CHARACTERIZATION OF THE BACTERIUM *SERRATIA MARCESCENS* AND MANAGEMENT OF SQUASH DISEASES

by

ZACHARY THOMAS MATTEEN

(Under the Direction of Elizabeth L. Little)

ABSTRACT

Cucurbit yellow vine disease (CYVD), caused by the squash bug-transmitted bacterium *Serratia marcescens*, causes vascular wilting and plant death. The goals of this thesis research were to 1) Characterize differences between CYVD strains and the closely-related strain R01-A; 2) Develop a reliable pathogenicity test which reproduces full disease symptoms; 3) Determine if biocontrol agents could suppress squash diseases; and 4) Evaluate which winter squash cultivars grew best in Georgia and whether cultivars differed in CYVD susceptibility. Sequence differences between CYVD strains and R01-A were identified and belong to a clade which may be a different species from other *S. marcescens* strains. An inoculation method using squash bug nymphs was comparable to needle inoculation and improved upon previous vector studies. Strains with antagonistic ability were identified, but biocontrol treatments had no effect *in vivo*. Several *Cucurbita moschata* cultivars performed well and differed in CYVD susceptibility.

INDEX WORDS: *Serratia marcescens*, cucurbit yellow vine disease, squash bug, downy mildew, powdery mildew, biological control, squash

CHARACTERIZATION OF THE BACTERIUM *SERRATIA MARCESCENS* AND MANAGEMENT OF SQUASH DISEASES

By

ZACHARY THOMAS MATTEEN

B.S.A., University of Georgia, 2015

B.A., University of Georgia, 2015

A Thesis Submitted to the Graduate Faculty of the University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2017

CHARACTERIZATION OF THE BACTERIUM *SERRATIA MARCESCENS* AND MANAGEMENT OF SQUASH DISEASES

by

ZACHARY THOMAS MATTEEN

Major Professor: Elizabeth L. Little

Committee: Brian H. Kvitko
Ronald R. Walcott

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2017

DEDICATION

I would like to dedicate this work to my parents, Rodney and Lisa, and my grandparents, Tom and Ordway, without whom I would not be where I am today.

ACKNOWLEDGEMENTS

I would first like to express my gratitude to Dr. Elizabeth Little for her guidance and encouragement throughout my project. Thank you for challenging and supporting me over the past two and a half years. I would like to thank my committee for their valuable input and suggestions, especially Dr. Brian Kvitko and his lab members for allowing me to utilize their lab. Many thanks to others in the department who helped make this work possible.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Squash origin and taxonomy

Squashes (including pumpkins and gourds) are thought to have originated in the New World and are one of four related species: *Cucurbita pepo*, *C. moschata*, *C. maxima*, and *C. argyrosperma*. The term 'pumpkin' has no exact botanical meaning and refers to winter squash cultivars that are used mainly for pies, animal forage, or decoration. Summer squash are the immature fruit of cultivars mainly in the species *C. pepo*. Winter squash are the mature fruit of any of the four *Cucurbita* species and the long storage time of these mature fruits, in some cases up to a year, was traditionally a very desirable trait as it allowed for consumption during the winter. Examples of *C. pepo* squash include most pie and carving pumpkins, delicata, acorn, and spaghetti squash. *C. moschata* includes cheese pumpkins, butternut, and tropical pumpkins. *C. maxima* includes giant pumpkins, kabocha, hubbard, buttercup, and 'North Georgia Candy Roaster'. *C. argyrosperma* includes cushaw squash, which are primarily cultivated for their seeds, vegetative parts, and as forage for livestock (McCreight 2017). Squash readily outcross within the same species, resulting in a wide variety of shapes and sizes when allowed to freely pollinate.

Squash production in the United States

In 2013, the U.S. produced 778,000 metric tons, ranking fourth worldwide in *Cucurbita* production (Nations 2012). The value of squash production in the United States totaled over $237 million dollars in 2013 (Agriculture 2013). Commercial winter squash production in the United States is largely centered in the Northeast and Southwest (USDA 2014). These cooler and/or more arid climates have more moderate climates and are less suitable for development of many cucurbit diseases and pests. A minor amount of commercial winter squash production, primarily pumpkins, occurs in the higher elevations of the Appalachian regions in the southeast, such as northern Georgia (USDA 2014). Commercial squash production in Georgia is largely limited to summer squash grown in late spring to early summer and early fall to avoid high temperatures and disease pressure (Keinath et al. 2017).

Consumer demand for winter squash has been increasing, particularly for locally grown fruit with unique flavors and appearances. Small-scale commercial squash production for direct marketing has increased rapidly in Georgia with the greatest numbers of growers near urban areas such as Atlanta, Athens, Macon, and Savannah. Many of these growers are using organic practices. Little to no information is available on which winter squash cultivars perform best under Georgia conditions and many of the commercially available winter squash cultivars are bred for conventional systems in northern or western climates. Winter squash are long season crops, and need to grow and produce in Georgia's hot and humid summers. Squashes are subject to several serious diseases and pests that flourish under high temperatures and humidity.

Pests and diseases of squash in Georgia

Economically important pests and diseases of squash in the southeastern United States include cucurbit downy mildew (*Pseudoperonospora cubensis*), powdery mildew (*Podosphaera*

xanthii), cucurbit yellow vine disease (CYVD, *Serratia marcescens*), various viruses such as Cucumber Mosaic, Watermelon Mosaic. and Papaya Ringspot, squash bugs (*Anasa tristis*), vine borers (*Melittia cucurbitae*), and pickleworm (*Diaphania nitidalis*) (Keinath et al. 2017). Downy mildew, an obligate parasite, is an economically important disease of cucurbits due to its annual occurrence and the ability to rapidly defoliate highly susceptible hosts. Oospores, the sexual survival spores, have not been reported in the United States. The pathogen survives on cucurbit hosts in the tropical regions of southern Florida and the Caribbean during the winter. Sporangia are dispersed long distances in rain events and gradually spread north during the growing season. The sporangia require at least two hours of free moisture to germinate and the infection periods are frequent in the humid eastern United States (McCreight 2017; Ojiambo et al. 2015)

Unlike downy mildew, powdery mildew infections are more prevalent in dry, but humid, conditions. The disease reduces photosynthesis, leading to reduced yield and fruit quality. If infestation is severe, the plants defoliate and sun scald can also occur on exposed fruit. Chasmothecia, the overwintering sexual structures that contain ascospores, are rarely seen. As an obligate parasite, the powdery mildew fungus must survive on living plants during the winter, either in greenhouses or tropical areas such as southern Florida (McCreight 2017).

CYVD is an emerging bacterial disease which is vectored by squash bugs (Pair et al. 2004). CYVD is characterized by yellowing and wilting of the foliage, followed by a sudden plant collapse. A diagnostic feature of CYVD is a browning of the phloem in the lower stem of collapsed plants (Bruton et al. 2003). If squash bugs are present, CYVD incidence can be high.

Pickleworm is the larvae of a tropical moth which migrates north during the summer from southern Florida where it overwinters. Larvae feed first on flower buds and later burrow

into maturing fruits (McCreight 2017). Pickleworms are particularly problematic in organic systems due to difficulties in control and zero tolerance in markets for holes in fruit.

Squash bugs are economically important pests of cucurbits with a feeding preference for squash, followed by melons and other cucurbits (McCreight 2017). Prolonged feeding by squash bugs, especially when populations are high, will lead to a condition called Anasa wilt in which leaves wilt and brown. Squash bug feeding on fruit results in blemishes and a reduction in quality. In years of high squash bug populations, whole plants may die due to feeding damage (Fargo et al. 1988; Neal 1993).

Organic pest and disease management

Organic management of cucurbit diseases uses a systems approach for producing healthy plants. A management plan starts with optimizing site and soil conditions, and nutrition. Optimizing pH and nutrient balances in the soil can influence disease severity (Dordas 2008). For management of specific crop problems, planting date and cultivar choice can be important preventative measures. Winter and summer squash are typically planted as early as possible to avoid high temperatures and lessen the impact of pests and diseases that occur during the summer such as downy mildew, viruses, and pickleworm (McCreight 2017).

The planting of resistant cultivars is one of the most effective disease and pest management strategies, particularly in long season crops where planting date cannot be easily manipulated. Availability of disease resistant squash cultivars is limited, especially for downy mildew and CYVD (Brett et al. 1961; Elsey 1985; Lebeda and Křístková 1993; McGrath and Staniszewska 1996; Novero et al. 1962; Vogt and Nechols 1993). High levels of resistance, as found with single gene resistance, are usually overcome eventually, especially with pests and

pathogens that produce multiple generations in a season (Margolies et al. 1998). Downy mildew in particular is increasing in importance with the development of new, more virulent races in the last decade (Holmes et al. 2014). In organic production, available fungicides have low efficacy and partially resistant cultivars are needed.

Crop rotation is a fundamental management practice in organic production and prevents the buildup of pests and diseases. However, for pathogens and pests that migrate each year from frost-free climates or other overwintering sites, such as the causal agents of downy and powdery mildew, and pickleworms, other methods must be used to manage primary inoculum. Crop rotation or squash-free periods can be effective for reducing squash bug numbers by limiting hosts on which to reproduce (McCreight 2017) and this may reduce overwintering squash bugs that carry CYVD. Employing drip irrigation reduces leaf wetness and the spread of foliar pathogens by splash dispersal. However, the effectiveness of drip irrigation can be limited in wet years and may not be economical for large acreages (McCreight 2017).

Biologically active soil forms the basis for successful organic production. Optimizing soil organic matter through the addition of high quality compost and green manure cover crops produces diverse microbial populations that increase plant health and resistance, and suppress plant pathogens and pests. The biological components can be increased on the above ground plant parts by growing a diversity of plants and avoiding the use of fungicides and insecticides. Biological control microbes are naturally occurring fungi and bacteria that are selected and marketed for their disease-suppressive abilities. While isolated fungi or bacteria are not as effective as a complex community of interacting microbes, the biocontrol organisms can be effective in some circumstances against foliar, fruit, and root diseases of cucurbits (Gafni et al. 2015; Maleki et al. 2011; Postma et al. 2009; Singh and Rao 1995). Selection of effective

biocontrol microbes usually starts with *in vitro* screening of candidates for antagonistic activity before testing in the field (Pliego et al. 2011). Several studies have investigated the use of *S. marcescens* as a biocontrol candidate for fungal diseases due to chitinase production (Ordentlich et al. 1988; Someya et al. 2000; Someya et al. 2005b).

Biological control organisms lessen disease impact by using a direct, mixed-path, or indirect form of antagonism (Pal and McSpadden Gardener 2006). Direct forms of antagonism include predation and parasitism, such as the nematode bacterial parasite *Pasteuria penetrans* (Chen and Dickson 1998). Mixed-path antagonism involves production of one or more extracellular products such as antibiotics, lytic enzymes, or unregulated metabolites such as ammonia (Pal and McSpadden Gardener 2006). The chitinase and prodigiosin production in *S. marcescens* are mechanisms of mixed-path antagonism employed in controlling plant diseases (Ordentlich et al. 1988; Someya et al. 2005a). Indirect mechanisms of antagonism include competition through nutrient acquisition or niche occupation, such as nonpathogenic mutants of *Acidovorax citrulli*, causal agent of bacterial fruit blotch, reducing disease by niche competition with pathogenic strains (Johnson et al. 2011). Another form of indirect antagonism is induction of resistance in host plants through detection of pathogen-associated molecular patterns (PAMP) or phytohormone-mediated induction (Choudhary et al. 2007; Someya et al. 2005a)

Induced resistance can be either induced systemic resistance (ISR) or systemic acquired resistance (SAR). SAR is mediated by salicylic acid, and results in the production of pathogenesis-related proteins such as lytic enzymes, cell wall-reinforcing proteins, or induced localized cell death (Pal and McSpadden Gardener 2006). SAR inducers have been utilized in control of *Phytophthora capcisi* and Cucumber Mosaic Virus (CMV) in squash (Koné et al. 2009; Mayers et al. 2005). ISR is usually mediated through jasmonic acid, ethylene, or a

combination of both, and ISR is induced most commonly through application of nonphytopathogenic rhizobacteria (Pal and McSpadden Gardener 2006; Shoresh et al. 2010).ISR inducers, such as *Bacillus subtilis*, have been employed in control of cucurbit powdery mildew (García-Gutiérrez et al. 2013). Studies have shown some *S. marcescens* strains are able to induce ISR to diseases in non-cucurbit hosts (Lavania et al. 2006; Press et al. 1997; Someya et al. 2002), but no studies have been conducted on the potential use of non-pathogenic *S. marcescens* strains for controlling diseases in squash.

Squash bugs

Squash bugs are sap-sucking true bugs in the family Coreidae and order Hemiptera, and are widely distributed throughout the United States (Wadley 1920). The squash bug typically completes its entire life cycle in six to eight weeks and has five nymphal instar stages (Fargo and Bonjour 1988). Nymphs show a strong tendency to aggregate, especially when young, and both adults and nymphs are cryptic and often hide during the day (Nechols 1987; Palumbo et al. 1991).

Squash bugs overwinter as adults in the soil, leaf litter, old buildings, woodpiles, debris or any other location that keeps them warm and dry in a reproductive diapause stage based on photoperiod (Adam 2006). Approximately half of adults enter diapause late August or early September in the southeast with oviposition slowing and finally ceasing approximately 30 days after onset of diapause (Fielding 1988). After emerging from overwintering sites, adult squash bugs travel unknown distances to find newly planted cucurbits and begin laying eggs within seven to ten days after terminating diapause (Nechols 1987; Wadley 1920). Adult squash bugs have up to three new generations in a season depending on climate with overwintering adults and

adults of the first new generation often overlapping their reproductive period (Adam 2006; Decker and Yeargan 2008; Nechols 1987).

In conventional growing systems, squash bugs are managed through insecticide use. Squash bugs are more challenging to control in organic systems due to a lack of efficacious organic insecticides and the use of mulches that offer hiding places for the cryptic squash bug (Cranshaw et al. 2001). Cultural practices such as removing debris in which adults can overwinter, crop rotation to slow buildup of squash bug populations, and early planting are commonly employed (Wadley 1920; Woodson and Fargo 1991). Trap cropping, where a sacrificial first planting is used as a lure to keep squash bugs off subsequent crops, works best for less-preferred squash bug hosts such as cantaloupes and cucumbers since squash bugs have a preference for and survive better on squash and pumpkins; however, squash bugs have feeding preferences among squash cultivars as well (Bonjour et al. 1993; Bonjour et al. 1990).

A few winter squash cultivars such as green striped cushaw and 'Waltham Butternut' appear to be less attractive and less susceptible to squash bug feeding damage compared to summer squash (Novero et al. 1962; Vogt and Nechols 1993). Some squash cultivars have minor resistance, but the resistance can break down under high squash bug pressure. Other control measures should be used with squash bug resistant cultivars and cultivars should be rotated (Margolies et al. 1998).

Squash bugs as a vector of CYVD

Squash bugs, an economically important pest on its own, is also the vector of CYVD, caused by the bacterium *S. marcescens* (Pair et al. 2004). An early study by Bextine et al. (2001) found that insect exclusion prevented the development of CYVD and concluded that an insect

vector played a role in the disease cycle. Pair et al. (2004) confirmed the squash bug as the vector following successful inoculation of plants with CYVD using squash bugs.

Squash bugs utilize piercing-sucking mouthparts to feed on the xylem of cucurbits, but they often probe the phloem as well prior to feeding (Adam 2006). When feeding occurs on plants during the first four weeks after planting, the causal agent of CYVD can be transmitted, although symptoms do not occur until fruiting initiates (Besler 2014). The squash bug is the source of primary inoculum as the bacterium is retained in squash bug adults during winter diapause. The bacterium is believed to be circulative and propagative within the hemocoel of its vector due to persistence through molting events and the long retention period during diapause, but the bacterium is not transmitted transovarial to the next generation of squash bugs (Pair et al. 2004; Wayadande et al. 2005).

Nymphs are capable of transmitting the bacterium and causing CYVD, although the relative importance of each life stage of the squash bug in transmitting CYVD is not known (Pair et al. 2004). Presence of the bacterium within all nymphal instars and adults has been confirmed using both culturing and PCR-based assays. Besler found that 5-50% of overwintering adults collected from two organic growing sites near Athens, Georgia carried the bacterium (Besler 2014). Therefore, reducing squash bug numbers is an effective method of reducing CYVD incidence. The use of floating row covers to exclude squash bugs, and other vectors, has been shown to reduce CYVD incidence. Row covers must be removed as soon as flowering commences to ensure good pollination and yield (Besler 2014; Cartwright et al. 1990).

Characterization of the causal agent of CYVD

CYVD was first observed in Texas and Oklahoma in 1988 (Bruton et al. 2003). Following numerous attempts to isolate and identify the pathogen, Avila et al. (1998) used PCR and sequencing to determine that the causal agent of CYVD was a prokaryote closely related to *S. marcescens*. Completion of Koch's postulates 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* (Rascoe et al. 2003; Zhang et al. 2003).

CYVD is an unusual disease complex and little is known about how this disease emerged and how the bacterium causes plant death. CYVD is the only plant disease vectored by squash bugs and the only non-fastidious bacterial pathogen that causes a phloem disease. *S. marcescens* is a highly diverse species that survives in many ecological niches, including as insect and human pathogens, endophytes, and in soil and water. CYVD is the only major plant disease caused by this bacterium.

Using DNA-DNA hybridization, Zhang et al. (2003) found CYVD strains to have a relatedness of 76% with the *S. marcescens* type strain, which is close to the 70% relatedness required for species level identity. The CYVD strains were 94-100% identical to each other and closely related to two rice endophyte strains, R02-A and R01-A, at 90% and 82% relatedness, respectively (Zhang et al. 2003). Suppressive subtractive hybridization revealed 48 unique inserts and two gene clusters that were present in CYVD strains but lacking in strain R02-A, a closely related nonphytopathogenic strain. These clusters included a fimbrial-gene cluster that was proposed to be part of a genome island and a potential virulence factor (Zhang et al. 2005). Two genes in this cluster, *fimA* and *fimH*, which encode the major fimbrial rod subunit and fimbrial adhesin, respectively, were disrupted via gene deletion. 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 (Luo 2007). Mechanisms for pathogenicity, including effectors and effector delivery systems, additional virulence factors, and pathogenicity determinants have not been identified in CYVD strains.

Based upon assays using the model nematode system *Caenorhabditis elegans,* virulence factors in human pathogenic *S. marcescens* strains include production of three groups of molecules*:* hemolysins, siderophores, and lipopolysaccharides, as well as certain extracellular products such as chitinase and structures related to adherence (Kurz et al. 2003; Rodrigues et al. 2006). Hemolysin degrades or ruptures red blood cells in humans and animals, having a cytotoxic effect, often through vacuolation (Hertle et al. 1999). Hemolysins as pathogenicity factors in plant pathogens are not well investigated, although a virulence factor identified in *Pseudomonas aeruginosa* infection on *Arabidopsis thaliana* is related to hemolytic activity (Rahme et al. 2000).

Siderophores are low molecular weight, mostly ferric specific ligands secreted under iron deficient conditions, which together with membrane receptor proteins, form part of a high-affinity iron transport system (Neilands 1981). Siderophore production in *S. marcescens* helps with growth and colonization under stressful conditions (Khilyas et al. 2016; Rodrigues et al. 2006). Lipopolysaccharide (LPS), a compound responsible for the biological activity of certain endotoxins, is located in the outer membrane of Gram-negative bacteria and is comprised of three regions: lipid A, the O-antigen and the core. The O-antigen, a repetitive saccharide chain, is the most important immunogenic component determining the O-serotype of bacteria. O-antigen affected the adherence capability, the antibiotic susceptibility, and the efficiency of transformation of *S. marcescens* (Palomar et al., 1995).

Most extracellular products seem to be universal for *S. marcescens* strains, and do not appear to play a significant role as virulence factors in human pathogenic strains, but adherence was positively correlated with pathogenicity in clinical serotypes (Aucken and Pitt 1998). Two classes of adhesins have been suggested for clinical *S. marcescens* strains, mannose-resistant (MR) and mannose-sensitive (MS) pili (Reid and Sobel 1987). While the role of adhesins is unknown in CYVD *S. marcescens* strains, a type I fimbrial gene cluster has been identified, and may play a role in adherence to plant cell walls, biofilm formation, or colonization of squash bug hosts (Zhang et al. 2005).

Project Objectives

1. Characterize CYVD strains of *S. marcescens* using whole genome sequencing

1A. Determine and characterize unique regions in CYVD strains and R01-A

1C. Evaluate phylogeny of *S. marcescens* strains with whole genome sequences

2. Use squash bug nymphs to inoculate plants with CYVD strains of *S. marcescens*

2A. Develop plant inoculation method using squash bug nymphs

2B. Compare nymph inoculation method to needle inoculation method

2C. Determine if nymphs can take up the non-pathogenic *S. marcescens* strain W11

3. Evaluate bacterial strains as biocontrol agents of cucurbit diseases

3A. Examine antagonistic abilities of bacterial strains against CYVD strains *in vitro*.

3B. Evaluate application methods and disease suppressive abilities of potential biocontrol strains

4. Evaluate winter squash cultivars for disease and pest resistance, yield, and fruit quality

4A. Determine resistance levels of cultivars to downy mildew, powdery mildew, and CYVD

4B. Count squash bug and pickleworm numbers to determine cultivar feeding preferences

4C. Evaluate fruit quality and yield of cultivars

Literature Cited

Adam, K. L. 2006. Squash bug and squash vine borer: Organic controls. ATTRA-National Sustainable Agriculture Information Service.

Agriculture, U. S. D. o. 2013. Squash Production, measured in dollars. in: National Statistics for Squash, National Agricultural Statistics Service.

Aucken, H. M., and Pitt, T. L. 1998. Antibiotic resistance and putative virulence factors of *Serratia marcescens* with respect to O and K serotypes. J. Med. Microbiol. 47:1105-1113.

Avila, F. J., Bruton, B. D., Fletcher, J., Sherwood, J. L., Pair, S. D., and Melcher, U. 1998. Polymerase chain reaction detection and phylogenetic characterization of an agent associated with yellow vine disease of cucurbits. Phytopathology 88:428-436.

Besler, K. R. 2014. Epidemiology and management of cucurbit yellow vine disease, and characterization of the causal agent *Serratia marcescens*. University of Georgia, Athens, GA.

Bextine, B., Wayadande, A., Bruton, B., Pair, S., Mitchell, F., and Fletcher, J. 2001. Effect of insect exclusion on the incidence of yellow vine disease and of the associated bacterium in squash. Plant Dis. 85:875-878.

Bonjour, E. L., Fargo, W. S., and Rensner, P. E. 1990. Ovipositional preference of squash bugs (Heteroptera: Coreidae) among cucurbits in Oklahoma. J. Econ. Entomol. 83:943-947.

Bonjour, E. L., Fargo, W. S., Al-Obaidi, A. A., and Payton, M. E. 1993. Host effects on reproduction and adult longevity of squash bugs (Heteroptera: Coreidae). Environ. Entomol. 22:1344-1348.

Brett, C. H., McCombs, C. L., and Daugherty, D. M. 1961. Resistance of squash varieties to the pickleworm and the value of resistance to insecticidal control. J. Econ. Entomol. 54:1191-1197.

Bruton, B., Mitchell, F., Fletcher, J., Pair, S., Wayadande, A., Melcher, U., Brady, J., Bextine, B., and Popham, T. 2003. *Serratia marcescens*, a phloem-colonizing, squash bug-transmitted bacterium: Causal agent of cucurbit yellow vine disease. Plant Dis. 87:937-944.

Cartwright, B. C., Palumbo, J. S., and Fargo, W. 1990. Influence of crop mulches and row covers on the population dynamics of the squash bug (Heteroptera: Coreidae) on summer squash. J. Econ. Entomol. 83:1988-1993.

Chen, Z. X., and Dickson, D. W. 1998. Review of *Pasteuria penetrans*: Biology, ecology, and biological control potential. J. Nematol. 30:313-340.

Choudhary, D. K., Prakash, A., and Johri, B. N. 2007. Induced systemic resistance (ISR) in plants: mechanism of action. Indian J. Microbiol. 47:289-297.

Cranshaw, W., Bartolo, M., and Schweissing, F. 2001. Control of squash bug injury: Management manipulations at the base of pumpkin. Southwest. Entomol. 26:147-150.

Decker, K. B., and Yeargan, K. V. 2008. Seasonal phenology and natural enemies of the squash bug (Hemiptera: Coreidae) in Kentucky. Environ. Entomol. 37:670-678.

Dordas, C. 2008. Role of nutrients in controlling plant diseases in sustainable agriculture: A review. Agron. Sustain. Dev. 28:33-46.

Elsey, K. D. 1985. Resistance mechanisms in *Cucurbita moschata* to pickleworm and melonworm (Lepidoptera: Pyralidae). J. Econ. Entomol. 78:1048-1051.

Fargo, W. S., and Bonjour, E. L. 1988. Developmental rate of the squash bug, *Anasa tristis* (Heteroptera: Coreidae) at constant temperatures. Environ. Entomol. 17:926-929.

Fargo, W. S., Rensner, P. E., Bonjour, E. L., and Wagner, T. L. 1988. Population dynamics in the squash bug (Heteroptera: Coreidae)-squash plant (Cucurbitales: Cucurbitaceae) system in Oklahoma. J. Econ. Entomol. 81:1073-1079.

Fielding, D. J. 1988. Photoperiodic induction of diapause in the squash bug, *Anasa tristis*. Entomol. Exp. Appl. 48:187-193.

