EPIDEMIOLOGY AND MANAGEMENT OF CUCURBIT YELLOW VINE DISEASE, AND CHARACTERIZATION OF THE CAUSAL AGENT *SERRATIA MARCESCENS*

by

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(Under the Direction of Elizabeth L. Little)

ABSTRACT

Cucurbit yellow vine disease (CYVD), caused by the squash bug-transmitted bacterium *Serratia marcescens*, causes a vascular wilt leading to plant death. The goals of this research were to 1) confirm the presence of CYVD in Georgia; 2) characterize the genetic diversity of phytopathogenic and nonpathogenic strains of *S. marcescens*; and 3) understand the vector/bacterium interactions and, using row covers, the effect of plant age on disease incidence. *S. marcescens* strains were recovered from symptomatic plants and confirmed as the CYVD pathogen. Genetic analysis indicated that CYVD-associated strains of *S. marcescens* recently diverged from a common ancestor. Epidemiological studies showed that overwintering squash bugs frequently harbored the pathogen and could acquire it during any instar. Row cover trials demonstrated that plant age is positively correlated with disease resistance and that flowering is the optimal time for row cover removal.

INDEX WORDS: *Serratia marcescens*, cucurbit yellow vine disease, squash bug, multilocus sequence analysis, rep-PCR, row cover

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DEDICATION

I would like to dedicate this work to my parents, Rich and Bev; my wife, Kate; and my siblings, Steve and Kristen. Thank you all.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

History and importance of pumpkin and summer squash. Pumpkin (*Cucurbita pepo* and *C. maxima*) and summer squash (*C. pepo*) are cultivated crops that belong to the family Cucurbitaceae. Both of these New World species originated in Central to South America, whereas other economically important cucurbits such as melons and cucumber originated in Africa and India and are considered Old World species (33). New World and Old World cucurbits share a multitude of diseases, such as downy mildew (*Pseudoperonospora cubensis*), bacterial wilt (*Erwinia tracheiphila*), and gummy stem blight (*Didymella bryoniae*) (38).

China is the leading producer of pumpkins, squash, and gourds where production reached 7 million metric tons in 2012. The United States is the fifth largest producer of pumpkins, squash, and gourds and produced approximately 0.90 million metric tons in 2012 (2).

Several plants in the family Cucurbitaceae, including cucumber *(Cucumis sativus),* cantaloupe *(Cucumis melo),* pumpkin and winter squash *(Cucurbita pepo* and *Cucurbita maxima),* summer squash *(Cucurbita pepo)* and watermelon *(Citrullus lanatus var. lanatus)* are of economic importance in the United States. In 2013, 0.31 million tons of summer squash and 0.57 million tons of pumpkin were harvested and valued at 237.10 and 149.94 million dollars, respectively (United States Department of Agriculture National Agriculture Statistics Service). Commercial vegetable production in Georgia in 2012 represented 6.7% of the total farm gate receipts with a value of $936 million. Watermelon was the most valuable cucurbit crop in Georgia with a value of $160 million. Squash (both summer and winter) production was valued at $44 million, with zucchini representing over half of the production value. Large-scale

vegetable production in Georgia is concentrated in the south central part of the state on the sandy soils of the coastal plain with Colquitt, Echols, and Tift Counties ranked 1, 2 and 3, respectively (35).

In recent years small-scale commercial vegetable production for direct marketing has been increasing rapidly in all areas of the state, although the greatest numbers of the fresh market growers are found near urban centers such as Atlanta, Athens, Savannah, Macon, and Columbus. Much of this produce is grown using organic methods. While the value of production is small in terms of total farm gate value, much of this new production may be under-reported due to the non-traditional methods of marketing. The USDA reported in 2011 that 23 certified organic vegetable farms in Georgia had total gross sales of $2,761,182 (1). However, most of the growers in Georgia that use organic methods of production are not certified organic. Currently in Georgia, there are 124 farms with certified naturally grown certification and 154 farmer's markets (up from 9 in 2003) (1, 3). Most of these farms also participate in Community Supported Agriculture (CSA) programs and provide weekly shares to their clientele. The small diversified fruit and vegetable production that typifies local market growers has a unique set of needs, especially when coping with pest pressures. One of the problems to emerge for organic vegetable growers in Georgia has been cucurbit yellow vine disease.

History of cucurbit yellow vine disease. Cucurbit yellow vine disease (CYVD) was first observed in Texas and Oklahoma in 1988 where it caused a decline of squash and pumpkin that was characterized by a yellowing and wilting of plants (12). Three years later the disease caused a collapse of many of the early-season watermelons in central Texas and Oklahoma, although it was several years before the causal agent was identified (6). Bruton *et al.* made thousands of fungal and bacterial isolation attempts from 1991-1995 from symptomatic and asymptomatic

cucurbits; however no single organism was consistently recovered from symptomatic plants. Electron microscopy revealed a clogging of the phloem tubes that was consistent with phloem discoloration, as well as rod-shaped organisms within the phloem sieve tube elements. This led scientists to consider the possibility of a fastidious, phloem-limited prokaryote as the causal agent (11). Around the same time Avila *et al.* used PCR and sequencing to determine that the causal agent of CYVD was likely a prokaryote in the class Gammaproteobacteria and was closely related to *Serratia marcescens* (6). Eventually a protocol was developed through which the bacterium could be consistently isolated from symptomatic plants and maintained in culture (12). Completion of Koch's Postulates, sequencing and analysis of the 16S rDNA and *groE* genes, as well as rep-PCR and DNA-DNA hybridization were then used to show that the causal agent of CYVD was *S. marcescens* (26, 37).

Although many attempts to control CYVD failed, the application of foliar insecticides lowered disease incidence and led researchers to consider the possibility of an insect vector playing a role in the pathogen life cycle. To test this theory, row covers were used to exclude insects from squash plants for 40 or 50 days and plants were subsequently screened for the presence of the pathogen. The authors found that insect exclusion prevented the development of CYVD and concluded that an insect vector must play a role in the disease cycle (7). Since squash bugs are a major pest of cucurbits, Pair *et al.* tested them as a potential pathogen vector. Overwintering squash bugs were collected from under hardwood litter in forested areas and allowed to feed on summer squash. A percentage of plants developed characteristic CYVD symptoms and *S. marcescens* was isolated and confirmed via PCR for these plants, thereby implicating the squash bug as the pathogen vector (24). In a subsequent study, an artificial feeding system was deployed whereby squash bugs could acquire the pathogen by feeding on

squash cubes that were vacuum-infiltrated with *S. marcescens*. Using this system, 12% of adult squash bugs acquired and transmitted the pathogen to squash plants, although the pathogen was never re-isolated from any of the plants in the experiment (31).

Disease symptoms. The first characteristic symptom to appear on an affected plant is a slight yellowing of leaves. This is followed by continued yellowing of leaves and vines and a wilt that usually occurs within two weeks after symptom onset. Wilting is often gradual and occurs approximately 2 weeks before harvest, although a rapid wilt at flowering and fruit set has been observed on occasion. The most diagnostic symptom is a honey-brown discoloration of the phloem tissue that occurs just above and below the soil line in the crown area of affected plants (12).

Causal agent of CYVD. *S. marcescens*, the causal agent of CYVD, is a ubiquitous, rod-shaped, facultative anaerobic, gram-negative enterobacterium with strains occupying diverse ecological niches (11, 12, 34). Bacterial colonies are round, smooth, entire, convex, creamy white, and range from 1.5 to 2.0 mm after 4 days of growth on nutrient agar at 28°C (12).

Pathogen vector. Transmission experiments have shown that CYVD strains of *S. marcescens* are vectored by the squash bug, *Anasa tristis*. Squash bugs are true bugs (Hemiptera) that are characterized by the presence of long piercing and sucking mouthparts that extend out from the front of the head (13). When threatened, squash bugs will release a foul smelling chemical that consists of the volatile compounds trans-2-decenal and trans-2-octenal (15). Stink bugs also release volatile chemicals and squash bugs are often considered to be a type of stink bug. However, squash bugs are in the family Coreidae and are more closely related to leaf-footed bugs such as *Leptoglossus* spp. than to other stink bugs (Pentatomidae) like the brown marmorated stink bug (*Halyomorpha halys*).

Squash bugs are the biggest single threat to squash and pumpkin production in the United States and can also pose a serious threat to watermelon production (20). Bonjour *et al.* found that squash bug ovipositional preference and nymphal survivorship increased in the following order: cantaloupe, cucumber, watermelon, squash, and pumpkin (8, 9). Although squash bugs exhibit a decreased preference for watermelon and certain varieties of winter squash such as butternut (cv. Waltham) and acorn (cv. Royal), studies have shown that they are able to completely overcome resistant varieties in as little as five generations (19, 27). In addition to *Anasa tristis*, which occurs throughout the United States and southern Canada, there also exists the horned squash bug (*Anasa armigera*). The horned squash bug occurs in the eastern United States and exhibits the same crop preferences as the squash bug, although it generally prefers wild cucurbits (29).

S. marcescens is the only known plant pathogen that is vectored by the squash bug. *S. marcescens* is acquired by squash bugs during feeding and/or probing of host plants prior to feeding (12). Studies have shown that nymphs can acquire the bacterium using an artificial feeding system, although it is unknown whether the nymphs can acquire and transmit the bacterium in nature (31). While squash bugs are thought to primarily feed on the xylem, they have also been shown to probe phloem tissue and this is likely when CYVD infection occurs (10, 21). Acquired bacteria will persist in the hemocoel of the insects through molting events and periods of hibernation (24, 31). It is thought that the bacterium is circulative and propagative due to persistence through molting events and long periods of retention within the vector during diapauses followed by successful inoculations of host plants (17, 31).

Primary inoculum for CYVD comes from squash bugs that have retained the bacterium over the winter and emerge in the spring to feed on and infect early-season cucurbit crops (24). The adults lay clusters of eggs on the undersides of the leaves after emergence. Eggs hatch after

seven to nine days and nymphs emerge and begin feeding. Nymphs will molt through five instars prior to emerging as adults. The entire life cycle takes six to eight weeks (13). Squash bugs will have one generation per year in the northern United States and Canada and two to three generations in the southern United States (14, 25). Adults will begin to enter diapause late in the growing season. Squash bugs that molt to adults after July will begin to enter diapause in the fall and overwinter with the pathogen (13, 24). Photoperiod is the key regulator of diapause induction in squash bugs and populations are locally adapted to the photoperiod typical of their latitude (22). The exact photoperiod for diapause induction has not been determined for squash bugs in Georgia, though it likely occurs in late August or September. Squash bugs overwinter under crop debris, clods of soil, man-made debris piles, and leaf litter in areas adjacent to cucurbit fields (5, 13).

Pathogenesis. While the causal agent for CYVD was not immediately identified, the honey-brown discoloration of the phloem seen in affected plants was positively correlated with the disease. Scanning electron microscopy revealed plugged phloem sieve tubes while transmission electron microscopy confirmed the presence of bacteria within the phloem (11). In a later study, suppressive subtractive hybridization revealed two gene clusters that were present in CYVD strains, but lacking in closely related nonphytopathogenic strains. These clusters included a fimbrial-gene cluster that was proposed to be part of a genome island and a potential virulence factor. Two genes present in the fimbrial-gene cluster were *fimA* and *fimH*, which encode the major fimbrial rod subunit and fimbrial adhesin, respectively (36). In a subsequent study, the *fimA* and *fimH* genes were disrupted via gene deletion. Electron microscopy of the resulting mutants showed an absence of fimbriae in the $fimA^-$ mutant, but not the $fimH^-$ mutant. Pathogenicity tests showed a reduction in virulence for both mutants, although they retained the

ability to cause disease, suggesting that there may be additional virulence factors involved in pathogenesis (17). Much about the process of pathogenesis, including effectors and effector delivery systems, additional virulence factors, and pathogenicity determinants is currently unknown.

Diversity of *S. marcescens.* *S. marcescens* is an ecologically and genetically diverse bacterium that can survive as a water saprophyte, soil saprophyte, insect pathogen, plant pathogen, and opportunistic human pathogen (11, 12, 34, 37). Studies have shown that CYVD strains of *S. marcescens* differ from those isolated from other ecological niches (26, 37). Fatty acid methyl ester (FAME) analysis indicated a significant difference between the fatty acid compositions of CYVD strains and all other *Serratia* spp. in the available database and, in some cases, placed CYVD strains closer to *S. fonticola*, *S. plymuthica*, and *S. odorifera*. A BIOLOG test showed substrate utilization profiles that were significantly different between CYVD and non-CYVD strains. In this test, four *S. marcescens* strains were used, including a human-associated strain, a cotton root endophyte, and two strains associated with CYVD. The human-associated strain and cotton root endophyte strain shared nearly identical substrate utilization profiles and had the ability to metabolize 30 substrates that the CYVD strains could not (26).

Repetitive elements based polymerase chain reaction (rep-PCR) relies on interspersed repetitive sequences commonly found in prokaryotic genomes. These repetitive sites can be used as primer binding sites that can amplify differently sized DNA fragments internal to the repetitive sequences in order to create a fingerprint pattern for a given bacterial strain (18, 30). Rep-PCR experiments indicated that CYVD strains of *S. marcescens* isolated from different cucurbit hosts, geographic locations, and growing seasons were identical and formed a group that was separate from all other *S. marcescens* strains. The most closely related strains using this

method included three rice endophytes (R01-A, R02-A, R03-A), a cotton endophyte (G01-A), and a human pathogen (H02-A).

DNA-DNA hybridization is a molecular technique that measures the binding affinity between the whole genomic DNA of two bacterial strains and is therefore an indirect measurement of the sequence similarity between two strains. In this method, the genomic DNA of two bacterial strains is incubated together to allow the two strands of DNA to hybridize. The melting point of the heterologous double-stranded DNA is then measured to determine how tightly the two strands are bound (i.e., the similarity of the DNA of the two bacterial strains) (16). This method has been used for species delineation in bacteria with the accepted definition of a separate species being less than 70% DNA-DNA relatedness and with greater than 5°C ΔT_m (32). DNA-DNA hybridization studies were used to identify the bacteria recovered from CYVD affected plants as *S. marcescens*. When the authors compared CYVD-associated *S. marcescens* strains to non-CYVD strains they found that the DNA-DNA relatedness ranged from 69% to 90% while the ΔT_m ranged from 1.0 to 3.5 (37). While these data suggested that the CYVD-associated bacteria were indeed *S. marcescens*, they also revealed a great amount of divergence at the genomic level. The CYVD strains were most closely related to a group of *S. marcescens* strains that contained endophytes from cotton (G01-A) and rice (R01-A) as well as a human-associated strain (H02-A) (37).

Distribution and impact of CYVD. CYVD has been identified in Arkansas, Colorado, Connecticut, Kansas, Massachusetts, Michigan, Missouri, Nebraska, Ohio, Tennessee, Alabama, and Georgia (17, 23, 28). Susceptible hosts include squash, watermelon, pumpkin, and cantaloupe. Pumpkin and squash are more susceptible to infection than cantaloupe and watermelon, although this could largely be due to feeding and ovipositional preference exhibited

by squash bugs (9, 12). CYVD has been severe in areas with abundant watermelon production and limited squash and pumpkin production (12, 24). Losses from this disease can vary drastically from year to year from a few plants to an entire field (12). Aside from the widespread losses documented in Texas and Oklahoma, there is relatively little information on the impact and seasonality of the disease where it has been reported. Sikora reported 20% disease incidence in summer squash and 25-30% disease incidence in watermelon in eastern Alabama in June of 2010 (28). Bruton *et al.* noted that late-season cucurbits, especially watermelon, had a lower incidence of CYVD. As of 2014, CYVD had not been reported on watermelon in Georgia, despite watermelon production exceeding all other cucurbit production combined (United States Department of Agriculture National Agriculture Statistics Service).

Disease management. Current disease management practices are aimed at controlling the squash bug vector. The application of insecticides is the most widely utilized strategy for the control of squash bugs and can be effective, although adult squash bugs are notoriously difficult to manage. Squash bugs tend to aggregate on the underside of leaves and at the base of squash plants where the insects can avoid foliar insecticides. The common use of plastic mulch in squash production gives squash bugs a place to shelter and can lead to greater increases in populations. Targeting the first generation of nymphs with insecticides can mitigate heavy infestations later in the growing season and keep the adult squash bugs from migrating to later plantings (13). There are several cultural practices for squash bug control and these can be utilized to reduce the use of chemical insecticides. Removal of the crop debris and old fruit that are attractive to squash bugs immediately after harvest can reduce overwintering populations (13). The use of trap crops to lure squash bugs away from high value cucurbits has been proposed, but not thoroughly investigated. In this system, trap crops that are highly attractive to

squash bugs are planted earlier than the main crop on field borders close to the overwintering sites of squash bugs or around plantings of susceptible crops. The trap crops intercept the squash bugs and can then be treated with a systemic pesticide or mechanically destroyed to reduce squash bug numbers (5). Homeowners or small scale growers can trap squash bugs by placing boards on the ground which will attract congregating squash bugs and allow for easy destruction (13). Researchers have found that row covers can be used to physically exclude squash bugs from crops and limit infestations, although covers must be removed at flowering to allow for pollination (4). It is currently unknown if row covers removed at flowering provide enough protection for acceptable control of CYVD.

Project Objectives

1. Cucurbit yellow vine disease etiology

1A. Determine the causal agent of squash collapse in Georgia

1B. Develop an effective pathogenicity test

1C. Evaluate the capacity of *S. marcescens* to survive and move within the host

2. Characterization of *S. marcescens* using rep-PCR and multilocus sequence analysis

3. Epidemiology and Management of CYVD

3A. Determine the temporal frequency of *S. marcescens* occurrence in *Anasa tristis*

3B. Evaluate the capacity of the horned squash bug, *Anasa armigera* to transmit *S. marcescens*

3C. Understand the seasonal dynamics of CYVD incidence through disease monitoring

3D. Determine the relationship between plant age and susceptibility to *S. marcescens*

3E. Evaluate the capacity of row covers to mitigate CYVD

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CHAPTER 2

CUCURBIT YELLOW VINE DISEASE ETIOLOGY[1]

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Abstract

In June 2012, a number of squash plants wilted and died in a field trial at the UGA Horticulture Farm. Several of these plants had symptoms that were consistent with cucurbit yellow vine disease (CYVD), caused by the squash bug (*Anasa tristis*) transmitted bacterium *Serratia marcescens.* Since first described in 1988 in Texas and Oklahoma, CYVD had been confirmed in several states but not in Georgia. To determine the causal agent of the squash collapse, vascular tissues from the lower stem of 21 affected plants were macerated in buffer and plated onto bacterial growth media. Individual bacterial colonies were compared with a known strain of the CYVD bacterium using biochemical and molecular methods. The presence of CYVD-specific strains of *S. marcescens* was confirmed in seven plants. Several inoculation methods were tested under greenhouse conditions to complete Koch's postulates. Bacterial inoculation via an inoculation fork was the only method that yielded disease symptoms, although the bacterium was able to colonize plant tissues endophytically without causing disease when inoculated with a syringe and hypodermic needle. In order to quantify the ability of the bacterium to colonize the host, plants were inoculated with *S. marcescens* P01, *S. marcescens* W11, *Xanthomonas cucurbitae* 12-3, or *Pseudomonas syringae* pv. *tomato* 88-84 using a syringe and hypodermic needle. Four weeks after inoculation, tissues were sampled at four points along the lower stem and CFU per gram of plant tissue were calculated. *S marcescens* colonized and multiplied to high populations in the plants at all sampling points more effectively than the other bacteria.

