CHARACTERIZING *XYLELLA FASTIDIOSA*, ITS GENETIC DIVERSITY AND VIRULENCE IN SOUTHERN HIGHBUSH BLUEBERRIES

by

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(Under the Direction of Jonathan E. Oliver and Miguel Cabrera)

ABSTRACT

Known for its wide host range, *Xylella fastidiosa* (Xf) is an emerging plant pathogenic bacterium of global importance. This pathogen causes bacterial leaf scorch (BLS) on southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids), a relatively new disease affecting this important fruit crop in the southeastern United States. In Georgia, USA, few Xf isolates from blueberry have been studied. This thesis investigated 1) the presence of the bacterium through multiple field surveys of blueberry plantings and the genetic diversity of obtained isolates; 2) the capability of such isolates to induce BLS in blueberry after artificial greenhouse inoculations; and 3) the association of observable BLS symptoms with actual pathogen presence in field conditions. Novel Xf genetic types capable of causing significant BLS symptoms were isolated from naturally infected blueberries. However, reliable prediction and management strategies for this disease still need to be devised.

INDEX WORDS: *Xylella fastidiosa*, Bacterial leaf scorch, blueberry, Southern highbush blueberry, *Xylella fastidiosa* subspecies *multiplex*, *Xylella fastidiosa* subspecies *fastidiosa*

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DEDICATION

To my father, the first person that comes up to my mind whenever they ask me ‘who's your best friend?’.

To my mother, who humbly endured my long absence and understood me for what I wanted to achieve.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Blueberries are increasing in importance as an agricultural commodity in Georgia. After their first introduction to the state in the 1950s, farmers, extension personnel, and the University of Georgia have worked relentlessly to help this commodity expand both production and value (Scherm and Krewer 2003). As an example of this exponential growth, 2004 marked the year when blueberries surpassed peaches in farm gate value, outclassing the fruit historically associated with Georgia, the "peach" state (Boatright and McKissick 2005, 2006). Since then, blueberry production has steadily increased, ranking consistently in recent years as one of the top ten agricultural commodities of Georgia and one of the top commodities in the fruits and nuts sector, second only to pecans (Wolfe and Stubbs 2017). Georgia farm gate values for blueberries are consistently over $200 million each year in Georgia (Wolfe and Stubbs 2018) and set records of over $300 million in 2013 and 2014 (Wolfe and Stubbs 2014, 2015).

Even though blueberries are planted throughout the state of Georgia, most acreage is concentrated in the southern Flatwoods (Scherm et al. 2001), specifically in the lower Coastal Plain characterized by sandy, acidic soils with a high water table – ideal for blueberry production (Scherm and Krewer 2003). Commercial types of blueberries grown in Georgia are the native rabbiteye blueberry (*Vaccinium virgatum* Aiton; syn. *Vaccinium ashei* Reade), northern highbush blueberries (*Vaccinium corymbosum*) and southern highbush (SHB) blueberries (*Vaccinium corymbosum* interspecific hybrids). Rabbiteye blueberries have traditionally represented the bulk

of southern production of blueberries for over a century, being well-adapted to high temperature and humidity, generally resistant to most pests and diseases, easy to grow, very productive, and yielding fruits with excellent firmness and shelf-life (Fonsah et al. 2008; Scherm and Krewer 2003). Northern highbush blueberries (native to the eastern coast of the United States), which have specific winter chilling requirements, do not do well in the warmer Georgia regions; their limited acreage is confined to the mountain regions in the northernmost part of the state (Krewer and NeSmith 2006). SHB blueberries, on the other hand, are the results of crosses between the northern highbush blueberry species (*Vaccinium corymbosum*) and native southern blueberries and are a relatively recent introduction (dating back to the mid-1990s). The recent market success of SHB blueberry varieties derives mostly from their ability to bloom and ripen much earlier than traditionally grown rabbiteye blueberry varieties. This particular trait has sparked considerable interest among Georgia growers and encouraged further increases in blueberry production tailored more towards fresh fruit sales rather than the processed fruit market, allowing SHB production to take advantage of a potentially lucrative market window early in the season (i.e. April and May) (Fonsah et al. 2007; Scherm and Krewer 2003).

Impressive yields can be obtained in the southern Georgia climate when SHB blueberries are managed carefully, particularly when planted in high-density cropping systems with either the right soil or using raised pine bark mulched beds (Krewer et al.). The establishment of this new SHB blueberry industry brought in a series of new agronomic and management challenges arising from more stringent requirements and factors that pertain to SHB blueberry bushes themselves, being much less forgiving plants compared to rabbiteye blueberries: first, the need for moist but well-drained, unamended sandy soils with a relatively high organic matter content (at least 3%) and relatively low pH (no higher than 5.5); second, an increased susceptibility to

spring freezes due to very early blooming; and third, an increased susceptibility to weeds and diseases, such as *Phytophthora* root rot, leaf spots and dieback diseases which called for different, more careful management choices that had not been needed with rabbiteye blueberries (Scherm and Krewer 2003; Scherm et al. 2001). Several new diseases were also observed such as necrotic ringspot caused by tobacco ringspot virus and blueberry ringspot virus (Chang et al. 2009).

In these circumstances, in 2004, a new disease was first observed and reported affecting (SHB) blueberry in Georgia. Initial symptoms of this disorder were marginal scorch-like necrotic burns on leaves, followed by defoliation, leaving twigs and stems naked with a yellow “skeletal” appearance (Brannen et al. 2016; Chang et al. 2009). The resemblance of such notable symptoms to other somewhat similar leaf-scorching disorders observed in the southern US and known to be caused by the bacterial agent *Xylella fastidiosa* (Xf), prompted scientists at the University of Georgia to test for the presence of this microorganism in symptomatic blueberries. Positive isolation of Xf cultures and final fulfillment of Koch’s postulates confirmed the bacterium's association and phytopathogenicity on blueberry, and the disease was named bacterial leaf scorch (BLS), specifically BLS of blueberry (Chang et al. 2009). BLS of blueberry has been a common sight in Florida as well (Harmon and Hopkins 2009) and is now regarded as a potential concern for any blueberry-producing region of the southeastern US.

We want to recall very briefly a few key aspects regarding Xf as a pathogen in general and the present threat it poses to plant health worldwide, given the minimal information available regarding the management of this disease that has been translated into practical uses to effectively counter the Xf issue. These points will be raised in more detail when introducing each specific chapter of our study.

Xf is a Gram-negative bacterium (Phylum: *Proteobacteria*) of the class *Gammaproteobacteria*, quite related to xanthomonads (*Xanthomonas* spp.), a typical group of phytopathogenic prokaryotes, but it is phenotypically and genotypically different enough from these close relatives to merit separate genus status (Wells et al. 1987). A xylem-limited plant pathogen, Xf is a vector-transmitted bacterium with a wide host range that includes both crops and ornamentals, mostly perennials and shade trees, and even wild plants. Economically important crops or ornamentals with Xf-induced diseases include grapevine with Pierce's disease (arguably the most famous and studied Xf-plant pathosystem, given its importance in epidemics in North America) (Davis et al. 1978; Hopkins 1989), citrus with citrus variegated chlorosis (another significant Xf-related problem, for the citrus industry, in South America) (Chang et al. 1993), almond with almond leaf scorch (Davis et al. 1980), oleander with oleander leaf scorch (Purcell et al. 1999), coffee with coffee leaf scorch (de Lima et al. 1998), pecan with pecan leaf scorch (Sanderlin and Heyderich-Alger 2000) and peach with phony peach disease (Davis et al. 1981b, a; Wells et al. 1983). These last two hosts are probably the best historically known, in addition now to blueberry, when it comes to Xf diseases in Georgia. Even so, these are just some of the common examples among the many known potential hosts on which Xf can infect and cause disease. To make matter worse, several wild plants (weeds, shrubs and trees) have been found to harbor the bacterium, often without visible symptoms (Purcell and Saunders 1999).

Transmission of inoculum occurs by sap-sucking xylem-feeding insects such as leafhoppers (family Cicadellidae), also known as sharpshooters, and spittlebugs (family Cercopoidea), able to actively penetrate and feed on plant tissue through their stylet mouthparts (Janse and Obradovic 2010). In turn, Xf is capable of attaching and reproducing in the insect's foregut, becoming persistent in those individuals that do not shed the cuticle anymore in their

final adult stage (Brlansky et al. 1983; Purcell and Finlay 1979). Once bacterial cells are transmitted to a healthy plant, bacterial multiplication and adhesion to xylem vessels occurs by forming biofilms and attachment proteins (fimbrial and afimbrial adhesins), centers of cell aggregations that clog water passages and restrict sap flow (Chatterjee et al. 2008). Following pathogen invasion, physiological responses from the host plant likely occur to isolate the infection site by developing plant secretions (tyloses and gums), thus self-inducing and exacerbating the blockage of xylem vessels (Hopkins 1989). For this reason, it has been suggested that issues associated with Xf generally involve water stress-like symptoms, as in the case of the several "leaf scorching" diseases typically observed in Xf-infected plants (including blueberry), which are typified by leaf necrosis, wilting, defoliation and eventually plant death (Hopkins 1989). However, biochemical and biophysical processes involved in pathogen-host interactions leading to disease are yet to be fully understood, and it is assumed that infection of a host plant by Xf does not automatically imply an obvious development of symptoms (Purcell and Saunders 1999).

Xf genetics and the proposed host specificity determined by its genetic variability constitute another topic of research related to this organism. Xf is the only species in the genus *Xylella* but at least four subspecies are commonly accepted according to DNA-DNA hybridization and phylogenetic studies: Xf subsp. *fastidiosa*, Xf subsp. *multiplex*, Xf subsp. *pauca* and Xf subsp. *sandyi* (Scally et al. 2005; Schaad et al. 2004; Schuenzel et al. 2005). This subdivision into subspecies, with strains found infecting multiple specific hosts that were unique to one subspecies, has warranted the hypothesis that isolates of Xf show host specialization (Janse and Obradovic 2010). For example, Pierce's disease of grapevine is only caused by Xf subsp. *fastidiosa*, phony peach disease by Xf subsp. *multiplex* and citrus variegated chlorosis by

Xf subsp. *pauca*. Even so, as mentioned before, host-pathogen interaction mechanisms remain unknown and so far no reliable loci have been identified for investigating this host specificity capability, provided it exists (Almeida and Nunney 2015). The resolution provided by current genotyping and phenotyping methods that employ multi-locus approaches, while useful for taxonomy studies, are probably not enough for investigating the source of host specificity regulation (Sicard et al. 2018). To this purpose, full-genome sequencing projects of several new Xf isolates are expected to bring a wealth of information that will undoubtedly provide further insight into the genetics of this pathogen, in addition to the few available full genome sequences already available (Sicard et al. 2018). It should be kept in mind though that molecular techniques, while a powerful tool for diagnostics and preliminary investigation, comprise just the first step in gaining preliminary insight into Xf and do not provide a comprehensive understanding of the pathogen in a given situation.

What we can safely conclude is that the emergence of diseases induced by Xf is the result of a complex interaction of multiple factors. Biological aspects, genetics, infection dynamics, environmental and ecological factors (e.g. a multitude of diverse hosts and vectors) inevitably interact together with great variability, making the study of such a pathosystem very challenging, despite the fact that the basic understanding of Xf has improved substantially since the early 2000s (Almeida and Nunney 2015; Chatterjee et al. 2008; Purcell 2013; Retchless et al. 2014; Sicard et al. 2018). New emerging diseases in recent years have characterized Xf as an infamous plant pathogen of global importance on an ever-increasing number of crops. The best, and most worrying example, is the case of olive quick decline syndrome, a new, severe disease outbreak that rapidly occurred in southern Italy following a recent introduction (Saponari et al. 2013), effectively elevating the Xf threat to Europe for the first time, outside the boundaries of the

Americas to which the bacterium was believed to be limited. Based upon the apparent lack of preparedness for managing Xf epidemics on new hosts (e.g. olive or blueberry), a call for research efforts to elucidate Xf in diverse scenarios and ecological situations is expected to occur worldwide (Almeida et al. 2019). In each of these scenarios, the problem is understanding Xf on a case-by-case basis and accounting for the many knowledge gaps pertaining to situations where Xf diseases are emerging: what are the factors involved in terms of specificity for a given commercial crop of interest affected by Xf? What are the specific vectors and the epidemiology dynamics involved in the transmission of Xf? What are the factors ultimately determining the emergence of significant symptoms? Answering basic questions like these while focusing on a specific crop with a specific Xf-related problem would provide useful insight that could be turned into solid solutions for management and disease control.

Rationale and Project Objectives

For this work, we have considered some of these knowledge gaps that still impair the design of real control strategies to motivate our goals regarding our study of this pathogen on blueberry in Georgia, specifically. Several reasons may be at the root of Xf emergence in blueberry and these likely include favorable warm climatic conditions, abundance of endemic insect vectors capable of transmitting infectious inoculum to healthy plants, a biological shift of the causal agent to a new potential, attractive host and an increasing acreage of susceptible SHB blueberry varieties. Significant losses have been estimated at 70% over a 10-year period for commercial blueberry plantings affected by Xf (P. Brannen, personal communication). Increasing management costs for BLS have also been reported due to the long-term negative effects that strategies for controlling Xf have on revenues coming from blueberry plantings. The

visual identification and eradication of infected blueberry bushes ('roguing') represents one of the only practical methods available today to mitigate disease incidence in the field (J. Oliver, personal communication). Replanting takes multiple seasons to reach a profitable enough maturity, and in the meantime any infected plant that still appears without symptoms (i.e. with latent infection) may act as a source of Xf inoculum, only waiting to be spread by apt vectors. The effectiveness of insecticide applications has not been evaluated yet for blueberry, but it is feared that the generalist nature of Xf vectors in Georgia (e.g. leafhoppers that are extremely polyphagous, including the widespread glassy-winged sharpshooter, *Homalodisca vitripennis*) will make it difficult to control individuals that constantly fly between crop plants and wild hosts with no specific feeding preference (Tertuliano et al. 2010; Tertuliano et al. 2012). Preliminary work exploring blueberry resistance to Xf in the field confirmed the lethality and dispersal of BLS in susceptible SHB blueberry cultivars commonly planted such as ‘Rebel’, ‘Star’ and ‘FL 86-19’, and established that there is indeed some degree of varietal resistance, or at least tolerance to Xf (Brannen et al. 2008). Host plant resistance would likely be the best management tactic for managing BLS and Xf; however, while present knowledge about potential for resistance to Xf does appear promising, no breeding program is actively developing Xf-resistant blueberry cultivars (Brannen et al. 2016).

Before devising any practical applications to manage the pathogen effectively, basic questions regarding BLS on blueberry, and its dangerous causal agent, Xf, have to be accounted for to solve this issue affecting such a profitable agricultural commodity. This project was born out of consideration for the present situation where minimal information is available for the BLS-Xf complex, and attempted to provide additional insight and useful material that could have an impact on future directions for managing Xf in blueberry in Georgia. Given the global

significance of Xf in recent years, any perspectives, reasons, points and information yielded in studying Xf within this niche crop of the southeastern US, may turn into useful knowledge for studying this pathogen more broadly in other situations and scenarios. The objectives of this thesis were the following:

1. Investigate the distribution of Xf in blueberry plantings in Georgia and its genetic diversity by first obtaining new isolates from infected plants.
2. Determine the differences in infection and virulence capability of these isolates in inducing BLS in blueberry through artificial inoculation trials.
3. Evaluate BLS symptom severity in field conditions and its association with pathogen dispersal and incidence over time.

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CHAPTER 2

NATURAL INFECTION OF SOUTHERN HIGHBUSH BLUEBERRY (*VACCINIUM CORYMBOSUM* INTERSPECIFIC HYBRIDS) BY *XYLELLA FASTIDIOSA* SUBSP. *FASTIDIOSA*[1]

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Abstract

Xylella fastidiosa (Xf) is an emerging insect-vectored, xylem-limited, bacterium that can cause disease on several economically important fruit and tree crops including almond, blueberry, citrus, grapevine, peach, and pecan. On blueberry, Xf causes bacterial leaf scorch (BLS), which is prevalent in the southeastern US. This disease, previously reported to be caused by Xf subsp. *multiplex* (Xfm), can result in rapid plant decline and death of southern highbush (SHB) blueberry cultivars. In 2017, a survey of blueberry plantings in southern Georgia (USA) confirmed the presence of Xf-infected plants in eight of nine sites examined, and seven isolates were cultured from infected plants. Genetic characterization of these isolates through single-locus and multi-locus sequence analysis revealed that three isolates from two sites belong to Xf subsp. *fastidiosa* (Xff), with significant similarity to isolates from grapevine. After these three isolates were artificially inoculated onto greenhouse-grown SHB blueberries (cv. “Rebel”), symptoms typical of BLS developed, and Xff-infection was confirmed through genetic characterization and re-isolation of the bacterium to fulfill Koch’s postulates. As all previously reported Xf isolates from blueberry have been characterized as Xfm, this is the first time that isolation of Xff has been reported from naturally infected blueberry plantings. The potential impact of Xff isolates on disease management in blueberry requires further exploration. Furthermore, given that isolates from both Xfm and Xff were obtained within a single naturally infected blueberry planting, blueberry in southern Georgia may provide opportunities for intersubspecific recombination between Xff and Xfm isolates.

Introduction

Xylella fastidiosa (Xf) is a xylem-inhabiting, gram negative, fastidious, plant pathogenic bacterium that infects a wide variety of host plants. In recent years, this pathogen has emerged as a considerable issue on an increasing number of perennial crops and trees (Almeida et al. 2019; Almeida and Nunney 2015; Chatterjee et al. 2008). Economically important agricultural crops that are affected by Xf include almond, citrus, coffee, grapevine, peach, pecan, plum and, more recently, blueberry and olive, as well as several shade trees and ornamentals like elm, maple, oak, oleander and sycamore (Chang et al. 2009; Chatterjee et al. 2008; Hopkins and Purcell 2002; Loconsole et al. 2014; Purcell 2013). In addition, many wild plants (wild grasses, sedges, vines and trees) are known to act as natural reservoirs and may carry the pathogen often without symptoms (Janse and Obradovic 2010; Purcell and Saunders 1999). Transmission of Xf is mediated by xylem sap-feeding vectors, including leafhoppers, sharpshooters (family Cicadellidae) and spittlebugs (family Cercopoidea), in a complex biological and ecological plant-vector-pathogen interaction that involves bacterial retention and multiplication within the vector's foregut and subsequent dispersal to another plant during xylem feeding (Brlansky et al. 1983; Purcell and Finlay 1979; Purcell et al. 1979).

Bacterial leaf scorch (BLS) of blueberry was first observed in 2004 and later confirmed to be caused by Xf in the blueberry production region of southern Georgia (Chang et al. 2009) and Florida (Harmon and Hopkins 2009). Disease symptoms include marginal chlorosis and subsequent scorching (necrotic burns) of leaves, premature defoliation, and dieback, ultimately leading to death of the entire blueberry bush (Brannen et al. 2016; Chang et al. 2009). Xf has been observed to most severely affect southern highbush (SHB) blueberry (*Vaccinium corymbosum* interspecific hybrids), with varying disease incidence and severity across different

cultivars (Brannen et al. 2008); however, rabbiteye blueberries (*V. ashei*) can also be infected and show symptoms in some cases (Ferguson et al. 2017). Several sharpshooter leafhoppers are believed to vector Xf in blueberry plantings in the southeastern US, with the glassy-winged sharpshooter (*Homalodisca vitripennis*) being the most frequently observed (Tertuliano et al. 2012).

Based upon genetic characterization of the 16S-23S intergenic spacer (ITS) region and multi-locus sequence typing analyses (MLST), the species Xf has been shown to be composed of multiple genetically distinct subspecies (Nunney et al. 2014; Parker et al. 2012; Scally et al. 2005; Schaad et al. 2004). These subspecies include Xf subsp. *fastidiosa* (Xff), isolated from grape, almond, alfalfa and maple, Xf subsp. *pauca* (Xfp), isolated from citrus, coffee, and olive, Xf subsp. *sandyi* (Xfs) isolated from oleander, and Xf subsp. *multiplex* (Xfm), isolated from various hosts including peach, pecan, plum, almond, blueberry, and several shade trees. Genetic differences between and within subspecies are assumed to determine the host range of individual isolates; however, the exact genetic determinants of Xf pathogenicity on certain hosts are not known. Cross-inoculations with isolates from different subspecies have shown that Xff isolates from grapevine and alfalfa do not infect peach or citrus and Xfm isolates from peach do not infect grapevine or citrus (Hopkins and Purcell 2002; Janse and Obradovic 2010). Likewise, Xfp isolates from South American citrus do not seem to colonize coffee and vice versa (Almeida et al. 2008; Nunney et al. 2013). Furthermore, host range tests with Xfm isolates from tree species recently showed that each Xf strain has a unique host range (Nunney et al. 2019). By contrast, some plant hosts can be naturally infected by multiple subspecies of Xf, as isolates from both Xfm and Xff have been found causing almond leaf scorch (Almeida and Purcell 2003). Despite the documented host specificity differences between isolates, the limited variation within genes

used in Xf phylogenetic studies (e.g. MLST) are unlikely to explain the differences in biological traits and infectivity among Xf isolates (Almeida and Nunney 2015; Killiny and Almeida 2011; Parker et al. 2012).

Even though bacterial leaf scorch of blueberry is known to cause severe, widespread yield reductions in the southeastern US blueberry production region within Georgia (#2 in blueberry production nationally) and Florida (#8 nationally), only a relatively small number of Xf isolates have been cultured from in-field symptomatic blueberry plants and characterized genetically. Among the reported blueberry Xf isolates characterized to date (Hopkins et al. 2012; Nunney et al. 2014; Oliver et al. 2015; Oliver et al. 2014; Parker et al. 2012), all have been reported to belong to a single clade of Xfm consisting of intersubspecific recombinants (Nunney et al. 2014). Nonetheless, Xff is prevalent (likely endemic) in the southeastern US, being isolated from grapes (*Vitis* sp.) and muscadines (*Vitis rotundifolia*) as well as elderberry and lupine (Hopkins and Purcell 2002; Parker et al. 2012), and artificial inoculation tests on SHB blueberry with several different Xff and Xfm isolates have shown the capability of both subspecies to cause leaf scorching symptoms in controlled greenhouse conditions (Oliver et al. 2015: Burbank et al. 2019).

The objective of this study was to better understand the distribution and diversity of Xf in blueberry plantings in southern Georgia. To that end, we surveyed nine blueberry plantings in southern Georgia and attempted to culture the pathogen from infected blueberry plants. Obtained isolates were initially genetically characterized using the *rpoD* gene. This was followed by more extensive characterization using two different multi-locus sequence typing approaches. Based upon our findings, we carried out Koch's postulates with some of the isolates obtained. Our results clearly show that isolates from both Xfm and Xff are present in naturally infected

blueberry plantings in southern Georgia, suggesting that SHB blueberry may be a permissive host for multiple Xf subspecies and accordingly provide opportunities for intersubspecific recombination and the emergence of new virulent Xf strains. Furthermore, these findings have the potential to impact management of Xf in blueberry production fields in the southeastern US.

Materials and Methods

Field survey and isolation of Xf. In September 2017, plantings of SHB blueberry in three counties in Georgia were surveyed for the presence of Xf. In total, nine sites were surveyed including three sites each in Bacon, Pierce, and Ware counties in Georgia. At each of the nine sites, leaf samples were collected from at least five plants per site showing typical leaf scorch symptoms (Fig. 2.1). Total DNA was extracted from 3 to 5 midribs and petioles of the collected leaf samples using a modified CTAB protocol (Doyle and Doyle 1987). The presence of Xf was initially verified by PCR testing using the RST31/33 primer pair (Minsavage et al. 1994), and subsequently confirmed using the HL5/6 primer pair (Francis et al. 2006). PCR was carried out using a S1000^{TM} Thermal Cycler (Bio-Rad, Hercules, CA) with a GoTaq® (Promega®, Fitchburg, WI) PCR kit, with the following reagents: 10 µl 2x GoTaq® Green Master Mix, 2 µl each of forward and reverse primers (10 µM for HL5/6 and 100 µM for RST31/33), 1 µl DNA (1-3 ng/µl) and 5 µl dH_2O. PCR conditions were as follows: 5 min at 94°C followed by 34 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec, and concluding with 72°C for 2 min. Appropriate positive and negative controls were included, and amplified products were visualized by gel electrophoresis on 1% agarose gels (1X TAE buffer) stained with GelRed® Nucleic Acid Stain (Biotium, Fremont, CA) using a Gel Doc^{TM} XR+ (Bio-Rad, Hercules, CA) gel imager.

After confirmation of Xf infection via PCR, attempts were made to isolate Xf from the midribs and petioles of symptomatic blueberry leaf tissue. Xf was isolated on Periwinkle Wilt (PW) and PW+ media according to the protocol previously described by Davis et al. (1981). Following DNA extractions from bacterial biomass via a modified CTAB protocol (Doyle and Doyle 1987), isolates were confirmed as Xf by PCR using the RST31/33 primer pair as described above. Glycerol stocks were prepared by scraping Xf isolates from plates, suspending them in a PD2 and 20% glycerol solution (Davis et al. 1980), and placing them at -80°C for long-term storage.

