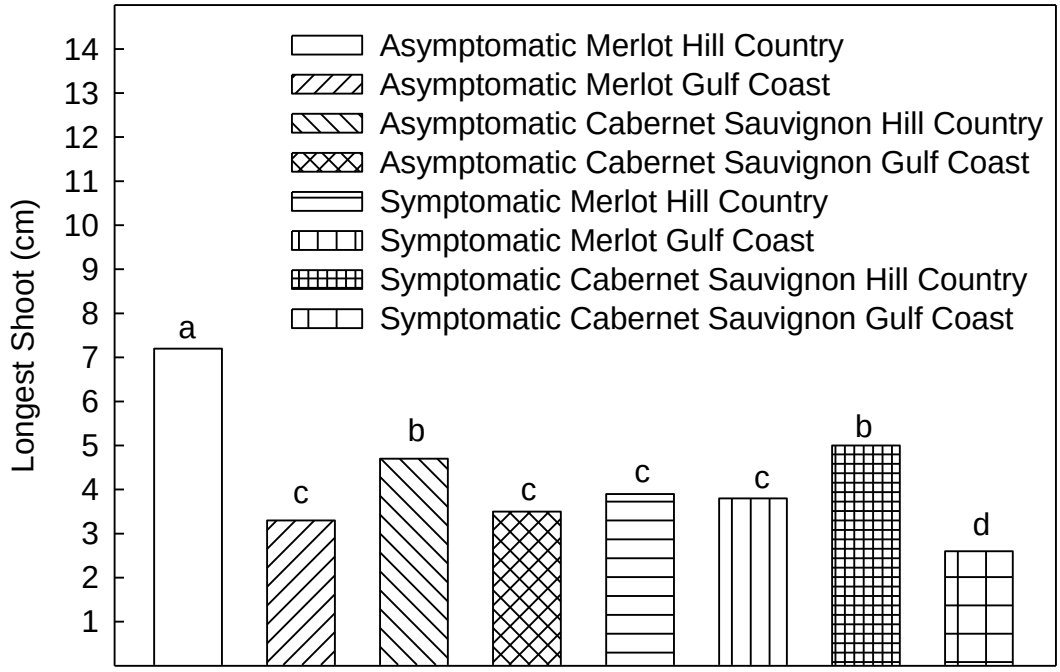

Figure 13. Symptomatic and asymptomatic cuttings were collected from potentially infected vines previously evaluated for Pierce's disease epidemiology by Dr. David Appel (Texas A&M University, College Station, TX) from 2004 to 2007. In trial one, an interaction between all three treatments (symptomology, cultivar, and location) influenced longest shoot. Asymptomatic Merlot Hill Country cuttings had longer shoots when compared to all other cuttings.

Chapter IV

Conclusions

In this study, *V. vinifera* cuttings were taken from vines which had been evaluated for PD symptoms the previous three years. Although *X. fastidiosa* vine status at the time of collection was unknown, cuttings from vines appearing symptomatic or asymptomatic were successfully propagated. Testing using ELISA and QRT-PCR confirmed *X. fastidiosa* in four (two asymptomatic/ two symptomatic) of 733 cuttings originally collected.

This experiment has demonstrated that grapevine cuttings infected with *X. fastidiosa* can be propagated to produce a healthy looking nursery plant that could be sold as clean nursery stock. Hence, acquiring nursery stock without prior testing of the vines may result in the introduction of diseased vines into the vineyard. Infected vines in the vineyard could serve as an additional source for xylem-feeding insects which may spread PD to uninfected established vines.

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Appendix

Appendix A

Krawitzky's ELISA Protocol

Prepare Buffer:

General Extraction Buffer 3 (GEB3): General extraction buffer is used to dilute and extract samples. It is used at a sample to buffer ratio of 1:10 (Weight: Volume)

For Example: 1 gram = 10 ml
.5 grams = 5 ml

To make (Y) GEB3 sample extract buffer, add (W) to (X) and mix into a smooth slurry. While mixing, slowly add the remaining volume of water. Add (Z) to the solution. Stir for 30 minutes.

