INCIDENCE OF *XYLELLA FASTIDIOSA* IN OKLAHOMA, A SURVEY OF POTENTIAL INSECT VECTORS, AND IDENTIFICATION OF POTENTIAL PLANT RESERVOIR HOSTS

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INCIDENCE OF *XYLELLA FASTIDIOSA* IN OKLAHOMA, A SURVEY OF POTENTIAL INSECT VECTORS, AND IDENTIFICATION OF POTENTIAL PLANT RESERVOIR HOSTS

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Abstract: The bacterium *Xylella fastidiosa* is the causative agent of specific plant diseases associated with peach, plum, oleander, almond, elm, oak, pecan, sycamore, and Pierce's disease of grapes. The bacterium is transmitted by xylem sap-feeding insects. In recent years, Pierce's disease was detected in eight counties in central and northeastern Oklahoma, prompting further investigation of the disease epidemiology in this state. I surveyed Oklahoma for xylem sap-feeding insects to determine the species composition within vineyards, nurseries, and pecan orchards and whether or not they harbored *X. fastidiosa*. A second objective involved determining the natural inoculativity of insect vectors commonly occurring in vineyards throughout the state. In the third objective, I surveyed common weeds in vineyards to identify potential reservoir hosts of *X. fastidiosa*. Weedy plants sampled in this study are known or suspected food sources of xylem sap-feeding insect vectors, are commonly found in Oklahoma vineyards, and have been implicated as potential reservoirs for *X. fastidiosa*. Immunocapture polymerase chain reaction was used to test all insect and plant samples in this study. I found the following sharpshooters in my survey: *Xyphon flaviceps*, *Draeculacephala navicula***,** *D. robinsoni*, *Graphocephala coccinea*, *G. versuta*, *G. hieroglyphica*, *Cuerna costalis*, *Oncometopia orbona*, *Homalodisca vitripennis*, *Prosapia bicincta*, and *Clastoptera xanthocephala*. The three most frequently captured species in vineyards and tree nurseries included *G. versuta*, *C. xanthocephala*, and *O. orbona*. From a subsample of insects screened for *X. fastidiosa*, 2.4% tested positive for the bacterium. Field-collected *G. versuta* inoculated *X. fastidiosa* to susceptible indicator plants, ragweed and alfalfa. A higher percentage of alfalfa became infected than ragweed following inoculation periods. Very few plants tested positive and none of the plants testing positive are considered new hosts of *X. fastidiosa*. These results indicate that weedy reservoir hosts may not be a contributing factor to the epidemiology of Pierce's disease in Oklahoma. From this study, I found that insect vectors were present and persistently captured from vineyards, nurseries and pecan orchards. The most predominant insect vector, *G. versuta*, was most abundant in June and July and was infective and competent to spread *X. fastidiosa* to susceptible plant host.

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CHAPTER I

INTRODUCTION

Xylella fastidiosa Wells et al. (1987) is a bacterial plant pathogen that causes Pierce's disease (PD) of grape, citrus variegated chlorosis (CVC), phony peach disease (PPD), alfalfa dwarf, almond leaf scorch (ALS), oleander leaf scorch (OLS), and leaf scorch of pecan, plum, and many species of shade trees. Although *X. fastidiosa* can infect more than 100 plant species in almost 50 families, it is not pathogenic in all plant hosts (Schaad et al. 2004).

Xylella fastidiosa is not transmitted from plant to plant without an insect vector (Purcell and Hopkins 1996). However, more recent studies have shown that inoculation of trees by needle injection is possible (Sanderlin 2005). The bacterium can also be transmitted through use of infected scion of pecan (Sanderlin and Melanson 2006), pruning shears (Krell et al. 2007), or propagation of infected grape cuttings (Robacker and Chang 1992). Several subspecies of *X. fastidiosa* are infectious to one or several plant species and in some cases inhabit the plant without causing symptoms. For instance, the subspecies that causes symptoms in plum will not induce symptoms in grapevines (Schaad et al. 2004). Common symptoms include leaf scorch characterized

by cell death of the leaves beginning at the leaf margins with a chlorotic band separating dead from healthy tissue. *Xylella fastidiosa* subsp. *multiplex* is responsible for disease in peach, plum, almond, elm, pigeon grape, sycamore, and other trees. *Xylella fastidiosa* subsp. *pauca* causes disease in citrus and coffee while subsp. *fastidiosa* causes disease in grape, alfalfa, almond, and maples (Schaad et al. 2004). Finally, subsp. *sandyi* is associated with disease in oleander, daylily, *Jacaranda* sp., and magnolia (Hernandez-Martinez et al. 2007, Almeida et al. 2008). Recently, researchers proposed a fifth subspecies that causes scorching symptoms in chitalpa, *X. fastidiosa* subsp. *tashke* (Randall et al. 2009). Although Schaad et al. (2004) grouped *X. fastidiosa* into subspecies, the bacterium was originally grouped into strains, a useful identification scheme that is still commonly used (Varani et al. 2012).

In Oklahoma, *X. fastidiosa* subsp. *multiplex* that causes bacterial leaf scorch (BLS) has been detected in American elm, sycamore, mulberry, red oak, porcelain berry, and giant ragweed (Dominiak and Olson, 2006). Subspecies *fastidiosa* was reported to be limited to warmer climates and pose little threat to Oklahoma (Feil and Purcell 2001). However, *X. fastidiosa* subsp. *fastidiosa* that causes PD has been detected in Oklahoma (Smith et al. 2009), warranting investigation of PD in this state. Smith and Dominiak-Olson (2009) used temperature thresholds established by Anas et al. (2008) that tracks historical weather data to determine the relative risk for PD infection in Oklahoma. Low-risk vineyards were those with surface air temperatures at or below -12 °C for two to three days. Based on ten years of surface air temperature data, the authors predicted that Craig Co. in northeastern Oklahoma is at low risk for survival of the pathogen that causes PD but Tulsa, Payne, Lincoln, and Kiowa counties are at moderate risk (i.e., surface air temperatures $\leq$ -12 °C for less than two days). All other grape-producing counties did not reach the minimum threshold and were

considered at no-risk for incidence of pathogen winter survival. They found that all counties were considered at moderate risk in 2007 (Smith and Dominiak-Olson 2009).

Xylella fastidiosa is transmitted primarily by xylem sap-feeding insects including many members of the Order Hemiptera: spittle bugs (Cercopidae), sharpshooters (Cicadellidae: Cicadellinae), cicadas (Cicadidae), and tube-building spittlebugs (Machaerotidae) (Almeida et al. 2005). Also, *X. fastidiosa* has been detected in many species of treehoppers (Membracidae) in Virginia and New Jersey (Huang et al. 2006, Zhang et al. 2011). Glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) (formerly *H. coagulata* Say), is the major vector of two economically important diseases caused by *X. fastidiosa*, PD and CVC. Glassy-winged sharpshooters possess traits that enable them to transfer *X. fastidiosa* more readily among plants. They have greater flight capabilities than that of smaller sharpshooters and they feed on a broad range of plants (i.e., 100 plant species in 30 families) (Costa et al. 2006). In addition, GWSS will feed on woody tissue whereas other sharpshooter species tend to feed on green plant tissue (Almeida 2007). The ability to feed on dormant woody grape plants allows GWSS to inoculate the bacterium into plant tissues that are more protected from adverse weather, thus increasing the likelihood of bacteria successfully overwintering in the plant (Almeida 2007). This sharpshooter has been found in Oklahoma tree nurseries in small numbers, but has yet to be collected from vineyards in this state (Overall et al. 2010).

In general, *X. fastidiosa* inhabits and multiplies in the foregut of sharpshooter vectors, specifically within the cibarium and precibarium (Almeida and Purcell 2006). The foregut lining is shed during ecdysis and as a result, any bacterial cells adhering to the foregut lining are lost (Purcell and Finlay 1979). Further, the bacterium is not transferred from females to

their eggs (i.e., transovarial transmission) and if acquired by adult leafhoppers, is transmitted for the life of the vector (Freitag 1951).

I have initiated a statewide survey of Oklahoma tree nurseries, pecan orchards, and vineyards for insect vectors of *X. fastidiosa*. Previous studies in this state have identified leafhopper species in vineyards (Mulder et al. 2003); however, little is known of the leafhopper complex within tree nurseries. Although previous studies have documented the presence of xylem sap-feeding leafhoppers in vineyards, little is known of the capabilities of those leafhoppers to transmit *X. fastidiosa* among plants. There is no known cure for grapevines or shade trees infected with *X. fastidiosa*. Since there is no way to control diseases associated with *X. fastidiosa*, growers must rely on management tactics that prevent spread of the pathogen. Oklahoma grape growers and nurserymen need to know the risk of diseases caused by *X. fastidiosa* developing in their vineyards or nurseries, how to manage the disease once it's found, and how to prevent further spread within and among plants. Thus, my overarching objective is to identify major vectors of *X. fastidiosa* in Oklahoma vineyards and tree nurseries, determine what percentage of those vectors carry *X. fastidiosa*, whether they can transmit the bacterium to healthy vines or trees, and identify weedy host plants in and around vineyards and nurseries that could serve as reservoir hosts of *X. fastidiosa*. Specifically, I aim to:

1. Determine the distribution and peak abundance of potential insect vectors of *X. fastidiosa*, the causal agent of Pierce's disease and bacterial leaf scorch, in Oklahoma vineyards and tree nurseries.

2. Determine the ability of identified vectors to transmit *X. fastidiosa* to healthy, susceptible plants.

3. Identify weedy plant species within and near vineyards and tree nurseries that could serve as sources of *X. fastidiosa*.

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CHAPTER II

LITERATURE REVIEW

In the late 1800's, scorch-like symptoms on grape leaves and stunted growth of peach and plum were observed in the United States, Mexico, and Argentina, yet a real connection was lacking among these plant disease symptoms (Pierce 1892, Turner and Pollard 1959a, Chang and Yonce 1987). The etiological agent causing leaf scorch and stunting in the infected plants was thought to be a virus due to the difficulty in culturing and graft transmissibility of the pathogen (Turner and Pollard 1959a, Hopkins 1977). In the 1970's, a similar rickettsia-like organism was found to be likely responsible for leaf scorch and stunted growth in grape, peach, plum, almond, periwinkle, and alfalfa based on morphological and serological tests (Goheen et al. 1973, Hopkins et al. 1973, Hopkins 1977, McCoy et al. 1978, Raju et al., 1981). Once suspected to be a virus, a rickettsia-like organism, a mycoplasma-like organism, and even a gram-positive bacterium (Schaad et al. 2004), the pathogen was isolated from grape and described as a gram-negative, aerobic, rod-shaped bacterium (Davis et al. 1978). Wells et al. (1987) described the

bacterium in greater detail and proposed the name, *Xylella fastidiosa*. The bacteria grow slowly with doubling times of 9 h to 2.3 days (Wells et al. 1987). Bacterial cells are non-motile rods and aflagellate; however, filamentous cells have been documented on artificial media. They have been referred to as "rickettsia-like" due to cell wall structure that appears "rippled," which is characteristic of bacteria in the families Rickettsiaceae and Legionellaceae. Circular colonies observed on media have been described as opalescent with sizes ranging from 0.6 mm to 1.5 mm, depending on colony age (Wells et al. 1987). *Xylella fastidiosa* is considered fastidious (i.e., requires specialized media for growth) and is strictly limited to plant xylem. Other examples of xylem-limited bacteria include *Pseudomonas syzygii* and *Clavibacter xyli* that cause Sumatra disease of cloves and ratoon stunting disease of sugarcane, respectively (Purcell and Hopkins 1996). *Xylella fastidiosa* belongs to the widespread family Xanthomonadaceae, which contains one other plant pathogenic genus of bacteria, *Xanthomonas*. Xanthomonadaceae consists of gram-negative proteobacteria and contains approximately 22 genera (Mhedbi-Hajri et al. 2011).

Plant Colonization

Xylella fastidiosa colonizes and multiplies in host plant xylem. Xylem transports water upward to the distal tissues for metabolic use and is composed of vessels of dead, lignified cells that are joined by bordered pits or channels. The bordered pits have a membrane that restricts large objects from passing (Newman et al. 2003). *Xylella fastidiosa* cells will attach to the vessel wall and multiply, subsequently forming a biofilm that, at high titers, can block the passage of water, which leads to leaf scorch (Newman et

al. 2003). To better visualize the bacterial communities in xylem vessels, Newman et al. (2003) genetically engineered colonies of *X. fastidiosa* that expressed a green fluorescent protein (Gfp). They found that *X. fastidiosa* colonizes the host plant as solitary cells or small colonies in more than 80% of infected vessels. Bacterial movement between vessels occurred via pit membranes and was important for colonization. Artificial blockage of the vessels was found to interfere with bacterial colonization (Newman et al. 2003). Interestingly, smaller bacterial colonies contained all live cells whereas heavily colonized vessels contained a greater number of dead cells, supporting the hypothesis that complete vessel blockage is detrimental to the bacteria. This suggests that *X. fastidiosa* is predominantly endophytic and occasionally or accidently blocks xylem vessels (Chatterjee et al. 2008a). Within a susceptible host plant, *X. fastidiosa* is able to colonize and multiply, subsequently blocking the passage of water and inducing symptom development (Chatterjee et al. 2008a). McElrone et al. (2003) showed that *X. fastidiosa* also induces low shoot hydraulic conductance by blocking the vessels. Furthermore, stress imposed by drought may increase development of symptoms.

Given the wide host range of *X. fastidiosa*, most plants infected are not susceptible but support low populations with little inter-vessel movement (Purcell and Saunders 1999). Host plants of *X. fastidiosa* can be grouped into three general categories based on the fate of the bacteria within that host: propagative or non-propagative, systemic or non-systemic, and pathological or non-pathological. *Xylella fastidiosa* is able to multiply within a propagative host, move between xylem vessels in a systemic host, and cause observable symptoms in a host affected pathogenically (Purcell and Saunders 1999).

Bacterial Colonization of Insect Foregut

Bacterial colonization differs in plant and insect hosts. In plant hosts, *X. fastidiosa* attaches to the xylem vessel wall in any orientation and forms a matrix-enclosed community. In contrast, bacteria in insects attach in a polar arrangement to the cuticle, forming a mat-like community (Newman et al. 2004). Colonies of *X. fastidiosa* inhabit the foregut of insect vectors, more specifically, the longitudinal groove that leads to the esophagus and cibarium (Alves et al. 2008) and must be able to withstand the fast flow of the xylem fluid being ingested by the insect (Silva et al. 2011). The bacterial cells attach laterally to insect foregut cuticle via carbohydrate-binding proteins, such as adhesins HxfA, and HxfB (Killiny and Almeida 2009). In fact, *X. fastidiosa* has numerous genes encoding adhesions and hemaglutinins, which reflect the ability of bacterial cells to adhere to two differing substrates (insect foregut and plant xylem vessel wall). Adhesins are important for biofilm formation and aggregation of cells in both the insect vector and plant host (Chatterjee et al. 2008a) and are conserved among gram-negative bacteria that colonize diverse hosts (Mhedbi-Hajri et al. 2011). After initial attachment to the cuticle of the insect foregut, bacterial titers increase and cells in the center of the colony become attached in a polar fashion, likely via type I pili, which increases the absorption of nutrients (Killiny and Almeida 2009). Almeida and Purcell (2006) detected *X. fastidiosa* cells on the precibarium of the blue-green sharpshooter, *Graphocephala atropunctata* (Signoret), and found that colonization of the precibarium was important for successful inoculation of the plant.

Jackson et al. (2008) studied the vector, *H. vitripennis*, and found that the size of the bacterial community within the foregut does not increase or decrease the inoculation rate of the bacteria. Plant variables may affect insect transmission. Daugherty et al. (2011) found that GWSS prefers to feed on healthy grapevines or those that are infected but asymptomatic. This is important because if GWSS preferred to feed on unhealthy and symptomatic grape vines, this could increase the spread and incidence of PD in vineyards (Daugherty et al. 2011). Further, Rashed et al. (2011) found that transmission efficiency of GWSS was not affected by different grape cultivars or the titers of *X. fastidiosa* within petioles. These results did not support previous observations by Hill and Purcell (1997) that linked higher bacterial populations in the plant to increased transmission rates.

Insect Inoculation

Xylella fastidiosa gains entry into the plant via feeding by xylem sap-feeding sharpshooters and spittlebugs. These insects have piercing-sucking mouthparts characterized by mandibular and maxillary stylets that are sheathed by the labium. Xylem fluid is drawn up through the stylets into the precibarium, then to the cibarium (i.e., sucking pump) before finally reaching the esophagus (Backus 1988). The precibarium is a narrow canal that leads to the cibarium (Backus 1988) and is the site of bacterial attachment and colonization (Almeida and Purcell 2006). Purcell and Finlay (1979) studied *Draeculacephala minerva* Ball and *G. atropunctata* (Signoret) and reported a non-circulative transmission mechanism (i.e., pathogen is not internalized) that did not require a latent period (i.e., insects were able to transmit bacteria after a 1-hour

acquisition period). The non-circulative transmission mechanism was supported further by their observation that nymphs lost the ability to transmit bacteria after molting. *Xylella fastidiosa* is considered to be propagative since it is able to reproduce within the insect vector foregut. In fact, it is the only known insect-transmitted pathogen that is both non-circulative and propagative (Backus et al. 2009). Based on electrical penetration graph studies of *H. vitripennis,* Backus et al. (2009) proposed the ingestion (salivation and egestion) hypothesis to explain pathogen transmission. In this model, insects acquire the bacterium via ingestion of bacteria-contaminated plant juices and inoculation of a healthy plant occurs through a combination of both salivation and egestion. Sharpshooter saliva is secreted into the plant through the salivary canal and then is quickly sucked back into the anterior foregut through the food canal. In the food canal, saliva passes forcefully over the bacterial biofilm due to actions of the pre-cibarial valve and cibarium pump. Finally, the bacteria are dislodged and subsequently egested into the plant (Backus et al. 2012).

Plant Host Entry and Infection

Whole-genome sequencing of *X. fastidiosa* subsp. *pauca* (Simpson et al. 2000) and subsp. *fastidiosa* (Van Sluys et al. 2003) enabled investigators to identify gene sequences responsible for host entry and infection. Based on sequences, *X. fastidiosa* is closely related to *Xanthomonas campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*, which are both pathogens of vascular tissue that gain entry into the plant via hydathodes (i.e., terminal extensions of xylem vessels). Unlike *X. fastidiosa* that is strictly limited to xylem vessels, *Xanthomonas* spp. are able to colonize the mesophyll layers after the

initial colonization of xylem tissue. Subspecies of *X. fastidiosa* and *Xanthomonas* spp. share similar gene sequences involved in host entry, yet have varying mechanisms of host interactions based on colonization of differing plant tissue (i.e., xylem and mesophyll cells, respectively) (Chatterjee et al. 2008a). A draft genome sequence of a non-pathogenic strain, EB92-1 (i.e., infectious without inducing symptoms) was recently published. Comparing this draft sequence to that of the pathogenic Temecula1 strain of *X. fastidiosa* subsp. *fastidiosa*, researchers were able to identify ten genes involved in pathogenicity (Zhang et al. 2011b).

Xylella fastidiosa lacks a type III secretion system, found in *Xanthomonas* spp., that is important to combat responses of the host plant defense (Chatterjee et al. 2008a). Plant defense is activated by detecting pathogen-associated molecular proteins (PAMP). The lack of the type III secretion system in *X. fastidiosa* is reasonable since the bacteria are not known to make contact with living plant tissue (i.e., they inhabit non-living xylem vessels). *Xylella fastidiosa* possesses a type II secretion system, also found in *Xanthomonas* spp., that is involved in secreting enzymes that degrade plant cell walls, including endoglucanases, xylanases, xylosidases, and a single polygalacturonase, which are considered "effecters" and thought to be involved in degradation of plant host pit membranes. This secretion system is important for movement of colonizing bacteria between xylem vessels. A polygalacturonase-deficient mutant of *X. fastidiosa* subsp. *fastidiosa* was unable to colonize grape plants and cause disease symptoms (Chatterjee et al. 2008a).

Xylella fastidiosa has type IV pili located at one end of the rod-shaped cells. Type IV pili are long and involved in twitching motility that enables upstream migration.

Upstream migration can explain the ability of *X. fastidiosa* to move long distances from the point of inoculation (Meng et al. 2005, Li et al. 2007). Twitching motility describes bacterial movement over moist surfaces in search of new colonization surfaces and is independent of flagella. This form of motility is characteristic of gram-negative bacteria primarily and is important in host colonization and subsequent biofilm formation (Mattick 2002). Type IV pili are involved in attachment to host surfaces, a critical precursor to biofilm formation (Li et al. 2007). *Xylella fastidiosa* also has shorter type I pili that are associated with cell wall attachment (Chatterjee et al. 2008a) and influence the density of the biofilm formed (Li et al. 2007). Type I pili are located at the same bacterial cell pole as type IV pili (Van Sluys et al. 2003). Types I and IV pili are involved in auto-aggregation that is an additional precursor to biofilm formation (De La Fuente et al. 2008). Type I pili are not involved in twitching motility (Li et al. 2007) but do influence speed of movement (De La Fuente et al. 2007).

Cell-Cell Signaling and Biofilm Formation

In their natural environments bacteria form biofilms, which provide protection from toxins such as antibiotics, allow for better rates of conjugation (i.e., horizontal gene transfer), and provide a means to store energy in exopolysaccharides (EPS). Biofilms may be composed of a single species or multiple species of bacteria (Watnick and Kolter 2000). *Xylella fastidiosa* cells aggregate and form biofilms in both the insect vector and plant host, and also form biofilms in culture. Secretion of EPS (fastidium gum) is important in the formation of biofilms based on studies that show mutants lacking an operon that encodes EPS were unable to form functional biofilms (Wulff et al. 2008).

Exopolysaccharides serve many functions including structural integrity and attachment of *X. fastidiosa* to host surfaces and neighboring bacterial cells. Enzymes are localized and stabilized by EPS thickness, allowing them to degrade plant cell walls and aid in digestion of xylem pit membrane barriers. The degradation of cell walls facilitates systemic movement of *X. fastidiosa* within the host plant (Roper et al. 2007).

Within the plant, pH influences aggregation of bacterial cells and formation of the biofilm (Wulff et al. 2008). Variations in biofilm clumping in liquid broth have been observed among the strains of *X. fastidiosa*. Strains from coffee, citrus, and grape tended to form more clumps in liquid media than strains from plum, almond, and elm (Marques et al. 2002). Zaini et al. (2009) were able to culture *X. fastidiosa* in media supplemented with xylem sap and found that its presence increased biofilm formation. Additionally, Cheng et al. (2009) found that the presence of xylem sap from PD-susceptible plants in media better supported bacterial growth and biofilm formation compared to that grown in media supplemented with xylem sap from PD-resistant plants. Based on additional in vitro studies, they found the following cell wall components promoted biofilm formation: laminarin, xylan, and k-carrageenen. Cheng et al. (2010) observed that *X. fastidiosa* grown in media produced extracellular DNA and postulated its importance in planktonic growth, biofilm formation, and viability of cells. They found that extracellular DNA had a positive effect on planktonic growth and biofilm formation but were associated with less viable cells. Rodrigues et al. (2008) evaluated the effects of copper, an antimicrobial compound, on bacterial biofilms and planktonic cells. They found that cells within a biofilm were less affected by copper than those that were planktonic. Based on these results, it is speculated that bacteria within the biofilms use a more complicated

mechanism to resist the effects of antimicrobials as opposed to simply acting as a barrier to diffusion of the compounds (Rodrigues et al. 2008).