Gafni, A., Calderon, C. E., Harris, R., Buxdorf, K., Dafa-Berger, A., Zeilinger-Reichert, E., and Levy, M. 2015. Biological control of the cucurbit powdery mildew pathogen *Podosphaera xanthii* by means of the epiphytic fungus *Pseudozyma aphidis* and parasitism as a mode of action. Front. Plant Sci. 6:1-11.

García-Gutiérrez, L., Zeriouh, H., Romero, D., Cubero, J., de Vicente, A., and Pérez-García, A. 2013. The antagonistic strain *Bacillus subtilis* UMAF6639 also confers protection to melon plants against cucurbit powdery mildew by activation of jasmonate- and salicylic acid-dependent defence responses. Microb. Biotechnol. 6:264-274.

Hertle, R., Hilger, M., Weingardt-Kocher, S., and Walev, I. 1999. Cytotoxic action of *Serratia marcescens* hemolysin on human epithelial cells. Infect. Immun. 67:817-825.

Holmes, G. J., Ojiambo, P. S., Hausbeck, M. K., Quesada-Ocampo, L., and Keinath, A. P. 2014. Resurgence of cucurbit downy mildew in the United States: A watershed event for research and extension. Plant Dis. 99:428-441.

Johnson, K. L., Minsavage, G. V., Le, T., Jones, J. B., and Walcott, R. R. 2011. Efficacy of a nonpathogenic *Acidovorax citrulli* strain as a biocontrol seed treatment for bacterial fruit blotch of cucurbits. Plant Dis. 95:697-704.

Khilyas, I. V., Shirshikova, T. V., Matrosova, L. E., Sorokina, A. V., Sharipova, M. R., and Bogomolnaya, L. M. 2016. Production of siderophores by *Serratia marcescens* and the role of MacAB efflux pump in siderophores secretion. BioNanoScience 6:480-482.

Koné, D., Csinos, A. S., Jackson, K. L., and Ji, P. 2009. Evaluation of systemic acquired resistance inducers for control of *Phytophthora capsici* on squash. Crop Protect. 28:533-538.

Kurz, C. L., Chauvet, S., Andres, E., Aurouze, M., Vallet, I., Michel, G. P., Uh, M., Celli, J., Filloux, A., De Bentzmann, S., Steinmetz, I., Hoffmann, J. A., Finlay, B. B., Gorvel, J. P., Ferrandon, D., and Ewbank, J. J. 2003. Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by *in vivo* screening. EMBO J. 22:1451-1460.

Lavania, M., Chauhan, P. S., Chauhan, S. V. S., Singh, H. B., and Nautiyal, C. S. 2006. Induction of plant defense enzymes and phenolics by treatment with plant growth–promoting rhizobacteria *Serratia marcescens* NBRI1213. Curr. Microbiol. 52:363-368.

Lebeda, A., and Křístková, E. 1993. Resistance of *Cucurbita pepo* and *Cucurbita moschata* varieties to cucurbit downy mildew (*Pseudoperonspora cubensis*). Plant Var. Seeds 6:109-114.

Luo, G. 2007. Phytopathogenicity, virulence factors, and in planta movement of *Serratia marcescens*. PhD dissertation. Oklahoma State University.

Maleki, M., Mokhtarnejad, L., and Mostafaee, S. 2011. Screening of rhizobacteria for biological control of cucumber root and crown rot caused by *Phytophthora drechsleri*. Plant Pathol. J. 27:78-84.

Margolies, D. C., Nechols, J. R., and Vogt, E. A. 1998. Rapid adaptation of squash bug, *Anasa tristis*, populations to a resistant cucurbit cultivar. Entomol. Exp. Appl. 89:65-70.

Mayers, C. N., Lee, K. C., Moore, C. A., Wong, S. M., and Carr, J. P. 2005. Salicylic acid-induced resistance to Cucumber mosaic virus in squash and *Arabidopsis thaliana*: contrasting mechanisms of induction and antiviral action. Mol. Plant Microbe Interact. 18:428-434.

McCreight, J. D. 2017. Compendium of cucurbit diseases and pests. Pages 1-9 in: Compendium of cucurbit diseases and pests. A. P. Keinath, W. M. Wintermantel and Z. T.A., eds. APS Press, St. Paul, Minn.

McGrath, M. T., and Staniszewska, H. 1996. Management of powdery mildew in summer squash with host resistance, disease threshold-based fungicide programs, or an integrated program. Plant Dis. 80:1044-1052.

Nations, F. a. A. O. o. t. U. 2012. Pumpkins, Squash, and Gourds. Statistical Division.

Neal, J. J. 1993. Xylem transport interruption by *Anasa tristis* feeding causes *Cucurbita pepo* to wilt. Entomol. Exp. Appl. 69:195-200.

Nechols, J. 1987. Voltinism, seasonal reproduction, and diapause in the squash bug (Heteroptera: Coreidae) in Kansas. Environ. Entomol. 16:269-273.

Neilands, J. B. 1981. Iron absorption and transport in microorganisms. Annu. Rev. Nutr. 1:27-46.

Novero, E. S., Painter, R. H., and Hall, C. V. 1962. Interrelations of the squash bug, *Anasa tristis*, and six varieties of squash (*Cucurbita* spp.). J. Econ. Entomol. 55:912-919.

Ojiambo, P. S., Gent, D. H., Quesada-Ocampo, L. M., Hausbeck, M. K., and Holmes, G. J. 2015. Epidemiology and population biology of *Pseudoperonospora cubensis*: a model system for management of downy mildews. Annu. Rev. Phytopathol. 53:223-246.

Ordentlich, A., Elad, Y., and Chet, I. 1988. The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. Phytopathology 78:84-87.

Pair, S. D., Bruton, B. D., Mitchell, F., Fletcher, J., Wayadande, A., and Melcher, U. 2004. Overwintering squash bugs harbor and transmit the causal agent of cucurbit yellow vine disease. J. Econ. Entomol. 97:74-78.

Pal, K. K., and McSpadden Gardener, B. 2006. Biological control of plant pathogens. Plant Health Instruct. 1117:1-25.

Palumbo, J. C., Fargo, W. S., and Bonjour, E. L. 1991. Colonization and seasonal abundance of squash bugs (Heteroptera: Coreidae) on summer squash with varied planting dates in Oklahoma. J. Econ. Entomol. 84:224-229.

Pliego, C., Ramos, C., de Vicente, A., and Cazorla, F. M. 2011. Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. Plant Soil 340:505-520.

Postma, J., Stevens, L. H., Wiegers, G. L., Davelaar, E., and Nijhuis, E. H. 2009. Biological control of *Pythium aphanidermatum* in cucumber with a combined application of *Lysobacter enzymogenes* strain 3.1T8 and chitosan. Biol. Control 48:301-309.

Press, C. M., Wilson, M., Tuzun, S., and Kloepper, J. W. 1997. Salicylic acid produced by *Serratia marcescens* 90-166 is not the primary determinant of induced systemic resistance in cucumber or tobacco. Mol. Plant-Microbe Interact. 10:761-768.

Rahme, L. G., Ausubel, F. M., Cao, H., Drenkard, E., Goumnerov, B. C., Lau, G. W., Mahajan-Miklos, S., Plotnikova, J., Tan, M. W., Tsongalis, J., Walendziewicz, C. L., and Tompkins, R. G. 2000. Plants and animals share functionally common bacterial virulence factors. Proc. Natl. Acad. Sci. U. S. A. 97:8815-8821.

Rascoe, J., Berg, M., Melcher, U., Mitchell, F. L., Bruton, B. D., Pair, S. D., and Fletcher, J. 2003. Identification, phylogenetic analysis, and biological characterization of *Serratia marcescens* strains causing cucurbit yellow vine disease. Phytopathology 93:1233-1239.

Reid, G., and Sobel, J. D. 1987. Bacterial adherence in the pathogenesis of urinary tract infection: a review. Rev. Infect. Dis. 9:470-487.

Rodrigues, A. P., Holanda, A. R., Lustosa, G. P., Nobrega, S. M., Santana, W. J., Souza, L. B., and Coutinho, H. D. 2006. Virulence factors and resistance mechanisms of *Serratia marcescens*. A short review. Acta Microbiol. Immunol. Hung. 53:89-93.

Shoresh, M., Harman, G. E., and Mastouri, F. 2010. Induced systemic resistance and plant responses to fungal biocontrol agents. Annu. Rev. Phytopathol. 48:21-43.

Singh, R., and Rao, S. N. 1995. Biological control of *Aphis gossypii* Glover on cucurbits by a parasitic wasp T*rioxys indicus* Subba Rao & Sharma. Biol. Agric. Hortic. 12:227-236.

Someya, N., Nakajima, M., and Akutsu, K. 2005a. Potential of an antagonistic bacterium S*erratia marcescens* strain B2 for the biological control of cucumber damping-off disease. Biocontrol Sci. 10:101-104.

Someya, N., Nakajima, M., Hibi, T., Yamaguchi, I., and Akutsu, K. 2002. Induced resistance to rice blast by antagonistic bacterium, *Serratia marcescens* strain B2. J. Gen. Plant Pathol. 68:177-182.

Someya, N., Nakajima, M., Watanabe, K., Hibi, T., and Akutsu, K. 2005b. Potential of *Serratia marcescens* strain B2 for biological control of rice sheath blight. Biocontrol Sci. Tech. 15:105-109.

Someya, N., Kataoka, N., Komagata, T., Hirayae, K., Hibi, T., and Akutsu, K. 2000. Biological control of cyclamen soilborne diseases by *Serratia marcescens* Strain B2. Plant Dis. 84:334-340.

USDA. 2014. 2012 USDA census of agriculture. United States Department of Agriculture, Washington, DC.

Vogt, E. A., and Nechols, J. R. 1993. Responses of the squash bug (Hemiptera: Coreidae) and its egg parasitoid, *Gryon pennsylvanicum* (Hymenoptera: Scelionidae) to three *Cucurbita* cultivars. Environ. Entomol. 22:238-245.

Wadley, F. M. 1920. The squash bug. J. Econ. Entomol. 13:416-425.

Wayadande, A., Bruton, B., Fletcher, J., Pair, S., and Mitchell, F. 2005. Retention of cucurbit yellow vine disease bacterium *Serratia marcescens* through transstadial molt of vector *Anasa tristis* (Hemiptera: Coreidae). Ann. Entomol. Soc. Am. 98:770-774.

Woodson, D. W., and Fargo, S. W. 1991. Interactions of temperature and squash bug density (Hemiptera: Coreidae) on growth of seedling squash. J. Econ. Entomol. 84:886-890.

Zhang, Q., Melcher, U., Zhou, L., Najar, F. Z., Roe, B. A., and Fletcher, J. 2005. Genomic comparison of plant pathogenic and nonpathogenic *Serratia marcescens* strains by suppressive subtractive hybridization. Appl. Environ. Microbiol. 71:7716-7723.

Zhang, Q., Weyant, R., Steigerwalt, A. G., White, L. A., Melcher, U., Bruton, B. D., Pair, S. D., Mitchell, F. L., and Fletcher, J. 2003. Genotyping of *Serratia marcescens* strains associated with cucurbit yellow vine disease by repetitive elements-based polymerase chain reaction and DNA-DNA hybridization. Phytopathology 93:1240-1246.

CHAPTER 2

CHARACTERIZATION OF CUCURBIT YELLOW VINE DISEASE STRAINS OF *SERRATIA MARCESCENS* USING WHOLE GENOME SEQUENCING[1]

[1] Matteen, Z. T. and Little, E. L. 2017. To be submitted to *Plant Disease*.

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. *S. marcescens* is a diverse species found in many ecological niches. In previous genetic studies, CYVD strains of *S. marcescens* formed a group separate from non-CYVD strains and were most closely related to two rice endophytes, R01-A and R02-A. The mechanism for how CYVD strains cause disease is unknown. The genomes of CYVD strains, R01-A, and nonpathogenic *S. marcescens* strain W11 were sequenced, aligned, and the predicted coding sequences (CDS) were determined in unique regions. CYVD strains contained 37 unique regions and 73 missing regions compared to strain R01-A. The genomes of CYVD strains were reduced in size by 0.26-0.39 Mb compared to the next smallest *S. marcescens* strain in the NCBI Genome Database and 0.78-0.91 Mb smaller compared to R01-A. Strain SB03 contained five insertion sequences not present in other CYVD strains. Using average nucleotide identity (ANI), CYVD strains and two non-pigmented, endophytic strains, R01-A and 90-166, were placed in a clade separate from other *S. marcescens* strains evaluated. Based on ANI values, the CYVD clade and some of the other *S. marsecens* strains may belong to a separate *Serratia* species.

Introduction

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 (Bruton et al. 2003). Avila et al. (1998) used sequence analysis to determine that the causal agent of CYVD was a prokaryote closely related to *Serratia marcescens*. Completion of Koch's postulates, 16S rDNA and *groE* sequence analysis, as well as rep-PCR and DNA-DNA hybridization, were used to show that the causal agent of CYVD was *S. marcescens* (Rascoe et al. 2003; Zhang et al. 2003).

PCR using primers that anneal to repetitive extragenic palindromic elements (rep-PCR), Zhang et al. (2003) determined CYVD strains formed a closely related cluster separate from non-CYVD strains of *S. marcescens*, but were most closely related to plant endophytic strains. Through DNA-DNA hybridization, Zhang et al. (2003) found CYVD strains to have a relatedness of 76% with the *S. marcescens* type strain, which is close to the 70% required for species level identity. The CYVD strains were 94 to 100% identical to each other and closely related to two rice endophyte strains, R02-A and R01-A, at 90% and 82% relatedness, respectively.

Using suppressive subtractive hybridization, Zhang et al. (2005) discovered 48 unique sequences in CYVD strain Z01-A compared with the endophytic strain R02-A. Putative genes were predicted for the sequences using BLASTX, with many of the sequences coding for the synthesis of bacterial surface molecules such as type 1 pili and O-antigens, a structural component of lipopolysaccharides. By creating fosmid libraries and sequencing the products of the hybridization, at least one phage cluster and a likely genome island containing fimbrial genes were present in CYVD strain Z01-A. The organization of these fimbrial genes was similar to that

of *Escherichia coli*, except one of the regulatory genes, *fimE*, was truncated (Zhang et al. 2005). Zhang (2004) observed pilus-like structures in Z01-A, but not in R02-A, when using transmission electron microscopy, indicating truncation of the regulator gene *fimE* does not disrupt production of pili. Luo (2007) later disrupted two genes in this fimbrial cluster, *fimA* and *fimH*, which encode the major fimrial rod subunit and fimbrial adhesion, respectively. Pathogenicity tests showed a reduction in virulence for both mutants, although they retained the ability to cause disease, suggesting additional virulence factors.

Using rep-PCR, Besler and Little (2017) found that CYVD strains from Georgia formed a closely related group separate from non-CYVD strains. Rep-PCR revealed genetic variation between Georgia CYVD strains and W01-A, a CYVD strain from Texas. In addition, a CYVD strain isolated from a squash bug, SB03, was found to share similarities with the *S. marcescens* type strain ATCC 13880. In the same study, multilocus sequence analysis (MLSA) of six housekeeping genes revealed that SB03 was closely related to the type strain at the *icd* loci and identical to the other CYVD strains at the other loci. While the results of this study appear to indicate distinct differences between *S. marcescens* strains, MLSA and rep-PCR examine only limited regions of the genome.

Advances in sequencing technologies now allow for the routine use of whole genome sequencing to answer questions on gene function and phylogeny, and to confirm the results of previous genetic studies. Average nucleotide identity (ANI) is a method commonly used with large sequences to determine phylogenetic relationships and bacterial speciation. ANI uses pairwise comparisons of open reading frames (ORFs) which share at least 30% sequence identity and for which at least 70% of the fragment can be aligned. DNA-DNA hybridization (DDH) has been used to estimate bacterial genome relatedness although DDH is laborious and difficult to

replicate between labs. ANI can be computed using genomes of only draft quality and ANI values correspond well to DDH values (Konstantinidis and Tiedje 2005; Richter and Rosselló-Móra 2009). Computation of ANI among *S. marcescens* strains used in previous studies would validate the relationships determined using MLSA, rep-PCR, and DDH. In addition, whole genome *S. marcescens* sequences available in the NCBI genome database can be included in genomic analyses.

Questions remain on the genetic determinants of pathogenicity that separate the CYVD from the non-CYVD *S. marcescens* strains. Common pathogenicity factors among phytopathogenic bacteria include secretion systems and their effectors, various toxins, exopolysaccharides, and siderophores, many of which are regulated by quorum-sensing systems (Abramovitch and Martin 2004; Alfano and Collmer 1996; Pfeilmeier et al. 2016). Based upon assays using the model nematode system, *Caenorhabditis elegans*, virulence factors in human pathogenic *S. marcescens* strains include production of three groups of molecules: hemolysins, siderophores, and lipopolysaccharides, as well as certain extracellular products such as chitinase and structures related to adherence (Kurz et al. 2003; Rodrigues et al. 2006).

Comparative genomics of pathogenic and nonpathogenic strains has become an efficient method of identifying possible pathogenicity factors in bacteria as well as providing insights into evolution. Putative pathogenicity determinants and growth factors have now been found in *Listeria monocytogenes*, *Pseudomonas* spp., *Xanthomonas campestris* pv. *vesicatoria*, and another atypical plant pathogen, *Pantoea ananatis* (De Maayer et al. 2014; Glaser et al. 2001; Loper et al. 2012; Thieme et al. 2005).

The objective of this study was to compare the genomes of CYVD and non-CYVD strains of *S. marcescens* and characterize unique sequences. A second objective was to determine

the genetic relationships between CYVD and non-CYVD strains using ANI, and to compare the results with previous studies that used MLSA and rep-PCR.

Materials and Methods

DNA preparation and sequencing

The DNA of ten *S. marcescens* strains (Table 2.1) was extracted using PureGene Bacto-Yeast Kit B (Qiagen, Hilden, Germany) from bacterial cultures grown in Luria-Bertani (LB) broth for 24 h in an orbital incubator shaker (New Brunswick Scientific I26) at 28°C. DNA quality was checked on a 0.7% Tris-acetate EDTA (TAE) agarose gel and concentration was determined using an Eppendorf BioSpectrometer (Eppendorf, Hamburg, Germany). At least 300 ng of DNA was suspended in 55 µL Tris-EDTA (TE) buffer and DNA samples were submitted to the Georgia Genomics Facility (University of Georgia, Athens, GA) for NGS DNA library prep. Sequencing was performed using an Illumina MiSeq PE250 platform with an insert size of 550 bp and targeted sequence coverage of greater than 30.

Sequence assembly and annotation

Raw sequence data was assessed for quality with FASTQC version 0.11.4 (Andrews 2010). Ends of sequenced reads with a Phred quality score below 20 and common synthetic sequence adapter sequences were trimmed with Trimmomatic version 0.32 (Bolger et al. 2014). Strains were assembled *de novo* using SPAdes version 3.10.0, a prokaryotic genome-specific assembler program based upon construction of de Bruijn graphs using automated k-mer sizes (Bankevich et al. 2012). The de Bruijn graphs are constructed based upon the overlap of sequences of various sizes, also known as k-mers. The contigs in the assemblies of highest

quality were then ordered using the MAUVE Contig Mover tool against the reference genome *S. marcescens* WW4 (accession number: CP003959.1) from the NCBI Genome database. The ordered contigs were annotated with the Rapid Annotation using Subsystem Technology (RAST) pipeline (version 2.0) which utilizes algorithms to identify protein-encoding and rRNA and tRNA predicted coding sequences (CDS), and assigns functions to the CDS in the genome based upon model bacterial systems (Aziz et al. 2008; Overbeek et al. 2014).

Genome alignment and characterization

The draft genomes of R01-A, P1, SB01, and Z07 were aligned using progressiveMAUVE and examined for sequence differences (Darling et al. 2010). Regions with low similarity between CYVD strains and R01-A were estimated for size and location within the genomes. The CDS found only in the three CYVD strains or in the non-CYVD strain R01-A were determined by the annotations provided by RAST. Protein sequences of these CDS were compared using the RAST Seed Viewer to visualize the relative size of insertions and deletions in relation to the whole genome (Overbeek et al. 2014).

Genome sequences of the three CYVD strains and R01-A were searched using effectiveDB for CDS similar to known sequences for type III and type IV secretion systems and effectors (Eichinger et al. 2016) . For type VI systems, a BLAST search was conducted with a *VgrG* protein sequence from *Vibrio cholerae* against the *S. marcescens* genomes. Homology of additional *S. marcescens* genes in insertion or deletion regions was determined using NCBI BLAST.

Characterization of CYVD strain SB03

The draft genomes of SB03, P1, SB01, and Z07 were aligned using progressiveMAUVE and examined for sequence differences (Darling et al. 2010). Regions with low similarity between the four CYVD strains were estimated for size and location within the genomes. Annotation files for the unique regions in SB03 were examined to determine what predicted coding sequences (CDS) were present. SB03 was aligned with the type strain ATCC13880 (NCBI accession number NZ_JMPQ00000000.1) to determine which unique regions found in SB03 were shared with ATCC13880.

Phylogeny based upon average nucleotide identity

The sequences of the ten *S. marcescens* strains sequenced in this study (Table 2.1) and eight other *S. marcescens* strains (Table 2.2) from the NCBI genome database, including strains used in previous CYVD genetic studies, were submitted to the web-based ANI-Matrix (Kostas lab, Georgia Institute of Technology, Atlanta, GA) located at http://enve-omics.ce.gatech.edu/g-matrix/. ANI uses a pairwise analysis between each strain based upon open reading frames (ORFs) that share at least 30% sequence identity and for which at least 70% of the fragment can be aligned. Two phylogenies based upon ANI were generated using an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis: One with all 18 *S. marcescens* strains and another using only the eight CYVD strains. Trees were exported to MEGA version 7.0.26 for editing.

Results

Genome assembly and annotation

The ordered, annotated contigs of four CYVD strains P1, Z07, SB01 and SB03 (Table 2.3) produced draft genomes ranging from 4.47 Mb to 4.60 Mb. The genome of strain SB03 was 33 Kb larger than the genome of the next largest strain, P1 (Table 2.3). The CYVD strains showed a reduction in genome size by 0.78 to 0.91 Mb compared to the 5.38 Mb rice endophyte R01-A. The CYVD-strain genomes were the smallest among the *S. marcescens* strains examined, with the next smallest genome in the NCBI database belonging to the 4.86 Mb strain FG194 (NCBI accession CP003942.1), an isolate from a leaf-cutter ant fungus garden. GC content of CYVD (59.3%) and R01-A (59.2%) genomes was within 0.1% and both were within the 58.3-60.2% GC content typical of *S. marcescens*. The CYVD strains had between 4,383 and 4,423 CDS, while the larger R01-A had 5,001 CDS.

Genome alignment and characterization

Comparison of the genomes of CYVD strains P1, Z07, and SB01 with the rice endophyte R01-A revealed 37 unique regions ranging from 749 to 46,890 bp shared by all three CYVD strains but not with R01-A (Figures 2.1 and 2.2). The larger unique CYVD regions were predominated by phage-related CDS. Eight of the insertions contained phage-related elements, two insertions had CDS for the production of a surface layer protein and adhesins, at least four insertions contained elements related to polysaccharide synthesis, three insertions contained toxin-antitoxin systems such as Toxin A and *HicAB*, one insertion contained CDS for hemolysin activation and secretion, and another contained elements for the efflux of homoserine lactones

(Figure 2.3). The remaining 18 unique sequences contained CDS for metabolism and antibiotic resistance, or the function could not be determined.

An additional 119,310 bp of sequences unique to the CYVD strains were placed at the end of the genome following contig reordering against the reference *S. marcescens* strain. Within this region is a cluster 27,793 bp in length of *fim* adhesion proteins as well as two sequences related to enterobactin exporter *EntS*, a siderophore, an additional *HicAB* toxin cassette, a putative exported protein YPO2521, several proteins of unknown function, and several plasmid elements such as plasmid partitioning protein *ParA*, *RecA*, resolvase and *RlgA*, and a DNA nicking endonuclease *TraR*. The GC content of this region is considerably lower than the rest of the genome at 48.8%.

Seventy-three unique regions were found in the non-CYVD strain R01-A compared to CYVD strains with sizes ranging from 587 to 74,171 bp (Figures 2.4 and 2.5). The R01-A unique sequences consist mainly of long sequences related to metabolism, siderophore production, and surface structures. Based upon CDS annotations, 20 of these regions are involved in the metabolism of amino acids or carbon sources, four regions are involved in polysaccharide production and 16 regions have surface features such as membrane proteins, fimbriae, and a type VI secretion system (Figure 2.5). Twelve of these regions contained hypothetical proteins of unknown function.

Neither the CYVD strains or R01-A possess sequences that share high similarity to known coding sequences for type II secretion systems (T2SS), type III secretion systems (T3SS), or type IV secretion systems (T4SS). All three CYVD strains appear to have one gene cluster 17.8 kb or more in length consisting of at least 14 CDS related to production of a type VI secretion system (T6SS) and include type VI-associated genes such as *vgrG*, *clpB*, *impABCDGH*

proteins surrounded by hypothetical proteins. This cluster is present in 17 other *S. marcescens* strains in the NCBI database. A second ~26.1 kb putative T6SS gene cluster was found in R01-A and contained at least 22 CDS sequences including *impABCDEFGHIJKM* proteins, a serine/threonine protein kinase, *vgrG*, *clpB*, and several additional proteins related to the T6SS. This larger gene cluster is conserved among 28 other *S. marcescens* strains in the NCBI database. The 17 *S. marcescens* strains from the NCBI database that contain the smaller T6SS gene cluster, also possess similar sequences to the larger, more complete T6SS gene cluster found in R01-A. CYVD strains are the only strains possessing the smaller T6SS gene cluster but not the larger T6SS gene cluster.

