2016

Characterization of Xylella fastidiosa in rabbiteye blueberry

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CHARACTERIZATION OF *XYLELLA FASTIDIOSA* IN RABBITEYE BLUEBERRY

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Plant Pathology and Crop Physiology

by
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December 2016

ACKNOWLEDGEMENTS

There are many people to thank for their assistance with this project. The late Dr. Don Ferrin took me on as a student to study a topic of interest to both of us. I appreciate the opportunity that he gave me. Dr. Chris Clark kindly adopted me as his student after Dr. Ferrin passed. I greatly appreciate his allowing me to continue working on this project, even though blueberries were not his area of focus. His plant pathology knowledge and skills, as well as his blueberry harvesting labor, have been fundamental to this work. In addition to Dr. Clark, I am thankful to have had Drs. Barbara Smith, Charlie Johnson, Clayton Hollier, Mike Stout, and Stephen Harrison on my committee. Dr. Smith not only provided advice on this project but also provided financial support through the USDA.

I greatly appreciate the support and learning experiences that faculty, staff members, and students at LSU have provided. Special appreciation is expressed to Mary Hoy, Emily Ringelman, Jacob Boudreaux, and Favio Herrera; Drs. Lawrence Datnoff, Randy Sanderlin, Rebecca Melanson, Raj Singh, Vinson Doyle, Charlie Overstreet, Melinda Butler, David Picha, James Geaghan, Bin Li, and Brian Marx; and Tim Burks, Deborah Xavier, Carolyn Savario, Claudette Oster, Richard Davis, Rebecca Sweany, and Yenjit Raruang.

A number of people from other universities contributed valuable advice on this project, also. Drs. Harald Scherm and Phil Brannen, of the University of Georgia, along with Dr. Rock Christiano and Renee Allen, deserved special recognition. Dr. Mark Black and Alfred Sanchez of Texas A&M and Dr. Penny Perkins-Veazie and Bill Cline of North Carolina State University also provided much appreciated help. A huge thank you goes to Cliff and Susan Muller for their cooperation. I appreciate the involvement of all of the other cooperating growers, as well.

Finally, thank you to my parents, who have provided me with a strong educational foundation and enduring love and support.

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ABSTRACT

Xylella fastidiosa colonizes the xylem of a wide range of plants and causes symptoms in many of them. It was of interest to determine how widespread *X. fastidiosa* was and what its potential impact might be in rabbiteye blueberry. *X. fastidiosa* was detected from two of 17 Louisiana orchards from which samples were collected. A yield study was conducted at one of these orchards, and mean yields of *X. fastidiosa*-positive plants were 55% and 62% less than those of *X. fastidiosa*-negative plants in 2013 and 2014, respectively. Average berry weight was also lower in *X. fastidiosa*-positive plants. However, plants that were *X. fastidiosa*-negative in 2013 remained so until 2015, suggesting that the bacterium does not spread rapidly in established orchards. Regular testing of plants that had tested positive for *X. fastidiosa* revealed that root sap was more reliable than stem sap for detection by real-time PCR. Detection by ELISA using leaf petiole/midrib tissue or, in the winter, shoot terminals, was not as consistent as detection in root sap by real-time PCR. However, it was comparable or better in some months and was consistently as good as or better than detection in stem sap by real-time PCR, except in April, when detection by ELISA in petiole/midrib tissue cannot be relied upon. Genotypes of *X. fastidiosa* identified from rabbiteye blueberry in Louisiana include combinations of alleles that are believed to have resulted from recombination between *X. fastidiosa* subspecies *multiplex* and *fastidiosa*. One genotype (ST 42) found at two Louisiana orchards was previously identified from southern highbush blueberry in Georgia. Inoculation of seven rabbiteye cultivars and one southern highbush cultivar with an *X. fastidiosa* isolate from rabbiteye blueberry did not result in detected infection within nine months. *X. fastidiosa* warrants awareness as a possible cause of yield loss and plant decline in rabbiteye blueberry. Rabbiteye blueberry likely harbors a genotype of *X. fastidiosa* that is pathogenic to southern highbush blueberry, so growers should

take care to use clean planting material if introducing rabbiteye plants into an orchard that contains susceptible southern highbush cultivars.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Xylella fastidiosa Wells et al. is well known as the bacterial pathogen that causes Pierce's disease of grapes. It also causes peach phony disease (Wells et al. 1987), almond leaf scorch (Davis et al. 1980), citrus variegated chlorosis (Hartung et al. 1994), coffee leaf scorch (de Lima et al. 1998), leaf scorch of shade trees (Hopkins and Purcell 2002), oleander leaf scorch (Purcell et al. 1999), and various other plant diseases (Hopkins and Purcell 2002). Recently, it has been associated with olive quick decline syndrome (Cariddi et al. 2014). While some *X. fastidiosa*-caused diseases have been recognized for a long time (e.g., Hutchins 1933; Pierce 1892), culture of the bacterium was not reported until 1978 (Davis et al. 1978), and it was named as recently as 1987 (Wells et al. 1987). In 2000, Simpson et al. published the genome sequence of *X. fastidiosa*, and, as Hopkins and Purcell (2002) point out, it "was the first nonviral plant pathogen to have its genome completely sequenced."

X. fastidiosa-caused diseases are found primarily in tropical and subtropical areas and have traditionally been found only in the Western Hemisphere (Hopkins and Purcell 2002), although the bacterium has also been found in the Kosovo region (Berisha et al. 1998), and, more recently, in Italy (Cariddi et al. 2014) and France (EPPO 2016). *X. fastidiosa*-caused diseases are especially prevalent in the southeastern United States (Hopkins and Purcell 2002). Proposed reasons for the bacterium's success in this region's grapes, for example, include the presence of the glassy-winged sharpshooter (*Homalodisca vitripennis*), a vector of *X. fastidiosa*; a long growing season; and warm nights (Hopkins and Purcell 2002).

Since 2004, researchers in Georgia and Florida have confirmed *X. fastidiosa* as the cause of a leaf scorch disease of southern highbush blueberries (*Vaccinium corymbosum* interspecific

hybrids) (Chang et al. 2009; Harmon and Hopkins 2009). Bacterial leaf scorch symptoms described on southern highbush plants include marginal leaf necrosis, thin twigs on new growth, leaf drop, stem yellowing, and plant death (Brannen et al. 2016). In a greenhouse trial in Georgia, inoculation of rabbiteye blueberry (*Vaccinium ashei* Reade = *V. virgatum* Aiton) plants with *X. fastidiosa* resulted either in no detected infection, in cultivar Premier, or in local colonization of two of six 'Powderblue' plants. In infected 'Powderblue' plants, symptoms did not progress past the inoculated stem within a fourteen month period (Chang et al. 2009). However, *X. fastidiosa* has been detected in samples from rabbiteye blueberry orchards in Louisiana that have experienced noticeable plant stunting and/or plant death (D. Ferrin, personal communication; M.H. Ferguson, unpublished data). Rabbiteye blueberry comprises the majority of the ≈3000 acres of blueberry plantings in Louisiana and Mississippi (E. Stafne, personal communication; USDA-NASS 2014).

1.2 *X. fastidiosa genotypes*

X. fastidiosa is a gram-negative bacterium in the class Gammaproteobacteria and the family Xanthomonadaceae (Brenner et al. 2005). Species in the genus *Xanthomonas* are among the closest relatives of the genus *Xylella* (Rodriguez-R et al. 2012; Wells et al. 1987). While *X. fastidiosa* has been the only accepted species in *Xylella* (Brenner et al. 2005), Su et al. (in press) have proposed a new species, *X. taiwanensis*, in association with a strain from pear in Taiwan. Research by others bolsters the case for separating this genotype from *X. fastidiosa* (Marcelletti and Scortichini 2016).

Within *X. fastidiosa*, there are two validly published subspecies, *X. fastidiosa* subsp. *fastidiosa* and *X. fastidiosa* subsp. *multiplex* (Bull et al. 2010; Schaad et al. 2004a, 2004b). A third, *X. fastidiosa* subsp. *pauca*, was described at the same time as the previous two, but

requirements for culture deposition have reportedly not been met (Bull et al. 2010; Schaad et al. 2004b). Subspecies *morus* (Nunney et al. 2014c), *sandyi* (Schuenzel et al. 2005), and *tashke* (Randall et al. 2009) have since been proposed. However, Marcelletti and Scortichini (2016) argue, based on analyses using extensive portions of strain genomes, that strains previously classified as subspecies *sandyi* and *morus* actually belong in subsp. *fastidiosa*. No strain identified as *X. fastidiosa* subsp. *tashke* strain was included in their analysis.

Scally et al. (2005) developed a multilocus sequence typing (MLST) scheme, modified by Yuan et al. (2010), which allows *X. fastidiosa* strains to be genotyped below the subspecies level. The MLST approach involves typing strains based on the exact nucleotide sequences at seven housekeeping loci (Scally et al. 2005). Each combination of nucleotides is assigned an allele number, and each combination of seven alleles is a different sequence type (ST) (Scally et al. 2005). An additional locus, *pilU*, is sometimes utilized in conjunction with the MLST loci (Nunney et al. 2014a; Yuan et al. 2010). Parker et al. (2012) took a different multilocus approach, which they call multilocus sequence analysis of environmentally mediated genes (MLSA-E), to characterizing *X. fastidiosa* isolates. Their approach involved nine genes that had, on average, higher ratios of non-synonymous to synonymous substitution rates than those used in the Yuan et al. (2010) MLST scheme. In other words, nucleotide differences in alleles at these loci more often resulted in codes for different amino acids, suggesting that these sequences reveal more genotypic variation (Hartwell et al. 2004; Parker et al. 2012).

Brady et al. (2012) have proposed another alternative to MLST called multilocus melting type (MLMT), which distinguishes between *X. fastidiosa* subspecies *fastidiosa*, *multiplex*, and *sandyi* based on melting temperatures following PCR amplifications at nine loci. It offers advantages over MLST in terms of speed and cost but only distinguishes to the subspecies level,

and alleles that are different from those in the reference strains of the subspecies may result in melting temperatures that do not correspond to those of the reference strains.

X. fastidiosa strains that have been characterized after being found naturally occurring in blueberry have been identified as subsp. *multiplex* or, in one case, have been equally closely matched to strains in subsp. *multiplex* and subsp. *sandyi* (Nissen 2010; Nunney et al. 2013; Parker et al. 2012). Nissen (2010) found that, within a 520 nucleotide segment of the 16S-23S rDNA intergenic spacer (ITS) region, two *X. fastidiosa* isolates from southern highbush blueberry were more similar to strains M12 and Dixon (both from almond), which are indicated as subsp. *multiplex* by Nunney et al. (2013), and to *X. fastidosa* subsp. *sandyi* strain Ann-1 (from oleander) than to a subsp. *pauca* strain or two subsp. *fastidiosa* strains.

The seven isolates of blueberry origin that have previously been typed by the MLST scheme have been identified as ST 42 or 43 (Nunney et al. 2013). Isolates identified as ST 42 were all from blueberry in Georgia, while those identified as ST 43 were from either Florida or Georgia. ST 43 isolates have only been identified from blueberry, while ST 42 isolates have also been found in giant ragweed and western soapberry in Texas.

STs 42 and 43 are deemed to be part of *X. fastidiosa* subsp. *multiplex* but believed to have been derived from a recombination event between strains in *X. fastidiosa* subsp. *multiplex* and *X. fastidiosa* subsp. *fastidiosa* (Nunney et al. 2014a). Recombination between strains is believed to be one way in which *X. fastidiosa* becomes able to infect new hosts (Nunney et al. 2014a). Nunney et al. (2014a) has suggested that an *X. fastidiosa* subsp. *fastidiosa* ancestor of the recombinant *X. fastidiosa* subsp. *multiplex* group came to the United States from Central America and "disappeared" after recombining with an *X. fastidiosa* subsp. *multiplex* strain.

STs 42 and 43 have the same alleles at four MLST loci (*holC*, *malF*, *nuoL*, and *petC*) and *pilU* but differ at loci *leuA*, *cysG*, and *gltT* (Nunney et al. 2014a). Alleles at the four MLST loci at which STs 42 and 43 are the same are *X. fastidiosa* subsp. *multiplex* alleles (Nunney et al. 2013; Yuan et al. 2010). On the other hand, allele 9 at the *pilU* locus differs at only one nucleotide from allele 1, which, outside of the putatively recombinant group, is found in *X. fastidiosa* subsp. *fastidiosa* strains from the United States and Costa Rica (Nunney et al. 2014a).

At *cysG*, STs 42 and 43 have different alleles (Nunney et al. 2013). Allele 12 at locus *cysG* in ST 42 is the same as an allele found in *X. fastidiosa* subsp. *fastidiosa* isolates from coffee in Costa Rica (Stouthamer and Nunney 2016), while allele 18 at locus *cysG* in ST 43 is considered a recombinant allele (Nunney et al. 2014a). Alleles 12 and 18 at *cysG* only differ at five nucleotides (Stouthamer and Nunney 2016). Outside of the recombinant group, the *cysG* allele most similar to allele 18 is allele 14, from *X. fastidiosa* subsp. *fastidiosa*, which differs at only three nucleotides (Nunney et al. 2014a; Stouthamer and Nunney 2016). Strains with *cysG* allele 14, like *cysG* allele 12, have been found in coffee in Costa Rica (Stouthamer and Nunney 2016). In the part of allele 18 that is believed to have come from *X. fastidiosa* subsp. *fastidiosa*, the sequence is identical to the respective portions of both alleles 12 and 14 (Nunney et al. 2014c).

ST 42 and ST 43 have different alleles at locus *gltT* – allele 3 in ST 42 and allele 7 in ST 43 – but both of these are considered *X. fastidiosa* subsp. *multiplex* alleles (Nunney et al. 2014a). The *leuA* allele 6 in ST 42 differs by two nucleotides from allele 3, which is considered an *X. fastidiosa* subsp. *multiplex* allele and is the one found in ST 43 (Nunney et al. 2014a). While these are very similar, *leuA* allele 9, from *X. fastidiosa* subsp. *fastidiosa*, only differs from allele

6 by six nucleotides, and the analysis of Nunney et al. (2014a) suggests that the origin of *leuA* allele 6 is uncertain.

By the MLSA-E analysis of Parker et al. (2012), seven *X. fastidiosa* strains from blueberry, including one from rabbiteye blueberry, grouped together in what they called clade A of *X. fastidiosa* subsp. *multiplex*. Nunney et al. (2013) included 13 of the 21 *X. fastidiosa* subsp. *multiplex* isolates used by Parker et al. (2012), including several of blueberry origin, in their study and found that the six that Parker et al. (2012) called clade A were all part of the group that Nunney et al. (2013) considered recombinant. Besides the blueberry isolates, clade A contained one from almond, which was typed by Nunney et al. (2013) as ST 27. ST 27 differs from ST 42 at three loci and from ST 43 at two (Nunney et al. 2013). The putative clade within clade A in which the blueberry and almond isolates occur appears as a sister to a ragweed isolate, which was typed by Nunney et al. (2013) as ST 42.

1.3 Symptoms of *X. fastidiosa*

Xylella fastidiosa causes different symptoms in different plants. These range from marginal leaf scorch in grape, pecan, and shade trees; to stunting in peach and ragweed; and chlorotic areas on foliage in citrus variegated chlorosis (Davis et al. 1978; Hopkins and Purcell 2002; Mizell n.d.; Sanderlin and Heyderich-Alger 2000; Wells et al. 1987). Purcell and Hopkins (1996) relate the accumulation of xylem-limited bacteria in roots and lower stems to stunting diseases and the build-up in leaf petioles and veins to leaf scorch diseases. Likewise, they associate bacterial concentrations in the trunk or branches with "die-back and general decline" (Purcell and Hopkins 1996).

In grape, symptom development occurs when the population of *X. fastidiosa* is high and the bacteria are present in numerous xylem vessels (Chatterjee et al. 2008). Furthermore, plants

with *X. fastidiosa*-caused diseases exhibit leaf scorch symptoms most severely in late summer, when one might expect the greatest water stress (Chatterjee et al. 2008). Work by Holland (2013) has shown that several plant parts of a bacterial leaf scorch-resistant southern highbush cultivar generally had higher rates of hydraulic conductance than the respective parts of moderately or highly susceptible cultivars when asymptomatic, suggesting that the resistant cultivar had an inherently better ability to conduct water.

Sun et al. (2013) showed numerically greater hydraulic conductivity in Pierce's disease-resistant grape (*Vitis* spp.) genotypes than susceptible ones when mock-inoculated with buffer, and the difference in hydraulic conductivity between resistant and susceptible genotypes was much greater when they had been inoculated with *X. fastidiosa*. The extent of tylosis formation is a possible reason for differing levels of hydraulic conductivity and symptom expression in infected plants, with genotypes that develop more tyloses being ones that are more susceptible to Pierce's disease (Sun et al. 2013).

However, it is not clear that occlusion of xylem by bacteria or as a result of plant responses is wholly responsible for symptoms (Chatterjee et al. 2008). Work by Choi et al (2013), for example, shows that symptoms in grape differ between *X. fastidiosa* infection and drought stress. Lee et al. (1982) reported that bacteria-free filtrate from cells of the yet unnamed bacteria causing Pierce's disease, almond leaf scorch, and/or alfalfa dwarf caused symptoms in various grape cultivars, as well as almond and plum. It appears that there were toxins in two fractions resulting from chromatography, one of which caused wilting, primarily, and another that caused necrosis. They mention that induction of a xylem-blocking plant response and/or compounds that physically blocked the xylem could play a part in causing wilting. Recently, work by Nascimento et al. (2016) suggested that a lipase/esterase protein (LesA) produced by *X.*

fastidiosa subsp. *fastidiosa* plays a substantial part in producing leaf scorch symptoms in grapes affected by Pierce's disease.

Bacterial leaf scorch symptoms described on southern highbush plants include marginal leaf necrosis, thin twigs on new growth, leaf drop, stem yellowing, and plant death (Brannen et al. 2016). In FL 86-19 southern highbush plants, symptoms progressed from marginal necrosis to yellow stems and leaf loss, on both inoculated and, later, non-inoculated branches (Chang et al. 2009). Leaflessness combined with yellowed stems is characteristic of blueberry plants with bacterial leaf scorch, and these symptoms help differentiate between this disease and other biotic and abiotic causes of plant decline (Brannen et al. 2016). Southern highbush blueberry plants typically do not die in the same year that they begin to show symptoms but do eventually die (Brannen et al. 2016). Southern highbush blueberry plants that have died from bacterial leaf scorch may be more scattered, while plants affected by root rot are frequently clustered in the field (Brannen et al. 2016).

Rabbiteye blueberry symptoms noted in Louisiana and suspected of being associated with *X. fastidiosa* include plant death, shoot dieback, lack of productivity in re-growth after severe pruning, marginal necrosis, leaf reddening, and extensive defoliation combined with yellow stems (T. Avant and D. Ferrin, personal communication; M. H. Ferguson, unpublished data), similar to what has been described on southern highbush blueberry in Georgia (Brannen et al. 2016).