Similar to other pathogenic bacteria, *X. fastidiosa* is able to control self-gene expression in a cell density-dependent fashion (Chatterjee et al. 2008a). The ability to control gene transcription is essential for pathogens that have intricate life cycles and inhabit multiple hosts (Almeida et al. 2012). Newman et al. (2004) found that cell-to-cell signaling is important for regulating both insect transmission and virulence. *X. fastidiosa* uses cell-to-cell signaling within both insect vectors and host plants, which is mediated by a fatty acid diffusible signaling factor (DSF). The signaling system controls production of adhesins and EPS (Chatterjee et al. 2008b). Mutated *X. fastidiosa* cells are unable to produce DSF or colonize the insect vector foregut and are not transmitted to plants due to lowered production of adhesins and deficiencies in biofilm formation (Newman et al. 2004, Chatterjee et al. 2010, Almeida et al. 2012). However, these same mutated *X. fastidiosa* cells are virulent in the plant host. Wild type *X. fastidiosa* cells are able to produce sufficient amounts of extracellular enzymes and type IV pili that are critical in xylem vessel-vessel movement but are not virulent (Chatterjee et al. 2010). The DSF-mediated cell-to-cell signaling system is critical in maintaining a balance between successful insect transmission of the pathogen while promoting colonization and spread within the plant host (Newman et al. 2004, Chatterjee et al. 2010). Almeida et al. (2012) suggest that multiple DSF molecules may coordinate the signaling system via regulation and pathogenicity factors for colonization of both plant and insect hosts. Chatterjee et al. (2010) found that cyclic di-GMP is critical to both extracellular and intracellular signaling. When concentration of cyclic di-GMP is low, DSF accumulates

while cell density increases. This leads to a change in the bacterial phenotype from planktonic that is able to colonize the plant to an adhesive state that is able to be transmitted by the insect vector (Chatterjee et al. 2010). A better understanding of interactions between *X. fastidiosa* cells within vectors and host plants will elucidate effective disease management strategies (Guilhabert and Kirkpatrick 2005).

Differentiation of Subspecies of *Xylella fastidiosa*

Although Wells et al. (1987) grouped several strains of *X. fastidiosa* into one species, there is evidence that multiple subspecies exist based on differing media requirements, DNA analysis, and pathogenicity studies (Chen et al. 2000a, b, Schaad et al. 2004). Early investigators were limited in detection capabilities and relied on partial DNA detection such as restriction fragment length polymorphism (RFLP) (Chen et al. 1992), randomly amplified polymorphic DNA (RAPD) analysis (Chen et al. 1995), repetitive extragenic palindromic element-polymerase chain reaction (REP-PCR) and RAPD analysis (Hendson et al. 2001), PCR amplification of genomic DNA fragments, and enzyme-linked immunosorbant assay (ELISA) (Minsavage et al. 1994). The development of PCR enabled researchers to sequence whole genomes, which led to more reliable tools for detection of pathogens.

A ribosomal gene sequence, 16S rDNA, has been extensively used to compare *X. fastidiosa* strains from diseased plants and establish genetic relatedness of strains (Chen et al. 2000a, b, Chen et al. 2002, Huang and Sherald 2004). In addition to the 16S rDNA, other genes have been targeted to detect *X. fastidiosa* including an enzyme encoding gene sequence, XF1968, and a protein coding gene sequence, XF2543. These sequences were

used to successfully detect and differentiate the subspecies *fastidiosa* and *sandyi* in GWSS adults (Costa et al. 2006). Investigators have developed primers that target the *gyrase B* (*gyrB*) gene in *X. fastidiosa*, which can also be used to differentiate subspecies since each have differing genetic sequences; yet, overall the gene sequence is conserved, allowing use of specific primers. Using real-time (RT) PCR coupled with fluorescent SYBR® Green dye, Bextine and Child (2007) were able to distinguish Pierce's disease, almond leaf scorch, and oleander leaf scorch strains. Bextine et al. (2008) have targeted multiple *TonB* genes, which encode outer membrane proteins, and the *zonula occludens toxin* (*ZOT*) gene to differentiate *X. fastidiosa* subspecies present in insect vectors using RT-PCR. These researchers are working to develop a simple and reliable method to differentiate subspecies that does not require sequencing of the strains.

Multilocus sequence typing (MLST) can be used to differentiate strains of *X. fastidiosa*. This method involves sequencing short regions of housekeeping genes that are conserved (Yuan et al. 2010, Nunney et al. 2012, Parker et al. 2012). Multilocus sequence typing has been used in typing of human pathogens and other plant pathogens such as *Pseudomonas syringae* and *Ralstonia solanacearum* and is regarded as powerful and reliable for differentiating species (Nunney et al. 2012). Nunney et al. (2012) were able to detect contamination in the first published sequence of *X. fastidiosa* subsp. *pauca*, Ann-1 genome. Based on their MLST analysis of Ann-1 genome and the Dixon genome (X. *fastidiosa* subsp. *fastidiosa*), they found the former deposited sequences were contaminated with the latter. They are currently re-sequencing the Ann-1 genome for deposit in GenBank (Nunney et al. 2012). They also determined that a commercially available strain, American Type Culture Collection (ATCC) 700964 Temecula1 (*X.*

fastidiosa subsp. *fastidiosa*), was more similar to *X. fastidiosa* subsp. *multiplex*. This supports the use of MLST to differentiate strains of *X. fastidiosa* as well as ensure the integrity of identities of strains used in research (Nunney et al. 2012). There is evidence that *X. fastidiosa* is able to exchange DNA among subspecies and that more than one subspecies can be found co-inhabiting the same plant or insect vector, which can increase genetic diversity (Kung and Almeida 2011). Researchers have recently compiled all sequenced *X. fastidiosa* genomes into a public-accessible database (Varani et al. 2012).

Today, four separate subspecies of *X. fastidiosa* are recognized based on genotype, serological differences, and host ranges. *Xylella fastidiosa* subsp. *multiplex* is responsible for disease in peach, plum, almond, elm, pigeon grape, sycamore, and other trees. *Xylella fastidiosa* subsp. *pauca* causes disease in citrus and coffee. *Xylella fastidiosa* subsp. *fastidiosa* causes disease in grape, alfalfa, almond, and maples (Schaad et al. 2004). A fourth, subsp. *sandyi*, is associated with disease in oleander, daylily, Jacaranda, and magnolia (Hernandez-Martinez et al. 2007, Almeida et al. 2008). While the subspecies grouping is commonly recognized as *X. fastidiosa*, six strains can be delineated based on highly conserved housekeeping genes (Scally et al. 2005). The following are strains with published and accessible genomes: citrus variegated chlorosis (9a5c), Pierce's disease (Temecula1), almond leaf scorch and Pierce's disease (M23), and almond leaf scorch (M12). There are two additional strains that cause oleander leaf scorch (Ann1) and almond leaf scorch (Dixon); however, sequenced genomes are not published (Varani et al. 2012). Recently, researchers proposed a fifth subspecies that causes scorching symptoms in chitalpa, *Chitalpa tashkentensis*, which is a common tree in New Mexico, Arizona, and California landscapes. The proposed name of the new

subspecies is *X. fastidiosa* subsp. *tashke (*Randall et al. 2009). Pear leaf scorch is the only disease caused by *X. fastidiosa* that occurs in Taiwan. Based on sequence variability of the 16S rRNA gene and 16S-23S ITS sequences, Su et al. (2012) proposed that strains causing pear leaf scorch in Taiwan may belong to a new subspecies and should be further studied. It has been difficult to group the strains into definitive subspecies or pathovars because many strains are cross-infective (i.e., two strains cause disease in the same plant host but are genetically distinct and the infection is not always reciprocal) (Hendson et al. 2001, Almeida and Purcell 2003a).

Background of Diseases Caused by *X. fastidiosa* in North America

Pierce's Disease

The earliest account of disease in grapes caused by *X. fastidiosa* occurred in the late 1800's and was referred to as California vine disease by N. B. Pierce (Pierce 1892). The cause of the disease was unknown. Pierce (1892) described symptoms of the disease, which typically manifested in the fall, and variations were seen in different grape varieties. Most diseased varieties showed characteristic symptoms of marginal leaf necrosis separated from the healthy leaf tissue by a yellow chlorotic band. Not all varieties displayed the characteristic yellow banding (e.g., 'Riesling'). Symptoms in grape canes appeared as distinct green areas due to unequal hardening of stems (Pierce 1892). Although PD was first described by a California specialist, PD was originally suspected to be native to the southeastern United States based on presence of PD-tolerant wild grape varieties in that region (Purcell 1977). More recently, Nunney et al. (2010) provided evidence that *X. fastidiosa* subsp. *fastidiosa* is native to Central America. Their

findings are based on a lack of genetic variability of North American strains of *X. fastidiosa* subsp. *fastidiosa* compared to Central American strains. Nunney et al. (2010) proposed that the subspecies that causes Pierce's disease likely was introduced into southern California via the importation of coffee plants from Central America in the late to mid-1800's. Alternatively, the pathogen that causes Pierce's disease could have been introduced into Texas or Florida and subsequently spread to California (Nunney et al. 2010). In the early 1900's, PD devastated vineyards in Orange County, California, prompting growers to relocate. Regular outbreaks of PD continued to occur in the coastal valleys of northern California and in the Central Valley (Goheen et al. 1973). By the twentieth century, occurrence of PD had significantly decreased until a recurrence of PD in California in the mid 1930's prompted renewed efforts to study the epidemiology of the disease and the renaming of California vine disease to Pierce's disease (Hewitt et al. 1946).

In Florida, sixteenth-century Spanish settlers attempted to plant varieties of European grape, *Vitis vinifera*, based on success of native grape species. However, all attempts to establish exotic varieties failed due to what investigators considered "degeneration" four centuries later. Investigators soon realized that symptoms described as "degeneration" in Florida were similar to those described for PD in California (Stoner 1953). At the time, the pathogen responsible for disease symptoms had yet to be identified.

Although Pierce (1892) observed bacterial bodies associated with PD, the pathogen was assumed to be a virus based on graft transmissibility and the difficulty in culturing the pathogen (Goheen et al. 1973, Hopkins 1977). Through ongoing studies,

investigators learned that tetracycline soil treatments reduced symptoms in PD-infected vines and concluded that the etiological agent may be a mycoplasma-like organism (MLO) based on previous observations that MLO's were negatively affected by tetracycline (Hopkins and Mortensen 1971). Three years later, investigators reported the observation of rickettsia-like organisms inhabiting PD-infected grape plant xylem and proposed the bacteria were related to those in the family Rickettsiae (Goheen et al. 1973, Hopkins and Mollenhauer 1973). Using serological assays, Raju et al. (1981) determined that the pathogen causing PD in the United States and Central America, almond leaf scorch, alfalfa dwarf, phony peach, plum leaf scald, and decline of citrus were all related. Development of detection methods such as ELISA enabled scientists to detect *X. fastidiosa* in other host plants (Raju et al. 1980, Nome et al. 1981).

In the early 1940's, leafhoppers were identified as vectors of the pathogen, *X. fastidiosa*, that causes PD and alfalfa dwarf in California (Hewitt et al. 1946). Initially, insects were the primary suspects based on the patterns of disease occurrence in alfalfa fields. From transmission studies, four sharpshooters were found to transmit *X. fastidiosa* subsp. *fastidiosa* in California: *D. minerva, Xyphon* (=*Carneocephala*) *fulgida* (Nott.), *Helochara delta* Oman, and *Neokolla circullata* (Baker) (Hewitt et al. 1946). Additional leafhopper vectors included *Cuerna occidentalis* Oman and Beaver, *C. yuccae*, and *Homalodisca liturata* Ball. Numerous spittlebug (Cercopidae) species were also found to transmit *X. fastidiosa* subsp. *fastidiosa* and included *Aphrophora angulata* Ball, *A. permutata* Uhler, *Clastoptera brunnea* Ball, and *Philaenus leucophthalmus* (Linnaeus). *Draeculacephala minerva, G. atropunctata,* and *X. fulgida* were considered the most important vectors in California due to their widespread distribution (Freitag et al. 1952).

Once the connection was made between PD of grape in California and "degeneration" of grapes in Florida, leafhoppers were identified as vectors of agents causing "degeneration" in Florida and those identified include *Oncometopia nigricans* (Walker), *H. vitripennis* (Germar), and *X. flaviceps* (Stoner 1953, Adlerz and Hopkins 1979). In the 1990's, the glassy-winged sharpshooter (GWSS) was first found in California (Sorensen and Gill 1996) and within ten years populations of GWSS had increased in vineyards, quickly spreading PD (Hopkins and Purcell 2002).

To date, PD limits the grape-growing industries in California with losses exceeding $10 million in one region, the Temecula Valley (Myers et al. 2007). In addition, PD limits production in Texas and North Carolina (Myers et al. 2007, Mitchell et al. 2009). Pierce's disease has been detected in vineyards across the state of Virginia and in vineyards that had previously been considered low risk for the disease (Wallingford et al. 2007). Several cases have been reported in Oklahoma (Smith et al. 2009) and a recent outbreak has occurred in New Mexico (Randall et al. 2007). Pierce's disease is limited by cold winters and, therefore, restricted to warmer climates (Purcell 1997); however, warming trends seem to be contributing to its range expansion northward in the southeastern United States (Anas et al. 2008). Ongoing programs are in place in all states to develop integrated pest management strategies. In a Florida vineyard, they found that infecting young vines with strains of *Xylella* that do not cause PD helped to control the incidence of PD on those vines (Hopkins 2005). Plant breeding programs have led to development of tolerant grape cultivars by incorporating resistant genes from grape cultivars that are native to the southeastern United States (e.g., muscadine) (Hopkins and Purcell 2002). Similar varieties have recently been planted in

California, Texas, and Alabama to evaluate the quality of the fruit and juice produced (Walker et al. 2011). Another approach to developing PD-resistant grape varieties involves genetically engineering varieties that express antimicrobial proteins that target and suppress *X. fastidiosa* subsp. *fastidiosa* (Dandekar et al. 2012).

Insect vectors are also targeted in muti-tactic IPM programs. For instance, in the California Central Valley, PD occurred on vines planted at the vineyard edge near pastures or hayfields. These crop habitats contained species that served as primary food sources for *X. fulgida* and *D. minerva.* The grape plants were "accidental feeding hosts" for these sharpshooters when they were displaced from their preferred habitat due to harvest or mowing (Purcell and Frazier 1985, Hopkins and Purcell 2002). In the coastal region of California, the incidence of PD was greater on vines that were adjacent to water sources such as streams or irrigated landscapes, which are preferred habitats of *G. atropunctata* (Hopkins and Purcell 2002) and contain elderberry and blackberry, which are proven reservoirs for strains of *Xylella fastidiosa* that cause PD (Baumgartner and Warren 2005). In both examples, infected vines were limited to localized areas and it was less likely that late-season infections would develop and would not survive winter temperatures (Almeida and Purcell 2003c). After the introduction of the larger GWSS PD became a chronic problem in California since this species can feed on woody tissues, allowing bacteria a better chance of winter survival (Almeida and Purcell 2003c). There are ongoing studies seeking to understand the biology of GWSS and develop more effective IPM programs that limit its spread in California and these studies are discussed in greater detail later.

Almond Leaf Scorch

Almond leaf scorch (ALS) is caused by *X. fastidiosa* subsp. *fastidiosa* (Schaad et al. 2004) and was first described in California in the mid 1970's. However, it may have been present since the 1930's (Mircetich et al. 1976). Initial symptoms include leaf scorch followed by reduction in fruit production and eventual death within two years (Cao et al. 2011). Due to sporadic occurrence of ALS in California, it has not been a major limitation to almond production.

In California almond orchards, the primary insect vector of ALS is *D. minerva*, which does not preferentially feed on almond. Therefore, growers can eliminate or reduce weedy plants that are nearby the orchard to decrease occurrence of leafhopper vectors (Sisterson et al. 2012). It was thought that GWSS might pose a significant threat to almond orchards based on its impact on grape production in the central valley of California. However, it was found that GWSS did not efficiently transmit the pathogen that causes ALS and that spread can be reduced by removing diseased trees (Almeida and Purcell 2003b). Long-term studies of infected almond orchards revealed that a majority of diseased trees lived up to 7 years and had reduced yields of up to 40%. Based on this, growers may choose not to remove infected trees from an orchard, especially since secondary infection is low (Sisterson et al. 2012). Selection of less susceptible cultivars is recommended for growers planting new almond trees (Daane et al. 2011).

As mentioned, three of the *X. fastidiosa* strains caused ALS and of those, one strain caused both PD and ALS (Varani et al. 2012). Almeida and Purcell (2003a) were able to identify three strains that caused ALS in California, two different strains isolated from almond that caused ALS, and one isolated from grape that caused PD. They found

that neither almond-isolated strain caused PD; however, the isolate from grape caused PD and ALS. Although cross-infectivity is likely with the strain that caused PD and ALS, almonds do not suffer from increased disease incidence when planted adjacent to PD-infected vineyards (Almeida and Purcell 2003b)

Citris Variegated Chlorosis and Coffee Leaf Scorch

Citrus variegated chlorosis (CVC) and coffee leaf scorch (CLS) are caused by *X. fastidiosa* subsp. *pauca* (Schaad et al. 2004). Almeida et al. (2008) concluded they are genetically and biologically distinct because strains from CVC multiplied within coffee but did not induce symptoms. Likewise, CLS strains did not survive in citrus. Symptoms of CVC are irregular chlorotic areas over the leaf surface and stunted fruit. Coffee leaf scorch symptoms are stunted leaves and fruits (Hopkins and Purcell 2002). In Brazil, sharpshooters that transmit CVC include *Dilobopterus costalimai*, *Acrogonia terminalis*, *Acrogonia citrine*, and *O. facialis* (Alves et al. 2008). Studies have indicated that *X. fastidiosa* bacterial cells were found in the "cibarial pump chamber" of *A. citrine*, *O. facialis*, and *D. costalimai* (Alves et al. 2008).

Citrus variegated chlorosis was first reported in Brazil in the late 1980's (Chang et al. 1993). Today, one-third of citrus trees in Brazil are infected with CVC. The risk posed by *X. fastidiosa* to the Brazilian citrus industry led to efforts to sequence its genome. Both CVC and CLS are major problems for producers of those commodities in Central America and northern South America. In order to control the spread of CVC in Brazil, growers prune infected branches, use insecticides that target the vectors, and produce seedlings in covered greenhouses to exclude vectors (Rodrigues et al. 2008).

Currently, regulatory agencies are taking precautions to prevent the introduction of *X. fastidiosa* subsp. *pauca* into the United States (Hopkins and Purcell 2002).

Oleander Leaf Scorch

Oleander, *Nerium oleander* L., is used in California landscapes due to its minimal upkeep requirements and low cost. In the mid 1990's, investigators observed death of both mature and newly planted oleander bushes (Purcell et al. 1999). Estimated potential loss due to infected roadside oleander exceeds $50 million for the California Department of Transportation (Bethke et al. 2001). Through efforts to identify the causal agent using ELISA, Purcell et al. (1999) detected *Xylella fastidiosa* subsp. *sandyi*. Symptoms of oleander leaf scorch begin as chlorotic blotches at the leaf margins that progress toward the midrib and eventually lead to leaf necrosis that is most prominent at the leaf tips (Purcell et al. 1999). Bethke et al. (2001) evaluated insecticides against GWSS and found that acetamiprid, imidacloprid, and thiamethoxam were effective in limiting the spread of *X. fastidiosa* from infected to healthy oleander.

Peach and Plum Disease

Phony peach disease (PPD) was first observed in Georgia in the late 1800's and was thought to be caused by a virus. Twenty-five years after the first observation of this malady, the disease had spread to orchards in six counties in Georgia. Twenty years later the disease had spread to Texas, Illinois, Kentucky, and North Carolina (Turner and Pollard 1959a). Symptomatic peach trees tend to foliate and flower prematurely in the spring and defoliate later in the fall than trees void of disease. Diseased trees produce

less fruit that is usually much smaller than that of healthy trees and the fruit color of diseased trees is generally more intense. Similar symptoms were observed in other *Prunus* spp. including wild plum and apricot (Turner and Pollard 1959a).

Early investigators noted that the pathogen could be spread through root grafts; therefore, it was suspected that a soil-dwelling arthropod could be responsible for spreading the pathogen and resulting in increased disease expression (Turner and Pollard 1959a). However, symptoms developed in the upper canopy of peach trees and sampling efforts were expanded to include sap-sucking insects. Fifty years after first observing PPD, several species of insects in the family Cicadellidae were identified as vectors in Florida and South Carolina orchards, including *H. vitripennis*, *O. orbona*, *C. costalis*, *H. insolita*, and *G. versuta* (Turner and Pollard 1959a, Kalkandelen and Fox 1968).

Plum leaf scald (PLS) is a problem in Japan and was first reported in Argentina (Chang and Yonce 1987). Chang et al. (1987) found that symptoms can be reduced with oxytetracycline trunk injections. Through ongoing investigations into PD of grapes, a "rickettsia-like" organism was identified as the probable etiological agent responsible for that disease (Goheen et al. 1973, Hopkins and Mollenhauer 1973). Symptomology of PPD was similar to that of PD, prompting the use of electron microscopy to identify a similar organism associated with PPD (Hopkins et al. 1973).

Currently, PPD and PLS limit the life of orchards in Florida and no cure exists. Controlling leafhopper vectors with insecticides is ineffective for PPD and PLS. However, removal of infected trees and eliminating reservoir hosts both within and near orchards are effective in minimizing disease occurrence (Mizell et al. 2008).

Bacterial Leaf Scorch of Shade Trees

Since 1931, bacterial leaf scorch (BLS) has been observed in American elm, *Ulmus americanus* L, in the southeastern United States as far north as Washington, D.C. (Sherald et al. 1994). Prior to associating the ailment with PD of grape, it was surmised that environmental factors led to the development of scorching. Early investigators identified rickettsia-like bacteria as the causal agent of elm, oak, and sycamore leaf scorch (Hearon et al. 1980).

Bacterial leaf scorch detected in sugar maple,*Acer saccharum* Marsh, and sweetgum, *Liquidambar styraciflua* L., in Kentucky was caused by *X. fastidiosa* based on ELISA tests (Haartman and Jarlfors 1996). Other plants with confirmed cases of BLS in Kentucky include oak species (bur, pin, red, and shingle) and sycamore (Haartman and Jarlfors 1996). In Washington, D. C., leaf scorch of mulberry, *Morus alba* L., has been detected and found to be related to the bacterium that causes PD of grapes. Based on serological tests, Kostka et al. (1986) concluded the mulberry-isolated *X. fastidiosa* strains were more similar to strains that cause PD than those isolated from sycamore, elm, and oak. In the 1980's, sycamores, *Platanus occidentalis* L., in South Carolina, Washington, D.C., Texas, and Louisiana were observed to have leaf scorch symptoms and tests confirmed the causal agent was *X. fastidiosa* (Sherald et al. 1983, Haygood and Witcher 1988). Bacterial leaf scorch of shade trees occurs throughout the southeastern United States including the Gulf and Mid-Atlantic States. Elm leaf scorch has been reported as far north as Ontario, Canada (Sherald 1999). In Oklahoma, BLS caused by *X. fastidiosa* subsp. *multiplex* has been detected in American elm, mulberry, red oak, giant ragweed, and sycamore (Dominiak and Olson 2006).