Introduction

Cucurbit yellow vine disease (CYVD), caused by the bacterium *S. marcescens*, is a disease of squash, watermelon, pumpkin, and cantaloupe that was first observed on squash and pumpkin in Texas and Oklahoma in 1988, and on watermelon in Oklahoma in 1991. Disease symptoms include yellowing, wilting, and a honey-brown discoloration of the phloem. Wilting is usually gradual and occurs approximately two weeks before harvest, although a rapid wilt at flowering and fruit set has been observed on occasion. Disease losses can range from a few plants to an entire field in affected areas (4, 5). CYVD-causing strains of *S. marcescens* are vectored by the squash bug (*Anasa tristis*), which overwinters with the pathogen and emerges in the spring to infect early-season cucurbit plantings (21).

During the past several years, growers in the Georgia Piedmont have reported a condition affecting squash that was characterized by a yellowing and wilting of plants that began around the time of flowering. Bacterial wilt caused by *Erwinia tracheiphila* was initially suspected as the potential causal agent, although a lack of bacterial streaming in wilted plants and an absence of symptoms in nearby cucumber plantings led to the consideration of an alternative causal agent (32). A honey-brown discoloration of the phloem indicated that the disease could be CYVD caused by *S. marcescens* (5).

S. marcescens strains that cause CYVD are unique among plant pathogens in that they are phloem-associated and cause a vascular wilt but they have cell walls and can be cultured on media. Other phloem-associated plant pathogens are obligate pathogens and do not grow on standard bacterial growth media (8). Completion of Koch's postulates for obligate, vector-transmitted plant pathogens can be challenging (1). Since *S. marcescens* grows well in culture,

several attempts have been made to establish an inoculation protocol that yields acceptable and consistent rates of disease incidence without relying on the squash bug vector (5, 18).

The current inoculation protocol for CYVD uses an inoculation fork that consists of five minuten pins spaced evenly apart and soldered to a piece of 10-gauge copper wire (5, 17). For inoculations, 20 µl of bacterial suspension at 1 x 10^9 to 1 x 10^{11} CFU is pipetted onto the stem at the junction of the stem and cotyledon and this area is subsequently stabbed with the inoculation fork 6-15 times so that the fork penetrates both cotyledons as well as the apical meristem (5, 18). Although this technique yields disease, it has several drawbacks. This method often leads to the development of symptoms that are not typical of the disease in the field, such as stunting and leaf cupping. Phloem browning, which is the most diagnostic symptom of CYVD, is often absent in plants inoculated in this manner even when they are PCR-positive for the pathogen (18). This technique also requires a very high bacterial titer for symptom development and has proven to be inconsistent in inciting disease with rates ranging from 17% - 60% (5, 18).

In this study, preliminary inoculation attempts to prove pathogenicity revealed that CYVD-causing strains of *S. marcescens* survived and colonized host plants endophytically without causing symptoms. The evolutionary origins of CYVD-associated *S. marcescens* are unknown, although several methods of genetic analysis have indicated that the most closely related strains are plant endophytes (22, 31). Determining the endophytic capabilities of *S. marcescens* may give additional insights into the evolutionary origins of CYVD strains.

The objectives of this research were to determine the causal agent of squash collapse in Georgia using biochemical and molecular methods, develop an effective and reproducible pathogenicity test for *S. marcescens*, and determine the ability of *S. marcescens* to colonize and move within host plants.

Materials and Methods

Pathogen isolation. In the summer of 2012 several plants in a field plot of pumpkin (cv. New England Pie) and summer squash (cv. Costata Romanesco and cv. Zephyr) at the University of Georgia Durham Horticulture Farm started showing symptoms approximately one week prior to fruiting that were characteristic of CYVD, including leaf yellowing, wilting, and a honey-brown discoloration of the phloem (Fig. 2.1). Isolations were attempted from twenty-one symptomatic plants, although not all plants exhibited the browning phloem symptom. Bacteria were isolated by excising small sections (0.1 g) of phloem tissue from the lower stem and crown. The tissue was surface sterilized for two min in 0.83% NaOCl and macerated using a microtube pestle (Kimble-Chase Kontes, Vineland, NJ) in a 1.5 ml Eppendorf tube containing 0.5 ml 0.01M phosphate buffered saline (PBS). Ten microliters of the resulting suspension was streaked onto nutrient yeast dextrose agar (NYDA) plates and incubated at room temperature for three days (5). Colonies were compared visually to a known CYVD-causing strain (W01-A) of *S. marcescens* (Table 2.1). Colonies resembling the known strain were sub-cultured several times to ensure purity and stored in 15% glycerol at -80ºC.

Pathogen identification. Bacterial colonies resembling *S. marcescens* were isolated from seven plants and a representative strain of the bacterium from each plant was subjected to biochemical and molecular diagnostic tests. The Gram-reaction was determined using the KOH test (25). BBL DrySlide Oxidase Slides (Becton Dickinson, Sparks, MD) were used to determine the presence of cytochrome oxidase (15). Hugh and Leifson's oxidative-fermentative (OF) medium was used to test for the ability of the strains to grow under anaerobic conditions (14, 23). Substrate utilization profiles using BIOLOG GN2 MicroPlates (BIOLOG, Hayward, CA)

for gram-negative bacteria were determined for CYVD *S. marcescens* strains P01 and W01-A, and for the non-CYVD rice endophyte R01-A strain.

For molecular identification, DNA was extracted from the seven strains recovered from symptomatic plants. Bacteria were grown in 5 ml Luria broth for 24 h at 28°C on a shaker (200 rpm). Bacterial genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Amplification of the 16S rDNA gene was carried out using primers YV1 (GGGAGCTTGCTCCCCGG) and YV4 (AACGTCAATTGATGAACGTATTAAGT) which are specific for all strains of *S. marcescens* (30). PCR was performed on a Mastercycler Personal (Eppendorf, Hauppauge, NY) using the following amplification conditions: 5 min at 95°C; 35 cycles of 40 s at 95°C, 60 s at 60°C, 90 s at 72°C, and 1 final extension cycle of 5 min at 72°C (30). PCR reaction volume was 25 µl which included 12.5 µl *Taq* PCR Master Mix (Qiagen, Valencia, CA), 8.5 µl distilled deionized water, 1.5 µl template DNA, 1.25 µl 10 µM forward primer YV1, and 1.25 µl 10 µM reverse primer YV4. PCR products were electrophoretically separated in a gel containing 1.5% GenePure agarose (Bioexpress, Kaysville, UT) and ethidium bromide at 100 µg/l. PCR products that yielded amplicons of expected length were prepared for sequencing using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Sanger sequencing was performed by the University of Georgia Genomics Facility using a 3730xl 96-capillary DNA Analyzer (Applied Biosystems, Foster City, CA). Sequences were visualized and aligned using Geneious v7.1.4 (Biomatters, Auckland, New Zealand) and compared to *S. marcescens* WW4 (accession CP003959.1) using Basic Local Alignment Search Tool (BLAST).

The strains were also tested using the CYVD-strain specific *S. marcescens* PCR primers A79F (CCAGGATACATCCCATGATGAC) and A79R (CATATTACCTGCTGATGCTCCTC) primers. These primers target a region of the genome that is present in CYVD strains, but absent

in all non-phytopathogenic strains tested (30). The PCR reaction volume was 25 µl, which included 12.5 µl *Taq* PCR Master Mix (Qiagen, Valencia, CA), 8.5 µl distilled deionized water, 1.5 µl template DNA, 1.25 µl 10 µM forward primer A79F and 1.25 µl 10 µM reverse primer A79R. PCR was performed on a Mastercycler Personal (Eppendorf, Hauppauge, NY) using the following amplification conditions: 5 min at 95°C; 35 cycles of 40 s at 95°C, 60 s at 60°C, 90 s at 72°C, and 1 final extension cycle of 5 min at 72°C (30). PCR products were electrophoretically separated in a gel containing 1.5% GenePure agarose (Bioexpress, Kaysville, UT) and ethidium bromide at 100 µg/l.

Pathogenicity test: Pumpkin seeds (cv. New England Pie, Johnny's Seeds, ME) were planted in 10.16 cm diameter pots and maintained in a greenhouse prior to inoculations. *S. marcescens* CYVD strain P01, which was identified using the methods outlined above, was grown in Luria broth for 24 hours at 28° on a shaker (200 rpm) and re-suspended in 0.01M PBS at a concentration of 1 x 10^8 CFU/ml. Plants were inoculated approximately seven days after planting at the expanded cotyledon stage by placing 20 µl of the bacterial suspension or 0.01M PBS at the junction of the stem and cotyledon. Six plants were inoculated with P01 and one plant was inoculated with 0.01M PBS as a negative control. An inoculation fork was used to make ten penetrations through the inoculum, both cotyledons, and the apical meristem (5). Plants were transferred to 25.40 cm diameter pots and maintained in a greenhouse for eight weeks and observed for symptom development. Re-isolations were attempted from all plants and PCR with CYVD-specific primers was performed on recovered bacteria as previously described.

Two additional *S. marcescens* strains were included in the second pathogenicity experiment. *S. marcescens* SB03 was isolated from a squash bug and identified as a CYVD-causing strain using PCR with the A79 primers (Table 2.1). *S. marcescens* strain W01-A was

isolated from a symptomatic watermelon in Texas and was previously shown to be pathogenic on pumpkin (5). *S. marcescens* strains P01, SB03, and W01-A were grown in Luria broth for 24 hours at 28° on a shaker (200 rpm) and re-suspended in 0.01M PBS at a concentration of 1 x 10^8 CFU/ml. Pumpkin (cv. New England Pie) plants were inoculated at the expanded cotyledon stage by placing 20 µl of the bacterial suspension or 0.01M PBS at the junction of the stem and cotyledon. An inoculation fork was used to make ten penetrations through the inoculum, both cotyledons, and the apical meristem (5). Ten plants were inoculated for each bacterial strain and the negative control. Plants were transferred to 25.40 cm diameter pots and maintained in a greenhouse for eight weeks while they were observed for symptom development. Re-isolations were attempted from all plants and PCR with CYVD-specific primers was performed on recovered bacteria as previously described.

***S. marcescens* survival and colonization of the host.** Two experiments were conducted to evaluate the capacity for *S. marcescens* to survive and colonize the host. For the first experiment, pumpkin seeds (cv. New England Pie) were planted in 10.16 cm diameter pots and maintained in a greenhouse prior to inoculations. *S. marcescens* P01 was grown in Luria broth for 24 hours at 28°C on a shaker (200 rpm) and re-suspended in 0.01M PBS at a concentration of 1 x 10^8 CFU/ml. *Pseudomonas syringae* pv. *tomato*, which was included in this experiment as a nonpathogenic control, was grown in nutrient broth for 24 hours at 28° on a shaker (200 rpm) and re-suspended in 0.01M PBS at a concentration of 1 x 10^8 CFU/ml. Plants were inoculated at the first true leaf stage, approximately one week after planting. Hypocotyls were infiltrated 3 cm above the soil line with 100 µl of bacterial suspensions or 0.01M PBS using a syringe and hypodermic needle. For each strain, 18 plants were inoculated. Eleven plants were inoculated with 0.01M PBS as a negative control. Plants were transferred to 25.40 cm diameter pots and

maintained in a greenhouse for four weeks. Isolations were made as described above from vascular bundles at the following four locations relative to the point of inoculation: 2.5 cm below, at inoculation point, 2.5 cm above, and 5.0 cm above. Ten-fold serial dilutions were made and 100 µl of each dilution was plated in triplicate onto LB (*S. marcescens*) or NYDA (*P. syringae*). Bacterial colonies were enumerated and populations were calculated and expressed as CFU/g plant tissue and as log transformations of these values. A one-way ANOVA (PROC GLM; SAS, SAS Institute, Cary, NC) was conducted on log transformed values to determine the significant difference in bacterial populations between sampling points.

Two additional strains were included in the second experiment, *S. marcescens* W11 and *Xanthomonas cucurbitae* 12-3 (Table 2.1). *S. marcescens* W11 is a watermelon endophyte and *X. cucurbitae* is the causal agent of bacterial leaf spot of cucurbits (3). *P. syringae* pv. *tomato* and *X. cucurbitae* 12-3 were grown in nutrient broth for 24 h at 28° on a shaker (200 rpm) and re-suspended in 0.01M PBS at a concentration of 1 x 10^8 CFU/ml. Plant hypocotyls were infiltrated 3 cm above the soil line with 100 µl of bacterial suspensions or 0.01M PBS using a syringe and hypodermic needle. For each strain, 14 plants were inoculated. Ten plants were inoculated with 0.01M PBS as a negative control. Plants were transferred to 25.40 cm diameter pots and maintained in a greenhouse for four weeks. Isolations were made as described above and 100 µl of each serial dilution was plated in triplicate onto LB agar (*S. marcescens*) or NYDA (*P. syringae* and *X. cucurbitae*). Bacterial colonies were enumerated and populations were calculated and expressed as CFU/g plant tissue and as log transformations of these values. A one-way ANOVA (PROC GLM; SAS, SAS Institute, Cary, NC) was conducted on log transformed values to determine the significant difference in bacterial populations between sampling points for each stain and at each sampling point between strains.

Results

Pathogen identification. A bacterium was consistently isolated from 5/10 pumpkin and 2/11 summer squash plants that was morphologically similar to the known CYVD strain of *S. marcescens*. Bacterial colonies were smooth, circular, entire, convex, and non-pigmented. All CYVD-associated strains were Gram-negative using the KOH test, oxidase negative, and facultatively anaerobic using Hugh and Leifson's OF medium (14, 23). Substrate utilization profiles constructed using BIOLOG identified the *S. marcescens* rice endophyte strain R01-A as *S. marcescens* with a probability of 100% and a similarity ranking of 0.866, with a similarity ranking of 0.500 or greater being required for reliable species identification. CYVD-associated strains P01 and W01-A yielded identical substrate utilization profiles that were different from the rice endophyte strain and were identified as most similar to *Tatumella ptyseos* and *S. marcescens* with similarity rankings of 0.154 and 0.120, respectively. These similarity rankings are considered to be too low to be reliable. While W01-A and P01 utilized 18 of the 96 substrates, R01-A utilized 74 of the 96 substrates including 18 that were utilized by the CYVD-associated strains.

PCR using the YV1 and YV4 primers, which are specific for all *S. marcescens* strains, produced the expected 452 bp fragment for the seven strains tested and the known CYVD strain. Sequence analysis of this fragment showed that the 452 bp region from the 16S rDNA gene of all seven strains and the known CYVD strain shared a 100% sequence identity with *S. marcescens* WW4 (accession CP003959.1), a strain that was isolated from a paper machine in Taiwan (6). PCR using the A79F and A79R primers, which are specific for CYVD strains of *S. marcescens*, produced the expected 338 bp amplicon for the seven strains tested and the previously identified

CYVD strain, W01-A, further confirming the seven strain as CYVD-associated strains of *S. marcescens* (Fig. 2.2)(30).

Pathogenicity test. In the first trial, symptoms characteristic of CYVD, including yellowing, wilting, and a honey-brown discoloration of the phloem were observed on four out of six plants inoculated with *S. marcescens* strain P01, but not the negative control plant (Table 2.2). CYVD strains of *S. marcescens* were recovered from all four symptomatic plants, but not from the asymptomatic plants or the negative control.

In the second trial, all of the plants exhibited some degree of yellowing. CYVD strains of *S. marcescens* were recovered from 6 out of 10, 5 out of 10, and 2 out of 10 plants that were inoculated with W01-A, P01, and SB03, respectively (Table 2.2). In all cases, the presence of phloem discoloration was positively correlated with pathogen recovery from inoculated plants. *S. marcescens* was not recovered from any plants with normal-appearing phloem or the negative controls.

***S. marcescens* survival and colonization of the host.** In the first experiment, the CYVD strain of *S. marcescens* was recovered from all four sampling points with concentrations ranging from 2.0×10^2 to 2.0×10^6 CFU/g plant tissue (Table 2.3). No bacteria were recovered from plants inoculated with *P. syringae* pv. *tomato*. For plants inoculated with *S. marcescens* P01, detection frequencies were greater at and below the point of inoculation than above the point of inoculation. Bacterial concentrations were also numerically higher at and below the point of inoculation, although no significant differences were found. No symptom development was observed in any plants.

In the second experiment, bacteria were recovered from three of the four sampling points in plants that were inoculated with *S. marcescens* P01 with concentrations ranging from 8.0×10^2

to 1.1 x 10^5 CFU/g plant tissue (Table 2.4). No bacteria were recovered from plants at 5.0 cm above the inoculation point. Detection frequencies were greater at and below the point of inoculation than above the point of inoculation. Bacterial concentrations were significantly greater 2.5 cm below the inoculation point than 2.5 cm above the inoculation point. For *S. marcescens* W11, bacteria were recovered at and below the inoculation point with concentrations ranging from 4.1 x 10^4 to 2.0 x 10^5 CFU/g plant tissue. For *X. cucurbitae*, bacteria were recovered at and below the point of inoculation, with concentrations ranging from 1.2 x 10^3 to 6.0 x 10^4 CFU/g plant tissue. No significant differences in bacterial concentrations were found between sampling points for *S. marcescens* W11 or *X. cucurbitae*. Overall, no significant differences in bacterial concentrations were found between *S. marcescens* P01, *S. marcescens* W11, and *X. cucurbitae* at or below the inoculation point. No symptoms were observed and no bacteria were recovered from plants inoculated with *P. syringae* pv. *tomato*.