Characterization of CYVD strain SB03

Comparisons between the sequences of CYVD strains P1, Z07, SB01 and SB03 revealed five regions found in SB03 ranging from 1,148 to 8,429 bp that were not found in the three other CYVD strains (Table 2.4). Of these sequences, two regions: a 8,429 bp region containing CDS related to pyruvate and sulfur metabolism and a 1,148 bp region containing CDS of unknown function, were shared with the *S. marcescens* type strain ATCC13880. The three remaining unique regions in SB03 contain CDS related to: production of a type IV pilus and chitin utilization, CDS of unknown function, and no CDS. An additional 25,964 bp of unique, mostly phage-related CDS, were placed at the end of the SB03 genome following contig reordering against the reference *S. marcescens* strain. None of this region was shared with ATCC13880.

Phylogeny based upon average nucleotide identity

ANI among all CYVD strains (Figures 2.7 and 2.8) was very high (>99.97%). CYVD strains were most closely related to the plant endophytic strains R01-A and 90-166 with average ANI values of 98.2% and 97.7%, respectively (Figure 2.9). CYVD strains shared ANI values of ~95% with strains ATCC 13880, B3R3, W11, and WW4. Strains 1274, AS1, and Ano2 had ANI values below the generally accepted species level cutoff of 95% compared to CYVD strains and other non-CYVD strains evaluated, indicating these strains may belong to a separate species (Figure 2.7).

Discussion

All of the sequenced CYVD strains had reduced genomes of approximately 0.8 Mb when compared with the rice endophyte R01-A. Many of these deletions include CDS related to metabolism, such as arginine, ornithine, and other carbon sources, and may explain some of the reduced capacity of CYVD strains to utilize carbon resources as shown by Rascoe et al. (2003) Genome reduction is common among highly specialized organisms, particularly pathogenic organisms (Merhej et al. 2013; Moran 2002). Loss of genes no longer needed due to niche specialization can improve ecological fitness. In some cases, antivirulence factors are also lost, resulting in a nonpathogenic organisms becoming pathogenic (Merhej et al. 2013). Further investigation of the regions missing from CYVD strains may provide insight into the evolution of pathogenicity, including the loss of antivirulence genes involved in regulation of metabolism, biofilm synthesis, and lipopolysaccharide modification (Bliven and Maurelli 2012).

The unique CYVD regions contain potential pathogenicity factors including siderophores, polysaccharides, toxins, and certain adhesins. However, some of these sequences,

such as one insertion containing at least three CDS related to polysaccharide synthesis, seem to be shared by numerous *S. marcescens* strains. The absence of these sequences in strain R01-A may be due to host specialization of the rice endophyte. CYVD strains encode four unique toxins: two *VapBC* cassettes, toxin A and one *HicAB* cassette in the genome island region. *VapBC* cassettes are found in R01-A, but more pairs are present in CYVD strains. Multiple copies of the *VapBC* cassette are found in other bacterial species and the cassette is thought to function as a post-transcriptional regulator of RNA due to its RNA-cleaving properties (McKenzie et al. 2012). *VapBC* is not known to serve any function in bacterial virulence. Toxin A was found in many human pathogenic and insect-associated *S. marcescens* strains in the NCBI genome database although the function of this toxin is not defined in the literature.

Other CDS, such as those related to hemolysins, may contribute to virulence despite the typical association of hemolysins with human pathogenic *S. marcescens* strains (Hertle et al. 1999; Kurz et al. 2003). Hemolysins contributed to virulence in a model system using *Arabidopsis thaliana* (Rahme et al. 2000). Hemolysins assist in iron-acquisition and may play a role as siderophores in plant-associated bacterium. CYVD strains also contain two genes related to homoserine lactone efflux. Other *S. marcescens* strains use homoserine lactones via quorum sensing to regulate adhesion and the production of several extracellular products such as prodigiosin, polysaccharides, and proteases (Bakkiyaraj et al. 2012; Labbate et al. 2007). The additional homoserine lactone efflux genes in CYVD strains may be regulating cellular functions important for colonization and pathogenicity.

Several insertion regions in CYVD strains may assist in colonizing squash bugs including CDS for lipopolysaccharide biosynthesis protein WzxC, an O-antigen acetylase, and a galactosephosphotransferase. All of these sequences are only found in one other *S. marcescens*

strain, AS1, which was isolated from a mosquito gut. A single surface layer protein was also found with similarity both to strain ASI and an isolate from a pine wilt disease-associated nematode. Polysaccharides function in biofilm formation and may allow bacteria to more effectively colonize their insect hosts (Limoli et al. 2015).

Based upon the draft genomes of the CYVD strains and R01-A, no type II (T2SS) or type III secretion systems (T3SS) are present, which is further supported by the inability of CYVD strains and R01-A to elicit an HR response on tobacco (data not shown). T3SS and T3SS effectors, which are common among phytopathogenic bacterium, are infrequently found in *S. marcescens*, and are believed to be acquired through horizontal gene transfer (Li et al. 2015). However, type VI secretion systems are present in CYVD strains and R01-A. T6SS and T6SS effectors are employed for anti-eukaryotic or anti-bacterial competition and are shown to contribute to virulence in *Pantoea ananatis*, a pathogen of onion and pineapple, and *Ralstonia solanacearum*, causal agent of bacterial wilt of solanaceous crops and others, and *Erwinia amylovora*, causal agent of fire blight (Shyntum et al. 2015; Tian et al. 2017; Zhang et al. 2014)

Li et al. (2015) found at least three variations of Type VI secretion systems (T6SS), termed T6SS-a, T6SS-b, and T6SS-c, in *S. marcescens* strain FS14. Unlike *S. marcescens* FS14, the T6SS-related gene cluster in CYVD strains and R01-A are not surrounded by mobile DNA elements, and do not appear to be horizontally-acquired. The larger T6SS-related gene cluster found in R01-A, but not CYVD strains, was believed to be conserved in *S. marcescens* and has a protein arrangement most similar to T6SS-a, and only differs in the number of hypothetical proteins, which was variable in the strains looked at by Li et al. (2015). The smaller of the T6SS-related gene clusters is most similar to T6SS-b, but does not appear to have a *TssM* (*ImpL*) protein on the end of the cluster. The *TssA* through *TssM* proteins are considered core

components of the T6SS (Zoued et al. 2014). Without the most critical of these components: *TssJ*, *TssL*, and *TssM*, the T6SS may not be fully functional (Felisberto-Rodrigues et al. 2011; Ma et al. 2009). The loss of *TssM* from the T66S-b gene clusters of CYVD strains and R01-A may have resulted in loss of function; however, the uncharacterized hypothetical proteins at the end of the gene cluster could mimic the ATPase and energizing activity of *TssM* (Ma et al. 2009). *S. marcescens* utilizes T6SS to target bacterial competitors (Murdoch et al. 2011). By enabling bacteria to outcompete competitors, T6SS could be considered a virulence factor and a contributor to pathogenesis although some T6SS secretion systems have also been shown to target eukaryotic hosts (Coulthurst 2013). In most other plant pathogenic bacteria, T6SS only slightly contributes to virulence (Kamber et al. 2017; Mattinen et al. 2008).

T6SS-b was found in both CYVD strain P1 and non-CYVD strain R01-A and, if functional, may be used for competition and defense rather than pathogenicity. The absence of T6SS-a in CYVD strains may be another example of genome reduction due to specialization. Since CYVD strains inhabit either the plant phloem or the squash bug, they may have lost genes that contribute to competitive success in the environment including T6SS-a which is utilized for inter-bacterial competition (Murdoch et al. 2011). Further work is needed to determine the function of these T6SS, particularly T6SS-b.

The genome island containing a type I fimbriae gene cluster located by Zhang et al. (2005) using suppressive subtractive hybridization was placed at the end of the genome sequences in this study. Zhang et al. (2005) determined that the region was likely of chromosomal origin rather than of plasmid origin. Mobile elements and a lower GC content in this region point to a possible horizontal acquisition. This region contains two enterobactin *EntS* siderophores that are unique to CYVD strains based on a BLAST search. The region also

contains an additional *HicAB* toxin-antitoxin cassette. While bacteriostatic, this cassette has not been shown to contribute to virulence in *Pseudomonas aeruginosa* (Li et al. 2016). Luo (2007) showed that type 1 fimbriae deficient mutants of a CYVD strain had reduced virulence. This group of fimbriae genes appears in three other *S. marcescens* strains in the NCBI genome database: two human pathogenic strains and one strain isolated from *Agave sisalana*.

Earlier phylogenetic analyses detected little to no genetic difference between CYVD strains, but consistently placed a pair of rice endophytes, R01-A and R02-A, as the most closely related non-CYVD strains (Rascoe et al. 2003; Zhang et al. 2003). These relationships were confirmed by a later study (Besler and Little 2017) which also found some genetic differences between SB03, a CYVD strain isolated from a squash bug, and the remaining CYVD strains. Average nucleotide identity (ANI) revealed few differences among CYVD strains although the more distant relationships previously determined using MLSA between CYVD strains and non-CYVD *S. marcescen*s strains by Besler and Little (2017) were similar to the results of the ANI analysis. Based upon ANI values, the non-pigmented, plant-associated 90-166, R01-A, and CYVD strains all form a clade separate from other members of the species. ANI between the CYVD clade and the other more distantly related *S. marcescens* strains is close to or below the 95% level commonly used for determining different species. As more *S. marcescens* strains are sequenced and added to the NCBI genome database, taxonomy within *S. marcescens* may be reevaluated and the CYVD clade may become a separate species.

ANI works best with complete genomes (Konstantinidis and Tiedje 2005), and may not be sensitive enough to differentiate closely related strains. The genetic differences determined in this study between SB03 and the other CYVD strains are mainly due to five sequence insertions totaling 16.2 kb which were not utilized in ANI analysis. SB03 is the largest genome among the

CYVD strains, and previous studies have shown a slight reduction in virulence (Besler 2014). Unique areas in SB03 may be more ancestral regions that have not yet been lost during genome reduction. Similarities to ATCC1388, shown in previous studies (Besler and Little 2017), were largely due to two insertions containing CDS of unknown function. However, smaller base pair changes are likely present as well. Finding smaller, more hypervariable regions within the genome that are still conserved among CYVD strains may better define genetic variability among CYVD strains.

Further insights into the origins and diversity of CYVD strains can be achieved with further analyses of these and additional CYVD strain sequences. Sequencing using primers designed to cover the variable regions found in this study would allow for validation and characterization of the unique insertions and deletions in CYVD strains. Based on the lack of T2SS, T3SS, and T4SS, CYVD strains likely rely on a novel pathogenicity mechanism that may have evolved during niche specialization and genome reduction. The results of this genomic analysis challenge the current taxonomy of *S. marcescens* and support the wide genetic differences previously determined using DDH. CYVD strains possess the smallest genomes of any of the *S. marcescens* strains available in the NCBI genome database, and the genetic differences calculated using ANI may support the separation of CYVD and related strains from *S. marcescens*.

Literature Cited

Abramovitch, R. B., and Martin, G. B. 2004. Strategies used by bacterial pathogens to suppress plant defenses. Curr. Opin. Plant Biol. 7:356-364.

Alfano, J. R., and Collmer, A. 1996. Bacterial pathogens in plants: Life up against the wall. Plant Cell 8:1683-1698.

Andrews, S. 2010. FastQC: a quality control tool for high throughput sequence data.

Avila, F. J., Bruton, B. D., Fletcher, J., Sherwood, J. L., Pair, S. D., and Melcher, U. 1998. Polymerase chain reaction detection and phylogenetic characterization of an agent associated with yellow vine disease of cucurbits. Phytopathology 88:428-436.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G. D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., and Zagnitko, O. 2008. The RAST Server: Rapid Annotations using Subsystems Technology. BMC Genomics 9:75-75.

Bakkiyaraj, D., Sivasankar, C., and Pandian, S. K. 2012. Inhibition of quorum sensing regulated biofilm formation in *Serratia marcescens* causing nosocomial infections. Bioorg. Med. Chem. Lett. 22:3089-3094.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., and Pevzner, P. A. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19:455-477.

Besler, K. R. 2014. Epidemiology and management of cucurbit yellow vine disease, and characterization of the causal agent *Serratia marcescens*. University of Georgia, Athens, GA.

Besler, K. R., and Little, E. L. 2017. Diversity of *Serratia marcescens* strains associated with cucurbit yellow vine disease in Georgia. Plant Dis. 101:129-136.

Bliven, K. A., and Maurelli, A. T. 2012. Antivirulence genes: insights into pathogen evolution through gene loss. Infect. Immun. 80:4061-4070.

Bolger, A. M., Lohse, M., and Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114-2120.

Bruton, B., Mitchell, F., Fletcher, J., Pair, S., Wayadande, A., Melcher, U., Brady, J., Bextine, B., and Popham, T. 2003. *Serratia marcescens*, a phloem-colonizing, squash bug-transmitted bacterium: Causal agent of cucurbit yellow vine disease. Plant Dis. 87:937-944.

Coulthurst, S. J. 2013. The Type VI secretion system – a widespread and versatile cell targeting system. Res. Microbiol. 164:640-654.

Darling, A. E., Mau, B., and Perna, N. T. 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One 5:e11147.

De Maayer, P., Chan, W. Y., Rubagotti, E., Venter, S. N., Toth, I. K., Birch, P. R. J., and Coutinho, T. A. 2014. Analysis of the *Pantoea ananatis* pan-genome reveals factors underlying its ability to colonize and interact with plant, insect and vertebrate hosts. BMC Genomics 15:1-14.

Eichinger, V., Nussbaumer, T., Platzer, A., Jehl, M. A., Arnold, R., and Rattei, T. 2016. EffectiveDB--updates and novel features for a better annotation of bacterial secreted proteins and Type III, IV, VI secretion systems. Nucleic Acids Res. 44:D669-D674.

Felisberto-Rodrigues, C., Durand, E., Aschtgen, M.-S., Blangy, S., Ortiz-Lombardia, M., Douzi, B., Cambillau, C., and Cascales, E. 2011. Towards a structural comprehension of bacterial type VI secretion systems: Characterization of the TssJ-TssM complex of an *Escherichia coli* pathovar. PLOS Pathog. 7:e1002386.

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., Berche, P., Bloecker, H., Brandt, P., Chakraborty, T., Charbit, A., Chetouani, F., Couvé, E., de Daruvar, A., Dehoux, P., Domann, E., Domínguez-Bernal, G., Duchaud, E., Durant, L., Dussurget, O., Entian, K. D., Fsihi, H., Portillo, F. G.-D., Garrido, P., Gautier, L., Goebel, W., Gómez-López, N., Hain, T., Hauf, J., Jackson, D., Jones, L. M., Kaerst, U., Kreft, J., Kuhn, M., Kunst, F., Kurapkat, G., Madueño, E., Maitournam, A., Vicente, J. M., Ng, E., Nedjari, H., Nordsiek, G., Novella, S., de Pablos, B., Pérez-Diaz, J. C., Purcell, R., Remmel, B., Rose, M., Schlueter, T., Simoes, N., Tierrez, A., Vázquez-Boland, J. A., Voss, H., Wehland, J., and Cossart, P. 2001. Comparative genomics of *Listeria* species. Science 294:849-852.

Hertle, R., Hilger, M., Weingardt-Kocher, S., and Walev, I. 1999. Cytotoxic action of *Serratia marcescens* hemolysin on human epithelial cells. Infect. Immun. 67:817-825.

Kamber, T., Pothier, J. F., Pelludat, C., Rezzonico, F., Duffy, B., and Smits, T. H. M. 2017. Role of the type VI secretion systems during disease interactions of *Erwinia amylovora* with its plant host. BMC Genomics 18:1-12.

Konstantinidis, K. T., and Tiedje, J. M. 2005. Genomic insights that advance the species definition for prokaryotes. Proc. Natl. Acad. Sci. U. S. A. 102:2567-2572.

Kurz, C. L., Chauvet, S., Andres, E., Aurouze, M., Vallet, I., Michel, G. P., Uh, M., Celli, J., Filloux, A., De Bentzmann, S., Steinmetz, I., Hoffmann, J. A., Finlay, B. B., Gorvel, J. P., Ferrandon, D., and Ewbank, J. J. 2003. Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by *in vivo* screening. EMBO J. 22:1451-1460.

Labbate, M., Zhu, H., Thung, L., Bandara, R., Larsen, M. R., Willcox, M. D. P., Givskov, M., Rice, S. A., and Kjelleberg, S. 2007. Quorum-sensing regulation of adhesion in *Serratia marcescens* MG1 is surface dependent. J. Bacteriol. 189:2702-2711.

Li, G., Shen, M., Lu, S., Le, S., Tan, Y., Wang, J., Zhao, X., Shen, W., Guo, K., Yang, Y., Zhu, H., Rao, X., Hu, F., and Li, M. 2016. Identification and characterization of the HicAB toxin-antitoxin system in the opportunistic pathogen *Pseudomonas aeruginosa*. Toxins 8:113-125.

Li, P., Kwok, A. H. Y., Jiang, J., Ran, T., Xu, D., Wang, W., and Leung, F. C. 2015. Comparative genome analyses of *Serratia marcescens* FS14 reveals its high antagonistic potential. PLoS ONE 10:e0123061.

Limoli, D. H., Jones, C. J., and Wozniak, D. J. 2015. Bacterial extracellular polysaccharides in biofilm formation and function. Microbiol. Spectrum 3:1-30.

Loper, J. E., Hassan, K. A., Mavrodi, D. V., Davis, E. W., II, Lim, C. K., Shaffer, B. T., Elbourne, L. D. H., Stockwell, V. O., Hartney, S. L., Breakwell, K., Henkels, M. D., Tetu, S. G., Rangel, L. I., Kidarsa, T. A., Wilson, N. L., van de Mortel, J. E., Song, C., Blumhagen, R., Radune, D., Hostetler, J. B., Brinkac, L. M., Durkin, A. S., Kluepfel, D. A., Wechter, W. P., Anderson, A. J., Kim, Y. C., Pierson, L. S., III, Pierson, E. A., Lindow, S. E., Kobayashi, D. Y., Raaijmakers, J. M., Weller, D. M., Thomashow, L. S., Allen, A. E., and Paulsen, I. T. 2012. Comparative genomics of plant-associated *Pseudomonas* spp.: Insights into diversity and inheritance of traits involved in multitrophic interactions. PLOS Genet. 8:1-24.

Luo, G. 2007. Phytopathogenicity, virulence factors, and *in planta* movement of *Serratia marcescens* Oklahoma State University, Stillwater, OK.

Ma, L. S., Lin, J. S., and Lai, E. M. 2009. An IcmF family protein, ImpLM, is an integral inner membrane protein interacting with ImpKL, and its walker a motif is required for type VI secretion system-mediated Hcp secretion in *Agrobacterium tumefaciens*. J. Bacteriol. 191:4316-4329.

Mattinen, L., Somervuo, P., Nykyri, J., Nissinen, R., Kouvonen, P., Corthals, G., Auvinen, P., Aittamaa, M., Valkonen, J. P., and Pirhonen, M. 2008. Microarray profiling of host-extract-induced genes and characterization of the type VI secretion cluster in the potato pathogen *Pectobacterium atrosepticum*. Microbiology 154:2387-2396.

McKenzie, J. L., Robson, J., Berney, M., Smith, T. C., Ruthe, A., Gardner, P. P., Arcus, V. L., and Cook, G. M. 2012. A VapBC toxin-antitoxin module is a posttranscriptional regulator of metabolic flux in mycobacteria. J. Bacteriol. 194:2189-2204.

Merhej, V., Georgiades, K., and Raoult, D. 2013. Postgenomic analysis of bacterial pathogens repertoire reveals genome reduction rather than virulence factors. Brief. Funct. Genomics 12:291-304.

Moran, N. A. 2002. Microbial minimalism: Genome reduction in bacterial pathogens. Cell 108:583-586.

Murdoch, S. L., Trunk, K., English, G., Fritsch, M. J., Pourkarimi, E., and Coulthurst, S. J. 2011. The opportunistic pathogen *Serratia marcescens* utilizes type VI secretion to target bacterial competitors. J. Bacteriol. 193:6057-6069.

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Parrello, B., Shukla, M., Vonstein, V., Wattam, A. R., Xia, F., and Stevens, R. 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res. 42:D206-D214.

Pfeilmeier, S., Caly, D. L., and Malone, J. G. 2016. Bacterial pathogenesis of plants: future challenges from a microbial perspective: Challenges in bacterial molecular plant pathology. Mol. Plant Pathol. 17:1298-1313.

Rahme, L. G., Ausubel, F. M., Cao, H., Drenkard, E., Goumnerov, B. C., Lau, G. W., Mahajan-Miklos, S., Plotnikova, J., Tan, M. W., Tsongalis, J., Walendziewicz, C. L., and Tompkins, R. G. 2000. Plants and animals share functionally common bacterial virulence factors. Proc. Natl. Acad. Sci. U. S. A. 97:8815-8821.

Rascoe, J., Berg, M., Melcher, U., Mitchell, F. L., Bruton, B. D., Pair, S. D., and Fletcher, J. 2003. Identification, phylogenetic analysis, and biological characterization of *Serratia marcescens* strains causing cucurbit yellow vine disease. Phytopathology 93:1233-1239.

Richter, M., and Rosselló-Móra, R. 2009. Shifting the genomic gold standard for the prokaryotic species definition. Proc. Nat. Acad. Sci. U. S. A. 106:19126-19131.

Rodrigues, A. P., Holanda, A. R., Lustosa, G. P., Nobrega, S. M., Santana, W. J., Souza, L. B., and Coutinho, H. D. 2006. Virulence factors and resistance mechanisms of *Serratia marcescens*. A short review. Acta Microbiol. Immunol. Hung. 53:89-93.

Shyntum, D. Y., Theron, J., Venter, S. N., Moleleki, L. N., Toth, I. K., and Coutinho, T. A. 2015. *Pantoea ananatis* utilizes a type VI secretion system for pathogenesis and bacterial competition. Mol. Plant-Microbe Interact. 28:420-431.

Thieme, F., Koebnik, R., Bekel, T., Berger, C., Boch, J., Buttner, D., Caldana, C., Gaigalat, L., Goesmann, A., Kay, S., Kirchner, O., Lanz, C., Linke, B., McHardy, A. C., Meyer, F., Mittenhuber, G., Nies, D. H., Niesbach-Klosgen, U., Patschkowski, T., Ruckert, C., Rupp, O., Schneiker, S., Schuster, S. C., Vorholter, F. J., Weber, E., Puhler, A., Bonas, U., Bartels, D., and Kaiser, O. 2005. Insights into genome plasticity and pathogenicity of the plant pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* revealed by the complete genome sequence. J. Bacteriol. 187:7254-7266.

Tian, Y., Zhao, Y., Shi, L., Cui, Z., Hu, B., and Zhao, Y. 2017. Type VI secretion systems of *Erwinia amylovora* contribute to bacterial competition, virulence, and exopolysaccharide production. Phytopathology 107:654-561.

Zhang, L., Xu, J., Xu, J., Zhang, H., He, L., and Feng, J. 2014. TssB is essential for virulence and required for type VI secretion system in *Ralstonia solanacearum*. Microb. Pathog. 74:1-7.

Zhang, Q. 2004. Genetic analysis of *Serratia marcescens*, the causal agent of cucurbit yellow vine disease. Oklahoma State University, Stillwater, OK.

Zhang, Q., Melcher, U., Zhou, L., Najar, F. Z., Roe, B. A., and Fletcher, J. 2005. Genomic comparison of plant pathogenic and nonpathogenic *Serratia marcescens* strains by suppressive subtractive hybridization. Appl. Environ. Microbiol. 71:7716-7723.

Zhang, Q., Weyant, R., Steigerwalt, A. G., White, L. A., Melcher, U., Bruton, B. D., Pair, S. D., Mitchell, F. L., and Fletcher, J. 2003. Genotyping of *Serratia marcescens* strains associated with cucurbit yellow vine disease by repetitive elements-based polymerase chain reaction and DNA-DNA hybridization. Phytopathology 93:1240-1246.

Zoued, A., Brunet, Y. R., Durand, E., Aschtgen, M. S., Logger, L., Douzi, B., Journet, L., Cambillau, C., and Cascales, E. 2014. Architecture and assembly of the type VI secretion system. Biochim. Biophys. Acta 1843:1664-1673.

Tables

Table 2.1. Source, collection year, and geographic origin of *Serratia marcescens* strains sequenced using Illumina MiSeq in this study. CYVD = cucurbit yellow vine disease.

***S. marcescens* strains**	**Source, host**	**Date collected**	**Geographic origin**
R01-A[a]	Rice endophyte	Unknown	Philippines
W11[b]	Watermelon endophyte	2013	US, Georgia
CYVD strains			
P01	Pumpkin	2012	US, Georgia
SB01	Squash Bug	2013	US, Georgia
SB03	Squash Bug	2013	US, Georgia
SB04	Squash Bug	2015	US, Georgia
S02	Yellow Summer Squash	2013	US, Georgia
S07	Yellow Summer Squash	2015	US, Georgia
Z07	Zucchini	2013	US, Georgia
W01-A[a]	Watermelon	1999	US, Texas

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

Table 2.2. Source, accession numbers, and geographic origin of *Serratia marcescens* strains from the NCBI genome database used in this study.