Bacterial leaf scorch of pecan, *Carya illinoinensis* (Wangenhein) K. Koch, was first observed in Louisiana in 1972 and was thought to be caused by a fungus until Sanderlin and Heyderich-Alger (2000) found that *X. fastidiosa* was the causative agent. Symptoms of BLS of pecan manifest in June and progressively increase into the fall. Marginal necrosis of the leaflets is typical beginning on the older leaflets at the basal portion of the rachis and progresses toward younger leaflets. Leaflet abscission follows the same pattern of symptom development. It is common to observe bare rachises with terminal leaflets remaining attached (Sanderlin and Heyderich-Alger 2000). Sanderlin and Heyderich-Alger (2003) found that defoliation of leaflets caused by BLS decreased nut and kernel weight by 10-13% and 14-19%, respectively. They speculated that over time, as bacteria migrate through the pecan tree, yield could be impacted significantly. Since pecan BLS is transmissible from grafts of scion wood, pecan growers can treat scions with hot water to reduce occurrence of disease (Sanderlin and Melanson 2008).

Symptoms of BLS of shade trees are similar yet vary in the development of symptoms among species. All symptoms typically manifest in late June to July and increase into the fall (Sherald 2007). Symptoms in mulberry include necrotic distal areas of the leaf with a yellow chlorotic band separating dead tissue from green tissue (Henneberger et al. 2004). Although leafhoppers have been shown to transmit *X. fasidiosa* that cause PPD and PD and can be captured from pecan orchards, until recently few studies have demonstrated insect transmission of *X. fastidiosa* to shade trees (Sherald 2007). Sanderlin and Melanson (2010) verified insect transmission of *X. fastidiosa* strains that cause BLS from infected to uninfected pecan trees in the laboratory by the

sharpshooters, *H. vitripennis*, *H. insolita*, and *C. costalis*, and spittlebugs, *Clastoptera achatina* and *Lepyronia quadrangularis*.

Insect Vectors and Their Biology

Primary vectors of *X. fastidiosa* are in the insect family Cicadellidae (Hemiptera: Auchenorrhyncha). Two tribes within this family are Proconiini and Cicadellini, which are xylem sap-feeders and known vectors of *X. fastidiosa* (Redak et al. 2004). In addition to sharpshooters, multiple spittlebug species (Cercopidae) and a species of cicada (Cicadidae) are able to transmit *X. fastidiosa* (Redak et al. 2004). Multiple species of treehoppers (Membracidae) have tested positive for carrying *X. fastidiosa*, yet there are no studies demonstrating transmission of the pathogen by these insects (Huang et al. 2006, Zhang et al. 2011a).

All members of Cicadellidae (sharpshooters) have piercing-sucking mouthparts with specialized muscles that enable them to feed on xylem fluid, which has negative pressure potential. Thus, sharpshooters have evolved larger muscles and, therefore, enlarged clypeal regions to house those muscles (Redak et al. 2004). In addition, minimal nutrients are present in xylem fluid, adding a nutritional hurdle for sharpshooters. Xylem transports water and electrolytes up through the plant into distal tissues. This fluid is limited in organic nutrients including non-essential amino acids. Similar to aphids that utilize bacterial symbionts in the genus *Buchnera*, sharpshooters have symbiotic gut bacteria that aid in providing necessary nutrients. For example, GWSSs use co-symbionts *Baummania* sp. and *Sulcia* sp. to provide cofactors and essential amino acids, respectively (Wu et al. 2006).

Hemiptera: Cicadellidae: Cicadellinae: Proconiini

***Homalodisca vitripennis* (Germar)**

Glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) (formerly, *H. coagulata* (Say)) is the most important vector of diseases caused by *X. fastidiosa*. This large sharpshooter is native to the southeastern United States (Nielson 1968) and has spread to southern California (USA) (Sorensen and Gill 1996), French Polynesia (Tahiti) (Hoddle 2004), and Hawaii (USA) (Almeida 2007). In California, GWSS transmits bacteria that cause PD of grapes and leaf scorch in almonds and oleander. In Texas and Florida, GWSS is a major vector of the pathogen that causes PD, PPD, and PLS. The ability of *H. vitripennis* to transmit *X. fastidiosa* has been extensively studied due its importance as a vector. Glassy-winged sharpshooters transmit *X. fastidiosa* that cause PPD and PD at 15 and 32% efficiency, respectively (Redak et al. 2004). Almeida and Purcell (2003c) found that GWSS transmits *X. fastidiosa* to grapevines at a lower efficiency than that of *G. atropunctata*, which is native to California. However, the rate at which it spreads *X. fastidiosa* can be high due to its ability to feed on and inoculate dormant woody tissue, its increased dispersal ability, and its large host range (Almeida and Purcell 2003c, Blua and Morgan 2003, Almeida 2007).

Glassy-winged sharpshooters feed on numerous plant species and favors sunflower, hollyhock, okra, lambsquarter, cotton, corn, and cowpeas. Woody plants preferred by GWSS include; oak, ash, silktree, crapemyrtle, and peach (Turner and Pollard 1959b). Early instars prefer herbaceous plants while older instars and adults feed primarily on woody plants (Turner and Pollard 1959b). In its native range, GWSS

overwinters as adults within underbrush of wooded areas from February to March and moves to different hosts including shrubs, peach trees, sunflower, and hollyhock in March and April. Females lay their eggs below the abaxial leaf epidermis of both herbaceous and woody species (the former is most common). The ovoid eggs are deposited singly in neat rows of 3 to 28 eggs. After egg deposition, females cover the egg mass with brochosomes (i.e., white chalky substance) secreted from the anus. Females mate once and eggs hatch in 12 days. The nymphal stages range in duration from 30 to 70 days. Adults can survive for up to three months. Two, possibly three generations per growing season have been observed in Missouri with the first generation beginning in March or April and the second or third appearing as late as September (Turner and Pollard 1959b).

Early investigators observed that GWSS feeds throughout mild winters and does not hibernate. When temperatures drop to 9 ºC, these sharpshooters are unable to fly and drop to the ground; however, they are able to withstand overnight freezing temperatures (Pollard and Kaloostian 1961). Previous studies have shown that GWSSs cease feeding when temperatures reach 10 ºC and death occurs after extended periods (15 days) in temperatures below 10 ºC (Johnson et al. 2006). Adults will feed on "evergreen plants" throughout the winter months in Texas (Lauzière and Sétamou 2009). Hoddle (2004) modeled possible overwintering habitats for GWSS that included the southeastern tip of Oklahoma. Although it is unknown if GWSS could survive Oklahoma winters, a small number of specimens was captured from tree nurseries in the northeastern part of Oklahoma (Overall et al. 2010).

In California, research has increased in developing control methods for GWSS, including biological, chemical, and mechanical methods. Insecticides that have been targeted include those that reduce GWSS feeding, thereby minimizing the opportunity for acquisition and transmission (Bethke et al. 2001). In California, the neonicotinoids, imidacloprid and dinotefuran, are effective for GWSS control (Byrne and Rosa 2008). Mechanical control methods include use of a screen barrier to limit dispersal of GWSS into vineyards. Blua et al. (2005) found that a barrier fastened from shade cloth five meters in height deterred up to 70% of GWSS from crossing or even approaching the barrier. Researchers have used symbiotic control to reduce the ability of GWSS to transmit *X. fastidiosa* by introducing the transformed bacterial symbiont, *Alcaligenes xylosoxidans* var. *denitrificans* (S1A*xd*), into insect foreguts by way of insect feeding (Ramirez et al., 2008). The bacterial symbionts have been genetically altered to introduce antibiotics that specifically interfere with the pathogenicity of *X. fastidiosa*.

There has been focus on biological control of GWSS in California where this invasive pest threatens billions of dollars in economic loss in almonds ($2.8 billion), grape ($4 billion), and stone fruit ($1 billion) industries (Son et al. 2012). Glassy-winged sharpshooter is known to be parasitized by several species of egg parasitoids in the genus *Gonatocerus* (Hymenoptera) (Triapitsyn et al. 2003). The most commonly released natural enemies are in the family Mymaridae and include *Gonatocerus ashmeadi* Girault, *G. trigutattus* Girault, a few other species in the genus *Gonatocerus,* and *Anagrus epos* Girault (Son et al. 2012). Of those released, *G. ashmeadi* and *G. trigutattu*s were successfully established in California and both have significant potential to reduce numbers of *H. vitripennis* (Gutierrez et al. 2011, Son et al. 2012). Recent studies in

California have focused on understanding the oviposition behavior and egg stages of *H. vitripennis* and a closely related native species, *H. liturata* Ball (smoke tree sharpshooter), to ultimately develop more efficient methods to rear and release egg parasitoids of GWSS (Al-Wahaibi and Morse 2009). Using temperature- and solar radiation-based models, Gutierrez et al. (2011) found that these two parasitoids do not affect the overall geographic distribution of GWSS, but do limit the abundance of adults by up to 90% throughout California.

***Paraulacizes irrorata* (Fabricius)**

Paraulacizes irrorata (F.) (formerly, *Cicada irrorata* Fabricius) is native to southeastern and central United States and Mexico (Young 1968). Mason and Yonke (1971) studied the life history of *P. irrorata* in Missouri. The following plants were listed as food sources for *P. irrorata*: Virginia wildrye, tall thistle, horseweed, *Cirsium sp*., prickly lettuce, wholeleaf rosinweed, and cultivated sorghum (Mason and Yonke 1971). This species overwinters in the adult stage in fallen plant debris near woody plants. At the onset of spring, adults migrate from wooded areas onto grass species and non-woody plants. Nymphs feed on herbaceous plants. In Missouri, investigators were able to capture adult bugs from June until October and observed large populations returning to woody habitats as temperatures decreased. They observed one generation per year in Missouri (Mason and Yonke 1971).

Mason and Yonke (1971) studied the life history of *P. irrorata* on sunflower in the laboratory. Females insert their eggs under the epidermis of the stems between the cotyledon and regular leaves. Eggs are laid "side by side" in rows that run the length of

the stem. On average, one female can oviposit over 250 eggs in her lifetime of 178 days. The egg stage lasts an average of 11 days. A period of 50 days elapses from eclosion until the adult stage. Males require an additional week to complete development. Early instars feed on the abaxial leaf surface and are sessile. Fifth instars and adults feed on stems of sunflower (Mason and Yonke 1971).

In Florida, the egg masses of *P. irrorata* were found to be parasitized by a wasp, *G. fasciatus* Girault (Hymenoptera: Mymaridae). This gregarious wasp will also parasitize the eggs of *H. vitripennis* and *O. orbona* (Tipping et al. 2006).

Paraulacizes irrorata has been captured from vineyards in Texas (Lauzière et al. 2008) and North Carolina (Villanueva et al. 2008). It is most commonly captured in tree nurseries in Oklahoma (Overall et al. 2010). In Louisiana, few species were collected from pecan orchards and of those species tested; two individuals of *P. irrorata* did not transmit *X. fastidiosa* (Sanderlin and Melanson 2010).

***Oncometopia orbona* (Fabricius)**

Oncometopia orbona (Fabricius) was formerly classified as *O. undata* F. and is commonly referred to as the broad-headed sharpshooter. It is distributed throughout the eastern United States from Florida to Maryland and south from Missouri and Texas to northern Mexico (Turner and Pollard 1959b, Neilson 1968). Broad-headed sharpshooters feed on almost 50 plant species in 25 families and will oviposit on 18 plant species (Nielson 1968). This sharpshooter prefers sunflower, hollyhock, okra, lambsqaurter, ash, oak, silktree, and crapemyrtle. Peach is preferred in spring and fall (Nielson 1968). Caged females inserted cylindrical eggs under the epidermis of sunflower leaves and then

cover each with brochosomes using their hind tibiae. The brochosomes originated from anal secretions and, after egg deposition, powdery brochosome material was replaced within five minutes. Six to 20 eggs may be laid next to each other by one female. Nymphs emerge 7 to 10 days after oviposition. Under field conditions in Missouri, the development period from egg stage through adult emergence requires 75 days**.** Hibernation occurs in the adult stage and females begin laying eggs in April. It is speculated that two generations are possible in Missouri. Females mate once and continue to lay fertilized eggs for 30 days (Swain 1936). Under laboratory conditions, females mate once and the egg stage lasts 12 days (Turner and Pollard 1959b). Dependent on conditions when a particular generation developes, the nymphal stage lasts from 40 to 57 days and adults live up to three months (Neilson 1968).

Oncometopia orbona eggs are parasitized by *Gonatocerus fasciatus* Girault (Triapitsyn et al. 2003). There are no other accounts of natural enemies for this insect. *Oncometopia orbona* has been captured in vineyards in Texas, Oklahoma, North Carolina, and Virginia (Buzombo et al. 2006, Lauzière et al. 2008, Villanueva et al. 2008, Overall et al. 2010, Wallingford and Pfeiffer 2012). Myers et al. (2007) studied the natural infectivity of field-collected *O. orbona*, confirming transmission of *X. fastidiosa* to grapevines. Turner and Pollard (1959a) reported the ability of *O. orbona* to transmit *X. fastidiosa* that causes PPD to peach trees with 33% efficiency. Zhang et al. (2011a) captured *O. orbona* from infected oak trees in New Jersey and found that of those collected, 14% tested positive for *X. fastidiosa*; however, they did not test the ability of this species to transmit *X. fastidisoa* that causes BLS.

***Cuerna costalis* (Fabricius)**

Cuerna costalis (F.) is distributed in the southeastern United States with a range extending from Texas to New York. This species is commonly referred to as the lateral-lined sharpshooter (Nielson 1968). Lateral-lined sharpshooters feed on grasses throughout the year and feed on other plant species in the spring including cotton, sunflower, ragweed, and young peach trees. Adults overwinter beneath compact grass in Georgia and emerge to reproduce in January and February. Females deposit eggs on blades of Johnsongrass and bermudagrass. Eclosion occurs in April (Turner and Pollard 1959b, Nielson 1968). Turner and Pollard (1959b) successfully reared this species in the laboratory on Johnsongrass. Adults mated once and were able to lay eggs continuously. Time from eclosion through all nymphal stadia ranged from 57 to 82 days, depending on the generation, and adults lived up to 80 days. Three generations per year were observed.

Lateral-lined sharpshooter is commonly found in vineyards in Texas (Lauzière et al. 2008) and North Carolina (Villanueva et al. 2008). This species is also commonly found in Oklahoma vineyards and tree nurseries (Mulder et al. 2003, Overall et al. 2010). It has been shown to effectively transmit *X. fastidiosa* that causes PPD with 25% efficiency (Redak et al. 2004). Although very few numbers of insects were tested, lateral-lined sharpshooter was able to transmit pecan leaf scorch (Sanderlin and Melanson 2010).

Hemiptera: Cicadellidae: Cicadellinae: Cicadellini

***Graphocephala versuta* Say**

Graphocephala versuta Say was formerly classified under the genus, *Tettigonia*, and is commonly known as the versute sharpshooter. The distribution of this species is the southern United States ranging as far north as Illinois and west to Texas (Neilson 1968). Adults overwinter in woody habitats and move to herbaceous perennials in March. Females oviposit on the adaxial leaf surface of ragweed, sunflower, okra, and other hosts. Four generations per year were observed in a laboratory setting on cowpea (Turner and Pollard 1959b). Egg incubation lasts from 6 to 11 days, depending on seasonal conditions. Nymphal development ranged from 36 to 42 days, depending on the generation, and adults live up to 42 days (Turner and Pollard 1959b).

The versute sharpshooter is the predominant species captured in Texas vineyards, comprising 21% of all xylem feeders (Lauzière et al. 2008). Similarly, this species is the predominant leafhopper captured on yellow sticky cards in Virginia (Wallingford and Pfeiffer 2012). This species has been captured in Oklahoma vineyards and tree nurseries (Mulder et la. 2003, Overall et al. 2010). Previous transmission studies have shown transfer of strains that cause PPD with 29% efficiency (Redak et al. 2004). Myers et al. (2007) evaluated the natural infectivity of *G. versuta* in North Carolina and demonstrated transmission of *X. fastidiosa* to grapevines. These researchers were able to detect *X. fastidiosa* in 63% of the 2,198 insects trapped in one vineyard over a two-year period. In Texas vineyards, *X. fastidiosa* was detected in 13% of *G. versuta* collected (Mitchell et al. 2009). Zhang et al. (2011a) found that 20% of *G. versuta* collected from BLS-infected oak trees tested positive for *X. fastidiosa*.

In Texas vineyards, *G. versuta* is one of three predominantly captured insects that are potential vectors of PD; the other two are GWSS and the sunflower spittlebug,

Clastoptera xanthocephala Germar (Morano et al. 2010). Morano et al. (2010) evaluated extensive insect frequency data (of the three most captured species) against environmental gradients (i.e., elevation, annual precipitation, cold hardiness, and one ecoregion environmental variable) to explain differences in state-wide frequencies and identify favorable or unfavorable environmental conditions. Based on their results, they found that *G. versuta* was more common in regions with higher annual precipitation.

***Graphocephala coccinea* (Forster)**

Graphocephala coccinea (Forster) has been captured in Ontario, Canada, and in the United States from Maine to Florida and west to California and Mexico. In the United States, this species feeds on blackberry, forsythia, pittosporum**,** cup plant, wholeleaf rosinweed, rose, thistle, milkweed, goldenrod, and fragrant sumac (Young 1977). Detailed life history studies of *G. coccinea* are lacking. Morcos (1948) observed the overwintering habits of this insect at the Imperial College Field Station, London. He reported the hibernation of eggs in rhododendron sepals. Typical of sharpshooters, eggs were inserted below the epidermis of plant tissue. Eggs were laid in groups of 2 to 10 in a "fan-like" arrangement (Morcos 1948).

This insect is commonly found in vineyards in Texas, North Carolina, and Oklahoma (Lauzière et al. 2008, Villanueva et al. 2008, Overall et al. 2010). There are no transmission studies establishing this insect as a vector of diseases caused by *X. fastidiosa*. However, a recent study on BLS of oak reported numerous *G. coccinea* tested positive for *X. fastidiosa* (Zhang et al. 2011a).

***Graphocephala hieroglyphica* (Say)**

Graphocephala hieroglyphica (Say) was formerly classified as *Tettigonia hieroglyphica* and *Cicadella hieroglyphica*. Hackman (1922) documented phenology and biology of this sharpshooter from both lab and field observations. Host plants include willow, poplar, broad-leafed milkweed, and giant ragweed. Willow is the preferred host, hence this insect is often referred to as the willow sharpshooter. Adults overwinter within leaf debris and other material on the ground. Adults become somewhat active and sunbathe on warm days in January. Overwintering adults emerge mid-February and initiate feeding on newly budded willow. Large groups were observed to gather on one stem. Adults begin reproducing in April and females deposit their eggs underneath the epidermal tissue on the adaxial leaf surface either as single eggs or in rows. Females oviposit in both poplar and willow. The time required for all nymphal stadia averaged 60 days (Hackman 1922).

This species is commonly captured in vineyards in Texas (Lauzière et al. 2008) and vineyards and tree nurseries in Oklahoma (Overall et al. 2010). Previous studies have confirmed that *G. hieroglyphica* transmits *X. fastidiosa* that causes PPD and PD (Redak et al. 2004).

***Xyphon flaviceps* Riley**

Xyphon flaviceps (Riley), or yellowheaded leafhopper, was formerly classified under the genus, *Carneocephala*. This species is distributed in the southeast and midwestern states of the United States and ranges as far north as Wisconsin and west to

New Mexico. Little is known of the biology of the yellowheaded leafhopper. It has been reported to primarily feed and reproduce on bermudagrass (Neilson 1968).

Xyphon flaviceps is common in Texas vineyards (Lauzière et al. 2008) and Oklahoma vineyards and tree nurseries (Overall et al. 2010). The yellowheaded leafhopper transmits *X. fastidiosa* to grapevines and alfalfa in Florida (Stoner 1953).

Hemiptera: Cercopidae

***Prosapia bicincta* (Say) and *Clastoptera xanthocephala* Germar**

The twolined spittlebug, *P. bicinta* (Say), is a pest of bermudagrass and other grasses. The immature stages feed on grass roots and stolons (Beck 1963). Adults feed on numerous plant species including alfalfa, blackberry, wild cherry, morning glory, peach, and ragweed (Pass and Reed 1965). Although commonly collected in Oklahoma, twolined spittlebug is unlikely to be a significant insect vector in transmission of disease since it is a poor flyer; however, it does have a large plant host range (Pass and Reed 1965).

The sunflower spittlebug, *Clastoptera xanthocephala* Germar, is frequently captured in Oklahoma (Overall et al. 2010). The sunflower spittlebug is abundant in Texas vineyards and has been shown to carry *X. fastidiosa* (Mitchell et al. 2009). In Texas, it is more often found in vineyards that are in higher elevated ecoregions such as the Texas Hill Country (Morano et al. 2010).

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CHAPTER III

DISTRIBUTION, PEAK ABUNDANCE, AND INFECTION STATUS OF POTENTIAL INSECT VECTORS OF *XYLELLA FASTIDIOSA*, THE CAUSAL AGENT OF PIERCE'S DISEASE OF GRAPES AND BACTERIAL LEAF SCORCH OF SHADE TREES IN OKLAHOMA

Abstract

Xylella fastidiosa Wells et al. is the causative agent of many plant diseases including those of peach, plum, oleander, almond, elm, oak, pecan, sycamore, and Pierce's disease of grapes. This pathogen is transmitted by xylem sap-feeding insects. I surveyed Oklahoma for potential insect vectors to determine the species composition of vineyards, nurseries, and pecan orchards and whether or not they harbor *X. fastidiosa*. I sampled several locations including three nurseries, seven vineyards, and two pecan orchards in different regions of the state. Yellow sticky traps were used to sample insect fauna at each location. Insects were removed from cards and screened for the presence of *X. fastidiosa* using immunocapture-PCR. I found the following sharpshooters in my survey: *Xyphon flaviceps*, *Draeculacephala navicula*, *D. robinsoni*, *Graphocephala coccinea*, *G. versuta*, *G. hieroglyphica*, *Cuerna costalis*, *Oncometopia orbona*, *Homalodisca vitripennis* (Hemiptera: Cicadellidae: Cicadellinae), *Prosapia bicincta*,

and *Clastoptera xanthocephala* (Hemiptera: Cercopidae). The three most frequently captured species in vineyards and tree nurseries include *G. versuta*, *C. xanthocephala*, and *O. orbona*. From a subsample of insects screened for *X. fastidiosa*, 2.4% tested positive for the bacterium.