1.4 *X. fastidiosa* in Plant Hosts

X. fastidiosa lives within the xylem of plants and, it appears, moves from vessel to vessel via pit membranes that the bacteria alter with degradative enzymes (Chatterjee et al. 2008; Pérez-Donoso et al. 2010). *X. fastidiosa* lacks a flagellum, but Type IV pili permit *X. fastidiosa* to

move via twitching against the flow of xylem sap, as well as acropetally (Meng et al. 2005). Meanwhile, short, Type I pili and other adhesins, such as hemagglutinins, contribute to attachment to surfaces and other *X. fastidiosa* cells, as well as to the formation of biofilms, to which extracellular polysaccharides (EPS) also contribute (De La Fuente et al. 2007; Feil et al. 2007; Guilhabert and Kirkpatrick 2005; Li et al. 2007; Meng et al. 2005; Roper et al. 2007).

X. fastidiosa engages in quorum sensing via a diffusible signaling factor (DSF) (Ham 2013). This DSF is involved in determining what growth characteristics *X. fastidiosa* expresses and thus whether it moves relatively widely in the plant or grows in aggregates that are more readily acquired by a vector (Chatterjee et al. 2008). The DSF, which would be present at higher levels when the number of *X. fastidiosa* cells present is greater, appears to favor attachment and the formation of biofilms (Chatterjee et al. 2008). Work by Baccari et al. (2014) showed that, when DSF is lacking (i.e., in bacteria mutated in the DSF synthase gene *rpfF*), *X. fastidiosa* produces a suppressor of attachment. It has been proposed that the formation of large colonies of cells (aggregates) in xylem vessels aids in transmission by insect vectors (Chatterjee et al. 2008). Under other circumstances, formation of aggregates would likely be deleterious to the survival of the bacteria, since both bacteria and plant cells need nutrients and thus need xylem fluid moving past them in order to survive (Chatterjee et al. 2008).

X. fastidiosa is somewhat unique among plant pathogenic bacteria in that it lacks DNA sequences that have been associated with Type III secretion system genes (Chatterjee et al. 2008; Simpson et al. 2000). Simpson et al. (2000) and Chatterjee et al. (2008) have suggested that this deficiency is related to the relative lack of interaction of *X. fastidiosa* with living plant cells.

The gene expression work of Choi et al. (2013) shows that that grape plants' response to *X. fastidiosa* is not just a response to drought stress but includes responses associated with

biological organisms. Work by Rodrigues et al. (2013) suggests that *X. fastidiosa*-resistant citrus plants initially show responses associated with necrotrophic organisms or abiotic stressors and later exert defenses corresponding to biotrophic organisms. As plant defense responses to biotrophic and necrotrophic organisms or abiotic stressors have been found to interact with one another (Pieterse et al. 2009), the way in which a plant responds to *X. fastidiosa* relates to how *X. fastidiosa* infection could impact or be impacted by other pathogens or stressors.

1.5 Spread of *X. fastidiosa* Between Plants

X. fastidiosa moves from plant to plant via the mouthparts of Hemipteran insects in the families Cicadellidae (sharpshooters; subfamily Cicadellinae) and Cercopidae (spittlebugs) (Chatterjee et al. 2008; Redak et al. 2004). It is reported that some cicadas (Cicadidae) can also transmit the bacterium (Krell et al. 2007; Redak et al. 2004). If acquired by a nymph (juvenile) of one of its insect vectors, *X. fastidiosa* does not remain in it until the adult stage, as it is carried in the foregut, the lining of which is shed when insects molt (Chatterjee et al. 2008; Gullan and Cranston 2010). However, if acquired by the insect as an adult, it may remain in the vector, where it multiplies, for the rest of the insect's life (Chatterjee et al. 2008; Severin 1949). There is reportedly no waiting period between when the bacteria are acquired and when they can be transmitted to another plant (Chatterjee et al. 2008).

While a variety of Hemipteran insects can transmit *X. fastidiosa* in one or more crops (Krell et al. 2007; Redak et al. 2004), the author is not aware of any research done to determine which vectors are able to transmit *X. fastidiosa* in blueberry, specifically. It has been noted that the glassy-winged sharpshooter was the most often seen leafhopper in southern highbush blueberry orchards in Georgia (Tertuliano et al. 2012). Ma et al. (2010) tracked populations of both the glassy-winged sharpshooter and another sharpshooter, *Graphocephala versuta*, in three

rabbiteye blueberry plantings in Alabama. While greater numbers of *G. versuta* were identified in central and northern Alabama, more glassy-winged sharpshooters were found in the southernmost location (Mobile). The findings of Ma et al. (2010) suggest that the glassy-winged sharpshooter prefers several other fruit crops, including muscadines, bunch grapes, and Satsuma mandarins, to rabbiteye blueberry plants, but the numbers trapped in peaches and blueberries were similar.

Holland (2013) showed that *X. fastidiosa* can be transmitted vertically in softwood cuttings of southern highbush blueberry plants. The 'Star' plants from which cuttings were taken were symptomatic, but the cuttings themselves were not symptomatic at the time of propagation. Based on several trials, a total of ≈5% of cuttings tested positive. Some evidence for transmission of *X. fastidiosa* by seed from citrus plants with CVC has been produced (Li et al. 2003), but substantial evidence also suggests that bacteria in or on seed do not result in persistent infection or diseased plants (Coletta-Filho et al. 2014; Hartung et al. 2014).

Besides being vectored by insects and transmitted by propagation, in some cases, *X. fastidiosa* can also by transmitted by grafting. Transmission by grafting has been demonstrated in, for example, citrus (Hartung et al. 1994) and pecan (Sanderlin and Melanson 2008). He et al. (2000) demonstrated that *X. fastidosa* can move between sweet orange trees via naturally-formed root grafts, also. Pruning tools appear to be another possible method of transmission, as *X. fastidiosa* was detected in one of 21 grape plants on which cuts were made, following multiple cuts on an infected plant (Krell et al. 2007).

The rate of spread of *X. fastidiosa* within orchards would impact a calculation of the economics of removing infected plants (Sisterson et al. 2008). Sisterson et al. (2012) monitored the increase in the incidence of infected almond (*Prunus dulcis*) trees in two orchards and used a

Monte Carlo simulation to analyze the proximity of new infections to original infections. It appeared that the increase in infected trees followed a linear rather than an exponential pattern, suggesting little within-orchard spread. Also, it did not appear that the location of previous infections influenced the location of new infections, supporting the idea that trees can be retained without a great risk of contributing to new infections. These results contributed to a conclusion that it may not necessarily be beneficial to remove infected plants from orchards that will be replanted relatively soon. It was acknowledged by Sisterson et al. (2008), however, that the glassy-winged sharpshooter could cause more within-orchard spread.

In a pecan (*Carya illinoinensis*) study in Louisiana, none of 10 originally noninfected plants of a susceptible cultivar, Cape Fear, became infected during a three year study period (Sanderlin and Heyderich-Alger 2003). In a younger pecan orchard, the infection incidence in the same susceptible cultivar increased from 5 to 64% over a period of six years, and rates of infection in less susceptible cultivars increased from 0 to 2.6 or 10.5% during the same period (Sanderlin et al. 2009). The former orchard was ≈25 years old at the beginning of the study, while trees in the latter were 14 years old when that study began. However, a statistical analysis of the pattern of spread in the latter orchard, over a period of three years, did not provide conclusive evidence that locations of infected trees impacted the locations of future infections (Li et al. 2011; Sanderlin et al. 2009), even though the glassy-winged sharpshooter is known to be present in Louisiana (Sanderlin and Melanson 2010).

In an area of California where the glassy-winged sharpshooter was present, however, Park et al. (2006) found that grapevines with Pierce's disease were frequently close to dead vines and/or locations from which older vines had been removed. Before the glassy-winged sharpshooter was present, *X. fastidiosa* is believed to have been transmitted to grapevines in

California primarily from plants other than grape, and the temporal pattern of spread was of the "simple interest" type (Hopkins and Purcell 2002; Purcell 2013). After this vector arrived, though, *X. fastidiosa* appeared to spread between grapevines (Hopkins and Purcell 2002). Movement of *X. fastidiosa* between citrus trees is considered important in citrus variegated chlorosis, with patterns of increase consistent with secondary spread (Purcell 2013).

1.6 Plant Yield and Survival in the Presence of *X. fastidiosa*

The impact of a pathogen results largely from its impact on yield quantity and quality. In some crops, like susceptible cultivars of grape (*Vitis vinifera*), death of plants infected with *X. fastidiosa* takes place quickly enough that the proportion of yield reduction that individual infected plants experience, while they remain alive, is likely of minor interest (Smith et al. 2014). Southern highbush blueberry plants with bacterial leaf scorch sometimes die quickly, as well, once symptoms are observed (Brannen et al. 2016). Even in crops in which *X. fastidiosa* does not cause rapid plant death, death may occur at a higher rate in infected than in noninfected plants.

In almond in California, symptomatic/infected trees of one cultivar had estimated yields 37 or 40% lower, depending on the study period in question, than those of trees lacking symptoms of almond leaf scorch disease (ALSD) (Sisterson et al. 2008, 2012). For a second cultivar, average estimated yields of symptomatic/infected trees were 17 or 19% lower than those of asymptomatic trees (Sisterson et al. 2008, 2012). In 13 of a total of 18 year-orchard-cultivar combinations, the estimated yields of symptomatic trees were significantly lower than those of asymptomatic trees (Sisterson et al. 2008, 2012).

Authors also addressed tree mortality of several cultivars in two orchards (Sisterson et al. 2012). Nine percent of trees tested and confirmed to be *X. fastidiosa*-positive in the first years of

six- to seven-year surveys of two orchards were found to be dead in the final years of the surveys, and it is expected that a higher proportion would have been so if some had not been removed. Only 1% of initially asymptomatic trees were dead.

Sanderlin and Heyderich-Alger (2003) studied pecan bacterial leaf scorch (PBLS) in Louisiana and report comparisons of estimated yield in ten symptomatic/*X. fastidiosa*-positive and ten asymptomatic/*X. fastidiosa*-negative pecan trees over three years. They made initial plant selections based on presence or absence of symptoms and tested them for *X. fastidiosa* in August of the first year, using enzyme-linked immunosorbent assay (ELISA), and again in October of the third year. Kernel weight was significantly lower (13 to 18%) in nuts from infected than noninfected trees each year for three years. Total nut weight was significantly lower in two years and marginally lower in a third ($P = 0.08$). In Brazil, sweet orange (*Citrus* sp.) plants inoculated with *X. fastidiosa* prior to planting yielded ≈20% less than ones that were not inoculated before planting, over an eight year period (Gonçalves et al. 2011).

1.7 Detection of *X. fastidiosa*

When and how *X. fastidiosa* can be reliably detected are questions pertinent to those learning the biology of the pathogen and developing strategies for managing disease. Rates of detection can depend on the time of year, the plant part sampled, and the detection method. Nissen (2010) isolated *X. fastidiosa* from petiole or petiole/midvein tissue and stem sap of southern highbush blueberry plants in both July and October. It appears that ≈12% of isolation attempts from symptomatic plants resulted in culture of the bacterium in both July and October. Most positive isolations came from petiole tissue rather than stem sap in July, while similar numbers came from petiole/midrib tissue and stem sap in October.

In work by Holland et al. (2014) with southern highbush blueberry plants of three cultivars or lines and four symptom classes, the incidence of detection by real-time PCR was greater in several cases (in all symptom classes for 'Bluecrisp' and in the asymptomatic class for FL 86-19) in stem growth that was one year old and/or older than in stem growth produced during the current season. Detection incidence was greater in stem growth more than one year old than in roots, for asymptomatic 'Bluecrisp' and FL 86-19 plants, and higher in year-old stem growth than in roots, for moderately symptomatic 'Star' plants. However, incidence was higher in roots than in current season's growth for moderately and severely symptomatic 'Bluecrisp' plants. While differences among plant parts with respect to detection incidence were not found for moderately symptomatic FL 86-19, when *X. fastidiosa* was detected, the bacterial titer was higher in roots than in current season's stem growth and stem growth more than one year old.

In two years of monthly attempts to isolate xylem-borne bacteria from twigs of plum (*Prunus salicina*) with symptoms of plum leaf scald, a disease later attributed to *X. fastidiosa* (Wells et al. 1987), Chang and Yonce (1987) experienced a trend of low rates of success in the spring and relatively high rates of success in fall and/or early winter, in Georgia. From roots, bacteria were successfully cultured from 20 to 50% per tree in each month during which samples were taken (November through May of one season).

Wells et al. (1980) found that bacterial concentrations were much higher in roots than twigs of peach (*Prunus persica*), in orchards with peach phony symptoms. It appears that the "twigs" they used were the terminal portions of shoots, made up of most recent growth. Bacterial concentrations were significantly greater in roots in May than in February, August, or November, and significantly greater in twigs in May than in February or November. In samples

taken from one tree, Wells et al. (1980) observed especially high numbers of bacteria in extracts from roots from the northwest quadrant of the tree.

In another peach study, bacteria were found in xylem sap from all or almost all (≥ 96%) sampled 4-cm root segments of trees that showed symptoms of phony peach disease, while the proportion of root segments from asymptomatic trees in which at least one bacterium was observed ranged from 4 to 70% (Aldrich et al. 1992). A significant relationship was not found between the *X. fastidiosa* observation rate and either root diameter class or the distance of the root segment from the trunk, and bacteria were observed in similar proportions of root extracts from each quadrant of symptomatic trees. The percentages of roots of asymptomatic trees from which bacteria-containing extracts were obtained were variable among quadrants, but the orientations of the quadrants are not indicated.

In *Vitis labrusca* grapevines on which Pierce's disease symptoms had been observed, the percentages of xylem vessels in which bacteria were observed by light microscopy were numerically greater in leaf veins in June and July than in the other months in which samples were taken (March, April, and October; Hopkins 1981). Rates of observation of bacteria were greater in petioles from April through October, and greater in stems during March and June, but low in April in the latter (Hopkins 1981). Bacteria were found in a higher proportion of xylem vessels in petioles and/or leaf veins than in stems, except in March, when bacteria were not found in leaf veins or petioles (Hopkins 1981). Hopkins and Thompson (1984) found that the percentage of xylem vessels in which bacteria were observed increased greatly between May and July (in leaf veins and stems) in a *V. labrusca* cultivar and between July and September (in leaf veins and petioles, or the former plus stems) in two muscadine grape (*V. rotundifolia*) cultivars.

In elm trees that developed leaf scorch, Sherald and Lei (1991) detected *X. fastidiosa* in extracts of petioles from 67% of trees in June and from 94% of trees in September, using ELISA. When ELISA was performed on extracts of stem tissue (previous year's growth) and petioles (current year's growth) collected from sycamore and elm trees in early May, stem tissue extracts consistently resulted in higher absorbance values (Sherald and Lei 1991).

In sycamore in Georgia, the combined average detection rate, by ELISA, from roots and shoots dropped dramatically (from almost 60% to ≈20%) between early February and early March and did not start to consistently rise again until July (Henneberger et al. 2004). Isolation rates from stems were highest between December and February and in August and September. They were lowest in April and stayed relatively low until July.

Costa et al. (2004) tested several types of plants during 11 or 12 months of the year using ELISA. They detected *X. fastidiosa* in at least some oleander (*Nerium oleander*) and Spanish broom (*Spartium junceum*) plants during each month of the year but failed to detect it in any grape (*V. vinifera*) or almond plants between February and April (grape) or February and May (almond). They also compared detection by ELISA and immunocapture polymerase chain reaction (IC/PCR) in grape plants in which symptoms had been observed during the previous season. While rates of detection by the two methods were similar in June, in May, *X. fastidiosa* was detected in 15 of 25 samples by ELISA but 0 of 25 samples by IC/PCR.

Bextine and Miller (2004) compared detection of *X. fastidiosa* by ELISA and PCR in both plant tissue (stem tissue and associated petiole and leaf vein tissue) and xylem sap of grape (*V. vinifera*) and oleander plants. In asymptomatic grape plants, they found higher rates of detection in xylem sap than in tissue, when utilizing either ELISA or traditional PCR. In symptomatic oleander plants, *X. fastidiosa* was detected at a higher rate by PCR using tissue than

by PCR using xylem sap. This may have been due, at least in part, to the fact that there were difficulties in obtaining xylem sap from oleander plants. In other cases, there was no significant difference between detection rates in xylem sap and plant tissue. When overall rates of detection by ELISA and PCR were compared, significant differences were not found for either grape or oleander.

Several observations can be made about detection of *X. fastidiosa* in deciduous, woody plants based on these studies. Detectability in aboveground plant parts generally appears more reliable in late summer, fall, and/or winter and relatively poor during some period in the late winter and/or spring. The findings of Wells et al. (1980) in peach are an exception, as bacterial populations were low in November and greatest in May, the one month during the spring when they sampled. It is possible that a period when populations would have been lower had passed by that time. The most reliable plant part for detection may vary by crop and time of year, but it does appear that detection is less frequent in recent growth.

1.8 Management of *X. fastidiosa*-caused Diseases

Management strategies that have been recommended for one or more *X. fastidiosa*-caused diseases include the use of resistant or tolerant crop genotypes, use of clean plants, removal of infected plants or possible alternative hosts, application of insecticides, and cultural practices specific to the respective crops (e.g., Brannen et al. 2016; Gonçalves et al. 2014; Mizell n.d.; Smith et al. 2014). The use of plants tolerant or, especially, resistant to plant diseases is a desirable management approach. When cultivars with tolerance or resistance are available, plant disease problems can be addressed proactively, at the time of planting, hopefully preventing future yield or quality losses and management expenses. Hopkins and Purcell (2002) have

asserted that resistance is "[t]he only feasible control for [the *X. fastidosa*-caused Pierce's disease] in southeastern United States and parts of California…."

In southern highbush blueberry, Chang et al. (2009) found differences between selection FL 86-19 and cultivar Southern Belle, with respect to the percentage of plants infected by *X. fastidiosa* and the rate and extent of symptom development. Consistent with observations from the field (Brannen et al. 2016), FL 86-19 appeared more susceptible to *X. fastidiosa*-caused bacterial leaf scorch of blueberry. 'Emerald', on the other hand, is considered resistant, and 'Star' is considered moderately susceptible (Tertuliano et al. 2012). In Florida, researchers observed that 'Star' seemed to be affected more severely than 'Windsor', with regard to the percentage of symptomatic plants and severity of symptoms (Harmon and Hopkins 2009).

While southern highbush blueberry cultivars show differing levels of susceptibility to *X. fastidiosa*, inoculation of rabbiteye blueberry plants with *X. fastidiosa* resulted either in no detected infection, in cultivar Premier, or in local colonization of two of six 'Powderblue' plants (Chang et al. 2009). In infected 'Powderblue' plants, symptoms did not progress past the inoculated stem within a 14 month period. Anecdotal reports from Louisiana suggest that, in rabbiteye blueberry fields where *X. fastidiosa* has been detected, 'Tifblue' has exhibited the most noticeable growth problems (C. Muller and R. Sanderlin, personal communication; M.H. Ferguson, unpublished data). In addition to the use of resistant or tolerant genotypes, the use of clean plants is important both for the sake of the yields of the plants themselves and to prevent the introduction of inoculum (Gonçalves et al. 2011).