Discussion

Our results confirmed that the squash wilt and collapse observed in the Georgia Piedmont was CYVD caused by the squash bug-transmitted bacterium *S. marcescens*. BIOLOG results showed that CYVD-causing strains of *S. marcescens* had drastically reduced metabolic capabilities when compared to the rice endophyte R01-A. Rascoe *et al.* also reported a reduction in metabolic capabilities when they compared two CYVD-causing strains to a cotton endophyte and a human-associated strain (22). Minor differences in metabolic capabilities have been described among strains within a single bacterial species and have even been used to differentiate pathovars among strains of *Pseudomonas syringae* (27). Tan *et al.* previously constructed substrate utilization profiles for strain R01-A and two saprophytic *S. marcescens* strains using

the Biotype 100 System and discovered a loss of metabolic capabilities in R01-A for two substrates and a gain of metabolic capabilities for two substrates (26). In contrast, CYVD-causing strains of *S. marcescens* showed a drastic reduction in substrate utilization, utilizing only 18 of the 96 substrates while R01-A utilized 74 of the 96 substrates. R01-A utilized all 18 substrates that were consumed by the CYVD-associated strains, indicating a potential loss of function in CYVD strains.

The decreased substrate utilization capacities observed in CYVD strains of *S. marcescens* may be the result of reductive evolution (22). This process occurs frequently in obligate endosymbionts and is characterized by a loss of genes that are redundant or whose gene products are unnecessary in the highly specialized niche occupied by that organism (2). Pieritti *et al.* found drastic differences when they compared the genome sizes of several plant pathogens in the family Xanthomonadaceae. Strains that were not strictly xylem-limited, such as *Xanthomonas campestris* pv. *campestris* and *X. axonopodis* pv. *vesicatoria* possessed genomes of typical size for bacteria, ranging from 5.1 to 5.2 Mb. Conversely, strains that were strictly xylem limited, such as *X. albilineans* and *Xylella fastidiosa* possessed greatly reduced genomes ranging from 2.5 to 3.8 Mb. Reductive evolution has also been reported for phloem-limited pathogens, including *Phytoplasma asteris*, where it has resulted in the loss of genes that were once thought to be essential for autonomously replicating cells (20). Whole genome sequencing of a CYVD-causing strain of *S. marcescens* would allow for comparative analysis with available *S. marcescens* genomes and would indicate if widespread genome loss has occurred in CYVD strains.

Koch's postulates were completed for this pathogen, although no plants collapsed in greenhouse experiments. In the second experiment all of the plants, including the negative

controls, showed some degree of yellowing. This may have been the result of a prolonged growth period in the greenhouse and a powdery mildew infection. In previous studies, plant collapse was not reported using this inoculation protocol when plants were sampled four weeks after inoculation. In our experiments, plants were sampled after eight weeks to allow for greater symptom development but plant collapse was not observed. This could be due to the low stress environment of the greenhouse, which was temperature controlled and essentially devoid of other pathogens and pests, including squash bugs. Additionally, the pumpkin plants in this study were not fruiting as extensively as typical pumpkins in the field and the fruit were slow to develop. If fruit initiation and maturation are key stressors that lead to plant decline associated with CYVD, then a lack of plant collapse in the greenhouse would be expected (5).

S. marcescens is a cosmopolitan species that exists in a variety of habitats and has been found to be associated with plants, insects, and humans (10). Several endophytic strains have been recovered from a variety of plant species, including cotton, rice, summer squash, tomato, and watermelon (5, 11, 12, 24). In many cases, endophytic strains of *S. marcescens* have been shown to provide significant benefits to host plants, including increased cold tolerance, plant growth promotion, and disease suppression through direct antagonism of plant pathogenic organisms (7, 12, 24). Genetic analysis using several methods indicated that CYVD-causing strains of *S. marcescens* were most closely related to a rice endophyte strain (R01-A) (22, 31).

Our data suggest that CYVD-causing strains of *S. marcescens* behave endophytically when inoculated directly into plant hypocotyls using a syringe and hypodermic needle. *S. marcescens* P01 was detected as far as 5.0 cm from the point of inoculation and occurred in tissue that had not yet formed at the time of inoculation (Table 2.3). Endophytic bacteria have been found to vary greatly in their capacity for in planta movement, with typical distances

ranging from 15 cells to several centimeters (13). The vast majority of endophytes have been recovered from lower stems or roots. While endophytic systemic spread has been documented for some bacteria, this occurs much less frequently than localization within a specific plant tissue or area of a plant (13, 19). In plants where systemic spread occurs, bacterial populations are generally much greater in lower stems and roots than in more aerial plant parts (13, 16, 19). Although P01 was recovered from as far as 5 cm above the inoculation point, the bacteria had a tendency for downward movement and concentrations were significantly higher below the inoculation point than above.

To gain a better understanding of the capacity of CYVD strain P01 to survive and colonize pumpkin plants, we included a watermelon endophyte (W11) and *Xanthomonas cucurbitae* 12-3 in the second experiment. Although it is currently unknown whether *X. cucurbitae* can move systemically in cucurbit hosts, several other xanthomonads move systemically in their hosts, including *X. arboricola* pv. *pruni* and *X. axonopodis* pv. *phaseoli* (1). Detection frequencies were higher for *S. marcescens* P01 than for W11 or *X. cucurbitae* at and below the inoculation point, although no significant differences were found in bacterial concentrations between strains (Table 2.4). Additionally, P01 was the only strain recovered above the point of inoculation, providing more support for this strain's ability to colonize the vascular tissues.

Although endophytic populations vary greatly between species, host, and in planta location; the average population density of introduced endophytes typically ranges from 10^3 to 10^5 CFU/g plant tissue (13). Gyaneshwar *et al.* inoculated rice seedlings with *S. marcescens* R01-A, a rice endophyte, and sampled plants 14 days after inoculation. They found population densities of 10^6 and 10^5 CFU/g plant tissue in root and stem sections, respectively (12). When we

sampled plants four weeks after inoculation with P01 we found an average population density of 10^5 CFU/g plant tissue at and below the inoculation point and average population densities ranging from 10^2 to 10^3 CFU/g plant tissue above the inoculation point.

Plant pathogenic bacterial populations tend to be significantly higher than endophytic populations (13). Tsiantos *et al*. found that disease only occurred on tomato when populations of *Clavibacter michiganensis* subsp. *sepedonicus* reached the threshold of 10^7 CFU/g tissue (29). Population densities as high as 10^{10} CFU/g tissue have been observed for *Ralstonia solanacearum* in tomato, eggplant, and pepper (9). Trivedi *et al.* used qPCR to determine population densities of the phloem-limited bacterium *Candidatus* Liberibacter asiaticus and found values ranging from 10^5 to 10^9 CFU/g plant tissue (28). Population thresholds necessary for symptom development have not been determined for CYVD-causing strains of *S. marcescens*. It is possible that, given enough time, P01 could have reached a concentration that might have led to symptom development and disease. This is unlikely, however, since bacteria were not introduced directly into the phloem tissue where they normally occur in diseased plants and *S. marcescens* is not known to invade phloem tissue without the aid of the squash bug vector or through severe tissue wounding with an inoculation fork. Furthermore, Luo demonstrated that *S. marcescens* could survive in the intercellular space between parenchyma cells and in xylem vessels, both of which are commonly colonized by endophytic bacteria (13, 18). It is more likely that *S. marcescens* is surviving endophytically in these locations, although whole vascular bundles were sampled and not individual components such as xylem, phloem, or parenchyma cells. Sampling of individual vascular components or repetition of this experiment with a GFP-tagged strain would give additional insights into the endophyte-like behavior of CYVD-causing strains of *S. marcescens*.

Overall, our results revealed that the observed plant collapse affecting summer squash and pumpkin in Georgia was CYVD caused by the squash bug-transmitted bacterium *S. marcescens*. This unique bacterium showed a greatly reduced ability to utilize substrates when compared to a rice endophyte of the same species, and this reduction may be the result of a prolonged association with phloem sieve tube elements that has led to a loss of unnecessary or redundant genes. In greenhouse inoculation studies CYVD strain P01 exhibited endophytic growth patterns and population densities while failing to incite disease, providing further evidence that CYVD-causing strains of *S. marcescens* may have descended from a plant endophyte.

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Tables

Table 2.1. Species, strains, and origins of bacteria used in this study.

Species, strains	Source, host	Date collected	Geographic origin
S. marcescens, non-CYVD			
R01-A[a]	Rice endophyte	Unknown	Philippines
WW4 (CP003959.1)	Paper machine	2013	Taiwan
W11[b]	Watermelon endophyte	2013	US, Georgia
S. marcescens, CYVD			
P01	Pumpkin	2012	US, Georgia
SB03	Squash bug	2013	US, Georgia
W01-A[a]	Watermelon	1999	US, Texas
Pseudomonas syringae pv. *tomato* 88-84	Tomato	Unknown	US, Georgia
Xanthomonas cucurbitae 12-3[b]	Pumpkin	2013	US, Georgia

[a] Strains provided by J. Fletcher, Oklahoma State University
[b] Strains provided by B. Dutta, University of Georgia

Table 2.2. Results of the pathogenicity test for *S. marcescens* strains that were PCR-positive using CYVD strain-specific primers. Pumpkin plants were inoculated with *S. marcescens* CYVD strains P01, SB03, W01-A, or 0.01M PBS and sampled after eight weeks. Phloem discoloration was observed in 43% of plants and *S. marcescens* was recovered from all of these samples, but none of the samples with healthy phloem or the negative control.

Inoculum species, strain	% of plants positive for *S. marcescens*	% of plants with discolored phloem tissue
Experiment 1		
S. marcescens P01	66.67	66.67
0.01M PBS	0.00	0.00
Experiment 2		
S. marcescens P01	50.00	50.00
S. marcescens SB03	20.00	20.00
S. marcescens W01-A	60.00	60.00
0.01M PBS	0	0

Table 2.3. Detection frequency and bacterial population densities at four sampling points from plants that were inoculated with *S. marcescens* P01 or *Pseudomonas syringae* pv. *tomato* at a concentration of 10^8 CFU. Detection frequencies represent the percentage of plants (n = 18) from which bacteria were recovered at each sampling point. Bacterial population densities are reported as log transformed values of the mean CFU/g plant tissue for each sampling point. Letters indicate significant differences in population densities between sampling points.

Inoculum species, strain	Distance and location from inoculation point (cm)	Detection frequency	mean $\log_{10}$ CFU/g plant tissue	Standard error
Serratia marcescens P01	2.5 below	0.33	4.73a	0.49
	0	0.39	4.23ab	0.32
	2.5 above	0.17	2.97c	0.35
	5.0 above	0.11	3.35abc	0.05
Pseudomonas syringae pv. *tomato*	2.5 below	0.00	-	-
	0	0.00	-	-
	2.5 above	0.00	-	-
	5.0 above	0.00	-	-
0.01M PBS buffer	No bacteria recovered			

Table 2.4. Detection frequency and bacterial population densities at four sampling points from plants that were inoculated with *S. marcescens* P01, *S. marcescens* W11, *X. cucurbitae*, or *Pseudomonas syringae* pv. *tomato* at a concentration of 10^8 CFU. Detection frequencies represent the percentage of plants (n = 14) from which bacteria were recovered at each sampling point. Bacterial population densities are reported as log transformed values of the mean CFU/g plant tissue for each sampling point. Letters indicate significant differences in population densities between sampling points.

Inoculum species, strain	Distance and direction from inoculation point (cm)	Detection frequency	mean $\log_{10}$ CFU/g plant tissue	Standard error
Serratia marcescens P01	2.5 below	0.43	4.48a	0.25
	0	0.57	4.20ab	0.19
	2.5 above	0.14	3.50b	0.60
	5.0 above	0.00	-	-
Serratia marcescens W11	2.5 below	0.29	5.15a	0.01
	0	0.29	4.88a	0.15
	2.5 above	0.00	-	-
	5.0 above	0.00	-	-
Xanthomonas cucurbitae	2.5 below	0.36	4.24a	0.21
	0	0.36	3.94a	0.29
	2.5 above	0.00	-	-
	5.0 above	0.00	-	-
Pseudomonas syringae pv. *tomato*	2.5 below	0.00	-	-
	0	0.00	-	-
	2.5 above	0.00	-	-
	5.0 above	0.00	-	-
0.01M PBS buffer	No bacteria recovered			

Figures

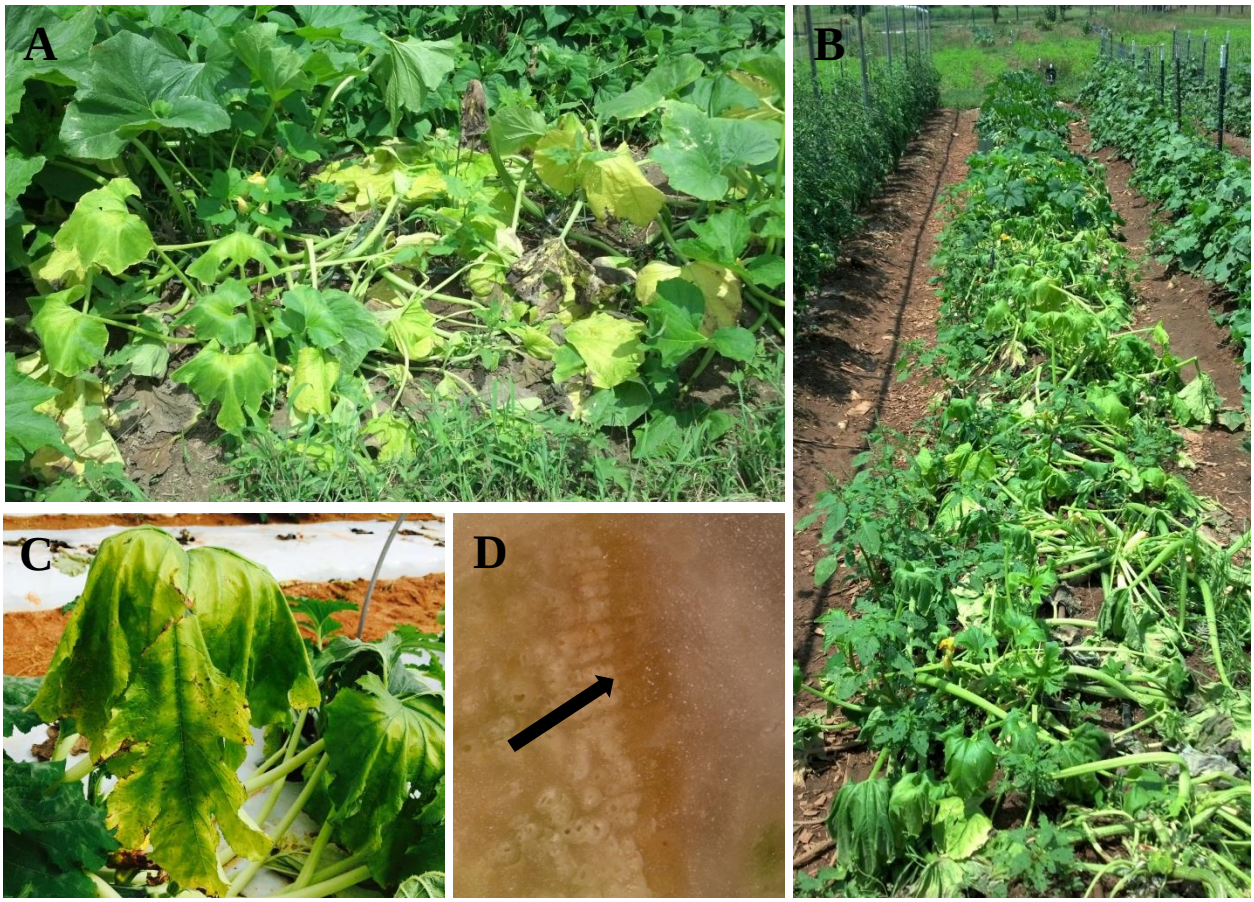

Figure 2.1. Symptoms of CYVD caused by the bacterium *S. marcescens*. Symptoms include a yellowing of leaves and wilting that typically coincides with flowering and fruit set (A-C). The most diagnostic symptom is a honey-brown discoloration of the phloem (arrow) (D).