Introduction

Xylella fastidiosa is a bacterial plant pathogen that infects the host xylem fluid and causes many diseases including Pierce's disease (PD) of grape, citrus variegated chlorosis, phony peach disease, alfalfa dwarf, and leaf scorch diseases of almond, oleander, plum, and shade trees. Common symptoms include leaf scorch, characterized by cell death of the leaves beginning at leaf margins with a chlorotic band separating dead from healthy tissue, or stunting, both of which can eventually lead to plant death. There are four commonly recognized subspecies of *X. fastidiosa*. Pierce's disease is caused by *X. fastidiosa* subsp. *fastidiosa* and bacterial leaf scorch (BLS) is caused by *X. fastidiosa* subsp. *multiplex*, both of which have been documented in Oklahoma. Two other subspecies not found in Oklahoma are *X. fastidiosa* subsp. *pauca* and *X. fastidiosa* subsp. *sandyi*, which cause disease in citrus/coffee and oleander, respectively (Schaad et al. 2004). Subspecies of *X. fastidiosa* can be differentiated based on DNA sequences and host ranges. For instance, the subspecies that causes leaf scorch in shade trees does not cause PD in grapes (Schaad et al. 2004). *Xylella fastidiosa* is commonly grouped into strains since the many subspecies are cross-infective in the same plant host (Hendson et al. 2001, Almeida and Purcell 2003).

In Oklahoma, *X. fastidiosa* subsp. *multiplex* has previously been identified in American elm, sycamore, mulberry, red oak, porcelain berry, and giant ragweed (Dominiak and Olson 2006, Olson et al. 2006). Pierce's disease has been predicted to be limited to warmer climates and thus poses little threat to Oklahoma grape growers; however, recently it has been detected in eight counties in central and northeastern Oklahoma (Jen Olson, *pers. comm.*) (Fig. 1). Smith and Dominiak-Olson (2009) used temperature thresholds outlined by Anas et al. (2008) to determine potential risk for PD in Oklahoma. Low-risk vineyards were those with surface air temperatures at or below -12 °C for two to three consecutive days. Using temperature data from the past decade, Smith and Dominiak-Olson (2009) found that Tulsa, Payne, Lincoln, and Kiowa Counties in Oklahoma were at medium risk for PD (i.e., surface air temperatures ≤ -12 °C for less than two consecutive days). In fact, both Tulsa and Payne Counties have had reports of PD in commercial vineyards (Jen Olson, *pers. comm*).

Xylella fastidiosa is transmitted primarily by xylem sap-feeding insects including spittlebugs (Cercopidae), sharpshooters (Cicadellidae: Cicadellinae), and cicadas (Cicadidae) (Redak et al. 2004, Krell et al. 2007). *Xylella fastidiosa* has been detected in treehoppers (Hemiptera: Membracidae) in Virginia and New Jersey (Huang et al. 2006, Zhang et al. 2011); however, studies are lacking that demonstrate treehoppers are able to transmit *X. fastidiosa*. In addition to insect transmission, *X. fastidiosa* can be transmitted vine to vine via use of contaminated pruning shears (Krell et al. 2007). Pecan bacterial leaf scorch, caused by *X. fastidiosa* subsp. *multiplex*, can be transmitted through use of infected rootstock (Sanderlin and Melanson 2006, Melanson et al. 2012). The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar), is the major vector of

X. fastidiosa subsp. *fastidiosa* that causes PD in Texas and California, a costly disease for grape growers in both states. Glassy-winged sharpshooter possesses traits that enable it to transfer *X. fastidiosa* more readily among plants. It has a greater flight capability than that of smaller sharpshooters and it feeds on a broad range of plants (i.e., 100 plant species in 30 families) (Costa et al. 2006). In addition, GWSS feeds on woody tissue whereas other sharpshooter species tend to feed on green plant tissue (Almeida 2007). The ability to feed on dormant, woody plant tissue allows GWSS to inoculate the bacterium into plant tissues that are more protected from adverse weather, thus increasing likelihood of bacteria successfully overwintering in the plant (Almeida 2007). This sharpshooter species has been found in an Oklahoma tree nursery and pecan orchard in small numbers, but has yet to be collected from vineyards in this state (Overall et al. 2010).

In general, *X. fastidiosa* inhabits and multiplies in the foregut of insect vectors, specifically the cibarium. The foregut lining is shed during the molting process, and as a result, any bacterial cells adhering to the foregut lining are also lost (Almeida and Purcell 2006). Although the bacteria replicate within the insect foregut, there is no latent period required between acquisition and inoculation (Purcell and Finlay 1979). Further, the bacterium is not transferred from females to their eggs (i.e., transovarial transmission) and if acquired by adult leafhoppers, is transmitted for the life of the vector (Freitag 1951). To manage the spread of *X. fastidiosa*, researchers have investigated the use of neonicotinoid and pyrethroid insecticides that target insect vectors of *X. fastidiosa* among an array of other integrated pest management (IPM) tactics. In Texas and Florida, researchers have found that *X. fastidiosa* subsp. *fastidiosa* is spread vine to vine by insect

vectors; therefore, insecticide applications target vectors within the vineyard (Kamas et al. 2000). In North Carolina vineyards, systemic neonicotinoid insecticides (e.g., acetamiprid, imidacloprid, and dinotefuran) and a pyrethroid insecticide (i.e., fenpropathrin) are effective in reducing the number of insect vectors, primarily *Graphocephala versuta* (Villanueva et al. 2008). In California where *X. fastidiosa* is introduced into the vineyard from bacterial populations that overwinter in GWSS (Park et al. 2006), growers use insecticides against the GWSS in citrus orchards (Kamas et al. 2000). The GWSSs overwinter in citrus, which is both a food and reproductive host (Park et al. 2006).

From 2008 to 2010, I conducted a statewide survey of Oklahoma vineyards, tree nurseries, and pecan orchards for insect vectors of *X. fastidiosa*. A previous study in this state identified leafhopper species in vineyards (Mulder et al. 2003); however, little is known of the leafhopper complex within tree nurseries. Furthermore, insects captured previously from vineyards were not tested for the presence of *X. fastidiosa*.

There is no known cure for grapevines or shade trees infected with *X. fastidiosa*. Since there is no way to control diseases associated with *X. fastidiosa*, growers must rely on management tactics that prevent spread of the pathogen, such as insecticide applications that target insect vectors, or removal of diseased vines or trees. The results from this study will be used to develop IPM tactics to limit the spread of *X. fastidiosa* in Oklahoma vineyards and tree nurseries.

Materials and Methods

Sampling Locations

Insect sampling was conducted from March until September of 2008, 2009, and 2010 in selected vineyards, tree nurseries, and pecan orchards throughout Oklahoma. In 2008, I sampled three tree nurseries (Custer, Cherokee, and Oklahoma Counties) and two vineyards (Wagoner and Payne Counties). In 2009, I added three additional vineyards (Jackson, Canadian, and Greer Counties) and a pecan, *Carya illinoensis* (Wagenh.) K. Koch, orchard (Marshall County). In 2010, I did not sample vineyards in Jackson, Greer, and Canadian counties due to the low number of xylem sap-feeding insects captured. I added four vineyards in 2010 with vines that tested positive for PD in Creek, Tulsa, and Lincoln counties. I added a second pecan orchard in Lincoln County in 2010. To account for the different number of sampling locations for each year, the total number of insects was averaged over the number of sampling locations. This was done with the seasonal phenology graphs only.

Yellow sticky cards (Whitmire Micro-Gen Research Laboratories, Inc., St. Louis, MO) measuring 12.2 cm X 7.6 cm were used to sample insect fauna within sample sites. In nurseries and pecan orchards, cards were placed near the canopy bottom (1 m above the ground) by securing them to 1.5-m bamboo sticks with clothes pins. In vineyards, cards were secured to vine support cables with clothes pins at mid-canopy level, approximately 1.5 m above ground. Twelve traps were placed at each site, with eight traps spaced evenly around the field perimeter and the remaining four traps placed on diagonals within the field. Sampling area was approximately 1 ha for each location. All sticky cards were changed weekly or bi-weekly, depending on the schedule of cooperating growers, and collected cards were stored at -20 °C until further analysis. Insects were identified, carefully removed using sanitized forceps, and placed

individually in microcentrifuge tubes for later analysis. All leafhoppers and spittlebugs were identified to genus and species prior to removal from cards. Insect identifications were verified by the Plant Disease and Insect Diagnostic Lab, Oklahoma State University. Voucher specimens for all species were deposited in the K.C. Emerson Entomology Museum, 127 Noble Research Center, Oklahoma State University.

Vineyards were composed of mostly French-American hybrids (*Vitis* species) and *Vitis vinifer*a grapes, which are both highly susceptible to PD (Kamas et al. 2000). An exception is the vineyard in Wagoner County that was planted primarily with 'Cynthiana' (*Vitis aestivalis*), a North American native grape that is tolerant to PD (Kamas et al. 2000). Tree nurseries were composed of the following shade trees: oak (*Quercus* spp.), elm (*Ulmus* spp.), willow (*Salix* spp.), sycamore (*Platanus occidentalis* L.), maple (*Acer* spp.), redbud (*Cercis canadensis* L.), and birch (*Betula* spp.). Pecan orchards were planted with the cultivar, 'Pawnee' (Marshall Co.) or 'Stuart' (Lincoln Co.)

Immunocapture-Polymerase Chain Reaction (IC-PCR)

To determine which insects harbored *X. fastidiosa,* I used immunocapture-polymerase chain reaction (IC-PCR), which is a method of detection that incorporates immunological techniques that are sensitive and specific to target pathogens. Further, this approach does not require DNA extraction, thus reducing the presence of contaminants that may interfere with traditional PCR (Pooler et al. 1997, Peroni et al. 2008). Micro-titer plate wells were coated with 50 µl of *X. fastidiosa* capture antibody diluted (1:100) in a 1X carbonate coating buffer (Agdia Inc., Elkhart, IN). Plates were incubated overnight at 4 °C in a plastic container. After incubation, plates were washed

three times with phosphate buffered saline-tween (PBS-T) and blotted dry before adding macerated insects. Insects were placed in 1.2-ml bead beater tubes (Life Sciences Products, Inc., Frederick, CO) that were previously filled with 0.25 g glass beads (BioSpec Products, Inc., Bartlesville, OK). To each tube, 300 µl of 0.05 M Tris-Cl was added and the samples were disrupted for 30 seconds using a Mini-BeadBeater-8 (BioSpec Products, Inc.). Tubes were centrifuged briefly to remove material from the lid. To each well, 50 µl of the sample was added and incubated overnight at 4 °C. After sitting for 24 h, wells were washed eight times with PBS-T and blotted dry before adding 50 µl of nuclease-free water to each well. To release antibody-DNA complexes bound to wells, the plate was heated at 100 °C for 5 minutes. DNA samples were amplified with primers that target a 733-base pair region of the *X. fastidiosa* genome (Minsavage et al. 1994). All PCR reactions (25 µl) contained 12.5 µl of EconoTaq PLUS GREEN 2X Master mix (Lucigen, Middleton, WI), 2.5 µl of each primer (RST33, RST31) (5 µM), 2.5 µl of DNA, and 5 µl of nuclease-free water. For those insects collected in 2010, a primer pair was used that amplified a smaller fragment, which improved PCR results. These PCR reactions (25 µl) contained 12.5 µl of EconoTaq PLUS GREEN 2X Master mix (Lucigen, Middleton, WI), 2.5 µl of each primer (OUTF1, OUTR1) (5 µM), 2.5 µl of DNA, and 7.5 µl of nuclease-free water (Bextine and Child 2007). After amplification, samples were visualized using a 1% agarose gel in 1X TAE buffer (2 µl of ethidium bromide stock [10 mg/ml] added to the gel) placed under UV light.

Sequencing

For those insects testing positive for the presence of *X. fastidiosa*, the amplified product was purified using the E.Z.N.A. Cycle-Pure Kit (Omega Bio-Tek Inc., Norcross, GA). Many of the positive samples had degraded, which meant few samples yielded quality sequences for comparison to those in the databases. In addition, many of the positive samples exhibited weak bands on the gel, likely resulting from low numbers of bacteria in the insect foregut. Of those samples that yielded sufficient DNA following PCR clean-up, the purified product was submitted to Oklahoma State University Recombinant DNA/Protein Resource Facility for automated sequencing. After sequencing, Mega4 (The Biodesign Institute, Tempe, AZ) was used to align the forward and reverse sequences when both were available. In many samples, only the forward sequence was available for comparisons**.** Sequences were compared to *X. fastidiosa* DNA sequences stored in GenBank using BLASTN (National Center for Biotechnology Information, Bethesda, MD)**.**

Statistical Analysis of Vector Abundance

Vector abundance was compared between vineyards and tree nurseries within the same week of sampling. In addition to comparing sums of the three most commonly captured insects from vineyards versus nurseries, comparisons were made of vector abundance at the interior and perimeter of each site within each week of sampling. Transformed data did not have a normal distribution (PROC UNIVARIATE, SAS 9.3, SAS Institute, 2010); therefore, I used a nonparametric analysis, the Kruskal-Wallis test,

to compare vineyards versus nurseries and interior versus perimeter at each sampling site (PROC NPAR1WAY, SAS 9.3, SAS Institute, 2010).

Results and Discussion

Insect Vector Abundance

The following xylem sap-feeding insects were recovered in this survey: *Xyphon flaviceps* (Riley), *Draeculacephala navicula* Hamilton, *D. robinsoni* Hamilton, *Graphocephala coccinea* (Forster), *G. versuta* Say, *G. hieroglyphica* (Say), *Cuerna costalis* (F.), *Oncometopia orbona* (F.), *Paraulacizes irrorata* (F.), *Homalodisca vitripennis* (Hemiptera: Cicadellidae: Cicadellinae) (Figs. 1 and 2), *Prosapia bicincta* (Say), and *Clastoptera xanthocephala* Germar (Hemiptera: Cercopidae) (Fig. 3). Three species were captured in nurseries that did not occur in vineyards: *H. vitripennis*, *D. robinsoni*, and *P. bicincta* (Tables 1 and 2). The three most frequently captured species were *G. versuta*, *O. orbona, and C. xanthocephala*. *Graphocephala versuta* was the most abundant species in vineyards in all years and nurseries in 2010 (Tables 1, 2). Similarly, *G. versuta* was one of three xylem sap-feeding insects commonly captured in twenty Texas vineyards located in thirteen counties, along with *C. xanthocephala* and *H. vitripennis* (Lauzière et al. 2008). In ten vineyards spanning five geographic regions of Virginia, the two most common species captured were *G. versuta* and *O. orbona* (Wallingford and Pfeiffer 2012). Based on two years of sampling, in four North Carolina vineyards, predominant insect vectors were *G. versuta, O. orbona, and P. irrorata* (Myers et al. 2007).

I captured three glassy-winged sharpshooter (GWSS) adults in a tree nursery in Cherokee County in northeastern Oklahoma (Table 2), which indicates GWSS may be distributed farther north than predicted by previous climate models (Hoddle et al. 2004). However, the absence of GWSS in northeastern OK vineyards suggests that these specimens were transient and introduced with nursery stock from states that are within he insect's normal distribution range. In 2009, two additional adults were captured at a pecan orchard in Marshall County in Southeastern Oklahoma, which is within the predicted distribution range of GWSS (Hoddle 2004). Specimens of GWSS collected in this study were not tested to determine if they were carrying *X. fastidiosa*.

In Oklahoma tree nurseries and pecan orchards, the most abundant insect vectors of *X. fastidiosa* were *C. xanthocephala*, *O. orbona*, and *G. versuta* (Table 2). There was an increase in the number of all three species captured in the final two years of the study, 2009 and 2010. Similarly, Zhang et al. (2011) found that *G. versuta* was captured frequently from urban shade trees (*Quercus* spp.), although treehoppers including *Ophiderma definita* Woodruff, *Telamona monticola* (F.), and *Atymna querci* (Fitch) (Hemiptera: Membracidae) were the most commonly captured xylophagous insects (Zhang et al. 2011). The study by Zhang et al. (2011) along with a previous study by Huang et al. (2006) were the first to implicate Membracidae as potential vectors of *X. fastidiosa*.

In Louisiana pecan orchards, the pecan spittlebug, *Clastoptera achatina* Germar, has been shown to transmit pecan BLS to healthy pecan trees and based on its abundance in orchards, may play a significant role in transmission of *X.*

fastidiosa (Sanderlin and Melanson 2010). I encountered a related species in nurseries, *C. xanthocephala*, which could potentially transmit *X. fastidiosa* that causes BLS to trees although transmission has not been demonstrated for this species. *Clastoptera xanthocephala* is reported to feed on a variety of plants including trees, shrubs, and grasses but prefers sunflower and ragweed. This species has two generations per year in Florida (Ball 1927).

Insect Vector Seasonal Phenology

In vineyards, there were two distinct peaks of *G. versuta* in 2009 and two less distinct peaks in 2010, suggesting this species has two generations per year in Oklahoma (Fig. 5). In 2009, the first peak occurred during the third week of June. In 2010, *G. versuta* exhibited an initial peak in July and a second peak in July and August (Fig. 5). In nurseries, *G. versuta* had one major peak of abundance. Large numbers of this species were captured from the third week of June until late July for all years of the study (Fig. 6).

In Virginia vineyards, Wallingford and Pfeiffer (2012) found that *G. versuta* had one generation per year based on collection records from ten vineyards and the abundance of *G. versuta* peaked in mid-May to June (Wallingford and Pfeiffer 2012). In Texas vineyards, *G. versuta* appears to peak in late May to mid-June (Mitchell et al. 2009), whereas *G. versuta* peaked early July through the first of August in North Carolina vineyards (Myers et al. 2007). In Texas and Virginia, *G. versuta* peaks about one month earlier than what was observed in Oklahoma vineyards. In North Carolina, however, *G. versuta* peaks about one month later than it does in Oklahoma vineyards.

Turner and Pollard (1959) described the overwintering habit of *G. versuta* near peach orchards in Georgia. They found that *G. versuta* overwintered in wooded areas and at the onset of spring (late February to early March) moved to perennials, upon which it preferentially fed. It was most often observed feeding on ragweed, blackberry, cotton, and sunflower, but was rarely seen feeding on trees. Furthermore, it never showed preference for a certain plant host. In Georgia, *G. versuta* had three generations per year based on observations of lab-reared colonies. Those insects reared indoors exhibited a longer life span than those reared in outdoor cages (Turner and Pollard 1959). Myers et al. (2007) found that *G. versuta* had one generation per year in North Carolina, similar to that observed in Virginia. Likewise, in Texas vineyards, *G. versuta* had one peak in abundance throughout the season (Mitchell et al. (2009), which indicates one generation per year. Morano et al. (2010) found that *G. versuta* was more common in regions in Texas with higher annual precipitation based on extensive insect frequency data against environmental gradients (i.e., elevation, annual precipitation, cold hardiness, and one ecoregion environmental variable).

In vineyards, very few *O. orbona* were captured, which provided little information regarding seasonal phenology. In nurseries, *O. orbona* was captured more frequently and had two distinct peaks in abundance. In 2009 and 2010, the first peak occurred in early June followed by a second peak in late July to early August (Fig. 7). In Georgia pecan orchards, *O. orbona* had two and a partial third generation per year based on studies conducted indoors (Turner and Pollard 1959). In this study, it can be inferred from population peaks that *O. orbona* has two generation per year in Oklahoma.

In Virginia vineyards, Wallingford and Pfeiffer (2012) found that *O. orbona* had one generation per year based on collection records from ten vineyards and peaked in abundance from mid-May to late June then became scarce later in the season (Wallingford and Pfeiffer 2012). In Texas vineyards, this species is not often encountered (Mitchell et. al. 2009). Myers et al. (2007) reported one peak in abundance for *O. orbona* in vineyards, which occurred in mid-June. In Oklahoma vineyards, the peak abundance of this insect did not occur until August; however, other insects in general were infrequently encountered in all vineyards compared to trap counts of *G. versuta* (Table 1).

In vineyards, *C. xanthocephala* was rarely captured, except at one vineyard in 2010 where 18 individuals were captured the first week of August. In nurseries, large numbers of *C. xanthocephala* were captured in 2009 with two peaks occurring in early June and early July (Fig. 8).

Percentage of Insect Vectors Testing Positive for *X. fastidiosa*

From a subsample of insects captured in 2009-2010 from vineyards, nurseries, and pecan orchards, 2.4% tested positive for the presence of *X. fastidiosa* in their foreguts (Tables 3 and 4). Within all sites sampled, species testing positive for *X. fastidiosa* included; *O. orbona*, *G. versuta*, and *G. coccinea* (Tables 3 and 4). One specimen from a fourth species, *Cuerna costalis*, collected from nurseries tested positive for *X. fastidiosa* (Table 4). Although the percentage of collected vectors carrying *X. fastidiosa* was low, these results are significant because disease transmission only requires one infective individual feeding on several plants. Insects that tested positive were collected during

June and July, which corresponds to peak abundance of *G. versuta*, the predominant vector in Oklahoma vineyards (Fig. 5). In vineyards, 10.7% of *O. orbona* and 2.2% of *G. versuta* tested positive for *X. fastidiosa*. Although *G. coccinea* has not been shown to transmit *X. fastidiosa* in previous studies (Redak et al. 2004), my results revealed that 5.8% of *G. coccinea* screened from vineyards tested positive for *X. fastidiosa*.

In nurseries, four insect vectors tested positive for *X. fastidiosa*: *O. orbona*, *C. costalis*, *G. versuta*, and *G. coccinea*. None of the insects captured from pecan orchards tested positive for *X. fastidiosa*. Of the subsample of *O. orbona* tested, 2.9% were carrying *X. fastidiosa* (Table 4). Of those that were tested, 3.3% of *G. versuta*, 4.1% *G. coccinea*, and 2.7% *C. costalis* tested positive for *X. fastidisoa*.

Results from vineyard studies are comparable to those obtained in other states. In Texas vineyards, 12.1% of total insect vectors collected tested positive for *X. fastidiosa*. Of those testing positive, 13.3% of *G. versuta* tested positive for *X. fastidiosa* (Mitchell et al. 2009), which is a higher percentage than that obtained in this study. The difference is likely attributed to the infection status of the sampled vineyards. In Texas, Mitchell et al. (2009) sampled from six vineyards, three of which had large numbers of PD-infected vines. In contrast, I sampled from two vineyards in 2008 and four vineyards in 2009 that had no reports of PD-infected vines. In 2010, I sampled from five vineyards, three of which had recent reports of vines infected with *X. fastidiosa*. Thus, Mitchell et al. (2009) collected insects from PD-infected vineyards in a state with a history of the disease. Since the majority of insects I collected were from vineyards without reports of PD, it is reasonable to expect a higher rate of detection of *X. fastidiosa* from Texas vineyards.

Zhang et al. (2011) collected insects from urban shade trees (*Quercus* sp.) in New Jersey and surveyed potential insect vectors of *X. fastidiosa* that cause BLS. They tested the insects for presence of *X. fastidiosa* and found similar results to my study. Of the insects screened, 20% and 4% of *G. versuta* and *G. coccinea* tested positive for *X. fastidiosa*, respectively (Zhang et al. 2011). In my study of nurseries, 4% of *G. coccinea* screened tested positive for *X. fastidiosa* whereas only 3.3% of *G. versuta* screened were carrying the pathogen.