Genetic characterization of Xf isolates using *rpoD*. RST31/33 primer pair amplicons from PCR on total DNA extracted from symptomatic plant samples were purified using the E.Z.N.A.® Cycle Pure Kit (Omega Bio-Tek, Norcross, GA) and sequenced in both directions via Sanger sequencing by Eurofins Genomics (Louisville, KY). These primers amplify a portion of the RNA polymerase sigma-70 factor *rpoD* locus from Xf. Following isolation of the bacteria from symptomatic plant material, RST31/33 primer pair PCR amplicons (733 nts) were also sequenced in both directions from each of the bacterial isolates. Returned sequences for each amplicon were reviewed for signal quality on FinchTV 1.5.0 software (Geospiza, Inc.) and then imported into SeaView 4.6.4 software (Galtier et al. 1996; Gouy et al. 2010) for manual editing and alignment. Obtained sequences were analysed and aligned alongside additional *rpoD* Xf sequences imported from the GenBank database (www.ncbi.nlm.nih.gov/genbank) in July 2018 (Table 2.5). Phylogenetic trees were generated to compare all sequences using the "neighbour-joining" (NJ) method in MEGA7 (Kumar et al. 2016; Saitou and Nei 1987).

Genetic characterization of Xf isolates using MLSA-E genes. For additional genetic characterization of Xf isolates, PCR was carried out to amplify bacterial DNA using primers

(Table 2.1) for amplifying the nine "environmentally-mediated" genes responsible for host infection and virulence regulation described by Parker et al. (2012). Amplified products were visualized by agarose gel electrophoresis, purified, prepared, sequenced, and analysed as described for the *rpoD* genetic characterization above. Nine phylogenetic trees (one for each gene) were generated using the NJ method in MEGA7. Finally, a concatenated 9-gene sequence for each Xf isolate was assembled to allow for multi-locus alignment. Concatenated isolate sequences obtained in this study, as well as additional Xf sequences imported from the GenBank database (www.ncbi.nlm.nih.gov/genbank) in July 2018 (Table 2.6), were used to generate a single master phylogenetic tree using the NJ method in MEGA7.

Genetic characterization of Xf isolates using MLST genes. For the three isolates obtained from a single field site ('AlmaReb1', 'AlmaReb2', and 'AlmaReb3'), additional genetic characterization was carried out using the multi-locus sequence typing (MLST) approach reported previously by Scally et al. (2005) and Yuan et al. (2010). Primers were used to amplify seven additional Xf genes (Table 2.1) via PCR as described above. Amplified products were visualized by agarose gel electrophoresis, purified, prepared, sequenced, and analysed as described for the *rpoD* genetic characterization above. To determine MLST sequence types for each isolate, obtained sequences were compared to the Xf MLST sequence database (https://pubmlst.org/xfastidiosa) hosted at the University of Oxford (Jolley et al. 2004; Scally et al. 2005).

Inoculation of blueberry with Xff isolates. Three isolates ('AlmaReb1', 'AlmaReb2', and 'PierceMed1') obtained from blueberry and identified as belonging to Xff based upon phylogenetic analyses were used to inoculate SHB blueberries in the greenhouse. Blueberry plants originally grown from tissue culture (cv. "Rebel" [PPA18, 138]) (NeSmith 2008) were

obtained from Agri-Starts (Apopka, FL) and transplanted into a one-part sand, three-parts pine bark mulch mixture in 9.5 liter and 25.7 cm (diameter) pots. Plants were maintained in the greenhouse at 28-35°C under natural sunlight and watered as needed. Plants were fertilized twice, the first time 15 days after potting and the second time 3 months later, with a granular slow-release fertilizer (Osmocote® Smart Release® Plant Food Plus Outdoor & Indoor 15-9-12, The Scotts Company, Marysville, OH) at the manufacturer's recommended rate for indoor use. Between one and two months after initial transplanting, blueberry plants were inoculated with Xff isolates using a 1-mL tuberculin syringe according to the protocol described previously (Chang et al. 2009; Oliver et al. 2015). Xff isolates were grown out from previously prepared glycerol stocks on periwinkle wilt (PW) medium for 10 days at 28°C, then suspended in succinate-citrate-phosphate (SCP) buffer prior to inoculation (De La Fuente et al. 2013). In total, each Xff isolate was used to inoculate three blueberry plants and three plants were inoculated with SCP buffer only. Two weeks after the original inoculations, all plants were re-inoculated in the same manner to ensure infection.

PCR confirmation of greenhouse blueberry infection and re-isolation from symptomatic plants. After 120 days post-inoculation, three lower leaves were collected from the originally inoculated stems of all plants and tested for the presence of Xf by PCR using the RST31/33 primer pair. At 150 days post-inoculation, leaf samples were collected from Xf infected plants and attempts were made to re-isolate the bacterium using the protocol described previously for field sample isolations (Davis et al. 1981). Bacterial colonies obtained were purified on PW media and total DNA was extracted using the modified CTAB protocol described previously to allow for PCR confirmation that the obtained isolates were Xf. PCR confirmation was carried out using primer pairs RST31/33 and copB-F/R (Table 2.1), and

amplicons were sequenced via Sanger sequencing by Eurofins Genomics (Louisville, KY) to verify that the cultured bacteria matched the identity of the isolate originally used to inoculate each blueberry plant, respectively.

Results

Field survey and Xf isolation results. A total of 47 leaf samples of SHB blueberry from nine field sites with evident BLS symptoms (Fig. 2.1) were collected and subsequently tested for the presence of Xf using PCR. PCR testing of leaf tissue indicated Xf-positive samples from 8 of 9 (88.8%) blueberry sites and 22 of 47 (47%) collected samples (Table 2.2). The only site from which Xf-positive leaf material was not confirmed was site 2 in Pierce County, Georgia. Attempts to isolate the Xf bacterium from the collected samples resulted in the isolation of seven Xf isolates from blueberry. Three of these isolates (‘AlmaReb1’, ‘AlmaReb2’, and ‘AlmaReb3’) came from SHB cv. ‘Rebel’ at site 7 in Bacon County, three additional isolates (‘AlmaStar1’, ‘AlmaStar2’, ‘AlmaStar3’) came from cv. ‘Star’ at site 9 in Bacon County, and the seventh isolate (‘PierceMed1’) came from cv. ‘Meadowlark’ at site 1 in Pierce County (Table 2.3).

Genetic characterization of Xf isolates using *rpoD*. For the seven isolates obtained from blueberry, sequencing of the *rpoD* gene fragment unexpectedly revealed two groups of sequences: Group 1 (consisting of four isolates with identical sequences - ‘AlmaReb3’, ‘AlmaStar1’, ‘AlmaStar2’, and ‘AlmaStar3’) and Group 2 (consisting of three isolates with identical sequences - ‘AlmaReb1’, ‘AlmaReb2’, and ‘PierceMed1’). BLAST analysis of the resulting sequences versus Xf sequences in the GenBank nucleotide collection showed the most similarity (99.4%) between Group 1 isolates and Xfm isolate ‘M12’ [CP000941] from almond, while Group 2 isolates showed 100% identity with Xff isolate ‘Temecula1’ [AE009442] from

grapevine. By contrast, the *rpoD* fragments sequenced for the Group 1 isolates showed 98.6% identity to the sequences from Group 2 isolates. Sequences for the *rpoD* amplicon generated from the seven isolates in this study were deposited in GenBank as accession numbers MN590433 to MN590439. A phylogenetic tree produced using *rpoD* sequences (Fig. 2.2) showed three predominant clades: one consisting of isolates identified as Xfp from coffee, citrus, and other hosts; one consisting of isolates from grapevine and muscadines identified as Xff; and one consisting of isolates from blueberry, almond, and shade trees identified as Xfm. The Group 1 isolates from this study clustered with Xfm sequences from blueberry while the Group 2 isolates from this study clustered with Xff isolates from grapevine. Sequencing of the *rpoD* fragments amplified from the DNA samples extracted from the field plants (out of which the isolates were cultured originally) produced the same sequence result as the PCR using DNA from the isolated bacteria. From site 7 in Bacon County, where three isolates were obtained, *rpoD* sequences from the five Xf-positive plants resulted in sequences from two plants that matched Group 2 sequences and three that matched Group 1.

Genetic characterization of Xf isolates using MLSA-E. Sequences obtained from the seven isolates in this study for each of the nine MLSA-E genes were deposited in GenBank as accession numbers MN590440 to MN59502. MLSA-E analysis also supported the results from *rpoD* in that the seven cultured Xf isolates grouped into two main groups (Group 1 and Group 2). The pattern observed with the *rpoD* sequences (sequences were identical within each of the two groups) was consistent across eight of the nine sequenced MLSA-E genes. For the *copB* gene fragment sequence, isolate ‘AlmaReb2’ was found to have an extra 15-nucleotides starting at position #104 within the *copB* amplicon compared to the other Group 2 isolates, ‘AlmaReb1’ and ‘PierceMed1’. Further comparisons with available GenBank sequences for *copB* proved

‘AlmaReb2’ to be a new haplotype, different from any *copB* sequence previously obtained for Xf. In addition, once the concatenated sequences of all nine MLSA-E genes were examined, it was apparent that ‘AlmaReb1’ and ‘PierceMed1’ also represented new haplotypes as well, with a unique combination of sequences from *copB* and *xadA* – though neither the *copB* or *xadA* sequences obtained for these isolates was unique on its own.

Single-gene MLSA-E phylogenetic trees consistently showed that sequences from Group 1 isolates grouped with Xfm sequences and Group 2 isolates with Xff for each of the nine genes (data not shown). Furthermore, the master MLSA-E tree supported the classification of Group 1 isolates as Xfm and Group 2 isolates as Xff (Fig. 2.3). Group 2 isolates grouped most closely with haplotype ‘GR_GAFL’ from Parker et al. (2012), consisting of Florida and Georgia muscadine and grapevine isolates. Group 1 isolates were identical to Parker et al. (2012) haplotype ‘BLUEBERRY3’.

Genetic characterization of Xf isolates using MLST. Sequences obtained from isolates ‘AlmaReb1’, ‘AlmaReb2’, and ‘AlmaReb3’ for each of the seven MLST genes were deposited in GenBank as accession numbers MN590503 to MN590523. MLST gene sequences (Table 2.4) indicated that the Group 1 isolate ‘AlmaReb3’ was identical to Sequence Type 42 (ST42) which includes three Xfm isolates previously sequenced from blueberry and two Xfm isolates from giant ragweed and one from western soapberry (Nunney et al. 2013). Group 2 isolates ‘AlmaReb1’ and ‘AlmaReb2’ were identical to ST1 (Table 2.4) which includes, among others, the Xff type isolate from grapevine ‘Temecula1’.

Inoculation of blueberry with Xf and fulfillment of Koch’s postulates. Greenhouse-grown blueberry plants inoculated with Xff isolates ‘AlmaReb1’, ‘AlmaReb2’, and ‘PierceMed1’ developed typical BLS symptoms after seven weeks post-inoculation (Fig. 2.4).

Plant inoculation with each of the isolates, respectively, caused definitive symptoms. Somewhat more severe symptoms were observed in the plants inoculated with isolate 'AlmaReb2' compared to the plants inoculated with the other two Xff isolates tested. PCR testing of leaves indicated Xf infection of all Xf-inoculated plants at 120 days post-inoculation, while plants inoculated with only SCP buffer tested negative for infection. Sequencing of *copB* and *rpoD* PCR products amplified from plant DNA confirmed that the obtained sequences matched the sequences from the respective isolates originally used for inoculation of each plant. At 150 days post-inoculation, isolation attempts resulted in the successful re-isolation of Xf from two of the plants inoculated with 'AlmaReb2'. Using DNA extracted from the re-isolated Xf bacterial culture, PCR and sequencing of the *copB* fragment revealed the sequence of *copB* (including the 15 nt insert) to be the same as the *copB* from isolate 'AlmaReb2' used in the original inoculation of these plants. Though BLS symptoms and Xf-specific PCR on plant DNA clearly demonstrated Xf infection of plants inoculated with 'AlmaReb1' and 'PierceMed1', re-isolation attempts from plants inoculated with these isolates were not successful.

Discussion

Our findings clearly demonstrate that isolates from both Xff and Xfm are present in naturally infected SHB blueberry plantings in southern Georgia. Based upon our genetic and greenhouse characterization of isolates from blueberry, Xff isolates closely related to isolates from grapevine and muscadine are capable of causing BLS symptoms on SHB blueberry, and are infecting SHB in at least two southern Georgia counties. To our knowledge, this represents the first report of natural infection of blueberry by Xff and also apparently represents the first time

that isolates from both Xfm and Xff have been identified within a single planting of any crop in the southeastern US, despite the fact that both subspecies are endemic within the region.

In addition to these novel findings regarding the subspecies of Xf in blueberry, our genetic characterization of these seven isolates from blueberry alongside the other sequenced haplotypes from other hosts represents a substantial contribution to the pool of knowledge regarding Xf diversity in blueberry in its own right. Prior to this work, a total of eight other Xf isolates from blueberry had been characterized by MLSA-E (Oliver et al. 2014; Parker et al. 2012) with only seven reported Xf isolates from blueberry characterized by MLST (Nunney et al. 2013), and only a single *rpoD* sequence available from blueberry Xf (Van Horn et al. 2017). Accordingly, the three MLSA-E haplotypes reported from a single SHB planting in Bacon Co., Georgia (site 7) in this study (including one previously known haplotype and two novel haplotypes) represent as many unique Xf haplotypes as had been sequenced previously from blueberry over the past 15 years since BLS was identified as a disease of blueberry.

The identification of new Xf haplotypes and Xff isolates capable of naturally infecting blueberry, suggests that more work is needed to understand the diversity of blueberry Xf isolates and the epidemiology of this disease on blueberry in the southeastern US. The similarity between the Xff isolates characterized in this study and the Xff isolates previously characterized from grapevine and muscadine in Florida and Georgia is likely no coincidence, and suggests the possibility of movement of Xf between these crops. As muscadine is a commonly grown residential and commercial crop plant in southern Georgia and wild grape relatives are common weed species in and adjacent to blueberry plantings, it is possible that these *Vitis* sp. may serve as reservoirs for Xff isolates within blueberry plantings. To our knowledge, Xfm isolates have not been identified in grapevine or muscadines in the southeast, and their ability to infect *Vitis*

sp. has not been proven. Furthermore, despite the fact that Xff isolates were found infecting SHB blueberry in two different Georgia counties in our study, the prevalence of Xff isolates in blueberries remains unclear. Since the limited number of prior blueberry BLS studies did not identify Xff isolates in blueberry plantings, it may be that Xff isolates capable of infecting blueberries are relatively rare or that other barriers to transmission and infection of blueberries with Xff isolates exist. It is conceivable that differences in host resistance between blueberry cultivars, differences in vector preferences between *Vitis* sp. and blueberry cultivars, or differences in the ability of vector species to acquire from or transmit Xff isolates between blueberry plants may make Xff isolates less capable than Xfm isolates of causing epidemics on blueberry. Along this line, using glassy-winged sharpshooters, a recent study by Burbank et al. (2019) could not demonstrate transmission of a grapevine Xff isolate despite successful acquisition from artificially inoculated SHB blueberry in controlled conditions. Prior studies which have used Xff isolates from grapevine for artificial inoculation of blueberries in the greenhouse showed that Xff isolates are capable of causing symptoms on SHB blueberries (Oliver et al. 2015); however, this prior study also suggested that the Xff isolates from grapevine and elderberry show significantly less virulence on blueberry relative to Xfm isolates from blueberry. This indicates the possibility that the Xff isolates from blueberry characterized in our study may differ from the previous Xff isolates from grapevine and elderberry tested by Oliver et al. (2015) in terms of virulence on blueberry. It should be noted that while the greenhouse work carried out in fulfillment of Koch's postulates described herein was not set up to compare the virulence of Xfm and Xff isolates, the field plants from which the Xff isolates cultured in this study were obtained showed unambiguous, severe symptoms of leaf scorch that appeared to be as severe as symptoms on plants infected with Xfm isolates (data not shown). Ultimately, to

determine if the Xff isolates from this study differ in virulence or pathogenicity traits from Xfm isolates from blueberry, experiments under controlled conditions need to be carried out.

The genetics underlying host specificity differences between Xf subspecies remains unclear, despite renewed interest in Xf diseases and genetic studies following the recent emergence of this pathogen in Europe. It is likely that the genetic basis of Xf pathogenicity is a complex trait that will not be elucidated from the characterization of the handful of genes typically utilized for multi-locus sequence typing. Nonetheless, the identification and characterization of Xf isolates that are genetically similar yet differ in host specificity, or Xf isolates that are genetically distinct which are still capable of infecting a common host (such as those described here), would likely be valuable for elucidating the determinants of Xf pathogenicity. To this end, applying the latest genomic analysis approaches as well as Xf gene knockout/replacement technologies to further investigate the isolates described in this study may help us to better understand the determinants of Xf pathogenicity on blueberry.

In our study, isolates from both Xfm and Xff were obtained from a single planting of SHB blueberry. This has potential implications for both disease management and new Xf strain emergence. To our knowledge, efforts to breed for Xf resistance in blueberry have not been pursued by blueberry breeders to this point, despite the fact that host resistance would be a desirable means for managing this difficult disease. Currently, there are no known SHB cultivars that are unable to be infected with Xf; however, apparent differences in disease prevalence and severity between cultivars suggest that tolerance differences may be present within cultivated SHB varieties. Furthermore, rabbiteye blueberries appear to be infrequently affected by this disease, even when grown in close proximity to SHB that are severely impacted. It is likely that host genetic differences account for these differences in susceptibility, and that these could be

utilized in breeding efforts; however, differences between strains of Xf infecting blueberry need to be taken into account in any concerted breeding effort, as multiple haplotypes and subspecies present in a single planting could result in the failure or breakdown of resistance that is specific to a single isolate or strain. Also, as alluded to previously, it is possible that Xf movement between wild or cultivated *Vitis* sp. and blueberry may be possible, and the discovery of Xff isolates in naturally infected SHB suggests the possibility that management practices targeting infected alternate host plants or vectors may impact Xf-infection and spread within SHB blueberries. This bears further investigation as well. For Xf, recombination between subspecies is known to be a significant factor in adaption and movement to new host plants, and prior studies have indicated that the Xfm isolates previously identified from blueberry are intersubspecific recombinants between subspecies *multiplex* and *fastidiosa* (Nunney et al. 2014). Our identification of SHB plantings with both Xfm and Xff subspecies indicate that blueberry plantings in southern Georgia may provide opportunities for recombination between the two subspecies, which has been shown to occur artificially between Xff isolates from grape and Xfm isolates from blueberry (Kandel et al. 2017).

As a whole, our study clearly demonstrates that Xf isolates from both Xff and Xfm are present in southern highbush plantings in southern Georgia. This confirms the suggestion of prior artificial inoculation research that isolates from both subspecies are capable of causing BLS on blueberry, and suggests that movement of Xff from wild hosts (possibly muscadines and relatives) to cultivated blueberry may occur. Further studies regarding the epidemiological implications of these findings with respect to BLS movement on blueberry may reveal new opportunities for managing this devastating disease of blueberries in the southeastern US.

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Table 2.1. Primers used for PCR amplification and sequencing of genes characterized in this study.

Gene	Role in Study	Primers (F/R)	Amplicon Size (bp)	Reference
	Pathogen detection	HL5/6		Francis et al. 2006
rpoD	Pathogen detection & genetic characterization	RST-31/RST-33	733	Minsavage et al. 1994
acvB	Genetic characterization (MLSA-E)	acvB-F/acvB-R	743	Parker et al. 2012
copB	Genetic characterization (MLSA-E)	copB-F/copB-R	607-862	Parker et al. 2012
cvaC	Genetic characterization (MLSA-E)	cvaC-F/cvaC-R	330	Parker et al. 2012
fimA	Genetic characterization (MLSA-E)	fimA-F/fimA-R	557	Parker et al. 2012
gaa	Genetic characterization (MLSA-E)	gaa-F/gaa-R	1129	Parker et al. 2012
pglA	Genetic characterization (MLSA-E)	pglA-F/pglA-R	828/829	Parker et al. 2012
pilA	Genetic characterization (MLSA-E)	pilA-F/pilA-R	405	Parker et al. 2012
rpfF	Genetic characterization (MLSA-E)	rpfF-F/rpfF-R	825	Parker et al. 2012
xadA	Genetic characterization (MLSA-E)	xadA-F/xadA-R	1087/1108	Parker et al. 2012
cysG	Genetic characterization (MLST)	cysG-F/cysG-R	600	Scally et al. 2005
gltT	Genetic characterization (MLST)	gltT-F/gltT-R	654	Scally et al. 2005
holC	Genetic characterization (MLST)	holC-F/holC-R	379	Scally et al. 2005
leuA	Genetic characterization (MLST)	leuA-F/leuA-R	708	Scally et al. 2005
malF	Genetic characterization (MLST)	malF-F/malF-R	730	Scally et al. 2005
nuoL	Genetic characterization (MLST)	nuoL-F/nuoL-R	557	Scally et al. 2005
petC	Genetic characterization (MLST)	petC-F/petC-R	533	Scally et al. 2005

Table 2.2. PCR testing results from 2017 Georgia SHB blueberry survey.

Site ID	County	SHB Cultivars	Xf positives/total (%)
1	Pierce	Meadowlark, Star, Farthing	3/5 (60%)
2	Pierce	Star	0/5 (0%)
3	Pierce	Meadowlark	1/5 (20%)
4	Ware	Rebel	1/5 (20%)
5	Ware	Star, Rebel	3/5 (60%)
6	Ware	Rebel, Sweet Crisp, Farthing	3/7 (43%)
7	Bacon	Rebel	5/5 (100%)
8	Bacon	FL 86-19 (V1)	1/5 (20%)
9	Bacon	Star	5/5 (100%)
			Total 22/47 (47%)

Table 2.3. Xf isolates cultured from SHB blueberries and characterized in this study.

Site ID	County	SHB Cultivar	Isolate
1	Pierce	Meadowlark	PierceMed1
7	Bacon	Rebel	AlmaReb1
7	Bacon	Rebel	AlmaReb2
7	Bacon	Rebel	AlmaReb3
9	Bacon	Star	AlmaStar1
9	Bacon	Star	AlmaStar2
9	Bacon	Star	AlmaStar3

Table 2.4. MLST results for Xf isolates from site 7 in Bacon County, Georgia.

Isolate	Sequence Type	*cysG*	*gltT*	*holC*	*leuA*	*malF*	*nuoL*	*petC*
AlmaReb1	ST1	1	1	1	1	1	1	1
AlmaReb2	ST1	1	1	1	1	1	1	1
AlmaReb3	ST42	6	3	5	12	4	3	3

Table 2.5. GenBank accession numbers for all sequences used in *rpoD* phylogenetic analysis.

Subspecies	Haplotype	Isolate	Host (Species)	rpoD Accession
fastidiosa	–	5008968	Coffee (*Coffea arabica*)	KP769844
	–	Agsavage	Persimmon (*Diospyros kaki*)	MF581193
	–	BQa	Grape (*V. vinifera x V. labrusca*) "Black Queen"	KP241937
	–	CFBP8073	Coffee (*Coffea canephora*)	LKES01000001
	–	Cherry-XA	Cherry (*Prunus avium*)	MF401540
	ALMOND1	M23	Almond (*Prunus dulcis*)	CP001011
	EB_LUPINE	EB92-1	Elderberry (*Sambucus canadensis*)	AFDJ01000098
	GR_CA3	Temecula1	Grape (*Vitis vinifera*)	AE009442
	GR_CATX1	GB514	Grape (*Vitis vinifera*)	CP002165
	GR_CATX2	ATCC 35879	Grape (*Vitis vinifera*)	JQAP01000004
		Stag's Leap	Grape (*Vitis vinifera*) "Chardonnay"	LSMJ01000002
	GR_GAFL	DSM 10026	Grape (*Vitis* sp.)	FQWN01000002
multiplex	–	ATCC 35871	Plum (*Prunus domestica*)	AUAJ01000003
	–	AZ01	Chitalpa (*Chitalpa tashkentensis*)	EU714200
	–	AZ03	Chitalpa (*Chitalpa tashkentensis*)	EU714201
	–	AZ04	Chitalpa (*Chitalpa tashkentensis*)	EU714202
	–	Griffin-1	Red Oak (*Quercus rubra*)	AVGA01000018
	–	NJPE1	Pin Oak (*Quercus palustris*)	EU334069
	–	NM02	Chitalpa (*Chitalpa tashkentensis*)	EU714204
	–	XF-281	Milkwort (*Polygala myrtifolia*)	MF401541
	ALMOND_SPARTIUM*	CFBP8417	Spartium (*Spartium junceum*)	LUYB01000001
		CFBP8418	Spartium (*Spartium junceum*)	LUYA01000001
		Dixon	Almond (*Prunus dulcis*)	AAAL02000001
	ALMOND3	M12	Almond (*Prunus dulcis*)	CP000941
	BLUEBERRY3	BB01	Blueberry (*Vaccinium corymbosum*)	MPAZ01000016

Table 2.5 (continued). GenBank accession numbers for all sequences used in *rpoD* phylogenetic analysis.