***S. marcescens* strains**	**Accession number**	**Source or Host**	**Geographic origin**
1274	CP019927.2	*Agave sisalana*	Brazil
90-166	NZ_LCWI0000000.1	Cotton Endophyte	Indonesia
Ano2	NZ_MJVC00000000.1	Mosquito Gut	US, Michigan
AS1	CP010584.1	Mosquito Gut	China
ATCC 13880	NZ_JMPQ00000000.1	Pond Water	Unknown
BR3R	CP013046.2	Corn Whorl Rot	China
SM39	AP013063.1	Human	Japan
WW4	CP003959.1	Paper Machine	Taiwan

Table 2.3. Size, number of contigs, and GC content of draft genomes of *S. marcescens* strains used for genome comparisons. Strains were assembled using SPAdes version 3.10.0 unless obtained from the NCBI genome database. GC content and number of coding sequences was predicted following annotation with RAST.

***S. marcescens* Strain**	**Number of Contigs**	**Genome size (bp)**	**Number of Coding Sequences**	**GC Content (%)**
P1	226	4,569,849	4,421	59.3
Z07	219	4,539,744	4,383	59.3
SB01	232	4,468,549	4,419	59.3
SB03	233	4,602,935	4,423	59.3
R01-A	91	5,379,362	5,001	59.2
ATCC13880*	62	5,092,452	4,761	59.8

*Sequence obtained from NCBI Genome database (accession number NZ_JMPQ00000000.1)

Table 2.4. Size and function of predicted coding sequences (CDS) in unique sequences found in CYVD strain SB03 but not in three other sequenced CYVD strains. Unique sequences in SB03 which were shared with ATCC13880 were determined following alignment using MAUVE.

Unique Regions only found in SB03		
Size (bp)	**# of CDS**	**Predicted Function**
2,910	4	Type IV pilus and chitin utilization
2,156	0	N/A
1,735	1	Unknown
Unique Regions in SB03 Shared with ATCC13880*		
Size (bp)	**# of CDS**	**Predicted Function**
8,249	6	Pyruvate and sulfur metabolism
1,148	2	Unknown

*Sequence obtained from NCBI Genome database (accession number NZ_JMPQ00000000.1)

Figures

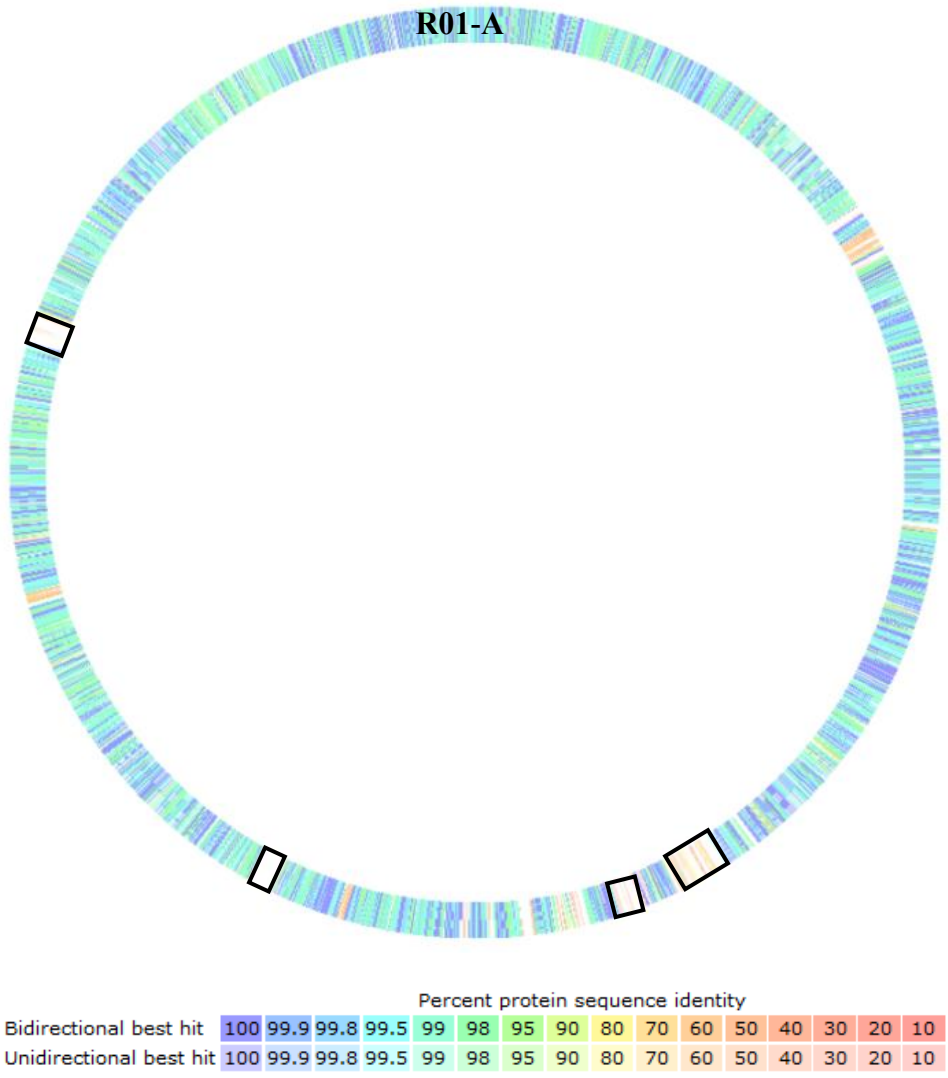


Figure 2.1. Comparison of predicted coding sequences (CDS) in R01-A to CYVD strain P1 based upon protein sequence. Predicted genes in *de novo* assembled genomes of R01-A were compared to P1 using RAST. P1 is used as the reference genome. The ring is constructed based upon CDS from P1 and the colors indicate whether CDS are found in R01-A. Genes of no similarity or low similarity are white or orange/red, respectively, and indicate insertion sequences found in P1 with no or low similarity matches in R01-A. Boxed regions correspond to phage-related insertions.

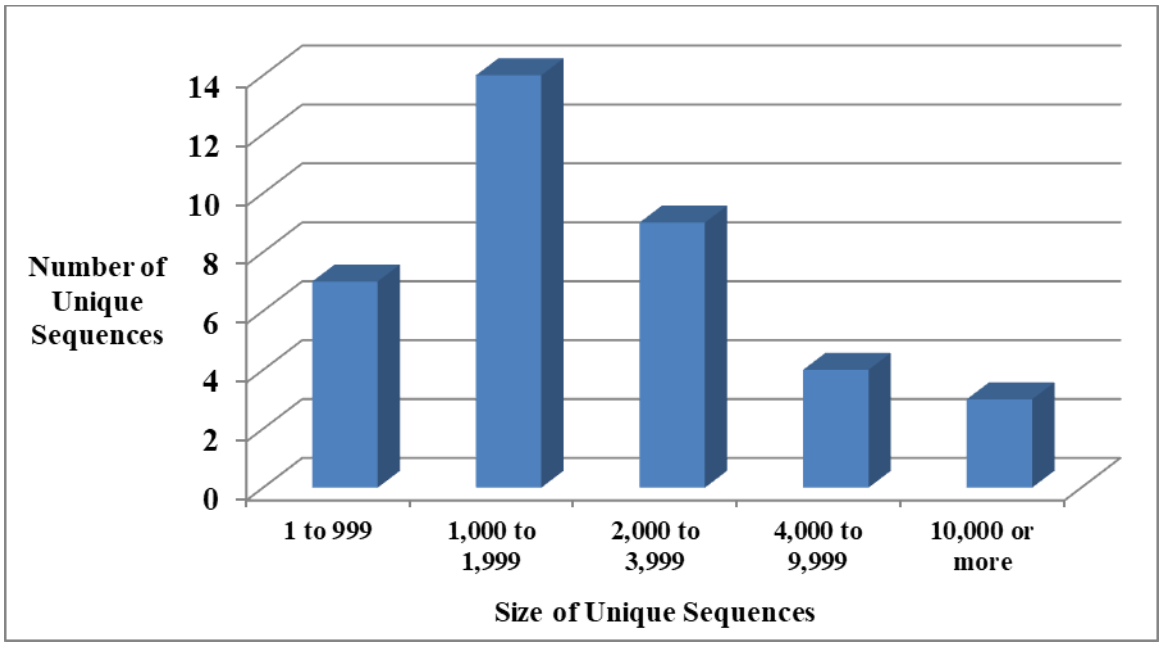


Figure 2.2. Histogram of sizes of sequences unique to CYVD strains. Three CYVD strains: P1, Z07, and SB01 were aligned to the rice endophyte R01-A using MAUVE to determine unique regions. Unique regions were sorted by size.

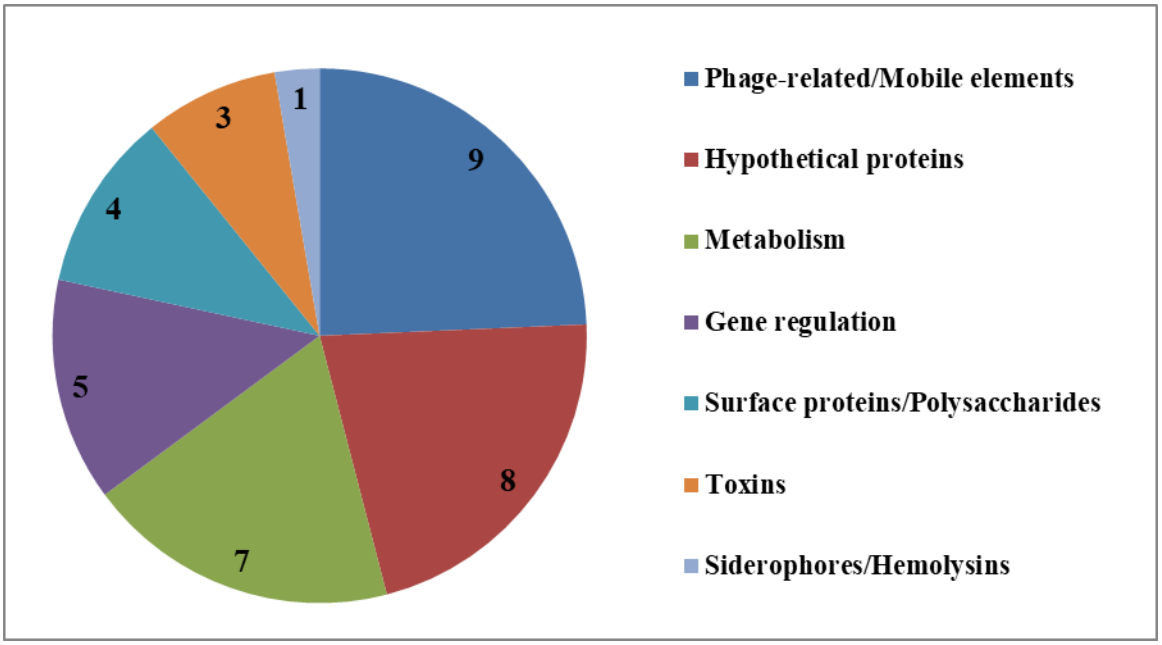


Figure 2.3. Predicted function of coding DNA sequences (CDS) in regions found only in CYVD strains when compared to non-CYVD strain R01-A. Predicted function of CDS was determined by annotation using RAST. Regions containing predicted functions in multiple categories were sorted based on the predicted function of the majority of CDS in the region. Number of unique regions is listed for each category.

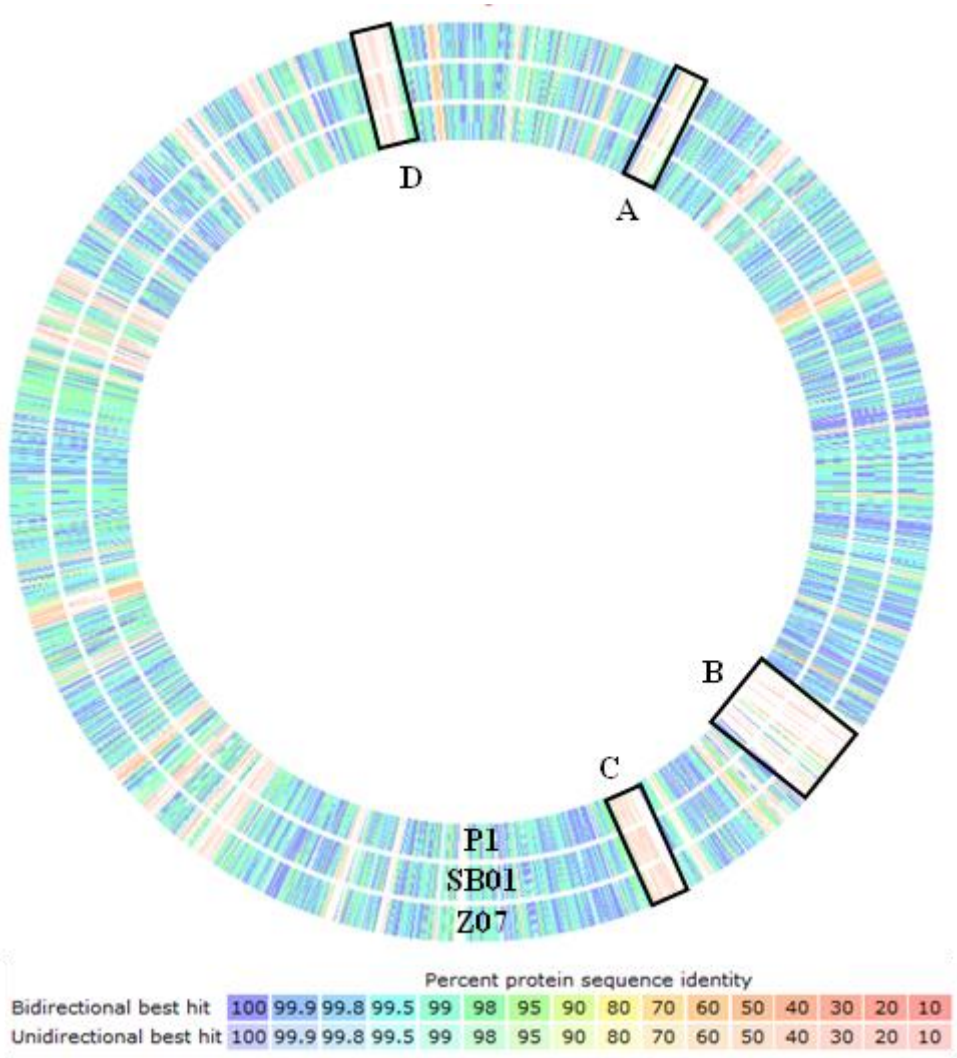


Figure 2.4. Comparison of predicted coding sequences (CDS) in CYVD strains to R01-A based upon protein sequence. Predicted genes in *de novo* assembled genomes of CYVD strains P1 (inner ring), SB01 (middle ring), and Z07 (outer ring) were compared to R01-A using RAST. R01-A is used as the reference genome. The ring is constructed based upon CDS from R01-A and the colors indicate whether CDS are found in CYVD strains. Genes of no similarity or low similarity are white or orange/red, respectively, and indicate sequences only found in R01-A with no or low similarity matches in CYVD strains. Boxed and labeled regions correspond to deletions: (A) type I fimbriae and hypothetical proteins (B) plasmid elements and toxins (C) polysaccharides, metabolism, and outer membrane proteins, (D) metabolism and polysaccharide synthesis.

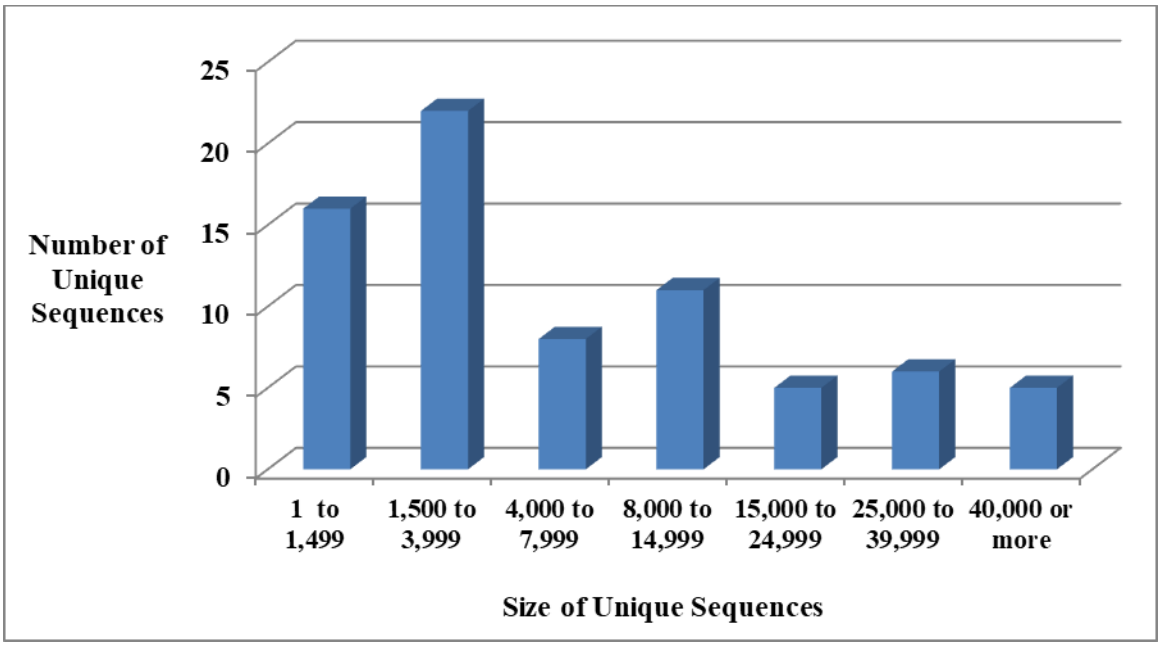


Figure 2.5. Histogram of sizes of sequences unique to R01-A and absent in CYVD strains. Three CYVD strains were aligned to the rice endophyte R01-A using MAUVE to determine unique regions. Size of regions found only in R01-A was determined and regions were sorted by size.

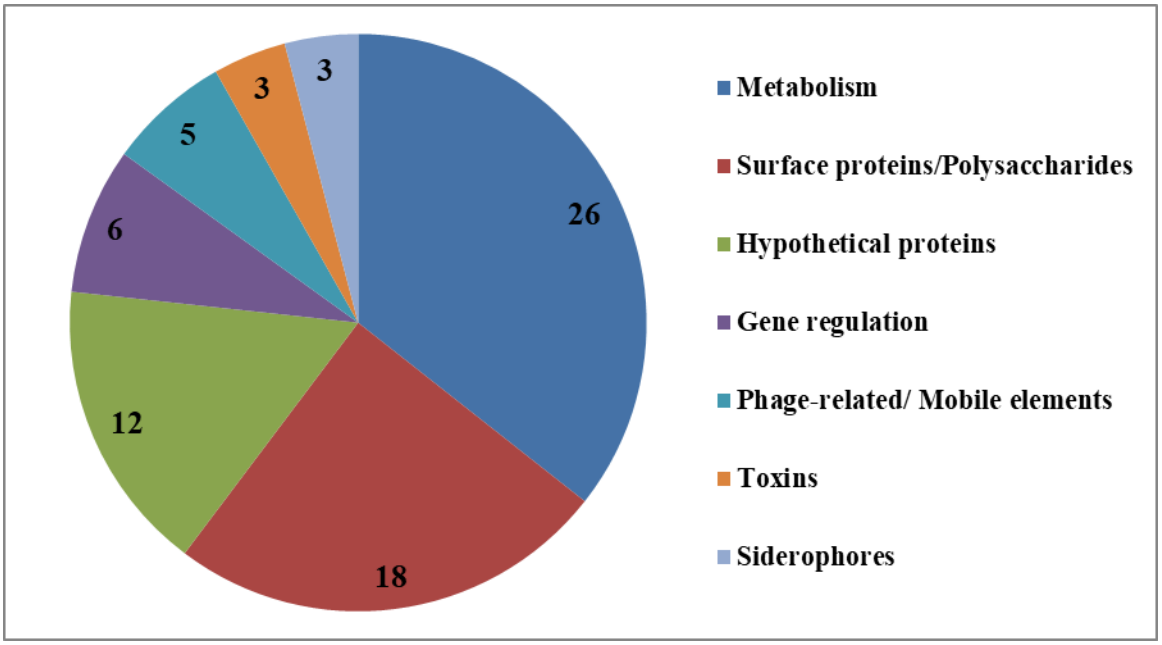


Figure 2.6. Predicted function of coding DNA sequences (CDS) in unique regions in R01-A strains compared to CYVD strains. Predicted function of CDS was determined by annotation using RAST. Unique regions containing predicted functions in multiple categories were sorted based on the predicted function of the majority of CDS in the region. Number of unique regions is listed for each category.

AS1	Ano2	1274	SM39	WW4	W11	B3R3	ATTC13880	R01-A	90-166	W01-A	SB03	SB04	SB01	S07	S02	P1	Z07	
100	100	98.9	96.0	94.6	94.7	94.6	94.9	93.9	93.9	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.5	AS1
100	100	98.9	96.0	94.6	94.6	94.7	94.9	94.0	93.9	94.5	94.4	94.4	94.4	94.4	94.4	94.4	94.4	Ano2
98.9	98.9	100	96.1	94.7	94.6	94.6	94.8	94.0	93.9	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.5	1274
96.0	96.0	96.1	100	95.0	95.0	95.0	95.1	94.1	94.0	94.4	94.5	94.5	94.5	94.5	94.5	94.5	94.5	SM39
94.6	94.6	94.7	95.0	100	99.1	98.2	96.6	95.1	95.1	95.1	95.1	95.1	95.1	95.1	95.1	95.1	95.1	WW4
94.7	94.6	94.6	95.0	99.1	100	98.2	96.6	95.0	95.1	95.1	95.1	95.1	95.1	95.1	95.1	95.1	95.1	W11
94.6	94.7	94.6	95.0	98.2	98.2	100	96.7	94.8	94.9	95.0	95.0	95.0	95.0	95.0	95.0	95.0	95.0	B3R3
94.9	94.9	94.8	95.1	96.6	96.6	96.7	100	94.9	94.8	95.0	95.0	95.0	94.9	95.0	95.0	95.0	95.0	ATCC13880
93.9	94.0	94.0	94.1	95.1	95.0	94.8	94.9	100	98.0	98.2	98.2	98.2	98.2	98.2	98.2	98.2	98.2	R01-A
93.9	93.9	93.9	94.0	95.1	95.1	94.9	94.8	98.0	100	97.7	97.7	97.7	97.7	97.7	97.7	97.7	97.7	90-166
94.5	94.5	94.5	94.4	95.1	95.1	95.0	95.0	98.2	97.7	100	100	100	100	100	100	100	100	W01-A
94.5	94.4	94.5	94.5	95.1	95.1	95.0	95.0	98.2	97.7	100	100	100	100	100	100	100	100	SB03
94.5	94.4	94.5	94.5	95.1	95.1	95.0	95.0	98.2	97.7	100	100	100	100	100	100	100	100	SB04
94.5	94.4	94.5	94.5	95.1	95.1	95.0	94.9	98.2	97.7	100	100	100	100	100	100	100	100	SB01
94.5	94.4	94.5	94.5	95.1	95.1	95.0	95.0	98.2	97.7	100	100	100	100	100	100	100	100	S07
94.5	94.4	94.5	94.5	95.1	95.1	95.0	95.0	98.2	97.7	100	100	100	100	100	100	100	100	S02
94.5	94.4	94.5	94.5	95.1	95.1	95.0	95.0	98.2	97.7	100	100	100	100	100	100	100	100	P1
94.5	94.4	94.5	94.5	95.1	95.1	95.0	95.0	98.2	97.7	100	100	100	100	100	100	100	100	Z07

Color	ANI Range
	96.0% or more
	95.0 to 95.9%
	94.0 to 94.9%
	Less than 94.0%

Figure 2.7. Matrix of average nucleotide identity (ANI) between *Serratia marcescens* genome sequences. ANI was computed from pairwise comparisons of open reading frames (ORFs) which shared at least 30% identity and which could be aligned for 70% of their length using the web-based ANI-Matrix (Kostas lab, Georgia Institute of Technology) located at http://enve-omics.ce.gatech.edu/g-matrix/. Cutoff values for determining members of the same species are most commonly 95 to 96%.