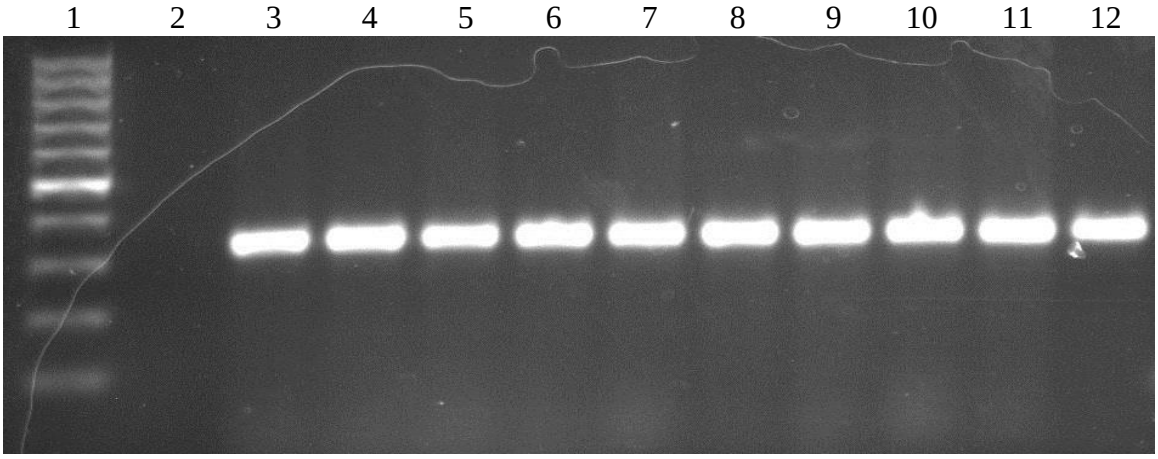

Figure 2.2. PCR amplification using A79F/R CYVD-specific primers on DNA extracted from bacteria that were recovered from symptomatic squash plants. Lane1, 100 bp DNA ladder; lane 2, negative control; lane 3, W01-A; lane 4, W01-C; lane 5, Z02; lane 6, P01; lane 7, P02; lane 8, P04; lane 9, Z01; lane 10, P03; lane 11, S5; lane 12, W01-A

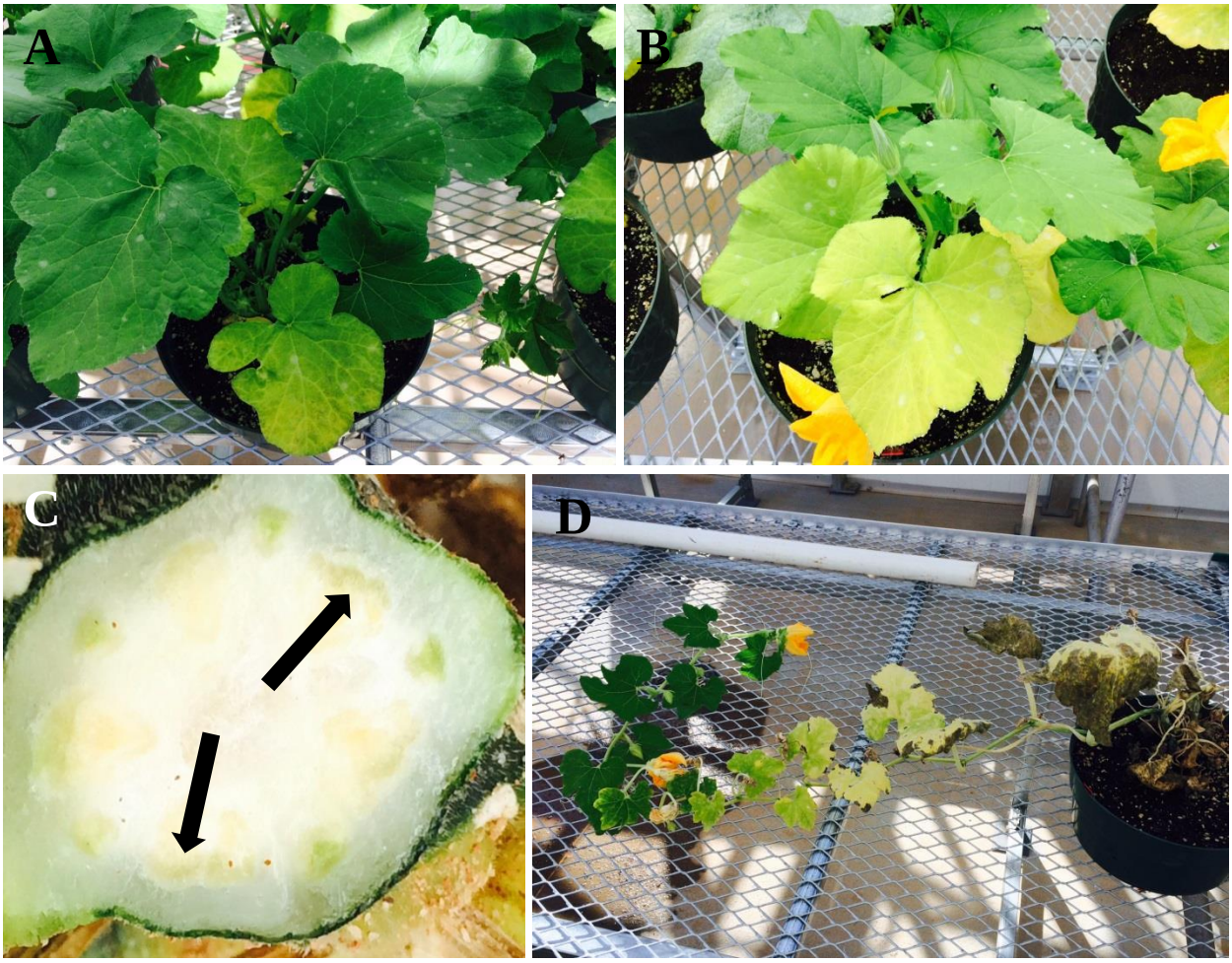

Figure 2.3. Plant symptoms observed from the pathogenicity test. Plant were inoculated with *S. marcescens* CYVD strains P01, SB03, or W01-A. Yellowing was observed on all plants (A, B, and D). Phloem discoloration (arrows) (C) was observed in 43% of plants and *S. marcescens* was only recovered from plants with phloem discoloration.

CHAPTER 3

CHARACTERIZATION OF PLANT PATHOGENIC AND NON-PATHOGENIC STRAINS OF *SERRATIA MARCESCENS* USING rep-PCR AND MULTILOCUS SEQUENCE ANALYSIS[2]

[2] Besler, K. R. and Little, E. L. 2014. To be submitted to *Phytopathology*.

Abstract

Cucurbit yellow vine disease (CYVD) caused by the squash bug (*Anasa tristis*) transmitted bacterium *Serratia marcescens* was first observed in Oklahoma and Texas in 1988. In 2012, CYVD was confirmed in Georgia where it causes substantial losses in organic squash production. *S. marcescens* is a highly diverse species found in many ecological niches. In previous studies, CYVD strains of *S. marcescens* form a distinct group separate from non-CYVD strains using biological and molecular characterization techniques including repetitive element sequence-based PCR (rep-PCR) but not multilocus sequence analysis (MLSA). MLSA is useful in determining genetic distances between closely related organisms. Genetic diversity of CYVD and non-CYVD strains of *S. marcescens* was compared using both rep-PCR and MLSA. For rep-PCR, the DNA of each strain was subjected to PCR using the BOX-A1R primer. Products were resolved on an agarose gel, bands were scored using BioNumerics software, and a phylogenetic tree was generated. Results indicated that the CYVD strains were diverse and formed a cluster separate from non-CYVD strains. In order to better understand this genetic variation, MLSA was performed on the DNA from the same strains. PCR primers were designed to amplify DNA fragments from six housekeeping genes. Product sequences were aligned and concatenated, and a phylogenetic tree was constructed. MLSA revealed a 100% similarity across all six loci for all CYVD strains with the exception of one strain, suggesting a recent divergence of plant pathogenic strains from a common ancestor. For both methods, CYVD strains clustered tightly and separately from non-plant pathogenic *S. marcescens* strains and were most closely related to a rice endophyte.

Introduction

Cucurbit yellow vine disease (CYVD), caused by the bacterium *S. marcescens*, is a disease of squash, watermelon, pumpkin, and cantaloupe that was first observed on squash and pumpkin in Texas and Oklahoma in 1988. Disease symptoms include yellowing, stunting, wilting, and a honey-brown discoloration of the phloem. Wilting is usually gradual and occurs approximately two weeks before harvest, although a rapid wilt at flowering and fruit set has been observed on occasion. Disease losses can range from a few plants to an entire field (8, 9). CYVD-causing strains of *S. marcescens* are vectored by the squash bug (*Anasa tristis*), which overwinters with the bacterium and emerges in the spring to infect early-season cucurbit plantings (29).

S. marcescens is an ecologically and genetically diverse bacterium in the family Enterobacteriaceae that can survive as a water and soil saprophyte, insect pathogen, plant endophyte and pathogen, and opportunistic human pathogen (8, 9, 42, 47). Studies have shown that CYVD strains of *S. marcescens* differ from those isolated from other ecological niches (34, 47). Fatty acid methyl ester analysis indicated a significant difference between the fatty acid composition of CYVD strains and all other *Serratia* spp. in the available database and, in some cases, placed CYVD strains closer to *S. fonticola*, *S. plymuthica*, and *S. odorifera* than to *S. marcescens*. A BIOLOG test showed substrate utilization profiles that were significantly different between CYVD and non-CYVD strains. In this test, four *S. marcescens* strains were used, including a human-associated strain, a cotton root endophyte, and two strains associated with CYVD. The human-associated strain and cotton root endophyte strain shared nearly identical substrate utilization profiles and had the ability to metabolize 30 substrates that the CYVD strains could not (34). Zhang *et al.* used molecular methods, including DNA-DNA

hybridization and rep-PCR to help describe these atypical differences and to definitively place the causal agent of CYVD in the species *S. marcescens* (47).

DNA-DNA hybridization (DDH) is a molecular technique that measures the denaturing temperature between the hybridized DNA of two bacterial strains and is therefore a measurement of binding affinity and indicates the number of nucleotide sequences shared by the two strains (21). This method has been used as a standard for bacterial species delineation with the accepted definition of a separate species being less than 70% DNA-DNA relatedness and with greater than 5°C change in melting temperature (ΔT_m) between the heterologous DNA duplex and the homologous DNA duplex (41). DDH studies were used to show that the bacterium recovered from CYVD affected plants was *S. marcescens*. These experiments compared CYVD-associated *S. marcescens* strains to non-CYVD strains and found that the DDH values ranged from 69% to 90% while the ΔT_m ranged from 1.0 to 3.5 (47). Although these data suggested that the CYVD-associated bacteria were indeed *S. marcescens*, they also revealed a great amount of divergence at the genomic level. In this study using DDH, CYVD strains were most closely related to a group of *S. marcescens* strains that included endophytes from cotton and rice as well as a human-associated strain (47).

Although DDH is still widely used for bacterial species demarcation, several drawbacks have resulted in the search for alternative methods. DDH requires technical expertise and is time consuming and labor intensive when compared to PCR-based techniques such as rep-PCR and gene sequencing (17). Because of this, DDH is only performed in specialized laboratories and only when other methods of differentiation are inadequate. Additionally, DDH has not been universally applied to all bacteria and, in some cases, strains that share >70% DDH values have been placed into separate species or genera (22). The biggest drawback of DDH is the lack of

reproducibility which has made the construction of a shared reference database impossible (17). Recently, researchers have been testing alternative methods for species delineation that are reproducible and that yield discrete data that can be easily shared between labs. These methods often involve multigene or whole genome sequencing. Goris *et al.* found that a 70% DDH value corresponded to 95% average nucleotide identity (ANI) when they examined several bacterial genera and concluded that ANI could replace DDH for strains with available genome sequences (17). Parkinson *et al.* found agreement in species differentiation for several xanthomonads when they compared DDH values to partial sequences of the *gyrB* gene, indicating that single gene or multigene sequencing may be a potential tool for species demarcation (31).

Repetitive sequence-based polymerase chain reaction (rep-PCR) was first described by Versalovic *et al.* in 1994 as a quick, inexpensive, and effective way to genotype bacteria (40). This method was widely adopted and has been used extensively by plant pathologists for species, subspecies, and pathovar differentiation (2, 6, 7, 23, 24, 33). Rep-PCR relies on interspersed repetitive DNA sequences commonly found throughout prokaryotic genomes (26). The three conserved repetitive DNA sequences that are most commonly used to generate genomic fingerprints are BOX elements, enterobacterial repetitive intergenic consensus (ERIC) sequences, and repetitive extragenic palindromic (REP) sequences. Primers based on these sequences work by annealing to the repetitive elements throughout the genome. This is followed by PCR amplification of the DNA that is internal to the repetitive sequences and generates 10 to 30 DNA fragments of varying size (200 bp to 6 kb) per bacterial strain. DNA fragments are then separated on an agarose gel to yield a unique genomic fingerprint for a given strain that can be compared to other strains to determine phylogenetic relationships. BOX, REP, and ERIC will all generate different fingerprints for a particular strain and are often used in conjunction to build

phylogenetic tress (24, 40). Previous rep-PCR experiments indicated that CYVD strains of *S. marcescens* isolated from different cucurbit hosts, geographic locations, and growing seasons were identical and formed a group that was separate from all other *S. marcescens* strains. The most closely related strains using this method included three rice endophytes (R01-A, R02-A, R03-A), a cotton endophyte (G01-A), and a human pathogen (H02-A) (47).

Multilocus sequence typing (MLST) is a method used for the characterization of strains of bacteria that relies directly on sequence data from 6 to 10 housekeeping genes. Housekeeping genes are necessary for basic cellular functions and, as a result, are constitutively expressed in all the cells of an organism regardless of tissue type, developmental stage, or external stimuli (15). Because their metabolic functions are crucial for cell maintenance and survival, housekeeping genes are under stabilizing selection that is characterized by a greater number of synonymous substitutions than non-synonymous substitutions. Over time, rare mutational events leading to synonymous substitutions will accumulate at housekeeping loci and result in unique allele combinations between closely related strains. This makes housekeeping genes ideal for MLST, which measures genetic diversity based on nucleotide polymorphisms across several loci (27, 28).

For MLST, housekeeping gene fragments of 400 to 600 bp are PCR amplified using primers designed from the nucleotide sequence flanking the gene fragment of interest. The amplified fragments are sequenced and non-identical fragments are arbitrarily assigned an allele number. A single nucleotide difference can result in a new allele number at a given locus. The combination of allele numbers across all the loci for a single strain results in a sequence type. Sequence types, which can represent several thousand base pairs of information, are logged into a public database. MLST, which was originally designed to identify virulent strains of *Neisseria*

meningitides, has several advantages over earlier characterization methods. Since MLST analysis is based on sequence data, the results are highly reproducible between labs. Sequences are entered into a database that is updated as new information becomes available (27, 28). In addition to clinical isolates, several important plant pathogens have been characterized using MLST (12, 16, 18, 19, 36, 44). Castillo *et al*. used MLST to draw conclusions about the evolutionary history of *Ralstonia solanacearum*. In this study, five housekeeping and three virulence-related genes were sequenced and concatenated and a phylogenetic tree was constructed. *R. solanacearum* was found to contain four deeply separated evolutionary lineages that were the result of geographic isolation and spatial distance (12). Bull *et al.* used MLST to characterize several strains of *Pseudomonas syringae*. *P. syringae* is a bacterial pathogen of numerous plant species and novel isolates are typically delineated into pathovars based on the results of extensive and tedious host range testing, as well as biochemical and nutritional profiling. Bull *et al.* were able to rapidly identify isolates as *P. syringae* pv. *coriandricola* and *P. syringae* pv. *apii* through the sequencing and analysis of four housekeeping genes.

One drawback of MLST is that a great deal of information can be lost when assigning allele numbers and subsequent sequence types, especially with an organism that is poorly defined or particularly diverse. With MLST, a new allele type can result from a single nucleotide polymorphism within that gene fragment and this will produce a new sequence type. This method is highly sensitive and extremely useful when typing clinical or plant pathogenic isolates that are very closely related (10, 16, 28). With unusually diverse organisms such as *S. marcescens* this can result in an overabundance of sequence types, making it difficult to draw meaningful conclusions about evolutionary histories. Do *et al.* used MLST to characterize 135 strains of *Streptococcus mutans* and found 121 unique sequence types, although many individual

loci were identical across multiple strains (14). In this case, direct examination of sequences or, at the very least, the allelic profiles would be needed to tell which strains cluster together. For these reasons, multilocus sequence analysis (MLSA) is often used separately or in conjunction with MLST. MLSA is similar to MLST, except that the analysis is based directly on DNA sequences instead of allele numbers and sequence types (1). This method is especially useful for the CYVD-associated strains of *S. marcescens,* which have significant sequence differences when compared to non-CYVD strains using other molecular techniques (46, 47).

The objectives of this study were to quantify the genetic diversity between non-CYVD and CYVD-associated strains of *S. marcescens* using rep-PCR and MLSA and gain a better understanding about the evolutionary history and origin of CYVD.

Materials and Methods

Bacterial strains: Thirty-three strains of *Serratia* spp. were examined in this study (Table 3.1) of which 32 strains were *S. marcescens* and one was *S. plymuthica*. Twenty-four of the *S. marcescens* strains were CYVD-associated and isolated from pumpkin, summer squash, watermelon, or squash bugs. All CYVD strains originated in Georgia, except for W01-A, which was isolated from a Texas watermelon. Strains W11 and On-01 were endophytes isolated in Georgia from watermelon and onion, respectively. The type strain was isolated from pond water. R01-A was a rice endophyte and I06-A was an insect pathogen. Data for strains SM39 (accession AP013063.1), WW4 (accession CP003959.1), and *Serratia plymuthica* (accession CP006566.1) were obtained from GenBank (5).

Gene determination and sequencing for MLSA. An MLST scheme developed for the enteric bacterium *E. coli* was used to identify housekeeping genes in *S. marcescens* that were

candidates for sequencing and analysis (Table 3.2) (43). Candidate genes were screened by comparing nucleotide polymorphisms between *S. marcescens* WW4 (accession CP003959.1) and *S. marcescens* Db11 (accession HG326223.1) at potential loci using Basic Local Alignment Search Tool (BLAST) (3). Housekeeping genes chosen for sequencing and analysis included *adk, fumC, gyrB, icd, mdh,* and *recA*. Selected genes had a similarity between 96% and 98% when comparing the two available sequences, which corresponded to between 8 and 20 polymorphisms per 500-600 bp gene fragments. Primers for amplification were designed to amplify housekeeping gene fragment lengths between 507 and 634 bp from the complete genome of *S. marcescens* WW4 using Primer3 primer design software (35, 39).

PCR was conducted for 30 strains of *S. marcescens* using primers designed from six housekeeping genes. Reaction volume was 25 µl, which included 12.5 µl *Taq* PCR Master Mix (Qiagen, Valencia, CA), 8.5 µl distilled deionized water, 1.5 µl template DNA, 1.25 µl 10 µM forward primer and 1.25 µl 10 µM reverse primer. PCR was performed on a Mastercycler Personal (Eppendorf, Hauppauge, NY) using the following amplification conditions: 5 min at 95°C; 30 cycles of 30 s at 95°C, 40 s at 60°C, 60 s at 72°C, and 1 final extension cycle of 5 min at 72°C (46). For the *icd* and *recA* genes an annealing temperature of 55°C was used instead of 60°C. PCR products were electrophoretically separated in a gel containing 1.5% GenePure agarose (Bioexpress, Kaysville, UT) and ethidium bromide at 100 µg/l. PCR products that yielded amplicons of expected length were prepared for sequencing using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Sanger sequencing was performed by the University of Georgia's Georgia Genomics Facility using a 3730xl 96-capillary DNA Analyzer (Applied Biosystems, Foster City, CA).

Sequence analysis. The sequences for the six housekeeping gene fragments were visualized and aligned using Geneious v7.1.4. (Biomatters, Auckland, New Zealand). The sequences were concatenated and neighbor-joining and maximum likelihood phylogenetic trees were constructed based on 3001 bp using MEGA v6.06 (38). A neighbor-joining tree was also constructed for the *icd* gene. The percentage of replicate trees containing the same taxa that clustered together was calculated using a bootstrap test with 500 replicates. The evolutionary distances were described as the number of base pair substitutions per site and were computed using the Maximum Likelihood method (37).

Rep-PCR. The DNA of 30 *S. marcescens* strains (Table 3.1) was compared using rep-PCR with the BOX-A1R primer (Integrated DNA Technologies, Coralville, IA) (40). Amplification was performed with each PCR reaction consisting of 1 PuReTaq Ready-To-Go PCR bead (GE Healthcare, Waukesha, WI), 21.5 µl distilled deionized water, 2.0 µl template DNA, and 1.5 µl 25 µM BOX-A1R primer for a total of 25 µl per reaction. PCR was performed on a Mastercycler Personal (Eppendorf, Hauppauge, NY) using the following amplification conditions: 2 min at 95°C; 30 cycles of 3 s at 94°C, 30 s at 92°C, 1 min at 50°C, 8 min at 65°C and 1 final extension cycle of 8 min at 65°C. PCR products were electrophoretically separated in a gel containing 1.5% GenePure agarose (Bioexpress, Kaysville, UT). The gel was run at 70 volts for 7.5 h at 4°C before staining for 30 min in 500 ml of water containing 250 µl ethidium bromide at 100 µg/l. The gel was then de-stained for 30 min and bands were visualized using a UVP Biospectrum 300 Imaging System (UVP LLC, Upland, CA).