Removal of infected crop plants and/or possible alternative hosts is recommended for managing some *X. fastidiosa*-caused diseases (Brannen et al. 2016; Mizell n.d.; Smith et al. 2014). For phony peach disease (PPD), management recommendations include planting away

from other peach orchards and plum trees, eliminating wild plum trees in the area, and rogueing infected peach trees from young orchards (Mizell n.d.). However, rogueing of infected plant material has not always been found or predicted to be helpful (Hewitt 1949; Sisterson et al. 2008).

Using an economic model and three years of estimated yield data, Sisterson et al. (2008) concluded that it would sometimes be more economical to keep infected almond trees instead of removing them. Their calculations were based on the assumption that there was little spread of *X. fastidiosa* between trees, and they acknowledge that the glassy-winged sharpshooter could cause more within-orchard spread. One factor to consider when deciding whether or not to rogue is how soon one expects to replace the orchard (Sisterson et al. 2012). If an orchard is to be replaced soon, new trees would likely not produce enough yield, in comparison with the potential yield of infected trees, to justify the cost of replacement (Sisterson et al. 2012).

Hewitt et al. (1949) concluded that removing symptomatic grape vines did not significantly reduce the incidence of newly apparent Pierce's disease infections in California vineyards. However, the studies on which this conclusion was based were done prior to the introduction of the glassy-winged sharpshooter into the area. The glassy-winged sharpshooter is believed to change the epidemiology of Pierce's disease so that spread of enduring *X. fastidiosa* infections occurs between grape plants, as well as from plants outside of the vineyard (Hopkins and Purcell 2002).

Removal of symptomatic parts of plants is effective in some cases. Removal of symptomatic tissue from individual plants has been found to be useful in reducing further disease development in individual citrus trees with citrus variegated chlorosis (Purcell 2013), and Queiroz-Voltan et al. (2006) have suggested that severe pruning of coffee trees may be helpful in

managing *X. fastidiosa* in some situations. However, work by Holland et al. (2014) suggests that this approach is not likely to be effective in blueberry, as *X. fastidiosa* can often be found in the lower stems, and sometimes roots, before symptoms appear.

The host ranges of the *X. fastidiosa* strains that are pathogenic to the crop of interest can be expected to impact the efficacy of practices such as rogueing of alternative hosts and utilization of clean planting material. Wild and cultivated plants in which detection of *X. fastidiosa* has been reported are numerous and include, among many others, annual bluegrass (*Poa annua*), blackberry (*Rubus* sp.), crape myrtle (*Lagerstroemia indica*), daylily (*Hemerocallis* sp.), ginkgo (*Ginkgo biloba*), groundsel tree (*Baccharis halimifolia*), Japanese honeysuckle (*Lonicera japonica*), nandina (*Nandina domestica*), oleander, peppervine (*Ampelopsis arborea*), ragweed (*Ambrosia* spp.), Southern magnolia (*Magnolia grandiflora*), sweet orange (*Citrus sinensis*), sweetgum (*Liquidambar styraciflua*), Virginia creeper (*Parthenocissus quinquefolia*), western soapberry (*Sapindus saponaria*), wild mustard (*Brassica* sp.), yaupon holly (*Ilex vomitoria*), and multiple grape (*Vitis* spp.) and oak (*Quercus* spp.) species (Buzombo et al. 2006; Costa et al. 2004; Hartung et al. 1994; Hopkins and Adlerz 1988; Nunney et al. 2013; Shapland et al. 2006; Wong et al. 2004). Hopkins and Purcell (2002) have suggested that there are likely to be more hosts of *X. fastidiosa* than have been identified. However, there appears to be a good deal of host specificity among strains of *X. fastidiosa*, and understanding the host range of strains is of great importance (Harris and Balci 2015; Hopkins and Purcell 2002; Nunney et al. 2013).

Application of insecticides has been recommended for managing vectors in blueberry plantings (Brannen et al. 2016). While the author is not aware of any work that has been done related to insecticide efficacy specifically related to management *X. fastidiosa* in blueberry, it is expected that insecticides would be useful in a system in which secondary spread occurs, as it is

expected to be where the glassy-winged sharpshooter is present (Hopkins and Purcell 2002). However, application of insecticides is not recommended for phony peach disease, which also occurs where the glassy-winged sharpshooter is present, as it has not been determined to be effective (Mizell n.d.).

1.9 Interacting Factors or Alternative Causes

While *X. fastidiosa* is a pathogen of southern highbush blueberry and a possible pathogen of rabbiteye blueberry, a number of other agents can cause disease in blueberry, as well. *Phytophthora cinnamomi* Rands causes root rot in blueberry, which can result in yellowing of leaves and leaf loss in rabbiteye cultivars (Milholland 1995). Ring nematode (*Mesocriconema* sp.) has been investigated as a possible factor in what has been called blueberry replant disease (Jagdale et al. 2013). Several viruses cause ring spot or ring blotch symptoms on blueberry plants, although it is not clear that any of these are problematic in rabbiteye blueberry (Martin et al. 2012). It also has been asserted that plant stress can promote expression of symptoms in plants infected with *X. fastidiosa* (Hopkins and Purcell 2002).

1.10 Research Justification and Objectives

Prior to this research, there was concern about the possible impact of *X. fastidiosa* on rabbiteye blueberry production. However, information was needed about its prevalence and any associated yield or fruit quality losses. It was of interest whether rabbiteye blueberry might serve as an alternative host of the bacterium for susceptible southern highbush blueberry cultivars and what plants might serve as alternative hosts for *X. fastidiosa* in rabbiteye blueberry. Also, it was of interest whether some rabbiteye blueberry cultivars were more resistant or tolerant to *X. fastidiosa* than others. Finally, knowledge of when and where in the plant the bacterium was

likely to be detected was needed in order to help understand when and how it is best to sample when trying to detect the pathogen in the field and to know when false negatives are more likely.

Objectives of this research were to determine the following:

- The prevalence of *X. fastidiosa* infections in rabbiteye blueberry plants in Louisiana.
- What *X. fastidiosa* strains are found in rabbiteye blueberry plants.
- Whether there is an association between *X. fastidiosa* infection and yield and/or fruit quality loss in rabbiteye blueberry.
- To what extent *X. fastidiosa* spread within a rabbiteye orchard.
- When and how the bacterium can be reliably detected in rabbiteye blueberry.
- If some cultivars of rabbiteye blueberry are more resistant or tolerant to *X. fastidiosa* than others.

CHAPTER 2: *XYLELLA FASTIDIOSA* IN RABBITEYE BLUEBERRY PLANTS AND WILD HOSTS IN LOUISIANA

2.1 Introduction

Wild and cultivated plants in which detection of *X. fastidiosa* Wells et al. has been reported are numerous and include, among many others, annual bluegrass (*Poa annua*), blackberry (*Rubus* sp.), crape myrtle (*Lagerstroemia indica*), daylily (*Hemerocallis* sp.), ginkgo (*Ginkgo biloba*), groundsel tree (*Baccharis halimifolia*), Japanese honeysuckle (*Lonicera japonica*), nandina (*Nandina domestica*), oleander (*Nerium oleander*), peppervine (*Ampelopsis arborea*), ragweed (*Ambrosia* spp.), Southern magnolia (*Magnolia grandiflora*), sweet orange (*Citrus sinensis*), sweetgum (*Liquidambar styraciflua*), Virginia creeper (*Parthenocissus quinquefolia*), western soapberry (*Sapindus saponaria*), wild mustard (*Brassica* sp.), yaupon holly (*Ilex vomitoria*), and multiple grape (*Vitis* spp.) and oak (*Quercus* spp.) species (Buzombo et al. 2006; Costa et al. 2004; Hartung et al. 1994; Hopkins and Adlerz 1988; Shapland et al. 2006; Wong et al. 2004). Hopkins and Purcell (2002) have suggested that there are likely to be more hosts of *X. fastidiosa* than have been identified. However, there appears to be a good deal of host specificity among strains of *X. fastidiosa*, and understanding the host range of strains is of great importance (Harris and Balci 2015; Hopkins and Purcell 2002; Nunney et al. 2013). Host range is likely to impact the efficacy of practices such as rogueing of alternative hosts and utilization of clean planting material.

Within *X. fastidiosa*, there are two validly published subspecies (*fastidiosa* and *multiplex*) and at least four proposed subspecies (*morus*, *pauca*, *sandyi*, and *tashke*) (Bull et al. 2010; Nunney et al. 2014c; Randall et al. 2009; Schaad et al. 2004a, 2004b; Schuenzel et al. 2005), although Marcelletti and Scortichini (2016) argue, based on analyses using extensive portions of

strain genomes, that strains previously classified as subspecies *sandyi* and *morus* actually belong in subsp. *fastidiosa*. *X. fastidiosa* strains that have been characterized after being found naturally occurring in blueberry have been identified as subsp. *multiplex* or, in one case, has been equally closely matched to strains in subsp. *multiplex* and subsp. *sandyi* (Nissen 2010; Nunney et al. 2013; Parker et al. 2012). Nissen (2010) found that, within a 520 nucleotide segment of the 16S-23S rDNA intergenic spacer (ITS) region, two *X. fastidiosa* isolates from southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids) were more similar to strains M12 and Dixon (both from almond), which are indicated as subsp. *multiplex* by Nunney et al. (2013), and to *X. fastidosa* subsp. *sandyi* strain Ann-1 (from oleander) than to a subsp. *pauca* strain or two subsp. *fastidiosa* strains. Nunney et al. (2013) placed all seven *X. fastidiosa* isolates of blueberry origin that were included in their study into a group that, while considered to be part of subsp. *multiplex*, they believed to have arisen from recombination with subsp. *fastidiosa* (Nunney et al. 2014a).

Scally et al. (2005) developed a multilocus sequence typing (MLST) scheme, modified by Yuan et al. (2010), which allows *X. fastidiosa* strains to be genotyped below the subspecies level. The MLST approach involves typing strains based on the exact nucleotide sequences at seven housekeeping loci (Scally et al. 2005). Each combination of nucleotides is assigned an allele number, and each combination of seven alleles is a different sequence type (ST) (Scally et al. 2005). An additional locus, *pilU*, is sometimes utilized in conjunction with the MLST loci (Nunney et al. 2014a; Yuan et al. 2010). Parker et al. (2012) took a different multilocus approach, which they call multilocus sequence analysis of environmentally mediated genes (MLSA-E), to characterizing *X. fastidiosa* isolates. Their approach involved nine genes that had, on average, higher ratios of non-synonymous to synonymous substitution rates than those used in

the Yuan et al. (2010) MLST scheme. In other words, nucleotide differences in alleles at these loci more often resulted in codes for different amino acids, suggesting that these sequences reveal more genotypic variation (Hartwell et al. 2004; Parker et al. 2012).

The seven blueberry isolates that have been typed by the MLST scheme have been identified as ST 42 or 43, which were among strains that were deemed to be recombinant subsp. *multiplex* (Nunney et al. 2013). By the MLSA-E analysis of Parker et al. (2012), seven strains from blueberry, including one from rabbiteye blueberry (*Vaccinium ashei* Reade = *V. virgatum* Aiton), grouped together in what they called clade A of *X. fastidiosa* subsp. *multiplex*. Nunney et al. (2013) included 13 of the 21 *X. fastidiosa* subsp. *multiplex* isolates (from blueberry and other hosts) used by Parker et al. (2012), and they found that the six that Parker et al. (2012) called clade A were all part of the group that Nunney et al. (2013) considered recombinant.

While these findings suggest the possibility that a narrow range of *X. fastidiosa* subsp. *multiplex* genotypes occur in naturally infected blueberry plants, southern highbush blueberry plants expressed symptoms of leaf scorch following inoculation with *X. fastidiosa* subsp. *fastidiosa* isolates from elderberry and lupine, as well as *X. fastidiosa* subsp. *multiplex* isolates from almond and blackberry, in work by Hopkins et al. (2012). Oliver et al. (2015) found that four subsp. *fastidiosa* isolates from two species (grape and elderberry) were able to colonize southern highbush blueberry cultivar Rebel plants, although the symptom severity they caused, as measured by area under disease progress curve (AUDPC) values, was less than those caused by two of three isolates of blueberry origin. The authors acknowledge that these results, which were the product of artificial inoculation, might not reflect what happens in nature.

Objectives of this study were to (i) determine the prevalence of *X. fastidiosa* infections in rabbiteye blueberry plants in Louisiana, and (ii) determine what *X. fastidiosa* strains are found in

rabbiteye blueberry plants. The latter objective relates to the practical questions of what plants might serve as alternative hosts for *X. fastidiosa* in rabbiteye blueberry and whether rabbiteye blueberry might serve as an alternative host of the bacterium for susceptible southern highbush blueberry cultivars.

2.2 Materials and Methods

2.2.1 Collection and Testing of Plant Samples

To assess the prevalence of *X. fastidiosa* in blueberry plantings, leaf, terminal shoot, and/or stem samples were collected from rabbiteye blueberry plants in 20 orchards in Louisiana and Mississippi, between Nov. 2012 and Feb. 2016. Samples were also collected from one or more wild or cultivated plants other than blueberry at four of these locations and one additional location in Louisiana. If there was a question about the identity of a plant, a sample was taken to the Louisiana State University Herbarium for confirmation.

Samples for enzyme-linked immunosorbent assay (ELISA) were generally composite samples consisting of leaves from a number of bushes in the orchard, although leaves from individual plants were sometimes tested separately. Petioles and lower midribs were cut out and tested using a double antibody sandwich ELISA assay for *X. fastidiosa* (Agdia, Inc., Elkhart, IN). In a limited number of cases when samples were collected at times when detection in leaves was not expected to be reliable, terminal shoot tissue was collected and tested by ELISA. In ELISA tests, there were two technical replicates (wells) for each sample, except as noted in the results. Absorbance was quantified using a Microplate Autoreader EL311 (BioTek Instruments, Inc., Winooski, VT). An absorbance of 0.100 or higher, relative to a buffer control, at 630 nm was used as the criterion for a positive sample, similar to the threshold (0.100 at 620 nm) used by Chang et al. (2009).

At several locations, stems, and on one occasion roots, were collected for testing of DNA from sap by real-time polymerase chain reaction (PCR). After returning from the field, sections were cut from stem or root samples using lopping shear blades wiped between samples with 70% ethanol (EtOH), and a vise was used to squeeze sap from the resulting piece (Holland et al. 2014). A basal section of each stem was used first. If insufficient sap was available from that section, subsequent sections, which sometimes included sections of branches from the main stem, were used. Sap was pipetted into autoclaved microcentrifuge tubes for storage at -70°C until DNA was extracted.

There were a limited number of cases in which DNA was extracted from leaf petiole/midrib tissue or from stem tissue, and these are noted in the results. After petioles/midribs or stems were cut out or cut up, respectively, they were stored at -20°C. Tissue was then ground in liquid nitrogen, using mortars and pestles, and again stored at -20°C until DNA was extracted.

DNA was extracted from sap using CTAB methods based on those described in Li et al. (2008) or Porebski et al. (1997), or by using the PowerPlant Pro DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). When the kit was used, FastPrep-24 Lysing Matrix A 2 ml tubes (MP Biomedicals, Solon, OH), as used in the CTAB extractions, were sometimes substituted for the tubes included with the kit. When DNA was extracted from petiole/midrib or stem tissue, the CTAB method based on Li et al. (2008) was used. Extracted DNA was stored at -70°C or, in a few cases, -20°C. Real-time PCR was performed using Applied Biosystems Power SYBR Green PCR Master Mix (Life Technologies Corporation, Carlsbad, CA) or SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA), and EFTu_3 forward and reverse primers, as published by Holland et al. (2014). Samples were tested using either one

or two technical replicates. Real-time PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) thermocycler using the following program: 10 min at 95°C; 40 cycles of 10 s at 95°C and 1 min at 60°C; and a dissociation step of 15 s at 95°C, 20 s at 60°C, and 15 s at 95°C (Holland et al. 2014). Positive samples had C_t values of 35 or less and melting temperatures between 77.2 and 78.3°C, when the Applied Biosystems master mix was used and between 81.2 and 82.4°C when the Bio-Rad master mix was used. Samples were re-tested if there was a conflict between wells with respect to results for the sample or if a sample tested positive but the amplification curve appeared atypical. If continuing conflicts occurred when testing in duplicate, a sample was tested in triplicate, and if two of three wells were positive, the sample was considered positive. Until positive samples from blueberry were detected, an extract of *X. fastidiosa* from grape (A-08; Melanson et al. 2012) was used as a positive control.

2.2.2 Isolation of *Xylella fastidiosa*

Sap from a 'Tifblue' rabbiteye blueberry stem from a commercial orchard in East Feliciana Parish, LA, was pipetted aseptically onto modified periwinkle wilt medium (Sherald et al. 1983). Growth suspected of being *X. fastidiosa* was transferred into 100 µl sterile distilled water, from which 10 µl drops were then deposited onto modified periwinkle wilt medium. After ≈1 month bacteria were scraped into 100 µl sterile distilled water, and DNA was extracted from 50 µl of this suspension and tested using real-time PCR with EFTu_3 primers, as previously described. The culture that tested positive for *X. fastidiosa*, LA-Y3, was maintained on modified periwinkle wilt and CS-20 (Chang and Walker 1988) media. After several transfers, four single colonies were selected and henceforth transferred separately.

2.2.3 Characterization of *X. fastidiosa* in Blueberry by Multilocus Sequence Typing

To determine what *X. fastidiosa* genotypes are found in rabbiteye blueberry, DNA extracted from the following sources was utilized for multilocus sequence typing: (i) *X. fastidiosa* isolate LA-Y3C, (ii) petiole/midrib tissue from 'Tifblue' rabbiteye blueberry plants in the same orchard from which LA-Y3 was obtained, and (iii) stem tissue of a rabbiteye blueberry plant a St. Landry Parish, LA, orchard. The East Feliciana Parish orchard was planted in the late 1970s or early 1980s, while the St. Landry Parish orchard was planted in the late 1980s. DNA was subjected to PCR using primers for loci *cysG*, *gltT*, *holC*, *leuA*, *malF*, *nuoL*, *petC*, and *pilU*, based on the protocol outlined in Yuan et al. (2010). The first seven loci are part of the MLST scheme for *X. fastidiosa*, as modified by Yuan et al. (2010). Between 0.7 and 5 µl of template DNA was used in each 25 µl reaction. Forward and reverse primers (0.75 µl of 10 µM), 0.25 µl of DreamTaq DNA Polymerase and 2.5 µl of 10x DreamTaq buffer (Thermo Scientific, Lithuania), 0.5 µl of 10 mM dNTPs (Promega, Madison, WI), and sterile water were also included in each reaction (Yuan et al. 2010).

PCR was performed on an Applied Biosystems 2720 Thermal Cycler (Life Technologies, Carlsbad, CA) using the following program: 3 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 65°C, and 1 min at 72°C; and 10 min at 72°C (Yuan et al. 2010). A 1% agarose gel and GelRed stain (Biotium, Hayward, CA) were used to check for the presence of products of the expected sizes. Beckman Coulter Genomics (Danvers, MA) or Macrogen USA (Rockville, MD) sequenced PCR products using the Sanger method. Products were either purified prior to sending by using the QIAquick PCR Purification Kit (QIAGEN Inc, Valencia, CA) or purified using an enzymatic method by the company performing sequencing.