Subspecies	Haplotype	Isolate	Host (Species)	rpoD Accession
multiplex	MILKWORT*	CFBP8416	Milkwort (*Polygala myrtifolia*)	LUYC01000004
	SYCAMORE2*	Sy-VA	Sycamore (*Platanus occidentalis*)	JMHP01000013
pauca	–	5044483	Coffee (*Coffea arabica*)	KP769843
	–	ALM-1	Almond (*Prunus dulcis*)	KJ406262
	–	Alm2	Almond (*Prunus dulcis*)	KX093246
	–	CE1	Olive (*Olea eurpaea*) "Arauco"	KM206739
	–	citri2	Citrus (*Citrus* sp.)	KT625424
	–	CO-BZ	Coffee (*Coffea arabica*)	KT764081
	–	Ins46	Leafhopper (Insect) (*Philaenus spumarius*)	HG939491
	–	OL-G	Olive (*Olea europaea*) "Ogliarola"	HG532022
	–	OL-X2	Olive (*Olea europaea*) "Ogliarola"	HG532023
	–	OLS0479	Oleander (*Nerium oleander*)	LRVH01000069
	–	PW-1	Periwinkle (*Vinca* sp.)	KJ406260
	–	Uncultured X.f. sp.	Olive (*Olea europaea*)	HG939492
	–	Uncultured X.f. sp.	Leafhopper (Insect) (*Euscelis lineolatus*)	HG939506
	–	Uncultured X.f. sp.	Leafhopper (Insect) (*Neophilaenus campestris*)	HG939505
	–	X4-5	Weeds	HG532021
	–	Xf9	Olive (*Olea europaea*)	LM999922
	COFFEE_PLUM*	6c	Coffee (*Coffea arabica*)	AXBS02000010
		COF0324	Coffee (*Coffea* sp.)	LRVG01000028
		Pr8x	Plum (*Prunus* domestica)	CP009826
	COFFEE1*	3124	Coffee (*Coffea* sp.)	CP009829
		32	Coffee (*Coffea arabica*)	AWYH01000030
	COFFEE2*	CFBP8072	Coffee (*Coffea arabica*)	LKDK01000001

Table 2.5 (continued). GenBank accession numbers for all sequences used in *rpoD* phylogenetic analysis.

Subspecies	Haplotype	Isolate	Host (Species)	rpoD Accession
pauca	HIBISCUS*	Hib4	Hibiscus (*Hibiscus schizopetalus*)	CP009885
	OLIVE_COFFEE_OLEANDER*	CoDiRo	Olive (*Olea europaea*)	JUJW01000004
		COF0407	Coffee (*Coffea* sp.)	LRVJ01000111
		De Donno	Olive (*Olea europaea*)	CP020870
		OLS0478	Oleander (*Nerium oleander*)	LRVI01000038
		Salento-1	Olive (*Olea europaea*)	CP016608
		Salento-2	Olive (*Olea europaea*)	CP016610
	ORANGE1	9a5c	Sweet Orange (*Citrus sinensis*) "Valencia"	AE003849
		U24D	Citrus (*Citrus sinensis*)	CP009790
	ORANGE2*	CVC0251	Citrus (*Citrus sinensis*)	LRVE01000022
		CVC0256	Citrus (*Citrus sinensis*)	LRVF01000034
		J1a12	Citrus (*Citrus sinensis*)	CP009823
	ORANGE3*	11399	Citrus (*Citrus sinensis*)	JNBT01000011
	ORANGE4*	Fb7	Citrus (*Citrus* sp.)	CP010051
sandyi	–	5685010	Coffee (*Coffea arabica*)	KP769842
	–	CO-15	Coffee (*Coffea arabica)*	KT764084
	–	CO33026	Coffee (*Coffea arabica*)	KT764082
	–	CO33233	Coffee (*Coffea arabica*)	KT764083
	COFFEE3*	CO33	Coffee (*Coffea arabica*)	LJZW01000013
	MULBERRY1*	Mul-MD	Mulberry *Morus alba*)	AXDP01000011
	MULBERRY2*	MUL0034	Mulberry (*Morus alba*)	CP006740
	OLEANDER	Ann-1	Oleander (*Nerium oleander*)	CP006696

*Asterisk indicates new MLSA-E haplotype assigned in this study according to naming scheme for MLSA-E haplotypes previously identified by Parker et al. 2012.

Table 2.6. GenBank accession numbers for all sequences used in MLSA-E phylogenetic analysis.

Subspecies	Haplotype	Isolate	Host (Species)	*acvB*	*copB*	*cvaC*	*fimA*	*qaa*	*pglA*	*pilA*	*rpfF*	*xadA*
fastidiosa	ALMOND1	M23	Almond (*Prunus dulcis*)	CP001011	CP001011	CP001011	CP001011	CP001011	CP001011	CP001011	CP001011	CP001011
	EB_LUPINE	EB92-1	Elderberry (*Sambucus canadensis*)	AFDJ01000096	AFDJ01000015	AFDJ01000143	AFDJ01000015	AFDJ01000072	AFDJ01000053	AFDJ01000096	AFDJ01000091	AFDJ01000091
		EB 92-3	Elderberry (*Sambucus canadensis*)	JQ361931	JQ361980	JQ362029	JQ362078	JQ362127	JQ362176	JQ362225	JQ362274	JQ362323
		L95-1	Lupine (*Lupinus aridonum*)	JQ361932	JQ361981	JQ362030	JQ362079	JQ362128	JQ362177	JQ362226	JQ362275	JQ362324
	GR_CA1	Conn Creek	Grape (*Vitis vinifera*)	JQ361949	JQ361998	JQ362047	JQ362096	JQ362145	JQ362194	JQ362243	JQ362292	JQ362341
		Fetzer	Grape (*Vitis vinifera*)	JQ361950	JQ361999	JQ362048	JQ362097	JQ362146	JQ362195	JQ362244	JQ362293	JQ362342
		Preston	Grape (*Vitis vinifera*)	JQ361951	JQ362000	JQ362049	JQ362098	JQ362147	JQ362196	JQ362245	JQ362294	JQ362343
		Teme 1	Grape (*Vitis vinifera*)	JQ361952	JQ362001	JQ362050	JQ362099	JQ362148	JQ362197	JQ362246	JQ362295	JQ362344
		Teme 2	Grape (*Vitis vinifera*)	JQ361953	JQ362002	JQ362051	JQ362100	JQ362149	JQ362198	JQ362247	JQ362296	JQ362345
	GR_CA2	Teme 4	Grape (*Vitis vinifera*)	JQ361954	JQ362003	JQ362052	JQ362101	JQ362150	JQ362199	JQ362248	JQ362297	JQ362346
	GR_CA3	Temecula1	Grape (*Vitis vinifera*)	AE009442	AE009442	AE009442	AE009442	AE009442	AE009442	AE009442	AE009442	AE009442
	GR_CA4	Napa-Silverado	Grape (*Vitis vinifera*)	JQ361955	JQ362004	JQ362053	JQ362102	JQ362151	JQ362200	JQ362249	JQ362298	JQ362347
	GR_CA5	Hopland	Grape (*Vitis vinifera*)	JQ361956	JQ362005	JQ362054	JQ362103	JQ362152	JQ362201	JQ362250	JQ362299	JQ362348
	GR_CATX1	GB514	Grape (*Vitis vinifera*)	CP002165	CP002165	CP002165	CP002165	CP002165	CP002165	CP002165	CP002165	CP002165
		King3	Grape (*Vitis vinifera*)	JQ361933	JQ361982	JQ362031	JQ362080	JQ362129	JQ362178	JQ362227	JQ362276	JQ362325
		King4	Grape (*Vitis vinifera*)	JQ361934	JQ361983	JQ362032	JQ362081	JQ362130	JQ362179	JQ362228	JQ362277	JQ362326
		King5	Grape (*Vitis vinifera*)	JQ361935	JQ361984	JQ362033	JQ362082	JQ362131	JQ362180	JQ362229	JQ362278	JQ362327
	GR_CATX2	ATCC 35879	Grape (*Vitis vinifera*)	JQAP01000015	JQAP01000001	JQAP01000001	JQAP01000001	JQAP01000002	JQAP01000014	JQAP01000015	JQAP01000002	JQAP01000002
		GIL BEC 625	Grape (*Vitis vinifera*) "Viognier"	JQ361947	JQ361996	JQ362045	JQ362094	JQ362143	JQ362192	JQ362241	JQ362290	JQ362339
		SLO	Grape (*Vitis vinifera*)	JQ361948	JQ361997	JQ362046	JQ362095	JQ362144	JQ362193	JQ362242	JQ362291	JQ362340
		Stag's Leap	Grape (*Vitis vinifera*) "Chardonnay"	LSMJ01000011	LSMJ01000001	LSMJ01000001	LSMJ01000001	LSMJ01000002	LSMJ01000009	LSMJ01000011	LSMJ01000002	LSMJ01000002
	GR_FL	PD 92-8	Bunch Grape *Vitis* sp.) "BN6 Sou-1"	JQ361957	JQ362006	JQ362055	JQ362104	JQ362153	JQ362202	JQ362251	JQ362300	JQ362349
	GR_GA	CC PM 1	Grape (*Vitis vinifera*) "Petit Manseng"	JQ361958	JQ362007	JQ362056	JQ362105	JQ362154	JQ362203	JQ362252	JQ362301	JQ362350
	GR_GAFL	3SV-G	Grape (*Vitis* sp.)	JQ361936	JQ361985	JQ362034	JQ362083	JQ362132	JQ362181	JQ362230	JQ362279	JQ362328
		Alpha 3	Grape (*Vitis* sp.)	JQ361937	JQ361986	JQ362035	JQ362084	JQ362133	JQ362182	JQ362231	JQ362280	JQ362329
		CC Cab 1	Grape (*Vitis vinifera*) "Cabernet Sauvignon"	JQ361938	JQ361987	JQ362036	JQ362085	JQ362134	JQ362183	JQ362232	JQ362281	JQ362330
		DSM 10026	Grape (*Vitis* sp.)	FQWN01000001	FQWN01000008	FQWN01000010	FQWN01000008	FQWN01000017	FQWN01000012	FQWN01000001	FQWN01000004	FQWN01000004
		F2_3	Grape (*Vitis* sp.) "Vidal"	JQ361939	JQ361988	JQ362037	JQ362086	JQ362135	JQ362184	JQ362233	JQ362282	JQ362331
		Georgia Grape	Grape (*Vitis* sp.)	JQ361940	JQ361989	JQ362038	JQ362087	JQ362136	JQ362185	JQ362234	JQ362283	JQ362332
		PD 10-1	Muscadine Grape (*Vitis rotundifolia*) "Carlos"	JQ361946	JQ361995	JQ362044	JQ362093	JQ362142	JQ362191	JQ362240	JQ362289	JQ362338
		PD 95-1	Wild Grape (*Vitis* sp.)	JQ361945	JQ361994	JQ362043	JQ362092	JQ362141	JQ362190	JQ362239	JQ362288	JQ362337
		TS Mer 1	Grape (*Vitis vinifera*) "Merlot"	JQ361941	JQ361990	JQ362039	JQ362088	JQ362137	JQ362186	JQ362235	JQ362284	JQ362333

Table 2.6 (continued). GenBank accession numbers for all sequences used in MLSA-E phylogenetic analysis.

Subspecies	Haplotype	Isolate	Host (Species)	*acvB*	*copB*	*cvaC*	*fimA*	*qaa*	*pglA*	*pilA*	*rpfF*	*xadA*
fastidiosa	GR_GAFL	TS Vid 1	Grape (*Vitis* sp.) "Vidal"	JQ361942	JQ361991	JQ362040	JQ362089	JQ362138	JQ362187	JQ362236	JQ362285	JQ362334
		WM1_1	Grape (*Vitis vinifera*) "Mourvedre"	JQ361943	JQ361992	JQ362041	JQ362090	JQ362139	JQ362188	JQ362237	JQ362286	JQ362335
		WM1_2	Grape (*Vitis vinifera*) "Mourvedre"	JQ361944	JQ361993	JQ362042	JQ362091	JQ362140	JQ362189	JQ362238	JQ362287	JQ362336
multiplex	ALMOND2	ALS6	Almond (*Prunus dulcis*)	JQ361916	JQ361965	JQ362014	JQ362063	JQ362112	JQ362161	JQ362210	JQ362259	JQ362308
	ALMOND3	M12	Almond (*Prunus dulcis*)	CP000941	CP000941	CP000941	CP000941	CP000941	CP000941	CP000941	CP000941	CP000941
	ALMOND_ SPARTIUM*	Dixon	Almond (*Prunus dulcis*)	AAAL02000002	AAAL02000006	AAAL02000012	AAAL02000006	AAAL02000004	AAAL02000010	AAAL02000002	AAAL02000002	AAAL02000001
		CFBP8418	Spartium (*Spartium junceum*)	LUYA01000003	LUYA01000008	LUYA01000013	LUYA01000008	LUYA01000017	LUYA01000011	LUYA01000003	LUYA01000003	LUYA01000001
		CFBP8417	Spartium (*Spartium junceum*)	LUYB01000003	LUYB01000010	LUYB01000005	LUYB01000010	LUYB01000005	LUYB01000012	LUYB01000003	LUYB01000003	LUYB01000001
	BLUEBERRY1	BB 08-1	Blueberry (*Vaccinium* sp.) "Windsor"	JQ361926	JQ361975	JQ362024	JQ362073	JQ362122	JQ362171	JQ362220	JQ362269	JQ362318
		BB 08-3	Blueberry (*Vaccinium* sp.) "Star"	JQ361927	JQ361976	JQ362025	JQ362074	JQ362123	JQ362172	JQ362221	JQ362270	JQ362319
		BB I-10	Blueberry (*Vaccinium* sp.) "V1"	JQ361928	JQ361977	JQ362026	JQ362075	JQ362124	JQ362173	JQ362222	JQ362271	JQ362320
	BLUEBERRY2	BB I-64	Blueberry (*Vaccinium* sp.) "V1"	JQ361917	JQ361966	JQ362015	JQ362064	JQ362113	JQ362162	JQ362211	JQ362260	JQ362309
		BB I-80	Blueberry (*Vaccinium* sp.) "Star"	JQ361918	JQ361967	JQ362016	JQ362065	JQ362114	JQ362163	JQ362212	JQ362261	JQ362310
	BLUEBERRY3	Alma 311	Blueberry (*Vaccinium* sp.)	JQ361920	JQ361969	JQ362018	JQ362067	JQ362116	JQ362165	JQ362214	JQ362263	JQ362312
		AlmaEm3	Blueberry (*Vaccinium* sp.) "Emerald"	KF445148	KF445149	KF445150	KF437627	KF445151	KF445152	KF437628	KF445153	KF445154
		BB01	Blueberry (*Vaccinium corymbosum*)	MPAZ01000023	MPAZ01000015	MPAZ01000032	MPAZ01000015	MPAZ01000001	MPAZ01000004	MPAZ01000023	MPAZ01000023	MPAZ01000022
		SRBB	Blueberry (*Vaccinium virgatum*)	JQ361921	JQ361970	JQ362019	JQ362068	JQ362117	JQ362166	JQ362215	JQ362264	JQ362313
	ELM	BH Elm	American Elm (*Ulmus americana*)	JQ361960	JQ362009	JQ362058	JQ362107	JQ362156	JQ362205	JQ362254	JQ362303	JQ362352
	LUPINE	L95-2	Lupine (*Lupinus villosus*)	JQ361914	JQ361963	JQ362012	JQ362061	JQ362110	JQ362159	JQ362208	JQ362257	JQ362306
	MILKWORT*	CFBP8416	Milkwort (*Polygala myrtifolia*)	LUYC01000001	LUYC01000003	LUYC01000003	LUYC01000003	LUYC01000010	LUYC01000008	LUYC01000001	LUYC01000001	LUYC01000004
	OAK1	Oak 92-10	Water Oak (*Quercus nigra*)	JQ361930	JQ361979	JQ362028	JQ362077	JQ362126	JQ362175	JQ362224	JQ362273	JQ362322
		Oak 95-1	Turkey Oak (*Quercus cerris*)	JQ361929	JQ361978	JQ362027	JQ362076	JQ362125	JQ362174	JQ362223	JQ362272	JQ362321
	OAK2	Oak 92-6	Oak (*Quercus* sp.)	JQ361924	JQ361973	JQ362022	JQ362071	JQ362120	JQ362169	JQ362218	JQ362267	JQ362316
	PLUM	Georgia Plum	Plum (*Prunus* sp.)	JQ361925	JQ361974	JQ362023	JQ362072	JQ362121	JQ362170	JQ362219	JQ362268	JQ362317
	RAGWEED	VAL VAL 072 ext	Giant Ragweed (*Ambrosia trifida* var. *texana*)	JQ361919	JQ361968	JQ362017	JQ362066	JQ362115	JQ362164	JQ362213	JQ362262	JQ362311
	REDBUD	UVA 519-B	Redbud (*Cercis* sp.)	JQ361915	JQ361964	JQ362013	JQ362062	JQ362111	JQ362160	JQ362209	JQ362258	JQ362307
	SUMPWEED	LLA FAL 718 A	Narrow Leaf Sumpweed (*Iva angustifolia*)	JQ361912	JQ361961	JQ362010	JQ362059	JQ362108	JQ362157	JQ362206	JQ362255	JQ362304
	SUNFLOWER	GIL GRA 274 ext	Annual Sunflower (*Helianthus annuus*)	JQ361913	JQ361962	JQ362011	JQ362060	JQ362109	JQ362158	JQ362207	JQ362256	JQ362305
	SYCAMORE1	4rd +1	Sycamore (*Platanus* sp.)	JQ361922	JQ361971	JQ362020	JQ362069	JQ362118	JQ362167	JQ362216	JQ362265	JQ362314
		4rd +2	Sycamore (*Platanus* sp.)	JQ361923	JQ361972	JQ362021	JQ362070	JQ362119	JQ362168	JQ362217	JQ362266	JQ362315
	SYCAMORE2*	Sy-VA	Sycamore (*Platanus occidentalis*)	JMHP01000023	JMHP01000027	JMHP01000009	JMHP01000027	JMHP01000007	JMHP01000010	JMHP01000014	JMHP01000014	JMHP01000013
pauca	COFFEE1*	32	Coffee (*Coffea arabica*)	AWYH01000039	AWYH01000003	AWYH01000005	AWYH01000003	AWYH01000031	AWYH01000011	AWYH01000048	AWYH01000031	AWYH01000029
		3124	Coffee (*Coffea* sp.)	CP009829	CP009829	CP009829	CP009829	CP009829	CP009829	CP009829	CP009829	CP009829

Table 2.6 (continued). GenBank accession numbers for all sequences used in MLSA-E phylogenetic analysis.

Subspecies	Haplotype	Isolate	Host (Species)	*acvB*	*copB*	*cvaC*	*fimA*	*qaa*	*pglA*	*pilA*	*rpfF*	*xadA*
pauca	COFFEE2*	CFBP8072	Coffee (*Coffea arabica*)	LKDK01000010	LKDK01000012	LKDK01000007	LKDK01000012	LKDK01000002	LKDK01000011	LKDK01000003	LKDK01000002	LKDK01000001
	COFFEE_PLUM*	COF0324	Coffee (*Coffea* sp.)	LRVG01000023	LRVG01000054	LRVG01000136	LRVG01000054	LRVG01000133	LRVG01000055	LRVG01000023	LRVG01000133	LRVG01000063
	COFFEE_PLUM*	6c	Coffee (*Coffea arabica*)	AXBS02000016	AXBS02000004	AXBS02000004	AXBS02000003	AXBS02000010	AXBS02000006	AXBS02000016	AXBS02000010	AXBS02000010
	COFFEE_PLUM*	Pr8x	Plum (*Prunus* sp.)	CP009826	CP009826	CP009826	CP009826	CP009826	CP009826	CP009826	CP009826	CP009826
	HIBISCUS*	Hib4	Hibiscus (*Hibiscus schizopetalus*)	CP009885	CP009885	CP009885	CP009885	CP009885	CP009885	CP009885	CP009885	CP009885
	OLIVE_COFFEE_OLEANDER*	CoDiRo	Olive (*Olea europaea*)	JUJW01000002	JUJW01000001	JUJW01000001	JUJW01000001	JUJW01000003	JUJW01000001	JUJW01000002	JUJW01000003	JUJW01000004
		COF0407	Coffee (*Coffea* sp.)	LRVJ01000030	LRVJ01000099	LRVJ01000144	LRVJ01000099	LRVJ01000067	LRVJ01000163	LRVJ01000030	LRVJ01000067	LRVJ01000068
		De Donno	Olive (*Olea europaea*)	CP020870	CP020870	CP020870	CP020870	CP020870	CP020870	CP020870	CP020870	CP020870
		OLS0478	Oleander (*Nerium oleander*)	LRVI01000035	LRVI01000009	LRVI01000009	LRVI01000009	LRVI01000021	LRVI01000007	LRVI01000035	LRVI01000021	LRVI01000028
		Salento-1	Olive (*Olea europaea*)	CP016608	CP016608	CP016608	CP016608	CP016608	CP016608	CP016608	CP016608	CP016608
		Salento-2	Olive (*Olea europaea*)	CP016610	CP016610	CP016610	CP016610	CP016610	CP016610	CP016610	CP016610	CP016610
	ORANGE1	U24D	Citrus (*Citrus* sp.)	CP009790	CP009790	CP009790	CP009790	CP009790	CP009790	CP009790	CP009790	CP009790
		9a5c	Sweet Orange (*Citrus sinensis*) "Valencia"	AE003849	AE003849	AE003849	AE003849	AE003849	AE003849	AE003849	AE003849	AE003849
	ORANGE2*	J1a12	Citrus (*Citrus* sp.)	CP009823	CP009823	CP009823	CP009823	CP009823	CP009823	CP009823	CP009823	CP009823
		CVC0251	Citrus (*Citrus sinensis*)	LRVE01000002	LRVE01000118	LRVE01000118	LRVE01000118	LRVE01000045	LRVE01000015	LRVE01000003	LRVE01000045	LRVE01000104
		CVC0256	Citrus (*Citrus sinensis*)	LRVF01000023	LRVF01000048	LRVF01000048	LRVF01000048	LRVF01000108	LRVF01000075	LRVF01000106	LRVF01000108	LRVF01000094
	ORANGE3*	11399	Citrus (*Citrus sinensis*)	JNBT01000008	JNBT01000002	JNBT01000002	JNBT01000002	JNBT01000010	JNBT01000027	JNBT01000028	JNBT01000010	JNBT01000011
	ORANGE4*	Fb7	Citrus (*Citrus* sp.)	CP010051	CP010051	CP010051	CP010051	CP010051	CP010051	CP010051	CP010051	CP010051
sandyi	OLEANDER	Ann-1	Oleander (*Nerium oleander*)	CP006696	CP006696	CP006696	CP006696	CP006696	CP006696	CP006696	CP006696	CP006696
		MED PRI 047	Oleander (*Nerium oleander*)	JQ361959	JQ362008	JQ362057	JQ362106	JQ362155	JQ362204	JQ362253	JQ362302	JQ362351
	MULBERRY1*	Mul-MD	Mulberry (*Morus alba*)	AXDP01000008	AXDP01000022	AXDP01000019	AXDP01000022	AXDP01000009	AXDP01000002	AXDP01000008	AXDP01000010	AXDP01000013
	MULBERRY2*	MUL0034	Mulberry (*Morus alba*)	CP006740	CP006740	CP006740	CP006740	CP006740	CP006740	CP006740	CP006740	CP006740
	COFFEE3*	CO33	Coffee (*Coffea arabica*)	LJZW01000001	LJZW01000007	LJZW01000008	LJZW01000007	LJZW01000014	LJZW01000012	LJZW01000001	LJZW01000005	LJZW01000002

*Asterisk indicates new MLSA-E haplotype assigned in this study according to naming scheme for MLSA-E haplotypes previously identified by Parker et al. 2012.

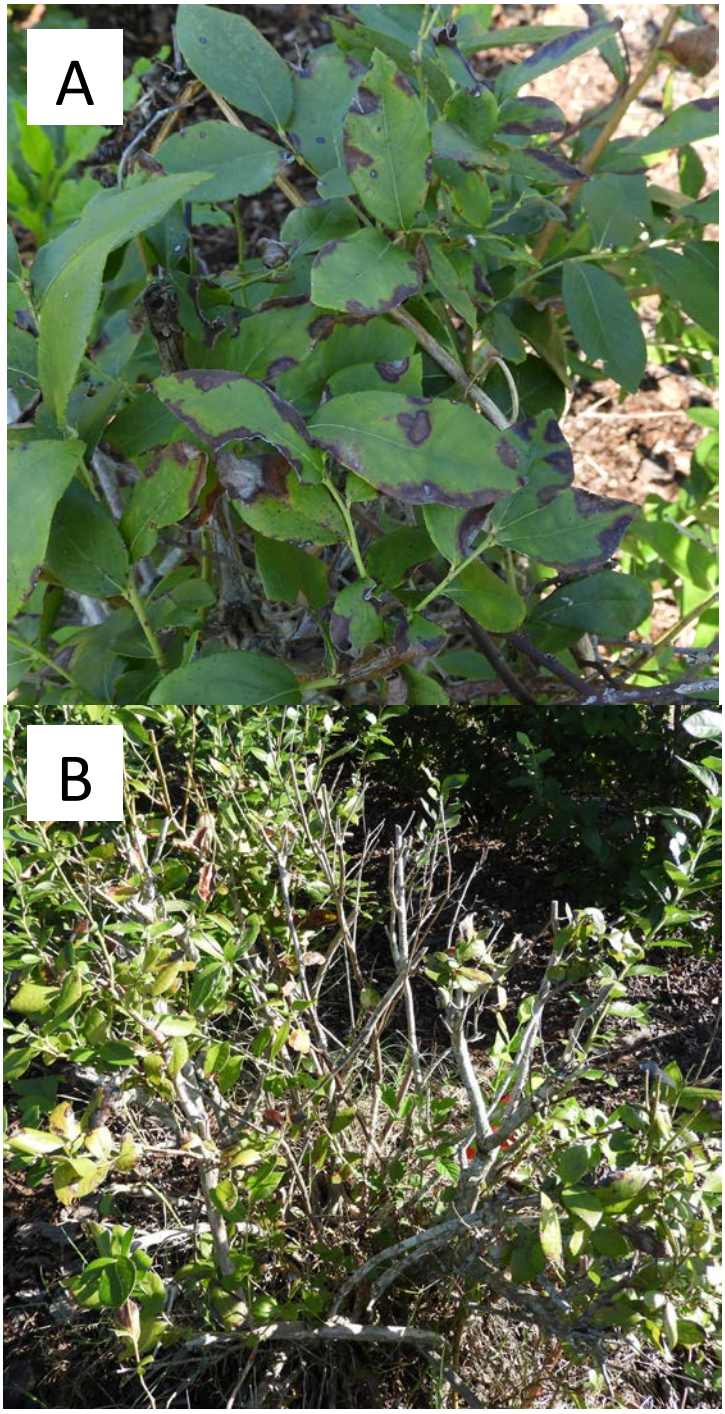

Figure 2.1. BLS symptoms observed during field survey. A. Leaf scorching symptoms and B. defoliation.