Comparisons of DNA fingerprint patterns were performed using BioNumerics software v2.0 (Applied-Maths, St. Martens-Latem, Belgium). Fingerprint profiles were aligned and bands were scored visually as positive or negative. A phylogenetic tree based on banding patterns was

constructed using the unweighted pairwise group method with arithmetic mean (UPGMA) cluster analysis and Dice's coefficient of similarity (13).

Results

MLSA Analysis. Phylogenetic trees constructed using the neighbor-joining and maximum likelihood methods yielded indistinguishable topologies (Figs. 3.1 and 3.2). All CYVD strains of *S. marcescens* formed a tight cluster and were separate from all non-CYVD strains with bootstrap support values of 97% and 99% for neighbor-joining and maximum likelihood phylogenies, respectively. With the exception of SB03, which was isolated from a squash bug, all CYVD strains had identical sequences at all six loci. SB03 shared identical sequences with CYVD strains at five of the six loci, but differed at the *icd* locus. At this locus, SB03 shared an identical sequence with the type strain which was isolated from pond water (Fig. 3.3). For the other five loci, SB03 differed from the type strain by an average of 15 nucleotide polymorphisms with a range of 6-26 polymorphisms.

Diversity was considerable between non-CYVD strains, which contained an average of 19 polymorphisms per gene fragment. On-01 (onion endophyte) and W11 (watermelon endophyte) shared identical sequences across all six gene fragments and formed a larger cluster with I06-A (insect pathogen) and WW4 (paper machine). SM39, a human clinical isolate, showed the most divergence of all non-CYVD strains. MLSA indicated that strain R01-A, a rice endophyte, was more closely related to CYVD strains than to non-CYVD strains. This strain differed from CYVD strains by an average of 6 polymorphisms per gene fragment and from other non-CYVD strains by an average of 14 polymorphisms per gene fragment.

Rep-PCR. Rep-PCR using the BOX-A1R primer revealed considerable variation among CYVD strains (Figs. 3.4 and 3.5). With the exception of SB03, all CYVD strains of *S. marcescens* formed a cluster separate from all non-CYVD strains. CYVD strain SB03 was most closely related to the type strain and was part of the non-CYVD group. Of the remaining CYVD strains, W01-A, which was isolated from a Texas watermelon plant, grouped separately from the Georgia CYVD strains but was more closely related than any non-CYVD strain. Similar to MLSA, endophytic strains On-01 and W11 were identical and formed a larger group that contained the insect pathogen I06-A. Rep-PCR data indicated that rice endophyte R01-A was most closely related to CYVD strains.

Discussion

With the exception of a single strain, both rep-PCR and MLSA grouped CYVD strains tightly together and separate from all non-CYVD strains. Our study revealed a surprising lack of genetic variation in CYVD-associated strains of *S. marcescens* across all six loci using MLSA. MLSA is based on the principles that govern bacterial reproduction and genetic variation (28). When a bacterial cell undergoes a non-lethal mutational event such as a base change, that mutation will be passed along to daughter cells and can continue to spread in a subset of the population through inheritance. This allows individual lineages of clonally reproducing organisms to be easily recognized based on patterns of variation in their sequence data. MLSA works by sequencing several, slowly evolving, housekeeping genes that are undergoing purifying selection and comparing nucleotide polymorphisms directly to determine evolutionary histories (27, 45). In this study, the number of polymorphic sites per gene ranged from 8 to 20 with a mean of 14 in the two published *S. marcescens* genomes used for gene selection. Similarly, the

number of polymorphic sites per gene among the seven non-CYVD strains ranged from 10 to 31 with a mean of 19. In sharp contrast, only one strain (SB03) of the 24 CYVD strains examined in this study contained genetic variation. This variation occurred at the *icd* locus and was characterized by five polymorphisms not present in the other 23 CYVD strains. Interestingly, SB03 shared an identical sequence with the type strain at this locus. Maiden *et al.* discovered a lack of genetic diversity similar to that reported here when they examined hyper-virulent strains of *Neisseria meningitides*. The six loci they examined were almost identical in the recently emerged hyper-virulent strains, whereas nonpathogenic strains exhibited a greater diversity characterized by numerous unique allele combinations (28). In this study, the lack of genetic variation revealed by MLSA indicates that CYVD-associated strains of *S. marcescens* may have diverged relatively recently and suggests that CYVD strains are derived from a single common ancestor (11, 28).

With the exception of CYVD strain SB03, rep-PCR clearly grouped CYVD-associated strains of *S. marcescens* in a tight cluster and separate from non-CYVD strains. Georgia CYVD-associated strains were further divided into three subgroups, each containing between six and nine strains. The hosts from which the Georgia CYVD strains were recovered did not appear to correlate with the subgroups; strains recovered from pumpkin and summer squash were present in each group. CYVD strain W01-A clearly grouped with the CYVD strains, although it was more distantly related and fell outside the three subgroups. This strain differed from the other CYVD strains in this study in that it was the only CYVD strain recovered outside of Georgia and the only CYVD strain recovered from watermelon. Although rep-PCR BOX banding patterns can differentiate pathovars for some plant pathogens, this is an unlikely scenario in this case since W01-A has been shown to be pathogenic on squash (9, 10, 20). Spatial distance and

geographic isolation have been shown to contribute significantly to genetic variation in several microbial species, including the globally distributed *Ralstonia solanacearum* (12, 30). Screening of additional isolates with varying geographic distributions might shed light on the effect of geography, if any, on genetic diversity.

CYVD strain SB03, which was isolated directly from a squash bug, clustered with non-CYVD strains and was most closely related to the type strain according to rep-PCR banding profiles (Fig 3.5). Similar results were found using MLSA where SB03 was found to be identical to all other CYVD strains at 5 out of 6 loci, but identical to the type strain at the *icd* locus (Fig. 3.3). MLSA and rep-PCR examine different regions of the genome so the relationship between SB03 and the type strain may be more complex than indicated by either method alone. Further examination of this strain, including rep-PCR with ERIC primers, sequencing and comparison of additional housekeeping genes, biological characterization, and phenotypic analysis could help determine if SB03 is an intermediary between a non-CYVD *S. marcescens* strain and the tightly clustered CYVD strains.

Several previous experiments, including rep-PCR, 16S rDNA and *groE* gene sequencing, and DNA-DNA hybridization have indicated that the rice endophyte R01-A was the most closely related strain to CYVD strains (34, 47). CYVD strains and R01-A are non-pigmented, whereas the bacterial colonies of most strains of *S. marcescens* are red in color due to the production of the pigment prodigiosin (25). Recently, it was demonstrated that a CYVD strain of *S. marcescens* was able to colonize squash endophytically without causing typical CYVD symptoms. The CYVD strain was found to colonize squash more effectively based on bacterial growth and movement within the host than a watermelon endophyte (W11), *Xanthomonas cucurbitae*, or *Pseudomonas syringae* pv. *tomato* (K. R. Besler, *unpublished data*). Our MLSA

results showed that strain R01-A is more closely related to CYVD-associated strains of *S. marcescens* than it is to non-CYVD strains, strengthening the argument that CYVD strains may have arisen from a plant endophyte.

We found that rep-PCR provided more discriminatory power than MLSA to differentiate between CYVD-associated strains of *S. marcescens* based on the genes selected. Rep-PCR relies on the amplification of regions between conserved repetitive sequences to which the primers bind. Since these repetitive sequences are dispersed throughout bacterial genomes, rep-PCR has the advantage of examining the entire genome (26, 40). MLSA, on the other hand, relies on extremely high resolution of a very limited portion of the genome which may lead to a loss in discriminatory power. This can be particularly problematic when dealing with very closely related bacterial strains (4). In a study examining the differentiation of *Salmonella enterica* strains, researchers found that MLST was able to identify 31 sequence types while rep-PCR identified 38 types and separated isolates according to serotype with higher discriminatory power than MLST (4). In this study, Rep-PCR also proved to be a more convenient and cost effective typing method compared to MLSA, since the cost of sequencing several genes across numerous strains can run in the thousands of dollars. MLSA involves PCR using multiple primer sets before the purified products are sent to a genomics facility for sequencing. Rep-PCR does not involve sequencing, and the analysis can be completed within 48 hours.

Although rep-PCR was able to differentiate between CYVD strains, this method gives no indication of where the variation lies in the genome due to the random annealing of the BOXA1R primer. In fact, many amplicons may represent noncoding regions of the genome. In contrast, MLSA produced a discrete data set based on nucleotide sequences of known genes that allowed us to identify and measure genetic variation. Additionally, MLSA provided a more

robust analysis based on 3001 base pairs that allowed for the accurate calculation of genetic distances. Dendrograms based on rep-PCR fingerprint profiles are calculated by scoring specific amplicons as present or absent and using the resulting binary data to construct a phylogeny (40). This analysis can be somewhat limiting since an acceptable fingerprint profile generally consists of 8 to 15 data points, or amplicons (40). Another major drawback of rep-PCR is its limited capacity for reproducibility and data portability, which is highlighted in this study (32). While we discovered genetic diversity among CYVD strains using rep-PCR with the BOXA1R primer, Zhang *et al.* found identical banding patterns for all CYVD isolates using the BOX, PGRS, and ERIC primers. This could be due to differences in the BOX primer. Versalovic *et al*. described three repetitive-based oligonucleotide primers that could be considered BOX primers (BOXA1R, BOXB1, and BOXC1) and it is unclear which primer was used by Zhang *et al.* (40, 47).

The results from this study showed that CYVD-associated strains of *S. marcescens* lacked diversity in the six housekeeping genes examined, suggesting that plant pathogenic strains recently diverged from a single common ancestor. Rep-PCR using the BOX primer revealed some diversity among CYVD strains, although it could not be determined where this diversity lies in the genome and whether it is manifested phenotypically. CYVD strain SB03, which grouped with the type strain using rep-PCR and at the *icd* locus using MLSA, could be an intermediate strain that bridges the gap between CYVD and non-CYVD strains. Further genomic and biological investigations, including the examination of additional *S. marcescens* strains, may shed light on the origins of this unique plant pathogen.

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Tables

Table 3.1. Source, collection year, and geographic origin of *Serratia* spp. strains used in this study. Accession numbers are included for strains where data was obtained from GenBank.

Species, strains	Source, host	Date collected	Geographic origin
S. marcescens, non-CYVD			
ATCC 13880 (type)[a]	Pond water	Unknown	Unknown
I06-A[a]	Unknown insect	1961	Unknown
On-01[b]	Onion endophyte	2013	US, Georgia
R01-A[a]	Rice endophyte	Unknown	Philippines
SM39 (AP013063.1)	Human	1999	Japan
WW4 (CP003959.1)	Paper machine	2013	Taiwan
W11[b]	Watermelon endophyte	2013	US, Georgia
S. marcescens, CYVD			
P01	Pumpkin	2012	US, Georgia
P02	Pumpkin	2012	US, Georgia
P03	Pumpkin	2012	US, Georgia
P04	Pumpkin	2012	US, Georgia
P05	Pumpkin	2013	US, Georgia
P06	Pumpkin	2013	US, Georgia
S5	Pumpkin	2012	US, Georgia
S02	Yellow summer squash	2013	US, Georgia
S04	Yellow summer squash	2013	US, Georgia
SB01	Squash bug	2013	US, Georgia
SB02	Squash bug	2013	US, Georgia
SB03	Squash bug	2013	US, Georgia
W01-A[a]	Watermelon	1999	US, Texas
Z01	Zucchini	2012	US, Georgia
Z02	Zucchini	2012	US, Georgia
Z04	Zucchini	2013	US, Georgia
Z05	Zucchini	2013	US, Georgia
Z06	Zucchini	2013	US, Georgia
Z07	Zucchini	2013	US, Georgia
Z08	Zucchini	2013	US, Georgia
Z09	Zucchini	2013	US, Georgia
Z10	Zucchini	2013	US, Georgia
Z12	Zucchini	2013	US, Georgia
Z13	Zucchini	2013	US, Georgia
S. plymuthica (CP006566.1)	Pumpkin endophyte	2009	Austria

[a] Strains provided by J. Fletcher, Oklahoma State University

[b] Strains provided by B. Dutta, University of Georgia

Table 3.2. Functions, primer sequences, and fragment lengths of genes used in multilocus sequence analysis.

Gene name	Gene function	Primer sequences (5' to 3'; forward/reverse)	Gene fragment length (bp)
adk	adenylate kinase	gggcgctggtaaaggtactc ctgccgcttctttgctgtag	536
fumC	fumarate hydratase	gcgcatttccgtatttcctc actccggatgggtattcagg	634
gyrB	DNA gyrase	gcggtaaattcgacgacaac tcatcgcggtacggaaac	567
icd	isocitrate/isopropylmalate dehydrogenase	cgttactaccagggcactcc gtcgccgttcaggttcatac	528
mdh	malate dehydrogenase	ggacgtggtgctgatttcc accagagacagaccgaaacg	507
recA	ATP/GTP binding motif	gcgctggatcctatctatgc cttcgccgtacatgatttgg	508

Figures

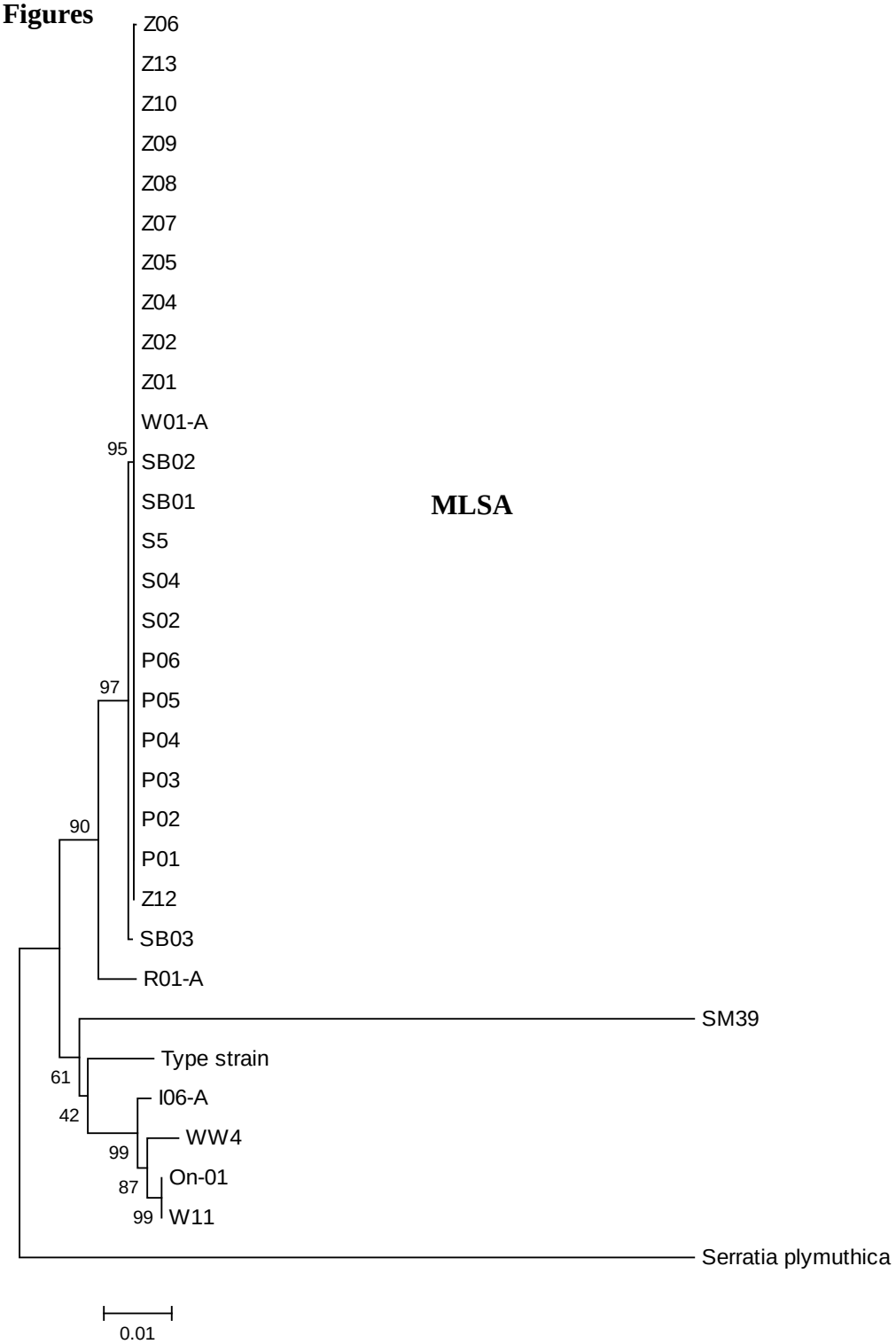

Figure 3.1. Neighbor-joining phylogeny of 31 *Serratia marcescens* strains and 1 *S. plymuthica* strain using MLSA. The tree was constructed using 3001 base pairs from six concatenated genes. Branch lengths represent evolutionary distances which were calculated using the Maximum Composite Likelihood method. The scale bar represents the number of nucleotide substitutions per site. Bootstrap values are depicted next to each branch.