Forward and reverse sequences were aligned using MAFFT software (http://mafft.cbrc.jp/alignment/server/). In MAFFT or Mesquite (Maddison and Maddison 2015), aligned forward and reverse sequences were viewed together. Sequences were then concatenated by joining the 3' end of the forward sequence with the reverse complement of the 3' end of the reverse sequence. If there was then a conflict between forward and reverse sequences that was unresolved by the editing process described above, chromatograms were viewed using Chromas (http://technelysium.com.au/wp/chromas/) to determine if one of the conflicting base calls appeared more reliable. If so, this nucleotide was utilized. For the seven MLST loci, the sequences were entered as queries in the *X. fastidiosa* MLST database (Stouthamer and Nunney 2016), using the BLAST function. The top allelic match, along with any mismatches or gaps, was noted for each locus. When concatenated sequences differed from established alleles, MAFFT was used to determine where they differed. For *pilU*, sequences were entered into the NCBI BLAST query tool, and the top *pilU* allele match was noted. One hundred percent identity at 545 nucleotides indicated a perfect match for a *pilU* allele.

2.3 Results

X. fastidiosa was detected in blueberry in two of the 17 orchards sampled in Louisiana, by both ELISA and real-time PCR (Table 2.1). In an ELISA test of one plant from an orchard in Lamar County, MS, the absorbance of one well was above 0.100, while the absorbance of the other one was below 0.100, and by the time it was retested, the results were not considered reliable due to the age of the extract, so results are considered ambiguous. No other positive samples were detected in Mississippi (Table 2.1).

Table 2.1. Tests for *X. fastidiosa* in blueberry plants from orchards in Louisiana and Mississippi.

	ELISA		Real-time PCR	
Parish (LA) or County (MS)	Result[z]	Date(s)[y]	Result	Date(s)
Beauregard (LA)	–[x]	3/2014		
Bossier (LA)	–	10/2014		
Caddo 1 (LA)	–	10/2014		
Caddo 2 (LA)	–	10/2014		
DeSoto 1 (LA)	–[x], –	5/2014, 10/2014	–	5/2014
DeSoto 2 (LA)	–	10/2014		
East Feliciana 1 (LA)	–	11/2012		
East Feliciana 2 (LA)[w]	+, +	7/2013, 8/2013	+, +	2/2013, 7/2013
East Feliciana 3 (LA)	–	8/2013	–	4/2013
Lamar 1 (MS)			–	2/2013
Lamar 2 (MS)	*[v]	7/2013	–	2/2013
Lincoln (LA)	–	10/2014		
Pearl River (MS)			–	2/2013
St. Landry (LA)	+	10/2014	+, +[u]	10/2014, 2/2016
St. Tammany 1 (LA)	–, –	11/2013, 10/2015	–	2/2013
St. Tammany 2 (LA)	–	11/2014		
Tangipahoa (LA)	–	11/2012		
Washington 1 (LA)	–	11/2014		
Washington 2 (LA)	–	11/2014		
Washington 3 (LA)	–	10/2015		

[z]A positive result (+) signifies that at least one sample taken from this location at the indicated time tested positive.
[y]Dates indicate the months in which samples were taken.
[x]This result is from ELISA on shoot tissue rather than leaf petiole/midrib tissue.
[w]Samples were collected from this location on numerous dates. For each method, only results from the first two sampling dates from which results were quantified are shown.
[v]There was disagreement between results of the two wells for one sample from this farm, so results were ambiguous.
[u]This result is from real-time PCR on DNA from stem tissue rather than sap.

In and around the perimeter of the East Feliciana Parish orchard where *X. fastidiosa* was detected in blueberry, several species of plants tested positive for *X. fastidiosa* by ELISA on one or more occasions (Table 2.2). These included American beautyberry, blackberry, Chinese privet, and wild cherry. However, in the cases in which samples from the same species were tested by real-time PCR, the results were negative. In a rabbiteye blueberry orchard at Bob R. Jones-Idlewild Research Station, also in East Feliciana Parish, a blackberry plant that showed symptoms of marginal leaf scorch tested positive by ELISA. An Elliott's blueberry (*V. elliottii* Chapm.) plant that grew in a residential yard in Washington Parish, LA, tested positive for *X. fastidiosa* by ELISA but not real-time PCR.

When sequences from MLST loci and *pilU* of *X. fastidiosa* from culture and infected plant samples were BLASTed into the MLST database (for MLST loci) or NCBI database (for *pilU*), exact matches were revealed for some sequences, and close matches were found for others (Table 2.3). For combinations of alleles that corresponded to a defined sequence type (ST), the ST is provided (Table 2.3). Sequences of one sample from the East Feliciana Parish orchard and one from a St. Landry Parish orchard corresponded to ST 42 and had *pilU* sequences identical to allele 9 (GenBank accession no. JX899410). The isolate and an additional plant sample from the East Feliciana Parish orchard had alleles corresponding to ST 42 and *pilU* allele 9, as well, except that *nuoL* sequences differed from the *nuoL* allele 3 sequence at one base, where sequence reads were very poor (data not shown). It is expected that resequencing would reveal exact matches for *nuoL* allele 3 and thus ST 42. Sequences of the *nuoL* locus from another single colony isolate, LA-Y3A, of the same culture from which LA-Y3C was derived revealed an exact match to *nuoL* allele 3, as well as to the other ST 42 alleles and *pilU* allele 9, except a high quality sequence from *cysG* was not obtained.

Table 2.2. Tests for *X. fastidiosa* in plants other than rabbiteye blueberry.

		ELISA		Real-time PCR	
Host	Location[z]	Result[y]	Date[x]	Result	Date
American beautyberry (*Callicarpa americana*)	E. Feliciana 2	+	8/2014		
American beautyberry	E. Feliciana 2	–	6/2016		
American beautyberry	Lincoln	–	10/2014		
American persimmon (*Diospyros virginiana*)	E. Feliciana 2	–	8/2014		
Blackberry (*Rubus* sp.)	E. Feliciana 1	+	8/2013		
Blackberry	E. Feliciana 2	0/2	4/2014		
Blackberry	E. Feliciana 2			0/3[w]	6/2014
Blackberry	E. Feliciana 2	1/3	8/2014		
Chinese privet (*Ligustrum sinense*)	E. Feliciana 2	–	8/2014		
Chinese privet	E. Feliciana 2	+	1/2015		
Chinese privet	E. Feliciana 2	2/2	2/2015		
Chinese privet	E. Feliciana 2	3/3	1/2016	–[w]	1/2016
Elliott's blueberry (*Vaccinium elliottii*)	Washington 4	+	6/2016	–[w]	6/2016
Plum (*Prunus* sp.)	DeSoto 1	–	5/2014		
Plum	E. Feliciana 1	–	8/2013		
Sparkleberry (*Vaccinium arboreum*)	E. Feliciana 2	–	1/2015		
Sweetgum (*Liquidambar styraciflua*)	E. Feliciana 2	–	1/2015		
Trumpet creeper (*Campsis radicans*)	E. Feliciana 2	+[v]	8/2014		
Virginia creeper (*Parthenocissus quinquefolia*)	DeSoto 1	–[v]	5/2014		
Water oak (*Quercus nigra*)	E. Feliciana 2	–	1/2015		
Wild cherry (*Prunus serotina*)	E. Feliciana 2	+	8/2014		
Wild cherry	E. Feliciana 2	–	1/2016		
Winged sumac (*Rhus copallinum*)	E. Feliciana 2	–	8/2014		
Yaupon holly (*Ilex vomitoria*)	E. Feliciana 2	+	1/2015		
Yaupon holly	E. Feliciana 2	2/2	2/2015		

(Table 2.2 continued)

		ELISA		Real-time PCR	
Host	Location[z]	Result[y]	Date [x]	Result	Date
Yaupon holly	E. Feliciana 2	3/3	1/2016	0/2[w]	1/2016

[z]Locations correspond to those noted in Table 2.1, except for the Washington Parish (LA) location, which was not included in Table 2.1.

[y]A positive result (+) indicates that one plant, or a composite sample from multiple plants of this kind, tested positive on this date. When multiple samples from individual plants were taken on one date, numerals are used. An entry of 2/3, for example, would indicate that three plants were tested and two tested positive.

[x]Dates indicate the months in which samples were taken.

[w]Plant tissue or sap from which DNA was extracted for real-time PCR were as follows: blackberry, stem and/or root sap; Chinese privet, petiole/midrib tissue and stem sap from the same plant (tested separately, same result); Elliott's blueberry, stem tissue; yaupon holly, petiole/midrib tissue from one plant and stem sap from another.

[v]Only one well rather than two was tested for these samples.

Table 2.3. *X. fastidiosa* alleles at multilocus sequences typing (MLST) and *pilU* loci.

		Alleles[x]								
Plant[z]	Location[y]	*cysG*	*gltT*	*holC*	*leuA*	*malF*	*nuoL*	*petC*	*pilU*	ST[y]
EF-C3	E. Feliciana 2	12	3	4	6	5	3	3	9	42
EF-H3	E. Feliciana 2	12	7/2[w]	4	3	5	3	3	9	–
EF-N3	E. Feliciana 2	12/1, 14/1[w]	7	7	6	5	4	3	9	–
SL-6	St. Landry	12	3	4	6	5	3	3	9	42

[z]All DNA sources were petiole/midrib tissue except SL-6, which was extracted from stem tissue.

[y]Locations correspond to those in Table 2.1.

[x]Alleles and STs correspond to those in the *X. fastidiosa* MLST database (Stouthamer and Nunney 2016) or, for allele *pilU* allele 9, GenBank accession number JX899410. Two MLST alleles are not found in the database. In these cases, entries contain the allele(s) in the MLST database that are the closest match(es), followed by the number of bases at which the alleles in this study differ from the established alleles. The entry 12/1, for example, indicates that the sequence differs by 1 nucleotide from allele 12.

Samples EF-H3 and EF-N3 both shared some alleles with ST 42 but differed at others. EF-H3 had *leuA* allele 3, and EF-N3 had *gltT* allele 7, *holC* allele 7, and *nuoL* allele 4. Two alleles, the *cysG* allele of EF-N3 and the *gltT* allele of EF-H3, did not match any that were defined in the MLST database. The EF-N3 *cysG* allele differed from alleles 12 and 14 by one base. It had an A at position 21, where allele 14 had a C, and a C at position 71, where allele 12 had an A. The *gltT* sequence of EF-H3 was most similar to *gltT* allele 7 but differed at two bases. It had a T at position 583 and an A at position 653, whereas *gltT* 7 had A and G at the respective positions.

2.4 Discussion

X. fastidiosa was detected in only two of 17 orchards sampled in Louisiana, in spite of the fact that samples were collected from most of these orchards in the late summer or fall, times when *X. fastidiosa* detection is expected to be relatively reliable (Chapter 3). Because at least one ELISA sample from each orchard was generally a composite sample taken from a number of bushes – and if not, a number of individual bushes were tested – the results from these are considered more reliable than the real-time PCR results, which were typically based on extracts of sap from stems of individual plants. Also, detection in stem sap during the time of year when several of these samples were taken is not especially reliable (Chapter 3). Samples from three orchards in Mississippi did not yield any unambiguously positive results. If the plant from one farm for which the wells in the ELISA test yielded conflicting results was indeed infected, the population of *X. fastidiosa* in that plant was likely low, and the bacterium did not appear to be prevalent in the orchard, since none of 19 other plants tested positive at this time. These results are encouraging, as they suggest that *X. fastidiosa* infections are not common in rabbiteye

blueberry orchards in Louisiana or, based on the limited number of orchards sampled, in southern Mississippi.

Several wild plants from around a blueberry orchard with *X. fastidiosa* infections tested positive by ELISA for *X. fastidiosa*. Chinese privet and yaupon holly tested positive on more than one occasion. It was interesting, though, that when these plants, or the Elliott's blueberry from another location, were tested by real-time PCR, they did not test positive for *X. fastidiosa*. The antibodies employed in the ELISA assay utilized for *X. fastidiosa* have been found to cross-react with *Pseudomonas syringae* pv. *syringae* and *Xanthomonas arboricola* pv. *pruni* (S. Lutes, Agdia, personal communication), so it is possible that these plants harbored epiphytic populations of one or both of these bacteria (Hattingh and Roos 1995; Lamichhane 2014). Wild cherry, in particular, has been identified as a source of epiphytic *P. syringae* pv. *syringae* (Gitaitis et al. 1985). Because *X. fastidiosa* is a xylem-limited bacterium, it was not thought necessary to surface sterilize or rinse leaves before testing by ELISA, but because the bacteria that might cause false positives by ELISA can live epiphytically (Hattingh and Roos 1995; Lamichhane 2014), rinsing of leaves to remove at least some epiphytic bacteria might be useful with respect to preventing false positives by ELISA (M. Butler, personal communication).

Some of the wild plants that tested positive for *X. fastidiosa* by ELISA have been previously reported as hosts. Buzombo et al. (2006) detected *X. fastidiosa* in yaupon holly in Texas by ELISA, PCR, and immunofluorescence. Hopkins and Adlerz (1988) reported detection of *X. fastidiosa* in and/or isolation of it from American beautyberry and blackberry. Wells et al. (1980) observed a small number of what were then referred to as "rickettsia-like bacteria" in extracts from roots and stems of wild cherry. However, when they tested bacteria from one year's root samples in an immunofluorescence assay, using antiserum to a phony peach strain of

what we now know as *X. fastidiosa* (Wells et al. 1987), positive results were not obtained. Hopkins and Adlerz (1988) did not isolate *X. fastidiosa* from a limited number of wild cherry from which attempts were made. Neither Elliott's blueberry nor Chinese privet has previously been reported as a host of *X. fastidiosa*. However, because of the lack of detection of *X. fastidiosa* by real-time PCR in either of these plants and the possibility of false positives by ELISA due to *P. syringae* pv. *syringae* and *X. arboricola* pv. *pruni*, these findings should not be construed as the establishment of these two plants as hosts of *X. fastidiosa*. Artificial inoculation could be performed to determine whether *X. fastidiosa* can infect these species.

One *X. fastidiosa* genotype present in rabbiteye blueberry in Louisiana is ST 42. This is one of two STs that have been previously identified from blueberry (Nunney et al. 2013; Stouthamer and Nunney 2016). All isolates of blueberry origin that have previously been identified as ST 42 have come from Georgia. ST 42 has also been identified from giant ragweed and western soapberry in Texas (Nunney et al. 2013).

The two samples that differed from ST 42 each contained one allele that did not match established alleles for the respective loci. The other alleles in these samples are found either in STs considered, like ST 42, to have resulted from recombination between *X. fastidiosa* subspecies *multiplex* and *fastidiosa*, or in both putatively recombinant and non-recombinant *X. fastidiosa* subsp. *multiplex* strains (Nunney et al. 2013; Nunney et al. 2014a; Nunney et al. 2014c; Yuan et al. 2010). Because these sequences were generated from plant samples, there is a possibility that more than one strain of *X. fastidiosa* was present, and isolation of the bacteria is needed to firmly establish the existence of new genotypes.

The alleles found in the EF-H3 sample were the same as those associated with ST 22, except that ST 22 has *gltT* allele 3 (Nunney et al. 2013). ST 22 has been identified from giant

ragweed and western ragweed in Texas (Nunney et al. 2013). The alleles identified from EF-N3, except at the *cysG* locus, are the same as those in ST 28, which has been identified from giant ragweed, annual sunflower, and seacoast sumpweed (Nunney et al. 2013). All of these ST 22 and ST 28 isolates came from Texas, where plants were collected around vineyards for *X. fastidiosa* isolation (Nunney et al. 2013). Additional efforts to type *X. fastidiosa* from wild plants might reveal that these STs are present in a number of other species.

Because a limited number of STs have been found in blueberry, and all of these appear likely to have resulted from recombination between two subspecies, this work supports the idea that there is some degree of host specificity within *X. fastidiosa*. However, the level of host specificity observed here is less than that found by Harris and Balci (2015), for example. They identified four *X. fastidiosa* STs from four genera of trees and determined that the STs were each specific to one tree genus, except that one elm sample contained a strain of the ST that was detected predominantly from oak species.

Considering that ST 42 has been found in giant ragweed and that recombinant genotypes similar to others identified from rabbiteye blueberry have been found in this and other Asteraceae family plants, the number of alternative hosts for strains of *X. fastidiosa* that infect blueberry may be considerable. Weed management compatible with good production practices could help minimize alternative hosts in the immediate vicinity of blueberry plants. Additional work to determine the STs of *X. fastidiosa* in wild plants would provide more insight on the likelihood of being able to aid management of *X. fastidiosa* by targeting certain plant genotypes for removal.

While Hopkins et al. (2012) and Oliver et al. (2015) found that some *X. fastidiosa* subsp. *fastidiosa* strains could infect southern highbush blueberry when inoculated, it appears that

persistent infections of blueberry by, for example, *X. fastidiosa* subsp. *fastidiosa* strains may not be common under natural conditions. Of course, even if infections by some strains do not persist for a long period of time, short-lived infections could give bacteria a chance to undergo recombination between or within subspecies (Oliver et al. 2015).

The finding of ST 42 in rabbiteye blueberry does suggest that rabbiteye blueberry may serve as an alternative host of strains that are able to infect susceptible southern highbush blueberry cultivars. There may be strains of *X. fastidiosa*, however, that are identical at MLST loci but have differences at other loci that influence their abilities to infect certain hosts. Cross-inoculation studies might add some clarity regarding the cross-infectivity of isolates from rabbiteye and southern highbush blueberry plants. However, the finding that even some *X. fastidiosa* subsp. *fastidiosa* strains can infect southern highbush blueberry plants under artificial conditions, while only *X. fastidiosa* subsp. *multiplex* sequence types have been found naturally occurring in blueberry, suggests that such studies do not necessarily reflect what happens under natural conditions (Hopkins et al. 2012; Nunney et al. 2013; Oliver et al. 2015; Parker et al. 2012). Considering that older rabbiteye plants, at least, do not always show distinctive symptoms of *X. fastidiosa* infection, growers are advised to take care to use clean planting stock when introducing rabbiteye plants into orchards in which susceptible southern highbush cultivars are grown.

CHAPTER 3: *XYLELLA FASTIDIOSA* IN A NATURALLY-INFECTED RABBITEYE BLUEBERRY ORCHARD

3.1 Introduction

Diseases caused by *Xylella fastidiosa* Wells et al. are especially prevalent in the southeastern United States (Hopkins and Purcell 2002). Proposed reasons for the bacterium's success in this region's grapes, for example, include the presence of the glassy-winged sharpshooter (*Homalodisca vitripennis*), a vector of *X. fastidiosa*; a long growing season; and warm nights (Hopkins and Purcell 2002). Besides Pierce's disease of grape, other *X. fastidiosa*-caused diseases found in the Southeast include phony peach disease, pecan bacterial leaf scorch, and oleander leaf scorch (Hopkins and Purcell 2002; Sanderlin and Heyderich-Alger 2000). Since 2004, researchers in Georgia and Florida have confirmed *X. fastidiosa* as the cause of a leaf scorch disease of southern highbush blueberries (*Vaccinium corymbosum* interspecific hybrids) (Chang et al. 2009; Harmon and Hopkins 2009). Inoculation of rabbiteye blueberry (*Vaccinium ashei* Reade = *V. virgatum* Aiton) plants with *X. fastidiosa* resulted either in no detected infection, in cultivar Premier, or in local colonization of two of six 'Powderblue' plants. In infected 'Powderblue' plants, symptoms did not progress past the inoculated stem within a fourteen month period (Chang et al. 2009). However, *X. fastidiosa* has been detected in samples from rabbiteye blueberry plants at farms in Louisiana that have experienced noticeable plant stunting and/or plant death (D. Ferrin, personal communication; M.H. Ferguson, unpublished data).