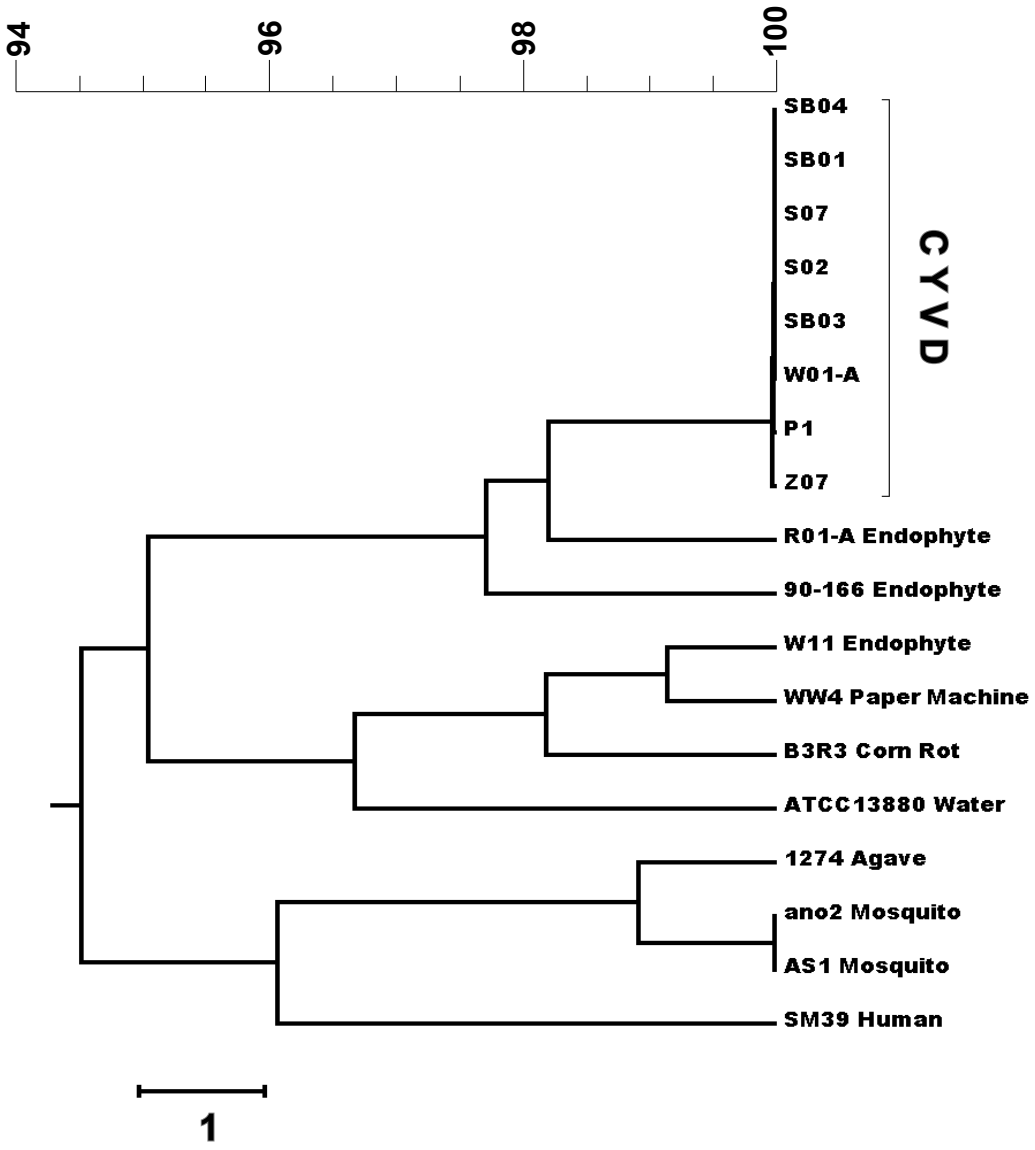


Figure 2.8. Phylogeny of 18 *Serratia marcescens* strains using average nucleotide identity (ANI) of genome sequences. Trees were generated using unweighted pair group method with arithmetic mean (UPGMA). Host or source of strains provided. Branch lengths reflect percent difference in ANI. ANI Values of 95% or more are generally considered of the same species.

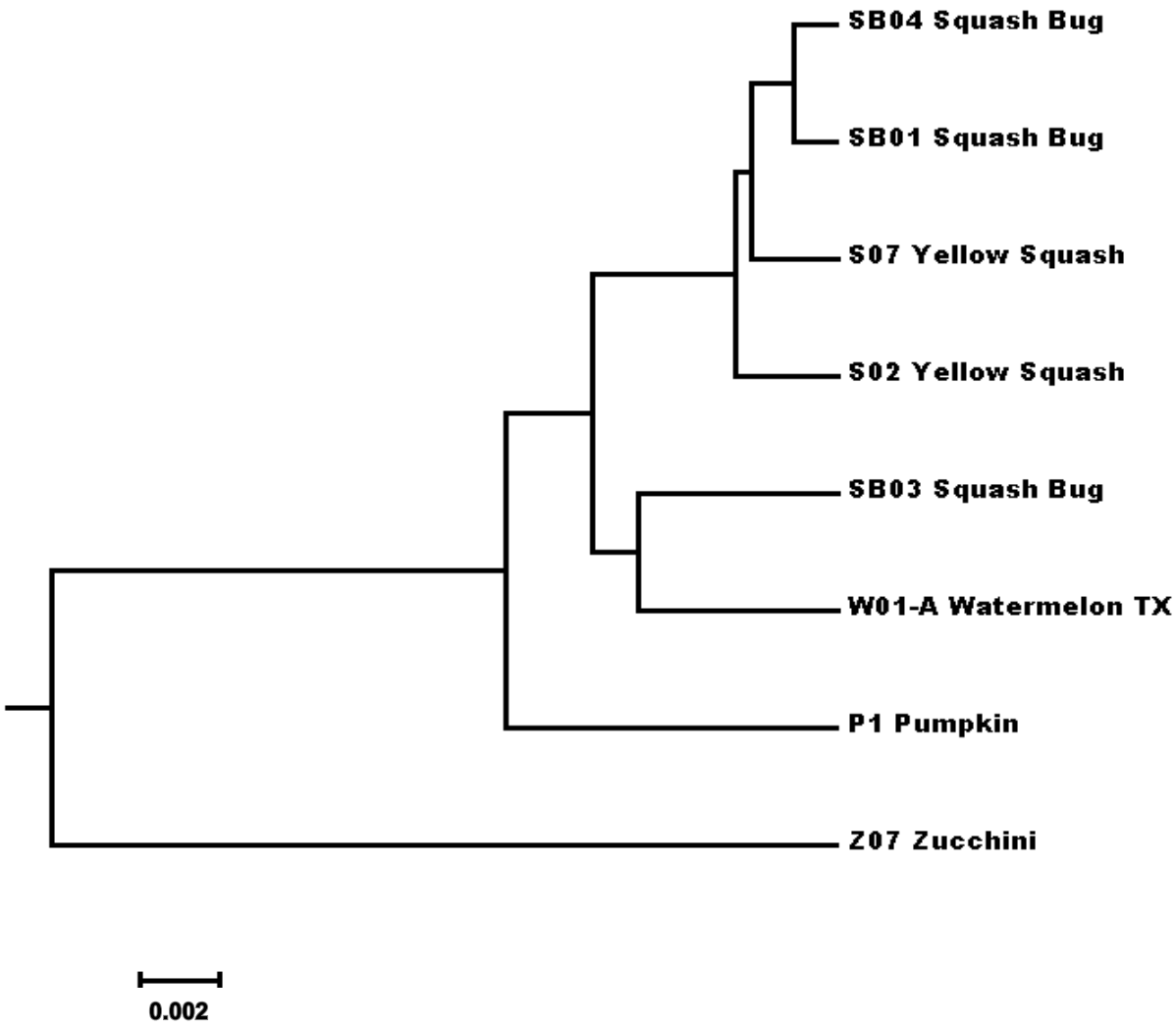


Figure 2.9. Phylogeny of eight *Serratia marcescens* cucurbit yellow vine disease strains using average nucleotide identity (ANI) of genome sequences. Trees were generated using unweighted pair group method with arithmetic mean (UPGMA). Host or source of strains provided. All strains were first isolated in Georgia, USA, unless otherwise noted. Branch lengths reflect percent difference in ANI.

CHAPTER 3

USE OF SQUASH BUG NYMPHS TO INOCULATE SQUASH PLANTS WITH CUCURBIT YELLOW VINE STRAINS OF *SERRATIA MARCESCENS*[2]

[2] Matteen, Z. T. and Little, E. L. 2017. To be submitted to *HortTechnology*.

Abstract

Cucurbit Yellow Vine Disease (CYVD), caused by the bacterium *Serratia marcescens*, causes a yellowing and wilting of cucurbit foliage followed by collapse and death. Two experimental inoculation methods have been used for CYVD: needle inoculation and adult squash bugs. Both methods are hampered by low efficiency and limited expression of disease symptoms. Needle inoculation used in this study yielded similar results to previous studies. An inoculation method was developed using second instar nymphs fed a bacterial culture. Inoculation using nymphs was performed alongside needle inoculation to compare efficacy and symptom expression. After inoculation, plants were planted in the field and covered initially with row covers to exclude insect feeding. CYVD strain P1 was recovered from 60.9-75% of nymphs used to inoculate plants. After eight weeks, lower stems of inoculated plants were examined for phloem browning, and CYVD strains were isolated. Plants in the first trial died from excessive squash bug feeding, but plants inoculated with nymphs in the second and third trial produced phloem browning at levels (33% and 43%, respectively) comparable to needle inoculated plants (57% and 43%, respectively). Symptoms of yellowing or wilting were not associated with plants exhibiting phloem browning. P1 was recovered from 100% of needle inoculated and 57 to 83% of nymph-inoculated plants. Second instar squash bug nymphs were fed the non-pathogenic, pigmented *S. marcescens* strain W11. W11 was recovered from 72.7-100% of fed nymphs and W11 may have potential as a biocontrol agent against CYVD strains within the squash bug.

Introduction

Cucurbit Yellow Vine Disease (CYVD), caused by the bacterium *Serratia marcescens*, causes a yellowing and wilting of cucurbit foliage followed by collapse and death of the entire plant. Internally, CYVD results in a browning of the phloem at the plant crown (Bruton et al. 1998). The pathogen is vectored persistently by squash bugs (*Anasa tristis*) and overwinters in adults (Pair et al. 2004). Squash bugs transmit the bacterium during probing or feeding on the vascular tissues of squash plants (Bruton et al. 2003). The bacterium is not transovarial but will persist through squash bug molting and all of the juvenile instar stages are believed to be capable of transmitting the bacterium (Wayadande et al. 2005). Squash bugs overwinter as adults in soil, leaf litter, old buildings, woodpiles, debris or any other location that will help keep them warm and dry (Adam 2006).

CYVD epidemiology and strain specificity is difficult to study, largely due to the lack of a reliable pathogenicity test. Current pathogenicity tests have low efficacy and usually fail to reproduce full disease symptoms. Two inoculation methods have been used for CYVD: needle inoculation and the use of squash bugs. The needle inoculation method uses fine gauge needles to stab repeatedly through a droplet of bacterial solution into the cotyledons and apical meristem of a young seedling (Bruton et al. 2003). Efficacy using this method varies from 20-60% and symptom expression takes at least six weeks (Besler 2014; Bruton et al. 2003). While Bruton et al. (2003) reported both wilting and phloem browning in CYVD-infected plants with needle inoculation, subsequent greenhouse inoculation studies produced phloem browning but no wilting and plant death (Besler 2014; Luo 2007).

In previous controlled experiments, the use of the squash bug vector to transmit the bacterium resulted in inconsistent CYVD symptom development with low efficiency. In a

greenhouse study (Pair et al. 2004), overwintering adults were collected and single adults were placed on squash plants at either the first true leaf stage or the 3-5 true leaf stage. Adults were left to feed until plants showed signs of stress or died, usually around seven days. The squash bugs were subsequently moved to a new plant. This was repeated so that at least four plants were exposed to a single squash bug. Plants were then grown for four weeks before observation for phloem browning, followed by bacterial isolation and confirmation. Based on the results, an estimated 20% of the collected squash bugs harbored the bacterium and the transmission efficiency for the CYVD bacterium ranged from 11 to 100%. Disease incidence for the two studies was 9.2% and 3.8% for the first true leaf or 3 to 5 true leaf stages, respectively, suggesting younger plants are more susceptible to infection.

In another study, squash bug adults were fed fruit cubes vacuum-infiltrated with a solution of a CYVD strain of *S. marcescens.* These adults were then evenly spaced on fourth true leaf stage plants under a row cover at a density of one adult per plant. At the end of the experiment 17% of plants developed phloem browning and tested positive for *S. marcescens* but only 12% of these plants exhibited full disease symptoms (Bruton et al. 2003). In another study, only 12% of adults, 10% of fifth instars and 2% of third instars that fed upon vacuum-infiltrated squash cubes were able to transmit the pathogen, although only phloem browning was observed in CYVD-positive plants (Wayadande et al. 2005).

Adult squash bugs are more difficult to utilize in these tests than nymphs as they can persist for long periods without feeding and are more damaging to plants, limiting the number of adults and exposure time on young plants. Adults will not feed on liquid diets (Bextine et al. 2003), and therefore must uptake the bacterium through vacuum-infiltrated squash cubes. However, adults may choose not to feed on squash cubes and standardizing the concentration of

the bacterium in fruit cubes is difficult. Nymphs in the second and third instar feed more frequently and may more readily take up a liquid culture than feed on squash cubes. Therefore, the use of second and third instar nymphal stages may result in more consistent results in transmission tests. In addition, young nymphs can be reared and used in larger numbers than adults due to the amount of space and fresh plant material needed over a longer period of time for adults.

The use of natural vectors has been a useful tool for studying insect-pathogen and pathogen-host interactions in many bacterial disease systems. Factors influencing host-pathogen interaction can be more easily studied in diseases which can be inoculated effectively without the use of a vector or for which inoculation with a vector is efficient, such as bacterial wilt of cucurbits (*Erwinia tracheiphila*), Stewart's wilt of corn (*Pantoea stewartii*), Pierce's disease of grape (*Xylella fastidiosa)*, and others (Ammar et al. 2014; Dutta et al. 2014; Sasu et al. 2010; Shapiro et al. 2014). However, the low efficiency, long incubation time, and inability to reproduce full symptoms using current CYVD inoculation methods have limited the ability to study the epidemiology and etiology. This study will compare the needle inoculation method with the use of squash bug nymphs in an effort to develop an improved inoculation method for CYVD.

Materials and Methods

Needle inoculation

Single colonies of *S. marcescens* strains P1, SB03, and R01-A (Table 3.1) were grown in 5 mL Luria-Bertani (LB) broth at 28°C for 18 h in an orbital incubator shaker (New Brunswick Scientific I26). The bacterium was pelleted with centrifugation and suspended in 0.01 M

phosphate buffered saline (PBS). Bacterial density was adjusted to 1 x 10^8 CFU/mL (Eppendorf BioSpectrometer) in PBS. 'New England Pie' pumpkin (*C. pepo*, Johnny's Seeds, ME) seedlings at the expanded cotyledon stage were inoculated by pipetting a 20 µL drop of the bacterial suspension or PBS at the juncture of the apical meristem and cotyledons, pinching together and bending the cotyledons at a 45º angle from the stem, and stabbing 20 times through the cotyledons, the suspension, and the apical meristem with an inoculation fork consisting of five small gauge needles (Figure 3.1). The fork was sterilized for 1 min in 0.83% sodium hypochlorite between treatments. Eight plants each were inoculated with P1, SB03, R01-A (a *S. marcescens* rice endophyte), or PBS. Plants were grown for eight weeks in the greenhouse in three gallon pots. The experiment was repeated twice.

Following eight weeks of growth, plants were examined for wilting or yellowing leaves. A stem piece from 1 cm below the cotyledon scars was surface sterilized in 0.83% sodium hypochlorite for 1 min, washed in sterilized water, and the phloem tissue was examined for discoloration. Two 50 mg pieces of vascular tissue were extracted and macerated using a microtube pestle in a 1.5 mL microcentrifuge tube containing 500 µL of PBS, and the suspension was streaked onto duplicate LB agar plates. Following incubation for three days at room temperature, bacterial colonies with characteristic morphology of *S. marcescens* were transferred to fresh LB plates. Four day old single bacterial colonies were boiled in 500 µL sterilized distilled water for 5 min. The identity of CYVD-strains was confirmed using PCR (Viriti 96-Well Thermal Cycler) and the CYVD strain-specific primers A79F (CCAGGATACATCCCATGATGAC) and A79R (CATATTACCTGATGCTCCTC) (Zhang et al. 2005).

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 using the following cycle: 5 min at 95°C; 35 cycles of 40 s at 95°C, 60 s at 60°C, 90 s at 72°C, and one final extension cycle of 5 min at 72°C. PCR products were electrophoretically separated in a 1.0% agarose gel (GenePure, Bioexpress, Kaysville, UT) with ethidium bromide at 100 µg/L. A sample was considered CYVD positive when a 338 bp fragment was observed.

Comparison of squash bug nymphs and needle inoculation

Squash bug eggs were collected from squash plants at the University of Georgia Horticulture Research Farm (Watkinsville, GA). Eggs attached to collected leaves were gently scraped off with a scalpel into two Magenta boxes (Sigma Aldrich, St. Louis, MO) containing a single folded paper towel dampened with sterilized water. The Magenta boxes were maintained on a 12 h day/12 h night cycle under fluorescent lights and at temperatures between 25ºC and 27ºC until hatching, usually within four to five days. First instars were fed surface-sterilized squash fruit and leaves for three to four days until they reached the second instar stage (Figure 3.2). Food was removed and nymphs were starved for 24 h. A 1 x 10^8 CFU/mL bacterial suspension of CYVD strain P1 was prepared with sterilized water as described above. The bacterial suspension was placed in a parafilm (Bemis Company, Inc., Neenah, WI) -covered 60 x 15 mm petri dish placed in a Magenta box (Figure 3.3). A separate group of nymphs was fed a sterilized water control prepared in a petri dish in the same manner. After exposure to the bacterium or water for 24 h, three nymphs were placed on 'Costata Romanesca' zucchini (*C. pepo*, High Mowing Seeds, VT) seedlings at the one to two true leaf stage for 72 h (Figure 3.4).

Eight plants were exposed to the squash bugs for each treatment. To compare with needle inoculation, eight plants each were needle inoculated with strain P1 or PBS as previously described.

Following feeding, nymphs were removed, surface sterilized in 70% EtOH for 1 min, washed in sterilized water, and macerated in 1.5 mL microcentrifuge tubes containing 1 mL PBS. The macerate was serially diluted to 1:100 in PBS and 100 µL of each dilution was spread on LB plates in duplicate. Following incubation for three days at room temperature, bacterial colonies with characteristic morphology of *S. marcescens* were transferred to fresh LB plates. Four day old single bacterial colonies were boiled in 500 µL sterilized distilled water for 5 min. The identity of CYVD-strains was confirmed using PCR (Viriti 96-Well Thermal Cycler) and the strain-specific primers A79F and A79R as previously described.

Inoculated plants were placed in the greenhouse for two days to recover from the inoculation before being planted in a certified organic plot at the University of Georgia Horticulture Research Farm in Watkinsville, GA. Plants were arranged in a completely randomized design with 46 cm in-row spacing and 3.0 m between rows. NatureSafe 10:2:8 fertilizer (Nature Safe, Cold Spring, KY) was incorporated at a rate of 6.8 kg per 55.7 m^2 before planting. The plants were immediately covered with Agribon+ Ag-15 polypropylene floating row covers (Polymer Group Inc., Charlotte, NC) to exclude squash bugs. 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. The experiment was repeated three times in the summer of 2016 approximately two weeks apart.

Floating row covers were removed after six weeks, and stems and roots were removed from plants at eight to nine weeks after planting. Stem pieces from 1 cm below the cotyledons

were sterilized and phloem tissue was examined for discoloration. Bacteria were extracted and identified as previously described.

Uptake of *S. marcescens strain* W11 by squash bug nymphs

Squash bug eggs were collected from a lab-maintained colony and were reared as previously described until they reached the second instar stage. Food was removed and nymphs were starved for 24 h. A 1 x 10^8 CFU/mL bacterial suspension in sterilized water of the endophytic, red pigmented *S. marcescens* strain W11 was prepared as previously described. Second instar squash bug nymphs that were hatched and reared in separate containers were fed either the bacterial suspension or sterilized water.

After exposure to the bacterium or water for 24 h, nymphs were exposed to squash fruit and leaves for three days. Single nymphs were placed in 1.5 mL microcentrifuge tubes, surface sterilized in 70% EtOH for 30 s, washed in sterilized water, and macerated in 1 mL PBS using a microtube pestle. The macerate was serially diluted to 1:1000 with PBS and 100 µL of each dilution was spread on LB plates in duplicate. Following incubation for three days at room temperature, pigmented *S. marcescens* colonies were enumerated and selected colonies were isolated onto fresh LB plates for identity confirmation as previously described.

Results

Needle inoculation

In trial 1, *S. marcescens* was recovered from eight of eight (100%) plants inoculated with R01-A, six of seven (85.7%) plants inoculated with P1 and seven of eight (87.5%) plants inoculated with SB03. In the second pathogenicity test, *S. marcescens* was recovered from six of

eight (75%) plants inoculated with R01-A, three of eight (37.5%) plants inoculated with P1, and three of eight (37.5%) plants inoculated with SB03 (Table 3.2). PCR confirmed that the bacterial colonies isolated from plants inoculated with P1 or SB03, but not R01-A, were CYVD strains of *S. marcescens*.

No differences were seen in foliar symptoms between inoculated and control plants in pathogenicity tests. In trial 1, three of seven plants (42.9%, one plant died) inoculated with P1 and two of eight (25%) plants inoculated with SB03 showed phloem browning in the lower stem. Plants inoculated with PBS or R01-A showed no phloem browning. In trial 2, two of eight plants (25%) inoculated with P1 and two of eight plants inoculated with SB03 exhibited phloem browning. Plants inoculated with PBS or R01-A showed no symptoms (Table 3.2).

Comparison of squash bug nymphs and needle inoculation

Several squash bug nymphs died during each inoculation attempt, although usually not until the final 24 h of the 72 h inoculation period. Following inoculation, 60.9 to 75% of nymphs were found to carry *S. marcescens* CYVD strains.

Upon removal of the row covers from the first set of inoculated plants, all plants died within a few days due to high heat and heavy squash bug feeding, and no data was collected. Most plants from the second and third set survived, although plant health was negatively affected by downy mildew and hot conditions. Fruit production initiated within a week after removal of row covers. Wilting began at eight weeks after planting in the majority of plants including those not carrying the pathogen.

All of the plants needle-inoculated with P1 were found to carry CYVD strains of *S. marcescens* compared to 57.1% and 83.3% of plants inoculated by nymphs in the second and

third trial, respectively (Table 3.3). Phloem browning was observed in 57.1% and 42.9% of the needle-inoculated plants in the second and third trial, respectively, and 42.9% and 33.3% of plants inoculated by nymphs, respectively. No correlation was seen between number of nymphs carrying the bacterium during feeding and development of CYVD symptoms.

Ability of squash bug nymphs to uptake endophytic *S. marcescens strain* W11

Pigmented *S. marcescens* colonies were recovered from 100% (n=14), 92% (n=25), and 72.7% (n=22) of nymphs in the three replications of the experiment at concentrations ranging from 1 x 10^3 CFU/mg to 4.81 x 10^5 CFU/mg tissue (Table 3.4). No contamination with pigmented *S. marcescens* was seen in nymphs fed only sterilized water. No difference in mortality was seen between bugs that took up W11 or only sterilized water.

Discussion

The use of second instar squash bug nymphs fed a bacterial solution instead of adult squash bugs fed squash cubes was an improved method of inoculating plants with CYVD strains of *S. marcescens* with a transmission efficiency of between 33 and 42%. The highest level reported in previous studies was 10% for nymphs and 17% for adults (Bruton et al. 2003; Pair et al. 2004; Wayadande et al. 2005). In this study, the level of transmission was similar to that achieved with needle inoculations although full symptom expression with either method was not achieved in the greenhouse or field.

The CYVD bacterium, unlike typical vascular bacterial pathogens such as *Ralstonia solanacearum*, causal agent of bacterial wilt of tomato, and *Erwinia tracheiphila*, causal agent of bacterial wilt of cucurbits (Brust 1997; Hayward 1991), can colonize plants endophytically

without causing disease symptoms (Besler 2014; Luo 2007). The endophytic relationship of *S. marcescens* strains with the cucurbit host was shown in this study. When inoculated with the CYVD bacterium, between 25 and 57% of plants developed the phloem browning characteristic of the disease, but the CYVD bacterium was isolated from between 38 and 100% of inoculated plants, meaning even apparently healthy plants were colonized by the bacterium. R01-A, a closely related endophytic strain isolated from rice (Besler and Little 2017; Zhang et al. 2003), did not cause phloem browning in this study but was able to colonize 75 to 100% of plants. CYVD strains are closely related to this plant endophytic strain and the origins of this pathogen may be as an endophyte that can cause disease symptoms when plants are physiologically stressed. In a previous needle inoculation study, Besler (2014) recovered the CYVD bacterium from plants exhibiting phloem browning at 2.5 cm below the site of inoculation at bacterial concentrations up to 2.0×10^6 CFU/g plant tissue showing that the bacterium not only reproduces in the plant but moves beyond the site of inoculation.

The mechanism for how CYVD strains cause disease symptoms and death in cucurbit hosts is currently unknown. The bacterium must be acquired when the plant is young but symptoms do not develop until onset of fruiting which may indicate that a certain concentration of the bacterium must be reached *in planta* and/or there are contributing stress factors such as continued squash bug feeding and/or channeling of resources to fruit production. Biofilm formation and adhesion, which aid in colonization of hosts, have previously been identified as density-dependent and regulated by quorum-sensing in *S. marcescens* (Bakkiyaraj et al. 2012; Labbate et al. 2007). Another contributing factor to disease may be the production of a phytotoxin. These toxins that are typically associated with disease symptoms are commonly employed among phytopathogens (Graniti 1991; Owens 1969; Scheffer and Livingston 1984).

The needle inoculation method failed to produce the foliar yellowing, wilting, or stunting reported in previous studies (Bruton et al. 2003; Luo 2007). Needle inoculated plants in this study only developed slight phloem browning as reported by Besler (2014). The phloem browning obtained from needle inoculated plants is not as pronounced as in diseased plants in the field. Hence there is potential for false positives, particularly in greenhouse grown plants that typically start declining after around six weeks in three gallon pots. When grown in the field, slightly more plants developed phloem browning (42.9 to 57.1%) than when grown in the greenhouse (25 to 42.9%). Plant growth in the field may elicit better symptom expression and the needle inoculation method could be further optimized to more reliably produce the phloem browning.