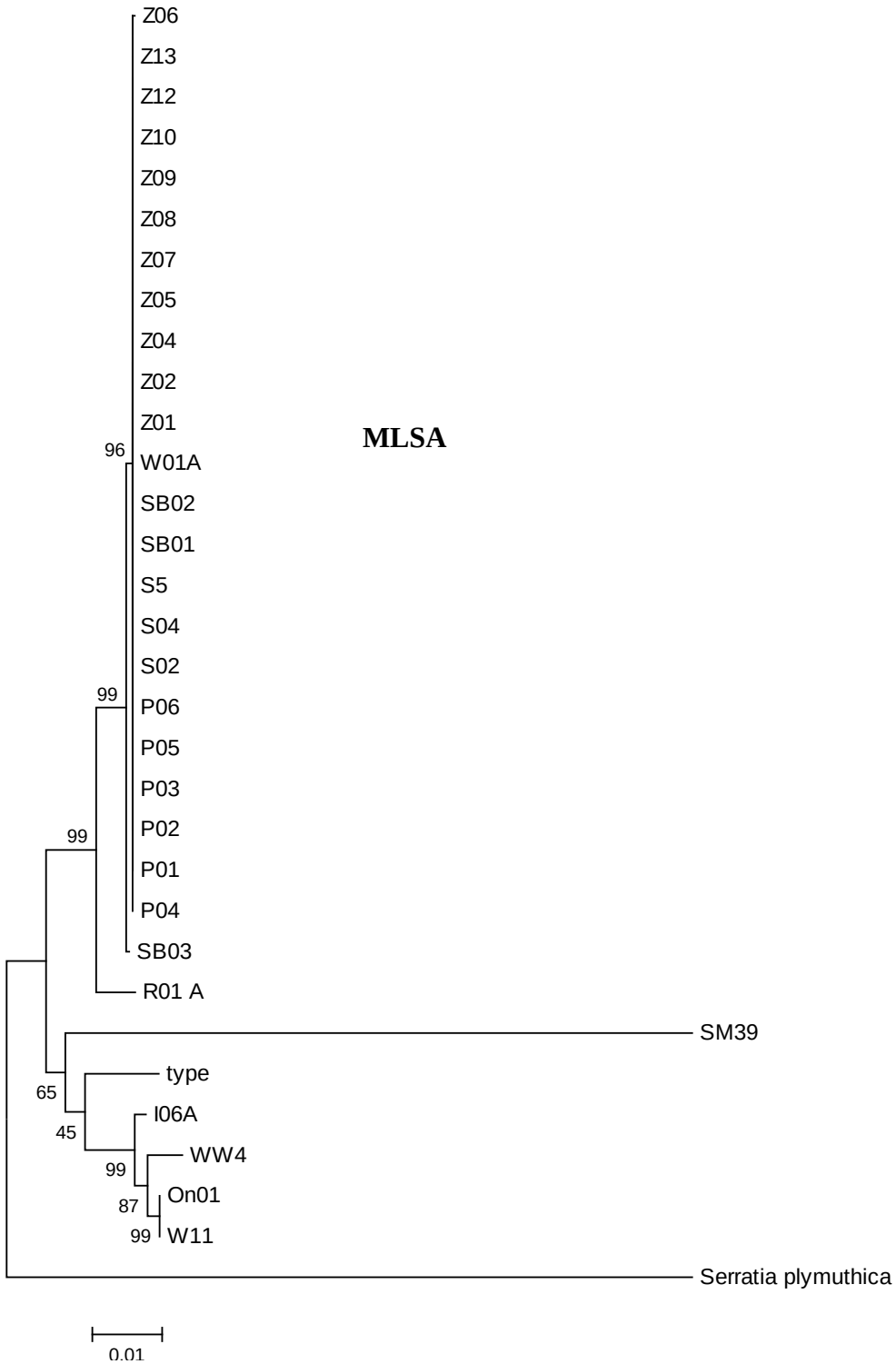

Figure 3.2. Maximum likelihood phylogeny of 31 *Serratia marcescens* strains and 1 *S. plymuthica* strain using MLSA. The tree was constructed using 3001 base pairs from six concatenated genes. Branch lengths represent evolutionary distances in nucleotide substitutions per site. Bootstrap values (500 replicates) are depicted next to each branch and represent the percentage of trees that clustered with the same taxa.

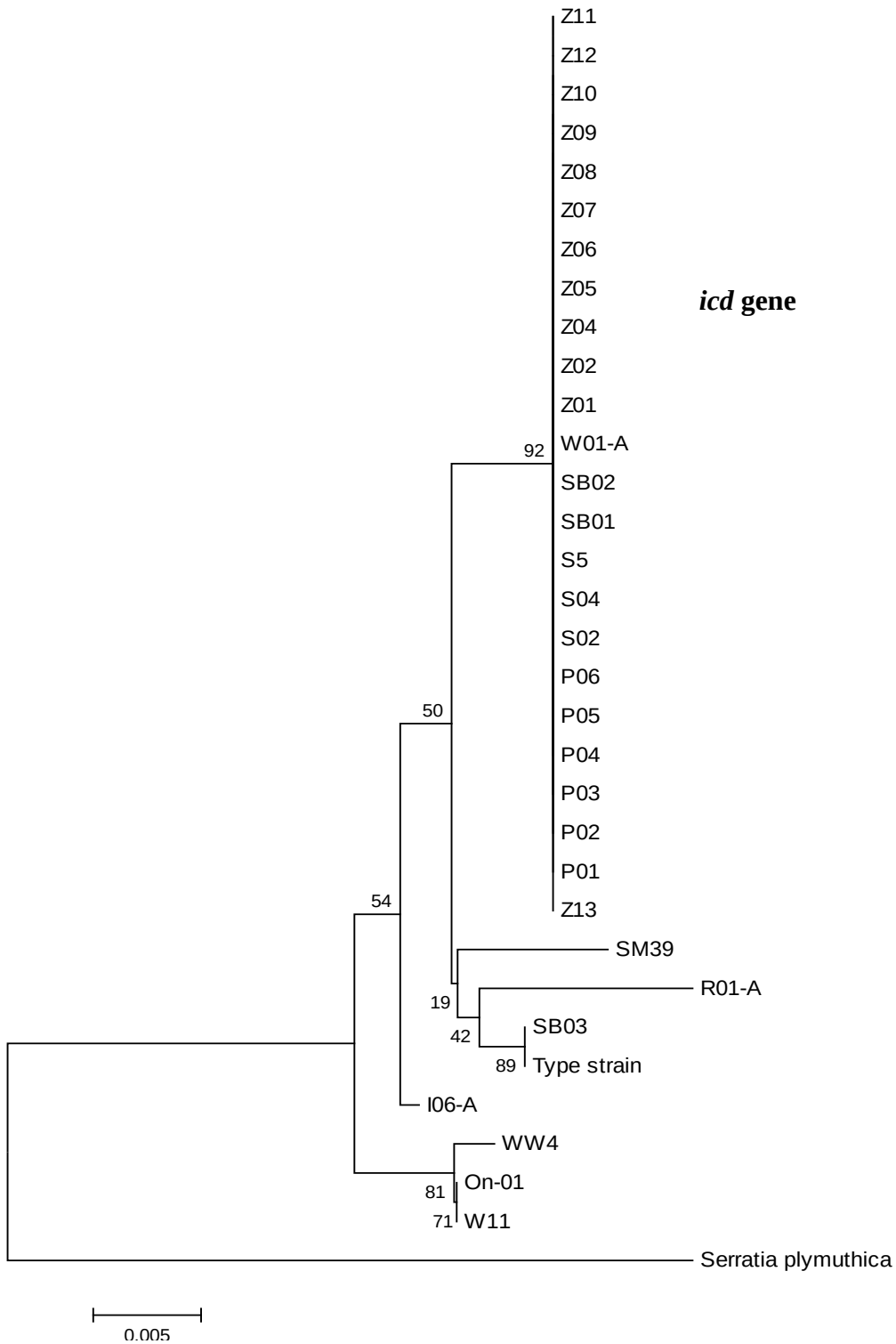

Figure 3.3. Neighbor-joining phylogeny of 31 *Serratia marcescens* strains and 1 *S. plymuthica* strain. The tree was constructed using 508 base pairs from the *icd* gene. Branch lengths represent evolutionary distances which were calculated using the Maximum Composite Likelihood method. The scale bar represents the number of nucleotide substitutions per site. Bootstrap values are depicted next to each branch.

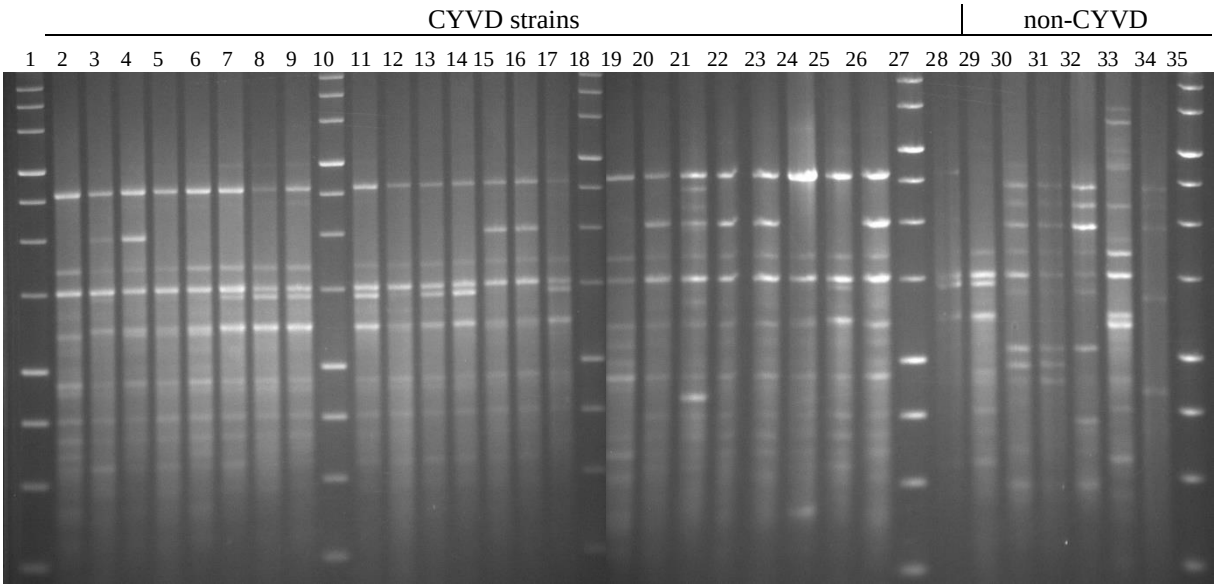

Figure 3.4. DNA fragment banding patterns from *S. marcescens* DNA amplified using repetitive element sequence-based PCR with the BOX-A1R primer. Lanes 1, 10, 18, 27, and 35 are the 1-kb DNA ladder. Lane 2, Z01; lane 3, Z02; lane 4, Z04; lane 5, Z05; lane 6, Z06; lane 7, Z07; lane 8, Z08; lane 9, Z09; lane 11, Z10; lane 12, Z11; lane 13, Z12; lane 14, Z13; lane 15, S02; lane 16, S04; lane 17, S5; lane 19, SB01; lane 20, SB02; lane 21, SB03; lane 22, P01; lane 23, P02; lane 24, P03; lane 25, P04; lane 26, P05; lane 28, P06; lane 29, W01-A; lane 30, W11; lane 31, On-01; lane 32, I06-A; lane 33, R01-A; lane 34, type strain;

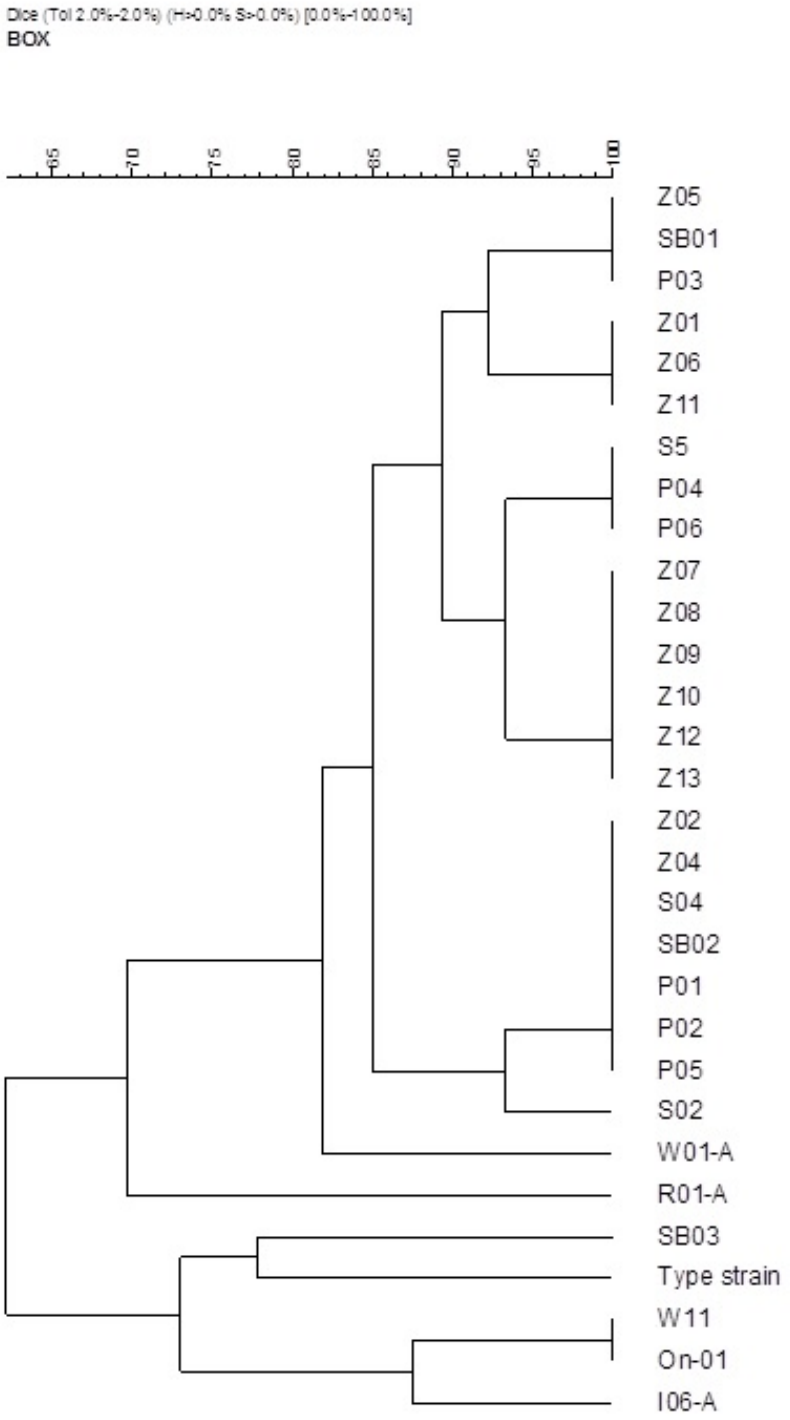

Figure 3.5. Phylogenetic tree constructed from the DNA banding patterns for *Serratia marcescens* strains using rep-PCR with the BOX-A1R primer. Tree was generated using UPGMA cluster analysis and Dice's coefficient.

CHAPTER 4

EPIDEMIOLOGY AND MANAGEMENT OF CUCURBIT YELLOW VINE DISEASE[3]

[3] Besler, K. R. and Little, E. L. 2014. To be submitted to *Plant Disease*.

Abstract

Cucurbit yellow vine disease (CYVD) causes plant decline and death in squash, pumpkin, watermelon, and cantaloupe. CYVD is caused by the squash bug-transmitted bacterium *S. marcescens*. To determine the frequency at which the vector harbors the pathogen, adult and juvenile squash bugs were collected during diapause and the growing season. DNA was extracted from squash bugs and screened for *S. marcescens* using PCR with CYVD strain-specific primers. The pathogen was detected in four out of five nymphal instars as well as adults from each sampling date. Overwintering squash bugs harbored the pathogen at the greatest frequency with a rate of 50%. The capacity of the horned squash bug (*Anasa armigera*) to transmit *S. marcescens* was examined using an artificial feeding system for pathogen acquisition. After acquisition, horned squash bugs were allowed to feed on squash seedlings for seven days. Plants were transferred to a field and covered with a row cover. No symptoms were observed on any of the squash fed upon by horned squash bugs. Two farm sites were visited during the 2013 and 2014 growing seasons to track the development of symptoms and incidence of CYVD for individual squash plantings. In 2013, CYVD incidence ranging from 12% to 93% were observed in squash planted in April and May, while no disease was detected in squash planted after May. In 2014 there was no CYVD observed at either site, although CYVD was present at other locations. A row cover study was initiated to study the relationship between plant age and susceptibility to CYVD, and to evaluate the effectiveness of row covers in reducing disease incidence. In this study, row covers were used to exclude the squash bug vector from squash plants for two, three, four, or five weeks. The untreated control received no row cover protection. In two out of three experiments disease incidence was significantly lower in plants that received row cover protection for three weeks or more, indicating that plant resistance to CYVD infection

may be positively correlated with plant age and that flowering is the optimal time for row cover removal.

Introduction

Cucurbit yellow vine disease (CYVD), caused by the bacterium *S. marcescens*, is a disease of squash, watermelon, pumpkin, and cantaloupe that was first observed on squash and pumpkin in Texas and Oklahoma in 1988. Disease symptoms include yellowing, stunting, wilting, and a honey-brown discoloration of the phloem. Wilting is usually gradual and begins approximately two weeks before harvest, although a rapid wilt at flowering and fruit set has been observed. Disease losses can range from a few plants to an entire field in affected areas (5, 6).

Transmission experiments have shown that CYVD strains of *S. marcescens* are vectored by the squash bug, *Anasa tristis* (6, 15, 20). Primary inoculum comes from squash bugs that emerge in the spring from overwintering sites and infect early-season cucurbit crops (15). Squash bugs are true bugs (Hemiptera) that belong to the family Coreidae and are characterized by the presence of long piercing and sucking mouthparts that extend out from the front of the head (7). Squash bugs were considered a major threat to pumpkin and squash production in the United States even before the discovery that they vector *S. marcescens* (11). This is the only known plant pathogen that is vectored by the squash bug (6).

S. marcescens is acquired by squash bugs during feeding and/or probing of host plants prior to feeding (6). Although squash bugs are thought to primarily feed on xylem (13), they have also been shown to probe phloem tissue (4) and this is likely how the CYVD bacterium is introduced (4, 13). Studies have shown that nymphs are able to acquire the bacterium using an artificial feeding system (20), although it is unknown whether nymphs can acquire the bacterium

and cause infections in the field. Acquired bacteria persist in the hemocoel of the squash bugs through molting events and periods of hibernation (15, 20). The vector/bacterium relationship is thought to be circulative and propagative due to persistence through molting events and long periods of retention within the vector followed by successful inoculations of host plants (10, 20). While squash bugs have been shown to vector CYVD-causing strains of *S. marcescens*, the pathogen has never been directly detected in the insects. Additionally, there is a limited understanding of how frequently they harbor the pathogen and how this frequency fluctuates throughout the year.

In addition to *Anasa tristis*, which occurs throughout the United States and southern Canada, there also exists the horned squash bug (*Anasa armigera*). The horned squash bug occurs in the eastern United States and exhibits the same crop preferences as the squash bug, although it generally prefers wild cucurbits (19). The capacity for the horned squash bug to acquire and transmit *S. marcescens* has not been determined.