Bacterial leaf scorch symptoms described on southern highbush plants include marginal leaf necrosis, thin twigs on new growth, leaf drop, stem yellowing, and plant death (Brannen et al. 2016). In FL 86-19 southern highbush plants, symptoms progressed from marginal necrosis

to yellow stems and leaf loss, on both inoculated and, later, non-inoculated branches (Chang et al. 2009). Leaflessness combined with yellowed stems is characteristic of blueberry plants with bacterial leaf scorch, and these symptoms help differentiate between this disease and other biotic and abiotic causes of plant decline (Brannen et al. 2016). Southern highbush blueberry plants typically do not die in the same year that they begin to show symptoms but do eventually die (Brannen et al. 2016).

Rabbiteye blueberry plant symptoms noted in Louisiana and suspected of being associated with *X. fastidiosa* include plant death, shoot dieback, lack of plant re-growth after severe pruning, marginal necrosis, leaf reddening, and extensive defoliation combined with yellow stems (T. Avant and D. Ferrin, personal communication; M. H. Ferguson, unpublished data), similar to what has been described in Georgia (Brannen et al. 2016).

However, there are other factors that may cause decline in rabbiteye blueberry bushes. *Phytophthora cinnamomi* Rands causes root rot in blueberry, which can result in yellowing of leaves and leaf loss in rabbiteye cultivars (Milholland 1995). Ring nematode (*Mesocriconema* sp.) has been investigated as a possible factor in what has been called blueberry replant disease (Jagdale et al. 2013). Several viruses cause ring spot or ring blotch symptoms on blueberry plants, although it is not clear that any of these are problematic in rabbiteye blueberry (Martin et al. 2012). It is important to consider such alternative causes of symptoms when assessing the possible impact of *X. fastidiosa* in rabbiteye blueberry.

In some crops, like susceptible cultivars of grape (*Vitis vinifera*), death of plants infected with *X. fastidiosa* takes place quickly enough that the proportion of yield reduction that individual infected plants experience, while they remain alive, is likely of minor interest (Smith et al. 2014). Southern highbush blueberry plants with bacterial leaf scorch sometimes die

quickly, as well, once symptoms are observed (Brannen et al. 2016). However, in other crops, like peach and citrus, infected plants can continue to survive but have reduced yield and altered fruit quality (Gonçalves et al. 2011; Hartung et al. 1994; Mizell, n.d).

There are crops for which yield loss in the presence and absence of *X. fastidiosa* has been compared. In almond (*Prunus dulcis*) in California, symptomatic/infected trees of one cultivar had estimated yields 37 or 40% lower, depending on the study period in question, than those of trees lacking symptoms of almond leaf scorch disease (ALSD) (Sisterson et al. 2008, 2012). For a second cultivar, average estimated yields of symptomatic/infected trees were 17 or 19% lower than those of asymptomatic trees (Sisterson et al. 2008, 2012). In 13 of a total of 18 year-orchard-cultivar combinations, the estimated yields of symptomatic trees were significantly lower than those of asymptomatic trees (Sisterson et al. 2008, 2012).

In pecan (*Carya illinoinensis*) in Louisiana, kernel weight was significantly lower (13 to 18%) in nuts from *X. fastidiosa*-infected than noninfected trees each year for three years (Sanderlin and Heyderich-Alger 2003). Total nut weight was significantly lower in two years and marginally lower in a third (Sanderlin and Heyderich-Alger 2003). In Brazil, sweet orange (*Citrus* sp.) plants inoculated with *X. fastidiosa* prior to planting yielded ≈20% less than ones that were not inoculated before planting, over an eight year period (Gonçalves et al. 2011).

How quickly *X. fastidiosa* spreads among plants in an orchard in which infected plants are found is another factor pertinent to management decisions. The rate of spread of *X. fastidiosa* within orchards would impact a calculation of the economics of removing infected plants (Sisterson et al. 2008). In one pecan study, none of 10 originally noninfected plants of a susceptible cultivar, Cape Fear, become infected during a three year study period (Sanderlin and Heyderich-Alger 2003). In an orchard with younger pecan trees, the infection incidence in the

same susceptible cultivar increased from 5 to 64% over a period of six years, and rates of infection in less susceptible cultivars increased from 0 to 2.6 and 10.5% during the same period (Sanderlin et al. 2009). However, an analysis of spread in the latter orchard did not provide conclusive evidence that locations of infected trees impacted the locations of future infections (Li et al. 2011; Sanderlin et al. 2009). Sisterson et al. (2012) found similar results in almond, contributing to a conclusion that it may not necessarily be beneficial to remove infected plants from orchards that will be replanted relatively soon. However, in an area of California where the glassy-winged sharpshooter was present, Park et al. (2006) found that grapevines infected with Pierce's disease were frequently close to dead vines and/or locations from which older vines had been removed.

Even in crops in which *X. fastidiosa* does not cause rapid plant death, death may occur at a higher rate in infected than in non-infected plants. In almond, 9% of trees tested and confirmed to be *X. fastidiosa*-positive in the first years of six- to seven-year surveys of two orchards were found to be dead in the final years of the surveys, and it is expected that a higher proportion would have been so if trees had not been removed (Sisterson et al. 2012). Only 1% of initially asymptomatic trees were dead (Sisterson et al. 2012).

When and how *X. fastidiosa* can be reliably detected are questions pertinent to researchers and those involved in plant diagnostics. Rates of detection can depend on the time of year, the plant part sampled, and the detection method. Nissen (2010) isolated *X. fastidiosa* from petiole or petiole/midvein tissue and stem sap of southern highbush blueberry plants in both July and October. It appears that ≈12% of isolation attempts from symptomatic plants resulted in culture of the bacterium in both July and October, with most positive isolations coming from leaf tissue in July and similar numbers coming from leaf tissue and stem sap in October.

In work by Holland et al. (2014) with southern highbush blueberry plants of three cultivars or lines and four symptom classes, the incidence of detection by real-time polymerase chain reaction (PCR) was greater in several cases (in all symptom classes for 'Bluecrisp' and in the asymptomatic class for FL 86-19) in stem growth that was one year old and/or older than in stem growth produced during the current season. Detection incidence was higher in stem growth more than one year old than in roots, for asymptomatic 'Bluecrisp' and FL 86-19 plants, and higher in year-old stem growth than in roots, for moderately symptomatic 'Star' plants. However, incidence was higher in roots than in current season's growth for moderately and severely symptomatic 'Bluecrisp' plants. While differences among plant parts with respect to detection incidence were not found for moderately symptomatic FL 86-19, when *X. fastidiosa* was detected, the bacterial titer was higher in roots than in current season's stem growth or stem growth more than one year old.

In two years of monthly attempts to isolate xylem-borne bacteria from twigs of plum (*Prunus salicina*) with symptoms of plum leaf scald, a disease later attributed to *X. fastidiosa* (Wells et al. 1987), Chang and Yonce (1987) experienced a trend of low rates of success in the spring and relatively high rates of success in fall and/or early winter, in Georgia. From roots, bacteria were successfully cultured from 20 to 50% per tree in each month during which samples were taken (November through May of one season).

Wells et al. (1980) found that bacterial concentrations were much higher in roots than twigs of peach (*Prunus persica*), in orchards with peach phony symptoms. It appears that the "twigs" they used were the terminal portions of shoots, made up of most recent growth. Bacterial concentrations were significantly greater in roots in May than in February, August, or November, and significantly greater in twigs in May than in February or November.

In another peach study, bacteria were found in xylem sap from all or almost all (≥ 96%) sampled 4 cm root segments of trees that showed symptoms of phony peach disease, while the proportion of root segments from asymptomatic trees in which at least one bacterium was observed ranged from 4 to 70% (Aldrich et al. 1992). A significant relationship was not found between the *X. fastidiosa* observation rate and either root diameter class or the distance of the root segment from the trunk.

In *Vitis labrusca* grapevines on which Pierce's disease symptoms had been observed, the percentages of xylem vessels in which bacteria were observed by light microscopy were numerically greater in leaf veins in June and July than in the other months in which samples were taken (March, April, and October; Hopkins 1981). Rates of observation of bacteria were higher in petioles from April through October, and higher in stems during March and June (Hopkins 1981). Bacteria were found in a higher proportion of xylem vessels in petioles and/or leaf veins than in stems, except in March, when bacteria were not found in leaf veins or petioles (Hopkins 1981). Hopkins and Thompson (1984) found that the percentage of xylem vessels in which bacteria were observed increased greatly between May and July (in leaf veins and stems) in a *V. labrusca* cultivar and between July and September (in leaf veins and petioles, or the former plus stems) in two muscadine grape (*V. rotundifolia*) cultivars.

In elm trees that developed leaf scorch, Sherald and Lei (1991) detected *X. fastidiosa* in extracts of petioles from 67% of trees in June and from 94% of trees in September, using enzyme-linked immunosorbent assay (ELISA). When ELISA was performed on extracts of stem tissue (previous year's growth) and petioles (current year's growth) collected from sycamore and elm trees in early May, stem tissue extracts consistently resulted in higher absorbance values (Sherald and Lei 1991).

In sycamore in Georgia, the combined average detection rate, by ELISA, from roots and shoots dropped dramatically (from almost 60% to ≈20%) between early February and early March and did not start to consistently rise again until July (Henneberger et al. 2004). Isolation rates from stems were highest between December and February and in August and September. They were lowest in April and stayed relatively low until July.

Costa et al. (2004) tested several types of plants during 11 or 12 months of the year using ELISA. They detected *X. fastidiosa* in at least some oleander (*Nerium oleander*) and Spanish broom (*Spartium junceum*) plants during each month of the year but failed to detect it in any grape (*V. vinifera*) or almond plants between February and April (grape) or February and May (almond). They also compared detection by ELISA and immunocapture polymerase chain reaction (IC/PCR) in grape plants in which symptoms had been observed during the previous season. While rates of detection by the two methods were similar in June, in May, *X. fastidiosa* was detected in 15 of 25 samples by ELISA but 0 of 25 samples by IC/PCR.

Bextine and Miller (2004) compared detection of *X. fastidiosa* by ELISA and PCR in both plant tissue (stem tissue and associated petiole and leaf vein tissue) and xylem sap of *V. vinifera* grape and oleander plants. In asymptomatic grape plants, they found higher rates of detection in xylem sap than in tissue, when utilizing either ELISA or traditional PCR. In symptomatic oleander plants, *X. fastidiosa* was detected at a higher rate by PCR using tissue than by PCR using xylem sap. This may have been due, at least in part, to the fact that there were difficulties in obtaining xylem sap from oleander plants. In other cases, there was no significant difference between detection rates in xylem sap and plant tissue. When overall rates of detection by ELISA and PCR were compared, significant differences were not found for either grape or oleander.

Several observations can be made about detection of *X. fastidiosa* in deciduous, woody plants based on these studies. Detectability in aboveground plant parts generally appears more reliable in late summer, fall, and/or winter and relatively poor during some period in the late winter and/or spring. The findings of Wells et al. (1980) in peach are an exception, as bacterial populations were low in November and greatest in May, the one month during the spring when they sampled. It is possible that a period when populations would have been lower had passed by that time. The most reliable plant part for detection may vary by crop and time of year, but it does appear that detection is less frequent in recent growth.

Objectives of this study included determining (i) whether there was an association between *X. fastidiosa* infection and yield and/or fruit quality loss in rabbiteye blueberry, (ii) to what extent *X. fastidiosa* spread within a rabbiteye orchard, and (iii) when and how the bacterium could be reliably detected. Associations of soil chemistry variables, *P. cinnamomi*, ring nematode, and ringspot symptom statuses with *X. fastidiosa* infection status were also sought.

3.2 Materials and Methods

3.2.1 Selection of Plants for Yield Comparison and Detection of *X. fastidiosa* in Yield Study Plants

Because *X. fastidiosa* is a slow growing organism, and it was not known whether an infection could be established or how long it would take for an infection to become established and to cause yield loss, this study was done utilizing naturally infected plants. Fifteen blocks of three plants were selected from within rows of 'Tifblue' rabbiteye blueberry plants at a commercial farm in East Feliciana Parish, LA. Within each block, the three plants were separated by a plant and/or a place where a plant was formerly located. Plants in each block were roughly uniform in size at the beginning of the study. Five plants were removed from the

study due to the determination, based on foliar appearance and/or time of bloom, that they were a cultivar other than 'Tifblue', leaving a total of 40 plants in the study. The orchard was originally planted in the late 1970s or early 1980s, making the orchard ≈30 to 35 years old at the time of harvest. The orchard was approximately 1 ha in size, was unirrigated, and had been managed using organic practices and harvested by hand.

In an initial effort to determine which plants were infected, one cane was cut from the base of each plant in Feb. 2013. After returning from the field, fresh cuts were made at the base of each stem and roughly 15 to 20 cm from the location of the first cut, using pruning loppers wiped between cuts with 70% ethanol (EtOH), based on a protocol described by Holland et al. (2014). A vise was used to squeeze sap from the resulting stem piece, and sap was pipetted into autoclaved microcentrifuge tubes. Sap was held for 2 days at ≈4°C and then at -70°C until DNA was extracted from 50 µl of sap, or less, if 50 µl were not available, using a PowerPlant Pro DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA).

Real-time PCR was performed using Applied Biosystems Power SYBR Green PCR Master Mix (Life Technologies Corporation, Carlsbad, CA) and EFTu_3 forward and reverse primers (Holland et al. 2014). An ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) thermocycler and the following program were used: 10 min at 95°C; 40 cycles of 10 s at 95°C and 1 min at 60°C; and a dissociation step of 15 s at 95°C, 20 s at 60°C, and 15 s at 95°C (Holland et al. 2014). One well (technical replicate) per plant was tested. Positive samples had C_t values of 35 or less and melting temperatures between 77.2 and 78.3°C. Until a positive sample from blueberry was detected, an extract of *X. fastidiosa* from grape (A-08; Melanson et al. 2012) was used as a positive control.

Leaf samples were then taken from plants in late July or early Aug. of 2013. Several leafy shoots of roughly 15 cm in length were cut from various locations on each plant. Samples were held at ≈5.6°C until shortly before petioles and lower midribs were cut from leaves using a scalpel blade, which was flamed between samples. A commercial ELISA kit using the double antibody sandwich method with polyclonal antibodies (Agdia, Inc., Elkhart, IN) was used to detect *X. fastidiosa* in 0.3 to 0.5 g of petiole and midrib tissue of the leaves. Samples were tested in duplicate. Absorbance was measured at 630 nm on a Microplate Autoreader EL311 (BioTek Instruments, Inc., Winooski, VT). An absorbance of 0.100 or higher, relative to a buffer control, was used as the criterion for a positive sample, similar to the threshold (0.100 at 620 nm) used by Chang et al. (2009).

Additional foliar samples (5 to 6 shoots/plant) were taken from all but two plants, on which leaves were no longer present, in Sept. of 2013. After excision with a flamed scalpel blade, petioles and lower midribs were held at -20°C until they were ground in liquid nitrogen using mortars and pestles. DNA was extracted from 0.04 to 0.05 g of tissue using the PowerPlant Pro DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). Real-time PCR was performed as described above for detection of *X. fastidiosa* in sap. Samples were tested in duplicate (two technical replicates).

Steps similar to those described above regarding selection and testing of blueberry plants were taken at Bob R. Jones-Idlewild Research Station, in East Feliciana Parish, LA. Twenty-three blocks of two plants were selected from within rows of 'Tifblue' rabbiteye blueberry plants that had been planted in 1996. Real-time PCR was performed on DNA extracts of sap from individual canes collected in Apr. 2013, and ELISA was performed using petiole/midrib tissue of

leaves collected in Aug. 2013. However, *X. fastidiosa* was not detected in any plants, and data collection at this location was discontinued after the first season.

At the commercial farm in East Feliciana Parish, annual sampling of the 40 yield study plants was repeated in Aug. 2014 and Sept. 2015, to determine if the infection incidence had increased, in comparison with that found in 2013. Each time, five to seven leafy shoots were collected. After returning from the field, samples were held at ≈5.6°C until shortly before petioles and lower midribs were excised. Some petioles and midribs from each plant were used for testing with a commercial ELISA kit (Agdia, Inc., Elkhart, IN), as previously described, while others were held at -20°C for DNA extraction. Tissue for DNA extraction was ground in liquid nitrogen, using mortars and pestles. DNA was extracted from 0.05 to 0.06 g of tissue using a cetyltrimethylammonium bromide (CTAB) protocol based on that of Li et al. (2008). DNA was dissolved in sterile nuclease free water and held at -70°C. Real-time PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA), along with the EFTu_3 primers and the machine previously described. Positive samples still had C_t values of 35 or less, but because a different master mix was used, the range for melting temperatures of positive samples was between 81.2 and 82.4°C. Samples were re-tested if there was a conflict between wells with respect to results for the sample or if a sample tested positive but the amplification curve appeared atypical.

3.2.2 Yield and Average Berry Weight

Fruit was harvested by hand once per week between 13 June and 5 July in 2013 and between 12 June and 10 July in 2014. During most weeks, it took more than one day to harvest fruit from all plants in the field. Each week's yield was weighed, and weekly yields were summed for each sample, to get total yield for each season. At the time of each harvest, a

subsample of berries was set aside for collection of average berry weight data and stored at ≈5.6°C. Twenty-five berries, or fewer if twenty-five berries were not available, were weighed, and mean berry weight was recorded for each plant following each harvest date. The overall mean of averages from each harvest date was used as the average berry weight for the season. If there were dates when a plant had fewer than 25 berries, the reduced number of berries was accounted for so that the weights from those dates did not disproportionally affect the seasonal average.

3.2.3 Soluble Solids and Titratable Acidity

During one week of harvest in June 2014, a subsample of berries from each plant was set aside for measurements of soluble solids concentration (SSC) and titratable acidity (TA). Berries were held at -70°C until the day of sample preparation. To prepare samples for measurements, berries were thawed, homogenized in a blender, and centrifuged in three to four ≈40 ml tubes per sample at ≈484 g for 15 min. The supernatant was filtered through a double layer of cheesecloth and centrifuged a second time at ≈830 g for 15 min, in 15 ml tubes.