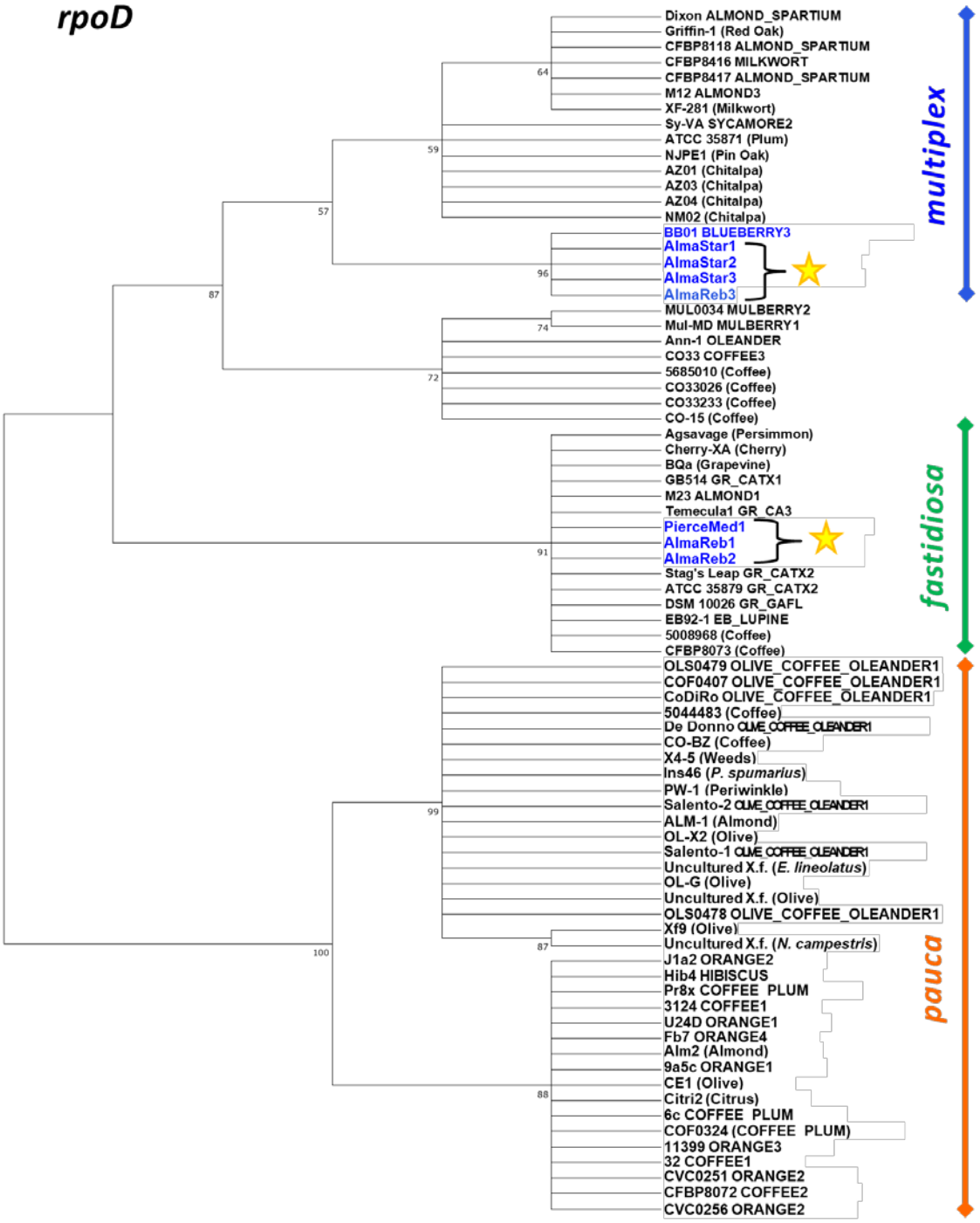

Figure 2.2. *rpoD* NJ phylogenetic tree showing unique haplotypes as well as individual isolates form this study. Branches were collapsed if bootstrap support <50%. Colored bars indicate major Xf subspecies groupings. Sequences from blueberry isolates are blue, and isolates from this work are indicated by a star.

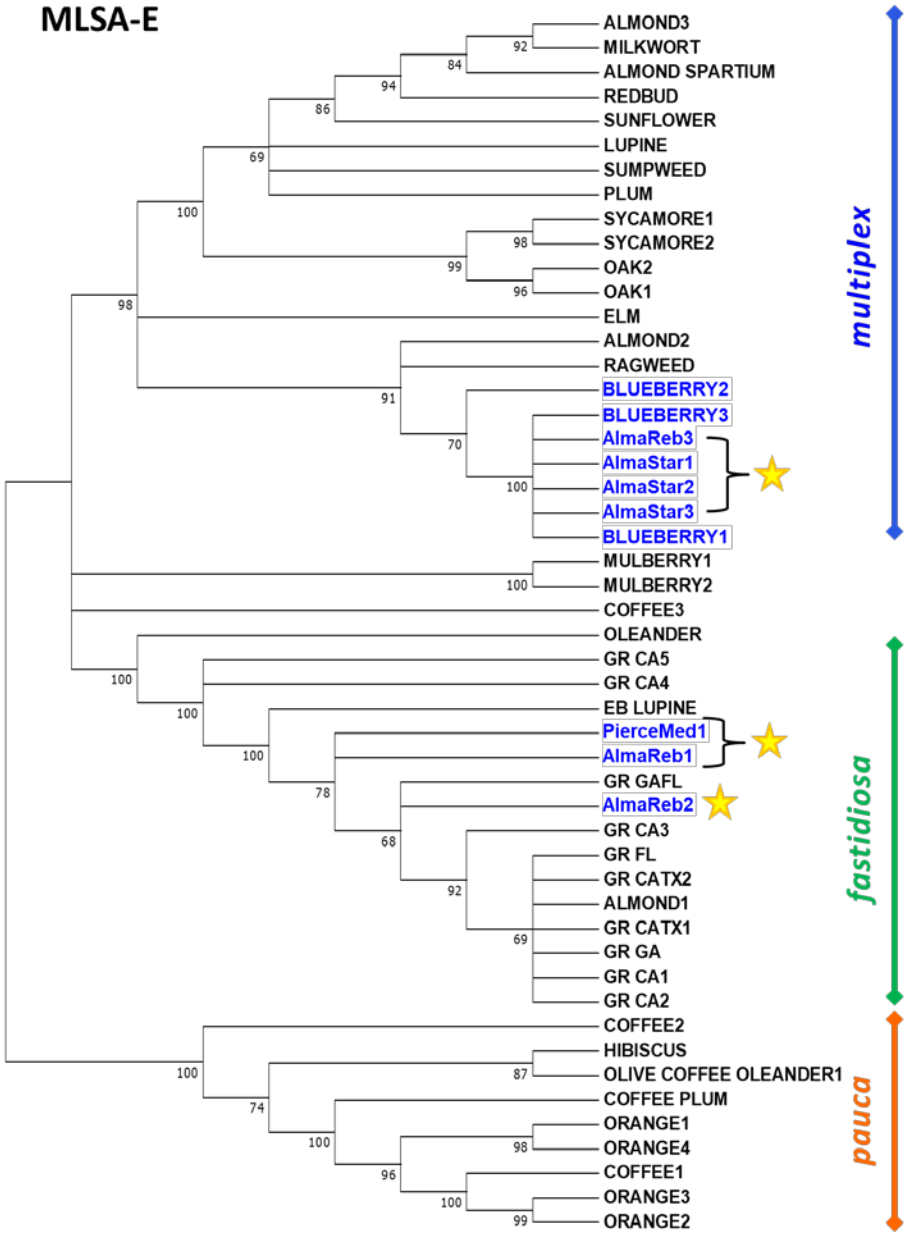

Figure 2.3. MLSA-E NJ phylogenetic tree showing unique Xf haplotypes as well as individual isolates from this study. Branches were collapsed if bootstrap support <50%. Colored bars indicate major Xf subspecies groupings. Sequences from blueberry isolates are blue, and isolates from this work are indicated by a star.

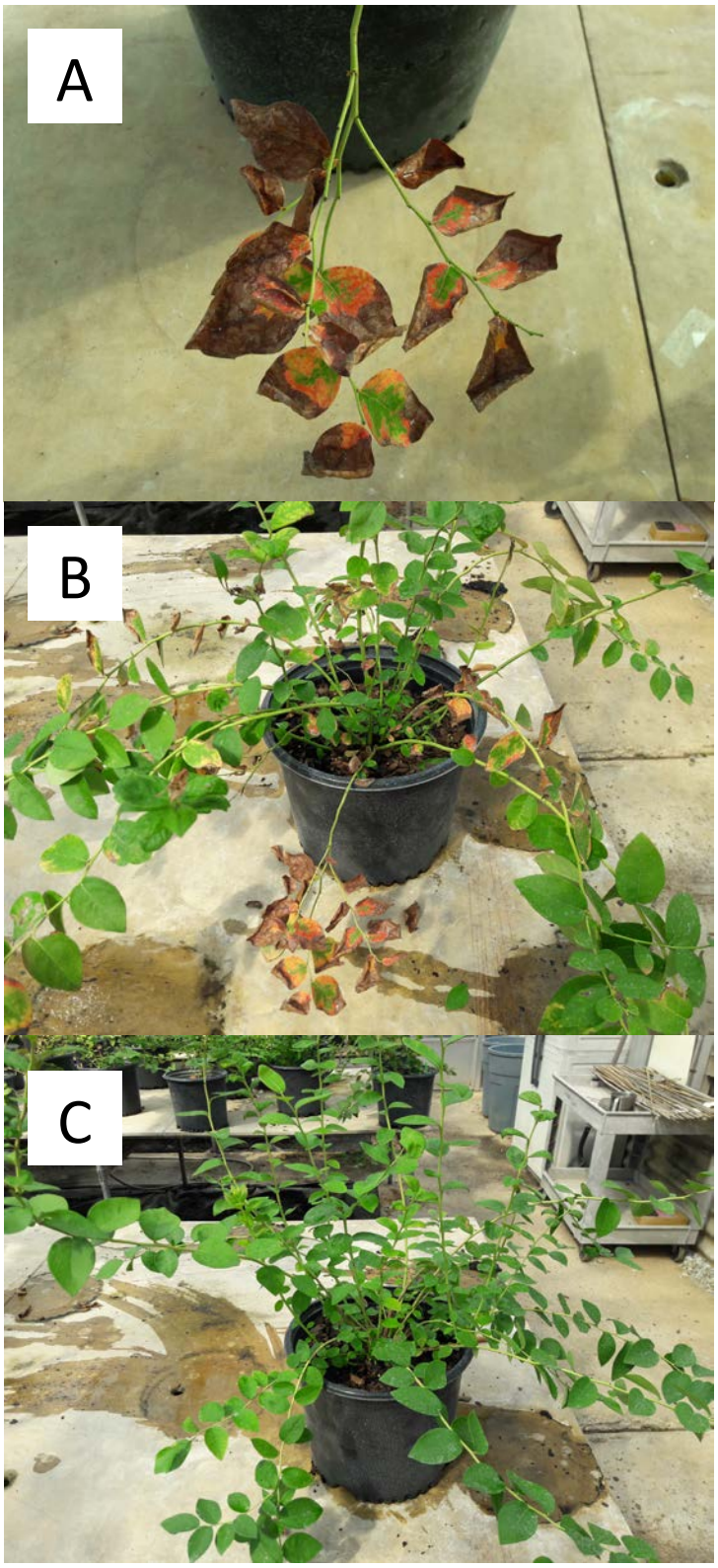

Figure 2.4. BLS symptoms induced by Xff on SHB blueberry cv. ‘Rebel’ in the greenhouse. A. Leaf scorch symptoms observed 90 dpi (days post inoculation) with Xff isolate ‘AlmaReb2’, B. Plant at 90 dpi with ‘AlmaReb2’, C. Plant at 90 dpi with inoculation buffer only.

CHAPTER 3

CHARACTERIZING THE RELATIVE VIRULENCE OF *XYLELLA FASTIDIOSA* SUBSP. *FASTIDIOSA* AND *XYLELLA FASTIDIOSA* SUBSP. *MULTIPLEX* ISOLATES FROM NATURALLY INFECTED GEORGIA BLUEBERRY IN GREENHOUSE CONDITIONS[1]

[1]Di Genova, D., and Oliver, J. E. 2019. To be submitted to Plant Disease.

Abstract

Xylella fastidiosa (Xf) is a xylem-limited bacterium capable of infecting multiple perennial fruit trees like grapevine, citrus, peach and almond. Significant epidemics have emerged recently on new susceptible hosts, including blueberry where Xf causes bacterial leaf scorch (BLS) primarily on southern highbush (SHB) blueberry varieties (*Vaccinium corymbosum* interspecific hybrids). Though isolates of Xf subsp. *multiplex* (Xfm) were formerly believed to be the only cause of BLS, recent findings have shown the presence of novel, genetically distinct isolates from Xf subsp. *fastidiosa* (Xff) in naturally infected blueberry plants in southeastern Georgia (USA). Little is known about the relative virulence of Xfm isolates versus these Xff isolates from naturally infected blueberry. To evaluate the relative virulence of several distinct genetic variants of Xff and Xfm from blueberry, greenhouse trials were carried out. Assessments were performed in three greenhouse experiments by artificially inoculating SHB blueberry cultivar 'Rebel' with Xfm and Xff isolates, respectively. Novel Xff isolates from blueberry were comparable in virulence to Xfm isolates in terms of ability to infect SHB blueberry cv. 'Rebel', symptoms produced, and timing of symptom development after infection. Results indicated that isolates from both Xfm and Xff have the potential to impact blueberry production in the southeastern US, and isolates from both subspecies should likely be used in resistance breeding efforts.

Introduction

Bacterial leaf scorch (BLS) is a significant disease affecting blueberry, primarily southern highbush (SHB) blueberry cultivars (*Vaccinium corymbosum* interspecific hybrids) in the southeastern US. BLS is caused by *Xylella fastidiosa* (Xf), a xylem-limited vector-transmitted bacterium capable of infecting a wide range of hosts in the plant world (Brannen et al. 2016; Chang et al. 2009; Hopkins and Purcell 2002). Much like other Xf-induced leaf "scorch" diseases, including Pierce's disease in grapevine, almond leaf scorch in almond or oleander leaf scorch in oleander (Janse and Obradovic 2010), symptoms typically associated with BLS of blueberry include a marginal leaf scorch with chlorosis or reddening of affected leaves. Often, the marginal necrosis may be bordered by a thick dark band separating the dead and healthy tissue. Symptoms may be either limited to single stems (especially in early disease stages) or uniformly distributed on the whole bush, likely dependent on the distribution of the infection throughout the xylem vessels of the plant. After initial scorching, premature leaf drop occurs, leaving bare thin twigs with a striking "skeletal", yellowish appearance. At later disease stages, stem dieback and whole wilting of the plant occur, often resulting in plant death in a matter of one to two years (Brannen et al. 2016; Chang et al. 2009; Harmon and Hopkins 2009).

The causal agent of BLS, Xf, is a versatile phytopathogenic bacterial species known to cause a range of diseases of economically important agricultural crops and ornamentals, especially in North America (Almeida et al. 2019). First and foremost, it is part of a complex pathosystem in which the bacterium itself, its hosts, its vectors and environment come together in a diverse interaction that results in disease that varies case by case (EFSA 2018; Jeger and Bragard 2019). Xf has been extensively studied for established, historically well-known diseases such as Pierce's disease on grapevine in North America or citrus variegated chlorosis on citrus in

South America. Nonetheless, the biotic and abiotic factors involved with the genetics, epidemiology and environmental interaction mechanisms of this bacterium are a long way from being satisfactorily accounted for (Daugherty and Almeida 2019). In fact, as time progresses, the emergence of the pathogen outside of the Americas (such as in the case of Europe - Italy, France and Spain) and its emergence on new crops (such as with BLS of blueberry) have revealed new aspects of Xf pathogenicity (Purcell 2013; Sicard et al. 2018). Unsurprisingly, whereas Xf was formerly known as a "relatively obscure plant pathogen" (Hopkins and Purcell 2002) restricted to only a handful of economically important hosts within a relatively large, yet well-defined region of the world, Xf is now regarded as a major, emerging global threat to plant health worldwide (Almeida et al. 2019; Almeida and Nunney 2015). A common framework of effective management strategies for Xf-induced diseases is likely to ultimately encompass a multitude of tactics exploiting diagnostic methods, chemical and biological control, breeding for host resistance, cultural practices and vector control; however, such a framework does not exist, as there are many knowledge gaps yet to be addressed regarding Xf epidemiology (Almeida et al. 2019; Saponari et al. 2019). As stated previously, such knowledge gaps are exacerbated by the constant appearance of new susceptible hosts and new vectors which often translates into emergence of new diseases; therefore, research efforts focusing on these situations are critical steps not only for disease control on a specific crop of interest, but also for contributing to an enhanced understanding of Xf from an interdisciplinary standpoint. In the case of blueberries, the aforementioned BLS disease is one of the several, relatively new challenges posed by Xf. Unsurprisingly, very little information is available on the bacterium affecting this specific crop, and current research efforts haven't been enough to be translated successfully into any effective management strategies capable of countering this new threat.

BLS was first observed in 2004 on blueberry in southern Georgia (USA), and was confirmed in 2006 to be caused by Xf (Chang et al. 2009). Not too long afterwards, BLS was first reported on blueberry in Florida as well (Harmon and Hopkins 2009), and has now been confirmed elsewhere in the southeast including Louisiana (Ferguson et al. 2017). Cultivars of SHB blueberry are generally considered to be most susceptible to the disease, showing acute symptoms in most cases. Varieties of rabbiteye blueberry (*Vaccinium virgatum* syn. *Vaccinium ashei*) have also been shown to be able to sustain Xf infections without symptoms (Brannen et al. 2016), but significant yield losses associated with BLS in rabbiteye blueberry have been reported in Louisiana (Ferguson et al. 2017). Variable degrees of susceptibility to infection have been observed between different SHB cultivars and further investigation of varietal resistance and tolerance has been recommended as a promising research focus for plant breeders and pathologists (Brannen et al. 2016; Brannen et al. 2008; Chang et al. 2009).

Xf is vectored by a seemingly large group of xylem sap-feeding leafhoppers and spittlebugs (Purcell et al. 1979). The most common species present in blueberry plantings in the southeastern US is the glassy-winged sharpshooter (*Homalodisca vitripennis*), and it is believed to be the most likely vector of Xf in blueberry fields; however, it showed negligible differences in settling behavior and preferential attraction to different cultivars of SHB blueberry tested (Tertuliano et al. 2012). Several isolates of Xf have been obtained and genetically characterized from different hosts in natural field conditions, including blueberry. The systematic taxonomy of Xf has identified at least four genetically distinct subspecies (subsp. *fastidiosa*, subsp. *multiplex*, subsp. *pauca*, subsp. *sandyi*) (Scally et al. 2005; Schaad et al. 2004), with blueberry isolates typically belonging to subsp. *multiplex* (Parker et al. 2012). This has led to the belief that the distinct groups of isolates within each subspecies differ in their ability to infect plant hosts. For

example, *X. fastidiosa* subsp. *fastidiosa* (Xff) is commonly known to cause Pierce's disease on grapevine while *X. fastidiosa* subsp. *pauca* (Xfp) is typically regarded as the causal agent of citrus variegated chlorosis; the Xff subspecies is unable to infect citrus and the Xfp subspecies is unable to infect grapevine (Hopkins and Purcell 2002; Janse and Obradovic 2010). Even though host specificity mechanisms are far from being understood and fully demonstrated in the case of Xf, within this framework, cases of cross-infection caused by different subspecies on the same host are rare, if not reported at all (Almeida and Purcell 2003). While it is arguable that genetic differences may influence the host specificity of Xf (Killiny and Almeida 2011; Van Sluys et al. 2003), many other complex factors related to the host and environment that impact the pathogen virulence are likely involved in the actual emergence of Xf-induced diseases (Almeida and Nunney 2015; Chatterjee et al. 2008; Hopkins and Purcell 2002).

While Xff is widespread in the southeastern US, until recently (Di Genova et al. 2019) all isolates obtained from blueberry with evident BLS symptoms have been identified as Xfm. Other isolates within the Xfm subspecies have been historically known in the region to cause phony peach disease in peach, plum leaf scald in plum and pecan leaf scorch in pecan (Cochran et al. 1951; Sanderlin and Heyderich-Alger 2000; Wells et al. 1983); however, isolates from blueberry appear to belong to a distinct clade within Xfm. Recently Xff isolates were successfully cultured from symptomatic leaves of blueberry plants naturally infected with BLS (Di Genova et al. 2019). Genetic characterization of multiple loci of these new isolates revealed three different haplotypes from both Xfm and Xff (Di Genova et al. 2019). The exact origin of these novel blueberry genotypes of Xf remains unknown; however, previous phylogenetic work has formulated the hypothesis regarding the possibility of inter- and intra-subspecific recombination between isolates, causing new genetic variation and resulting in a shift to new hosts (Nunney et

al. 2014a; Nunney et al. 2014b). A previous study revealed that Xff isolates from grapevine and elderberry can cause symptoms on SHB blueberry in the greenhouse, albeit with significantly less virulence than Xfm isolates from blueberry (Oliver et al. 2015). Furthermore, virulence differences exist even among blueberry isolates (Oliver et al. 2015), and, at the same time, cultivars of SHB blueberry seem to vary in susceptibility to Xf infection, showing variable levels of disease incidence in field conditions (Brannen et al. 2008).

Characterizing the relative virulence and host range of Xf isolates at our disposal is one of the next logical steps in achieving a better understanding of the potential of this pathogen to cause disease (Oliver et al. 2015). From this perspective, the recent identification of Xff isolates from naturally infected blueberry makes this objective even more compelling. Further *in planta* testing for virulence expression of such isolates showing different phylogenetic profiles with subtle molecular variability across multiple genes, even within the same subspecies of Xf, is necessary to understand their true impact in the emergence of BLS (Di Genova et al. 2019; Parker et al. 2012). In this study, we artificially inoculated SHB blueberry plants of one cultivar over three greenhouse trials to evaluate the infection capabilities and virulence of novel Xff isolates obtained and characterized previously by Di Genova et al. (2019), alongside other Xfm isolates originally isolated from naturally infected SHB blueberry plantings in south Georgia.

Materials and Methods

Xf isolates. Five Xf isolates (‘AlmaReb1’, ‘AlmaReb2’, ‘AlmaReb3’, ‘AlmaStar1’, and ‘PierceMed1’) were screened for virulence differences across three greenhouse experiments (Table 3.1). Among these five isolates, two were Xfm (isolates ‘AlmaReb3’ and ‘AlmaStar1’) while the other three were Xff (isolates ‘AlmaReb1’, ‘AlmaReb2’, and ‘PierceMed1’) which

were all isolated from SHB blueberry in Georgia (Di Genova et al. 2019). The three Xff isolates had been previously characterized according to MLSA-E and were found to group into two new unique haplotypes (Di Genova et al. 2019). About 20 days prior to plant inoculations, all isolates were plated on periwinkle wilt (PW) from glycerol stocks prepared previously for storage at -80°C. Plated isolates were grown at 28°C and the biomass was transferred once onto new plates after 10 days, and finally scraped and suspended in a succinate-citrate-phosphate buffer according to the protocol previously described (De La Fuente et al. 2013). Cell concentrations were standardized at approximately 10^8 cells/ml across all bacterial suspensions as previously described by Oliver et al. (2015).