CYVD has been identified in Arkansas, Colorado, Connecticut, Kansas, Massachusetts, Michigan, Missouri, Nebraska, Ohio, Tennessee, Alabama, and Georgia (10, 14, 18). Aside from the widespread losses documented in Texas and Oklahoma, there is relatively little information on the impact and seasonality of the disease where it has been reported. Sikora reported 20% disease incidence in summer squash and 25-30% disease incidence in watermelon in eastern Alabama in June of 2010 (18). Bruton *et al.* noted that late-season cucurbits, especially watermelon, had a lower incidence of CYVD (6). There is currently no information about the distribution, seasonality, or impact of CYVD on squash plantings in Georgia.

Several studies have been conducted that examined plant age versus susceptibility to a given pathogen. Kus *et al*. showed that Arabidopsis becomes more resistant to *Pseudomonas*

syringae pvs. *tomato* and *maculicola* as plants mature due to the production of age-related resistance (ARR) antimicrobial compounds (9). Conversely, Miller demonstrated that onion leaves became increasingly susceptible to *Alternaria porri* as they aged and that newly emerged leaves were more susceptible on older plants than newly emerged leaves on younger plants (12). Relatively little is known about the susceptibility of plants to CYVD strains of *S. marcescens* as it relates to the age or growth stage of the plant. A previous greenhouse study using a mechanical inoculation procedure indicated that susceptibility to disease may decrease as the plant matures. In this study, approximately 60% of plants became infected when inoculated at the non-expanded cotyledon stage, whereas 10% of plants became infected when inoculated at the first true leaf stage (6). It is unclear whether these results are an indication of age-related resistance in the field, since CYVD has been observed in squash and pumpkin that were transplanted into the field after the first true leaf stage (6).

Current CYVD management practices emphasize controlling the squash bug vector. The application of insecticides is the most widely utilized strategy for the control of squash bugs and can be effective, although adult squash bugs are notoriously difficult to manage. In organic systems, insecticide options are generally much less effective and have no residual activity. Squash bugs tend to aggregate at the base of squash plants and on the underside of leaves where foliar insecticides get low coverage (7). The common use of plastic mulch in cucurbit production provides shelter for the squash bugs and can lead to increases in populations (8). Targeting the first generation of nymphs with insecticides can mitigate heavy infestations later in the growing season and keep the adult squash bugs from migrating to later plantings (7). Cultural practices are also effective in reducing squash bug populations. Removal and destruction of crop debris and old fruit immediately after harvest can reduce overwintering populations (7). The use of trap

crops to lure squash bugs away from high value cucurbits has been proposed but not thoroughly investigated. In this system, trap crops that are highly attractive to squash bugs are planted earlier than the main crop on field borders close to overwintering sites of squash bugs or around plantings of susceptible crops. Trap crops intercept the squash bugs and can then be treated with an insecticide or mechanically destroyed to reduce squash bug numbers (2). Small scale growers can trap squash bugs by placing boards on the ground. The squash bugs will congregate under the boards and are easy targets for destruction (7).

Researchers have found that row covers can be used to physically exclude squash bugs from crops and limit infestations, although they must be removed at flowering to allow for pollination (1). This management method was shown to be effective for controlling bacterial wilt of muskmelon caused by the cucumber beetle-transmitted bacterium *Erwinia tracheiphila* (17). Bextine *et al*. demonstrated that the CYVD pathogen was likely insect vectored by using row covers to exclude insects and prevent disease development, although this study did not examine yield or the effect of timing of row cover removal to mitigate CYVD (3).

Materials and Methods

Temporal frequency of *S. marcescens* in *Anasa tristis*. Adult squash bugs were collected from two farm sites with a history of cucurbit production and CYVD during both the diapause phase (winter) and in-season over a period of 18 months in 2013 and 2014. Squash bug nymphs representing all five instars were collected during June and July of 2013. Horned squash bugs (*Anasa armigera*) were collected in July of 2013. Site A was the University of Georgia Durham Horticulture Farm in Oconee County, GA and Site B was the University of Georgia UGArden in Clarke County, GA.

Squash bug DNA extraction was performed using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). For extractions, the head and prothorax were removed and placed in a 1.5 ml Eppendorf tube. The tube was filled with liquid nitrogen and the frozen insect tissue was ground into a fine powder using a microtube pestle. DNA was extracted according to the manufacturer's recommendations for the purification of total DNA from insects. DNA was also extracted from five horned squash bugs as described above. In addition to DNA extraction, nine squash bugs were subject to bacterial isolation via tissue maceration and plating. For isolations, the head and prothorax were removed and placed in a 1.5 ml Eppendorf tube filled with 1.0 ml 0.01M PBS. The tissue was ground using a microtube pestle and 100 µl of the resulting suspension was spread on Luria broth (LB) agar plates and incubated at room temperature for three days. Colonies resembling *S. marcescens* were transferred several times before the DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA).

Purified DNA was subjected to PCR using the A79 primers, which are specific for CYVD strains of *S. marcescens* (21). PCR reaction volume was 25 µl, which included 12.5 µl *Taq* PCR Master Mix (Qiagen, Valencia, CA), 8.5 µl distilled deionized water, 1.5 µl template DNA, 1.25 µl 10 µM forward primer A79F and 1.25 µl 10 µM reverse primer A79R. PCR was performed on a Mastercycler Personal (Eppendorf) using the following amplification conditions: 5 min at 95°C; 35 cycles of 40 s at 95°C, 60 s at 60°C, 90 s at 72°C, and 1 final extension cycle of 7 min at 72°C (21). PCR products were electrophoretically separated in a gel containing 1.5% GenePure agarose (Bioexpress, Kaysville, UT) and ethidium bromide at 100 µg/l. Squash bugs were scored as positive if the expected 338 bp product was detected.

Capacity of the horned squash bug, *Anasa armigera*, to transmit *S. marcescens*. Horned squash bugs, including both male and females, were collected in July of 2013 at the

University of Georgia Durham Horticulture Farm in Watkinsville, GA. Horned squash bugs were reared and maintained in a 0.9 x 0.5 x 0.8 m cage at ≈26°C with a photoperiod of 14:10 h light:dark and provided with 2 to 4 week old pumpkin (cv. New England Pie, Johnny's Seeds, ME) or summer squash (cv. Zephyr, Johnny's Seeds, ME) plants (20). The bugs were allowed to mate and the resulting adult offspring were used in transmission studies.

S. marcescens P01 was grown in 5 ml Luria broth for 24 h at 28°C on a shaker (200 rpm) and resuspended in sterile water to a concentration of 1 x 10^8 CFU. Zucchini fruit were surface sterilized with 70% ethanol and cut into small cubes (≈ 1 cm^3). The cubes were placed in a 125 ml Büchner flask connected to a vacuum and 5 ml of bacterial suspension or sterile water was added to the flask. The flask was sealed with a rubber stopper and the vacuum was engaged for 15 s and subsequently released, forcing the suspension into the squash cubes. The vacuum was engaged and released three more times as described (6, 20). The cubes were placed in a sterile petri dish, along with a single adult horned squash bug. Horned squash bugs were allowed to feed on infiltrated squash cubes for a period of 48 h then placed in a sterile petri dish with no food for 24 h. Horned squash bugs were transferred to squash plants (cv. Zephyr, Johnny's Seeds, ME) in the expanded cotyledon stage at a ratio of 1:1 squash bug to plant and allowed to feed for 7 days (15). Plants were transplanted to the field, covered with an Argibon Ag-15 Insect Barrier row cover, and observed for symptom development. Under each row cover there were 5 plants that were exposed to squash bugs that fed on *S. marcescens* infiltrated cubes and 5 plants that were exposed to squash bugs that fed on sterile water-infiltrated cubes. This experiment was repeated 5 times. At the conclusion of the inoculation procedure, DNA was extracted from the horned squash bugs and tested for the presence of CYVD strains of *S. marcescens* as described above.

Seasonal dynamics of CYVD incidence. Farms were visited during the 2013 growing season to assess the extent of CYVD. Two sites with high disease pressure were chosen for continual monitoring throughout 2013 and 2014 growing seasons. Site 1 was the University of Georgia UGArden in Clarke County, GA and Site 2 was a commercial organic farm in Oglethorpe County, GA. Visual observations were made throughout the season and symptomatic plants were sampled to confirm the presence of *S. marcescens*. Bacteria were isolated from symptomatic plants by excising small sections (0.1 g) of the brown phloem tissue from the lower stem. Tissue samples were surface sterilized for two minutes in 0.825% NaOCl and macerated in a 1.5 ml Eppendorf tube containing 0.5 ml phosphate buffered saline (PBS). Ten microliters of the resulting suspension was streaked onto LB agar plates and incubated at room temperature for three days (6). Colonies resembling a known CYVD-associated strain of *S. marcescens* were transferred several times to ensure purity.

DNA amplification was carried out using PCR with the A79F/R primers. For use in PCR, bacterial cells from a plate of LB were suspended in 0.5 ml 0.01M PBS to a concentration of approximately 1 x 10^5 CFU/ml and heated to 100°C for 10 minutes. PCR reaction volume was 25 µl, which included 12.5 µl *Taq* PCR Master Mix (Qiagen, Valencia, CA), 8.5 µl distilled deionized water, 1.5 µl bacterial suspension, 1.25 µl 10 µM forward primer A79F and 1.25 µl 10 µM reverse primer A79R. PCR was performed on a Mastercycler Personal (Eppendorf) using the following amplification conditions: 5 min at 95°C; 35 cycles of 40 s at 95°C, 60 s at 60°C, 90 s at 72°C, and 1 final extension cycle of 7 min at 72°C (21). PCR products were electrophoretically separated in a gel containing 1.5% GenePure agarose (Bioexpress, Kaysville, UT) and ethidium bromide at 100 µg/l. Samples were considered positive if a 338 bp product was observed (21).

Relationship between plant age and susceptibility to *S. marcescens* and capacity for row covers to mitigate CYVD. Three field trials of a row cover experiment were completed on certified organic land at the University of Georgia Durham Horticulture Farm during 2013 and 2014. Planting dates for the three trials were June 5, 2013, April 21, 2014, and July 25, 2014. Fields were fertilized with Nature Safe 10-2-8 (Nature Safe, Cold Spring, KY) at a rate of 1221.3 kg/hectare. A Latin square experimental design was used with four replications of four treatments with each replication consisting of 10 plants. Zucchini (cv. Costata Romanesco, Johnny's Seeds, ME) were directly seeded onto raised beds under white plastic mulch (TriEst Ag. Group, Inc.) with drip irrigation. Plants were spaced 0.6 m apart and row centers were 1.8 m apart. After seeding, each replication, excluding the control, was covered with Agribon+ Ag-15 Insect Barrier polypropylene spunbond row cover (Polymer Group Inc., Charlotte, NC) (Fig 4.2A). The row covers were supported by 1.25 cm wide metal conduit hoops that were driven approximately 15 cm into the ground. The row covers were sealed by covering the edges with soil and sand bags. Four treatments were used in 2013: 1) no row covers, 2) row covers removed two weeks post-emergence, 3) row covers removed four weeks post-emergence, and 4) row covers removed five weeks post-emergence. Treatments were altered slightly for 2014 based on data from the 2013 experiment and row covers were removed at two, three, and four weeks post-emergence. In trials two and three, squash fruit were harvested two to three times per week and weighed. Plants that developed symptoms characteristic of CYVD were immediately sampled and tested for the presence of *S. marcescens* as described above. Nine, seven, and nine plants were sampled in trials one, two, and three, respectively. At the conclusion of the trials two and three, one asymptomatic plant was sampled from each replication (n=16) and immediately tested for the presence of *S. marcescens* as described above. The percentage of plants that tested

positive for *S. marcescens* in each replication was calculated. Data were analyzed using a one-way analysis of variance (ANOVA). Pairwise comparisons were performed between all treatments using LSMEANS for PROC GLIMMIX (SAS, v9.4, Cary, NC) with $\alpha = 0.05$. Yield data were also subject to a one-way ANOVA using the same parameters.

Results

Temporal frequency of *S. marcescens* in *Anasa tristis*. Overwintering squash bugs were primarily found in piles of used shade cloth and other debris surrounding cucurbit fields. Squash bug populations experienced a dramatic decline following prolonged periods of heavy rain in July and August of 2013 and were absent from previously sampled plots. During the winter of 2014, no squash bugs were found in any of the previous year's overwintering sites or any other typical overwintering habitat such as leaf litter or crop debris piles. With the exception of the fourth instar, CYVD-causing strains of *S. marcescens* were detected in all squash bug growth stages with the highest frequency of detection (50%) in overwintering adults collected in 2013 (Table 4.1). Adults that had overwintered and were collected in June of 2013 showed a reduction in detection frequency of *S. marcescens* at Site A (23%) and Site B (10%) compared with the overwintering adult level of detection (Fig. 4.1). Adults that had overwintered and were collected in June of 2014 yielded similar detection frequencies with 23% and 6% of squash bugs harboring *S. marcescens* at Site A and Site B, respectively. Squash bugs collected in September of 2014 harbored *S. marcescens* at rates of 33% for Site A and 5% for Site B. Except for the 4th instar, *S. marcescens* was detected in at least one squash bug from each instar (Table 4.2). *S. marcescens* was detected in four out of five horned squash bugs.

Capacity of the horned squash bug, *Anasa armigera*, to transmit *S. marcescens*. No symptoms associated with CYVD were observed on plants fed upon by the horned squash bug. At the conclusion of the experiments visual inspection revealed phloem that was normal in appearance with no browning evident. PCR on extracted DNA showed that two of the six horned squash bugs that fed upon *S. marcescens*-infiltrated squash cubes were positive for the pathogen while none of the negative control insects were positive.

Seasonal dynamics of CYVD incidence. CYVD was confirmed in four out of the six counties sampled in this study. All of the positive samples were from the Georgia Piedmont and were isolated from summer squash or pumpkin. CYVD symptoms have not been reported in southern Georgia and *S. marcescens* has not been confirmed in the large-scale commercial watermelon and squash production areas of South Georgia. In 2013, losses due to CYVD occurred at Site 1 and Site 2 (Table 4.3). Site 1 had losses of 67% and 93% in two plantings and Site 2 had losses ranging from 12% to 23%, although several later plantings were unaffected. CYVD symptoms were not observed at either site in squash planted after May, 2013. In 2014, few squash bugs and no CYVD symptoms were observed at Site 1 or Site 2.

Relationship between plant age and susceptibility to *S. marcescens* and capacity for row covers to mitigate CYVD. CYVD was observed in all three row cover field trials across two years, although trial two in the spring of 2014 had a lower incidence of CYVD and fewer squash bugs than trial one (spring 2013) and trial 3 (summer 2014). In trials one and three, zucchini plants that were protected from squash bugs with row covers for at least three weeks had significantly ($P < 0.05$) less CYVD than plants not protected by a row cover and plants in which the row cover was removed two weeks after plant emergence (Fig. 4.3 A and C). In trial one, CYVD incidence was 13.9% and 10.3% for plants that received no row cover protection and

plants that were protected for two weeks, respectively. In trial three, CYVD incidence was 18.2% and 20.1% for plants that received no row cover protection and plants that were protected for two weeks, respectively. In trial two there was no significant difference in CYVD incidence among treatments, although incidence was numerically lower in plants that were protected from squash bugs for at least three weeks after emergence (Fig. 4.3B). In this experiment CYVD incidence was 7.69%, 7.69%, and 2.5% in plants that were covered for zero, two, and three weeks, respectively. CYVD was not observed in plants that were protected for four weeks. *S. marcescens* was recovered from nine, seven, and nine plants in trials one, two, and three, respectively. All of these plants exhibited characteristic CYVD symptoms and *S. marcescens* was confirmed using PCR with the A79F/R CYVD strain-specific primers (21). In experiment three, *S. marcescens* was recovered from two plants that exhibited CYVD-like yellowing and wilting, but no phloem discoloration. *S. marcescens* was not recovered from any of the 32 asymptomatic plants that were sampled at the conclusion of trials two and three.

Yield was not significantly ($P < 0.05$) affected in trial two during the spring of 2014 (Fig. 4.4A). Yield was significantly higher in plants that received row cover protection for at least three weeks in trial three during the late summer of 2014 when other disease and pest pressures were higher (Fig. 4.4B). Plants declined during July and August due to the effects of whiteflies, squash bugs, cucumber beetles, powdery and downy mildew, and viruses.

Discussion

Although transmission experiments have shown that squash bugs are the vector of CYVD-associated strains of *S. marcescens*, this study demonstrates for the first time that the pathogen can be detected in squash bugs (15, 20). In addition to direct bacterial isolation onto

media from squash bugs, PCR using CYVD-specific primers detected the pathogen in squash bug adults and in nymphs as early at the first instar. This increases the importance of targeting nymphs for control of CYVD since previous studies showed that nymphs that acquired *S. marcescens* artificially were able to retain the pathogen through molting events and subsequently infect plants (20). While the adult squash bugs that emerge from diapause are the primary source of inoculum for early-season outbreaks, their offspring are the principal disseminators of the pathogen for CYVD outbreaks in later cucurbit plantings. The targeting of second generation squash bug nymphs before maturity and dispersal may therefore be a practical management option to control the spread of the disease.

The bacterium was found in a high proportion of the adult squash bugs collected during the winter at sites with a previous history of CYVD, revealing the importance of adult squash bugs in perpetuating CYVD from one year to the next. The high frequencies of detection in overwintering adults may be the result of the pathogen life cycle. This bacterium is thought to be circulative and propagative in the squash bug vector and this could result in an increase in bacterial titer and detection frequency as squash bugs age with the pathogen. Interestingly, overwintering squash bugs collected in January and February harbored the pathogen more frequently than emerging adults collected in June. Although some strains of *S. marcescens* are known to be entomopathogenic, the effect of CYVD-associated strains on squash bug fitness is poorly understood. If *S. marcescens* causes a reduction in squash bug fitness, then overwintering squash bugs harboring the pathogen may be at a competitive disadvantage for resources or die sooner than the adults without the bacterium.

Four out of five horned squash bugs that were collected in the field in 2013 tested positive for *S. marcescens*, prompting a study to determine whether they could transmit the

pathogen. Adult horned squash bugs were able to acquire the pathogen by feeding on zucchini cubes that were infiltrated with *S. marcescens,* although they were unable to transmit the pathogen to squash seedlings. No CYVD symptoms were observed on any of the plants that were fed on by horned squash bugs. Horned squash bugs prefer wild cucurbits over agricultural varieties and are typically seen in low numbers relative to squash bugs (7). Additionally, CYVD has never been observed in wild cucurbits, increasing the likelihood that squash bugs and *S. marcescens* have coevolved to a greater extent than horned squash bugs and *S. marcescens* (10). Screening overwintering horned squash bugs for the pathogen would help determine if *S. marcescens* is capable of long term survival and propagation in horned squash bugs and would indicate the level of coevolution relative to squash bugs.