Positive samples were sequenced to determine which subspecies of *X. fastidiosa* were carried by insect vectors. One *G. versuta* collected from a vineyard in 2009 tested positive for *X. fastidiosa* that was 98% similar to *X. fastidiosa* subsp. *fastidiosa* GB514 (GenBank accession number CP002165.1; E Value 0), which causes PD in grape. In 2010, one *O. orbona* collected from a nursery and two *G. versuta* collected from vineyards were carrying *X. fastidiosa* that was 100% similar to *X. fastidiosa* strain M13 DNA gyrase subunit B (gyrB) gene, partial cds (GenBank accession number DQ223493.1; E Values $1e^{-137}$, $3e^{-140}$, and $4e^{-138}$, respectively). These three samples were also 99% similar to *X. fastidiosa* subsp. *fastidiosa* GB514 (GenBank accession number CP002165.1; E Values $2e^{-136}$, 3e-^{139}, and $5e^{-136}$, respectively). One *G. versuta* collected from a vineyard in 2010 was carrying *X. fastidiosa* that was 96% similar to *X. fastidiosa* subsp. *fastidiosa* GB514 (GenBank accession number CP002165.1; E Value:$7e^{-152}$). Another *G. versuta* collected from a nursery was 99% similar to this same subspecies (E Value $9e^{-130}$). Although only six samples were sequenced, many that were collected from vineyards were carrying the subspecies of *X. fastidiosa* that causes PD.

In Texas, Mitchell et al. (2009) found differences in infection status when comparing insects collected within vineyards to those that were collected outside of vineyards. *Graphocephala versuta* and *H. vitripennis* collected outside of vineyards were more likely to be carrying *X. fastidiosa* subspp. *multiplex* and *sandyi*, which do not cause PD in grapes. Vectors captured within the vineyard, however, were more likely to be carrying *X. fastidiosa* subsp. *fastidiosa* that causes PD (Mitchell et al. 2009). Thus, Mitchell et al. (2009) concluded that the spread of PD was from vine to vine since insects collected within vineyards were more likely to harbor *X. fastidiosa* subsp. *fastidiosa*. In my study, many of the positive insects were carrying *X. fastidios*a subsp. *fastidiosa*. This was true for both nursery and vineyard-collected insect vectors. In Oklahoma, more in-depth studies similar to that of Mitchell et al. (2009) are needed to better understand the disease cycle and identify viable vector and disease management programs. If secondary infection is the primary mode of pathogen spread, growers can successfully implement vector control that targets insects within the vines. However, if primary infection is the main source of *X. fastidiosa*, studies are needed that identify plant hosts that are nearby vineyards and are viable hosts to insect vectors and *X. fastidiosa*.

Insect Abundance Comparison

Nurseries versus Vineyards

I compared total numbers of the three most commonly captured species, *G. versuta*, *O. orbona*, and *C. xanthocephala*, from vineyards versus nurseries. For each weekly comparison in 2009 and 2010, there were no significant differences in abundance of *O. orbona* and *C. xanthocephala* between vineyards and nurseries. However, there

were fewer numbers of these insects captured overall compared to that of *G. versuta*. In 2009, significantly more *G. versuta* were captured from nurseries during the third week of June compared to those captured from vineyards (Table 5). In the same year, there were significantly more *G. versuta* in vineyards the last week of July and first week of August (Table 5). In 2010, significantly more *G. versuta* were captured from vineyards throughout July until the second week of August (Table 5).

In general, more *G. versuta* were captured in nurseries early in the season than later in the season (Table 5). This could reflect those observations by Turner and Pollard (1959) that *G. versuta* moved from woody overwintering sites into perennials early in the season. Understanding the seasonal phenology of *G. versuta*, the predominant insect vector in Oklahoma vineyards, is important for timely and cost-effective disease management. If grape growers know when to expect insect vectors moving into vines from overwintering sites, they can begin monitoring programs that coincide with vector emergence.

Interior versus Perimeter

Sticky cards were placed in a similar pattern at each vineyard, tree nursery, and pecan orchard. Four cards were placed within the interior of the crop (i.e., vines or trees) and located at least 20 ft. from the edge of the crop. The remaining eight cards were placed along the perimeter of the crop. I compared the total number of insects captured on the interior to those on the perimeter at each sampling site for each week of sampling. However, very few differences were observed for comparisons between the interior and perimeter at each location. There were no significant differences observed in captures of

O. orbona and *C. xanthocephala*. Significantly more *G. versuta* were captured from the interior compared to the perimeter at two vineyards (A&B) in July and the first week of August in 2009 (Table 6).

In Texas vineyards, Lauzière et al. (2008) carried out a similar study to identify changes in seasonality as well as the overwintering habits of insect vectors of *X. fastidiosa*. Exterior traps were placed in areas adjacent to the vines in nearby weedy habitats and interior cards were located within the vines. No differences were found in the number of *G. versuta* and *C. xanthocephala* captured in the surrounding environment compared to those captured within the vineyard. Based on these findings, they concluded that these two species did not preferentially feed upon or inhabit either area (Lauzière et al. 2008). In my studies, I did not examine host plant preference of the common insect vectors collected from vineyards. However, my findings suggest that *G. versuta* commonly feeds on grapes.

In nurseries, there were no significant differences in vector abundance in the interior versus perimeter for *O. orbona* and *C. xanthocephala*. On three of the six sampling dates, significantly more *G. versuta* were captured from the perimeter of nurseries compared to the interior at two locations (Table 6). As mentioned earlier, trees are not the preferred hosts of *G. versuta* (Turner and Pollard 1959). Thus, results obtained from sampling in nurseries suggest that *G. versuta* may be attracted to yellow sticky cards from the surrounding environment.

Conclusions

The three most frequently captured vector species were *G. versuta*, *O. orbona*, *and C. xanthocephala*; *G. versuta* was the most abundant species in both vineyards and nurseries. *Graphocephala versuta* was the major vector species collected in this survey, and several subsamples of the sharpshooter populations tested positive for *X. fastidiosa*. Based on three years of capture data from yellow sticky traps, *G. versuta* produces up to two generations per year in Oklahoma. For both nurseries and vineyards, most insects that tested positive for *X. fastidiosa* were collected in June and July, which coincided with peak abundance. Increased abundance of insect vectors can increase the likelihood of *X. fastidiosa* transmission due to a larger number of "inoculation events" subsequently leading to chronic infections (Daugherty and Almeida 2009). In perennial crops like grapes, the incidence of disease can be high even when the abundance of insect vectors is low (Almeida et al. 2005).

Vector abundance can be associated with incidence of PD in vineyards for disease cycles where primary infection is important (Purcell 1981). Primary infection occurs when insects infect vines with *X. fastidiosa* that was acquired from an outside source. For example, an early season peak in the abundance of blue-green sharpshooter (BGSS) in California's Napa Valley was correlated with transmission of PD and subsequent development of infections that persisted from year to year (i.e., chronic infection) (Purcell 1981). This early season transmission of *X. fastidiosa* to grape plants by a vector is thought to lead to a more established chronic infection in grape plants. This is attributed to the amount of time that bacteria have to multiply and translocate within the plant before the onset of winter (Almeida et al. 2005). In my studies, insects did not test

positive for *X. fastidiosa* when collected early in the season (i.e., March to May). In contrast, most insects testing positive were captured in June and July. In addition, I found that these insects were inoculative (i.e., able to transmit *X. fastidiosa* to a susceptible plant) (see Chapter 4). In Florida, Adlerz and Hopkins (1979) found that insect vectors were inoculative later in the season, when *X. fastidiosa* titers in the grape were sufficient for acquisition. This indicated that *X. fastidiosa* was being transmitted from vine to vine. Mitchell et al. (2009) found similar results in Texas. This is likely the scenario in Oklahoma vineyards based on the mid- to late-season abundance of infective vectors. In California, Feil et al. (2003) found that if inoculation occurs later in the season, affected vines were able to overcome the infection and did not exhibit PD symptoms the following year. Recovery from infection resulted from the combined effects of dormant-season pruning, thereby removing vines infected late in the season, and reduced pathogen survival due to cold winter temperatures (Feil et al. 2003). *Xylella fastidiosa* subsp. *fastidiosa* will not survive winter temperatures that fall below 4 ºC (Hoddle 2004). Presently, PD in Oklahoma has only recently been reported and little is known of the overwintering survival of *X. fastidiosa* in vines.

I collected many different insect species that are potential vectors of *X. fastidiosa* in vineyards. However, only a few species are thought to influence development of PD (Almeida et al. 2005). Other factors, such as transmission efficiency of the insect vector and ecological information (e.g., host preference, seasonality, and distribution) influence the disease cycle (Almeida et al. 2005). Host plant preference impacts the number of insects found on a particular plant and the length of time spent feeding on it (Almeida et al. 2005). In theory, feeding duration on a suitable host plant should be positively

correlated with inoculation of *X. fastidiosa*; however, development of PD in vineyards is influenced more by overwintering survival of the bacterium and the degree of success of initial colonization (Almeida et al. 2005).

Daugherty et al. (2010) provided recent evidence that within-plant feeding preference influences the rate of *X. fastidiosa* transmission by changing the vector's propensity for pathogen acquisition. For example, when BGSS was allowed to feed only in the canopy of alfalfa, its preferred feeding site, the acquisition rate of *X. fastidiosa* was low. When BGSS fed only on the base of the alfalfa stem, a non-preferred site, the vector acquired *X. fastidiosa* at a higher rate. The difference resulted from a higher incidence of *X. fastidiosa* in the lower stem of alfalfa compared to the canopy (Daugherty et al. 2010). Based on my comparisons of vectors captured from the interior versus the perimeter of vineyards, it is evident that grapes are a preferred and viable host of *G. versuta*, since more insects were found within the interior of grape vines. The preference for grape would positively influence the transmission of *X. fastidiosa* from vine to vine. These insects are most likely feeding on young plant growth based on observations of Turner and Pollard (1959), which would increase the likelihood of inoculation of *X. fastidiosa* (Almeida et al. 2005). Purcell (1981) found that older plant growth of some grape cultivars was less susceptible to *X. fastidiosa* infection.

My studies generated important information about *X. fastidiosa* transmission in nurseries and pecan orchards; however, BLS has not been a major limiting factor for pecan and nursery production in Oklahoma. Indeed, pecan BLS has not been detected in our state to date (Jen Olson, *pers. comm.*) and no insects captured from pecan orchards were carrying *X. fastidiosa*. I determined that several insects captured from nurseries

were carrying *X. fastidiosa* subsp. *fastidiosa*, the subspecies that causes PD. Furthermore, insects captured in nurseries were more often captured at the perimeter of sampled trees, suggesting that nearby perennial and weedy hosts were vector sources. Modified trapping studies could elucidate the relationship among nursery trees, surrounding flora, and insect movement between them. Many species (i.e., *O. orbona* and *G. versuta*) are reported to move from wooded areas into crops throughout the season to optimize their nutrient-poor food source (Turner and Pollard 1959, Redak et al. 2004).

Currently, recommendations to grape growers include rogueing diseased vines and using sterile pruning shears during the growing season to limit secondary spread of the pathogen (i.e., vine-to-vine transmission). However, before recommending rogueing of plants it is important to consider if the infection cycle is primary or secondary. In California's Central Valley, transmission of *X. fastidiosa* to grapevines is primary and occurs when BGSS incidentally feeds on grapes that are planted near this species' preferred habitat, alfalfa pastures or irrigated lands. In cases where the infection is primary, there is little benefit in rogueing diseased plants (Sisterson and Stenger 2013). When the infection is secondary, however, plants should be rogued immediately to slow the spread of the pathogen (Sisterson and Stenger 2013).

Insecticides are useful if application timing coincides with peak occurrence of insect vectors in vineyards (Purcell and Frazier 1985). In Texas, insects transmit *X. fastidiosa* from infected vines to un-infected vines within the vineyard (Mitchell et al. 2009), and the use of neonicotinoid and pyrethroid insecticides are effective against sharpshooter vectors (Kamas et al. 2000). In my study, abundance of *G. versuta* first increased in early June and based on this peak, growers can effectively target this vector

with systemic insecticides provided there is a sufficient dose within the plant to kill the insects when they begin feeding. Targeting insect vectors for control can limit vine-to-vine spread of *X. fastidiosa*, which is likely occurring in Oklahoma vineyards.

The climate where the vines are grown is an important component that influences the disease cycle and should be considered when recommending management options. As mentioned, *X. fastidiosa* subsp. *fastidiosa* will not survive winter temperatures that fall below 4 ºC (Hoddle 2004). If vines are infected later in the season in colder regions, the bacteria are unable to gain a systemic infection that travels further into the plant where bacteria are protected from cold temperatures. This phenomenon is known as 'cold curing' (Lieth et al. 2012). In Oklahoma, late-season infections to vines is most likely occurring based on their mid- to late-season peak abundance that coincided with the time infected vectors are detected. Therefore, chronic infections of *X. fastidiosa* in vines are not expected. Climate change has potential to increase the likelihood that *X. fastidiosa* will establish chronic infections in Oklahoma grapes. Additionally, the range of GWSS, the key vector of PD, could expand further northward into Oklahoma (Garrett et al. 2006). Currently, many Oklahoma counties are considered low to moderate risk for the occurrence of PD (Smith and Dominiak-Olson 2009). If temperatures continue to increase, changes are likely in the distribution of key vectors and *X. fastidiosa* subsp. *fastidiosa*.

This study lays the foundation for research of PD and BLS in Oklahoma. Future studies should track yearly occurrence of PD in grapevines to determine if vines are able to recover from *X. fastidiosa* infection. In California, the recovery from infection resulted from the combined effects of dormant-season pruning and reduced pathogen

survival due to cold winter temperatures (Feil et al. 2003). In a Creek County vineyard, PD was first reported in 2009 and I observed that infection of those vines persisted throughout my study. The infection in those vines is chronic and not limited by winter-time temperatures. More research is needed that tracks pathogen survival in vines over time. In Oklahoma, grape production is a growing industry that went from 525 acres of grapes in 2005 (Stafne 2007) to over 600 acres in 2011 (Oklahoma Grape Industry Council, *pers. comm*.). Oklahoma growers preferentially plant *Vitis vinifera* cultivars (e.g., 'Cabernet Sauvignon' and 'Merlot') (Stafne 2007), which are susceptible to *X. fastidiosa* (Kamas et al. 2000). If the incidence of PD in Oklahoma increases, growers stand to experience significant loss of plants.

The predominant insect vector of *X. fastidiosa* in Oklahoma vineyards, tree nurseries, and pecan orchards, *G. versuta,* was most abundant in June and July. Of a subsample of insects captured in 2009-2010 from vineyards, nurseries, and pecan orchards, 2.4% tested positive for carrying *X. fastidiosa* in their foreguts. It was during their peak abundance when positive insects were captured. Insecticide application should be timed to coincide with peak insect activity to reduce the chance of pathogen transmission. More studies are needed on the life history of *G. versuta* in grapevines and shade trees. Little is known of its preferred food and oviposition plants. Furthermore, more research is needed in pecan orchards to determine what insect vector would likely be important in transmission of *X. fastidiosa*. Of the 933 G. *versuta* collected from tree nurseries and pecan orchards, only 51 were captured from pecan orchards. No insects collected from pecan orchards were carrying *X. fastidiosa*.

In Oklahoma, pecan BLS has not been documented. However, monitoring efforts should continue based on findings in Louisiana that have shown nut yield reductions are likely with chronic infection of *X. fastidiosa* (Sanderlin and Heyderich-Alger 2003). Oklahoma pecan acreage exceeds 140,000 and ranks second in total acreage to Texas in the United States (Smith 2009). Monitoring efforts for pecan BLS in our state should continue.

Bacterial leaf scorch in Oklahoma has been documented in American elm, sycamore, mulberry, red oak, and porcelain berry (Dominiak and Olson 2006, Olson et al. 2006) and has potential to negatively impact urban and commercially grown shade trees. Infected trees do not die immediately but tree life is shortened and the aesthetic quality is reduced (Sherald and Kostka 1992). Growers should rogue infected trees to reduce the chance of *X. fastidiosa* being transmitted to healthy trees. My studies are the first to document infective insect vectors that carry *X. fastidiosa* in tree nurseries in this state and based on my findings there is potential for the spread of *X. fastidiosa* in both small and large production operations.

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Table 1. Total numbers of xylem sap-feeding insects captured on sticky cards[a] in Oklahoma vineyards[b].

Species	Total			
	2008[c]	2009[d]	2010[e]	
Cicadellidae: Cicadellinae: Proconiini				**Total**
Paraulacizes irrorata	0	3	3	6
Oncometopia orbona	5	6	22	33
Cuerna costalis	3	2	3	8
Cicadellidae: Cicadellinae: Cicadellini				
Graphocephala versuta	57	609	1622	2288
Graphocephala coccinea	0	13	3	16
Graphocephala hieroglyphica	0	6	2	8
Draeculacephala navicula	0	0	8	8
Xyphon flaviceps	0	5	1	6
Cercopidae				
Clastoptera xanthocephala	2	4	37	43

[a] 12 sticky cards (12.2 X 7.6 cm) were used at each sampling location; Cards were replaced weekly or bi-weekly from March until September each year

[b] Vineyards were planted with French-American hybrids, *Vitis* spp., and *Vitis vinifera* cultivars; Wagoner Co. vineyard was planted with 'Cynthiana.' *Vitis aestivalis.*

[c] Sampled two vineyards in Payne and and Wagoner Counties

[d] Sampled five vineyards in Payne, Wagoner, Jackson, Canadian, Greer Counties

[e] Sampled six vineyards in Payne, Wagoner, Creek, Tulsa, and Lincoln Counties

Table 2. Total numbers of xylem sap-feeding insects captured on sticky cards[a] in Oklahoma tree nurseries[b] and pecan orchards[c].

Species	Total			Total
	2008[d]	2009[e]	2010[f]	
Cicadellidae: Cicadellinae: Proconiini				
Paraulacizes irrorata	43	52	20	115
Oncometopia orbona	121	430	376	927
Cuerna costalis	170	27	12	209
Homalodisca vitripennis	2	3	0	5
Cicadellidae: Cicadellinae: Cicadellini				
Graphocephala versuta	85	365	483	933
Graphocephala coccinea	18	16	14	48
Graphocephala hieroglyphica	25	1	4	30
Draeculacephala navicula	14	1	12	27
Xyphon flaviceps	15	6	0	21
Cercopidae				
Clastoptera xanthocephala	18	294	3	315
Prosapia bicincta	7	2	0	9

[a] 12 sticky cards (12.2 X 7.6 cm) were used at each sampling location; cards were replaced weekly or bi-weekly from March until September each year

[b] Tree nurseries were planted with oak (*Quercus* spp.), elm (*Ulmus* spp.), willow (*Salix* spp.), sycamore (*Platanus occidentalis*), maple (*Acer* spp.), redbud (*Cercis canadensis*), and birch (*Betula* spp.)

[c] Pecan orchards were planted with pecan (*Carya illinoensis*) cultivars 'Pawnee' (Marshall Co.) and 'Stuart' (Lincoln Co.)

[d] Sampled three tree nurseries in Custer, Cherokee, and Oklahoma Counties; did not sample pecan orchards

[e] Sampled three tree nurseries in Custer, Cherokee, and Oklahoma Counties; sampled one pecan orchard in Marshall County

[f] Sampled three tree nurseries in Custer, Cherokee, and Oklahoma Counties; sampled two pecan orchards in Marshall and Lincoln Counties

Table 3. Xylem sap-feeding insects tested for presence of *Xylella fastidiosa.*collected[a] from vineyards[b].

Species	2009[c] and 2010[d] Results			
	Sampled	Tested[e]	# Positive	% Positive
Cicadellidae: Cicadellinae: Proconiini				
Paraulacizes irrorata	6	6	0	0
Oncometopia orbona	28	28	3	10.7
Cuerna costalis	5	4	0	0
Homalodisca vitripennis	0	0	0	0
Cicadellidae: Cicadellinae: Cicadellini				
Graphocephala versuta	2231	946	21	2.2
Graphocephala coccinea	17	17	1	5.8
Graphocephala hieroglyphica	8	7	0	0
Xyphon flaviceps	6	1	0	0
Cercopidae				
Clastoptera xanthocephala	39	29	0	0
Total	2340	1038	25	2.4

[a] 12 sticky cards (12.2 X 7.6 cm) were used at each sampling location; cards were replaced weekly or bi-weekly from March until September each year

[b], All vineyards were planted with French-American hybrids (*Vitis* spp.) and *Vitis vinifera* cultivars, except the Wagoner Co. vineyard, which was planted with 'Cynthiana (*Vitis aestivalis)*

[c] Sampled five vineyards in Payne, Wagoner, Jackson, Canadian, Greer Counties

[d] Sampled six vineyards in Payne, Wagoner, Creek, Tulsa, and Lincoln Counties

[e] A subsample of the total number collected was tested using Immunocapture PCR

Table 4. Xylem sap-feeding insects tested for presence of *Xylella fastidiosa* collected[a] from tree nurseries[b] and pecan orchards[c].

Species	2009[d] and 2010[e] Results			
	Sampled	Tested[f]	# Positive[g]	% Positive
Cicadellidae: Cicadellinae: Proconiini				
Paraulacizes irrorata	72	62	0	0
Oncometopia orbona	806	724	21	2.9
Cuerna costalis	39	37	1	2.7
Homalodisca vitripennis	3	0	0	0
Cicadellidae: Cicadellinae: Cicadellini				
Graphocephala versuta	895	504	17	3.3
Graphocephala coccinea	29	24	1	4.1
Graphocephala hieroglyphica	5	3	0	0
Xyphon flaviceps	6	0	0	0
Cercopidae				
Clastoptera xanthocephala	299	299	0	0
Total	2154	1653	40	2.4

[a] 12 sticky cards (12.2 X 7.6 cm) were used at each sampling location; cards were replaced weekly or bi-weekly from March until September each year

[b] Tree nurseries were planted with oak (Quercus spp.), elm (Ulmus spp.), willow (Salix spp.), sycamore (Platanus occidentalis L.), maple (Acer spp.), redbud (Cercis canadensis L.), and birch (Betula spp.).

[c] Pecan orchards were planted with pecan (*Carya illinoensis*) cultivars 'Pawnee' (Marshall Co.) and ‘Stuart’ (Lincoln Co.)

[d] Sampled three tree nurseries in Custer, Cherokee, and Oklahoma Counties; sampled one pecan orchard in Marshall county

[e] Sampled three tree nurseries in Custer, Cherokee, and Oklahoma Counties; sampled two pecan orchards in Marshall and Lincoln Counties

[f] A subsample of the total number collected was tested using Immunocapture PCR

[g] No positive insects were collected from pecan orchards

Table 5. Comparison of *Graphocephala versuta* abundance collected from vineyards and nurseries[a].

Date[b]	Vineyards	Nurseries	K-W[c]	*P*
2009				
12 June	0[d]	49	4.1829	0.0408
19 June	2	153	8.8169	0.0030
26 June	195	75	0.1759	0.6749
10 July	36	44	2.2084	0.1464
17 July	16	15	0.2013	0.4027
24 July	217	16	3.9479	0.0469
7 Aug.	142	15	30.6594	0.0001
2010				
19 June	101	29	1.7489	0.186
26 June	19	50	0.6592	0.4168
3 July	167	96	2.2918	0.1301
10 July	570	31	7.5540	0.0060
19 July	110	18	6.4558	0.0111
24 July	176	9	9.2758	0.0023
31 July	113	9	9.0551	0.0026
7 Aug.	56	6	9.8553	0.0017
14 Aug.	148	5	8.6218	0.0033
21 Aug.	42	5	2.5662	0.1092
4 Sept.	47	1	5.3039	0.0213

[a] Nurseries include tree nurseries and pecan orchards

[b] Date that card was retrieved from field

[c] Kruskal-Wallace test statistic; *df*=1 for all tests

[d] Sums of *G. versuta* are significantly different at $P \leq 0.05$

Table 6. Comparisons of *Graphocephala versuta* collected from the interior and perimeter of vineyards and nurseries[a].