Trials using squash bug nymphs also failed to produce full CYVD symptoms, although incidence of phloem browning was comparable to needle inoculation, and more consistent. Full CYVD symptom expression may be dependent on other factors. In natural field infections, progression of wilt symptoms varies from one to several days and yellowing is sometimes not observed. The difficulty in achieving full disease symptoms using artificial inoculation methods may be due to insufficient concentrations of the bacterium being transmitted, not being inoculated in the correct tissues, or a lack of additional stress factors such as continual squash bug feeding. *S. marcescens* could also be part of a disease complex with another organism. Synergistic effects between microbes in inciting disease is well documented (Lamichhane and Venturi 2015; Le May et al. 2009).

Squash bugs readily acquired *S. marcescens* strain W11, a pigmented watermelon endophyte shown to be antagonistic against CYVD strains (Matteen, unpublished data), with 72.7 to 100% of nymphs taking up the bacterium. Interestingly, this is higher than the 60.9 to

75% uptake observed with CYVD strain P1. Colonies of W11 grew faster on LB plates than P1 and may be able to grow to higher concentrations in squash bugs. Additionally, the antimicrobial, red-pigmented compound prodigiosin (Williams 1973) produced by W11 and most *S. marcescens* strains, but not by CYVD strains, may be antagonistic to native squash bug bacteria, and this competitive edge may aid in colonization. The efficient uptake by squash bugs of strain W11, a potential biocontrol agent against CYVD strains, could be used to deliver W11 into plants or to suppress the CYVD bacterium within the squash bug.

Using squash bug nymphs as an inoculation method for CYVD does offer some benefits over other methods. Unlike the needle inoculation method, a lower percentage of asymptomatic plants carried the *S. marcescens* strains, possibly indicating inoculation by nymphs more frequently introduces the bacterium either at the required concentration or location to cause disease. Using nymphs appears to be more efficient than using adults, with a higher percentage of plants exhibiting symptoms. When compared to adults, native bacterial populations in nymphs hatched from eggs are likely lower and would offer less competition to CYVD strains following uptake. Nymphs reared for this study appeared to not survive for more than two days without feeding, whereas adults could go several days or more without plants. Therefore, nymphs have to regularly feed and will readily take up a bacterial solution, while adults may choose not to take up a liquid solution (Bextine et al. 2003).

Inoculation using squash bug nymphs requires optimization both in the uptake method, which yielded 60.9 to 75% of nymphs carrying the bacterium, and in transmission. The parafilm placed over the petri dish, even when thinly stretched, may be difficult for the stylets of small nymphs to penetrate. Placement of a cotton swab or another wicking material through the parafilm into the solution may provide easier access to the solution. While younger nymphs more

readily take up bacterial solutions, transmission studies by Wayadande et al. (2005) showed higher transmission rates in adults (12%), than fifth instar (10%), or third instar (2%) nymphs. Feeding nymphs while young and waiting until they reach an older instar or adult stage may provide better transmission efficiency and symptom expression.

Transmission studies by Bextine et al. (2001) showed the latent period of adults between uptake of the bacterium and transmission to plants to be as little as 1-2 days, although 4-6 days was more common. In this study, nymphs were placed almost immediately on plants following uptake of solutions due to the high mortality of nymphs, but further optimization of the latent and transmission periods may also increase transmission efficiency. The transmission period was limited by the cramped conditions within the 2 L bottle. The use of a larger mesh cage would allow for a longer transmission period and may improve transmission efficiency.

Overall, inoculating with squash bug nymphs provides a new method of transmitting CYVD strains into plants. The nymphs are easier to rear and manipulate, and the bacterial solution is an improvement over vacuum-infiltrated squash cubes. While the efficiency and disease expression could be improved, this method shows potential as a more reliable pathogenicity test which would facilitate future studies on the etiology and epidemiology of CYVD.

Literature Cited

Adam, K. L. 2006. Squash bug and squash vine borer: Organic controls. ATTRA-National Sustainable Agriculture Information Service.

Ammar, E.-D., Correa, V. R., Hogenhout, S. A., and Redinbaugh, M. G. 2014. Immunofluorescence localization and ultrastructure of stewart's wilt disease bacterium *Pantoea stewartii* in maize leaves and in its flea beetle vector *Chaetocnema pulicaria* (Coleoptera: Chrysomelidae). J. Microsc. Ultra. 2:28-33.

Bakkiyaraj, D., Sivasankar, C., and Pandian, S. K. 2012. Inhibition of quorum sensing regulated biofilm formation in *Serratia marcescens* causing nosocomial infections. Bioorg. Med. Chem. Lett. 22:3089-3094.

Besler, K. R. 2014. Epidemiology and management of cucurbit yellow vine disease, and characterization of the causal agent *Serratia marcescens*. University of Georgia, Athens, GA.

Besler, K. R., and Little, E. L. 2017. Diversity of *Serratia marcescens* strains associated with cucurbit yellow vine disease in Georgia. Plant Dis. 101:129-136.

Bextine, B. R., Wayadande, A., Pair, S. D., Bruton, B. D., Mitchell, F., and Fletcher, J. 2001. Parameters of *Serratia marcescens* transmission by the squash bug, *Anasa tristis*. Phytopathology 91:S8.

Bextine, B. R., Wayadande, A., Bruton, B. D., Pair, S. D., Mitchell, F., and Fletcher, J. 2003. Artificial feeding system for the squash bug, *Anasa tristis* (De Geer) (Heteroptera: Coreidae). Southwest. Entomol. 28:63-67.

Brust, G. E. 1997. Differential susceptibility of pumpkins to bacterial wilt related to plant growth stage and cultivar. Crop Protect. 16:411-414.

Bruton, B. D., Fletcher, J., D. Pair, S., Shaw, M., and Sittertz-Bhatkar, H. 1998. Association of a phloem-limited bacterium with yellow vine disease in cucurbits. Plant Dis. 82:512-520.

Bruton, B. D., Mitchell, F., Fletcher, J., Pair, S. D., Wayadande, A., Melcher, U., Brady, J., Bextine, B., and Popham, T. W. 2003. *Serratia marcescens*, a phloem-colonizing, squash bug-transmitted bacterium: causal agent of cucurbit yellow vine disease. Plant Dis. 87:937-944.

Dutta, B., Barman, A. K., Srinivasan, R., Avci, U., Ullman, D. E., Langston, D. B., and Gitaitis, R. D. 2014. Transmission of *Pantoea ananatis* and *P. agglomerans*, causal agents of center rot of onion (*Allium cepa*), by onion thrips (*Thrips tabaci*) through feces. Phytopathology 104:812-819.

Graniti, A. 1991. Phytotoxins and their involvement in plant diseases. Introduction. Experientia 47:751-755.

Hayward, A. C. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 29:65-87.

Labbate, M., Zhu, H., Thung, L., Bandara, R., Larsen, M. R., Willcox, M. D. P., Givskov, M., Rice, S. A., and Kjelleberg, S. 2007. Quorum-sensing regulation of adhesion in *Serratia marcescens* MG1 is surface dependent. J. Bacteriol. 189:2702-2711.

Lamichhane, J. R., and Venturi, V. 2015. Synergisms between microbial pathogens in plant disease complexes: a growing trend. Front. Plant Sci. 6:1-12.

Le May, C., Potage, G., Andrivon, D., Tivoli, B., and Outreman, Y. 2009. Plant disease complex: Antagonism and synergism between pathogens of the Ascochyta blight complex on pea. J. Phytopathol. 157:715-721.

Luo, G. 2007. Phytopathogenicity, virulence factors, and *in planta* movement of *Serratia marcescens* Oklahoma State University, Stillwater, OK.

Owens, L. D. 1969. Toxins in plant disease: Structure and mode of action. Science 165:18-25.

Pair, S. D., Bruton, B. D., Mitchell, F., Fletcher, J., Wayadande, A., and Melcher, U. 2004. Overwintering squash bugs harbor and transmit the causal agent of cucurbit yellow vine disease. J. Econ. Entomol. 97:74-78.

Sasu, M. A., Seidl-Adams, I., Wall, K., Winsor, J. A., and Stephenson, A. G. 2010. Floral transmission of *Erwinia tracheiphila* by cucumber beetles in a wild *Cucurbita pepo*. Environ. Entomol. 39:140-148.

Scheffer, R. P., and Livingston, R. S. 1984. Host-selective toxins and their role in plant diseases. Science 223:17-21.

Shapiro, L. R., Seidl-Adams, I., De Moraes, C. M., Stephenson, A. G., and Mescher, M. C. 2014. Dynamics of short- and long-term association between a bacterial plant pathogen and its arthropod vector. Sci. Rep. 4:4155-4162.

Wayadande, A., Bruton, B., Fletcher, J., Pair, S., and Mitchell, F. 2005. Retention of cucurbit yellow vine disease bacterium *Serratia marcescens* through transstadial molt of vector *Anasa tristis* (Hemiptera: Coreidae). Ann. Entomol. Soc. Am. 98:770-774.

Williams, R. P. 1973. Biosynthesis of prodigiosin, a secondary metabolite of *Serratia marcescens*. Appl. Microbiol. 25:396-402.

Zhang, Q., Melcher, U., Zhou, L., Najar, F. Z., Roe, B. A., and Fletcher, J. 2005. Genomic comparison of plant pathogenic and nonpathogenic *Serratia marcescens* strains by suppressive subtractive hybridization. Appl. Environ. Microbiol. 71:7716-7723.

Zhang, Q., Weyant, R., Steigerwalt, A. G., White, L. A., Melcher, U., Bruton, B. D., Pair, S. D., Mitchell, F. L., and Fletcher, J. 2003. Genotyping of *Serratia marcescens* strains associated with cucurbit yellow vine disease by repetitive elements-based polymerase chain reaction and DNA-DNA hybridization. Phytopathology 93:1240-1246.

Tables

Table 3.1. Sources of *Serratia marcescens* strains used in this study. Strains P1 was isolated from a cucurbit yellow vine disease (CYVD) infected squash plant and SB03 was isolated from a field-collected squash bug. CYVD-strains were confirmed using strain-specific primers A79F and A79R. Non-CYVD strains were confirmed using species-specific primers YV1 and YV4.

***S. marcescens* Strain**	**Source**	**Location of Isolation**	**Pathogenicity**
P1	**Pumpkin**	**Georgia, USA**	**CYVD-strain**
SB03	**Squash Bug**	**Georgia, USA**	**CYVD-strain**
R01-A[a] (IRBG501)	**Rice Endophyte**	**Philippines**	**Non-CYVD strain**
W11[b]	**Watermelon Endophyte**	**Georgia, USA**	**Non-CYVD strain**

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

Table 3.2. Results of pathogenicity test using needle inoculation. For each trial, eight pumpkin (*Cucurbita pepo* 'New England Pie') plants were inoculated with each *S. marcescens* strain: P1, SB03, R01-A, or phosphate buffered saline (PBS). After eight weeks in a greenhouse a cross section of the lower stem was examined for phloem browning. Isolations were made from phloem tissues and bacterial colonies characteristic of *S. marcescens* were confirmed using PCR and strain-specific primers.

CYVD Incidence Using Needle Inoculation		
Inoculum	**Plants Positive for *S. marcescens***	**Plants with Phloem Discoloration**
Trial I		
PBS	**0 of 8 (0.0%)**	**0 of 8 (0.0%)**
R01-A	**8 of 8 (100.0%)**	**0 of 8 (0.0%)**
P1	**6 of 7 (85.7%)**	**3 of 7 (42.9%)**
SB03	**7 of 8 (87.5%)**	**2 of 8 (25.0%)**
Trial II		
PBS	**0 of 8 (0.0%)**	**0 of 8 (0.0%)**
R01-A	**6 of 8 (75.0%)**	**0 of 8 (0.0%)**
P1	**3 of 8 (37.5%)**	**2 of 8 (25.0%)**
SB03	**3 of 8 (37.5%)**	**2 of 8 (25.0%)**

Table 3.3. Results of pathogenicity tests on zucchini (*Cucurbita pepo* 'Costata Romanesca') inoculated using either needle or squash bug nymphs during summer 2016. For each method, eight plants each were inoculated with *S. marcescens* strain P1 or phosphate buffered saline (PBS). The experiment was repeated three times. Inoculated plants were grown in the field under a floating row cover to exclude insects. The row cover was removed at six weeks and plants were assessed at eight weeks. Phloem browning was observed in cross sections of the lower stem. Bacterial colonies characteristic of *S. marcescens* were isolated and identity was confirmed using PCR and strain-specific primers. Plants from Trial 1 died due to excessive squash bug feeding and heat after removal of row covers.

Inoculation Method	Inoculum	Plants positive for *S. marcescens*	Percentage with Phloem Discoloration
Trial 1			
Needle	**P1**	**ND**	**ND**
Needle	**PBS**	**ND**	**ND**
Nymphs	**P1**	**ND**	**ND**
Nymphs	**Sterilized Water**	**ND**	**ND**
Trial 2			
Needle	**P1**	**7 of 7 (100.0%)**	**4 of 7 (57.1%)**
Needle	**PBS**	**0 of 8 (0.0%)**	**0 of 8 (0.0%)**
Nymphs	**P1**	**4 of 7 (57.1%)**	**3 of 7 (42.9%)**
Nymphs	**Sterilized Water**	**0 of 7 (0.0%)**	**0 of 7 (0.0%)**
Trial 3			
Needle	**P1**	**7 of 7 (100.0%)**	**3 of 7 (42.9%)**
Needle	**PBS**	**0 of 7 (0.0%)**	**0 of 7 (0.0%)**
Nymphs	**P1**	**5 of 6 (83.3%)**	**2 of 6 (33.3%)**
Nymphs	**Sterilized Water**	**0 of 7 (0.0%)**	**0 of 7 (0.0%)**

ND=No data taken. Samples were of low quality due to excessive squash bug feeding.

Table 3.4. Number of squash bug nymphs carrying endophytic strain W11 three days after uptake of a prepared culture. Nymphs were starved and fed a standardized culture of strain W11 or a sterilized water control. After three days, nymphs were crushed in PBS, diluted by 1:1000, and plated on to LB. Red colonies typical of W11 were then enumerated.

Number of Squash Bug Nymphs Carrying *S. marcescens* Strain W11 Three Days After Uptake		
Trial	**Inoculum**	
	W11	**Sterilized Water**
1	**14 of 14 (100%)**	**0 of 10 (0%)**
2	**23 of 25 (92.0%)**	**0 of 15 (0%)**
3	**16 of 22 (72.7%)**	**0 of 12 (0%)**

Figures

Figure 3.1. Inoculation fork used for inoculation of cucurbit seedlings with *S. marcescens*. The fork consists of a plastic bacterial loop handle attached to a melted plastic pen cap embedded with five fine-gauge needles.

Figure 3.2. Second instar nymphs (A) and newly molted adult squash bug (B). Squash bugs progress through five instar stages after hatching which last approximately 3, 9, 8, 7, and 9 days depending on temperature, with a total lifespan of 6-8 weeks.

Figure 3.3. Magenta box (Sigma Aldrich, St. Louis, MO) containing a petri dish with a culture of a CYVD *Serratia marcescens* strain. The petri dish is covered with thinly stretched Parafilm (Bemis Company, Inc., Neenah, WI) and is placed on a paper towel dampened with sterilized deionized water. Squash bug nymphs are placed inside on the Parafilm to feed.

Figure 3.4. Squash bug inoculations of plants by second instar nymphs. Three nymphs were placed in the containers on young plants for three days. Plants in six inch pots were covered with 2 L soda bottles that had the bottoms removed and had wire mesh secured with a rubber band over the top. (A) Zucchini seedlings covered with 2 L bottles and wire mesh and (B) Second instar nymphs feeding on seedling.

CHAPTER 4

EVALUATION OF BACTERIAL STRAINS AS BIOCONTROL AGENTS OF CUCURBIT DISEASES[3]

[3] Matteen, Z. T. and Little, E. L. 2017. To be submitted to *Plant Disease*.

Abstract

Studies on biological control of the cucurbit diseases downy mildew (*Pseudoperonospora cubensis*) and cucurbit yellow vine disease (CYVD, *Serratia marcescens*) are limited but may be useful in organic systems. Nonpathogenic *S. marcescens* strains are known to have antagonistic abilities against other microbes due to the production of the antimicrobial pigment prodigiosin. *In vitro* antagonistic assays against CYVD strain P1 were performed using nonpathogenic *S. marcescens* strains. Of the *S. marcescens* strains, W11, a watermelon endophyte, produced the largest zones of inhibition on Luria-Bertani (LB) agar. 'New England Pie' pumpkin (*C. pepo*) seedlings were removed from pots and the roots washed of soil. Roots were then soaked in W11 or PBS solution. The plants were inoculated one cm below the cotyledons using a syringe and needle with either CYVD strain P1 or phosphate buffered saline (PBS). After six weeks of growth in a greenhouse, stem lengths were measured and phloem tissues were sampled at three points along the lower stem and the roots for the presence of *S. marcescens* strains. No significant increase in stem length was observed. W11 was recovered more frequently in roots than stems, but was found at all sampling sites when recovered from the stem. P1 was consistently isolated at the inoculation site and 1 cm below, and inconsistently at 1 cm above. No significant reduction in P1 population size was observed in W11 treated plants. 'Costata Romanesca' zucchini (*C. pepo*) seed was soaked and roots were drenched with a solution of *S. marcescens* W11, *Bacillus mojavensis* RRC101, or PBS and were grown in the field. Plants were evaluated for downy mildew, powdery mildew, and CYVD. No significant difference was seen in treatments other than a 4% reduction in powdery mildew in W11-inoculated plants compared to RRC101-inoculated plants in trial 3.

Introduction

In recent years, there has been increased consumer demand for fresh locally grown fruit and vegetables. This has led to a resurgence of growers for the fresh local market in Georgia and across the country, and many of these growers are using organic practices. However, squash can be difficult to grow in a hot, humid climate. Economically important pests and diseases of squash in the southeastern United States include cucurbit downy mildew (*Pseudoperonospora cubensis*), powdery mildew (*Podosphaera xanthii),* cucurbit yellow vine disease (CYVD, *Serratia marcescens*), squash bugs (*Anasa tristis*), vine borers (*Melittia cucurbitae*), and pickleworm (*Diaphania nitidalis*) (Keinath et al. 2017).

Plant health management in organic systems depends on a systems approach with cultivar choice, site selection, and planting date, together with optimizing soil organic matter and nutrition. Summer squash is often grown either in early summer or fall to avoid the pests, diseases, and high temperatures of July and August (McCreight 2017). The planting of resistant cultivars is an effective disease and pest management strategy although availability of resistant cultivars of winter squash is limited (Elsey 1985; Lebeda and Křístková 1993; McGrath and Staniszewska 1996; Novero et al. 1962).

CYVD, a bacterial disease characterized by yellowing of leaves and wilting of plants, is vectored by the squash bug, and is an emerging disease of squash in Georgia (Besler and Little 2015). Squash bugs utilize piercing-sucking mouthparts to feed on the vascular tissues of cucurbits (Adam 2006). When feeding occurs on young plants, the causal agent of CYVD can be transmitted, although symptoms do not occur until fruiting initiates (Besler 2014). Crop losses from CYVD in organic production in Georgia have ranged from 12 to 93% (Besler and Little 2015). Management of the squash bug will reduce CYVD incidence but without the availability of effective insecticides in organic production growers must use multiple approaches, including

sanitation, crop rotation, early planting, trap cropping, and row covers (Alston and Barnhill 2008; Besler 2014).

Biological control microbes can be effective against a variety of cucurbit diseases (Gafni et al. 2015; Maleki et al. 2011; Postma et al. 2009; Singh and Rao 1995). Selection of biocontrol agents usually involves *in vitro* screening of candidates for antagonistic activity before testing in the field (Pliego et al. 2011). Several studies have investigated the use of *S. marcescens* as a biocontrol candidate for fungal diseases due to chitinase production (Ordentlich et al. 1988; Someya et al. 2000; Someya et al. 2005b), but few studies have been conducted on the potential use of non-pathogenic *S. marcescens* strains for controlling bacterial diseases.

Biological control organisms lessen disease impact by using either a direct, mixed-path, or indirect form of antagonism (Pal and McSpadden Gardener 2006). Direct forms of antagonism include predation and parasitism, such as the nematode bacterial parasite *Pasteuria penetrans* (Chen and Dickson 1998). Mixed-path antagonism involves production of one or more extracellular products such as antibiotics, lytic enzymes, or unregulated waste products such as ammonia (Pal and McSpadden Gardener 2006). The chitinase and prodigiosin production in *S. marcescens* are mechanisms of mixed-path antagonism employed in controlling plant diseases (Ordentlich et al. 1988; Someya et al. 2005a). Indirect mechanisms of antagonism include competition through nutrient acquisition or niche occupation, such as nonpathogenic mutants of *Acidovorax citrulli*, causal agent of bacterial fruit blotch, reducing disease by niche competition with pathogenic strains (Johnson et al. 2011). Another form of indirect antagonism is induction of resistance in host plants through detection of pathogen-associated molecular patterns (PAMP) or phytohormone-mediated induction (Choudhary et al. 2007; Someya et al. 2005a)

Induced resistance can be either induced systemic resistance (ISR) or systemic acquired resistance (SAR). SAR is mediated by salicylic acid, and results in the production of pathogenesis-related proteins such as lytic enzymes, cell wall-reinforcing proteins, or induced localized cell death (Pal and McSpadden Gardener 2006). SAR inducers have been utilized in control of *Phytophthora capcisi* and Cucumber Mosaic Virus (CMV) in squash (Koné et al. 2009; Mayers et al. 2005) ISR is usually mediated through jasmonic acid, ethylene, or a combination of both, and ISR is produced most commonly through application of nonphytopathogenic rhizobacteria (Pal and McSpadden Gardener 2006; Shoresh et al. 2010). ISR inducers, such as *Bacillus subtilis*, have been employed in control of cucurbit powdery mildew (García-Gutiérrez et al. 2013). Studies have shown some *S. marcescens* strains to be able to induce ISR to diseases in non-cucurbit hosts (Lavania et al. 2006; Press et al. 1997; Someya et al. 2002).

This study will test whether potential biocontrol bacteria have any antagonistic activity against CYVD strains of *S. marcescens* using agar plate assays. In addition, greenhouse and field studies will evaluate methods of introducing the biocontrol bacterium into the plant and whether these biocontrol candidates can promote plant growth and/or suppress disease.

Materials and Methods

In vitro antagonism assays

Non-pathogenic *S. marcescens* strains that were isolated from either squash bug or cucurbit hosts were compared to *Bacillus mojavensis* RRC101, a known biocontrol bacterium, and *Acidovorax citrulli* 00-1, the causal agent of bacterial fruit blotch, in an *in vitro* antagonistic assay against CYVD strain P1 (Table 4.1). Bacteria were grown for three days on Luria-Bertani

(LB) agar and single colonies were grown in LB at 28°C for 18 h. Cultures were centrifuged and the pelleted bacterium was resuspended in 0.01 M phosphate buffered saline (PBS). The density of a culture of strain P1 was adjusted to 1 x 10^6 CFU/mL while the other cultures were adjusted to 1 x 10^8 CFU/mL using a spectrophotometer (Eppendorf BioSpectrometer). Twenty five LB agar plates were each spread with 80 µL of the 1 x 10^6 CFU/mL P1 bacterial suspension and dried for 8 h. Five treated plates were each spotted in triplicate with 10 µL of strains W11, ZM01, ZM02, *A. citrulli* 00-1, *Bacillus mojavensis* RRC101, and a PBS control (Figure 4.3). After three days of growth, zones of inhibition from the edge of the spotted colonies were measured.

In vivo testing of biocontrol strain W11

To prepare inoculum, *S. marcescens* strains W11 and P1 were grown for at 28°C for18 h in LB, pelleted, and resuspended in PBS at 1 x 10^8 CFU/mL as previously described. 'New England Pie' pumpkin (*C. pepo*) seedlings at the expanded cotyledon stage were carefully uprooted, roots were washed of soil, and ten seedlings each were placed in large beakers containing either the W11 or PBS solution for 30 min. Following dipping, plants were replanted in labeled four inch pots. After four days, 100 µL of CYVD strain P1 or PBS was injected into the seedlings with a syringe and a needle (PrecisionGlide 30 gauge, Becton, Dickinson and Company, Franklin Lakes, NJ) at 1 cm below the cotyledons.

After four days, seedlings were transplanted into three gallon pots and grown for six weeks in a greenhouse in a completely randomized design. Plants stems were measured from the soil line to the end of the longest shoot. Length was measured in centimeters one day post

inoculation and again at three and six weeks. Growth between time points was calculated. The experiment was repeated three times.

After six weeks, plants were uprooted and trimmed at the third internode. Stems and main roots were sterilized in 0.83% sodium hypochlorite for 1 min and rinsed in sterilized water. Samples at the point of inoculation, 1 cm above and below the inoculation site, and from the vascular tissue of the main root at 2 cm below the soil line were taken in all plants to assay for colonization by W11 and P1. Tissue pieces weighing 0.1 g were macerated in 900 µL PBS and were serially diluted to 1:1000 in PBS. One hundred microliters of each dilution was plated on to LB media and *S. marcescens* P1 and W11 colonies were counted after four days. Single colonies with characteristic morphology of each bacterium were isolated on to new LB plates.