In 2013, losses due to CYVD were observed at both farm sites surveyed and ranged from 12% to 93%. Disease was severe early in the season but was not observed in squash planted after May. Prolonged heavy rains in June and July of 2013 appeared to have contributed to decreased squash bug populations during the summer of 2013. The decrease in squash bug populations resulted in much lower levels of overwintering squash bugs as determined by the inability to find squash bugs in the overwintering habitats where they were found in abundance the previous winter. This led to a complete absence of CYVD, as well as a marked reduction in squash bug populations, at both CYVD monitoring sites in 2014. CYVD was confirmed in 2014 at other locations, although at much lower frequencies than in 2013. Below average temperatures in January of 2014 may have also contributed to low squash bug populations in 2014. The average minimum temperature in January 2014 was -3.4°C, 3.7°C lower than the historical average (United States Department of Commerce NOAA). Both farms surveyed rotate their crops, which may have impacted the ability of the already reduced overwintering populations to locate

susceptible plants. CYVD was observed in multiple plantings in 2014 at the University of Georgia Durham Horticulture Farm. This location had numerous plots dedicated to cucurbit research throughout the growing season as well as year-round cucurbit production in greenhouses, which could have led to a greater incidence of CYVD when compared to the two surveyed farm sites.

While CYVD has devastated many squash plantings in northern Georgia, the disease has not affected the watermelon producing regions in South Georgia. This may be due to the use of insecticides to manage squash bugs. Many of the fresh market growers in the Georgia piedmont use organic methods of production and do not apply conventional insecticides. Squash bug preference may also play a role as squash is preferred over watermelon, although this does not account for the lack of CYVD in the large squash acreages grown in South Georgia (7). A third possibility is that *S. marcescens* does not yet occur in the squash bug populations in South Georgia. Screening squash bugs from these locations would give more insight into the distribution of *S. marcescens* in vector populations throughout Georgia and how it impacts disease incidence.

Row covers significantly decreased CYVD incidence in two of the three experiments, suggesting a positive correlation between plant age and resistance to CYVD. The phenomenon of age-related resistance (ARR) has been observed in numerous plant species across all pathogen groups, although there is no single mechanism that is known to be responsible for the transition from susceptible to resistant. ARR is often correlated with the transition from the vegetative phase to the floral phase, although it can also be related to plant age, leaf size, and the production of defense or signaling compounds (16). Kus *et al.* inoculated Arabidopsis plants with *Pseudomonas syringae* pv. *tomato* DC 3000 at 30 or 40 days after germination and found a ten-

fold increase in bacterial titer in plants inoculated at 30 days and a complete lack of disease symptoms in plants inoculated at 40 days. Resistance was directly correlated with the vegetative-floral phase transition and characterized by antimicrobial compounds that were dependent on a salicylic acid (SA)-dependent signaling pathway that was separate from the SA-dependent signaling pathway associated with systemic acquired resistance (9).

In this study, none of the plants that were protected from the squash bug vector for more than three weeks developed CYVD symptoms. In contrast, from 8% up to 20% of the plants that were protected for two weeks or less developed symptoms and tested positive for *S. marcescens*. A single plant (<1%) that was protected for three weeks developed CYVD symptoms and subsequently tested positive for *S. marcescens*. These results indicate that ARR may be induced during the vegetative-floral phase interface, which typically took place three to four weeks after plant emergence, and suggest that plant developmental stage, rather than plant age, is an indicator of plant resistance.

Another possible explanation for the reduction in CYVD in protected plants is that bacterial populations do not have sufficient time to reach the threshold population density necessary for disease development. *S. marcescens* is thought to cause disease in part by clogging the phloem of susceptible hosts and subsequently causing phloem necrosis (5). Zhang *et al.* used suppressive subtractive hybridization to discover a fimbrial-gene cluster that was proposed to be part of a genome island and a potential virulence region. Two genes included in the fimbrial-gene cluster were *fimA* and *fimH*, which encode the major fimbrial rod subunit and fimbrial adhesin, respectively (21). These proteins, which are absent in many nonpathogenic strains, aide in bacterial attachment to plant surface structures and have been shown to be virulence factors (10). If vascular clogging is the primary means of pathogenesis, then it is possible that high

bacterial titers are necessary for disease. Mechanical inoculations of squash in the non-expanded cotyledon stage with *S. marcescens* at 1 x 10^{10} CFU resulted in symptom development after four weeks at which point plants had not yet collapsed (6). These results suggest that bacteria may not reach disease-causing thresholds until several weeks after initial infection.

Yield was significantly affected in trial 3 during August and September of 2014. In this trial, yields were significantly greater for plants that received at least three weeks of row cover protection. In addition to CYVD incidence being significantly reduced in these treatments, zucchini were protected from several other cucurbit pests including the striped (*Acalymma vittatum*) and spotted (*Diabrotica undecimpunctata*) cucumber beetle, squash vine borer (*Melittia cucurbitae*), and pickleworm (*Diaphania nitidalis*). Heavy infestations of these insects, as well as squash bug feeding accompanied by CYVD, caused plant losses of 55% and 53% in plants that received no row cover protection and plants that were protected for two weeks, respectively. A reduction in vigor was observed in plants that were able to withstand heavy insect feeding (Fig. 4.2B)

Results from these experiments showed that a high proportion of squash bugs harbored *S. marcescens* throughout the year, with the highest detection rates in overwintering squash bugs. Nymphs could acquire the pathogen at any instar and have previously been shown to subsequently infect susceptible hosts (20). CYVD caused extensive losses in squash plantings in the Georgia Piedmont, but disease incidence was highly variable and appeared to be correlated with squash bug densities. CYVD has not yet been observed in squash or watermelon in South Georgia, despite significant production acreage in that region. Row cover studies indicated that plant age is positively correlated with resistance to CYVD and that the induction of resistance seems to coincide with the plants transition from the vegetative phase to the floral phase. Data

also showed that CYVD can be effectively managed with the use of row covers, and that plant flowering should be considered the indicator for row cover removal.

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Tables

Table 4.1. Detection frequency of *S. marcescens* in adult *Anasa tristis* collected in 2013 and 2014. PCR was conducted on extracted DNA using primers specific for CYVD-associated strains of *S. marcescens*.

Location	Detection frequency (%)			
	Jan/Feb 2013	June 2013	June 2014	September 2014
Site A	52.2	22.7	22.7	33.3
Site B	46.2	9.5	5.6	5.0

Table 4.2. Detection frequency of *S. marcescens* in juvenile *Anasa tristis* collected in July of 2013. PCR was conducted on extracted DNA using primers specific for CYVD-associated strains of *S. marcescens*.

Location	Detection frequency (%)				
	1st instar	2nd instar	3rd instar	4th instar	5th instar
Site A	8.3	0.0	66.7	none tested	33.3
Site B	0.0	25.0	5.6	0.0	0

Table 4.3. Location, planting dates, and CYVD incidence during the 2013 and 2014 growing seasons at two farm sites. Site 1 was the University of Georgia Durham Horticulture Farm in Oconee County, GA and Site 2 was a commercial organic farm in Oglethorpe County, GA.

Location	**Year**	**Date planted**	**Number of plants**	**CYVD incidence (%)**
Site 1	2013	05/13	15	67
		05/13	15	93
Site 2		04/13	175	12
		04/13	77	23
		05/13	63	0
		07/13	57	0
Site 1	2014	4/14	34	0
		5/14	67	0
		7/14	300	0
Site 2		04/14	346	0
		05/14	196	0
		05/14	79	0
		06/14	274	0

Figures

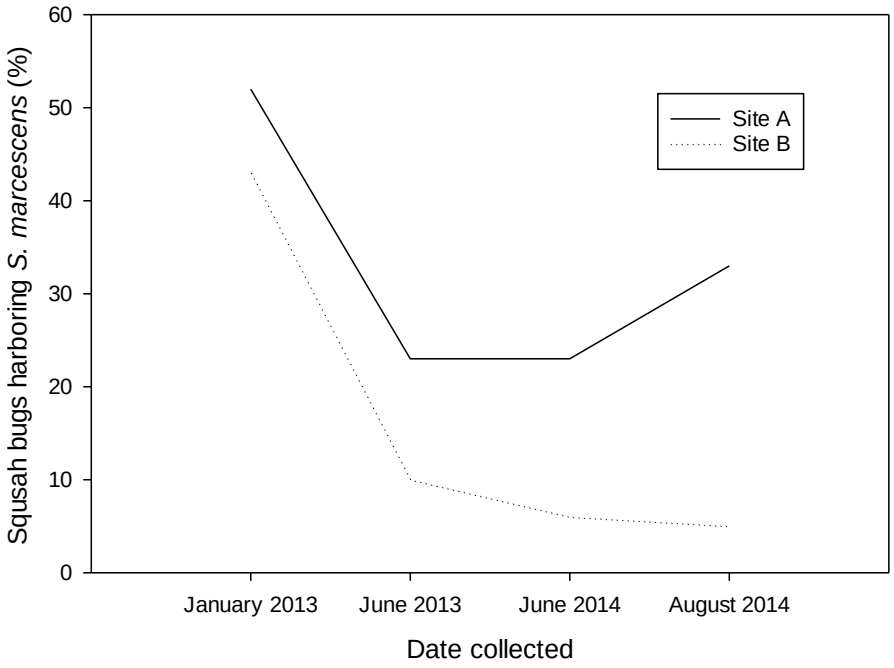

Figure 4.1. Detection frequency of *S. marcescens* in adult *Anasa tristis* over time. Site A was the University of Georgia Durham Horticulture Farm and Site B was the University of Georgia UGArden.

Figure 4.2. Images of row cover trials that were conducted three times from 2013 to 2014. Row covers were used to exclude the squash bug vector for two, three, four, or five weeks after plant emergence (A). Plants that were protected from squash bugs for more than three weeks (B, left row) had significantly less CYVD in two of three trials and significantly greater yields in one of two trials when compared to plants that were protected for zero (B, right row) or two weeks.

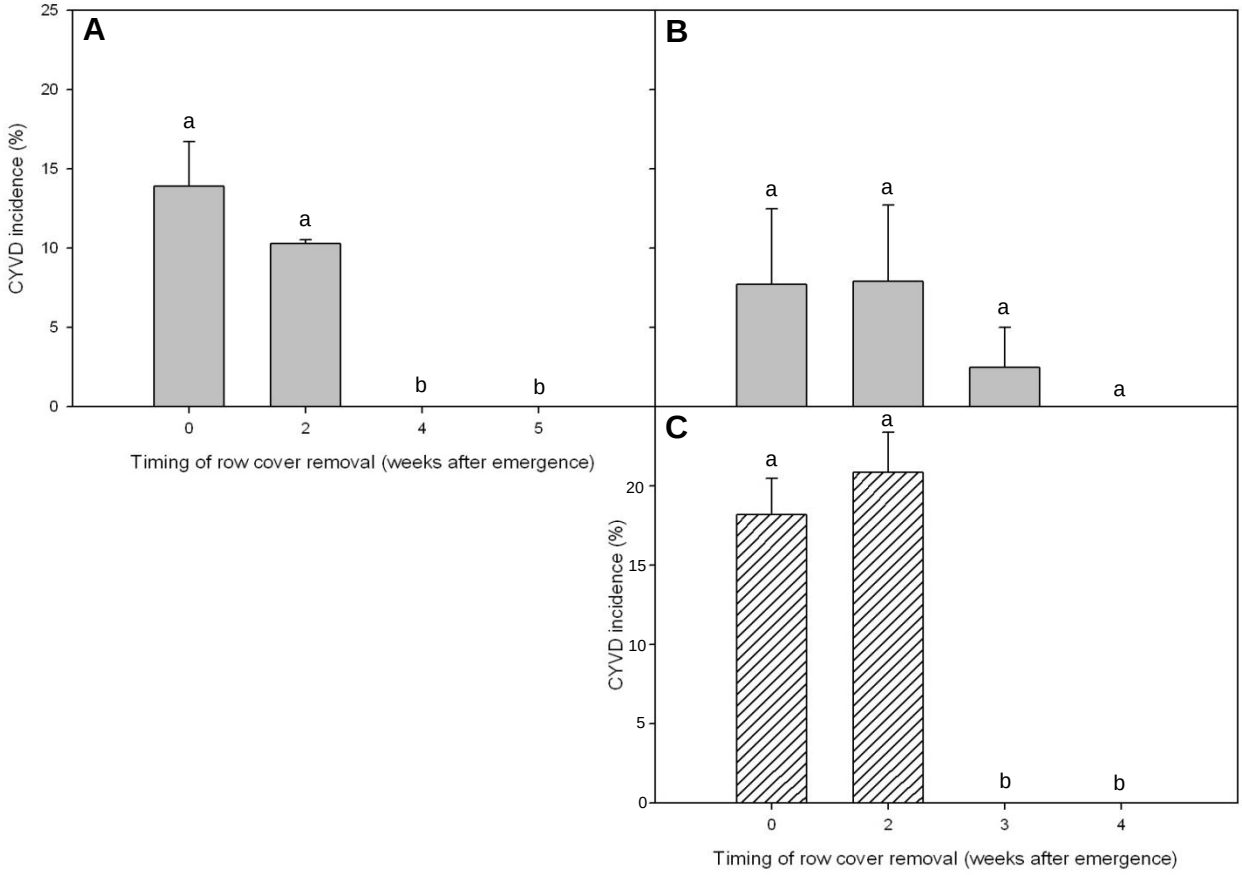

Figure 4.3. Percentage of zucchini plants positive for CYVD in the 2013-2014 row cover experiments. Row covers were removed at two, three, four, or five weeks after plant emergence. Zucchini were planted in June 2013 (A), April 2014 (B), and July 2014 (C). Error bars represent standard error. Letters denote significant differences between treatments according to the one-way ANOVA analysis ($\alpha = 0.05$).

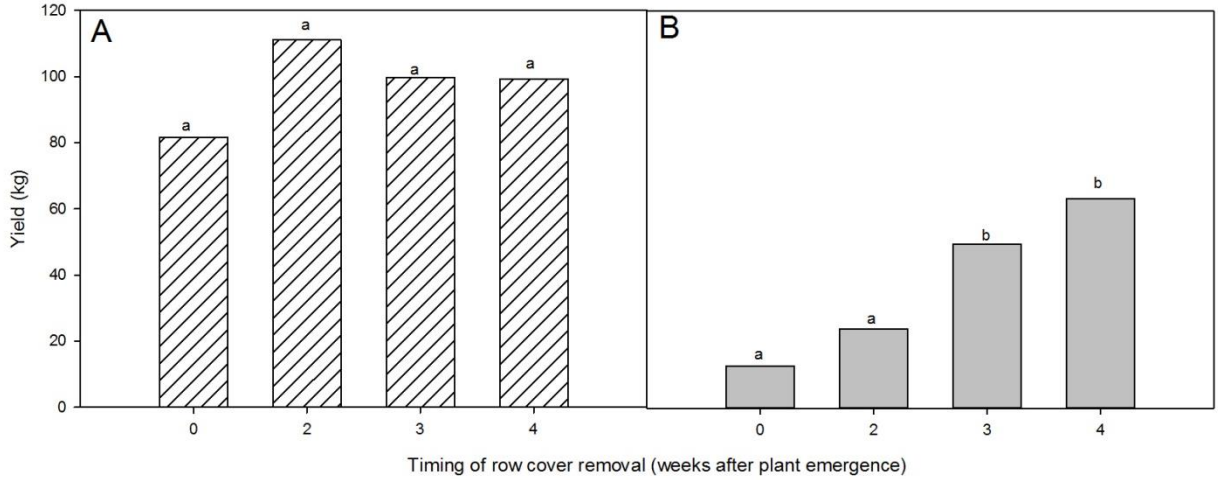

Figure 4.4. Total yield for each treatment from the 2013-2014 row cover studies. Squash bugs were excluded using row covers for zero, two, three, or four weeks. Zucchini were harvested in May - June of 2014 (A) and August – September of 2014 (B). Letters denote significant differences between treatments according to the one-way ANOVA analysis ($\alpha = 0.05$).

CHAPTER 5

CONCLUSIONS

The research presented in this thesis investigated the disease etiology, epidemiology, and the causal agent genetic diversity of cucurbit yellow vine disease (CYVD) caused by the squash bug-transmitted bacterium, *S. marcescens*. The first study determined that the causal agent of a squash collapse in the Georgia Piedmont was the squash bug-transmitted bacterium *S. marcescens*. This was the first time CYVD was confirmed in Georgia. In greenhouse inoculation studies CYVD strains of *S. marcescens* grew endophytically within pumpkin plants without inducing visible symptoms, suggesting that CYVD-causing strains of *S. marcescens* may have descended from a plant endophyte.

The second study used multilocus sequence analysis (MLSA) and repetitive sequence-based PCR (rep-PCR) to characterize the genetic diversity of 32 phytopathogenic and nonphytopathogenic strains of *S. marcescens* and 1 strain of *S. plymuthica*. The results demonstrated that CYVD-associated strains of *S. marcescens* lacked diversity in the six housekeeping genes examined, suggesting that plant pathogenic strains recently diverged from a single common ancestor. Rep-PCR revealed some diversity among CYVD strains, although we could not determine where this diversity existed in the genome and if it is manifested phenotypically. CYVD strain SB03, which grouped with the type strain using rep-PCR and at the *icd* locus using MLSA, could be an intermediate strain that bridges the gap between CYVD and non-CYVD strains.

The third study examined the disease epidemiology of CYVD, including vector/bacterium interactions, local disease impact, and the relationship between plant age and resistance to the CYVD pathogen. Results from these experiments showed that squash bugs harbored *S. marcescens* throughout the year, with the highest detection rates (50%) in overwintering squash bugs. Nymphs could acquire the pathogen at any instar and have previously been shown to subsequently infect susceptible hosts. CYVD caused extensive losses in squash plantings in the Georgia Piedmont, but disease incidence was highly variable and appeared to be correlated with squash bug densities. CYVD has not yet been observed in squash or watermelon in South Georgia, despite significant production acreage in that region. This may be due to the use of insecticides to manage squash bugs in commercial watermelon plantings in South Georgia, since many of the fresh market growers in the Georgia Piedmont use organic methods of production and do not apply conventional insecticides. Additionally, the squash bug populations in South Georgia may not harbor *S. marcescens*. Row cover studies indicated that plant age is positively correlated with resistance to CYVD and that the induction of resistance coincides with the plant's transition from the vegetative phase to the floral phase. Resistance could have resulted from the production of antimicrobial compounds or be due to low bacterial population densities in plants that were protected for three weeks or more. Data also showed that CYVD can be effectively managed with the use of row covers, and that plant flowering should be considered the indicator for row cover removal.