Greenhouse setup and plant inoculations. Inoculation trials were carried out based upon previous protocols (Chang et al. 2009; Oliver et al. 2015). Specifically, for each greenhouse trial, blueberry plants of SHB blueberry cultivar 'Rebel' [PPA18, 138: (NeSmith 2008)] originally grown from tissue culture and obtained from Agri-Starts (Apopka, FL) were used. In addition, in parallel to the third experimental replicate with 'Rebel', SHB cultivars 'Emerald' and 'Farthing' were also inoculated with Xf (described in Appendix B). Plants were transplanted into 9.5 liters and 25.7 cm (diameter) pots filled with a soil mixture of one part sand and three parts pine bark mulch. Plants were allowed to grow for 30 days prior to bacterial inoculum delivery. Plants were maintained in controlled conditions in the greenhouse at 28°C-35°C under natural sunlight, watered regularly as needed, and fertilized with granular slow-release fertilizer (Osmocote® Smart Release® Plant Food Plus Outdoor & Indoor 15-9-12, The Scotts Company, Marysville, OH) at the manufacturer's recommended rate for indoor use. Plants were initially arranged in a randomized block design with treatments corresponding to isolate used for inoculation. Each plant was inoculated using a 1-ml tuberculin syringe. The two largest stems

were pricked just above the soil line, and a droplet of bacterial suspension in succinate-citrate-phosphate buffer was placed on the hole to allow for xylem uptake as previously described by Chang et al. (2009). Seven replicate plants received each isolate treatment in each trial, and an inoculation buffer only and no-buffer controls were used as control treatments. The first inoculation of each plant was considered to be DAY_0 for the experiment and all references to days post inoculation (dpi) refer to this initial inoculation. Two weeks after the first inoculation, to ensure infection, each plant was reinoculated (in the same manner as before) with the same isolate it had received previously. For trial #1, 2, and 3, respectively, plants were first inoculated on Feb 6th, 2018, Nov 2nd, 2018 and May 24th, 2019.

Disease development and symptom scoring. After inoculation, plants were maintained in the greenhouse for 5 months and monitored for symptom development. Following the appearance of initial symptoms of BLS (marginal and veinal chlorosis or reddening), plants were scored weekly for disease development until the conclusion of each trial at 140 dpi. Symptoms were assessed visually according to the severity rating scale published previously by Oliver et al. (2015). Scored symptoms for BLS included leaf color changes (from yellowing to reddening to necrosis/heavy scorching), number of stems bearing symptomatic leaves, and overall symptom spread throughout the leaves of affected stems. At the conclusions of each trial, severity scores at each time point were used to calculate the Area Under Disease Progress Curve (AUDPC) for each plant based on the midpoint rule method (Campbell and Madden 1990). For Xf-inoculated plants, only Xf-infected plants (as determined by conventional PCR testing) were included in the analysis. Statistical analysis for all trials was performed in the R language for statistical computing (Ihaka and Gentleman 1996). ANOVA was used to summarize AUDPC results for all

three trials. For this dataset, significant differences between treatments were determined using Fisher's LSD test at $P < 0.05$.

Plant testing by PCR. Following symptom appearance and development, leaf samples were collected from each plant and tested for the presence of Xf. Three lower leaves were collected at 75, 105, and 135 dpi, which correspond to ~15, 45 and 75 days after first symptom appearance, respectively. Leaves 1, 3, and 5 from above the inoculation point were collected at 75 dpi and leaves 2, 4, and 6 were collected at 105 dpi. Leaves from the same plant and collection day were pooled into single samples. At 135 dpi three lower leaves were collected from control plants only to verify that these plants remained free of Xf throughout each trial. Total DNA extraction from leaf tissues was performed according to a modified cetyltrimethylammonium bromide (CTAB) protocol adapted for blueberry leaves (Christiano and Scherm 2011; Doyle and Doyle 1987). Extracted DNA samples were tested by means of conventional PCR using the RST31/33 Xf-specific primer pair, targeting the *rpoD* locus (Minsavage et al. 1994). PCR was setup with reagents and reaction conditions according to the same parameters previously described (Di Genova et al. 2019). Amplified products were loaded on a 1% agarose gel and examined by electrophoresis to check for Xf-positives according to a protocol previously described (Di Genova et al. 2019). Plants with detectable Xf amplicons using primer pair RST31/33 were considered to be infected with Xf.

Results

PCR confirmation of *X. fastidiosa* infection. Based upon PCR testing results, all Xf isolates examined were capable of infecting SHB cultivar 'Rebel'. Extraction and testing of leaf DNA by Xf-specific PCR revealed a high percentage of infection with all isolates across all three

replicate trials. The percentage of plants inoculated that were confirmed to be infected with Xf ranged from 75% (16/20 plants) with isolate 'PierceMed1' to 100% (20/20 plants) with isolate 'AlmaReb3' (Table 3.2). Most of the plants were confirmed by PCR to be infected with Xf at the first round of leaf sampling at 75 dpi (data not shown). In all three replicate trials, all plants from the two control treatments (buffer and no-buffer) remained free from Xf infection based upon PCR testing of leaves collected at 75, 105, and 135 dpi.

Disease progression and symptoms caused by *X. fastidiosa* isolates. In all three replicate trials, the first symptoms of BLS were observed between 57 and 62 dpi. Initial symptoms would often start as a faint yellow or red discoloration appearing mostly along either the veins or margins on the upper leaf surface, generally on those leaves closer to the inoculation point on the two originally inoculated stems (Fig. 3.1 A., B. and C.). Discoloration would then change to bright red and then into necrosis or leaf scorch, often with a thick dark line between the necrotic area and healthy tissue sometimes in a typical "oak-leaf" pattern before expanding to include the whole leaf (Fig. 3.1 D., E. and F.). As the disease progressed, symptoms generally spread first to other leaves throughout the entire length of the initially affected stems and eventually to uninoculated stems in the most severe cases before the end of each trial. The most severely affected stems and plants would show defoliation, stem dieback, and plant decline before the conclusion of each trial (Fig. 3.1 G., H. and I.). Generally, symptoms and their observed progression resembled what had been previously described in other studies with artificially inoculated blueberry plants in greenhouse conditions (Chang et al. 2009; Oliver et al. 2015). Occasionally, a yellowed or necrotic leaf was noted on the control plants, however, in all cases for the control plants, this did not progress and based upon the negative PCR testing results was likely just due to physical damage or nutritional issues rather than to disease. Nonetheless,

these minor “symptoms” were scored, due to their superficial similarity to the initial stages of BLS on blueberry.

All isolate treatments caused symptoms typical of BLS; however, differences between isolate treatments were visually observable in terms of symptom severity and progression (Table 3.3). Across all three replicate trials, treatments receiving isolates ‘AlmaReb3’ and ‘AlmaReb2’ were consistently among the most severely affected plants which showed the earliest severe BLS symptoms compared to the other isolate treatments. By contrast, isolates ‘AlmaReb1’ and ‘PierceMed1’ generally produced the least severe symptoms after infection, while ‘AlmaStar1’ performed somewhat in between the most severe and least severe isolates.

Discussion

In this study, we demonstrated that Xff isolates from naturally infected blueberry are comparable in virulence to Xfm isolates from blueberry in three replicate greenhouse experiments. Previously, Di Genova et al. (2019) had conclusively shown that three novel Xff isolates originally obtained in natural field conditions from symptomatic SHB blueberry plants were pathogenic on blueberry under greenhouse conditions by fulfilling Koch’s postulates; however, the relative virulence of the novel Xff isolates in comparison to more commonly identified Xfm isolates from blueberry was not determined at that time. Prior work by Oliver et al. (2015) with Xff isolates from grapevine and elderberry had indicated that Xff isolates can cause BLS symptoms on blueberry under greenhouse conditions, but in that study the Xff isolates used were significantly less virulent than most typical Xfm isolates from blueberry, suggesting that they may be less adapted as pathogens of that host. The results observed in the current study stand in stark contrast to the previous work by Oliver et al. (2015) and suggest that

the novel Xff isolates found on blueberry by Di Genova et al. (2019) are capable pathogens on blueberry in terms of their relative virulence versus Xfm isolates.

Based upon our observations, the timing of first symptom appearance was relatively similar across trials (57-62 dpi) and Xf isolates treatments. However, symptoms caused by more severe isolates such as 'AlmaReb3' and 'AlmaReb2' were more likely to occur consistently across all plants during that time while first symptom appearance with less severe isolates was somewhat more variable across inoculated plants. Therefore, the differences in AUDPC between the most and least severe groupings (i.e. 'AlmaReb2' and 'AlmaReb3' versus 'AlmaReb1' and 'PierceMed1') were largely due to the rate at which symptoms progressed after appearance during the 80 to 90 days before each trial concluded, rather than to large differences in the timing of initial symptom appearance per isolate. It is not known what would have happened if single trials had been continued for additional months or years, and it is possible that all isolates may have resulted in similarly severe symptoms or in plant death after a longer defined period. Generally, detectable Xf infection (based upon PCR) was tightly correlated with symptom occurrence, though one inoculated plant in each of the three greenhouse trials did develop symptoms despite no detection of Xf by PCR at 75 or 105 dpi. This was likely a "false negative" PCR result.

In terms of both the timing of the first symptom appearance in the greenhouse as well as the overall severity of symptoms observed in our experiment, Xff isolate 'AlmaReb2' and Xfm isolate 'AlmaReb3' were of comparable virulence in all three replicate trials. By contrast, Xfm isolate 'AlmaStar1' and Xff isolates 'AlmaReb1' and 'PierceMed1' were in a less virulent group in the three trials. Taken together, this suggests that based solely upon subspecies, Xff isolates cannot be distinguished from Xfm isolates based upon their virulence on blueberry in this study.

Within subspecies, it should be noted that ‘AlmaReb1’ and ‘PierceMed1’ are the same genetic haplotype based upon multi-locus sequence typing and they also performed similarly in terms of observed virulence, whereas isolate ‘AlmaReb2’ was a different haplotype within Xff than these two isolates and resulted in significantly more severe symptoms after infection. By contrast, ‘AlmaReb3’ showed relatively higher virulence than ‘AlmaStar1’ in our experiment, despite these two being the same multi-locus sequence typing haplotype. Since multi-locus sequence typing is based upon a small number of genes, it is possible (and quite likely) that isolates of the same haplotype may have genetic differences which differentially impact isolate virulence that are outside of those regions used for multi-locus sequence classification.

Based upon the resulting symptom severity in the greenhouse, our experiment suggested that the Xff isolates examined here may be better adapted to blueberry than Xff isolates examined previously by Oliver et al. (2015); however, it should be noted that other factors involved in Xf virulence remain unaccounted for in greenhouse settings. Several environmental factors as well as plant age were held constant in the greenhouse, and these factors are likely to impact initial infection probabilities as well as observed symptom severity in the field. We also did not examine the relative cell abundance or replication (using quantitative PCR) of each isolate within their host plant, which could have implications for symptom development after the 5-month period of study used here. In addition, the use of artificial inoculations would tend to eliminate differences between Xf strains in terms of their ability to be picked up and transmitted from plant to plant by insect vectors. As pointed out by Oliver et al. (2015), the pinprick method used to artificially inoculate plant stems (performed twice in each experimental replicate) virtually ensures direct delivery of bacterial cells and potentially makes successful infection (and therefore symptom expression) much more likely. In the field, insect vectors are responsible for

moving the pathogen from plant to plant, and differences in vector transmissibility both within and between species may ultimately account for the apparent differences in prevalence of these two subspecies on blueberry.

Previously, genetic characterization studies of several isolates have been conducted to investigate and differentiate the epidemiology of Xf across the many hosts it infects. Multi-locus sequence analysis methods have been used to clarify the taxonomy of Xf across subspecies (Scally et al. 2005; Schaad et al. 2004), and more recently to understand Xf evolutionary history and possible relationships between its subspecies and intersubspecific recombinants (Nunney et al. 2013; Nunney et al. 2010; Parker et al. 2012; Yuan et al. 2010). The isolates used in this study were characterized initially by Di Genova et al. (2019) using a multi-locus sequence typing approach. Isolates 'AlmaReb1' (Xff), 'AlmaReb2' (Xff), and 'AlmaReb3' (Xfm) used in this study were all isolated from the same blueberry planting in Alma, Georgia in 2017 (Di Genova et al. 2019); nonetheless, they represent two distinct subspecies of Xf and three unique genetic haplotypes. The observed genetic variability between these isolates from the same host across and within two different species (Xff and Xfm) was associated with apparent differences in pathogenicity in this greenhouse study. Even though the genetic basis of Xf pathogenicity is poorly understood, further genetic characterization of these isolates could provide new insights into Xf virulence mechanisms and host adaptation and, in the long term, aid efforts to develop new management tools to combat Xf. Whole genome sequencing of these isolates would help to discover genetic differences beyond those limited differences observed through multi-locus sequencing approaches and allow for more advanced studies of Xf pathogenicity on blueberry.

Based upon our findings, Xff can cause significant BLS symptoms on SHB blueberry, and poses a risk to this important and profitable fruit crop. There are many unknowns regarding

BLS on blueberry already, and the presence of two destructive subspecies has the potential to further complicate management of this disease. Alternate hosts for Xf strains capable of causing BLS on blueberry are currently not known; however, given that Xfm and Xff subspecies can have different host ranges, it is possible that the source of blueberry infection for these two subspecies may differ, with potential implications for management. Host resistance to Xf in blueberry, which has not been actively explored through breeding efforts, would be an ideal management tool, but according to our findings breeding efforts must take into account the presence of genetically distinct Xf strains on blueberry. Experiments are currently underway to determine if differences exist between the responses of existing cultivars to these unique isolates.

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Table 3.1. Xf isolates used in the three replicate greenhouse trials.

Isolate[z]	Xf subsp.	Isolation Host
AlmaReb1	Xff	*Vaccinium* "Rebel"
PierceMed1	Xff	*Vaccinium* "Meadowlark"
AlmaReb2	Xff	*Vaccinium* "Rebel"
AlmaReb3	Xfm	*Vaccinium* "Rebel"
AlmaStar1	Xfm	*Vaccinium* "Star"

[z] Source for all isolates: Di Genova et al. 2019.

Table 3.2. PCR testing results for Xf-inoculated plants from the three greenhouse trials.

Treatment	Trial #1[z]	Trial #2[z]	Trial #3[z]	Total[z]
AlmaReb1	4/7 (57%)	7/7 (100%)	6/7 (86%)	17/21 (81%)
PierceMed1	4/6 (67%)	7/7 (100%)	5/7 (71%)	16/20 (75%)
AlmaReb2	7/7 (100%)	7/7 (100%)	5/6 (83%)	19/20 (95%)
AlmaReb3	6/6 (100%)	7/7 (100%)	7/7 (100%)	20/20 (100%)
AlmaStar1	7/7 (100%)	6/7 (86%)	6/7 (86%)	19/21 (90%)
Buffer	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/21 (0%)
No-buffer	0/7 (0%)	0/6 (0%)	0/6 (0%)	0/19 (0%)

[z] PCR-positive plants/total surviving plants (percentage). Seven plants were inoculated per treatment per trial. Totals less than seven indicate plants that died prior to testing.

Table 3.3. AUDPC values for each treatment in each trial and cumulative AUDPC values for all three trials.

Treatment	AUDPC[z]			
	Trial #1[y]	Trial #2[y]	Trial #3[y]	Total[x]
AlmaReb1	219.62 de	790.0 b	316.08 c	488.53 b
PierceMed1	274.87 cd	795.78 b	555.10 bc	590.34 b
AlmaReb2	735.28 b	1054.78 a	719.30 ab	848.79 a
AlmaReb3	1055.75 a	891.28 ab	925.07 a	952.45 a
AlmaStar1	479.42 c	695.25 b	719.67 ab	633.44 b
Buffer	24.857 e	8.08 c	15.93 d	15.02 c
No buffer	20.071 e	6.57 c	9.50 d	13.85 c

[z]For inoculated plants, AUDPC values are calculated from Xf-positive plants only, as defined by PCR results.

[y]Means in each column followed by the same letter are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

[x]Corresponds to the average AUDPC across all three experimental replicates.

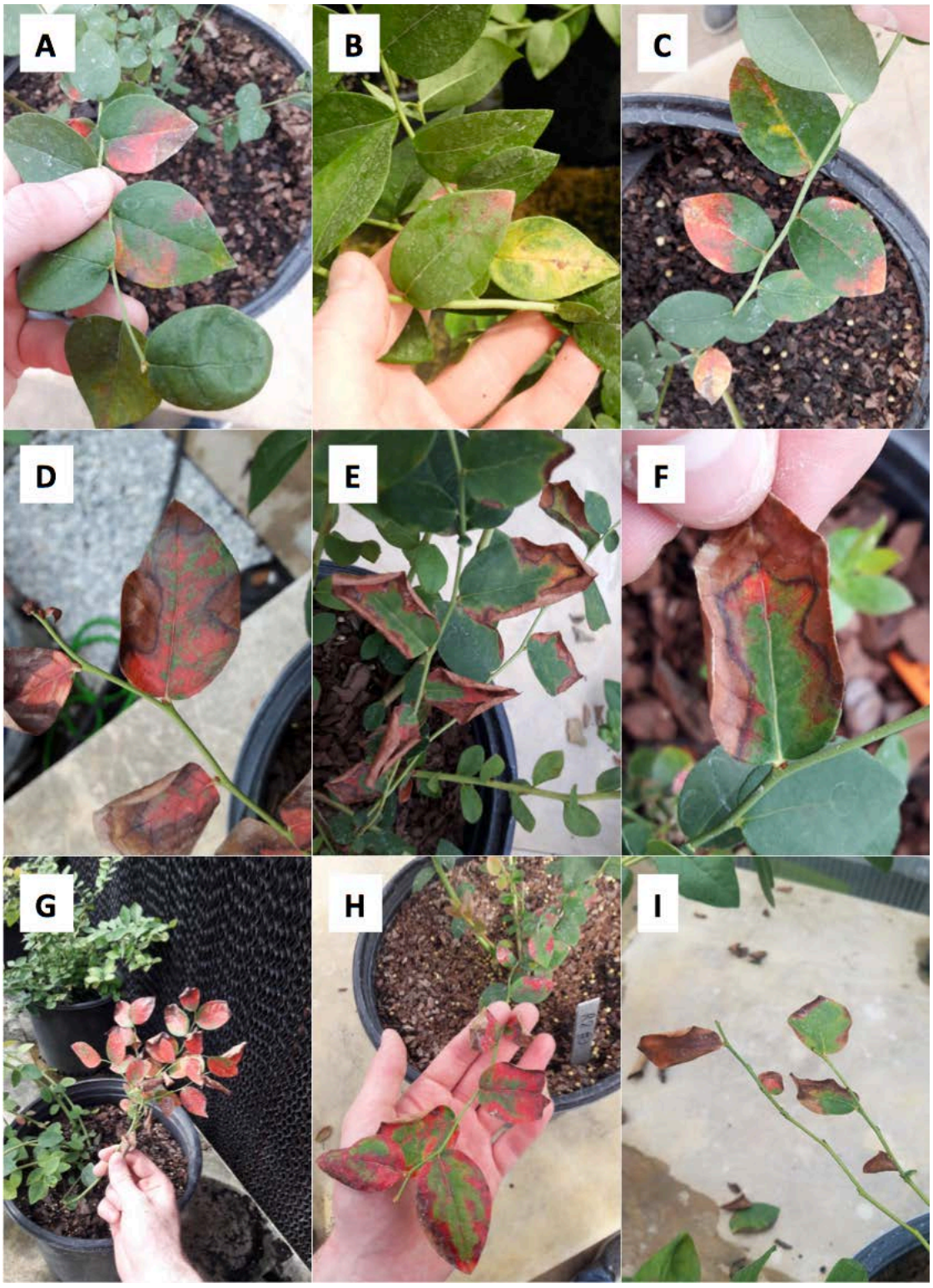

Figure 3.1. BLS symptoms observed during each greenhouse trial. A., B. and C. Initial faint discoloration and chlorosis on margins and veins of leaves located on the two originally inoculated stems. D., E. and F. Development of reddening followed by necrosis on leaf surfaces with development of typical dark band and "oak-leaf" pattern. G., H. and I. Symptom spread over the whole affected stem, with heavy scorching followed by defoliation.

CHAPTER 4

VISUAL ASSESSMENT OF BACTERIAL LEAF SCORCH SYMPTOM SEVERITY AND ITS ASSOCIATION WITH INCIDENCE AND YIELD IMPACTS OF *XYLELLA FASTIDIOSA* ON SOUTHERN HIGHBUSH BLUEBERRY IN GEORGIA, USA[1]

[1]Di Genova, D., Lewis, K. J., Smith, E. D., Severns, P. M., and Oliver, J. E. 2019. To be submitted to Plant Disease.

Abstract

Xylella fastidiosa (Xf) is an emerging vector-transmitted bacterial pathogen of significant concern, capable of infecting many economically important woody crops across the globe, including grape in North America, citrus in South America and olive in southern Europe. In recent years, Xf has been confirmed as the cause of bacterial leaf scorch of blueberry (BLS) in the southeastern US. This disease causes relatively rapid decline and death of susceptible southern highbush (SHB) blueberry cultivars. Currently, there are few practical management options for this disease on blueberry, and growers rely on the identification, removal, and replacement of symptomatic plants to limit losses. While initial investigations of Xf genetics, pathogenicity, and vector behavior have been performed on blueberry, many basic questions on its epidemiology, establishment, and dispersal dynamics in field conditions remain largely unanswered. To better understand the impacts and spread of BLS, SHB blueberry cultivars 'Rebel' and 'Star' were visually assessed for BLS symptom severity in plantings in Bacon County, Georgia, USA during Fall 2018 and 2019. Leaves were collected from these plants and tested for Xf via molecular testing. In addition, yield and fruit quality were assessed in Spring 2019 on a subset of plants assessed in 2018. Results of these assessments indicated an increase in observable disease severity over the two years, a significant association between visual assessment and Xf incidence, and a measurable impact of BLS disease on yield. These results provide preliminary information regarding the usefulness of visual assessment for the identification of Xf infection and implementation of management strategies in blueberry.

Introduction

Plant diseases caused by the bacterium *Xylella fastidiosa* (Xf) are well documented for several perennial crops (e.g. peach, plum, pecan) in the southeastern US (Sanderlin and Heyderich-Alger 2000; Wells et al. 1983). Within this geographic area, Xf is believed to be endemic and favored by warm climatic conditions and an abundance of vectors, namely leafhoppers (family Cicadellidae), particularly the glassy-winged sharpshooter (*Homalodisca vitripennis*), and spittlebugs (family Cercopoidea) that contribute to its survival (Hopkins and Purcell 2002). Over the last decade, a new disorder called bacterial leaf scorch (BLS) of blueberry was first reported affecting the increasing acreage of southern highbush (SHB) blueberry (*Vaccinium corymbosum* interspecific hybrids) in Georgia and Florida and later confirmed to be caused by Xf (Chang et al. 2009; Harmon and Hopkins 2009). Similar to other "leaf scorching" diseases associated with Xf infections (e.g. Pierce's disease, almond leaf scorch, plum leaf scald, pecan leaf scorch), BLS symptoms are characterized by marginal leaf burn or necrosis, leaf drop, and overall plant decline that can be mistaken for drought and water stress issues (Brannen et al. 2016). BLS symptoms of blueberry become more obvious once a plant starts to defoliate, usually on a single or a few localized branches of the bush, leaving bare stems and twigs with a distinctive yellow color, before severe defoliation and overall plant death occur, typically within 1-2 years after first symptom appearance (Brannen et al. 2016; Chang et al. 2009).

A xylem-limited, obligately vector-transmitted phytobacterium, Xf is currently regarded as one of the most important emerging plant pathogens worldwide (Almeida and Nunney 2015; Hopkins and Purcell 2002; Janse and Obradovic 2010). Despite being known for more than a century as the "classic" causal agent of Pierce's disease (PD) in grapevine, first observed in

California in 1892 (Pierce 1892), major interest from the scientific community on the characterization of this bacterial species, its etiology, biology and epidemiology was not common until the second half of the 20th century. A devastating PD epidemic after the establishment in California of a new invasive vector, the glassy-winged sharpshooter (Blua et al. 1999; Gill 1995), the successful in vitro culturing of Xf (Davis et al. 1978), and the emergence of citrus variegated chlorosis (CVC) disease in South America (Chang et al. 1993) generated a significant amount of research efforts focused on Xf on grapevine and citrus. As a result, these two crops have been by far the most studied hosts when it comes to the Xf pathosystem (Chatterjee et al. 2008; Sicard et al. 2018), even though the bacterium is actually known to infect some 350 plant species, many asymptomatically (EFSA 2018). Other economically important crops affected by Xf include almond (almond leaf scorch), coffee (coffee leaf scorch), peach (phony peach disease), plum (plum leaf scald), pecan (pecan leaf scorch) and even shade trees and ornamentals such as oak, maple and oleander (Hopkins and Purcell 2002; Janse and Obradovic 2010; Purcell and Hopkins 1996).