Date[b]	Site[c]	Interior	Perimeter	K-W[d]	*P*
Vineyards					
10 July 2009	A	13[e]	6	4.0415	0.0444
24 July 2009	B	132	75	3.8418	0.05
7 Aug. 2009	B	83	25	7.1042	0.0256
26 June 2010	A	13	6	4.4328	0.0353
10 July 2010	C	50	53	3.5908	0.0581
10 July 2010	A	15	7	4.8461	0.0277
31 July 2010	C	3	0	7.333	0.0068
14 Aug. 2010	C	23	6	7.3139	0.0068
Nurseries					
19 June 2009	D	7	26	0.3688	0.6135
26 June 2009	D	3	40	4.0113	0.0452
10 July 2009	D	3	20	2.6977	0.1005
26 June 2009	E	1	16	4.483	0.0342
10 July 2009	E	5	12	1.0023	0.3168
17 July 2009	E	0	15	4.3636	0.0367

[a] Nurseries did not include pecan orchards

[b] Date that card was retrieved from field

[c] Different letters indicate different sites; Such Cuonty Vineyard; B) County Vineyar,

[d] Kruskal-Wallace test statistic; *df*=1 for all tests

[e] Sums of *G. versuta* are significantly different at $P \leq 0.05$

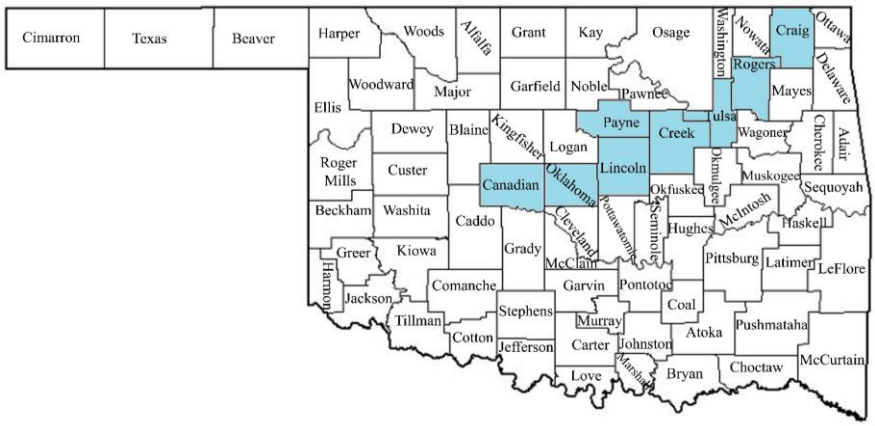

Figure 1. Counties with confirmed cases of Pierce's disease from 2008 to 2010 (source: Plant Disease and Insect Diagnostic Lab, Oklahoma State University).

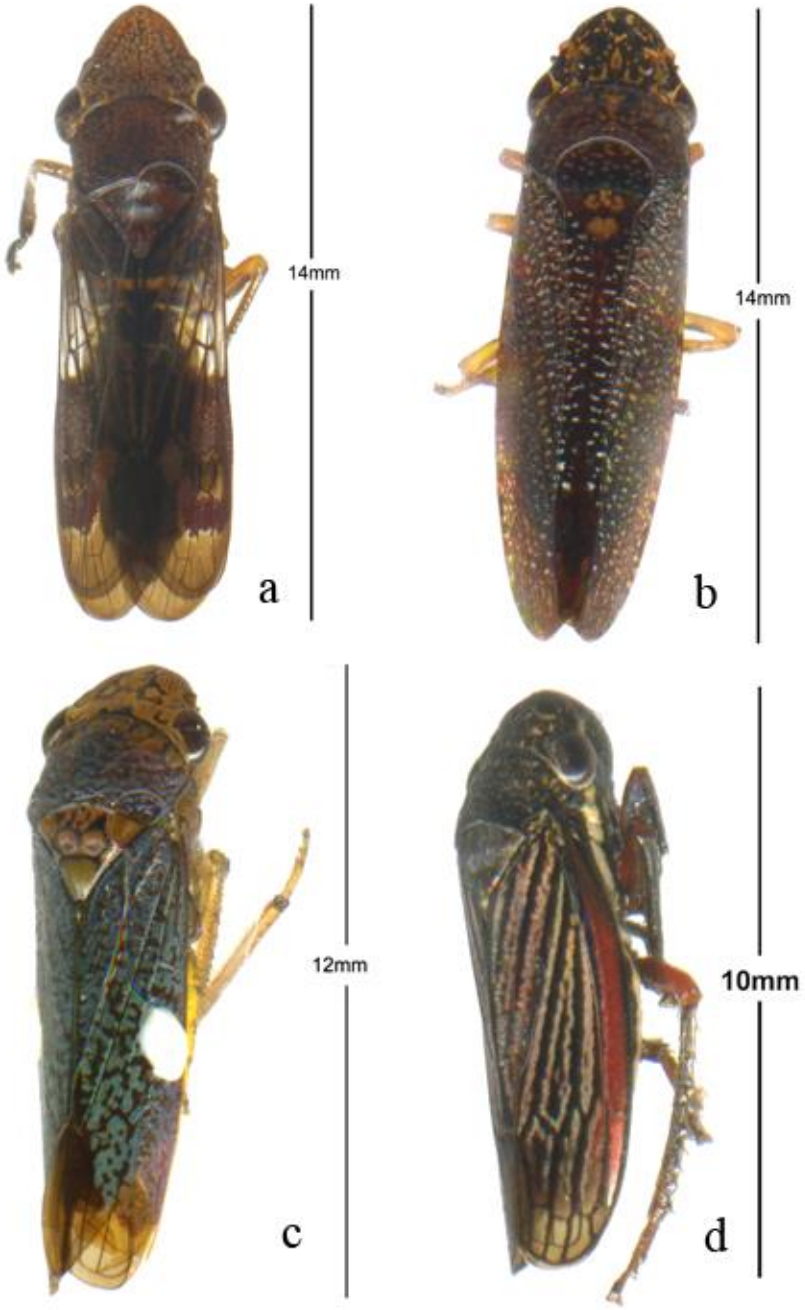

Figure 2.Insect vectors (Hemiptera:Cicadellidae:Cicadellinae:Proconiini) captured from vineyards, tree nurseries and pecan orchards in Oklahoma: a) *Homalodisca vintripennis*; b) *Paraulacizes irrorata*; c) *Oncometopia orbona*; d) *Cuerna costalis*.

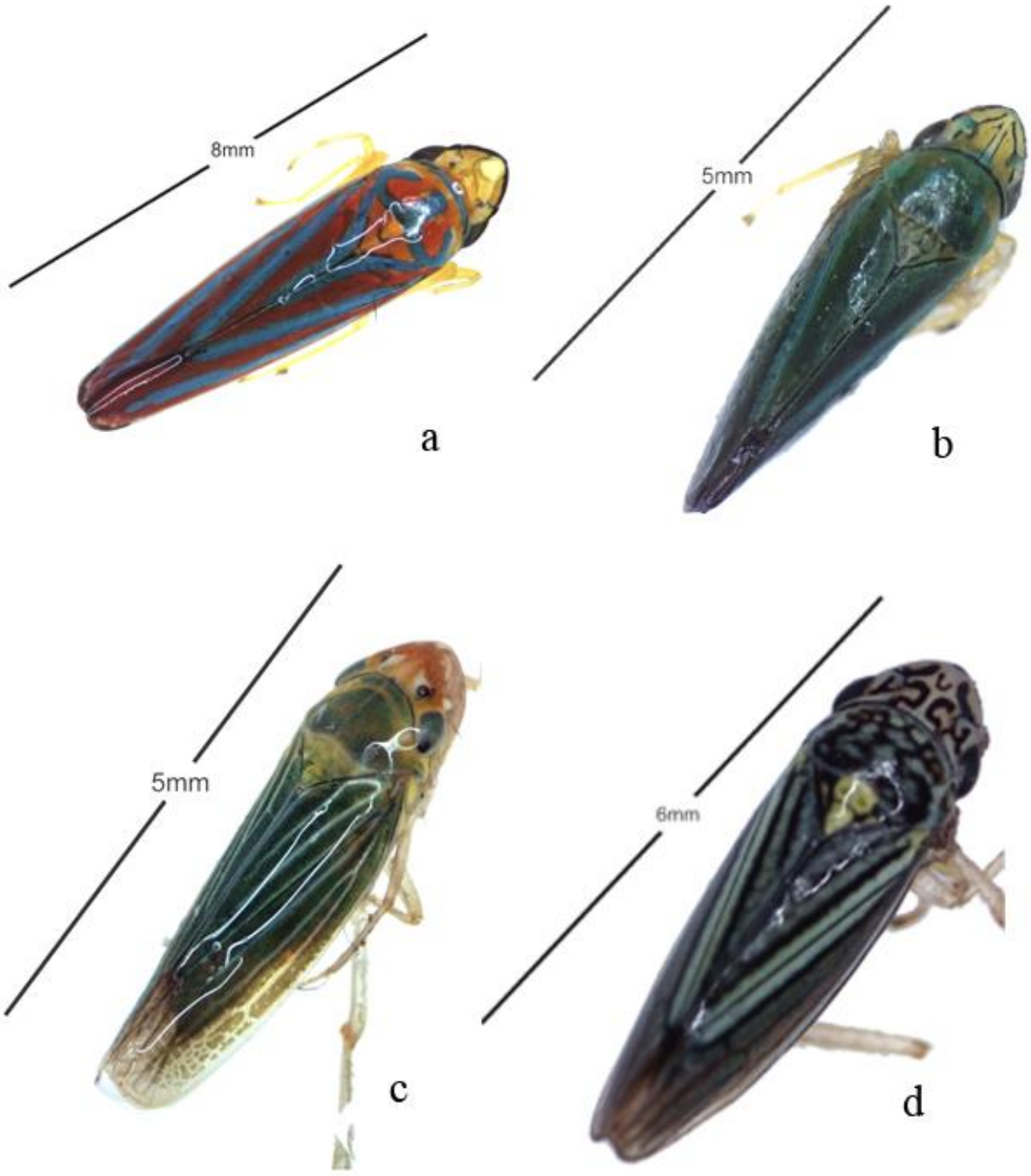

Figure 3. Insect vectors (Hemiptera:Cicadellidae:Cicadellinae: Cicadellini) captured from vineyards, tree nurseries and pecan orchards in Oklahoma: a) *Graphocephala coccinea*; b) *G. versuta*; c) *Xyphon flaviceps*; d) *G. heiroglyphica*.

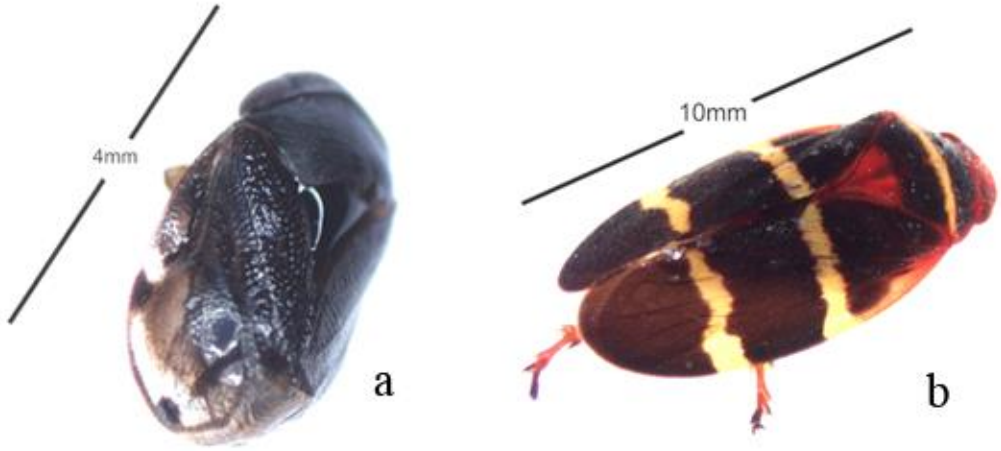

Figure 4. Insect vectors (Hemiptera:Cercopidae) captured from vineyards, tree nurseries and pecan orchards in Oklahoma: a) *Clastoptera xanthocephala*; b) *Prosapia bicincta*.

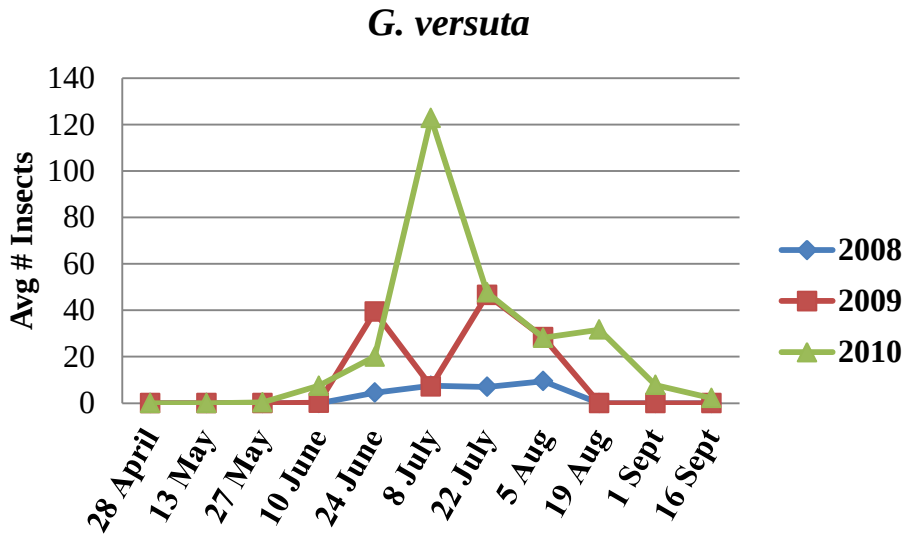

Figure 5. Seasonal phenology of *Graphocephala versuta* in vineyards; insect numbers are averaged by the number of sites sampled for that year (2 vineyards in 2008, 5 in 2009, and 6 in 2010).

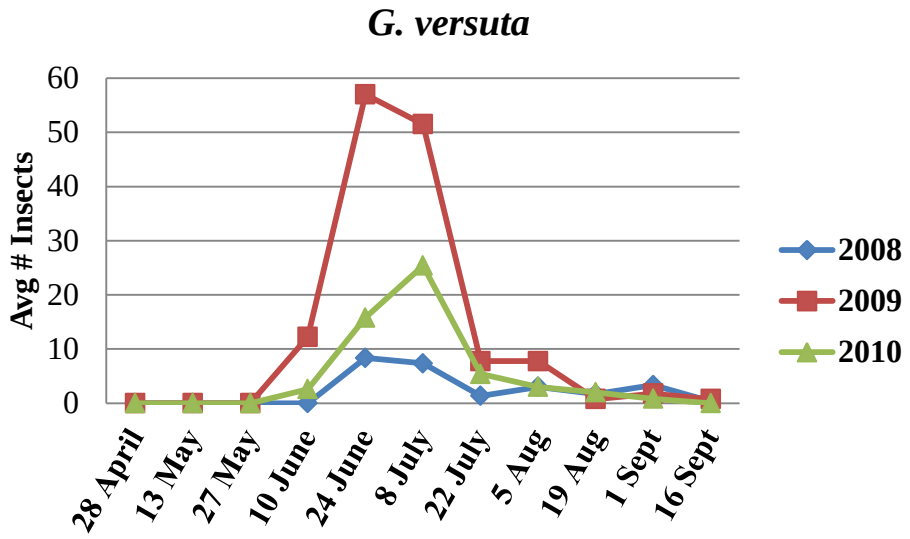

Figure 6. Seasonal phenology of *Graphocephala versuta* in tree nurseries and pecan orchards; insect numbers are averaged by the number of sites sampled for that year (3 in 2008, 4 in 2009, and 5 in 2010).

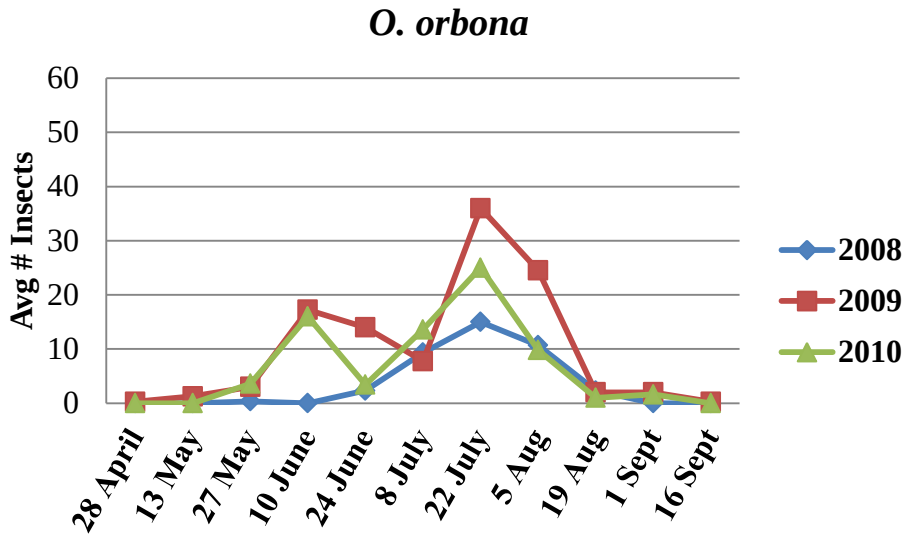

Figure 7. Seasonal phenology of *Oncometopia orbona* in nurseries; insect numbers are averaged by the number of sites sampled for that year (3 in 2008, 4 in 2009, and 5 in 2010).

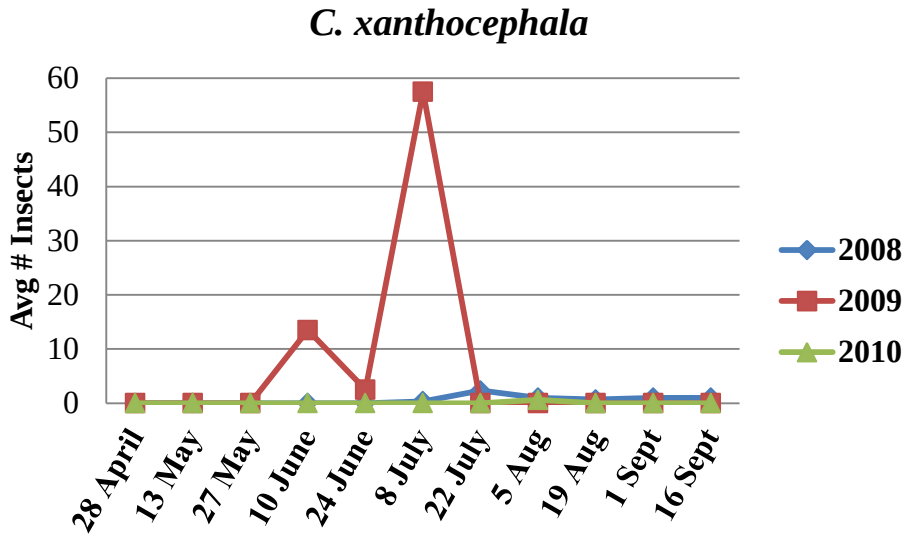

Figure 8. Seasonal phenology of *Clastoptera xanthocephala* in nurseries; insect numbers are averaged by the number of sites sampled for that year (3 in 2008, 4 in 2009, and 5 in 2010).

CHAPTER IV

THE NATURAL INOCULATIVITY OF INSECT VECTORS OF *XYLELLA FASTIDIOSA* COLLECTED FROM OKLAHOMA VINEYARDS

Abstract

Xylella fastidiosa subspecies *fastidiosa* is the causative agent of Pierce's disease of grape. This gram-negative bacterium is transmitted by xylem sap-feeding insects in the families, Cicadellidae and Cercopidae. In recent years, Pierce's disease of grape has been detected in eight counties in central and northeastern Oklahoma, prompting further investigation of the disease epidemiology in this state. My objective was to determine the natural inoculativity of commonly captured insect vectors occurring in vineyards. From August 2012 until May 2012, *Graphocephala versuta*, *G. coccinea*, *Paraulacizes irrorata*, *Oncometopia orbona*, *Cuerna costalis, and Entylia carinata* were collected from vineyards, taken back to the lab, and allowed an inoculation access period of seven days. Ragweed and alfalfa were selected as indicator plants since they are known hosts of *X. fastidiosa* subspp. *fastidiosa* and *multiplex*, are grown from seed, require little space, and grow rapidly. Following insect removal, indicator plants were held for two weeks before testing for insect inoculation of *X. fastidiosa*. Immunocapture-PCR was used to test all plant and insect samples in this study. Field collected *G. versuta* were inoculative

to both ragweed and alfalfa. Following inoculation, a higher percentage of alfalfa became infected than ragweed. The results will be used to aid integrated pest management programs that prevent or reduce further spread of Pierce's disease in Oklahoma vineyards.

Introduction

Xylella fastidiosa subspecies *fastidiosa* is a gram-negative bacterium that causes Pierce's disease (PD) of grape, a costly malady for the grape-growing industry in California and Texas. This pathogen is reported to be limited to warmer climates and poses little threat to Oklahoma (Feil and Purcell 2001). However, PD has been detected in several commercial vineyards in Oklahoma (Smith et al. 2009), warranting investigation of PD in this state (Fig. 1). While little is known about how PD arrived in Oklahoma and how it is spreading throughout the state, the disease is likely to increase in severity as it has in the southeastern U.S. over the past six years due to warmer winter temperatures (Anas et al. 2008). Indeed, Smith and Dominiak-Olson (2009) used established temperature thresholds and historical weather data to determine relative risk of PD surviving winter in five grape-producing counties of Oklahoma. Based on ten years of surface air temperature data, the authors conclude that Craig Co. in northeastern Oklahoma is at low risk for survival of the pathogen that causes PD, but Tulsa, Payne, Lincoln, and Kiowa Counties are at moderate risk (Smith and Dominiak-Olson 2009). The current economic losses associated with PD in Oklahoma are unknown due to the relatively recent history of the disease in the state. However, Texas has experienced millions of dollars in losses since 1990 due to PD, which limits the wine grape industry in the eastern part of that state (Kamas et al. 2000). Given the high value of the crop and the history of this disease causing serious economic harm in Texas, Oklahoma

growers stand to experience heavy losses. As of 2011, the industry has grown to over 600 acres of grapes, at least 60 licensed wineries, and 80 vineyards (Oklahoma Grape Industry Council, *pers. comm.*).