After four days of incubation at room temperature, single bacterial colonies were boiled in 500 µL sterilized distilled water for five minutes. The identity of CYVD-strains was confirmed using PCR (Viriti 96-Well Thermal Cycler) and the strain-specific primers A79F (CCAGGATACATCCCATGATGAC) and A79R (CATATTACCTGATGCTCCTC) that target unique CYVD strain sequences (Zhang et al. 2005). Identity of red strains was confirmed using PCR with YV1 (GGGAGCTTGCTCCCCGG) and YV4 (AACGTCAATTGATGAACGTATTAAGT) primers that are specific for *S. marcescens* (Zhang et al. 2005).

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 using the following cycle: 5 min at 95°C; 35 cycles of 40 s at 95°C, 60 s at 60°C, 90 s at 72°C, and one final extension cycle of 5 min at 72°C. PCR products were electrophoretically separated in a 1.0% agarose gel (GenePure,

Bioexpress, Kaysville, UT) and ethidium bromide at 100 µg/L. Fragments of 338 bp and 409 bp were considered positive for CYVD strain P1 and endophytic strain W11, respectively.

Activity of potential biocontrol strains against cucurbit diseases in the field

S. marcescens strain W11 and *Bacillus mojavensis* RRC101 were grown for three days on LB plates and selected single colonies were grown in LB at 28°C for 18 h. Cultures were centrifuged and density was adjusted to 1 x 10^8 CFU/mL in PBS as described previously. Three glass beakers were filled with 500 mL of the respective bacterial suspensions or a sterilized water control. Forty seeds of 'Costata Romanesca' zucchini (*C. pepo*, High Mowing Seeds, Wolcott, VT) were placed in each flask for an hour (Figure 4.1). Seeds were removed from the suspensions, placed on a paper towel to dry for 30 min, and were planted within an hour after drying.

Field trials were conducted at the UGA Horticulture Research Farm (Watkinsville, GA) and were managed using certified organic practices. A winter cover crop of crimson clover and rye was incorporated into the soil approximately six weeks prior to planting. Nature Safe 10:2:8 (Nature Safe, Cold Spring, KY) was broadcast in 1.5 m wide beds by hand at a rate of 1,221 kg/ha (6.8 kg/row). The fertilizer was tilled in with a rototiller along bed centers two weeks before planting. A single line of Chapin drip tape (Jain Irrigation Systems, Jalgaon, India) with a flow rate of 1.90 Lpm/30.5 m at a pressure of 0.70 kg/cm^2 was set up running across each bed with emitters spaced at 20.3 cm. Ground cloth (ProGro Supply, Willacoochee, Ga) was placed between rows. For each experimental repetition, seeds were planted with 46 cm in-row spacing split between two 13.7 m long and 1.5 m wide rows spaced 3.0 m apart in a completely randomized design (Figure 4.2). Treated seeds for each trial were planted on May 23rd, May 24th,

May 31st, and June 1st, and were grown for ten weeks. Following germination, a layer of wheat straw was placed around plants to conserve moisture and suppress weeds. Plants were irrigated for an hour in the morning three days a week until the fourth week after planting, after which plants were watered four days per week for 2 h in the morning.

S. marcescens strain W11 and *Bacillus mojavensis* RRC101 were grown for three days on Luria-Bertani (LB) agar and selected single colonies were grown in flasks of 25 mL LB at 28°C for 18 h. Density of cultures was adjusted to 1 x 10^8 CFU/mL in PBS as described previously. Fifty mL of W11, *Bacillus mojavensis* RRC101 or PBS were poured around the base of the zucchini plants at the expanded cotyledon stage a week after planting of each experimental repetition.

Diseased lower stems and roots of plants exhibiting typical CYVD symptoms of leaf yellowing and wilting, followed by plant collapse, were collected from the field, and a stem piece from the soil line to the cotyledon scars was surface sterilized in 0.83% sodium hypochlorite for 1 min, washed in sterilized water, and the phloem tissue was examined for discoloration. A 0.1 g piece of vascular tissue was extracted and macerated using a microtube pestle in a 1.5 mL microcentrifuge tube containing 500 µL of PBS, and the suspension was streaked onto duplicate LB agar plates. Following incubation for three days at room temperature, bacterial colonies with characteristic morphology of *S. marcescens* were transferred to fresh LB plates. Four day old single bacterial colonies were boiled in 500 µL sterilized distilled water for 5 min. The identity of CYVD-strains was confirmed using PCR (Viriti 96-Well Thermal Cycler) and the strain-specific primers A79F and A79R as described previously.

For evaluation of suppression of downy and powdery mildew, a modified version of the scale developed by Urban and Lebeda (2006) was employed. Six fully-expanded, mature leaves

were randomly selected from the canopy of each plant and rated on a scale of 0-5 with 0 representing no lesions, 1: 1 to 10%, 2: 11 to 24%, 3: 25 to 49%, 4: 50 to 99%, and 5: 100% leaf area affected. Disease ratings occurred at 50 days after planting (dap) on July 12th, 51 dap on July 14th, 47 dap on July 17th, and 47 dap on July 18th, for the four experimental repetitions.

Statistical analyses

All statistical analyses were run in R Statistical Software (R Foundation for Statistical Computing, Vienna, Austria). Scale numbers were transformed to midpoints of percentage ranges for each scale point prior to analyzing downy and powdery mildew severity. All analyses were run using a one way analysis of variance (ANOVA) and an α=0.05. Values were log-transformed prior to analysis if necessary. Multiple comparison tests were performed using Tukey's HSD.

Results

In vitro antagonism assays

Zones of inhibition for the three non-pathogenic pigmented *S. marcescens* strains ranged from 1 to 9 mm (Figure 4.3). The largest zones of inhibition were seen in *S. marcescens* strains ZM02 and W11 with a range of 4 to 9 mm. Zones of inhibition ranged from 1 to 4 mm for ZM01. No zone of inhibition was seen for the buffer control, *Acidovorax citrulli* 00-1 or *Bacillus mojavensis* RRC101. Since W11 has been characterized in previous molecular studies (Besler 2014) this strain was chosen for further evaluation.

In vivo testing of biocontrol strain W11

On average, *S. marcescens* strain W11 was recovered from 23.3% of the stems of plants inoculated with W11 and P1 and 30% of the stems of plants inoculated with only W11. W11 was isolated from 30 to 40% of the roots of the two treatments, respectively. When present in the stem, W11 was found at all three recovery points. CYVD strain P1 was recovered from the inoculation site and 1 cm below in all inoculated plants but was only recovered from 1 cm above the inoculation site in 53.3% of plants inoculated with only P1 and 73.3% of plants inoculated with both W11 and P1 (Table 4.2). Inoculation with W11 did not significantly reduce the population of strain P1 at 1 cm above in trials two (P=0.99) and three (P=0.58), but significantly lower (P=0.02) P1 populations were found at 1 cm above in trial one in plants not inoculated with W11. No significant differences in P1 numbers were seen at the inoculation site (P=0.55, P=0.99, P=0.99) or at 1 cm below the inoculate site (P=0.99, P=0.99, =0.77) (Table 4.2). Populations of P1 ranged from 0 to 1.60×10^4 CFU/mg plant tissue at 1 cm above, 6.50×10^2 to 3.68×10^5 CFU/mg at the site of inoculation, and 5.00×10^1 to 1.03×10^5 CFU/mg at 1 cm below. The highest concentration observed for W11 was 2.82×10^4 CFU/mg plant tissue in the roots and 9.2×10^3 CFU/mg plant tissue in the stem. No additional reduction in P1 population was seen in W11-treated plants where W11 was recovered than in plants where W11 was not recovered. Average stem growth over six weeks was higher in plants inoculated with W11 ($\bar{x}$=196.81 cm) than with the PBS control ($\bar{x}$=177.27 cm). Increase in stem growth was not significant (P=0.33) between treatments.

Activity of potential biocontrol strains against cucurbit diseases in the field

The first symptoms of downy and powdery mildew were observed on June 27th, and the first incidence of CYVD was observed on June 29th (Figure 4.4). Plectosporium blight, caused by

the fungus *Plectosporium tabacinum*, rapidly contributed to a decline in plant health (Figure 4.5), so plants could not be rated more than once for downy and powdery mildew. Average downy and powdery mildew severity were low during all four ratings (<15%). No significant difference was seen in downy mildew severity for trial 1 (P=0.63), trial 2 (P=0.52), trial 3 (P=0.30), or trial 4 (P=0.37, Figure 4.6). No significant difference was seen in powdery mildew severity in trial 1 (P=0.40), trial 2 (P=0.91), or trial 4 (P=0.27), but the *S. marcescens* treatment did offer better control of powdery mildew than the *B. mojavensis* treatment in trial 3 (P=0.02, Figure 4.7). There was no significant difference in CYVD control among treatments for trial 1 (P=0.91), trial 2 (P=0.80), trial 3 (P=0.07), or trial 4 (P=0.41, Figure 4.8).

Discussion

The red pigmented, prodigiosin-producing non-CYVD *S. marcescens* strains demonstrated an antagonistic effect against a CYVD strain of *S. marcescens* using an *in vitro* assay. Strains ZM02 and W11 produced the largest zones of clearing, and also are the deepest red of the three *S. marcescens* strains. Prodigiosin production is density dependent (Thomson et al. 2000), so the larger zones of clearing may be due to the fast growing red-pigmented strains producing more antimicrobial compounds. Prodigiosin as a mechanism of antagonism is possible, as the *A. citrulli* and *B. mojavensis* strains produce no prodigiosin or zone of inhibition. However, production of other antibiosis-related compounds, such as proteases, is also possible (Mota et al. 2017). More studies are needed to determine the exact mechanism of antagonism.

A statistically significant reduction of CYVD strain P1 was only observed in trial one at 1 cm above the inoculation point where lower P1 populations were recorded in plants that were not exposed to W11. This reduction was likely due to inconsistent colonization by P1 at 1 cm above

the inoculation site. CYVD strains were previously shown to more readily travel and colonize below points of inoculation than above and a similar trend in populations was seen in this study (Besler 2014). Populations of P1 at the three stem sampling points was highly variable regardless of sampling site or treatment and variability was likely due to inconsistent colonization using syringe and needle inoculation.

A significant increase in growth was not observed in pumpkins (*C. pepo* 'New England Pie') treated with *S. marcescens* W11 compared to control plants. Length of vines in all treatments was highly variable, with some stunting of growth seen in all treatments, likely due to damage sustained during uprooting and soaking. W11 was isolated more frequently from roots than stem tissue. Endophytic bacteria can enter through mechanical wounds, such as those created during uprooting and washing roots, but many endophytes secrete cell wall degrading enzymes or enter through natural openings created during lateral root formation (Compant et al. 2010). Once inside vascular tissues, endophytes can be disseminated to other tissues (Chi et al. 2005; Compant et al. 2005). W11 was able to enter roots and travel up the stem in 23.3% to 30% of inoculated plants and was isolated from vascular tissues, showing possible utilization of vascular tissues for dissemination. Further damage to the roots or a longer soaking period may be necessary for better introduction of the endophyte but could lead to further stunting of growth and plant death.

As soaking roots with W11 did not yield consistent colonization of plants, two additional application methods were utilized on field-grown plants: soaking seeds and a soil drench. A *Bacillus mojavensis* strain was selected for application as *Bacillus* spp. are commonly employed in biological control studies (García-Gutiérrez et al. 2013; Gardener 2009; Jiang et al. 2015; Romero et al. 2007). Inoculation with W11 and *B. mojavensis* RRC101 did not reduce severity or

incidence of any of the three disease evaluated compared to the PBS control. However, in one trial a statistically significant difference of approximately 4% in powdery mildew severity and 25% in CYVD incidence was observed between plants inoculated with RRC101 and W11, but this difference may be due to natural variation. Differences in severity of downy and powdery mildew between treatments would have possibly increased if ratings were performed later in the season. Severe damage to foliage and stems by Plectosporium blight developed under wet conditions and disease spread quickly in the field. To ensure ratable foliage was present, the four trials were rated for downy and powdery mildew before downy and mildew infestations had fully developed.

RRC101 was not recovered from the stems of any symptomatic plants during isolation of CYVD strains, but W11 or another pigmented *S. marcescens* strain was recovered from the lower stems of 10% of symptomatic plants inoculated with W11. Colonization of RRC101 and W11 on roots was not assayed. Inoculated populations of W11 and RRC101 were possibly not high enough to effectively compete against native microbes and colonize the rhizosphere. Rainy conditions may have also washed applied solutions away before colonization could occur. An additional study using vacuum infiltration of seeds or the addition of one or more soil drenches would possibly increase populations of RRC101 and W11 and offer better disease control.

Effectiveness of biological control agents can be reduced by environmental factors, host genotype, and host nutrition (Walters et al. 2013). Host genotype plays a significant role in which microbes dominate the rhizosphere and phyllosphere, usually through production of plant phenolics (Badri et al. 2013; Tucci et al. 2011). In studies on induction of plant defense genes and resistance phenotypes, host genotype greatly affected induction of resistance (Sharma et al.

2010; Walters et al. 2011). Microbes isolated from squash could be more effective biocontrol agents than RRC101, isolated from corn, and W11, from watermelon, which have not previously been shown to induce resistance or control cucurbit diseases.

Induction of resistance does not always produce noticeable disease control as plant defense genes are already partially or fully induced by native microbes. In studies on tomato and wheat, defense genes were already activated prior to application of SAR activators. Gene expression was increased further in tomato after application of an SAR activator, but gene expression was very high in wheat prior to application and no changes were seen in expression (Herman et al. 2007; Pasquer et al. 2005). The field in which the studies were conducted is maintained using organic practices which foster microbial diversity. Defense genes may already be activated by native microbes in the rhizosphere and application of RRC101 and W11 may not induce additional resistance.

Organic management of cucurbit diseases uses a systems approach for producing healthy plants. A biological control agent would be particularly useful for CYVD which is currently managed through the exclusion and elimination of the squash bug vector. Additional isolations from healthy squash hosts may yield microbes which are host-adapted and offer better suppression of disease than strains utilized in this study.

Literature Cited

Adam, K. L. 2006. Squash bug and squash vine borer: Organic controls. ATTRA-National Sustainable Agriculture Information Service.

Alston, D. G., and Barnhill, J. V. 2008. Squash bug. in: Utah Pest Fact Sheet Utah State University Extension, Logan, UT.

Badri, D. V., Chaparro, J. M., Zhang, R., Shen, Q., and Vivanco, J. M. 2013. Application of natural blends of phytochemicals derived from the root exudates of *Arabidopsis* to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. J. Biol. Chem. 288:4502-4512.

Besler, K. R. 2014. Epidemiology and management of cucurbit yellow vine disease, and characterization of the causal agent *Serratia marcescens*. University of Georgia, Athens, GA.

Besler, K. R., and Little, E. L. 2015. First report of cucurbit yellow vine disease caused by *Serratia marcescens* in Georgia. Plant Dis. 99:1175-1175.

Chen, Z. X., and Dickson, D. W. 1998. Review of *Pasteuria penetrans*: Biology, ecology, and biological control potential. J. Nematol. 30:313-340.

Chi, F., Shen, S.-H., Cheng, H.-P., Jing, Y.-X., Yanni, Y. G., and Dazzo, F. B. 2005. Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. Appl. Environ. Microbiol. 71:7271-7278.

Choudhary, D. K., Prakash, A., and Johri, B. N. 2007. Induced systemic resistance (ISR) in plants: mechanism of action. Indian J. Microbiol. 47:289-297.

Compant, S., Clément, C., and Sessitsch, A. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biol. Biochem. 42:669-678.

Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clement, C., and Ait Barka, E. 2005. Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. Appl. Environ. Microbiol. 71:1685-1693.

Elsey, K. D. 1985. Resistance mechanisms in *Cucurbita moschata* to pickleworm and melonworm (Lepidoptera: Pyralidae). J. Econ. Entomol. 78:1048-1051.

Gafni, A., Calderon, C. E., Harris, R., Buxdorf, K., Dafa-Berger, A., Zeilinger-Reichert, E., and Levy, M. 2015. Biological control of the cucurbit powdery mildew pathogen *Podosphaera xanthii* by means of the epiphytic fungus *Pseudozyma aphidis* and parasitism as a mode of action. Front. Plant Sci. 6:1-11.

García-Gutiérrez, L., Zeriouh, H., Romero, D., Cubero, J., de Vicente, A., and Pérez-García, A. 2013. The antagonistic strain *Bacillus subtilis* UMAF6639 also confers protection to melon plants against cucurbit powdery mildew by activation of jasmonate- and salicylic acid-dependent defence responses. Microb. Biotechnol. 6:264-274.

Gardener, B. B. M. 2009. Biocontrol of plant pathogens and plant growth promotion by *Bacillus*. Pages 71-79 in: Recent developments in management of plant diseases. U. Gisi, I. Chet and M. L. Gullino, eds. Springer Netherlands, Dordrecht.

Herman, M. A. B., Restrepo, S., and Smart, C. D. 2007. Defense gene expression patterns of three SAR-induced tomato cultivars in the field. Physiol. Mol. Plant Pathol. 71:192-200.

Jiang, C.-H., Wu, F., Yu, Z.-Y., Xie, P., Ke, H.-J., Li, H.-W., Yu, Y.-Y., and Guo, J.-H. 2015. Study on screening and antagonistic mechanisms of *Bacillus amyloliquefaciens* 54 against bacterial fruit blotch (BFB) caused by *Acidovorax avenae* subsp. *citrulli*. Microbiol. Res. 170:95-104.

Johnson, K. L., Minsavage, G. V., Le, T., Jones, J. B., and Walcott, R. R. 2011. Efficacy of a nonpathogenic *Acidovorax citrulli* strain as a biocontrol seed treatment for bacterial fruit blotch of cucurbits. Plant Dis. 95:697-704.

Koné, D., Csinos, A. S., Jackson, K. L., and Ji, P. 2009. Evaluation of systemic acquired resistance inducers for control of *Phytophthora capsici* on squash. Crop Protect. 28:533-538.

Lavania, M., Chauhan, P. S., Chauhan, S. V. S., Singh, H. B., and Nautiyal, C. S. 2006. Induction of plant defense enzymes and phenolics by treatment With plant growth–promoting rhizobacteria *Serratia marcescens* NBRI1213. Curr. Microbiol. 52:363-368.

Lebeda, A., and Křístková, E. 1993. Resistance of *Cucurbita pepo* and *Cucurbita moschata* varieties to cucurbit downy mildew (*Pseudoperonspora cubensis*). Plant Var. Seeds 6:109-114.

Maleki, M., Mokhtarnejad, L., and Mostafaee, S. 2011. Screening of rhizobacteria for biological control of cucumber root and crown rot caused by *Phytophthora drechsleri*. Plant Pathol. J. 27:78-84.

Mayers, C. N., Lee, K. C., Moore, C. A., Wong, S. M., and Carr, J. P. 2005. Salicylic acid-induced resistance to Cucumber mosaic virus in squash and *Arabidopsis thaliana*: contrasting mechanisms of induction and antiviral action. Mol. Plant Microbe Interact. 18:428-434.

McCreight, J. D. 2017. Compendium of cucurbit diseases and pests. Pages 1-9 in: Compendium of cucurbit diseases and pests. A. P. Keinath, W. M. Wintermantel and Z. T.A., eds. APS Press, St. Paul, Minn.

McGrath, M. T., and Staniszewska, H. 1996. Management of powdery mildew in summer squash with host resistance, disease threshold-based fungicide programs, or an integrated program. Plant Dis. 80:1044-1052.

Mota, M. S., Gomes, C. B., Souza Júnior, I. T., and Moura, A. B. 2017. Bacterial selection for biological control of plant disease: criterion determination and validation. Braz. J. Microbiol. 48:62-70.

Novero, E. S., Painter, R. H., and Hall, C. V. 1962. Interrelations of the squash bug, *Anasa tristis*, and six varieties of squash (*Cucurbita* spp.). J. Econ. Entomol. 55:912-919.

Ordentlich, A., Elad, Y., and Chet, I. 1988. The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. Phytopathology 78:84-87.

Pal, K. K., and McSpadden Gardener, B. 2006. Biological control of plant pathogens. Plant Health Instruct. 1117:1-25.

Pasquer, F., Isidore, E., Zarn, J., and Keller, B. 2005. Specific patterns of changes in wheat gene expression after treatment with three antifungal compounds. Plant. Mol. Biol. 57:693-707.

Pliego, C., Ramos, C., de Vicente, A., and Cazorla, F. M. 2011. Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. Plant Soil 340:505-520.

Postma, J., Stevens, L. H., Wiegers, G. L., Davelaar, E., and Nijhuis, E. H. 2009. Biological control of *Pythium aphanidermatum* in cucumber with a combined application of *Lysobacter enzymogenes* strain 3.1T8 and chitosan. Biol. Control 48:301-309.

Press, C. M., Wilson, M., Tuzun, S., and Kloepper, J. W. 1997. Salicylic acid produced by *Serratia marcescens* 90-166 is not the primary determinant of induced systemic resistance in cucumber or tobacco. Mol. Plant-Microbe Interact. 10:761-768.

Romero, D., de Vicente, A., Rakotoaly, R. H., Dufour, S. E., Veening, J.-W., Arrebola, E., Cazorla, F. M., Kuipers, O. P., Paquot, M., and Pérez-García, A. 2007. The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. Mol. Plant-Microbe Interact. 20:430-440.

Sharma, K., Butz, A., and Finckh, M. 2010. Effects of host and pathogen genotypes on inducibility of resistance in tomato (*Solanum lycopersicum*) to *Phytophthora infestans*. Plant Pathol. 59:1062-1071.

Shoresh, M., Harman, G. E., and Mastouri, F. 2010. Induced systemic resistance and plant responses to fungal biocontrol agents. Annu. Rev. Phytopathol. 48:21-43.

Singh, R., and Rao, S. N. 1995. Biological control of *Aphis gossypii* Glover on cucurbits by a parasitic wasp T*rioxys indicus* Subba Rao & Sharma. Biol. Agric. Hortic. 12:227-236.

Someya, N., Nakajima, M., and Akutsu, K. 2005a. Potential of an antagonistic bacterium S*erratia marcescens* strain B2 for the biological control of cucumber damping-off disease. Biocontrol Sci. 10:101-104.

Someya, N., Nakajima, M., Hibi, T., Yamaguchi, I., and Akutsu, K. 2002. Induced resistance to rice blast by antagonistic bacterium, *Serratia marcescens* strain B2. J. Gen. Plant Pathol. 68:177-182.

Someya, N., Nakajima, M., Watanabe, K., Hibi, T., and Akutsu, K. 2005b. Potential of *Serratia marcescens* strain B2 for biological control of rice sheath blight. Biocontrol Sci. Tech. 15:105-109.

Someya, N., Kataoka, N., Komagata, T., Hirayae, K., Hibi, T., and Akutsu, K. 2000. Biological control of cyclamen soilborne diseases by *Serratia marcescens* Strain B2. Plant Dis. 84:334-340.

Thomson, N. R., Crow, M. A., McGowan, S. J., Cox, A., and Salmond, G. P. 2000. Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia* is under quorum sensing control. Mol. Microbiol. 36:539-556.

Tucci, M., Ruocco, M., De Masi, L., De Palma, M., and Lorito, M. 2011. The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. Mol. Plant Pathol. 12:341-354.

Urban, J., and Lebeda, A. 2006. Fungicide resistance in cucurbit downy mildew – methodological, biological and population aspects. Ann. Appl. Biol. 149:63-75.

Walters, D. R., Ratsep, J., and Havis, N. D. 2013. Controlling crop diseases using induced resistance: Challenges for the future. J. Exp. Bot. 64:1263-1280.

Walters, D. R., Havis, N. D., Paterson, L., Taylor, J., and Walsh, D. J. 2011. Cultivar effects on the expression of induced resistance in spring barley. Plant Dis. 95:595-600.

Zhang, Q., Melcher, U., Zhou, L., Najar, F. Z., Roe, B. A., and Fletcher, J. 2005. Genomic comparison of plant pathogenic and nonpathogenic *Serratia marcescens* strains by suppressive subtractive hybridization. Appl. Environ. Microbiol. 71:7716-7723.

Tables

Table 4.1. Source of strains used in this study. Pigmented strains produce the red colored compound prodigiosin. Strains W11, ZM01, and ZM02 are non-cucurbit yellow vine, pigmented strains.

Strain	Host of Origin	Geographical Origin	Comments
***Serratia marcescens* P1**	**Pumpkin**	**GA, USA**	**CYVD strain, unpigmented**
***Serratia marcescens* W11**[a]	**Watermelon**	**GA, USA**	**Endophyte, pigmented**
***Serratia marcescens* ZM01**	**Summer squash**	**GA, USA**	**Endophyte, pigmented**
***Serratia marcescens* ZM02**	**Squash bug**	**GA, USA**	**Non-CYVD, pigmented**
***Bacillus mojavensis* RRC101**[b]	**Corn**	**Italy**	**Non pathogenic**
***Acidovorax citrulli* 00-1**[b]	**Citron**	**Unknown**	**Pathogen**

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

Table 4.2. Recovery of strains W11 and P1 from inoculated plants grown for six weeks. Roots of ten 'New England Pie' pumpkin seedlings in the cotyledon stage were dipped into a 10^8 CFU/mL suspension of endophytic strain W11 in PBS. The roots of control plants were dipped into PBS. Half the plants from each treatment were inoculated with either CYVD strain P1 or PBS. Bacteria were isolated and enumerated from 100 mg of vascular tissues at three locations on the stem: At the inoculation point and 1 cm above and below the inoculation point. A one-way ANOVA and multiple comparisons test using Tukey's HSD was run on the mean $\log_{10}$ CFU/mg plant tissue for strain P1 at each isolation site.