Blueberry is among the most recent group of novel hosts found to be affected by Xf-induced diseases (Chang et al. 2009), along with pear (Leu and Su 1993), avocado (Montero-Astua et al. 2008), chitalpa (Randall et al. 2009) and olive (Saponari et al. 2013). Despite the fact that blueberries are one of the most profitable fruit commodities in the southern US (Brannen et al. 2016), there are many knowledge gaps related to BLS on blueberry. Market trends in the southeastern US have been recently characterized by an increasing transition from traditional rabbiteye blueberry (*Vaccinium ashei*) to SHB blueberry cultivars (Scherm and Krewer 2003) that are, apparently, quite susceptible to BLS (Brannen et al. 2016; Brannen et al. 2008). Preliminary surveys and genetic analysis of a small number of bacterial isolates have been

performed to try to understand this new disease on a new host in an area where the causal agent was known to be already widespread (Di Genova et al. 2019; Nunney et al. 2014a; Nunney et al. 2014b; Oliver et al. 2015). At the moment, effective BLS management options in blueberry plantings are limited, other than suggested methods to slow epidemics down through chemical control of vectors, propagation of Xf-free clean plant material, and 'roguing' (removing) diseased plants in the field (Brannen et al. 2016; Holland et al. 2014). Eradication and destruction of infected blueberry bushes probably remains the most encouraged operation to perform in the field, as diseased SHB blueberries seem to decline and die rather quickly (Brannen et al. 2016); however, information is lacking regarding the extent of the role that single infected blueberry plants play in harbouring the bacterium and contributing to its spread within a planting. Brannen et al. (2016) noted that BLS-symptomatic plants are scattered throughout the field and that newly symptomatic plants are frequently adjacent to where another one died in the previous season. To make matter worse, inoculum from outside the planting may be present, but alternate hosts have not been identified for Xf strains that infect blueberry. In addition, native rabbiteye blueberry, can carry the bacterium in some cases (Brannen et al. 2016). Ferguson et al. (2017) identified yield losses in rabbiteye blueberry due to Xf infection, though it was also noted that the pathogen didn't spread rapidly within the rabbiteye planting over the multi-year duration of the experiment. In any case, information and case studies showing the spread of BLS and its underlying phenomena are scarce and basic questions regarding the disease cycle and actual impact of Xf in blueberry in the southeastern US still need to be answered before integrated management strategies for BLS can be developed and implemented. A potential research area of focus for BLS management involves breeding for resistant cultivars (Brannen et al. 2016), as different levels of disease incidence have been observed among some SHB blueberry cultivars

(Brannen et al. 2008), and work is currently underway to translate artificial *in planta* Xf inoculation tests into screening for varietal resistance in field conditions (Brannen et al. 2008; Chang et al. 2009; Holland and Scherm 2012; Hopkins et al. 2012; Oliver et al. 2015).

Dynamics, factors and epidemiological variables affecting the rate of Xf spread within naturally infected plantings have not been investigated for several important crops including blueberry. An array of proposed control strategies have been put forward for management of Pierce's disease, as reviewed by Kyrkou et al. (2018), from strategies to prevent new infections of Xf (e.g. insect vector control, wild host plant control, cross-protection methods) to exclusion principles to keep the bacterium out of vineyards (e.g. bacteriophage and antagonistic microorganisms, natural antibacterial and plant-stimulating compounds). However, even in this well-studied system (grapevine), management of Xf is still a challenge.

Identification of key parameters determining the spread of Xf-induced diseases, regardless of the host crop of interest, is a daunting task. Despite renewed interest in Xf and its induced diseases in recent years, biological and ecological data are still not enough for reliable modelling schemes and forecasting of Xf epidemics (Almeida and Nunney 2015). Host-plant-vector interactions and host plant specificity mechanisms have not been elucidated consistently or linked yet with the amount of work investigating the genetics of Xf and its variability (Almeida and Nunney 2015; Chatterjee et al. 2008; Retchless et al. 2014). Jeger and Bragard (2019) highlighted how the complexity of Xf inevitably involves many different subtle components, including its wide range of host plants and vectors, and therefore proposed a modelling perspective that shares an epidemiological approach common to any vector-borne disease, mainly plant virus diseases. We add however that such an objective becomes even more complicated in the case of a susceptible, yet important crop like SHB blueberry, where little to

no prior work has been carried out to try to estimate BLS spread and its severity at the field scale. As such, we believe that important insight could be gained by studying natural spread of Xf and how it acts in specific situations within an infected cropped field. For this initial work, we carried out a preliminary multi-year assessment of BLS severity in SHB blueberry plantings naturally infected by Xf in southern Georgia, and investigated visual assessment of symptoms, Xf incidence, and yield impacts.

Materials and Methods

SHB blueberry site selection. Three commercial SHB blueberry plantings were selected for field work. These three plantings were located on two different farms near Alma, Georgia, in an intensive agricultural area devoted to the production of blueberries. Farms were located in close proximity to one another, separated by a local highway, and previously identified as having both symptoms of BLS and presence of Xf (Di Genova et al. 2019). Farm #1 consisted of two plantings both arranged in an East-West direction (3.5 m between rows) with approximately 100 to 140 bushes in each row (0.75 m between plants). The first planting consisted of 20 rows of SHB blueberry cultivar ‘Rebel’ and the second planting, which was located immediately south of the first planting, consisted of 26 rows of SHB blueberry cultivar ‘Star’ (Fig. 4.1 A.). The ‘Star’ and ‘Rebel’ plantings were initially established in 2015, meaning that plants were 3 years old at the start of our work in 2018. Farm #2 consisted of multiple high-density rectangular plots (75 m by 10.5 m), adjacent to one another and separated by grass strips. Each high-density plot consisted of multiple parallel small rows arranged in a South-North direction (0.9 m between plants by 1.5 m between rows), with 10 to 14 ‘Rebel’ bushes per row (Fig. 4.1 B.). Both farms were managed with conventional practices and located on a sandy soil using raised beds that

were mulched with pinebark. Plantings in Farm #1 were drip-irrigated, while Farm #2 was irrigated using overhead sprinklers. Both farms utilized hand-harvesting in early spring, from early to late April.

Severity assessment scale. To evaluate BLS symptom severity, we employed a 4-tier severity scale (Table 4.1 and Fig. 4.2). A symptom severity ranking was assigned to each plant in each surveyed row/block according to visual evaluation of BLS symptoms. Rank "0", "1", "2" and "3" were assigned for healthy, lightly affected, moderately affected or severely affected plants, respectively. 'Healthy' ("0") indicated a plant that was non-symptomatic and visually sound while 'light' ("1") indicated the presence of leaf scorch or defoliation symptoms resembling BLS on a single isolated twig. 'Moderate' ("2") indicated a plant with at least two twigs with scorching and/or defoliation typical of BLS. 'Severe' ("3") indicated a plant where the majority of the stems were characterized by heavy leaf scorching and defoliation. Finally, dead bushes (i.e. with no visible leaves) were marked as such and were not evaluated in either year. Very young plants, recognized by being very small relative to the other bushes, were assumed to be replants and were not evaluated.

Severity assessments for Farm #1 and Farm #2. To evaluate BLS symptoms on plants for both 'Rebel' and 'Star' cultivars located in Farm #1, three rows per variety were randomly selected: rows #6, #13 and #17 within the 'Rebel' planting and rows #2, #7 and #26 within the 'Star' planting (Fig. 4.1 A.). In total, the three selected 'Rebel' rows included 364 plants, and the three selected 'Star' rows included 401 plants. Since the 'Rebel' planting located at Farm #2 was arranged in high-density beds, two of these beds, designated "Block 2" and "Block 3" were chosen. Within Block 3, a rectangular area of ten rows (row #1 to #10) consisting of 117 total plants was selected, and within Block 2 a rectangular area of ten rows (row #11 to #20)

consisting of 110 total plants was selected, for a total of 227 bushes (Fig. 4.1 B.) at farm #2. During September 2018 and again in September 2019, all plants within those rows and blocks were visually assessed for BLS symptoms and scored according to the 4-tier severity scale described above.

Sampling for molecular analysis within Farm #1. Following symptom evaluation, in order to test plant tissue for Xf infection, two to three shoots bearing leaves with visible scorching symptoms (or no symptoms in the case of asymptomatic plants) were sampled from at least five randomly selected plants from each severity rank, from each row (when available). Plants that were sampled in 2018 were resampled in 2019 for year to year comparisons on incidence on the sampled plants. In addition, new plants were randomly selected to represent each severity rank in 2019. Sampled plants were numbered and marked with flags to allow for resampling and return to each sample plant the following year.

Sampling for molecular analysis within Farm #2. For molecular analysis of plants within Farm #2, a different sampling scheme was employed in 2018 versus the scheme used for Farm #1. Sampling in 2018 from Farm #2 included five randomly chosen plants that had been rated as 'severe' ("3") within each block, followed by sampling of the 4 surrounding plants for each of these 10 plants (4 x 10 = 40 total plants), giving a final total of 50 plants sampled for testing from Farm #2 in 2018. For 2019, Farm #2 was sampled in the same manner as Farm #1, where five plants were randomly selected from each severity rank from each block. Symptomatic or asymptomatic shoots and leaves were sampled in the same way as described for Farm #1.

Molecular testing of field samples. After each sampling round, 3-4 leaves were picked from each sample and their midribs with petioles were cut with sterile razor blades for use in Total DNA extraction. Total DNA extraction from midrib tissue was performed according to a

modified CTAB extraction protocol adapted for blueberry leaves (Doyle and Doyle 1987; Christiano and Scherm 2011). Extracted DNA samples were tested by means of PCR using the RST31/33 Xf-specific primer pair (Minsavage et al. 1994), which is commonly employed to detect Xf presence. PCR reagents, sample preparation, and cycling parameters for amplification were as described by Di Genova et al. (2019). Amplified products were visualized using gel electrophoresis assay on a 1% agarose gel.

Symptom severity changes, association of severity with Xf incidence, and spatial distribution of disease. In order to evaluate our rating system and its use to assess disease, year over year severity changes and the relationship between our severity categories and Xf incidence (based on PCR testing results) were evaluated. The severity assessment results from 2018 and 2019 were evaluated as repeated measures of severity on each plant. Severity ratings for each plant at each site from 2018 were compared to severity ratings from 2019 using a paired t-test to assess year over year changes (https://mathcracker.com/t-test-for-paired-samples). To examine the relationship between Xf incidence and the visually assigned disease severity ratings, the percentages of Xf-positive samples from the 'Star' and 'Rebel' plantings from Farm #1 were treated as the incidence within that severity class and the association between severity class and the numbers of Xf-positive samples and Xf-negative samples collected was assessed using Pearson's chi-squared test (https://www.socscistatistics.com/tests/chisquare2/default2.aspx). In addition, for the two 'Rebel' high-density blocks from Farm #2, Moran's I was calculated to determine if there was spatial autocorrelation among the recorded disease severity ratings within the block (Gittleman and Kot 1990). Moran's I was calculated using function Moran.I (ape v5.3 package in R) (Paradis and Schliep 2019).

Yield and fruit quality assessment. For Farm #1, three plants from each of the two cultivars (‘Rebel’ or ‘Star’) were randomly chosen from the surveyed rows to represent each of the visually assessed disease severity levels (“0”, “1”, “2”, and “3”). From these plants, ripe fruit were harvested by hand in 2019 on 12 April (‘Rebel’ only), 16 April (‘Star’ and ‘Rebel’), 22 April (‘Star’ and ‘Rebel’), and 29 April (‘Star’ only). All ripe berries present on each plant were collected on each harvest date. Total yield and other fruit quality parameters were assessed as described by Smith (2016). Harvested berries were counted and weighed after culling to assess marketable berry weight. The experiment was analyzed within cultivar to avoid additional interactions using SAS’s 9.4 Proc GLM (SAS Institute Inc., Cary, NC, U.S.). Means were separated at $P < 0.05$ level using Fisher’s least significant difference (LSD) test.

Results

2018 symptom severity assessment results from Farm #1 and Farm #2. The results of the 2018 symptom assessment (Table 4.2, Table 4.3, Table 4.4, Table 4.5) showed differences in assessed severity between sites. Initial symptom assessment in 2018 within the ‘Rebel’ planting in Farm #1 (Table 4.2) showed a large number of diseased plants as well as a large number of plants that were either already dead or had been recently replanted. As a result, 106 out of 364 plants (29.1%) within the three assessed ‘Rebel’ rows were excluded from subsequent assessment given that they were dead or too small for comparison to other plants (replants). Within this planting, 70 of 364 (19.2%) plants were assessed as ‘healthy’ (“0” rating), 133 of 364 (36.5%) were assessed as having ‘light’ symptoms (“1” rating), 30 of 364 (8.2%) were assessed as having ‘moderate’ symptoms (“2” rating), and 25 of 364 (6.9%) were assessed as having ‘severe’ symptoms (“3” rating). Symptoms within the ‘Star’ planting in Farm #1 (Table 4.3)

were generally less severe than in the ‘Rebel’ planting. Within the three assessed rows, 23 of 401 (5.7%) were either already dead at the time of assessment or had recently been replanted. Among the remainder of the assessed ‘Star’ plants, these were assessed as 203 (50.6%) ‘healthy’, 120 (29.9%) ‘light’, 52 (13.0%) ‘moderate’, and 3 (<1%) ‘severe’.

At Farm #2, Block 2 and Block 3 each included only 1 dead plant (<1% of total). Assessed plants in Block 2 (Table 4.4) included 41 of 110 (37.3%) ‘healthy’, 36 (32.7%) ‘light’ 14 (12.7%) ‘moderate’, and 18 (16.4%) ‘severe’. For Block 3 (Table 5), assessed plants included 40 of 117 (34.2%) ‘healthy’, 49 (41.9%) ‘light’, 8 (6.8%) ‘moderate’, and 19 (16.2%) ‘severe’.

2019 symptom severity assessment results from Farm #1 and Farm #2. The 2019 symptom assessment showed changes in the observed diseased severities on numerous plants (Table 4.2, Table 4.3, Table 4.4, Table 4.5). In total, within Farm #1, 135 of the 258 ‘Rebel’ plants (52.3%) given 0-3 ratings in 2018 had the same symptom severity rating assigned in 2019 (data not shown). By contrast, 123 of these plants received different ratings in 2019, with 79 (30.6%) being assessed as having more severe symptoms and 44 (17.1%) receiving a lower (less severe) rating in 2019 than 2018. The vast majority of these “improvements” in assessed severity (42 of 44) were from ‘light’ (“1”) to ‘healthy’ (“0”). Within the ‘Star’ planting at Farm #1, the symptoms on 146 of 378 (38.6%) plants given 0-3 ratings in 2018 remained the same. By contrast, 232 of 378 of assessed ‘Star’ plants received a different rating in 2019 vs. 2018, with 180 (47.6%) being assessed as having more severe symptoms and 52 (13.8%) receiving a less severe rating in 2019 vs. 2018. In the case of ‘Star’, the majority (34 of 52, 65%) of plants showing “improvement” were rated as ‘moderate’ in 2018 and were assessed as ‘light’ (29 total) or ‘healthy’ (5 total) in 2019.

From Farm #2, 47 of 109 (43.1%) Block 2 plants and 56 of 116 (48.3%) Block 3 plants assessed in 2018 received the same severity rating in 2019. Of the 62 plants in Block 2 that received a different severity rating in 2019 versus 2018, the majority 51 of 62 (82.2%) were assessed as having more severe symptoms in 2019 versus 2018. Of the 60 plants in Block 3 whose ratings changed, the overwhelming majority 55 of 60 (91.7%) showed an increase in assessed severity in 2019.

Xf incidence on sampled plants. Among the Farm #1 'Rebel' plants sampled in 2018, results from PCR testing for Xf (Table 4.6) indicated a high Xf incidence within sampled plants assessed as 'severe' (13 of 15, 86.7%). In total from the sampled and tested plants, 8 of 15 (53.3%) plants assessed as 'moderate', 6 of 18 (33.3%) plants assessed as 'light', and 2 of 18 (11.1%) plants assessed as 'healthy' were confirmed by PCR to be Xf-infected in 2018. For Farm #1 'Star' plants (Table 4.6), a relatively lower incidence was noted among sampled plants with 3 of 3 (100%) 'severe', 4 of 15 (26.7%) 'moderate', 1 of 15 (6.7%) 'light', and 0 of 19 (0%) 'healthy' confirmed by PCR to be Xf-infected. For Farm #2, in which the original sampling was done differently in 2018, 7 of 10 (70%) 'severe' plants were confirmed to be Xf-infected by PCR (Table 4.7). Among the samples tested from plants surrounding these 'severe' plants: within Block 2, 3 of 4 (75%) 'severe', 3 of 3 (100%) 'moderate', 3 of 10 (30%) 'light', and 2 of 3 (66.7%) 'healthy' plants were confirmed to be Xf-infected, and within Block 3, 2 of 3 'severe' (66.7%), 1 of 2 'moderate' (50%), 1 of 5 (20%) 'light') and 4 of 10 (40%) 'healthy' were Xf-infected. For 2019, PCR testing results on samples collected are not yet available.

Symptom severity changes and association of severity with Xf incidence. Based upon paired t-testing of severity scores assigned in 2018 versus those assigned in 2019, a significant increase in assessed severity was noted in 2019 in all plantings on both farms ($P = 0.0001$ for

Farm #1 ‘Rebel’, $P < 0.0001$ for Farm #1 ‘Star’, and $P < 0.0001$ for Farm #2 ‘Rebel’). Comparison of the observed disease incidence (based upon molecular testing for Xf) in 2018 among the randomly sampled plants belonging to each severity class (Table 4.6) using Pearson’s chi-squared test indicated an association between assigned severity categories and numbers of Xf-positive and Xf-negative samples for the ‘Rebel’ planting at Farm #1 ($\chi^2 = 9.3093$, $P = 0.02545$). Since some of the cells in the contingency table of results for ‘Star’ were zero values, Pearson’s chi-squared test statistic could not be calculated in 2018 for this planting; however, these numbers visually also suggested an apparent relationship between assigned severity categories and Xf disease incidence based upon molecular testing. Furthermore, by adding 1 to all entries in the “Star” contingency table to eliminate zero values, Pearson’s chi-squared test statistic did indicate a significant association between severity category and Xf-incidence ($\chi^2 = 15.9594$, $P = 0.001156$). Since the planting at Farm #2 was not sampled randomly in 2018, the association between 2018 disease severity and Xf-incidence could not be assessed. Nevertheless, a high percentage of plants rated as ‘severe’ and ‘moderate’, were found to be infected with Xf based upon PCR results (Table 4.7).

Spatial autocorrelation of disease within high-density blocks. Using the 2018 and 2019 severity assessment information for the two high density blocks within the ‘Rebel’ planting of Farm #2 (Fig. 4.3), spatial autocorrelation among the assessed severities was examined. Assigned severity rankings of individual plants as well as their location within the block were used to assess whether symptom severity was non-random in its distribution within each block using Moran’s I test statistic. Based upon this analysis, there was found to be a significant correlation between symptom severities and locations of assessed plants within Block 2 in 2019

($P < 0.0001$) but not in 2018 ($P = 0.056$). No significant spatial autocorrelation was observed within Block 3 in either 2018 ($P = 0.87$) or 2019 ($P = 0.16$).

Yield and fruit quality assessment. In Spring 2019**,** fruit quality parameters and yield were determined for plants across the assigned severity categories from 2018 for both 'Star' and 'Rebel' plantings at Farm #1. In terms of total yield, at least numerically, average yield per plant decreased with increasing disease severity for both varieties, while some fruit quality parameters did not correlate with assessed disease severity (Table 4.8). Average yield from the 'Rebel' plants assessed as 'severe' (52.88 g) was observed to be 22% of yield from 'healthy' plants (237.53 g). For 'Star', average yield from plants assessed as 'severe' (109.11 g) was 37% of the yield from plants assessed as 'healthy' (294.53 g). In general, as only 3 replicates were included for each severity category from each variety, a high degree of variability was observed overall. Nonetheless, some assessed parameters indicated significant variation across the severity categories. For 'Rebel', average yield, cullage, marketable fruit, soluble solids, and several fruit color parameters were significant at $P < 0.05$ (Table 4.9). For 'Star', only soluble solids, and several fruit color parameters were significant.

Discussion

In this study, we used visual disease severity assessments, molecular detection of Xf infection, and evaluation of yield and fruit quality to investigate the impact and spread of BLS within commercial SHB blueberry plantings in southern Georgia over two years. We used visual disease severity assessments to estimate the year over year spread of disease, and saw a measurable increase in disease within all three plantings. By comparing our disease severity assessment results with results from Xf molecular detection, we were able to show a significant

relationship between Xf incidence and observable symptoms, suggesting a level of accuracy of visual severity assessment. In addition, in this work, disease severity assessed in the fall was correlated with yield and fruit quality during the next spring harvest. Taken together, these findings suggest that visual assessment of BLS disease may ultimately be of use to growers as they make decisions regarding the removal of diseased and unproductive plants for management of BLS disease.

Xf diseases are well-known to be difficult to manage, and SHB blueberry growers in the southeast are plagued by BLS on blueberry. Especially in intensive blueberry production regions (such as Bacon County, GA where the three plantings used in our study were located), BLS is known to cause significant plant mortality and impact yield. Growers faced with this issue often rely on 'roguing' of infected plants to eliminate infections from the field, or they ignore the situation entirely until the whole planting is no longer productive enough to justify its continued maintenance. In either case, growers visually assess disease issues to make judgements regarding individual plants and whole plantings.

Molecular detection tools for Xf, while reliable and decreasing in cost, are generally only used to confirm infections already suspected from visual observations. Though molecular detection methods are certainly more accurate than visual assessment (even considering occasional issues with "false negative" PCR testing results), such methods can be cost prohibitive to assess disease at the whole field scale and in most cases are just used to confirm disease on a subset of suspect plants. Furthermore, molecular detection of Xf often necessitates laboratory facilities and reliance on trained professionals and testing services, limiting grower access to these tools.

Differences between the establishment, management, and age of the two farms utilized for our studies are of note. At the start of our study, Farm #1 consisted of 3-year-old SHB 'Rebel' and 'Star' plants grown with typical wide row spacing and with drip irrigation. By contrast, Farm #2 consisted of 11-year-old SHB 'Rebel' plants grown in high-density pine bark beds and with overhead irrigation. Though both growers reported that vector management with insecticides was not consistently practiced, both farms have been generally well-managed. The manager at Farm #2 has practiced active 'roguing' and replacement of diseased plants throughout the extended life of the planting. Despite this, we observed an abundance of severe disease symptoms at both locations, and a high disease incidence among sampled plants, suggesting that both farms are being severely impacted by BLS. Within Farm #2, 23% of Block 3 plants and 29.1% of Block 2 plants were showing signs of moderate to severe disease in 2018; and, based upon conversations with the grower in 2019, due to the high level of disease within this planting, he is likely to remove the remaining plants in 2020 to start anew. At Farm #1, within the 'Rebel' planting which had only been established 3 years prior to 2018, we observed moderate to severe disease affecting over 15% of assessed plants in 2018 and over 22.2% in 2019. When these numbers are added to the large number of plants (29.1%) within this planting that had apparently died and been replaced prior to 2018 (again during the first 3 years after establishment), this adds up to >45% of the assessed rows being composed of unproductive or underproductive plants. Furthermore, if we extrapolate out, based upon the observed Xf incidences and the number of plants corresponding to each respective disease severity category within the rows we evaluated, we would estimate that over 35% of the 3-year-old plants within this planting were already infected with Xf in 2018. Given both the ability of Xf to be readily vectored from plant to plant, and the impact on yield documented here, this does not bode well

for the future productivity of this planting. The ‘Star’ planting at Farm #1, showed less severe disease than the ‘Rebel’ planting, but did have over 12% of the 3-year-old plants showing moderate to severe disease in 2018. Overall, BLS severity and Xf incidence appeared to be lower in the ‘Star’ planting than the ‘Rebel’ planting, possibly due to differences between the cultivars in terms of susceptibility or tolerance to Xf.

Ultimately, any evaluation system for scoring plants according to disease symptoms should be carefully examined for accuracy. Our study provided a preliminary effort in this, by using only a basic system that employs simple visual observation of BLS symptoms and attempts to correlate the symptom severity categorization with Xf incidence. While we did see a correlation between our visual symptom severity assessment and Xf-incidence, we did note a few things that could affect the accuracy of visual assessment of BLS on ‘Rebel’ and ‘Star’. In general, for ‘Rebel’, we noted that a significant proportion (31.6%) of plants assessed to have ‘light’ BLS symptoms in 2018 were ‘healthy’ based upon the 2019 assessment. Since Xf-infected blueberry plants are generally assumed to steadily decline following infection, this likely indicates that the initial stages of Xf-infection cannot by accurately diagnosed visually. This is not unexpected, as one of the difficulties with managing Xf diseases has been the presence of a latent period following infection where symptoms are not apparent (Purcell 2013). This suggests that additional tools for the assessment of BLS symptoms in blueberry plantings should be explored in order to better understand the differences between healthy plants and Xf-infected, asymptomatic plants in field conditions. In contrast, across the ‘Rebel’ plantings at both farms, we saw minimal examples (only 6 of 114) where plants assessed as ‘moderate’ or ‘severe’ in 2018 were assessed as being ‘light’ or ‘healthy’ in 2019. This suggests that we were able to reliably identify true BLS symptoms visually once they had become more severe on this cultivar.

This was not always the case for ‘Star’, as 34 of 52 plants (65.4%) assessed as ‘moderate’ in 2018 were assessed as ‘light’ or ‘healthy’ in 2019. This suggests that it may be harder to definitively determine whether the “scorch” and “defoliation” observed on ‘Star’ is due to BLS or some other cause (e.g. drought stress symptoms, opportunistic seasonal diseases, leaf spotting, defoliation). Generally, ‘Star’ has relatively “twiggy” growth and is known to easily show the impacts of drought stress. Furthermore, ‘Star’ is known to have moderate tolerance to Xf in comparison to ‘Rebel’ which is well-known to be severely impacted by Xf. Taken together, this might make ‘Star’ more problematic to assess visually and raises the question of whether other cultivars (not examined in our study) may likewise be even more difficult to accurately assess visually.

In this study, we also investigated the impact of BLS on fruit yield. Though our data comes from a single harvest season and only included three replicates per assessed severity category, the information generated by our research does help to illustrate the impact of BLS disease on plant productivity. Our data from both varieties indicated that BLS can have a significant impact on yield, reducing yield by 78% and 63%, respectively, on the most severely impacted ‘Rebel’ and ‘Star’ plants versus healthy plants. To help growers make informed decisions about BLS management and when to replant entire affected blocks, additional studies are necessary to characterize the full economic impact of lost blueberry production due to BLS.