While the Oklahoma grape and wine industry continues to expand, many pest and disease problems are arising that require scientific expertise. One of the most serious threats to Oklahoma vineyards is the recent increased incidence of PD. *Xylella fastidiosa* is transmitted by xylem sap-feeding insects including spittle bugs (Hemiptera: Cercopidae) and sharpshooters (Hemiptera: Cicadellidae: Cicadellinae) (Almeida et al. 2005). However, mechanical transmission can occur via pruning shears (Krell et al. 2007) or propagation of infected grape cuttings (Robacker and Chang 1992). Insect vectors carry bacteria within their foregut and adults are able to continuously transmit *X. fastidiosa* once they acquire the pathogen by feeding on infected host plants (i.e., persistent transmission) (Hopkins and Purcell 2002, Purcell and Finlay 1979). The foregut lining is shed during the molting process, and as a result, any bacterial cells adhering to the foregut lining are also lost (Purcell and Finlay 1979). Further, the bacterium is not transferred from females to their eggs (i.e., transovarial transmission) (Freitag 1951).

Within the host plant, the bacteria multiply, spread, and can eventually clog the water-conducting xylem tissues, which leads to symptoms of PD. Typical symptoms include marginal leaf necrosis, usually separated from healthy tissue by a discolored band, which varies with grape variety (Fig. 2a). Another characteristic symptom is the appearance of "matchsticks", or petioles that have remained attached to the stem after leaf drop (Fig. 2b). In Texas, susceptible grape plants will die from PD infection (Kamas et al. 2000) and, in extreme cases, entire vineyards may be lost (Mitchell et al. 2009).

Several potential vectors of *Xylella fastidiosa* occur in Oklahoma, including glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae) (Overall et al. 2010). Glassy-winged sharpshooter (GWSS) is the most important vector of the pathogen that causes PD in California and Texas because, relative to other sharpshooters, it has a wider host range, higher dispersal ability, and can feed on both woody and non-woody plant tissue (Almeida and Purcell 2003, Redak et al. 2004). The latter trait is significant because feeding on woody stems inoculates plant tissue that is not pruned, thus facilitating establishment of the pathogen in the plant (Almeida and Purcell 2003). While low numbers of GWSS have been recovered in Oklahoma (Overall et al. 2010), other potential sharpshooter vectors occur in much greater abundance. Of particular concern are versute sharpshooter, *Graphocephala versuta* (Say), and broad-headed sharpshooter, *Oncometopia orbona* (F.), which have been shown to transmit *X. fastidiosa* subsp. *fastidiosa* to grapes in North Carolina (Myers et al. 2007).

The objective of this study was to determine if commonly encountered insect vectors in Oklahoma vineyards are infected with and able to inoculate *X. fastidiosa* to healthy plants. It is difficult to predict and manage diseases that are transmitted by insect vectors; therefore, data on transmission efficiency and vector abundance over time are used to predict the incidence of PD in vineyards (Daugherty and Almeida 2009). Also important is monitoring for the appearance of inoculative insects in or near vineyards. If *X. fastidiosa* is transmitted to grape plants in early spring, the bacteria have a better chance of establishing a chronic infection by surviving cold winter temperatures (Almeida et al. 2005). The results obtained from this study, coupled with vector abundance data, will elucidate the disease transmission

cycle of PD in Oklahoma vineyards, providing growers practical knowledge to manage and prevent the spread of *X. fastidiosa*.

Materials and Methods

Natural Inoculatively Tests

Although grape would be an ideal plant to demonstrate inoculation of *X. fastidiosa*, it is impractical for this study because it requires long periods of time to grow and facilities large enough to hold a large number of vines. Rather, two greenhouse-grown surrogate plant species that are known to be susceptible to *X. fastidiosa* subspp. *fastidiosa* and *multiplex* were used. Alfalfa, *Medicago sativa* L., is susceptible to both subspecies (Lopes et al. 2009) and has been used as an indicator plant in a previous study (Hill and Purcell 1997). Ragweed, *Ambrosia artemisiifolia* L., is often infected with *X. fastidiosa* subsp. *multiplex* (Timmer et al. 1983, Dominiak and Olson 2006) and was included in this study based on previous findings that many of the insects captured were carrying both subsp. *multiplex* and subsp. *fastidiosa* (see Chapter 3). An advantage of working with these plants is that they grow quickly from seed, thus ensuring that they were not previously infected with the bacterium prior to inoculation studies. Insect vectors (*G. versuta*, *G. coccinea*, *O. orbona*, *Paraulacizes irrorata* (F.), *Cuerna costalis* (F.) (Hemiptera: Cicadellidae), and *Entylia carinata* Germar (Hemiptera: Membracidae)) were collected from four vineyards that had previous reports of PD-symptomatic vines. During this study, symptomatic vines were present at all vineyards sampled. Two of the study vineyards were in Lincoln County and the other two were in Creek and Payne Counties. Vineyards were visited bi-weekly from 24 May 2012 until 1 August 2012, corresponding to peaks in abundance of *G. versuta*, the most

common insect vector captured in vineyards as determined in previous studies. Insects were collected using a leaf blower (Model SH 85C, Stihl Inc., Virginia Beach, VA) that was modified to provide suction. Mesh bags (55 cm by 120 cm) were secured to the blower tube with rubber bands and served to capture live insects. At all sites, stands of ragweed were present and were primary sources of insect collections. Insects were collected from grass hosts within and between vines and from the vines. Collections were usually made between 10 am to 3 pm. During transport back to the laboratory, to minimize predation, live insects within the mesh bags were kept in a cooler.. Upon return to the lab, the insects were removed immediately using an aspirator. Insects were identified, counted, and separated into cohorts of the same species. The insect cohorts were kept in plastic vials (39 x 85 mm) that were modified to allow aspirator access. For the most abundant species, *G. versuta*, five to seven individuals were placed on indicator plants. Numbers of cohorts varied depending on how many insects were captured at all sites visited. For instance, if only two *C. costali*s were captured on one collecting trip, then only two insects were used in one test. Each cohort was immediately placed on indicator plants and enclosed in mesh-covered plastic cages (Fig. 3a). Cages measured 45 cm by 15 cm and fit over the plants that were grown in 10.16 cm diameter pots (4.9 fl. oz.) (American Plant Products and Services Inc., Oklahoma City, OK). Plants were grown in a greenhouse at 26 °C to 35 °C. After insects were placed on indicator plants, they were kept in an insect rearing room under a 14:10 L:D photoperiod at 20 °C to 22 °C. Insects were first placed on one ragweed plant and after an inoculation access period (IAP) of seven days, transferred to another cage containing one alfalfa plant (var. 'Good as Gold II'). Ragweed and alfalfa indicator plants were 4 weeks old, on average (e.g., 3 to 5 cotyledon stage). After the second seven-day IAP, insects were carefully removed from

plants using an aspirator, properly labeled, and stored at -20 °C for further analysis. When insects were removed or transferred, plants were inspected for egg masses (Fig. 3b) and nymphs, which were destroyed. To ensure plants were insect-free after IAPs, plants were exposed to CO_2 to kill any insect nymphs that may have been present. There were a total of 50 inoculation trials using both ragweed and alfalfa plants. Unexposed plants were included for each trial, 50 of each plant species. Cages were placed over the unexposed plant and they were kept in the rearing room along with the plants exposed to insects. Samples of petioles and leaves from exposed and unexposed plants were taken 10 days after the feeding period and tested for *X. fastidiosa* using immmunocapture polymerase chain reaction (IC-PCR) methods. Insects were tested also for *X. fastidiosa* using IC-PCR.

Immunocapture-Polymerase Chain Reaction (IC-PCR)

To test for the presence of *X. fastidiosa* in both insects and plants, immunocapture-polymerase chain reaction (IC-PCR) was used, which is a method of detection that incorporates immunological techniques that are sensitive and specific to target pathogens. Further, it does not require DNA extraction, thus reducing the presence of contaminants that may interfere with traditional PCR (Pooler et al. 1997, Peroni et al. 2008). Micro-titer plate wells were coated with 50 µl of *X. fastidiosa* capture antibody diluted (1:100) in a 1X carbonate coating buffer (Agdia Inc., Elkhart, IN). Plates were incubated overnight at 4 °C in a plastic container. After incubation, plates were washed three times with phosphate buffered saline-tween (PBS-T) and blotted dry before adding macerated insects or plants. Insects were placed in 1.2-ml bead beater tubes (Life Sciences Products, Inc., Frederick, CO) filled with 0.25 g glass beads (BioSpec Products, Inc., Bartlesville, OK). To each tube, 300

µl of 0.05 M Tris-Cl were added and the samples were disrupted for 30 seconds using a Mini-BeadBeater-8 (BioSpec Products, Inc.). Tubes were centrifuged briefly to remove material from the lid. Plant samples were placed in mesh sample bags (Agdia Inc., Elkhart, IN) and general extraction buffer (GEB) with Tween added at a rate of 0.1 ml per gram of plant material and macerated using a wooden hammer and/or drill press equipped with a tissue homogenizer (Agdia Inc., Elkhart, IN). Once insect or plant samples were thoroughly macerated, 50 µl of the sample was added to each well and incubated overnight at 4 °C. After sitting for 24 h, wells were washed eight times with PBS-T and blotted dry before adding 50 µl of nuclease-free water to each well. To release antibody-DNA complexes bound to wells, the plate was heated at 100 °C for 5 minutes. The DNA eluent was used directly in PCR. In order to amplify *X. fastidiosa* DNA, multiple primer sets were used that enabled differentiation of *X. fastidiosa* subsp. *fastidiosa* and *X. fastidiosa* subsp. *multiplex.* One primer pair encoded a 412 bp XF2542 gene of the *X. fastidiosa* subsp. *fastidiosa* strains that encoded a fimbrial protein. The second two primer pairs encoded a 512 bp gene fragment and a 638 bp fragment of the XF1968 gene that are found in strains that are grouped with *X. fastidiosa* subsp. *multiplex* (Hernandez-Martinez et al. 2006). PCR reactions (25 µl) contained 12.5 µl of EconoTaq PLUS GREEN 2X Master mix (Lucigen, Middleton, WI), 0.56 µl of each primer (0.25 µM), 5 µl of DNA eluent, 4.14 µl of nuclease-free water, and 5 µl of DNA eluent. After amplification, samples were visualized using a 1% agarose gel in 1X TAE buffer (2 µl of ethidium bromide stock [10 mg/ml] added to the gel) placed under UV light.

Sequencing

For those insects and plants testing positive, amplified products were recovered from the gel using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). The purified product was submitted to Oklahoma State University Recombinant DNA/Protein Resource Facility for automated sequencing. After sequencing, Mega4 (The Biodesign Institute, Tempe, AZ) was used to align the forward and reverse sequences, when both were available. In many samples, only the forward sequence was available for comparisons**.** Sequences were compared to *X. fastidiosa* DNA sequences stored in GenBank using BLASTN (National Center for Biotechnology Information, Bethesda, MD)**.**

Results and Discussion

Field collections of live insects yielded five known vector species, including *G. versuta*, *G. coccinea*, *P. irrorata*, *O. orbona,* and *C. costalis* (Table 1). Of these, *G. versuta* was the primary vector found to be naturally inoculative. Inoculative *G. versuta* were first captured 21 June and successfully inoculated ragweed and alfalfa with *X. fastidiosa* (Tables 2 and 3). *Graphocephala versuta* was naturally inoculative throughout the growing season beginning 21 June (Tables 2 and 3). Cohorts of *G. versuta* corresponding to 21 June, 28 June, and 12 July inoculated *X. fastidiosa* to more alfalfa plants than to ragweed (Tables 2 and 3). A tree hopper, *E. carinata*, was frequently captured from weeds adjacent to the vineyards, especially ragweed. Although this species has not been shown to transmit *X. fastidiosa*, I included it based on other studies that found various treehopper species carry the pathogen (Huang et al. 2006, Zhang et al. 2011). In my study, none of the *E. carinata* collected tested positive for *X. fastidiosa* (Table 1) and none successfully inoculated *X. fastidiosa* to ragweed or alfalfa (Tables 2 and 3). The two individuals of *P. irrorata* that

tested positive for *X. fastidiosa* (Table 1) did not inoculate the bacterium to any of the test plants (Table 2 and 3). This species is collected less frequently than that of *G. versuta* in vineyards. It is captured more often from tree nurseries in Oklahoma and previous studies have confirmed this species' ability to transmit *X. fastidiosa* that causes PD (Myers et al. 2007) and phony peach disease (Turner and Pollard 1959).

Based on results from the multiplex primers, it is evident that insects were able to support both *X. fastidiosa* subspp. *fastidiosa* and *multiplex*. Most insects that tested positive were not carrying sufficient titers of *X. fastidiosa* to exhibit bright bands on the gel, which made it difficult to discern which subspecies were likely present. There were two *G. versuta* that tested positive from the cohort of specimens collected on 21 June. These specimens carried *X. fastidiosa* subsp. *multiplex*. Two more *G. versuta* captured 12 July and 1 August were carrying *X. fastidiosa* subsp. *multiplex*. One *G. versuta* captured 26 July was carrying *X. fastidiosa* subsp. *fastidiosa*, based on presence of one band that was 412 bp (data not shown). I was unable to verify these findings via sequencing of the amplified products due to the low bacterial titers and subsequent low yield of DNA.

Plants that tested positive were infected with higher titers of *X. fastidiosa* than levels detected in insects. None of the unexposed plants tested positive for *X. fastidiosa*, which supports my findings that positive test plants became infected following feeding by insect vectors. Although ragweed has been reported to host populations of *X. fastidiosa* subsp. *multiplex* (Timmer et al. 1983, Dominiak and Olson 2006), I was able to recover both subspecies from this plant (Fig. 4); however, my study did not determine if the bacteria were systemic or propagative. Of the positive ragweed samples, all samples were carrying *X. fastidiosa* subsp. *multiplex*. Of the total testing positive, 30% also were infected with *X.*

fastidiosa subsp. *fastidiosa* based on presence of the 412 bp band. This was confirmed after sequencing a positive ragweed sample. The ragweed sample that tested positive for both subspecies yielded a 412 bp band that was 95% similar to *X. fastidiosa* subsp. *fastidiosa* GB514, complete genome (GenBank accession number CP002165.1; E Value $2e^{-131}$). The same ragweed sample yielded a 638 bp fragment that was 97% similar to the *X. fastidiosa* M12 complete genome (GenBank accession number CP000941.1; E Value 0). This sample also yielded a 512 bp fragment that was 98% similar to the *X, fastidiosa* M12 complete genome (E Value 0). *Xylella fastidiosa* M12 is a strain that causes almond leaf scorch but not PD (Varani et al. 2012).

All positive alfalfa samples were infected with *X. fastidiosa* subsp. *multiplex* after exposure to inoculative insects. Of the total testing positive, 30% also were infected with *X. fastidiosa* subsp. *fastidiosa*. An alfalfa sample that tested positive for both subspecies yielded a 412 bp band that was 94% *X. fastidiosa* subsp. *fastidiosa* GB514, complete genome (GenBank accession number CP002165.1; E Value $2e^{-131}$). This alfalfa positive sample also yielded 638 bp fragment that was 97% similar to *X. fastidiosa* M12 complete genome (E Value 0). The same sample yielded a 512 bp fragment that was 97% similar to *X. fastidiosa* M12 complete genome (E Value $2e^{-173}$). These results further support the findings that alfalfa was infected with both *X. fastidiosa* subspp. *fastidiosa* and *multiplex*.

All field-collected insects were first placed on ragweed for a seven-day IAP before being moved to alfalfa for a subsequent seven-day IAP. Throughout the experiments, more alfalfa plants were inoculated with *X. fastidiosa* than that of ragweed by field-collected *G. versuta* (Tables 2 and 3). Insects were allowed to feed on ragweed initially, allowing time for bacterial concentrations to build up in the plant and perhaps allowing previous uninfected

insects to acquire the bacteria. The same cohort of insects was immediately transferred to alfalfa and since there is no latent period between acquisition and inoculation (Purcell and Finlay 1979) those insects could have become infective after the initial IAP on ragweed. In fact, *X. fastidiosa* is the only insect-transmitted plant pathogen that does not require a latent period and is persistently transmitted (Almeida et al. 2005). For example, the BGSS is able to transmit *X. fastidiosa* after 24 hours of acquiring the pathogen (Purcell and Finlay 1979). Another likely situation is that alfalfa is simply more susceptible to *X. fastidiosa* infection than ragweed. In hindsight, it might have been a better option to allow each insect cohort access to one plant, either ragweed or alfalfa. However, the few insect vectors collected throughout the growing season limited the number of inoculativity trials.

For successful insect transmission, Hill and Purcell (1997) found that plants must support minimal *X. fastidiosa* concentrations of 10^4 CFU/g, which is sufficient for insect vector acquisition and subsequent inoculation into a noninfected host. They also found that as the bacterial concentration increased in plants (e.g., grape and blackberry) the rate of insect transmission increased (Hill and Purcell 1997). Adlerz and Hopkins (1979) found that overwintered adult vectors were not able to inoculate *X. fastidiosa* that causes PD; however, insects collected later in the season were inoculative. This suggests that high bacterial concentrations within host plants were necessary for insect acquisition and subsequent inoculation. Another important factor to predict transmission and incidence of PD in vineyards is to determine when inoculative vectors are abundant. If vines are inoculated with *X. fastidiosa* in early spring, the bacteria have a better chance of establishing a systemic infection that can survive winter conditions (Almeida et al. 2005). The recovery from late-season infection is a result of the combined effects of dormant-season pruning in which late-

season infected vines were removed and colder winter-time temperatures, which inhibit *X. fastidiosa* survival (Feil et al. 2003).

Insect vectors that have high numbers of bacterial cells in their forguts with *X. fastidiosa* lead to increased numbers of successful transmissions of *X. fastidiosa* to grapes (Daugherty and Almeida 2009). Adlerz and Hopkins (1979) found that insect vectors were inoculative later in the season in Florida vineyards when bacterial titers in grape were sufficient for *X. fastidiosa* acquisition. Likewise, in Texas vineyards, insects were carrying *X. fastidiosa* in their foreguts later in the season. Insects collected early season were not carrying *X. fastidiosa* (Mitchell et al. 2009). Mitchell et al. (2009) and Adlerz and Hopkins (1979) concluded that *X. fastidiosa* was being transmitted from vine-to-vine. In my study, *G. versuta* were not inoculative early in the season in Oklahoma. This species was inoculative later in the season, which coincided with its peak abundance (see Chapter 3). These findings indicate that *X. fastidiosa* is being spread vine-to-vine in Oklahoma vineyards.

Conclusions

Understanding the epidemiology is important when developing IPM programs. More in-depth studies that expand on the initial findings from this study should be conducted to better understand the interaction of the insect vectors with the vines (e.g., seasonal flight patterns and life cycle on grape). Studies similar to those of Purcell and Frazier (1985) would help identify preferred plant hosts, preferred habitats, dispersal patterns, and phenology of the insect vectors. This information would allow us better management options for PD in Oklahoma vineyards. In the southeastern U.S., the epidemiology of PD has been difficult to study compared to comprehensive studies in California vineyards. This difficulty

is due to the small size of vineyards, widespread and abundant habitats for insect vectors, and a large number of plant hosts of *X. fastidiosa* in the southeastern U.S. (Hopkins and Purcell 2002). Most vineyards in Oklahoma are small, typically one to five total acres (Stafne 2007). Also, the abundance and diversity of insect vectors of *X. fastidiosa* in Oklahoma vineyards (Overall et al. 2010) coupled with small vineyard size make it difficult to study patterns of PD infection in our state. Many food plants of insect vectors are known hosts of *X. fastidiosa*, such as blackberry and Virginia creeper (Hopkins and Adlerz 1988). These plants are common in Oklahoma landscapes; therefore, weed management in and near vineyards is important. Based on findings from this study, recommendations to growers include rogueing diseased vines and using sterile pruning shears during the active growing season to limit secondary spread of the pathogen (i.e., vine-to-vine spread). Insecticides can be useful if they are timed to coincide with peak occurrence of insect vectors in vineyards; however, based on the high mobility of insect vectors insecticides would provide only temporary control (Purcell and Frazier 1985). Although the mobility of vectors should be considered, systemic insecticides such as imidacloprid have been shown to reduce the spread of PD in vineyards (Krewer et al. 2002).

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Table 1. Insect vectors[a] used in inoculativity experiments that tested positive for *Xylella fastidiosa*.

Vector Species	24-May	6-Jun	21-Jun	28-Jun	12-Jul	17-Jul	26-Jul	1-Aug
Hemiptera: Cicadellidae								
Graphocephala versuta	0/1[b]	0/18	2/31	0/31	2/24	1/17	4/21	0/14
G. coccinea						0/22		
Cuerna costalis	0/9							
Paraulacizes irrorata		1/4		1/4	0/3			
Oncometopia orbona			0/2	0/1	0/1			
Hemiptera: Membracidae								
Entylia carinata		0/2			0/8	0/3		0/6

[a] Insects were collected from four vineyards weekly or bi-weekly from 24 March to 1 Aug. in 2011

[b] Ratio represents the number of insects that tested positive for *X. fastidiosa* over the total number of insects that were placed on plants

Table 2. Inoculation of *Xylella fastidiosa* to ragweed by insect vectors collected from four vineyards[a].

Vector Species	24-May	6-Jun	21-Jun	28-Jun	12-Jul	17-Jul	26-Jul	1-Aug
Graphocephala versuta	0/1[b]	0/3	1/5	2/7	2/5	0/3	5/6	1/5
G. coccinea						0/2		
Cuerna costalis	0/2							
Paraulacizes irrorata		0/1		0/1	0/1			
Oncometopia orbona			0/1	0/1	0/1			
Entylia carinata		0/1			0/2	0/1		0/1

[a] Vineyards were located in Payne, Lincoln, and Creek Counties

[b] Ratio represents number of plants that tested positive for *X. fastidiosa* over the total number of plants used in the inoculativity tests

Table 3. Inoculation of *Xylella fastidiosa* to alfalfa by insect vectors collected from four vineyards[a].

Vector Species	24-May	6-Jun	21-Jun	28-Jun	12-Jul	17-Jul	26-Jul	1-Aug
Graphocephala versuta	0/1[b]	0/3	4/5	4/7	5/5	0/3	5/6	1/5
G. coccinea						0/2		
Cuerna costalis	0/2							
Paraulacizes irrorata		0/1		0/1	0/1			
Oncometopia orbona			0/1		0/1			
Entylia carinata		0/1			0/2			0/1

[a] Vineyards were located in Payne, Lincoln, and Creek Counties

[b] Ratio represents number of plants that tested positive for *X. fastidiosa* over the total number of plants used in the inoculativity tests

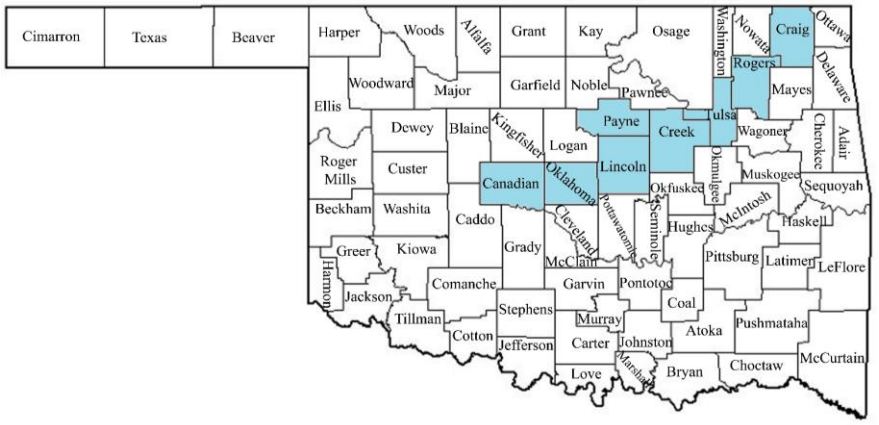

Figure 1. Counties with confirmed cases of Pierce's disease from 2008 to 2010 (source: Plant Disease and Insect Diagnostic Laboratory, Oklahoma State University).