Ten ml (or less, if 10 ml were not available) of the resulting juice was used for TA analysis, and the rest was held at ≈4°C, or at -20°C for longer periods, for SSC measurement. To measure TA, 0.1 N NaOH was added while stirring the blueberry juice, until the pH measured ≥ pH 8.1. The volume of 0.1 N NaOH used to raise the pH to ≥ 8.1 was measured. This volume was multiplied by 0.1 (to account for the normality of the NaOH solution) and 0.059 (to account for the formula weight and number of dissociable hydrogen ions of a succinic acid molecule [Ehlenfeldt et al. 1994], as well as a conversion from milliliters to liters) and divided by the volume of juice used (in mL), to get a TA value. Calculations were based on the properties of succinic acid, because Ehlenfeldt et al. (1994) found this acid to exist at the highest

concentration among measured acids (including citric, malic, and quinic, along with succinic) in fruit from rabbiteye cultivars in general and from 'Tifblue', specifically. SSC was measured using a Bellingham and Stanley RFM 80 Refractometer (Kent, UK).

3.2.4 Soil Sampling

Six soil cores were taken under the canopy of each bush, to a depth of ≈15 to 20 cm, in Nov. 2013. Samples for each plant were mixed and placed in a plastic bag, which was then sealed. Bags were stored at 14°C. Soil was analyzed for plant nutrient (Ca, Cu, K, Mg, P, S, and Zn) and Na content, along with pH, by the Louisiana State University Agricultural Center Soil Testing and Plant Analysis Lab.

3.2.5 Nematode Populations

A 100 ml portion of the soil collected in Nov. 2013 was assayed for nematodes. Nematodes were extracted using the semi-automatic elutriation and sugar centrifugation method (Byrd et al. 1976; Jenkins 1964). Briefly, using 100 ml of soil, nematodes were trapped in a 400 mesh sieve (38 μm openings). Sieve contents were stored in water at ≈4°C for at least 4 hours and then added with tap water to a ≈40 ml centrifuge tube. Contents were centrifuged for 5 min at ≈350 g. The supernatant was poured off, and a solution containing 454 g/L of sucrose was added to the tubes and stirred to mix with the contents. This mixture was centrifuged for 1 min ≈350 g and then poured through a 400 mesh sieve. Contents were washed into tap water and stored at ≈4°C until nematodes were counted by D. Xavier at the Louisiana State University Agricultural Center Nematode Advisory Service. Counts were converted to populations of nematodes per 500 cm^3.

3.2.6 Phytophthora Assay

From the soil collected in Nov. 2013, ≈120 ml was placed into plastic containers, and 200 ml of deionized water was added. Eight disks of Sasanqua camellia (*Camellia sasanqua*) leaf, which had been cut from leaves using a standard hole punch, were transferred to each container using forceps. The leaves, hole punch, and forceps had been sanitized with 70% EtOH prior to use. Three days after baiting, five to eight disks, from among those that were still floating on the surface of the water, were inserted under PARPH-V8 medium (one plate/sample) using sterilized forceps (Ferguson and Jeffers 1999). The medium differed from the original recipe in that 99% pure pentachloronitrobenzene (PCNB) was used as its source rather than Terraclor, and it was dissolved in 95% EtOH, adding 10 ml 95% EtOH per liter of medium. After five to seven days, designation of cultures as positive, negative, or uncertain for *Phytophthora* sp. was made based on hyphal morphology by R. Singh in the Louisiana State University AgCenter Plant Diagnostic Center. After eight days from the time leaf disks were plated, pieces of agar containing suspected *Phytophthora* sp. mycelia were transferred aseptically to PARPH. Antibiotic concentrations of the medium were like those in Ferguson and Jeffers (1999), but cornmeal agar was used and V8 was omitted, as in Jeffers and Martin (1986). As in the PARPH-V8 medium, the source of PCNB was 99% PCNB dissolved in 95% EtOH, rather than Terraclor. Samples for which the presence of *Phytophthora* sp. was still questionable were tested using a Phytophthora ImmunoStrip (Elkhart, IN).

In January 2015, additional soil samples were taken. Four soil cores were collected from within 1 m of the base of each yield study plant, to depths of ≈15 to 20 cm. Samples were placed in plastic bags, which were sealed and stored at ≈5.6°C. Samples were set out at room temperature for two or six days prior to the first and second rounds of baiting, respectively. For

each sample, ≈100 ml of soil was placed in a sandwich container, and 200 ml of distilled, deionized water was poured over it. Eight disks cut from *Camellia japonica* leaves were floated on top of the water in each container. Leaves had been dipped in 70% EtOH and allowed to dry, and disks had been cut using a standard hole punch that had been dipped in 70% EtOH and flamed.

Three days after each round of baiting began, baits were removed from the containers, using sterilized forceps that were dipped in 70% EtOH and flamed between samples. For each sample, five disks, from among those that had not sunk, were placed on a clean paper towel and blotted dry (Ferguson and Jeffers 1999). Forceps were used to insert the five disks into a selective medium. PARPH was used after the first round of baiting, while pimaricin was left out the second time, so that the medium was effectively ARPH. Antibiotic concentrations of the media were like those in Ferguson and Jeffers (1999), but cornmeal agar was used and V8 was omitted, as in Jeffers and Martin (1986).

Thirteen to fourteen days after inserting leaf disks into PARPH after the first round of baiting, medium from new plates of PARPH was overlaid on the original plates. There was no overlay step for plates from the second round of baiting. One to three pieces of agar (≈0.2 cm^2) containing possible *P. cinnamomi* hyphae were transferred to PARPH or ARPH media from the overlaid plates (from first round) ≈6.5 weeks after overlay or from original plates (from second round) ≈2 weeks after insertion of disks. Eleven days after the previous transfers, one to three pieces of agar (≈0.2 cm^2) containing possible *P. cinnamomi* hyphae were transferred to cV8A-10% (Jeffers 2007).

Between 13 and 15 days after transfer to cV8-10%, hyphae from ≈2 cm^2 were scraped from the plates and placed in 1.5 ml microcentrifuge tubes with 500 µl of 10 mM Tris-HCl (pH

7.5), based on the method of Kong et al. (2003). Tubes were placed in boiling water for 20 min and then centrifuged for three min. Supernatant (200 µl) was transferred to new microcentrifuge tubes, and these were stored at -20°C.

PCR using Lpv2 and Lpv3 primers was performed following the protocols outlined in Kong et al. (2003). Because DNA concentrations in the extracts were low, the volume of template in the PCR reaction was successively increased from 2 µl to 4 µl to 8 µl to 16.9 µl, if no product was found for a given sample at a lower concentration. Because a positive result with both primers is necessary to confirm a sample as *P. cinnamomi*, samples were run with Lpv3 primers at increased template concentrations only if positive results were obtained with the Lpv2 primers.

3.2.7 Ringspots

Leaves of yield study plants were observed in Aug. 2014 and Sept. 2015 for the presence of ringspots, to determine if a ringspot-causing virus was likely to be present.

3.2.8 Data Analysis

Data were analyzed in SAS 9.4 (SAS Institute Inc., Cary, NC). Analyses of variance (ANOVAs) were performed using PROC MIXED and the Kenward-Roger degrees of freedom method. When only one year of data was analyzed at one time, a likelihood ratio chi-square test was done in Microsoft Excel (Microsoft Corp., Redmond, WA) using -2 Res Log Likelihood values and the degrees of freedom of the covariance structures, to determine whether a better fit resulted from using combined or separate variances by plant infection status. If there was not a significant difference in the fits, the simpler model was used. Likewise, when two years of data were analyzed together, likelihood ratio chi-square tests were done to determine if a better fit resulted from using combined or separate variances by plant infection status or by year and if an

autoregressive covariance structure resulted in a better fit that the default covariance structure. In all cases, models with non-normal residuals distributions, according to a Shapiro-Wilk test ($\alpha = 0.05$), were not utilized. Ranked rather than raw data were used for SSC analysis, as residuals were non-normal when raw data were used. Once a satisfactory model for yield was determined, ANOVAs were also performed with *P. cinnamomi* status, 2014 ringspot status, ring nematode population, and soil chemistry variables (Ca, Cu, K, Mg, P, S, Zn, Na, or pH), individually, as covariables.

To determine whether the presence of *Phytophthora* spp. (as identified morphologically or by ImmunoStrips following 2013 sampling), *P. cinnamomi* (as identified by PCR following 2015 sampling), ring nematodes, or a ring spot-causing virus possibly predisposed plants to infection and/or colonization with *X. fastidiosa*, logistic analyses were performed using PROC GLIMMIX, in order to account for the arrangement of plants in blocks. A binary distribution was specified, and the Kenward-Roger degrees of freedom method was used. It was also considered desirable to determine if any soil chemistry variables (nutrient or Na concentrations, or pH) might be predisposing plants to *X. fastidiosa* infection and/or colonization, but because there were nine soil chemistry variables, these were subjected to a principal components analysis using PROC FACTOR in SAS. Once the top three factors were determined, the factor scores were also utilized as independent variables in a logistic analysis of infection incidence.

3.2.9 Seasonal Detectability of *X. fastidiosa* in Leaves, Shoot Sap, and Root Sap

Approximately every two months between Feb. 2014 and June 2016, plants were sampled to determine when and where in the plants *X. fastidiosa* could be detected. Sampling dates in 2014 were as follows: 27 Feb., 24 Apr., 27 June, 1 Sept., and 7 Nov. In 2015, dates were 2 Jan., 27 Feb., 29 Apr., 29 June, 8 to 9 Sept., and 13 Nov. In 2016, plants were sampled on 5 Jan., 29

Feb., 29 Apr., and 29 June. On the first three sampling dates (between Feb. and June 2014), two rabbiteye blueberry plants that were not part of the yield study but were in the same orchard and had previously tested positive for *X. fastidiosa*, after being noted as expressing possible symptoms of *X. fastidiosa* infection, were sampled. Beginning in Sept. 2014, after the final harvest of yield study plants, the nine yield study plants that had tested positive were included, so that a total of 11 plants were sampled on the remaining dates (between Aug. 2014 and June 2016).

Plant parts tested were as follows: (i) leaf petioles/midribs or, in January and February, terminal shoot growth; (ii) stems; and (iii) roots. Leaf petioles/midribs and terminal shoots were tested using ELISA. Sap was squeezed from stems and roots, and DNA extracted from the sap was tested using real-time PCR. Collection of leaves or shoot terminals, along with testing by ELISA, was done in a manner similar to that described above for sampling to determine if the number of infected plants had increased. Roughly 10 to 15 cm of terminal growth from five shoots per plant was typically collected, but the number of shoots was occasionally less if little foliage was available. In a very limited number of instances, multiple sections were taken from one shoot to get sufficient foliage. Very recent growth was avoided. Shoots that were sources of petioles/midribs or shoot pieces for ELISA were kept at ≈5.6°C until shortly before use.

Stems sampled for sap collection were ≈0.5 to 1.6 cm in diameter at the base, while roots were taken from within 1 m of the base of the plant and were ≈0.3 to 1.7 cm in diameter and ≈8 to 15 cm in length. During the three months (between Feb. and June 2014) when only two plants were sampled, three roots were taken from each plant, for a total of six roots. Stems and root pieces were kept at room temperature and ≈5.6°C, respectively, until sap was collected. Protocols for collection of sap, extraction of DNA, and real-time PCR were similar to those

described for determination of infection status in 2013. A basal section of each stem was used first, and if sufficient sap was not available from that section, subsequent sections, which sometimes included sections of branches from the main stem, were used. SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) was used for these samples, so the melting temperatures of positive samples were between 81.2 and 82.4°C. Samples were re-tested if there was a conflict between wells with respect to results for the sample or if a sample tested positive but the amplification curve appeared atypical. Samples were occasionally tested in triplicate if continuing conflicts occurred when testing in duplicate, and if two of three wells were positive, the sample was considered positive.

Sample numbers varied among plant parts and months, as indicated in the results, because the aboveground portions of some plants died during the course of the study, so that leaf and stem samples were no longer available from those plants. Also, in some months, sap could not be obtained from a stem and/or root sample from one or more plants.

3.3 Results

Only one plant in the commercial orchard in East Feliciana Parish tested positive for *X. fastidiosa* based on sap from stems collected in Feb. 2013. However, nine plants, including the one that previously tested positive, tested positive by ELISA of petiole/midrib samples collected in late July and early Aug. of 2013. While none of these tested positive by real-time PCR when petiole/midrib tissue was collected in Sept. 2013 and DNA was extracted using the PowerPlant Pro DNA Isolation Kit, all but one of these nine plants later tested positive by real-time PCR in 2014 and/or 2015, when the CTAB extraction method was used to extract DNA from petiole/midrib tissue.

The same leaf samples were tested by both ELISA and real-time PCR in Aug. 2014 and Sept. 2015, when testing to determine if the bacterium had spread to other yield study plants. In 2014, eight plants still had a sufficient number of leaves for testing by both methods, and samples from five plants tested positive by ELISA, while samples from six tested positive by real-time PCR. In 2015, outcomes of ELISA and real-time PCR tests were the same, with leaves from four of a total of six undefoliated plants testing positive by both methods. Plants that tested negative for *X. fastidiosa* by ELISA in 2013 continued to test negative by both ELISA and real-time PCR in 2014 and 2015.

By Apr. 2016, no foliage was present on four of the nine plants from the yield study in which *X. fastidiosa* had been detected. The yield study plants that consistently tested negative for *X. fastidiosa* had new growth at this time. Besides death of the above-ground parts of plants, symptoms noted in association with infected plants included foliar reddening and chlorosis and, less commonly, marginal necrosis. However, obvious symptoms were not always observed.

The nine plants that tested positive for *X. fastidiosa* yielded significantly less than the 31 that did not, with mean yields of *X. fastidiosa*-positive plants being 55% and 62% less than *X. fastidiosa*-negative plants in 2013 and 2014, respectively (Table 3.1). There was a significant ($P = 0.01$) infection status by year interaction with respect to yield, but observation of mean values for each year revealed that the trends were the same in both years (i.e., positive plants yielded less than half of what negative plants did), so yield data from both years were analyzed together. The difference between average berry weights was also significant (Table 3.1).

In berries harvested during one week in 2014, the SSC was higher in fruit from plants in which *X. fastidiosa* had been detected than in fruit from plants in which it had not been detected (Table 3.2). No significant difference in TA was found (Table 3.2).

Table 3.1. Yield of ripe blueberries per plant and average berry weight of 'Tifblue' rabbiteye blueberry plants in which *X. fastidiosa* was and was not detected.

	Yield/plant (kg)		Average berry weight (g)	
	2013	2014	2013	2014
X.fastidiosa-positive[z]	4.0 ± 1.4[x]	6.3 ± 2.3	1.2 ± 0.08	1.3 ± 0.07
X.fastidiosa-negative[y]	8.9 ± 0.6	16.7 ± 0.9	1.4 ± 0.01	1.4 ± 0.01
P-value[w]	<.0001		0.0507	

[z]N = 9 in both years for yield/plant and in 2013 for average berry weight. N = 6 in 2014 for average berry weight.

[y]N = 31 in both years, for both variables.

[x]Mean ± standard error

[w]*P*-values are for the comparison between *X. fastidiosa*-positive and negative plants. Years were analyzed together.

Table 3.2. Soluble solids concentrations and titratable acidity of berries harvested during one week of 2014 from 'Tifblue' rabbiteye blueberry plants in which *X. fastidiosa* was and was not detected.

	Soluble solids (%)	Titratable acidity (%)
X.fastidiosa-positive[z]	11.5 ± 0.5[x]	0.57 ± 0.02
X.fastidiosa-negative[y]	10.1 ± 0.1	0.57 ± 0.005
P-value	0.0217[w]	0.8324

[z]N = 6

[y]N = 31

[x]Mean ± standard error

[w]Ranks of soluble solid values were analyzed due to a lack of normality.

Phytophthora spp. was identified from soil around 78% of *X. fastidiosa*-positive plants and 77% of *X. fastidiosa*-negative plants. *P. cinnamomi* was detected in soil from around 56% of *X. fastidiosa*-positive plants and 23% of *X. fastidiosa*-negative plants. The results of logistic regression analyses to check for possible predisposing effects of *Phytophthora* spp. or *P. cinnamomi* on *X. fastidiosa* infection were not significant (P=0.9700 and P=0.1384, respectively). Ringspots were observed on 33% of *X. fastidiosa*-positive plants and 65% of *X. fastidiosa*-negative plants in Aug. 2014. The *P*-value (0.1943) resulting from logistic regression did not suggest an association between ringspot symptoms and *X. fastidiosa* infection status. Ringspots were not observed in Sept. 2015.

Mean population estimates of ring nematodes per 500 cm^3 soil were 572 and 419 for *X. fastidiosa*-positive and negative plants, respectively. Results of logistic regression analysis (P=0.3158) did not suggest that ring nematodes might predispose plants to infection by *X. fastidiosa*. The *P*-value for the "effect" of *X. fastidiosa* status on yield remained highly significant ($P < .0001$) when *P. cinnamomi* status, ringspot status, ring nematode population estimate, or any of the soil chemistry variables were included as covariables.

A principal components analysis revealed three factors that accounted for 74% of the variation in the soil chemistry data. Factor 1 accounted for 31% of the variation in the soil chemistry data, and the most highly correlated variables were Ca, Mg, and K. Factor 2 accounted for 29% of the variation, with P, Cu, and Zn being most highly correlated with it. Factor 3, with which S, Na, and pH were most highly correlated, accounted for 15% of the variation. The Factor 2 score was the only one that resulted in a significant *P*-value (P=0.0396) in a logistic analysis, and the variables most highly correlated with this factor were examined individually. Means and *P*-values for the logistic analyses utilizing these variables are shown in Table 3.3. Since pH was not one of the variables most highly correlated with Factor 2, results of logistic regression using pH as an independent variable are not shown. However, mean pH values for soil around *X. fastidiosa*-positive and negative plants were pH 4.76 and pH 4.83, which are near the middle of the range recommended for blueberries (Puls 1999).

Table 3.3. Concentrations of nutrients in soil around plants in which *X. fastidiosa* was (n = 9) and was not (n = 31) detected.

	X.fastidiosa-positive	*X.fastidiosa*-negative	*P*-value[z]
P (ppm)	30.8	24.1	0.1250
Cu (ppm)	1.62	1.35	0.0479
Zn (ppm)	1.63	1.41	0.0850

[z]*P*-values are from logistic analyses performed to determine if the concentration of this nutrient might predispose plants to infection/colonization by *X. fastidiosa*.

During Feb. and June 2014, two of the months when only two plants were sampled as part of the seasonal detection study, *X. fastidiosa* was detected in both plants in terminal shoot (February) or leaf petiole/midrib (June) tissue by ELISA. The bacterium was also detected in sap from stems of both plants, by real-time PCR, in February and June. In Apr. 2014, however, the bacterium was not detected in leaf tissue from either plant and was detected in only one of the two stems. *X. fastidiosa* was detected in all three root pieces of both plants in all three months, except for one piece sampled in June.

Between Sept. 2014 and June 2016, when a total of 11 plants were sampled on each date, detection in root sap ranged from 27% to 73% (Fig. 3.1). Detection rates in stem sap ranged from 0% to 38% (Fig. 3.1). Detection in leaf or shoot tissue by ELISA ranged from 0% to 80%, with no positive samples in either year in April (Fig. 3.1). Detection rates represent the number of positive samples as a percentage of the total samples of that kind (petiole/midrib or terminal shoot tissue, stem sap, or root sap) tested from that date.