Blueberry bushes, once planted, take at least two years to enter into full production output. The practice of roguing and replacing BLS-affected plants, while the only practical management option available in many cases, inevitably presents several critical weaknesses. The economic decision behind this practice is complex, considering the perennial nature of a blueberry planting, and it becomes even less useful if Xf is capable of becoming widespread on a

new planting in a matter of one or two seasons after planting (as apparently was the case with Farm #1 in our study, which was only three years old in 2018). Also, the PCR testing results from our study confirmed that several apparently 'healthy' Xf-infected plants were among the plants assessed in our study, decreasing the likelihood that roguing will be able to eliminate inoculum from the field. Furthermore, it is likely only a matter of time before those asymptomatic Xf-infected plants will start to develop symptoms, show reduced yields, and impact the productivity of the planting. For these reasons, identifying asymptomatic plants which harbor the pathogen may be critical, making management of Xf diseases a significant challenge.

It should also be noted that our study took place only over a 13-month period (September 2018 to September 2019). Given this relatively short interval, it would be informative to continue disease severity observations over a longer term. This would provide further insights into disease progression, spread, and the long-term impact of Xf on these SHB blueberry cultivars. Blueberry is a perennial crop, characterized by agronomic and management decisions that impact the production over multiple years, and issues such as Xf have repercussion for the long-term profitability of blueberry plantings. Nevertheless, the information collected in our study constitutes an important first step in understanding the impact of BLS in areas where Xf is present alongside intensive commercial production of blueberry.

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Table 4.1. Symptom severity descriptors adopted for BLS assessment on plants.

Severity Rank	% of Diseased Plant	Description
0 (Healthy)	0 %	– No visible symptom and/or healthy plant.
1 (Light)	Up to 10 %	– Isolated/scattered BLS symptoms (including questionable BLS symptoms). – Isolated stem and/or few leaves affected: scorching and defoliation.
2 (Moderate)	Up to 50 %	– Multiple stems affected: scorching and/or defoliation.
3 (Severe)	More than 50 %	– Multiple stems affected: diffused heavy scorching and/or heavy defoliation.
DEAD / REPLANT	N/A	– Completely defoliated/wilting plant (DEAD). – Very small bush relative to the other scored plants (REPLANT).

Table 4.2. Total number of plants within assessed symptom severity rankings in 2018 and 2019 for surveyed rows of cultivar ‘Rebel’ in Farm #1.

Severity	2018 (%)	2019 (%)
0 (Healthy)	70 (19.2%)	77 (21.2%)
1 (Light)	133 (36.5%)	100 (27.5%)
2 (Moderate)	30 (8.2%)	38 (10.4%)
3 (Severe)	25 (6.9%)	38 (10.4%)
DEAD / REPLANT	106 (29.1%)	111 (30.5%)
Total	**364**	**364**

Table 4.3. Total number of plants within assessed symptom severity rankings in 2018 and 2019 for surveyed rows of cultivar ‘Star’ in Farm #1.

Severity	2018 (%)	2019 (%)
0 (Healthy)	203 (50.6%)	74 (18.5%)
1 (Light)	120 (29.9%)	253 (63.1%)
2 (Moderate)	52 (13.0%)	37 (9.2%)
3 (Severe)	3 (< 1%)	14 (3.5%)
DEAD / REPLANT	23 (5.7%)	23 (5.7%)
Total	**401**	**401**

Table 4.4. Total number of plants within assessed symptom severity rankings in 2018 and 2019 in Block 2, Farm #2.

Severity	2018 (%)	2019 (%)
0 (Healthy)	41 (37.3%)	26 (23.6%)
1 (Light)	36 (32.7%)	34 (30.9%)
2 (Moderate)	14 (12.7%)	22 (20.0%)
3 (Severe)	18 (16.4%)	20 (18.2%)
DEAD / REPLANT	1 (< 1%))	8 (7.3%)
Total	**110**	**110**

Table 4.5. Total number of plants within assessed symptom severity rankings in 2018 and 2019 in Block 3, Farm #2.

Severity	2018	2019
0 (Healthy)	40 (34.2%)	20 (17.1%)
1 (Light)	49 (41.9%)	42 (35.9%)
2 (Moderate)	8 (6.8%)	22 (18.8%)
3 (Severe)	19 (16.2%)	24 (20.5%)
DEAD / REPLANT	1 (< 1%)	9 (7.7%)
Total	**117**	**117**

Table 4.6. Xf-incidence results by assessed severity rank from Farm #1 in 2018.

Cultivar	Severity	Tested	Positives (%)
Rebel	0 (Healthy)	18	2 (11.1%)
	1 (Light)	18	6 (33.3%)
	2 (Medium)	15	8 (53.3%)
	3 (Severe)	15	13 (86.7%)
Star	0 (Healthy)	19	0 (0%)
	1 (Light)	15	1 (6.7%)
	2 (Medium)	15	4 (26.7%)
	3 (Severe)	3	3 (100%)

Table 4.7. Xf-incidence results by assessed severity rank from Farm #2 in 2018.

Block	Severity	Tested	Positives (%)
2	0 (Healthy)	3	2 (66.7%)
	1 (Light)	10	3 (30.0%)
	2 (Medium)	3	3 (100%)
	3 (Severe)	4	3 (75.0%)
3	0 (Healthy)	10	4 (40.0%)
	1 (Light)	5	1 (20.0%)
	2 (Medium)	2	1 (50.1%)
	3 (Severe)	3	2 (66.6%)

Table 4.8. Quantitative yield data from yield assessment conducted on Farm #1.

Variety	Severity		Average Yield (g)	Cullage (g)	Marketable Fruit (g)	Individual Fruit (g)
Rebel	0 (Healthy)		237.53 a	105.36 a	132.18 b	1.8444 a
	1 (Light)		486.76 a	144.38 a	342.38 a	1.8333 a
	2 (Moderate)		162.17 ab	57.58 b	104.59 b	1.6333 a
	3 (Severe)		52.88 b	16.15 b	43.34 b	1.5125 a
		P	<0.0001	<0.0001	<0.0001	0.2687
		Rsq	0.491977	0.460772	0.449535	0.092561
		LSD	135.32	44.866	106.2	0.3993
Star	0 (Healthy)		294.53 a	84.31 ab	210.22 a	1.9 a
	1 (Light)		293.62 a	111.3 ab	219.03 a	2.1875 a
	2 (Moderate)		176.41 a	51.93 ab	146.5 a	1.7 a
	3 (Severe)		109.11 a	26.71 b	82.7 a	1.6222 a
		P	0.181	0.0929	0.2972	0.2626
		Rsq	0.13939	0.189912	0.113926	0.122657
		LSD	200.71	69.808	164.36	0.6092

Table 4.9. Qualitative yield data from yield assessment conducted on Farm #1.

Variety	Severity		Soluble Solids (Brix)	Acid (%)	Sugar/Acid Ratio	Color				
						Shade (L)	a	b	Chroma	Hue
Rebel	0 (Healthy)		10.425 b	0.53583 a	19.717 a	30.8857 b	1.6901 a	-2.9515 a	3.655 a	295.922 a
	1 (Light)		10.5 b	0.54 a	18.584 a	31.9992 a	1.8678 a	-3.5202 b	4.2328 a	298.362 a
	2 (Moderate)		11.0333 ab	0.60833 a	20.07 a	31.8082 a	1.5785 ab	-3.6592 b	4.2563 a	290.163 a
	3 (Severe)		12.125 a	0.62 a	20.07 a	31.9165 a	1.1958 b	-4.2235 c	4.4913 a	286.354 a
		P	0.0445	0.1656	0.8862	0.0016	0.0413	<0.0001	<0.0001	0.2563
		Rsq	0.28105	0.187599	0.025972	0.053626	0.029362	0.100076	0.096439	0.014533
		LSD	1.2035	0.0978	4.6	0.7744	0.4223	0.4832	0.387	12.341
Star	0 (Healthy)		11.02 bc	0.784 ab	14.9 ab	29.1253 b	2.449 a	-0.8954 a	3.038 b	230.67 b
	1 (Light)		10.375 c	0.75 ab	19.26 a	30.097 a	2.5778 a	-1.9133 bc	3.686 a	270.76 ab
	2 (Moderate)		11.86 ab	0.666 b	13.383 b	29.5408 ab	1.9338 b	-2.5956 c	3.6292 a	281.34 a
	3 (Severe)		12.0333 a	0.905 a	14.2 ab	29.5662 ab	1.9118 b	-1.3278 ab	3.3668 ab	239.37 ab
		P	0.0069	0.1495	0.1385	0.1249	0.0057	0.0001	0.0244	0.1197
		Rsq	0.52202	0.276189	0.283977	0.030642	0.065853	0.1041	0.049664	0.031156
		LSD	0.9385	4.897959	5.5342	0.7767	0.4591	0.7873	0.4634	48.984

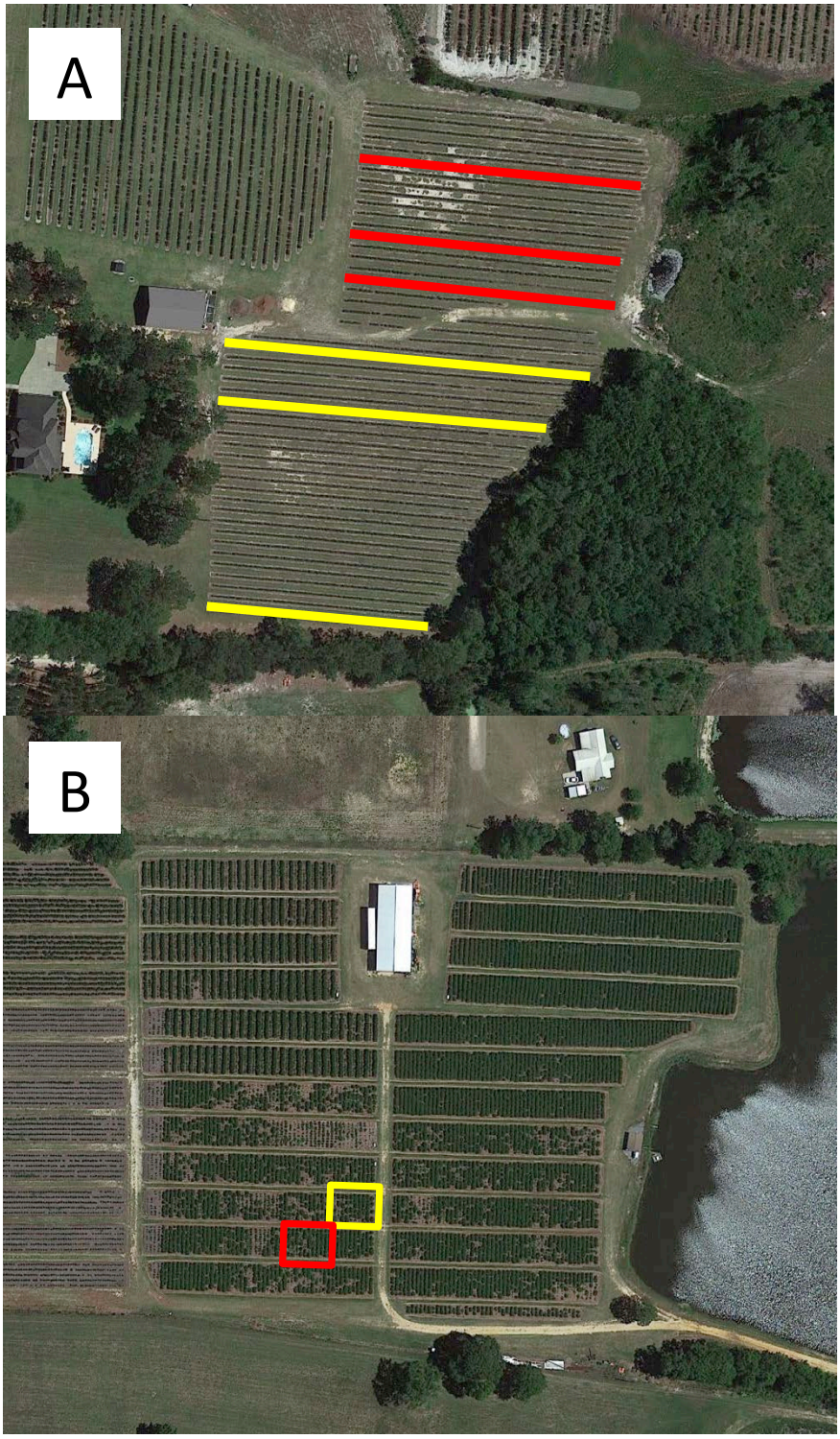

Figure 4.1. Aerial view of selected blueberry plantings, with surveyed rows or blocks highlighted. A. Farm #1 with SHB blueberry rows of cultivar ‘Rebel’ (in red) and ‘Star’ (in yellow). B. Farm #2 (SHB blueberry ‘Rebel’ only) with 10 high-density rows in each block, Block 2 (in red) and Block 3 (in yellow).

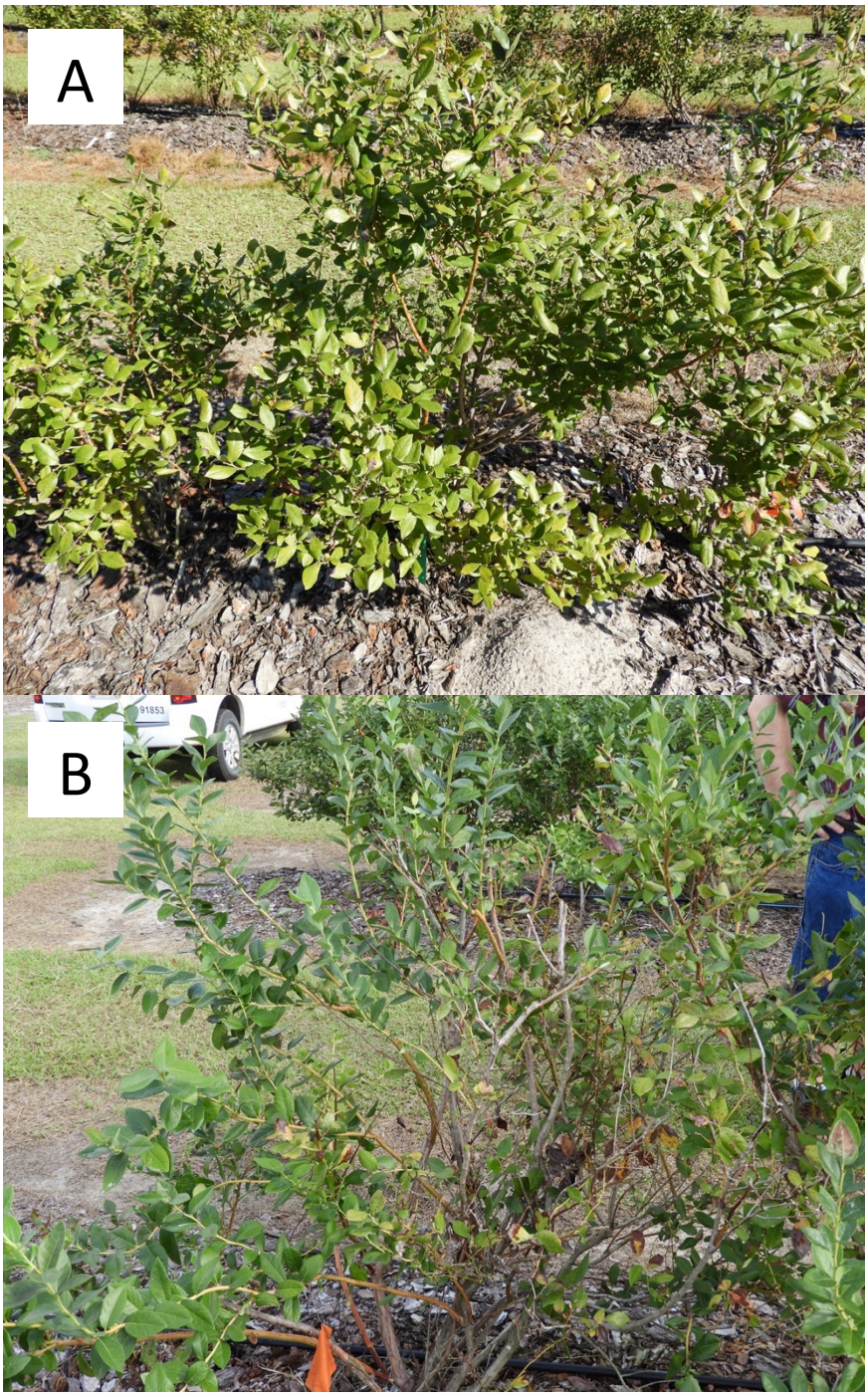

Figure 4.2. Examples of blueberry plants scored with the 4 different symptom severity ranks (all plants depicted here are SHB blueberry 'Rebel' later confirmed to be *X. fastidiosa*-positive by PCR, from Farm #1, Fall 2018). A. Asymptomatic/healthy bush, B. light symptoms, C. moderate symptoms and D. severe symptoms.

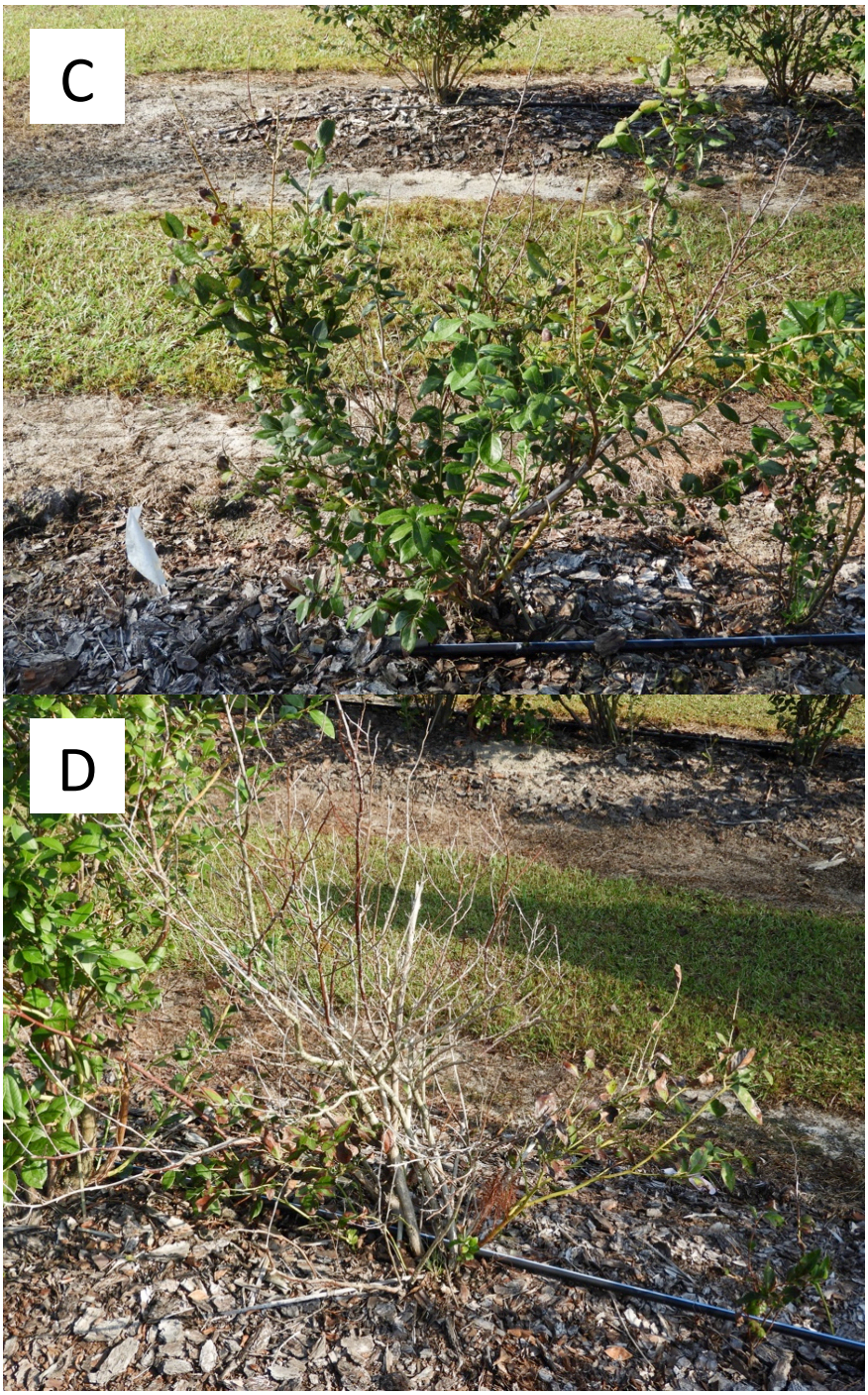

Figure 4.2 (continued). Examples of blueberry plants scored with the 4 different symptom severity ranks (all plants depicted here are SHB blueberry 'Rebel' later confirmed to be *X. fastidiosa*-positive by PCR, from Farm #1, Fall 2018). A. Asymptomatic/healthy bush, B. light symptoms, C. moderate symptoms and D. severe symptoms.

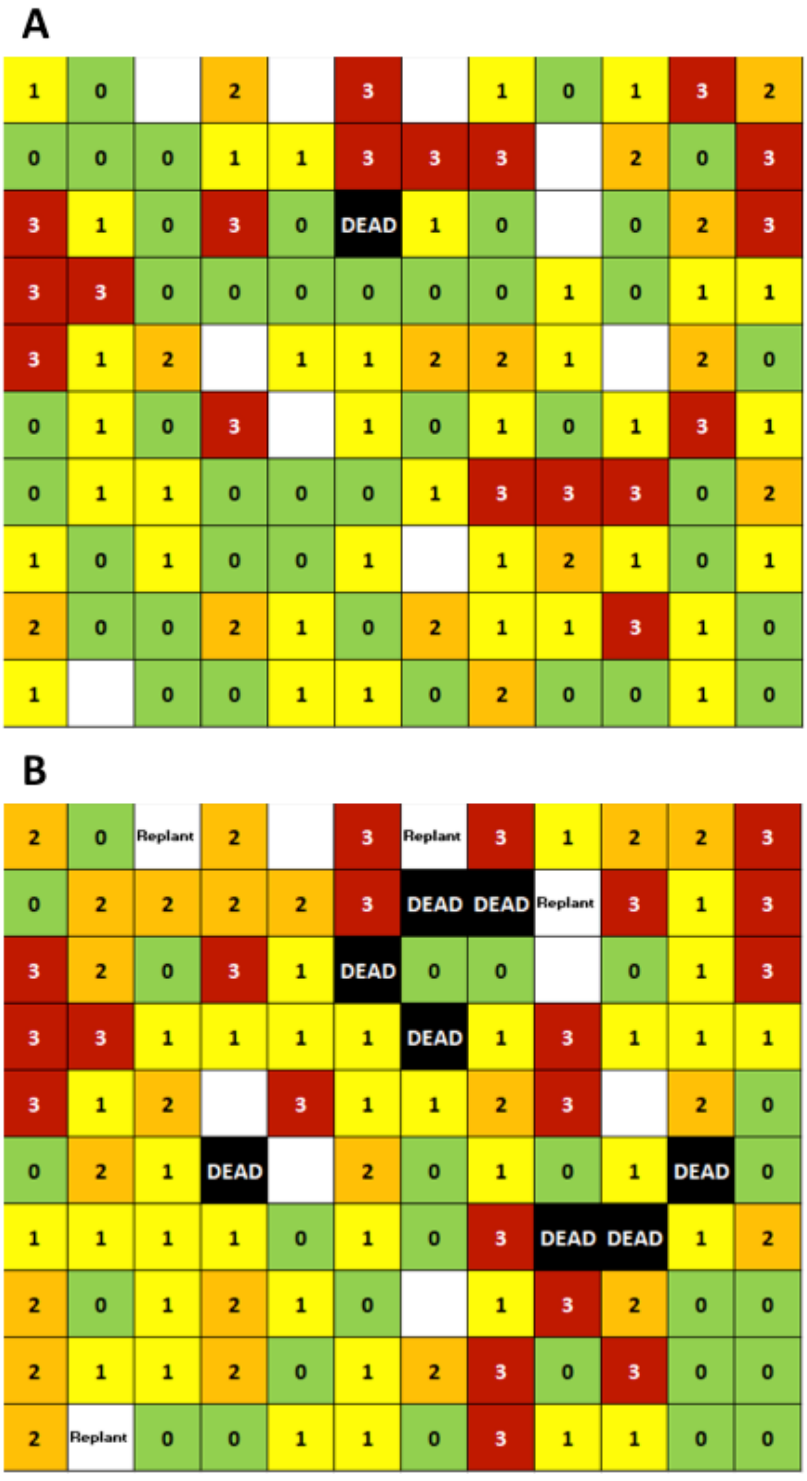

Figure 4.3. Assessed disease severity on plants located in Farm #2, with single blueberry rows arranged from left (South side of each block) to right (North side of each block). A. Block 2 (2018), B. Block 2 (2019), C. Block 3 (2018) and D. Block 3 (2019).