Figure 2. a) Foliar symptoms of Pierce's disease, leaf scorch; b) 'matchstick' symptom of Pierce's disease, attached petioles after leaf abscission.

Figure 3. a) Ragweed and alfalfa cages; b) egg mass of *Cuerna costalis.*

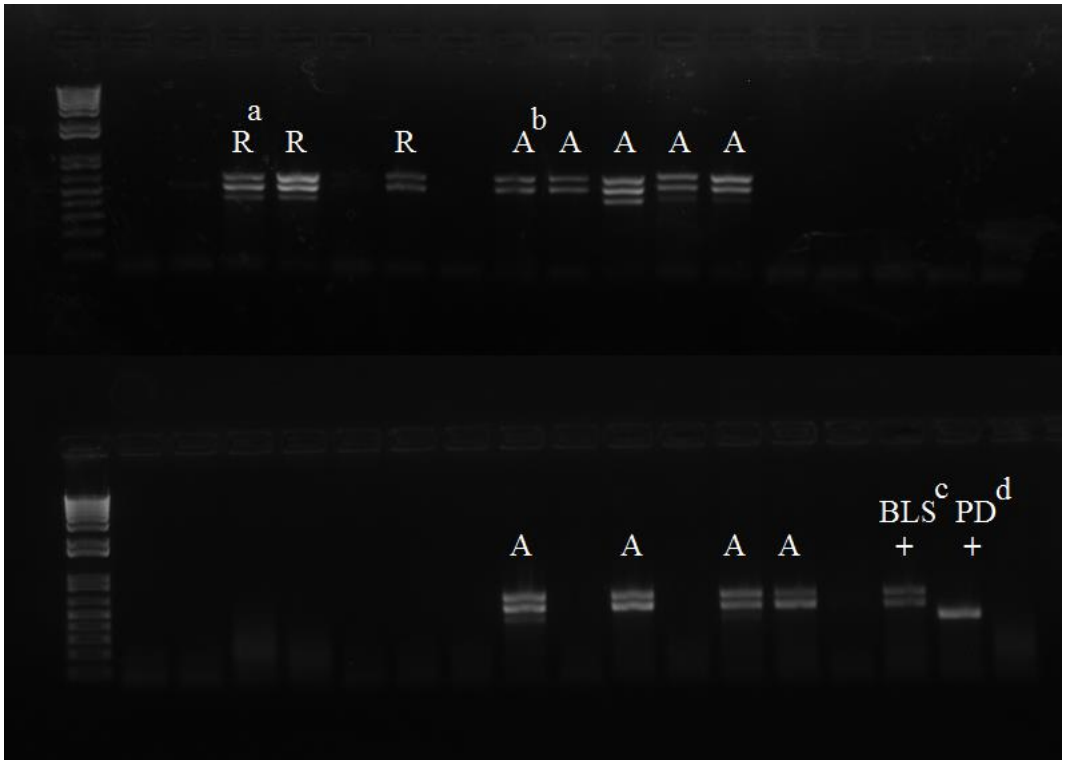

Figure 4. Gel image of results from multiplex PCR; a) positive alfalfa sample; b) positive ragweed samples; c) positive control, *X. fastidiosa* subsp. *Multiplex* based on presence of two bands, 512 and 638 bp; d) positive control, *X. fastidiosa* subsp. *fastidiosa*; based on presence of single band, 412 bp; 1kb plus ladder was used.

CHAPTER V

IDENTIFICATION OF WEEDY PLANTS ASSOCIATED WITH OKLAHOMA VINEYARDS THAT COULD SERVE AS SOURCES OF *XYLELLA FASTIDIOSA*

Abstract

Xylella fastidiosa is the causative agent of Pierce's disease of grape and many other plant diseases including leaf scorch of oleander, almond, pecan, and other shade trees, phony peach, and alfalfa dwarf. This gram-negative bacterium is transmitted by xylem sap-feeding insects in the families Cicadellidae and Cercopidae. Pierce's disease of grape has been detected in eight counties in central and northeastern Oklahoma. I surveyed common weeds in vineyards to identify potential reservoir hosts of *X. fastidiosa*. Weedy plants sampled in this study are known or suspected food sources of xylem sap-feeding insect vectors, are commonly found in Oklahoma vineyards, and have been implicated as *X. fastidiosa* hosts. I sampled from four vineyards in three counties in Oklahoma, all of which had previous reports of Pierce's disease. Immunocapture multiplex PCR was used to test plant samples. Plants testing positive were bermudagrass, woodsorrel, hop clover, giant ragweed, and

grape. Very few plants tested positive and none of the plants testing positive are new hosts of *X. fastidiosa*. These results indicate that weedy reservoir hosts may not be a contributing factor to the epidemiology of Pierce's disease in Oklahoma.

Introduction

Xylella fastidiosa subsp. *fastidiosa* is a gram-negative bacterium that causes Pierce's disease (PD) of grape, a devastating and costly disease in Texas and California. More recently, PD has been an increasing threat to vineyards in the southeastern United States (e.g., North Carolina and Virginia), coinciding with a warming climate (Anas et al. 2008). Since 2009, this bacterium has been found on PD-symptomatic grape vines in eight counties in central and northeastern Oklahoma (Jen Olson, *pers. comm.*). *Xylella fastidiosa* subsp. *fastidiosa* is not transmitted from plant to plant without an insect vector (Purcell and Hopkins 1996). However, the bacterium can be transmitted mechanically via pruning shears (Krell et al. 2007) or propagation of infected grape cuttings (Robacker and Chang 1992). Although *X. fastidiosa* has a wide and diverse host range, it is not pathogenic in all plant hosts (Schaad et al. 2004).

Insect vectors that transmit *X. fastidiosa* can feed on a wide range of plants throughout the growing season; therefore, it is important to test a variety of potential host plants for presence of the pathogen (Hopkins and Purcell 2002). These plants may occur within the vineyard or in surrounding habitat and can provide an inoculum source of *X. fastidiosa* (i.e., they are reservoir plant hosts). For instance, in the California Central Valley, PD occurred on vines planted at the vineyard edge near pastures or hayfields. These crop habitats contained weedy species that served as primary food sources for

Xyphon (=*Carneocephala*) *fulgida* (Nottingham) and *Draeculacephala minerva* (Ball), known vectors of *X. fastidiosa*. Grape plants were "accidental feeding hosts" for these sharpshooters when they were displaced from their preferred habitat due to harvest or mowing (Purcell and Frazier 1985, Hopkins and Purcell 2002). In California, the incidence of PD is greater on vines that are adjacent to water sources, such as streams or irrigated landscapes, which are preferred habitats of *Graphocephala atropunctata* (Signoret) (Hopkins and Purcell 2002). The preferred habitats of *G. atropunctata* also contain elderberry and blackberry, which are proven reservoir hosts of *X. fastidiosa* subsp. *fastidiosa* (Baumgartner and Warren 2005). Hopkins and Purcell (2002) suggest that *X. fastidiosa* subsp. *fastidiosa* is transmitted to vineyards from surrounding landscapes (i.e., primary transmission) and that vine-to-vine spread (i.e., secondary transmission) is not considered a major factor. In Texas, however, symptoms first appear on vines within the vineyard, indicating that secondary transmission of PD is significant (Kamas et al. 2000). Supporting these findings, Mitchell et al. (2009) found that insect vectors captured within Texas vineyards were carrying *X. fastidiosa* subsp. *fastidiosa* more often than those captured from weedy plants adjacent to the vineyard, which were carrying subspecies of *X. fastidiosa* that do not cause PD.

Several woody species occurring in or near Texas vineyards have tested positive for *X. fastidiosa* subsp. *fastidiosa*, many of which also occur in Oklahoma (e.g., holly and Japanese honeysuckle) (Buzombo et al. 2006). Florida surveys have identified elderberry, Virginia creeper, goldenrod, and blackberry as sources for primary PD infection to grapevines (Hopkins and Adlerz 1988). My objective was to identify plants that are fed upon by insect vectors and serve as sources of *X. fastidiosa* subsp. *fastidiosa*

in and around Oklahoma vineyards. This information will be used by growers to understand the disease cycle of *X. fastidiosa* and enable them to target specific weedy species for removal from the vineyard and surrounding habitat in order to limit the spread of PD.

Materials and Methods

Study Vineyards

In 2012, I surveyed the interior and edge habitat of four vineyards in Oklahoma that had reports of PD-positive vines in search of reservoir host plants of *X. fastidiosa*. Two of the study vineyards were in Lincoln County and the other two were in Creek and Payne Counties. Vineyards were visited once monthly corresponding to 24 May, 21 June, 26 July, 14 August, and 15 October, 2012. The Creek County vineyard was planted with 'Merlot', 'Cabernet Sauvignon', and 'Chardonnay' cultivars and was surrounded by woody areas on all sides. The Payne County vineyard was planted with 'Riesling', 'Merlot', and 'Chardonnay' cultivars and was surrounded by wooded areas on two sides and a weedy, roadside ditch on another. One Lincoln County vineyard was planted with 'Ruby Cabernet', 'Cabernet Sauvignon', and 'Muscat' cultivars and was adjacent to one weedy area that was mostly overtaken by kudzu. The remaining edges were grassy fields and a trailer park. The second vineyard in Lincoln County was planted with 'Shiraz', 'Sauvignon Blanc', 'Merlot', and 'Chardonnay' cultivars and was surrounded on three sides with heavily wooded areas and one grassy pasture. The vineyards ranged in size from 0.31 ha to 2.0 ha.

Plant Sampling Methods

At each vineyard, flags were used to mark two diagonal, 50-m transects that intersected near the center of the vineyard. A third 50-m transect was established perpendicular to the vineyard edge next to a riparian or woody area. Along each transect, three locations were randomly selected and marked off with a 1-m^2 quadrat constructed from PVC pipe (Fig.1a). I identified plant species within each quadrat and sampled those species that are known or suspected hosts of *X. fastidiosa* subsp. *fastidiosa*. All sampled plant species were present in all four vineyards. I collected a subsample of each plant species that was proportional to the number of plants present (i.e., 10% of total plants present) and tested each sample for the presence of *X. fastidiosa* using immunocapture-polymerase chain reaction (IC-PCR). I noted whether these plants were symptomatic or asymptomatic in the field and whether or not any insect vectors were observed feeding on the plant. Unfortunately, central Oklahoma experienced significant drought in 2012 and drought-stressed plants appeared symptomatic, confounding observations of *Xylella*-induced symptoms. Throughout the study, no known insect vectors were observed on or within the sample quadrats. Target host plants included woodsorrel, (*Oxalis* sp.), geranium (*Geranium* sp.), bermudagrass (*Cynodon dactylon* (L.) Pers.), plantain (*Plantago* sp.), grape (escaped *Vitis* spp.), Virginia creeper (*Parthenocissus quinquefolia* (L.) Planch.), and giant ragweed (*Ambrosia trifida* L.). The identity of all plant species was verified by Dr. Dwayne Elmore, Associate Professor, Natural Resources and Ecology Management, Oklahoma State University.

Plate Culture of Plant Samples

In addition to detecting the pathogen using IC-PCR, I attempted to culture the bacteria on nutrient agar. Within a day of collecting plant material, a small subsample of each sample was sterilized, cut into small pieces, and placed in 1.2-ml bead beater tubes (Life Sciences Products, Inc., Frederick, CO) filled with 0.25 g glass beads (BioSpec Products, Inc., Bartlesville, OK). The samples were homogenized for two minutes with a Mini-Beadbeater-1 (BioSpec Products, Inc., Bartlesville, OK). After briefly spinning the tubes to remove any material from the lid, 200 µl of each sample tube was placed on PD3 media, labeled, and incubated at 29 °C. Plates were viewed after two days of incubation and those with significant growth of fungi and other bacteria were discarded. After a two-week incubation period, one or two colonies per plate were removed using a bacterial loop and placed in a test tube containing 600 µl of succinate-citrate-phosphate (SCP) buffer. Each sample was vortexed briefly before adding 50 µl of each sample to plate wells for IC-PCR (described below) to determine if any colonies of *X. fastidiosa* formed, providing further evidence whether plants were or were not infected with the bacterium.

Immunocapture-Polymerase Chain Reaction (IC-PCR)

I used IC-PCR to test for the presence of *X. fastidiosa* in weedy plants. This detection method incorporates immunological techniques that are sensitive and specific to target pathogens. Further, it does not require DNA extraction, thus reducing the presence of contaminants, such as phenolics, that interfere with *Taq* polymerase, an integral enzyme in PCR (Pooler et al. 1997, Fatmi et al. 2005, Peroni et al. 2008). Micro-titer

plate wells were coated with 50 µl of *X. fastidiosa* capture antibody diluted (1:100) in a 1X carbonate coating buffer (Agdia Inc., Elkhart, IN). Plates were incubated overnight at 4 °C in a plastic container. After incubation, plates were washed three times with phosphate buffered saline-tween (PBS-T) and blotted dry before adding macerated plants. Plant samples were placed in mesh sample bags (Agdia Inc., Elkhart, IN) and general extraction buffer (GEB) with Tween (Agdia Inc., Elkhart, IN) added at a rate of 0.1 ml per gram of plant material and macerated using a wooden hammer and/or drill press equipped with a tissue homogenizer (Agdia Inc., Elkhart, IN) (Fig. 1b). Once plants were thoroughly macerated, 50 µl of the sample were added to each well and incubated overnight at 4 °C. After sitting for 24 h, wells were washed eight times with PBS-T and blotted dry before adding 50 µl of nuclease-free water to each well. To release antibody-DNA complexes bound to wells, the plate was heated at 100 °C for 5 minutes. The DNA eluent was used directly in PCR. In order to amplify *X. fastidiosa* DNA, multiple primer sets were used that enabled differentiation of *X. fastidiosa* subsp. *fastidiosa* and *X. fastidiosa* subsp. *multiplex.* One primer pair encoded a 412 bp XF2542 gene of the *X. fastidiosa* subsp. *fastidiosa* strains that encoded a fimbrial protein. The second two primer pairs encoded a 512 bp gene fragment and a 638 bp fragment of the XF1968 found in strains that are grouped with *X. fastidiosa* subsp. *multiplex* (Hernandez-Martinez et al. 2006). All PCR reactions (25 µl) contained 12.5 µl of EconoTaq PLUS GREEN 2X Master mix (Lucigen, Middleton, WI), 0.56 µl of each primer (0.25 µM), 4.14 µl of nuclease-free water, and 5 µl of DNA eluent. After amplification, samples were visualized using a 1% agarose gel in 1X TAE buffer (2 µl of ethidium bromide stock [10 mg/ml] added to the gel) placed under UV light.

Sequencing

For those plants testing positive, amplified products were recovered from the gel using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). The purified product was submitted to Oklahoma State University Recombinant DNA/Protein Resource Facility for automated sequencing. After sequencing, Mega4 (The Biodesign Institute, Tempe, AZ) was used to align the forward and reverse sequences, when both were available. In many samples, only the forward sequence was available for comparisons**.** Sequences were compared to *X. fastidiosa* DNA sequences stored in GenBank using BLASTN (National Center for Biotechnology Information, Bethesda, MD)**.**

Results and Discussion

In this study, I detected *X. fastidiosa* in only a few plants collected from the vineyard floor and near wooded or weedy perennial habitat (Table 1). Plants testing positive were bermudagrass, woodsorrel, hop clover, giant ragweed, and grape. The bermudagrass that tested positive for *X. fastidiosa* was collected in May. The positive woodsorrel, hop clover, and grape samples were collected in July. Of the two ragweed samples that tested positive, one was collected in August and the other in October. None of the plants testing positive are new hosts of *X. fastidiosa*. The supplemental method of plating macerated sterile plant samples on PD3 media yielded no positive samples (data not shown); however, many of the plates were significantly contaminated, which reduced the sample size. Of those plants testing positive, the bacterial titer was low resulting in very little PCR product. This made it difficult to obtain sequences to compare with gene

sequences in the GenBank database. The few plants testing positive indicates that under the conditions experienced in 2012 *X. fastidiosa* would not likely be brought into the vineyard by insect vectors via primary infection. Continued research should include documenting patterns of PD infection in individual vineyards over many years to further support the spread of inoculum from grapevine to grapevine via insect vectors.

In the southeastern U.S., the epidemiology of PD has been difficult to study compared to comprehensive studies in California vineyards. This difficulty is due to the small size of vineyards, widespread and abundant habitats for insect vectors, and a large number of plant hosts of *X. fastidiosa* (Hopkins and Purcell 2002). In many states, the occurrence and increased prevalence of PD is a recent development. Therefore, studies of PD in the southeastern U.S. have focused on understanding the dispersal of insect vectors and their transmission efficiencies of *X. fastidiosa*. In addition to understanding the biology of insect vectors, researchers have studied the occurrence of plant hosts in and around the vineyard that have potential to provide a source of *X. fastidiosa* (Hopkins and Purcell 2002).

Bactericides are useless against *X. fastidiosa* because the bacterium is protected within xylem vessels. Therefore, management strategies focus on preventing the pathogen from entering the vineyard or, if the vines are already infected, preventing its spread by removing infected plants and replanting with tolerant grape varieties (Kamas et al. 2000). The pattern of PD infection in the vineyard enables growers to understand the disease cycle in the vineyard. For instance, if more vines are infected on the edge of the vineyard, this would indicate the infection is primary (i.e., insect vectors are bringing in *X. fastidiosa* after acquiring it from adjacent or even distant plants (Park et al. 2011). As

mentioned, small vineyard size in Oklahoma makes this a difficult variable to model. In many Oklahoma counties, fewer than 10 acres of grapes are grown (Stafne 2007). Other factors that influence the incidence of PD in vineyards are vineyard age and susceptibility of vines to PD. Younger vines are more susceptible to chronic infection of *X. fastidiosa* and some cultivars, in general, are less susceptible to PD (Park et al. 2011). In Oklahoma, many of the vineyards are recently planted and the majority of the cultivars are *Vitis vinifera* (Stafne 2007) which are susceptible to *X. fastidiosa* (Kamas et al. 2000).

The climate where grapes are grown is another important component that influences the disease cycle. *Xylella fastidiosa* subsp. *fastidiosa* will not survive winter temperatures that fall below 4 ºC (Hoddle 2004). Likewise, the most important insect vector, *H. vitripennis*, is unable to survive temperatures that remain below 10 ºC for extended periods (15 days) (Johnson et al. 2006). If vines are infected later in the season in colder regions, the bacteria are unable to gain a systemic infection that travels further into the plant where bacteria are protected from cold temperatures and pruning practices. This phenomenon is known as 'cold curing' (Lieth et al. 2011). Global warming has potential to increase the likelihood of pathogens and their insect vectors surviving previously uninhabitable environments (Garrett et al. 2006). Interestingly, many Oklahoma counties are considered low to moderate risk for the occurrence of PD (Smith and Dominiak-Olson 2009) and *H. vitripennis* has been found in the northeastern part of Oklahoma, well beyond its predicted range (Overall et al. 2010). The GWSS was captured at a nursery and it is likely these individuals were transient (i.e., populations were inadvertently brought in are not established because of their inability to survive

winter conditions). However, if temperatures continue to increase GWSS would be able to survive and become a serious problem in this state.

Conclusions

Since 2009, reports of PD in Oklahoma have been increasing gradually. To help Oklahoma grape growers meet this new challenge, it is imperative that researchers continue to determine characteristics of the disease complex (insect vector/susceptible plant host/environment) that are unique to this state. Understanding the disease cycle/complex is important for the management of PD in vineyards. In California, weeds and other crops that are near vineyards influence the disease cycle of PD. For instance, in the Temecula Valley, the incidence of PD on grapevines that are grown near citrus orchards is much higher. This is because *H. vitripennis,* the most important vector of *X. fastidiosa*, reproduces on and overwinters in citrus trees before moving into vineyards (Blua and Morgan 2003).

Although I was unable to identify potential host plants of *X. fastidiosa* near vineyards, weed management is still vital. Reservoir hosts that have already been documented in previous studies as hosts of *X. fastidiosa* are present in Oklahoma landscapes such as Virginia creeper and blackberry (Hopkins and Adlerz 1988). Ragweed, which tested positive for *X. fastidiosa* in this study, is host to many of the vector species (Turner and Pollard 1959). Grape growers should remove any stands of ragweed, escaped or wild *Vitis* spp., and other common weeds that are within or near the vineyard. Additionally, future studies should investigate the phenomena of cold-curing in grapevines in Oklahoma, which would help growers better understand the risk of

disease on vines from year to year. It is well known that many *Vitis vinifera* grape cultivars are susceptible to PD and growers should choose to plant less susceptible varieties. Furthermore, it is imperative that growers sanitize pruning shears and use caution when obtaining root stock and cuttings from fellow viticulturists.

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Table 1. Plants collected from vineyards[a] and results from immunocapture-PCR testing for *X. fastidiosa.*

Plant	Total Plants Tested[b]	Number Positive[c]
Bermudagrass	214	1
Wood sorrel	118	1
Hop Clover	86	1
Giant ragweed	50	2
Plantain	49	0
Geranium	28	0
Grape	21	1
Virginia Creeper	17	0

[a] Samples were collected Monthly in May, June, July, August, and October from four Oklahoma vineyards (Payne, Lincoln, and Creek Counties)
[b] Immunocapture PCR was used to test all plant samples
[c] Samples that tested positive for *X. fastidiosa*

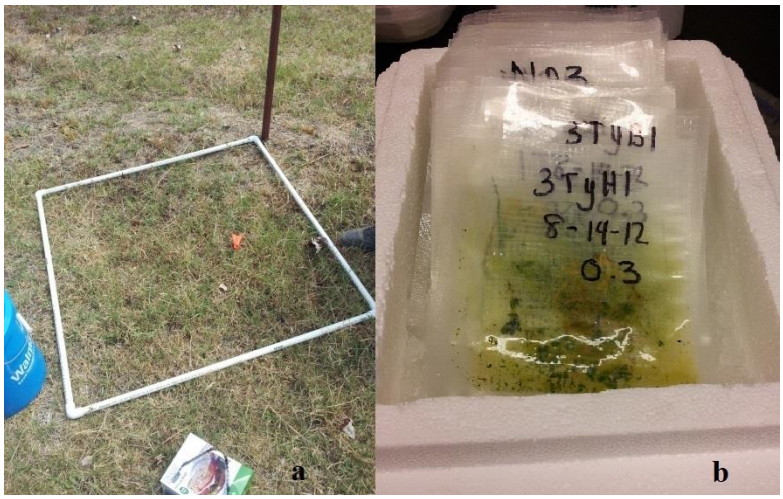

Figure. 1. A) One square meter quadrat used to sample sites along transects; b) macerated plant samples placed on ice.

VITA

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