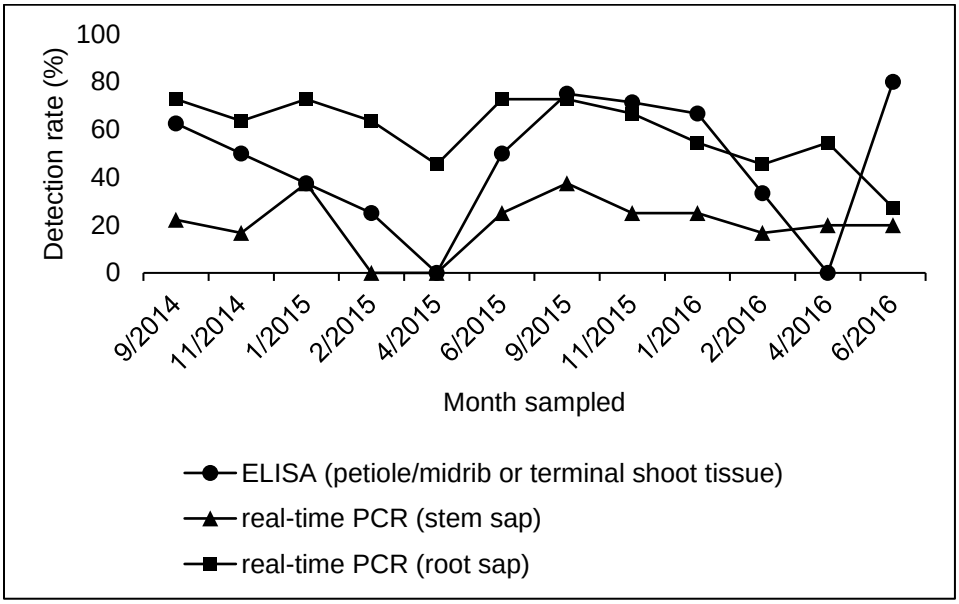

Fig. 3.1. Incidence of *X. fastidiosa* detection in multiple petioles/midribs or most recent shoot growth (in January and February), by ELISA; root sap by real-time PCR; and stem sap by real-time PCR. N ranged between 5 to 8 (ELISA), 4 to 9 (stem sap), and 9 to 11 (root sap).

3.4 Discussion

Results suggest that *X. fastidiosa* infection may cause a significant decline in productivity of 'Tifblue' rabbiteye blueberry plants but that spread is very limited. Results of ELISA tests in Aug. or Sept. 2013 indicated that nine of 40 plants were infected. *X. fastidiosa* was later detected by real-time PCR in all but one of these. The initial ELISA result from the one in which infection was not later detected may have reflected an infection that did not spread systemically in the plant. Six of the nine *Xylella*-positive plants were located in blocks with at least one other positive plant, seeming to suggest that spread between plants may be important. This is consistent with the findings of Park et al. (2006) in grape, in an area in which the glassy-winged sharpshooter was present, but contrasts with results of Sisterson et al. (2012) in almond and Li et al. (2011) in pecan. To further investigate the spatial relationships of infected plants in the field, distribution data could be subjected to analyses like those found in Park et al. (2006).

The fact that none of the 31 initially *X. fastidiosa*-negative plants within the yield study tested positive in 2014 or 2015 suggests that spread between plants, or spread followed by colonization to a degree that allows detection, is not rapid. It has been suggested that, in such a situation, using noninfected planting material may limit the number of infections in an orchard (Sanderlin and Heyderich-Alger 2003). However, it is possible that the infection incidence would increase more quickly in a younger orchard, and it is likely that new infections would eventually be detected if bushes were monitored for a longer period. In a pecan orchard that was ≈25 years old when data collection was initiated, Sanderlin and Heyderich-Alger (2003) did not detect new infections in 10 initially *X. fastidiosa*-negative pecan trees during the course of a three year study. However, in another pecan orchard in which trees were only 14 years old when the study began, infection incidence in trees of the same susceptible cultivar increased from 5 to

64% over a six-year period (Sanderlin et al. 2009). It cannot be assumed that spread would be as limited in a young rabbiteye blueberry orchard as it appears to be in the orchard surveyed in this study.

This study did not include monitoring of possible vectors, but the findings of Ma et al. (2010) suggest that the glassy-winged sharpshooter prefers other fruit crops, including muscadines, bunch grapes, and Satsuma mandarins, to rabbiteye blueberry plants. While it is not known that any of these crops were grown in the area of the blueberry orchard, many wild plants were in the vicinity, and if the glassy-winged sharpshooter prefers some of them to rabbiteye blueberry, a relatively low level of feeding activity of vectors might contribute to a low rate of spread in rabbiteye blueberry.

Four of the nine *X. fastidiosa*-positive plants appeared dead when survival was assessed in Apr. 2016. Since foliar, stem, and root samples were removed from *X. fastidiosa*-positive plants approximately every two months for two years, after the final yield study harvest, they differed in this respect from *X. fastidiosa*-negative plants by this time. However, based on plant vigor, it appeared that only one of the 31 *X. fastidiosa*-negative plants would have had no living aboveground growth at this time, had it been sampled in the same way.

Results clearly indicate that the detectable presence of *X. fastidiosa* is associated with yield loss in 'Tifblue' rabbiteye blueberry. Yields of *X. fastidiosa*-positive plants were less than half (45% and 38% in 2013 and 2014, respectively) of those of plants that tested negative in both years. These numbers correspond to yield reductions of 55% in 2013 and 62% in 2014 and are greater than losses or estimated losses reported in almond, pecan, or citrus (Gonçalves et al. 2011; Sanderlin and Heyderich-Alger 2003; Sisterson et al. 2008, 2012). To fully assess the

importance of *X. fastidiosa* in rabbiteye blueberry, it would be helpful to have data from other naturally infected orchards.

Average berry weight was lower in infected plants, as well. Only six of the nine *X. fastidosa*-positive plants had fruit in the second season, and those that did were plants that appeared to be in earlier stages of decline. The difference in average berry weight might have been greater if more *X. fastidiosa*-positive plants had produced fruit. The greater SSC observed in a limited sample of fruit from infected plants is consistent with what might be expected in plants with reduced water uptake owing to plugging of the xylem. Also, studies involving other fruit crops have sometimes shown that greater soluble solids concentrations were associated with reduced crop loads (Berkey et al. 2011; Einhorn et al. 2011; Serra et al. 2016). Researchers should be aware of a possible effect of *X. fastidiosa* in case unexplained variations in soluble solids are observed, for example, in a cultivar or breeding line evaluation of a plant species in which *X. fastidiosa* can be a chronic issue (P. Perkins-Veazie, personal communication).

Of the variables evaluated as possible predisposing factors for *X. fastidiosa* infection, it appears that neither *Phytophthora* spp., *P. cinnamomi*, nor ringspot symptoms are associated with *X. fastidiosa* infection status. Likewise, logistic analyses did not support hypotheses that levels of ring nematodes, soil pH, or concentrations of most soil nutrients or Na inclined plants to infection with *X. fastidiosa.* The *P*-value for Cu, however, was significant, and that for Zn was significant at $\alpha = 0.10$. Because these soil nutrient levels were naturally occurring rather than applied treatments, it is possible that the associations between higher levels of these two nutrients with infection status are coincidental, or that infected plants took up lesser amounts of them, leaving higher concentrations in the soil. Also, while results are shown for logistic analyses using Cu, Zn, and P, the fact that these are all associated with the same component in a

principal components analysis shows that they are not independent of each other. It is possible that one predisposes plants to infection while the concentrations of the others are simply correlated with the concentration of that predisposing factor. Additional research is needed to show any causal relationship between soil Cu or Zn concentration and plant susceptibility to *X. fastidiosa* infection.

The mean soil Cu and Zn concentrations for both *X. fastidiosa*-positive and *X. fastidiosa*-negative plants were well below the levels considered toxic for crops in general (Hardy et al. 2014). However, most recommendations related to Cu and Zn fertility in blueberries are based on foliar tissue levels (e.g., Hart et al. 2006; Puls 1999), which were not analyzed in this study. Additional work investigating a possible relationship between these nutrients and *X. fastidiosa* infection should take foliar concentrations into account.

Dutta et al. (2015) formulated models that associated plant tissue concentrations of Cu^{2+} and Zn^{2+}, along with Fe^{2+} and Mn^{2+}, and severity of bacterial leaf spot (causal agent: *Xanthomonas euvesicatoria*) in pepper. In related work, Rooks et al. (2015) found that the soil Cu:Fe ratio was associated with tomato spotted wilt virus severity in tobacco. In both cases, it is suggested that a relationship between concentrations of these nutrients and disease severity may be mediated by elements of the systemic acquired resistance response, including the expression of certain superoxide-dismutase genes and the *NPR1* gene (Dutta et al. 2015; Rooks et al. 2015). These studies provide some evidence of a possible relationship between soil Cu and plant predisposition to disease, and it would be interesting to see if the observed relationship between soil Cu concentration and *X. fastidiosa* infection status is more than coincidental.

Detection of *X. fastidiosa* was most consistent in root sap, by real-time PCR, although even with this method, the bacterium was only detected in an average of 66% of samples. There

was one plant in which *X. fastidiosa* was never detected in the roots or stems, even though it was detected in leaves in several months by ELISA and once in leaves by real-time PCR. Interestingly, *X. fastidiosa* was detected by real-time PCR in DNA extracts from root sap as many as 19 months after above-ground plant growth ceased to be observed. If a plant's death is suspected of being associated with *X. fastidiosa* infection, real-time PCR of DNA extracted from root sap might make it possible to determine whether the bacterium was present for some time after death of the above-ground part of the plant.

Results are in agreement with the finding by Wells et al. (1980) that bacterial counts were consistently higher in xylem sap from peach roots than from twigs. However, results differ from those of Holland et al. (2014). In their study of southern highbush blueberry plants, when there were statistical differences between the respective plant parts, incidence of detection was always greater in sap from stem sections at least one-year-old than in that from roots. In the current study, detection in stem sap was relatively unreliable. Differences in where and to what extent *X. fastidiosa* accumulates may be a reason for the difference in symptoms noted in southern highbush and rabbiteye blueberry. It is also possible that differences in levels of PCR-inhibiting compounds between the roots and shoots affected the differences in rates of detection.

The finding that detection was more consistent in the roots than stems is inconsistent with the suggestion by Purcell and Hopkins (1996) that "die-back and general decline" symptoms are associated with high concentrations of bacteria in the trunk or branches, while accumulation of bacteria in the roots is associated with stunt diseases, like phony peach. Of course, since Koch's postulates have not been completed for the cause of dieback and decline in rabbiteye blueberry, it is possible that a factor other than or in addition to *X. fastidiosa* is involved in causing these symptoms.

While detection by ELISA in leaf or terminal shoot tissue was not as consistent as detection in root sap by real-time PCR, it was comparable or better in some months and was consistently as good as or better than detection in stems by real-time PCR, except in April. The low rate of *X. fastidiosa* detection in stems, relative to leaves, was likely due in part to the fact that only one stem per plant was sampled, while leaves from multiple shoots were sampled, in cases when multiple living shoots existed. Furthermore, it is likely that not all *X. fastidiosa* cells exit stem and root pieces in sap when they are squeezed with a vise. French et al. (1977) found that using a higher concentration of potassium hydroxide instead of a lower concentration, or water, for vacuum extraction of bacteria resulted in greater numbers being observed in the extracted suspensions, suggesting that chemical treatment was needed to liberate additional cells. Collection of leaves is easier and faster than collection of roots, and testing with ELISA is less expensive and requires less equipment than testing with real-time PCR, making testing leaves with ELISA an attractive option in many cases. However, it is clear that detection in leaves by ELISA should not be relied upon in April, when new foliar growth is present.

Although detection rates by ELISA and real-time PCR in leaf petiole/midrib tissue were not compared during bimonthly sampling, the same leaf samples were tested by both ELISA and real-time PCR in Aug. 2014 and Sept. 2015. In tests of tissue sampled at these times, results of the two methods were the same or similar. Bextine and Miller (2004) found similar rates of detection of *X. fastidiosa* by ELISA and traditional PCR in both grape and oleander. Costa et al. (2004), likewise, found that rates of *X. fastidiosa* detection by ELISA and IC/PCR in grape with Pierce's disease were similar in June. In May, *X. fastidiosa* was detected by ELISA in 60% of samples, while it was not detected by IC/PCR in any (Costa et al. 2004). While both traditional and real-time PCR methods have been demonstrated to detect *X. fastidiosa* at approximately one

hundredth of the concentration at which ELISA detects the bacterium (Gambetta et al. 2005; Hill and Purcell 1995; Minsavage et al. 1995), inhibitors may interfere with detection by PCR, in blueberry particularly (Halpern and Hillman 1996; Nissen 2010).

The antibodies employed in the Agdia, Inc. (Elkhart, IN) ELISA assay for *X. fastidiosa* have been found to cross-react with *Pseudomonas syringae* pv. *syringae* and *Xanthomonas arboricola* pv. *pruni* (S. Lutes, Agdia, personal communication), so results of ELISA tests for *X. fastidiosa* should be interpreted somewhat cautiously. Costa et al. (2004) has suggested using ELISA first and then using PCR to verify positive results.

The finding that detection of *X. fastidiosa* in petioles/midribs is low in the spring, rises during the summer, and is relatively high during the late summer, fall, and/or winter is consistent with findings regarding detection in aboveground plant parts of other deciduous woody plant species, including elm, sycamore, almond, plum, and several types of grape (Chang and Yonce 1987; Costa et al. 2004; Henneberger et al. 2004; Hopkins and Thompson 1984; Sherald and Lei 1991). It seems intuitive that it would take time for bacteria to colonize new foliar growth, and it has been observed that poor bacterial growth seems to be associated with rapid plant growth (Hopkins and Thompson 1984). Results of observations of bacteria in peach twigs differed, however, in that higher numbers were observed in May than in Aug. or Sept. (Wells et al. 1980).

ELISA and real-time PCR could have detected living or dead *X. fastidiosa*, so results do not show that populations of living bacteria are high at all times when the bacteria were detected. However, the isolation work of Chang and Yonce (1987) and Henneberger et al. (2004) suggests that living *X. fastidiosa* cells are present in plum and sycamore in Georgia around the times when we detected it and that populations are low when we failed to detect it in leaves. If application of insecticides is to be considered as part of a management strategy for diseases caused by *X.*

fastidiosa, the times when bacterial populations are highest is an important consideration, as this is likely when the bacteria are most easily acquired by insect vectors (Hopkins et al. 1991). Of course, the populations of insect vectors at these times are also important considerations. We did not address this factor, but in Mobile, AL, which is at a latitude comparable to East Feliciana Parish, Ma et al. (2010) found that captures of the glassy-winged sharpshooter, the most populous sharpshooter, increased sharply between late May and early June and then decreased gradually until reaching a relatively low, but still detectable, level in late July to early August. Therefore, it seems that June and July would be the prime period for spread of *X. fastidiosa* by the glassy-winged sharpshooter. This encompasses the harvest season, which could be inconvenient but also means that those spraying for spotted winged drosophila (SWD) during the ripening and harvest periods may reduce vector numbers without making additional insecticide applications, if the insecticide applied for SWD is effective for sharpshooters. However, with the lack of spread observed in this orchard during the two years of monitoring, along with previous work suggesting that insecticide application was ineffective for *X. fastidiosa* management (Mizell n.d), insecticide application for vectors alone is of questionable value.

Research and extension workers, as well as growers, should be aware of *X. fastidiosa* as a potential yield- and survival-impacting factor in rabbiteye blueberry. Spread of infection between established plants was not detected but might occur more rapidly among younger plants. Detection of *X. fastidiosa* by ELISA in the spring is unreliable, and false negatives can be expected at this time. Besides additional studies on yield loss in naturally infected orchards, investigation of a possible predisposing effect of Cu or Zn on susceptibility of plants to infection by *X. fastidiosa* might be warranted.

CHAPTER 4: EVALUATION OF COMMON RABBITEYE BLUEBERRY CULTIVARS FOR RESISTANCE TO *XYLELLA FASTIDIOSA*

4.1 Introduction

The use of plants tolerant or, preferably, resistant to plant diseases is a desirable management approach. When cultivars with tolerance or resistance are available, plant disease problems can be addressed proactively, at the time of planting, hopefully preventing future yield or quality losses and management expenses. In grape (*Vitis* spp.), for example, it is known that certain species and hybrids are more tolerant or resistant to *Xylella fastidiosa* Wells et al. than others and that some cultivars within the generally susceptible *V. vinifera* species are less susceptible than others (Hopkins and Purcell 2002).

In southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids), Chang et al. (2009) found differences between selection FL 86-19 and cultivar Southern Belle, with respect to the percentage of plants infected by *X. fastidiosa* and the rate and extent of symptom development. Consistent with observations from the field (Brannen et al. 2016), FL 86-19 appeared more susceptible to *X. fastidiosa*-caused bacterial leaf scorch of blueberry. 'Emerald', on the other hand, is considered resistant, and 'Star' is considered moderately susceptible (Tertuliano et al. 2012). In Florida, researchers observed that 'Star' seemed to be affected more severely than 'Windsor', with regard to the percentage of symptomatic plants and severity of symptoms (Harmon and Hopkins 2009).

It does not appear that vector preference explains differences in susceptibility to bacterial leaf scorch observed among southern highbush blueberry cultivars (Tertuliano et al. 2012). Work by Holland (2013) has shown that several plant parts of a bacterial leaf scorch-resistant southern highbush cultivar generally had higher rates of hydraulic conductance than the

respective parts of moderately or highly susceptible cultivars when asymptomatic, suggesting that the resistant cultivar had an inherently better ability to conduct water. Sun et al. (2013) showed numerically greater hydraulic conductivity in Pierce's disease-resistant grape (*Vitis* spp.) genotypes than *X. fastidiosa*-susceptible ones when mock inoculated with buffer, and the difference in hydraulic conductivity between resistant and susceptible genotypes was much greater when they had been inoculated with *X. fastidiosa*. The extent of tylosis formation is a possible reason for differing levels of hydraulic conductivity and symptom expression in infected plants, with genotypes that develop more tyloses being ones that are more susceptible to *X. fastidiosa* (Sun et al. 2013).

While southern highbush blueberry cultivars show differing levels of susceptibility to *X. fastidiosa*, inoculation of rabbiteye blueberry (*Vaccinium ashei* Reade = *V. virgatum* Aiton) plants with *X. fastidiosa* resulted either in no detected infection in cultivar Premier, or in local colonization of two of six 'Powderblue' plants (Chang et al. 2009). In infected 'Powderblue' plants, symptoms did not progress past the inoculated stem within a 14 month period. However, *X. fastidiosa* has been detected in samples from rabbiteye blueberry plants at farms in Louisiana that have experienced noticeable plant stunting and/or plant death (D. Ferrin, personal communication; M.H. Ferguson, unpublished data). Anecdotal reports from Louisiana suggest that, in rabbiteye blueberry fields where *X. fastidiosa* has been detected, 'Tifblue' has exhibited the most noticeable growth problems (C. Muller and R. Sanderlin, personal communication; M.H. Ferguson, unpublished data).