C

0	1	0		1	3	2	0	1	1		1	1	
1	3	1	1	1	0	1	3	3	0	3	0		
0	0	1	3	1	1	3	1	0	0	3	3		
0	3	1	1	3	1	1			0	3		1	0
1	0	0	1	2	0		1	0	1	1	0		
0	1		3	0		2	0	1	3	1	2	2	2
	3	1	2	0		0	0	1		0	3	0	
3	0		0	1	0		1	1	0	0	0	1	2
3	1		0	1	1		1	1	1	1	1	0	1
1	3	0	1	0	1	0	0	0	0	DEAD	1	1	1

D

0	3	1		2	DEAD	3	1	1	0		2	1	
1	DEAD	1	3	1	1	2	DEAD	3	0	3	0		
0	2	2	DEAD	2	3	3	3	1	1	3	3		
1	3	2	2	3	1	1			DEAD	3	Replant	1	1
2	0	2	2	3	0		1	1	1	1	0		
0	1		3	1		3	1	3	3	1	2	1	2
	3	2	1	1		1	2	1		1	DEAD	0	
DEAD	2		1	2	0		2	DEAD	1	0	0	1	2
3	1		0	3	1		2	1	1	1	1	1	3
1	3	0	0	1	0	0	3	0	0	DEAD	2	2	1

Figure 4.3 (continued). Assessed disease severity on plants located in Farm #2, with single blueberry rows arranged from left (South side of each block) to right (North side of each block). A. Block 2 (2018), B. Block 2 (2019), C. Block 3 (2018) and D. Block 3 (2019).

CHAPTER 5

SUMMARY AND CONCLUSIONS

In this thesis our purpose was to explore key aspects and provide a basic understanding of Xf on blueberry, an emerging pathogen on a specific niche crop of significant economic importance for southern US fruit production. We surveyed, isolated and investigated multiple Xf isolates from SHB blueberry plantings in Georgia, USA and described their diversity, yielding interesting results about their genetics. Not only were we able to add a sizeable number of new isolates to the previous literature of Xf strains coming from blueberry, but we also characterized a significant number of new haplotypes from a single bacterial species within a single host, showing a consistent genetic differentiation among just a few analyzed genes. Most importantly, we determined that both subsp. *multiplex* and, unexpectedly, subsp. *fastidiosa*, infect blueberry in natural field settings.

We evaluated the potential impact of this new Xf subspecies in blueberry by testing the pathogenicity and virulence characteristics of our isolates in controlled greenhouse conditions. Significant disease levels were assessed from all our employed isolates, including those newly identified Xff isolates from blueberry, leading us to conclude that both Xff and Xfm isolates are adapted to cause disease on blueberry. Further investigation of host-pathogen interaction mechanisms and biological factors ultimately determining disease is needed to accurately explain the Xf pathogenicity. What we know for sure is that SHB blueberry is a susceptible host of

choice for Xf and the development of effective management tools to counter BLS should be a priority if we are to prevent disease losses that are likely to continue increasing.

Our investigation of Xf incidence in the field and BLS impact on SHB blueberry production addresses this need to management tools somewhat, by assessing the reliability of visual assessment of disease for the implementation of 'roguing'. Preliminary data on Xf incidence, estimated potential of infection and fruit yield losses, were intriguing, and demonstrated the association between BLS symptoms and yield losses. As a whole, it was clear from this work that visual assessment of disease is somewhat accurate, in severe cases, but probably is not sufficient for early identification of Xf in the field. Given the potential impact of latent infections, it is no wonder that Xf is so difficult to manage at a field level. Longer, multi-year assessments should be carried out on the basis of this preliminary work and novel methods for timely assessment of Xf infections should be devised and compared to simple visual diagnosis of BLS on plants within commercial fields.

This first report of Xff isolates capable of infecting SHB blueberry in the field, warrants additional research into the diversity of Xf, not only on this profitable crop of the southeastern US, but also in light of the supposed host specificity that has always characterized our previous knowledge of this pathogen (i.e. a phytobacterium capable of infecting only a specific range of hosts depending on certain subspecies). We found a notable exception to this assumed rule of thumb which is even more troubling given that Xf itself has spread to new continents with new epidemics and reports emerging in recent years of new hosts and notable damage to agricultural production, with economic and social repercussions. In this perspective, the case of Xf in blueberry represents a perfect example in which knowledge gaps regarding the biology, ecology and epidemiology of this pathogen have made it difficult to control this pathogen or reduce

losses due to disease. Following our objectives investigated in this study, we expect the following future directions for Xf and BLS in blueberry:

1. Further research into the genetic structure of Xf isolates from blueberry through whole genome sequencing techniques to better elucidate the basis for Xf pathogenicity.
2. Further investigations into the virulence and pathogen adaptability of Xf isolates from blueberry to different hosts, ideally trying to evaluate host response in different cultivars of blueberry itself, in light of future possibilities for breeding for host resistance to BLS.
3. Continued BLS assessment in the field for a multi-year study projection to reliably understand the association between observable BLS symptoms, Xf incidence, dispersal, impact on plant yield, and possibilities for early detection.

APPENDIX A

ISOLATION AND GENETIC CHARACTERIZATION OF ADDITIONAL *XYLELLA FASTIDIOSA* ISOLATES DURING FALL 2018

Rationale

The original survey of BLS-symptomatic blueberry plants in Fall 2017 (reported in Ch. 2) was extended in Fall 2018 to additional planting sites in additional counties in southern Georgia as well as to the fields scored for symptoms (from Ch. 4). The purpose here was to collect additional infected blueberry samples and obtain additional Xf isolates for genetic and greenhouse studies. Though the evaluation of the isolates obtained from 2018 survey are still in progress, we herein briefly explain the procedure used to collect and test the sampled plant material and detail the final bacterial isolation outcome and subsequent genetic characterization of the newly obtained Xf isolates from Fall 2018.

Field Survey, Sample Processing and Isolate Characterization

SHB blueberry plantings in Appling, Bacon, Pierce and Ware counties (southeast Georgia, USA) were surveyed to collect a large set of samples of twigs and leaves showing typical BLS symptoms. Within the four counties mentioned above, three, two, two and five blueberry plantings were surveyed (Table A.1), respectively, with a variable number of plant samples collected in each, depending on field size, BLS incidence, and previous reports and history of BLS within the site. All the plant material brought back to the lab was processed and tested for Xf presence by PCR assay using the RST31/33 Xf-specific protocol described previously (see Ch. 2; HL5/6 primer pairs not used here) (Table A.1). Similarly, isolation attempts for Xf-positive samples were attempted and any successfully grown bacterial colonies were processed, genetically typed, and stored as glycerol stocks as was done in 2017 (Ch. 2).

Genetic characterization of 2018 isolates was conducted according to the same protocol utilized in 2017 (see Ch. 2): specifically, PCR was used to amplify specific target loci, namely

rpoD, the nine MLSA-E loci (*acvB*, *copB*, *cvaC*, *fimA*, *gaa*, *pglA*, *pilA*, *rpfF*, *xadA*), and seven additional MLST loci (*cysG*, *gltT*, *holC*, *leuA*, *malF*, *nuoL*, *petC*) for a robust characterization of 2018 isolates showing unique genetic profiles. For 2018 isolates, *rpoD* sequences and MLSA-E sequences were used to generate a "neighbour-joining" (NJ) phylogenetic tree to visualize genetic differences between 2018 isolates, along with 2017 blueberry isolates, as done previously (see Ch. 2). Based upon the findings of this phylogenetic analysis, MLST sequences were obtained for select, unique isolates and compared versus the Xf MLST sequence database (https://pubmlst.org/xfastidiosa) hosted at the University of Oxford (Jolley et al. 2004; Scally et al. 2005), in order to match any corresponding MLST sequence type (ST) using the same procedure employed for the 2017 isolates (Ch. 2).

Results and Discussion

Bacterial isolations resulted in successful culturing of 17 new Xf isolates (Table A.2). Isolates from two new counties (Ware and Appling) and two new additional SHB blueberry cultivars (‘Sweet Crisp’ and ‘Emerald’) were obtained in 2018. More interestingly, genetic characterization of these new isolates from 2018 revealed unexpected variability among presently known Xf haplotypes from blueberry (sequence alignment data not shown). Characterization of the 2018 isolates by *rpoD* showed that isolate ‘ManSweetC1’ from Ware County had multiple base substitutions and insertions not previously observed for an Xf isolate. Further characterization of 2018 isolates by MLSA-E typing, resulted in the identification of additional unique genetic haplotypes within Xfm (Fig. A.1). In 2018, three new unique blueberry haplotypes were identified among the characterized isolates: ‘BLUEBERRY6’, ‘BLUEBERRY7’ and ‘BLUEBERRY8’. These blueberry haplotype names were assigned

according to the naming scheme adopted by Parker et al. 2012, after previous blueberry Xff isolates from Di Genova et al. 2019 were named as belonging to 'BLUEBERRY4' ('AlmaReb1' and 'PierceMed1') and 'BLUEBERRY5' ('AlmaReb2'), respectively. 'BLUEBERRY6' isolates 'AlmaReb4', 'AlmaReb5', 'AlmaReb6' and 'AlmaReb7' (all coming from the same site in Bacon County), possessed a common multi-base difference in the *cvaC* locus, resembling the same amplicon sequence previously observed for Xff isolates 'AlmaReb1', 'AlmaReb2' and 'PierceMed1' from 2017. 'BLUEBERRY7' included isolates 'BaxStar1', 'BaxStar3' and 'BaxStar4' (all coming from the same site in Appling County), which possessed a unique 1-base difference towards the end of the *acvB* amplicon that had not been observed previously, as well as a multi-base difference in the *rpfF* locus shared with the 'BLUEBERRY8' haplotype. Isolate 'ManSweetC1' was classified as 'BLUEBERRY8' and showed the most extensive differences versus other Xfm haplotypes. Differences included a 1-base insertion in *copB* and the same multi-base difference in *rpfF* (shared identically with 'BLUEBERRY7' as stated above). Intriguingly, the identified genetic differences within *rpfF* suggested a hybrid combination of the *rpfF* sequences previously observed from Xfm and Xff isolates from blueberry. This suggests potential recombination between isolates of the two subspecies and warrants further investigation. Roughly the first quarter of the amplicon showed the Xff *rpfF* sequence previously observed in Xff isolates 'AlmaReb1', 'AlmaReb2' and 'PierceMed1' while the rest of the amplicon was identical to the Xfm *rpfF* sequence previously observed in Xfm isolates 'AlmaReb3', 'AlmaStar1', 'AlmaStar2' and 'AlmaStar3'. Additional isolates belonging to the 'BLUEBERRY3' haplotype were also obtained in 2018, including 'AlmaReb8', 'AlmaReb9', 'AlmaStar4', 'AlmaStar5', 'AlmaStar6', 'BaxReb1', 'BaxReb2' and 'BaxStar2'. One additional 2018 Xf isolate, 'BaxEm1' was classified as belonging to the 'BLUEBERRY1' haplotype group.

Based upon the unique MLSA-E characterization of isolate ‘ManSweetC1’, further characterization was performed using the MLST approach (described in Ch. 2). The resulting sequence typing for the seven genes was compared to our previous findings from 2017 isolates (Table A.3). The MLST characterization classified isolate ‘ManSweetC1’ as Sequence Type 40 (ST40), which includes three Xfm isolates previously sequenced from purple leaf plum (*Prunus cerasifera*) and one Xfm isolate from elderberry (*Sambucus canadensis*) (Nunney et al. 2013). No Xf isolates from blueberry have previously been classified as Sequence Type 40.

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Table A.1. SHB blueberry sites surveyed and tested in Fall 2018.

Site ID	County	SHB Cultivar	Xf positives/total (%)
1	Appling	Rebel	5/5 (100%)
2	Appling	Emerald, Star	3/5 (60%)
3	Appling	Star	4/5 (80%)
4	Bacon	Rebel	27/50 (54%)
5	Bacon	Rebel, Star	37/118 (31%)
6	Pierce	Farthing, Meadowlark	1/5 (20%)
7	Pierce	Meadowlark, SuziBlue	1/6 (17%)
8	Ware	Rebel	0/5 (0%)
9	Ware	Farthing, Rebel, Sweet Crisp	2/10 (20%)
10	Ware	Rebel, Star	0/5 (0%)
11	Ware	Georgia Dawn	0/2 (0%)
12	Ware	KeeCrisp	0/1 (0%)

Table A.2. Xf bacterial isolates (listed by name) successfully cultured from plantings surveyed in Fall 2018.

County	SHB Cultivar	Isolate Name	Isolate Alias
Bacon	Rebel	AlmaReb4	R4
Bacon	Rebel	AlmaReb5	R5
Bacon	Rebel	AlmaReb6	R6
Bacon	Rebel	AlmaReb7	R7
Bacon	Rebel	AlmaReb8	R8
Bacon	Rebel	AlmaReb9	R9
Bacon	Star	AlmaStar4	S4
Bacon	Star	AlmaStar5	S5
Bacon	Star	AlmaStar6	S6
Appling	Emerald	BaxEm1	E1
Appling	Rebel	BaxReb1	R10
Appling	Rebel	BaxReb2	R11
Appling	Star	BaxStar1	S7
Appling	Star	BaxStar2	S8
Appling	Star	BaxStar3	S9
Appling	Star	BaxStar4	S10
Ware	Sweet Crisp	ManSweetC1	C1

Table A.3. MLST typing results for MLST-characterized ManSweetC1 isolate in 2018 versus 2017 isolates.

Isolate	Sequence Type	*cysG*	*gltT*	*holC*	*leuA*	*malF*	*nuoL*	*petC*
AlmaReb 1	ST1	1	1	1	1	1	1	1
AlmaReb2	ST1	1	1	1	1	1	1	1
AlmaReb3	ST42	6	3	5	12	4	3	3
ManSweetC1	ST40	18	7	7	6	5	3	3

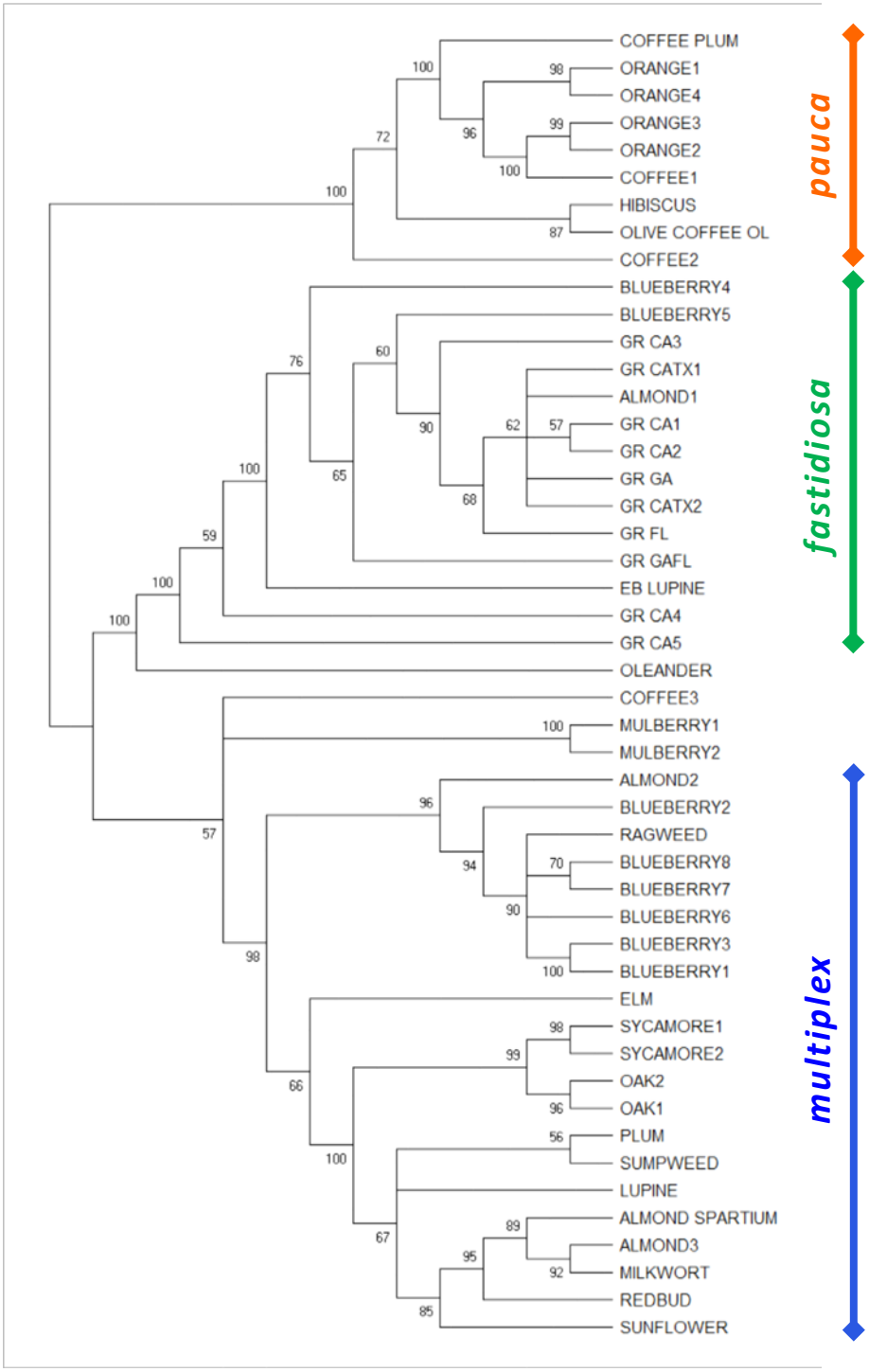

Figure A.1. MLSA-E NJ phylogenetic tree combining all unique *Xylella fastidiosa* haplotypes along with haplotypes of all isolates from blueberry isolated and characterized during both Fall 2017 and Fall 2018 surveys. Previous blueberry Xff isolates from Di Genova et al. 2019 were named as belonging to 'BLUEBERRY4' ('AlmaReb1' and 'PierceMed1') and 'BLUEBERRY5' ('AlmaReb2'), respectively. Tree branches were collapsed if bootstrap support <50%.

APPENDIX B

ADDITIONAL WORK AND FUTURE DIRECTIONS FOR VIRULENCE TESTING OF *XYLELLA FASTIDIOSA* ON SOUTHERN HIGHBUSH BLUEBERRY CULTIVARS

Rationale

As a follow-up to the greenhouse experiment presented in Ch. 3, the virulence of blueberry Xff isolates was assessed on additional cultivars of SHB blueberry, alongside cultivar 'Rebel' as previously described. After obtaining preliminary, yet valuable, information concerning the pathogenicity and virulence capabilities of several Xff isolates from blueberry, one possible next step was to lay the foundation for future work in investigating the response of different cultivars to these novel isolates. These greenhouse experiments are currently in progress, and are expected to add in parallel to the knowledge gained so far on Xf and BLS in blueberry.

Cultivar Choice, Greenhouse Organization, and Experimental Setup

Concurrently with the third experimental replicate designed for characterizing the virulence of Xf isolates on SHB cultivar 'Rebel' (described in Ch. 3), two additional SHB cultivars, 'Emerald' and 'Farthing' were used for virulence assessment. These two cultivars, 'Emerald' (US PP12,165 P2) and 'Farthing' (US PP19,341 P2), were chosen for the experiment as they are commonly utilized in blueberry production in the southeastern US. Transplants were potted, grown, and monitored in the greenhouse as described for 'Rebel' in Ch. 3, sharing the same greenhouse space and conditions as the 'Rebel' plants examined during the third experimental replicate presented in Ch. 3. The same Xf isolates used to infect 'Rebel' were also delivered to 'Emerald' and 'Farthing' with the same methods as previously described, and on the same day as 'Rebel' (May 24th, 2019). The same randomized block design was maintained, with each of the treatments delivered to seven replicate plants for each of the two cultivars, including buffer and no-buffer controls.

Symptom Scoring, Preliminary Results and Discussion

Monitoring of disease development and weekly scoring for symptoms on each of the new cultivars, starting on the first day of BLS symptom appearance, followed the same procedure described for the 'Rebel' plants evaluated in Ch. 3. The first symptoms of BLS were spotted 62 dpi (days post inoculation) for several 'Emerald' plants, with at least one plant showing symptoms per isolate treatment. Similar to the symptoms observed on 'Rebel', symptoms on 'Emerald' started initially as a faint reddish discoloration along the margins of leaves located on the two inoculated stems, close to the inoculation point. Of note, in contrast to 'Rebel', the discoloration appeared to be a darker red color (Fig. B.1). This could be a specific early infection response of 'Emerald' dictated by cultivar differences. Nevertheless, significant visible symptoms were observed on 'Emerald', with treatments 'AlmaReb3', 'AlmaStar1' and 'AlmaReb2' appearing to show the most severe amount of disease over the 5-month observation period (heavy discoloration, severe leaf necrosis, defoliation). Somewhat less severe symptoms were observed for treatments 'AlmaReb1' and 'PierceMed1' as the case was with 'Rebel'. This agrees with previous observations of symptoms on 'Rebel' and suggests that it is difficult to distinguish between the subspecies of Xf isolates based solely upon their virulence on blueberry (at least in greenhouse conditions). The timing of first symptom appearance, as well, was essentially the same for 'Emerald' and 'Rebel'. Further studies are necessary to understand mechanisms of Xf virulence expression and the pathogen's adaptation to different cultivars of SHB blueberry. Analysis of the AUDPC and testing for Xf incidence (PCR) followed according to the same scheme adopted for 'Rebel' plants.

By contrast, 'Farthing' plants, in general, did not show BLS-like discoloration or necrosis over the 5-month duration of the trial. Interestingly, only two 'Farthing' plants, both inoculated

with ‘AlmaReb3’, started to show good symptoms at about 115 dpi. These symptoms progressed towards more severe marginal leaf burns and necrosis typical of BLS which spread through the inoculated stems (Fig. B.2). All the other inoculated ‘Farthing’ plants remained asymptomatic just as with buffer and no-buffer plants. It is possible that other plants inoculated with ‘AlmaReb3’, or in general ‘Farthing’ plants inoculated with Xf would have resulted in appearance and development of typical, severe BLS symptoms (and plant death) after a longer incubation period in the greenhouse, past the 5-month observation timeframe adopted in our trial.

Nonetheless, based upon the apparently different degrees of observable symptoms, with virtually no disease reported for ‘Farthing’ plants, the possibility of host resistance to Xf should be further investigated for different cultivars of SHB blueberry. Given that only a single experiment with cultivars ‘Farthing’ and ‘Emerald’ has been carried out, it is premature to draw any final conclusions concerning the large fraction of asymptomatic plants in the ‘Farthing’ trial, as the 5-month duration of our symptom assessment may not be a long enough timeframe to evaluate symptoms for a disease that is known to take a long time to occur with long-term repercussions. Molecular testing of collected leaves of ‘Farthing’ plants at 75 and 105 dpi (same protocol applied throughout the three ‘Rebel’ trials in Ch. 3) confirmed Xf presence in only 2 out of the total Xf-treated plants, with both of these having been inoculated with the ‘AlmaReb3’ isolate, and only one showing BLS symptoms (Table B.1). Whether these results are due to a lack of initial infection or the presence of inhibiting factors during the chemical reactions involved during PCR requires further exploration. Furthermore, examination of the relative Xf cell concentration through quantitative PCR may provide important information regarding differential reproduction of Xf within the different cultivars – a factor that could impact BLS severity on these cultivars. Due to the very low number of ‘Farthing’ plants testing positive for

Xf by PCR within our experiment, AUDPC analysis was not performed for ‘Farthing’ plants. For ‘Emerald’, molecular testing revealed a much higher incidence of Xf infection (Table B.1) relative to ‘Farthing’, but a relatively lower incidence versus the Xf testing results for ‘Rebel’. AUDPC analysis for ‘Emerald’ was calculated for Xf-positive plants only, and suggested relatively higher virulence of the Xfm isolates versus the Xff isolates on this cultivar (Table B.2). Only the Xfm isolates ‘AlmaReb3’ and ‘AlmaStar1’ caused significantly more symptoms versus the uninoculated controls, whereas the average symptom severity produced by Xff isolates ‘AlmaReb1’, ‘AlmaReb2’, and ‘PierceMed1’ was not significantly different than the control treatments.

Table B.1. PCR testing results for Xf-inoculated ‘Farthing’ and ‘Emerald’ plants.

Treatment	Farthing[z]	Emerald[z]
AlmaReb1	0/6 (0%)	6/7 (85%)
PierceMed1	0/7 (0%)	4/7 (57%)
AlmaReb2	0/7 (0%)	3/7 (42%)
AlmaReb3	2/7 (28%)	5/7 (71%)
AlmaStar1	0/7 (0%)	5/7 (71%)
Buffer	0/7 (0%)	0/7 (0%)
No-buffer	0/6 (0%)	0/7 (0%)

[z]PCR-positive plants/total surviving plants (percentage). Seven plants were inoculated per treatment per trial. Totals less than seven indicate plants that died prior to testing.

Table B.2. AUDPC values for each treatment on ‘Emerald’ cultivar.

Treatment	AUDPC[zy]
AlmaReb1	97.51 bc
PierceMed1	241.50 abc
AlmaReb2	252.17 abc
AlmaReb3	520.50 a
AlmaStar1	356.00 ab
Buffer	10.86 c
No buffer	11.29 c

[z]For inoculated plants, AUDPC values are calculated from Xf-positive plants only, as defined by PCR results.

[y]Means in each column followed by the same letter are not significantly different according to Fisher’s LSD test ($P \leq 0.05$).

Figure B.1. Initial BLS discoloration and reddening symptoms on an ‘Emerald’ plant.

Figure B.2. BLS symptoms observed on one of the two symptomatic ‘Farthing’ plants at 140 dpi, showing typical leaf reddening and discoloration, marginal scorching and the dark band separating necrotic from healthy tissue.