Buffer Powder (X)	Distilled Water(Y)	(W)	Tween 20 (Z)
48 g	1000 ml (1L)	50 ml	20 ml or 20 g
24 g	500 ml (1/2L)	25 ml	10 ml or 10 g
12 g	250 ml (1/4 L)	12.5 ml	5 ml or 5 g
6 g	125 ml (1/8 L)	6.25 ml	2.5 ml or 2.5 g
3 g	62.5 ml (1/16 L)	3.125 ml	1.25 ml or 1.25 g
1.5 g	31.25 ml (1/32 L)	1.5625 ml	.625 ml or .625 g

Recommended to prepare enough buffers as needed for one day

Prepare Controls:

Add 2.0 ml sample extraction buffer (General Extraction Buffer) to reconstitute the Vial/bottle of lyophilized positive control. After preparing the positive and negative control vials, divide them into smaller portions. Dispense the controls into 1.5 mL Micro centrifuge tubes that can be capped and secured.

Preparing the Humid Box:

Prepare a humid box by lining an airtight container with a wet paper towel.

Preparing a Test Well Diagram:

Using the Multiskan software, Ascent, create a diagram of the plate by clicking on the layout tab under the general menu. Insert the name of the samples corresponding to the wells in which they are located into the Ascent software including the positive and negative controls.

Preparing samples:

If possible select petiole samples showing symptoms. Using a razor blade finely chop the petioles into small 1-2mm pieces. Once the petioles have been finely chopped, add sample extraction buffer. Add X grams of petiole pieces and X ml of sample extraction buffer into a test tube, remembering the 1:10 (g/ml) ratio. Using the Omni homogenize the sample. Next pour the test tube contents into a Falcon 50 mL conical centrifuge tube. Insert the conical centrifuge tube into the centrifuge. After the centrifuge has run for 4 minutes, using a 3 mL BD sterile syringe extract 2.5 mL of

supernatant from the 50 mL centrifuge tube. Insert the supernatant into 2- 1.5 micro centrifuge tubes. Cap the 1.5 micro centrifuge tube securely.

TEST PROCEDURE

Dispense sample:

Insert the Eppendorf pipette and a new sterile tip into the micro centrifuge tube. Dispense 100µl of prepared sample into only 1 test well. Using a new tip for each sample, dispense 100µl of positive control into the positive control wells and dispense 100µl of sample extraction buffer into the buffer wells. Dispose of the tips into a waste basket when finished. Label the diagram created in the Accent software. Repeat until the plate is full.

Incubate plate:

Set the plate inside the humid box and incubate for 2 hours at room temperature

Prepare enzyme conjugate:

Mix MRS with 1XPBST in a1:5 ratio. The volume of enzyme conjugate required depends on the number of test wells used, with 100µl needed per test well. To estimate the volume needed, prepare 1 ml for each 8-well strip, or 10 ml for each 96-well plate. Calculate the volume of enzyme conjugate needed based on the volume of enzyme conjugate diluents used and on the dilutions given on the bottles. Use a new sterile pipette tip for each bottle to prevent contamination. If the dilution given on the bottle is 1:100 and you are preparing 1 ml of enzyme conjugate you should first dispense 1 ml of enzyme conjugate diluents. Then add 10µl from the enzyme conjugate bottle to the enzyme conjugate diluents. After adding the concentrated enzyme conjugate to the diluent, it is important to mix the enzyme conjugate well.

Example:

10 mL PBST + 2 MRS (1:5) = 12 mL or 12000 µL (Total) = add 120 µL enzyme conjugate

$$\frac{10\ \mu L}{1000\ \mu L} = \frac{X}{12000\ \mu L}$$

Always prepare enzyme conjugate within 10 minutes before use.

Wash Plate:

When the plate has been incubated, wash the plate. Use a quick flipping motion to dump the well into a sink or a garbage can without mixing the contents. Fill all the wells to overflowing with the 1xPBST or tap water, and then quickly empty them again. Repeat 4 to 8 times.

After washing, hold the frame upside down and tap firmly on a folded paper towel to remove all droplets of wash buffer.

Add enzyme conjugate:

Dispense 100µl of prepared enzyme conjugate per well.