Susceptibility to *X. fastidiosa* varies among plant genotypes, and there are also differences among *X. fastidiosa* isolates with respect to their abilities to infect certain hosts, or the natural hosts in which they are found, and the severity of symptoms that they cause in hosts

that they do infect (e.g., Hopkins 1980; Nunney et al. 2013; Oliver et al. 2015; Prado et al. 2005). While findings suggest that the strains of *X. fastidiosa* found in blueberry under natural conditions may be limited to *X. fastidiosa* subsp. *multiplex* (Nunney et al. 2013; Parker et al. 2012), Oliver et al. (2015) showed that *X. fastidiosa* subsp. *multiplex* isolates from southern highbush blueberry differed with respect to the severity of symptoms that they caused and the concentrations they attained in southern highbush blueberry cultivar Rebel.

The objective of this work was to determine if some cultivars of rabbiteye blueberry were more resistant or tolerant to *X. fastidiosa* than others, using one strain isolated from southern highbush blueberry and one from rabbiteye blueberry.

4.2 Materials and Methods

4.2.1 Plant Material

Plants of rabbiteye blueberry cultivars Climax, Powderblue, Premier, Prince, and Tifblue in approximately 1 L biodegradable containers, as well as bareroot 'Brightwell' plants of 72 cell plug size, were obtained from Hartmann's Plant Company (Lacota, MI). Rabbiteye cultivar Ochlockonee and southern highbush cultivar Star plants were received in approximately 1 L plastic containers from Fall Creek Nursery Farm and Nursery, Inc. (Lowell, OR). 'Star' was included as a positive control, since it is considered moderately susceptible to bacterial leaf scorch (Tertuliano et al. 2012). All plants originated from tissue cultured stock. Plants were potted into #2 containers with medium composed of 1:1:1 ground bark : peat moss : sand, in Apr. 2014. Slow release 14-14-14 fertilizer was incorporated into the potting medium at a rate of ≈1.5 g/L prior to planting.

Plants were arranged on greenhouse benches in a factorial design with six replications. Within each replication, three plants of each cultivar were arranged randomly, except that one

'Brightwell' plant was missing from one replication. The three plants of each cultivar, within each replication, were for inoculation with an isolate of *X. fastidiosa* from a rabbiteye plant in Louisiana, an isolate from a southern highbush plant in Georgia, or for a mock-inoculated control. All plants were pruned to ≈60 cm in height and to four main, green stems of at least ≈25 cm in length in Aug. 2014, prior to the first inoculation attempt. Succulent shoots arising from the base were retained. Plants were fertilized occasionally with Peters Professional 21-7-7 Acid Special fertilizer (Everris NA Inc., Dublin, OH) and once with Fertilome Chelated Liquid Iron and Other Macronutrients (Voluntary Purchasing Groups, Inc., Bonham, TX). During the course of the study, flowers were removed from plants so that they would not produce fruit.

4.2.2 Inoculation in 2014

Cultures used included *X. fastidiosa* isolated from 'Tifblue' rabbiteye blueberry in East Feliciana Parish, LA (LA-Y3), and isolate GA-B2, which was isolated from a V1 (also known as FL 86-19 [Brannen et al. 2016]) southern highbush blueberry plant in Brantley County, GA, and provided by H. Scherm and A. Savelle of the University of Georgia. For the first inoculation attempt, bacteria from each culture were streaked onto five plates containing CS-20 medium (Chang and Walker 1988). After 15 days, bacteria were suspended in PW broth (Davis et al. 1981), and a ten-fold dilution series was performed in PW broth to 10^{-9} (Chang et al. 2009). Tubes were stored at 28°C and observed for color change.

On 23 and 24 Aug. 2014, after 51 to 52 days of incubation, a 20 µl drop of LA-Y3 or GA-B2 *X. fastidiosa* inoculum in PW broth or of the PW broth control was placed on one stem of each plant (Hopkins 1980). Concentrations were $\approx 10^4$ color-changing units for LA-Y3 and $\approx 10^3$ color-changing units for GA-B2, based on the quantification method of Chang and Zheng (1999) used in Chang et al. (2009). The stem was pierced with a 21-gauge needle to allow

uptake of the drop into the xylem (Chang et al. 2009). Plants were reinoculated in the same manner approximately two weeks later, on Sept. 7 to 8, when concentrations were again $\approx 10^4$ color-changing units for LA-Y3 and $\approx 10^3$ color-changing units for GA-B2.

Plants were sampled between 26 Oct. and 1 Nov. 2014, 63 to 70 days after the first inoculation and 48 to 55 days after the second inoculation. From each plant, twelve leaves were collected from near the bases of their respective shoots. After sampling, plants inoculated with the LA-Y3 culture and control plants were moved to an outdoor screen cage so that plants could undergo dormancy. The plants inoculated with the GA isolate were kept in the greenhouse due to USDA APHIS permit restrictions. Twelve leaves were again collected from plants inoculated with the LA culture or mock-inoculated, between 30 and 31 July, 2015, ≈11 months after being inoculated.

Leaves were stored at ≈4°C or, for longer term storage, -70°C, until petioles and midribs were cut out and stored at -20°C. Petioles and midribs were then ground in liquid nitrogen, using mortars and pestles. DNA was extracted from up to 0.1 g of tissue using a cetyltrimethylammonium bromide (CTAB) protocol based on that of Li et al. (2008). Extracted DNA was stored at -20°C until use. Real-time polymerase chain reaction (PCR) was used to check for the presence or absence of *X. fastidiosa* in extracted DNA. PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) and EFTu_3 forward and reverse primers (Holland et al. 2014). An ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) thermocycler and the following program were used: 10 min at 95°C; 40 cycles of 10 s at 95°C and 1 min at 60°C; and a dissociation step of 15 s at 95°C, 20 s at 60°C, and 15 s at 95°C (Holland et al. 2014). Samples were considered positive that had a C_t value of 35 or less and a melting temperature of 81.2 to 82.4°C. DNA extracts were tested

in duplicate and were re-tested if there was a conflict between results for the sample or if a sample tested positive but the amplification curve appeared atypical.

4.2.3 Inoculation in 2015

Because it appeared that the 2014 inoculations had not resulted in infection, plants were inoculated again in 2015. For this attempt a different method of inoculum preparation was used. Two single colony isolates of *X. fastidiosa* from the LA-Y3 culture were grown on CS-20 plates for 10 to 11 days and then suspended in succinate-citrate-phosphate (SCP) buffer (Hopkins 1980). On 2 Oct. 2015, bacteria from four plates of each of the two LA-Y3 single colony isolates were suspended in 6 ml of autoclaved SCP buffer, which resulted in a solution with an absorbance of 0.275 at 630 nm. This inoculum was used to inoculate LA plants (those previously inoculated with the LA-Y3 culture) in four of the six replications on 2 Oct. 2015. On the following day, growth from four plates of one LA-Y3 subculture and two of another LA-Y3 subculture were suspended in 3 ml of autoclaved SCP buffer, which resulted in an absorbance of 0.250 at 630 nm. This inoculum was used to inoculate LA plants in the other two replications on 3 Oct. 2015. Concentrations of inoculum are estimated to have been 2 to 3 x 10^4 CFU/ml, based on dilution plating. Five 10 µl drops of inoculum were deposited onto each plant (often one drop per stem), within 15 cm of the base of the plant. A 21-gauge needle was used to puncture the stem to allow uptake of inoculum into the xylem. The same procedure was used for mock inoculation with SCP buffer into control plants on 4 Oct. 2015.

Isolate GA-B2 was cultured on plates containing CS-20 medium for 10 days, and bacteria from 10 plates were scraped into 1.8 ml autoclaved SCP buffer. The resulting absorbance was 0.054 at 630 nm. This inoculum was used to inoculate GA plants (those previously inoculated with the GA-B2 culture) on 19 Oct. 2015, following the same protocol used on plants inoculated

with the LA-Y3 isolates, except that only two to three 10 µl drops were used for each plant in five replications, and one replication was not re-inoculated. Plating of inoculum did not reveal living GA-B2 colonies in the suspension in SCP buffer that was used for inoculation. However, living colonies were later observed on plates from which bacteria were transferred into the buffer, suggesting that bacteria used in inoculum were viable.

On 6 to 7 July 2016, sap was collected from stems that had been inoculated with the GA-B2 strain. It was not always possible to determine the location at which the stem had been inoculated, but an attempt was made to make a cut 1 to 3 cm above the point of inoculation. Pruning shears or loppers used to make the cuts were cleaned between cuts with a paper towel soaked in 70% ethanol. Based on the protocol described by Holland et al. (2014), sap was squeezed from the cut surface of the removed portion of the stem using a vise, pipetted from the cut surface of the stem into autoclaved microcentrifuge tubes, and stored at -70°C until the sap was used for DNA extraction. Sap collected from individual inoculated stems of plants from four of the five re-inoculated replications was combined for a total of 50 µl sap per plant. Equal amounts of sap were used from all stems from which sap was obtained, unless an adequate amount of sap was not available from one stem. In that case, additional sap from the other stems was used. DNA was extracted from sap using the PowerPlant Pro DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA).

Between 20 and 23 July 2016, twelve basal leaves were collected from among the stems inoculated with the LA isolate or mock-inoculated in Oct. 2015. Extraction of DNA and real-time PCR were performed on LA samples, as with leaf samples collected in 2014 and 2015. Because no LA samples tested positive, DNA was not extracted from control samples at this time. Real-time PCR, as previously described, was used to check for the presence or absence of

X. fastidiosa in extracted DNA from plants in the LA and GA treatments. Samples were retested in duplicate or triplicate if there was a conflict between wells with respect to results for a sample.

4.3 Results

No samples tested positive for *X. fastidiosa* in 2015 or 2016. Because of this, neither resistance nor tolerance could be evaluated. One 'Brightwell' plant inoculated with the GA-B2 strain died shortly after the first inoculation, but this is believed to have been due to trauma from the inoculation process, as the 'Brightwell' plants were very small at the time.

4.4 Discussion

It is not possible to evaluate whether differences in resistance exist among rabbiteye blueberry cultivars from this study because the pathogen was not detected in any of the inoculated plants. Considering the slow-growing nature of *X. fastidiosa*, it may colonize rabbiteye blueberry slowly. However, considering that plants in this study were tested 11 months after the 2014 inoculations and eight to 10 months after the 2015 inoculations, despite slow growth, the bacterium could have been expected to have reached detectable populations based on previous studies involving southern highbush and rabbiteye blueberry. In rabbiteye blueberry cultivar Powderblue, one plant tested positive 71 days after inoculation, while another first tested positive at 142 days after inoculation (Chang et al. 2009). Oliver et al. (2015) tested southern highbush blueberry plants 140 and ≈119 days after the first and second inoculations, respectively, and detected infection in 73% or more of plants into which *X. fastidiosa* strains of blueberry origin had been inoculated.

Viability of the inoculum may have been a problem for the 2014 inoculations. In an attempt to reach concentrations of 10^7 to 10^8 color-changing units, comparable to those used by Chang et al. (2009), bacteria were incubated in PW broth for 51 to 52 days before the first

inoculation attempt. Because color-changing units were used for relative quantification, the viability of inoculum was not tested by dilution plating. However, when *X. fastidiosa* was grown in PW broth for freezer preservation, it was found that colonies were recovered from bacteria in a suspension diluted to 10^{-1} and incubated for 18, 20, or 27 days but not in broth incubated for 55 days (unpublished data). Therefore, it is suspected that bacteria may have lost viability by the time they were used for the 2014 inoculations.

Even when bacteria were viable, the concentration may not have been high enough for successful establishment in plants. Although the absorbances of the suspensions containing LA-Y3 cultures in SCP buffer for the 2015 inoculation were 0.25 or higher at 630 nm, results of dilution plating suggest that the concentration of living *X. fastidiosa* cells was lower than the 10^7 to 10^8 CFU/ml that Hopkins (1984) associated with an absorbance of 0.25 at 600 nm. The absorbance of 0.054 at 630 nm, as well as the lack of growth after plating, suggests that the concentration of bacteria in the 2015 GA-B2 inoculum was low and the viability of the inoculum was low, or that bacteria were non-viable. It has been suggested that the number of bacteria present in an individual insect is not important when *X. fastidosa* is transmitted by a vector (Chatterjee et al. 2008), but Prado et al. (2005) found that pin-prick inoculations had higher rates of success when more bacteria were present in the inoculum. When attempting to inoculate *X. fastidiosa* isolated from citrus into *Citrus sinensis*, no plants inoculated with inoculum containing 10^3 or 10^5 CFU/ml, a range that includes the estimated density of the LA-Y3 inoculum used in this study in 2015, tested positive eight months after inoculation. Eleven and 49% of those inoculated with suspension containing 10^7 and 10^8 CFU/ml, respectively, tested positive at that time. When a coffee isolate was inoculated into coffee, no plants tested positive eight months

after inoculation with inoculum containing 10^4 CFU/ml, while 15, 31, and 34%, respectively, tested positive after inoculation with suspensions containing 10^6, 10^7, and 10^9 CFU/ml.

Another possible cause of difficulties was that bacteria had been maintained in media culture for relatively long durations. The LA-Y3 culture had only undergone four transfers prior to suspension in PW broth for the 2014 inoculation attempts, but it had undergone 16 transfers over a two year period prior to use for the 2015 attempt. Once the GA-B2 isolate was received from the University of Georgia, it was transferred two and eight times before inoculation of PW broth (2014) and SCP buffer (2015), respectively. Hopkins (1984) found that cultures of the Pierce's disease-causing bacterium typically caused less severe symptoms in grape after 18 months of weekly transfers.

The naturally fluctuating temperatures of the screenhouse where LA and control plants were grown beginning in the fall of 2014 could potentially have impacted infection, also. In a susceptible cultivar of olive, 90% of plants were considered systemically infected 12 months after inoculation in a greenhouse experiment, while only 30% of plants in a screenhouse study were considered systemically infected at that time (Saponari et al. 2016).

It was found that *X. fastidiosa* was detected more consistently in the roots than stems or leaf petioles/midribs of infected rabbiteye blueberry bushes (Chapter 3). Testing of roots might have been more likely to yield detections of *X. fastidiosa* sooner but would likely have been destructive to the containerized plants. Plants inoculated with the LA-Y3 cultures may be retested at a later date, in case infection occurred but colonization to the point of detectability in leaves is occurring slowly. Although previous studies on blueberry suggest that *X. fastidiosa* should have been detectable when tested, factors may have resulted in colonization occurring more slowly in this study. Colonization may be slower in more tolerant plants than more

susceptible ones (Smith et al. 2016), like some southern highbush blueberry cultivars. The use of 15 cm pots in Chang et al. (2009) suggests that the rabbiteye plants they used were likely smaller than the plants in this study were at the time of the 2015 inoculation. At that time, the plants in this study had been growing in #2 containers for ≈1.5 years. Their size may have resulted in requiring a longer time for infection to be detectable. Meanwhile, others are advised to avoid frequent transfers of *X. fastidiosa* in artificial media and to ensure that *X. fastidiosa* inoculum density is high, and discouraged from growing the bacterium in broth for an extended period prior to using it for inoculation.

CHAPTER 5: SUMMARY AND CONCLUSIONS

X. fastidiosa was detected in two of 17 rabbiteye blueberry orchards sampled in Louisiana. One strain found at both farms is of the same sequence type (ST; ST 42), according to the MLST scheme of Yuan et al. (2010), as one of two strains found in Georgia in southern highbush blueberry (Nunney et al. 2013). Two other genotypes identified from rabbiteye blueberry in Louisiana include combinations of alleles that, like ST 42, are believed to have resulted from recombination between *X. fastidiosa* subspecies *multiplex* and *fastidiosa* (Nunney et al. 2013; Nunney et al. 2014a). The genotypes found in this study have not been identified from other crop plants, but ST 42 has been found in giant ragweed and western soapberry (Nunney et al. 2013). The STs most similar to the other identified genotypes have been found in several wild plants in the Asteraceae family. The fact that a ST found in rabbiteye blueberry is the same as one found in southern highbush blueberry suggests that growers should take care not to bring infected rabbiteye blueberry plants into orchards with susceptible southern highbush cultivars. Inoculation of seven rabbiteye cultivars and southern highbush cultivar Star with *X. fastidiosa* isolated from rabbiteye blueberry in Louisiana failed to result in infection within nine months. Inoculum preparation procedures were likely responsible, at least in part, for this failure.

Yields of *X. fastidiosa*-positive 'Tifblue' rabbiteye blueberry plants were 55% and 62% less than those of consistently *X. fastidiosa*-negative plants in 2013 and 2014, respectively, and within three years of testing positive for *X. fastidiosa*, four of nine *X. fastidiosa*-positive plants lacked living above-ground growth. Because plants were naturally infected, causation of yield loss and plant death by *X. fastidiosa* is not proven. To fully assess the importance of *X. fastidiosa* in rabbiteye blueberry, it would be helpful to have data from other naturally infected

orchards. However, this finding suggests that research and extension workers, as well as growers, should be aware of *X. fastidiosa* as a potential yield- and survival-impacting factor in rabbiteye blueberry. Spread of infection was not detected in this study, but plants were ≈30 to 35 years old, and spread might occur more rapidly among younger plants.

Detection of *X. fastidiosa* by enzyme-linked immunosorbent assay (ELISA) using leaf petiole/midrib tissue or, in the winter, shoot terminals was not as consistent as detection in root sap by real-time PCR. However, it was comparable or better in some months and was consistently as good as or better than detection in sap by real-time PCR, except in April, when detection by ELISA of petiole/midrib tissue cannot be relied upon. Detection by real-time PCR using DNA from stem sap was relatively poor.

X. fastidiosa infects rabbiteye blueberry plants under natural conditions and is associated with yield loss in an orchard in Louisiana. However, it does not appear to spread rapidly between established plants. The small number of genotypes that have been found in southern highbush or rabbiteye blueberry suggests that using clean planting material may be helpful, although wild plants may harbor strains that infect blueberry, as well. The fact that rabbiteye and southern highbush plants host at least one of the same genotypes suggests that using clean plants is particularly important when planting rabbiteye plants near susceptible southern highbush cultivars.

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VITA

Mary Helen Ferguson grew up in Bogalusa, LA. She is the daughter of John and Sandra Ferguson. While she did not grow up on a farm, her family exposed her to agriculture through her Fisher-Price Play Family Farm, her grandparents' gardens, and her uncle's farm, as well as by sending her to camp at Strong River Camp and Farm. Mary Helen attended high school in Covington, LA, where she enjoyed her status as an outsider from comparatively rural Bogalusa and declared a desire to be a farm manager.

Mary Helen attended college at Birmingham-Southern College in Alabama and graduated with a degree in biology and a minor in math in 2004. While at Birmingham-Southern, she had opportunities to study at Educational Concerns for Hunger Organization in Florida and to work with urban gardens in Birmingham. After graduating from Birmingham-Southern, she studied horticultural science at North Carolina State University, doing research on compost use in strawberry production. She received her M.S. in 2006. For the next five years, Mary Helen served as an agricultural extension agent with responsibilities for horticulture and forestry in Randolph County, NC. In 2012, she returned to Louisiana and began working on her Ph.D. with Dr. Don Ferrin. After his passing, Dr. Chris Clark kindly adopted her as his graduate student.