Incubate plate:

Incubate the plate in the humid box for 2 hours at room temperature

Wash plate:

As before, wash the plate 4 to 8 times with 1xPBST or tap water.

Add TMB substrate:

Add 100µl of TMB substrate into each well.

Incubate plate:

Incubate the plate in a humid box for 35-40 minutes

Evaluate results:

Insert the well plate into the Multiskan EX Machine and create a measurement program by clicking on the measurement tab.

Make sure that the measurement program has the filter set at 650 nm.

Set the measurement mode to "Absorbance" with number of 40.

Start the ELISA optical density program.

Wells that turn to a blue color indicate a positive result. Wells that have no color represent a negative result

Appendix B

Dr. David Appel's Lab Protocol

Prepare Buffer:

General Extraction Buffer 3 (GEB3): General extraction buffer is used to dilute and extract samples. It is used at a sample to buffer ratio of 1:10 (Weight: Volume)

For Example: 1 gram = 10 ml

.5 grams = 5 ml

To make (Y) GEB3 sample extract buffer, add (W) to (X) and mix into a smooth slurry. While mixing, slowly add the remaining volume of water. Add (Z) to the solution. Stir for 30 minutes.

Buffer Powder (X)	Distilled Water(Y)	(W)	Tween 20 (Z)
48 g	1000 ml (1L)	50 ml	20 ml or 20 g
24 g	500 ml (1/2L)	25 ml	10 ml or 10 g
12 g	250 ml (1/4 L)	12.5 ml	5 ml or 5 g
6 g	125 ml (1/8 L)	6.25 ml	2.5 ml or 2.5 g
3 g	62.5 ml (1/16 L)	3.125 ml	1.25 ml or 1.25 g
1.5 g	31.25 ml (1/32 L)	1.5625 ml	.625 ml or .625 g

Recommended to prepare enough buffer as needed for one day

Preparing Greenhouse Samples:

Select petiole samples showing symptoms. Using a razor blade finely chop the petioles into small 1-2mm pieces. Once the petioles have been finely chopped, add sample extraction buffer. For most samples use the GEB3 ratio listed above to dilute the samples. Add X grams of petiole pieces and X ml of sample extraction buffer into a test tube, remembering the 1:10 (g/ml) ratio. Using the Omni homogenizer (Model: TH115) and the Omni hard tip (Model: 3_750), insert the tip into the test tube and turn on the homogenizer. Pour the test tube contents into a Falcon 50 ml conical centrifuge tube. Insert the conical centrifuge tube into the centrifuge. After the centrifuge has run for 4 minutes remove the centrifuge tube and using a 3 mL BD sterile syringe extract 2.5 mL of supernatant from the 50 mL centrifuge tube. Insert the supernatant into 2-1.5 micro centrifuge tubes. Cap the 1.5 micro centrifuge tube securely. Freeze until ready to use

To use samples: Remove plant xylem samples from the freezer and allow them to thaw out for 15-20 minutes.

Preparing Vineyard samples:

If possible select petiole samples showing symptoms. Using a razor blade finely chop the petioles into small 1-2mm pieces. Once the petioles have been finely chopped, add sample extraction buffer. For most samples use the GEB3 ratio listed above to dilute the samples. Add X grams of petiole pieces and X ml of sample extraction buffer into a micro centrifuge tube (USA Scientific, Ocala FL), remembering the 1:10 (g/ml) ratio. Insert the tubes into the ballbeater plate (Biospec products, Bartlesville, OK). Set

the timer for 2 minutes and press the start button. After the time has elapsed, remove the tubes and insert the tubes into the Eppendorf 5415 D centrifuge. Allow the Centrifuge to spin for 15 seconds and once the machine has stopped spinning, place the spun tubes into a Reversible micro centrifuge rack (Cole-Parmer, Vernon Hills, IL) Using a VWR pipette extract 5 µl of supernatant into each well. Dispose of each tip after each use.
To use samples: Remove plant xylem samples from the freezer and allow them to thaw out for 15-20 minutes.

Prepare PCR reagents:

Prepare 6000 µl (6ml) of PCR reagent by adding 3300µl (3.3ml) of 2X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 2500 µl (2.5 ml) of Nanopure H20, 54 µl of X. fastidiosa ITS-Forward Primer and X. fastidiosa ITS-Reverse primer (Integrated DNA technologies, Coralville Iowa),, and 135 µl of X. fastidiosa FAM/TAMRA Fluorogenic Probe (Integrated DNA technologies, Coralville Iowa), into the same 50 ml twist-cap tube.
Mix the contents of each tube with a Vortex Genie 2 for a few minutes to ensure that the primers and probes have completely homogenized.

Prepare AB 7300 Computer Program:

Turn on the AB 7300 and then turn on the AB Dell Desktop computer and wait until the desktop loads
Double-click the icon that reads "7300 System Software"
Click on "File," then click "New"
A New Document Wizard window will appear
Make sure that Assay reads: Absolute Quantification (Standard Curve)
Container: 96-Well Clear
Template: Blank Document
Run Mode: Standard 7300
Operator: 7300 User
Comments: SDS v1.3.1
Plate Name: (what you want the file to be called)
A window will appear which will ask to "Select Detectors," choose the Detector Name "X-Sample;" Description XYLELLA SA; Reporter "FAM;" Quench "TAMRA" and Press "Add" Make sure that the Positive Reference is set at "ROX"
Continuing with the "New document wizard," the next window will say "Set up Sample Plate"
Click on the square that is located above the A and to the left of the 1, so that the sample plate becomes highlighted
Check the box that is located to the left of "X-Sample" below the "Use" column
The highlighted area on the bottom should have inserted green boxes which contain a "U" into each well of the diagram
Click "Finish" to complete the diagram
Once "Finish has been clicked" a full screen diagram is created of the sample layout seen earlier in the "Set up Sample Plate" window

Note each cell which contains a "U" represents a potential sample, to insert positive or negative controls click once on the Hide/Show Well Inspector application icon located in the toolbar below Tools, Instruments, and Analysis
The Well Inspector window will open which will allow the change of sample cells to positive or negative control cells
Click on the cell that needs to be changed and enter a name for the cell in the "Sample Name" blank
If the cell will be used for a sample, make sure that under the "Task" column reads "Unknown".
If the cell will be used for a positive control, scroll over "unknown" and click once to open a pull down menu.
Scroll down and Highlight "Standard" for a positive control.
For a negative control follow the same protocol but instead of selecting "Standard" select "NTC" under the "task" column

Once complete the "U" on the well diagram should change to a "S" for a positive control and "N" for a negative control. If you added a name, the name should also appear on the well diagram under the "Setup Tab"
Print a hard copy of the well diagram for reference later while pipetting samples into each well
Click Save to update the file
Click once on the Instrument tab and click once on Stage 1 of the Thermal Cycler Protocol.
Once Stage 1 is selected and is highlighted press the "delete" key on the keyboard
Deleting the original Stage 1 of 4 will allow for 3 stages, the first being Stage: 1 Reps: 1 95.0°C 10:00; Stage: 2 Reps: 40 95.0°C 0:15; Stage 3: 60.0°C 1:00
When the AB 7300 has been loaded with a PCR plate, to start the machine press the "start" button
Click Save to update the file

Prepare Test Plate:

Remove frozen plant samples from the freezer and allow them to thaw
Using one MicroAmp^{tm} Optical 96-Well Reaction Plate (Applied Biosystems, Foster City, CA)
Pour the contents of the Tube containing the PCR reagent cocktail into a pipette tray
Pipette 45 µl of reagent mix into each well
Using the diagram created before with the AB software, Pipette 5 µl of sample into each well
After each sample dispose of the tip into a waste receptacle
When the entire plate is completed, seal the plate with a special adhesive tape designed for PCR and using a rubber spatula blade make sure the tape is firmly attached to the plate
Place the plate on the vortex genie and vortex the plate several times in each side of the plate

Check for air bubbles by looking under the plate and flick any wells that contains air bubbles
Spin for 1-2 minutes in a centrifuge
Check again for air bubbles and remove any bubbles by flicking the bottom of the well with bubble.
Open the program on the computer that was created earlier and load the plate into the AB7300 with the foam pad on top of the tray with the grey side down
Close the AB 7300 drawer and start the program
The Program will run for about one hour forty minutes.

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