Distance from inoculation point (cm)	Inoculum		Average Detection Frequency (%)		Mean $\log_{10}$ CFU P1/mg plant tissue		
	Dip	Syringe	P1	W11	Trial 1	Trial 2	Trial 3
1.0 above	PBS	P1	53.3	0	0.75b	1.13a	2.80a
	W11	P1	73.3	23.3	2.70a	1.47a	1.58a
	W11	PBS	0	30.0	0	0	0
	PBS	PBS	0	0	0	0	0
0	PBS	P1	100.0	0	4.88a	4.20a	4.35a
	W11	P1	100.0	23.3	3.92a	4.25a	3.88a
	W11	PBS	0	30.0	0	0	0
	PBS	PBS	0	0	0	0	0
1.0 below	PBS	P1	100.0	0	3.78a	3.59a	3.96a
	W11	P1	100.0	23.3	3.69a	3.84a	2.97a
	W11	PBS	0	30.0	0	0	0
	PBS	PBS	0	0	0	0	0

Figures

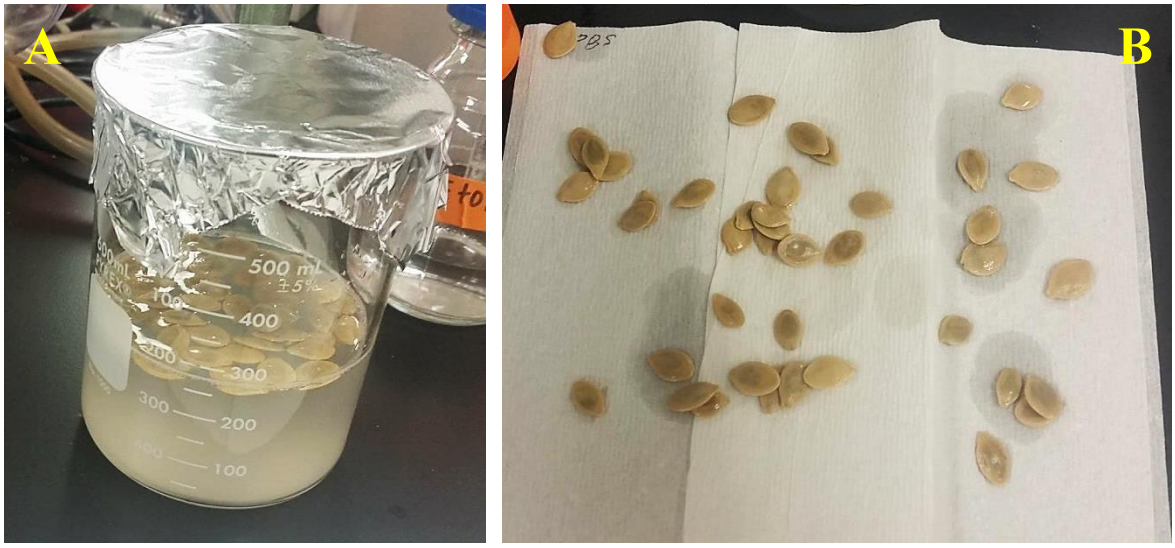


Figure 4.1. Seeds soaking in a solution of a biocontrol strain (A) and drying on a paper towel following soaking (B). Seeds were soaked for an hour in a bacterial solution prepared at 1 x 10^8 CFU/mL or phosphate-buffered sale (PBS) prior to planting directly in the field.

Figure 4.2. Zucchini plants utilized in field trials in 2017. Biocontrol agents were applied as a seed soak prior to planting and a soil drench one week after planting. Plots were laid out in a completely randomized design.

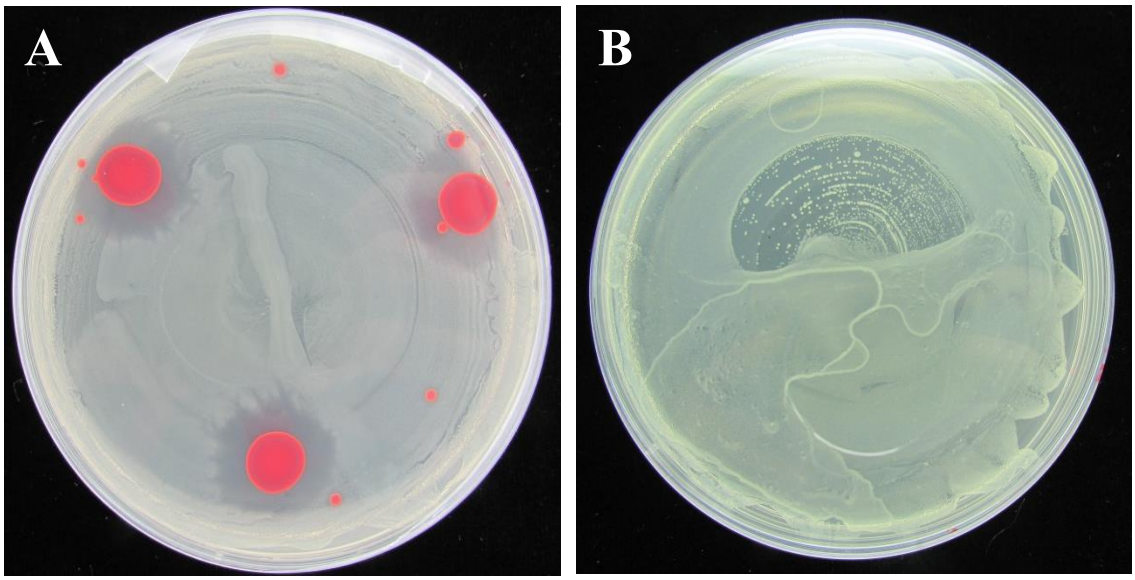


Figure 4.3. *In vitro* assays of antagonism of biocontrol candidates against CYVD strains. Density of P1 was adjusted to 1 x 10^6 CFU/mL in 0.01 M phosphate buffered saline (PBS) and other cultures were adjusted to 1 x 10^8 CFU/mL. Twenty five LB agar plates were each spread with 80 µL of P1 solution and dried for 8 h. Five treated plates were each spotted in triplicate with 10 µL of strains W11, ZM01, ZM02, *Acidovorax citrulli* 00-1, *Bacillus mojavensis* RRC101, and a PBS buffer control. After 3 days of growth, zones of inhibition from the edge of the spotted colonies were measured. Zones of inhibition for strain W11 produced against P1 (A). No activity by *A. citrulli* 00-1 against P1 (B).

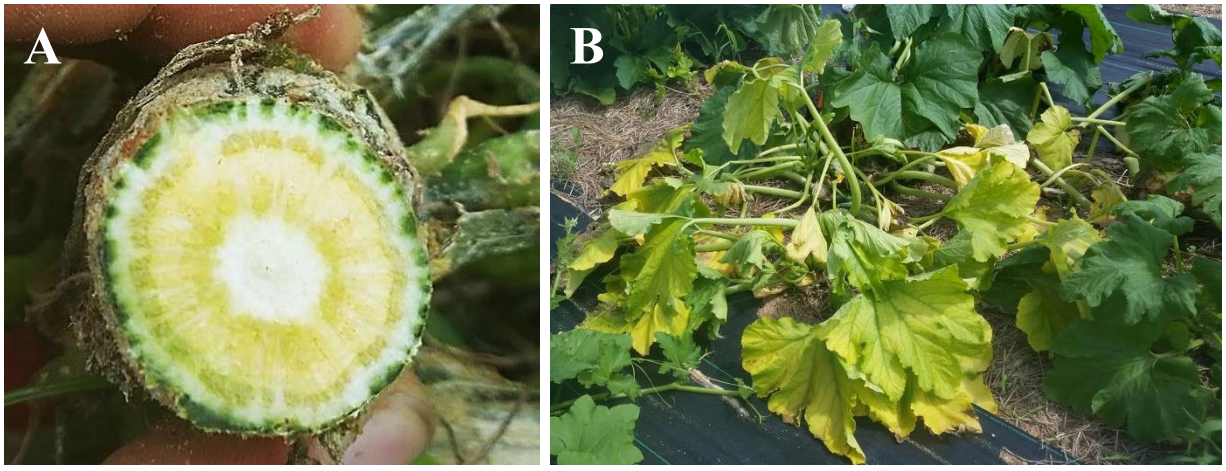


Figure 4.4. Cucurbit yellow vine disease (CYVD) symptoms on zucchini. Crown area of zucchini stem showing characteristic vascular browning (A). Collapsed zucchini plant showing wilting and leaf yellowing (B).

Figure 4.5. Plectosporium blight (*Plectosporium tabacinum*) symptoms on zucchini. Stems showing breaking and collapse characteristic of severe infestation (A). Typical spindle-shaped lesions on zucchini petioles (B).

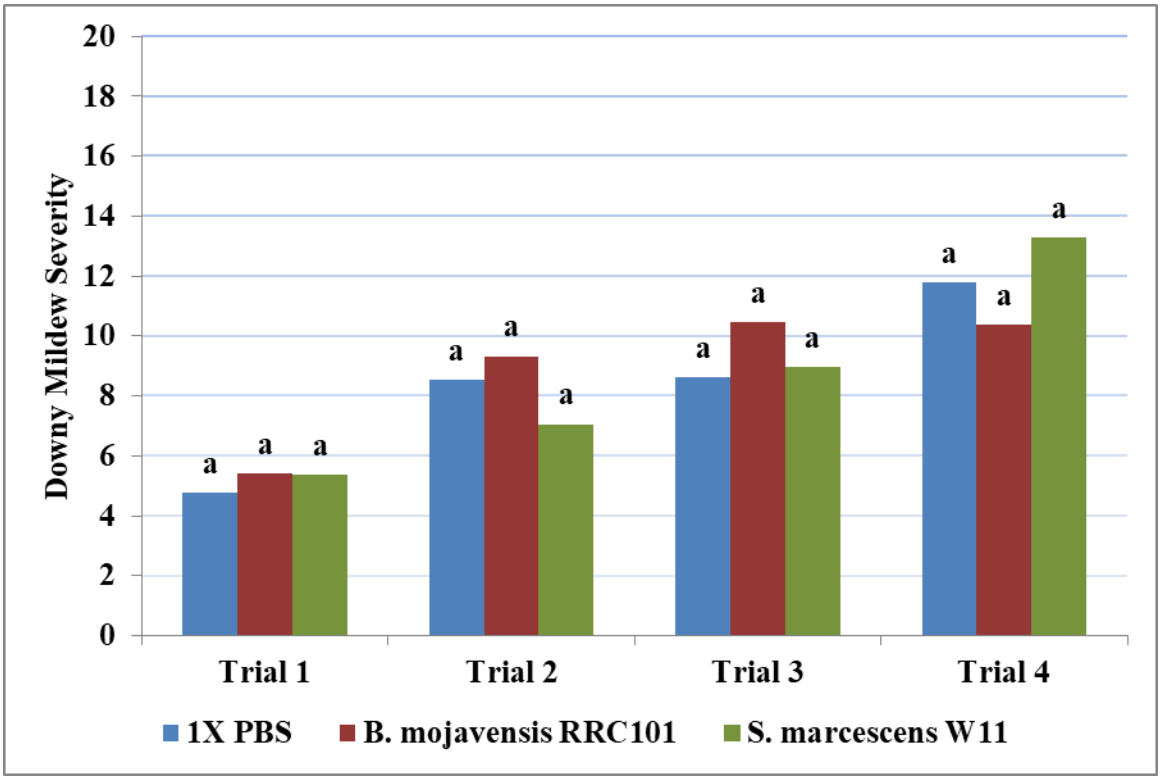


Figure 4.6. 2017 ratings of downy mildew on zucchini inoculated with biocontrol agents. Six fully expanded, mature leaves were randomly selected from each of the 20 replicated plants in each treatment and were rated for downy mildew on a scale of 0-5 with 0 representing no lesions, 1: 1 to 10%, 2: 11 to 24%, 3: 25 to 49%, 4: 50 to 99%, and 5: 100%. Scale numbers were converted to midpoint percentages for analyses. Ratings were performed at 50 days after planting (dap), 51 dap, 47 dap, and 47 dap for the four trials. Letters indicate significant differences in downy mildew severity between treatments based upon a one-way ANOVA and Tukey's HSD.

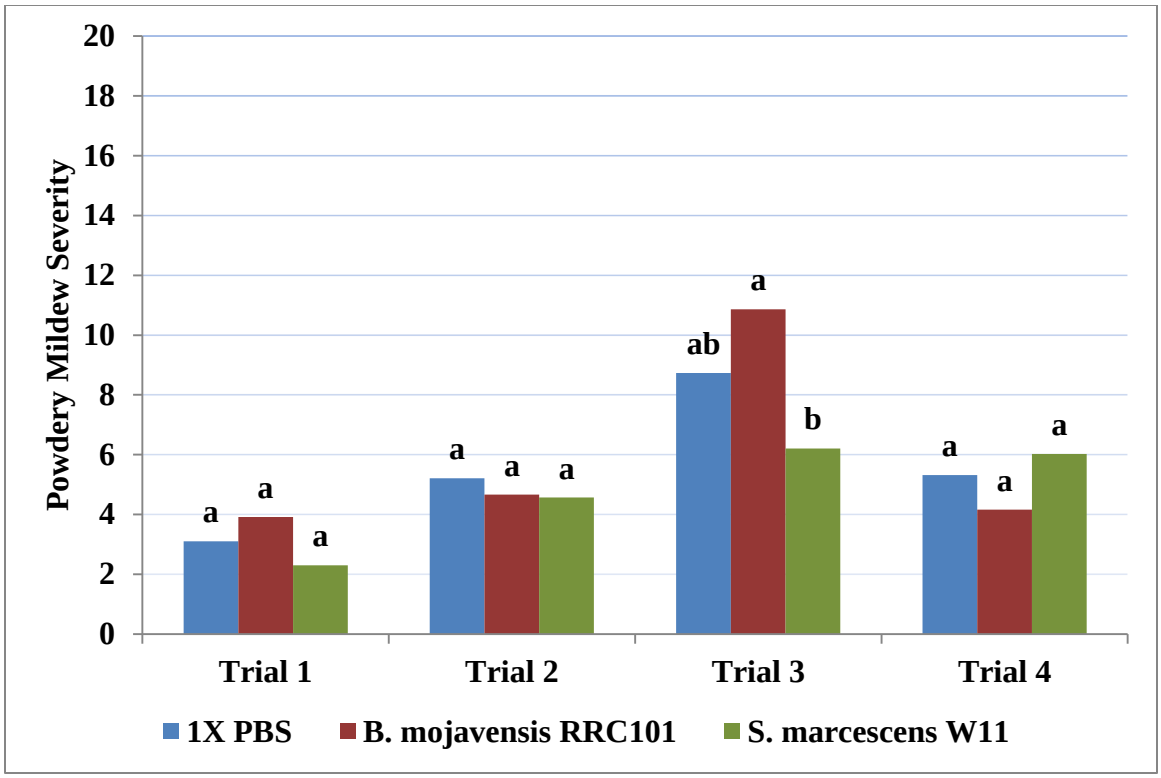


Figure 4.7. 2017 ratings of powdery mildew on zucchini inoculated with biocontrol agents. Six fully expanded, mature leaves were randomly selected from each of the 20 replicated plants in each treatment and were rated for powdery mildew on a scale of 0-5 with 0 representing no lesions, 1: 1 to 10%, 2: 11 to 24%, 3: 25 to 49%, 4: 50 to 99%, and 5: 100%. Scale numbers were converted to midpoint percentages for analyses. Ratings were performed at 50 days after planting (dap), 51 dap, 47 dap, and 47 dap for the four trials. Letters indicate significant differences in powdery mildew severity between treatments based upon a one-way ANOVA and Tukey's HSD.

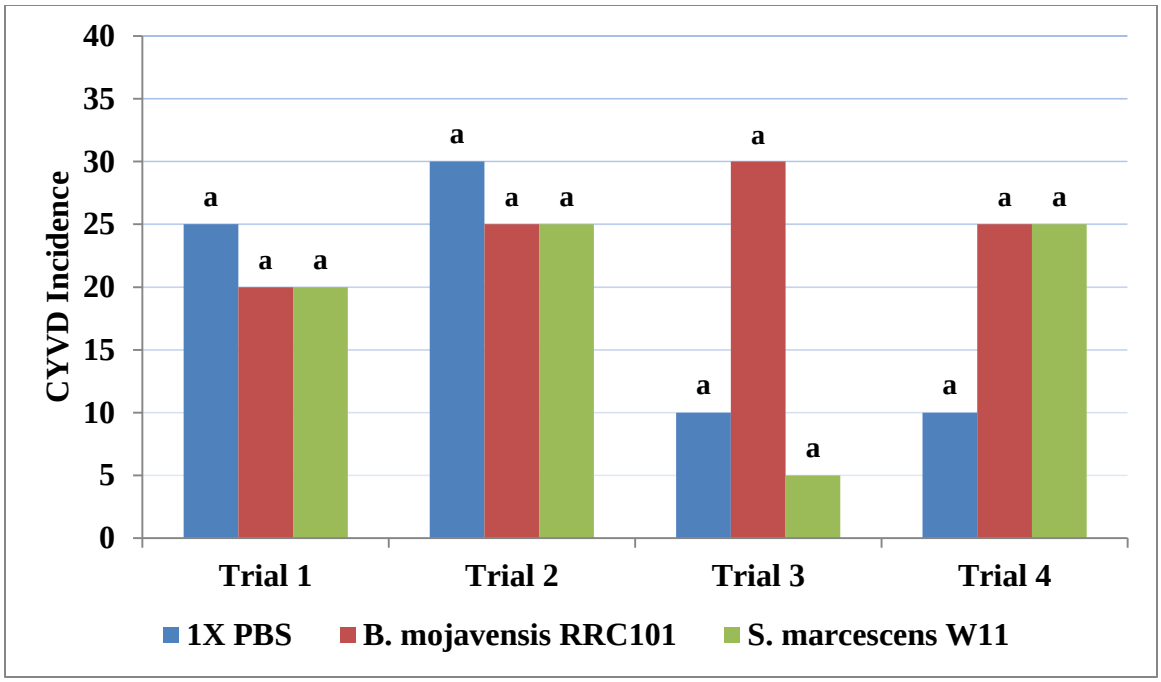


Figure 4.8. 2017 ratings of cucurbit yellow vine disease (CYVD) in zucchini inoculated with biocontrol agents. Stems and roots of plants developing yellowing or wilting were collected and evaluated for phloem browning. Stems were isolated on to LB and representative *S. marcescens* colonies were confirmed via PCR using CYVD strain-specific primers. Letters indicate significant differences in CYVD incidence between treatments based upon a one-way ANOVA and Tukey's HSD.

CHAPTER 5

EVALUATION OF WINTER SQUASH CULTIVARS FOR DISEASE AND PEST RESISTANCE, YIELD, AND FRUIT QUALITY[4]

[4] Matteen, Z. T. and Little, E. L. 2017. To be submitted to *HortScience*.

Abstract

Demand is increasing for locally grown winter squash (*Cucurbita* spp.) Squash growers in the southeast face numerous economically important pests and diseases such as downy mildew (*Pseudoperonospora cubensis),* powdery mildew (*Podosphaera xanthii),* cucurbit yellow vine disease (*Serratia marcescens*), squash bugs (*Anasa tristis),* and pickleworm (*Diaphania nitidalis*). Cultivars need to be identified that are adapted to Georgia growing conditions and have broad resistance against multiple pests. During the summers of 2015 to 2017, winter squash cultivars were grown in replicated studies at the Durham Horticultural Farm in Watkinsville, GA. Cultivars were evaluated for severity of downy and powdery mildew, incidence of CYVD, squash bug numbers, and pickleworm damage. Yield, Brix, and dry matter were determined with harvested fruit. 'Waltham butternut' and 'Metro P.M.R butternut' had significantly more downy mildew than the other cultivars. Powdery mildew severity was low and not statistically different on all cultivars all three years. CYVD incidence was not observed in year 1 and was low (0-25%), in year 2. In year 3, 'Waltham Butternut' and 'Delicata Zeppelin' had significantly more CYVD with 81.25-90% mortality. Squash bugs did not show a significant cultivar preference in year 2, but adults preferred 'North Georgia Candy Roaster' for feeding and 'Tan Cheese' for oviposition in year 3. Cultivars varied in susceptibility to pickleworm. Yield was low in year 2 with dry matter, Brix, and taste highest in Waltham Butternut. Yield was higher in year 3 for most cultivars with 'Seminole x Waltham F6' and 'Crowning' having the highest Brix values.

Introduction

Commercial winter squash production in the United States is largely centered in the Northeast and Southwest (USDA 2014). These cooler and/or more arid climates have more moderate climates and are less suitable for development of many cucurbit diseases and pests. A minor amount of commercial winter squash production, primarily pumpkins, occurs in the higher elevations of the Appalachian regions in the southeast, such as northern Georgia (USDA 2014). Commercial squash production in Georgia is largely limited to summer squash cultivars grown in late spring to early summer and early fall to avoid high temperatures and disease pressures (McCreight 2017)

Squashes (including pumpkins and gourds) are all thought to have originated in the New World and include four commonly cultivated species: C*ucurbita pepo*, *C. moschata*, *C. maxima*, and *C. argyrosperma*. The term 'pumpkin' has no exact botanical meaning and refers to winter squash cultivars that are used mainly for pies, animal forage, or decoration. Summer squash are the immature fruit of cultivars mainly in the species *C. pepo*. Winter squash are the mature fruit of four *Cucurbita* species and the long storage time of these mature fruits, in some cases up to a year, was traditionally a very desirable trait and allowed for consumption during the winter when most fresh vegetables were not available Examples of *C. pepo* squash include most pie and carving pumpkins, delicata, acorn, and spaghetti squash. *C. moschata* includes cheese pumpkins, butternut, and tropical pumpkins. *C. maxima* includes giant pumpkins, kabocha, hubbard, buttercup, and 'North Georgia candy roaster'. *C. argyrosperma* includes cushaw squash, which are primarily cultivated for their seeds and vegetative parts (McCreight 2017). Squash readily outcross within the same species, resulting in a wide variety of shapes and sizes when allowed to freely pollinate.

In recent years, there has been increased consumer demand for winter squash, particularly for locally grown fruit with unique flavors and appearances. In Georgia, there has been a resurgence of growers for the fresh local market and many of these growers are using organic practices. Little to no information is available on which squash cultivars perform best under Georgia conditions and many of the available commercial winter squash cultivars are bred for conventional systems in northern or western climates. Winter squash are long season crops, and are subject to several serious diseases and pests that flourish under heat and humidity. More information is needed on winter squash cultivars that perform well in organic systems in the southeast.

Economically important pests and diseases of squash in the southeastern United States include cucurbit downy mildew (*Pseudoperonospora cubensis*), powdery mildew (*Podosphaera xanthii)*, cucurbit yellow vine disease (CYVD, *Serratia marcescens*), squash bugs (*Anasa tristis*), pickleworm (*Diaphania nitidalis*), vine borers (*Melittia cucurbitae*), striped cucumber beetles (*Acalymma vittatum*), and spotted cucumber beetles (*Diabrotica undecimpunctata*) (McCreight 2017). Cucurbit downy mildew and powdery mildew overwinter in frost-free regions and spread northward annually, arriving in the southeast in June or July (McCreight 2017; Ojiambo et al. 2015). Cucurbit downy mildew, in particular, has seen a recent resurgence with greater disease severity and more destructive epidemics in the last decade due to the introduction of new races (Cohen et al. 2015).

Organic management of cucurbit foliar diseases uses a systems approach with cultural manipulations such as planting date, nutrition, and site conditions, together with the planting of resistant cultivars, if available. With the development of new, more virulent races, a greater reliance on the use of resistant cultivars is necessary for organic production (McGrath 2006

(McGrath 2004; McGrath and Shishkoff 2003). Pest and disease resistance may be found in species and cultivars locally adapted to the cultural and environmental conditions under which they are grown. Limited studies have examined downy mildew resistance in winter squash (Lebeda and Křístková 1993), and no studies have been published in the southeastern united states. Some open-pollinated cultivars have natural partial resistance to powdery mildew and there are a few available powdery mildew resistant F1 hybrids. (Lebeda and Křístková 1994),

Squash bugs are particularly damaging to cucurbits grown under organic conditions in the southeastern United States (Doughty et al. 2016; McCreight 2017). Squash bugs are sap-sucking true bugs in the family Coreidae and order Hemiptera and are widely distributed throughout the United States (Wadley 1920). The squash bug typically completes its entire life cycle in six to eight weeks and has five nymphal instar stages (Fargo and Bonjour 1988). There are up to three new generations of squash bugs in a season depending on climate (Adam 2006; Decker and Yeargan 2008; Nechols 1987). The economic management threshold is one adult per plant in non-organic systems, and adults are difficult to control even using conventional pesticides (Palumbo et al. 1991). Without the availability of effective organic-approved insecticides, control in organic production relies on removal of debris, crop rotation, early planting, trap cropping, and row covers (Alston and Barnhill 2008; Besler 2014).

Squash bugs overwinter as adults in a reproductive diapause stage near fields in the soil, leaf litter, old buildings, woodpiles, and debris (Adam 2006). After emerging from overwintering sites, adult squash bugs travel unknown distances to find newly planted squash on which to feed and lay eggs (Nechols 1987; Wadley 1920). Squash bugs are the vector for the bacterium *S. marcescens*, causal agent of cucurbit yellow vine disease (CYVD). The CYVD bacterium overwinters inside squash bug adults and is transmitted to the young squash plants in the spring.

The bacterium is not transmitted transovarial to the nymphs. The newly hatched nymphs acquire the bacterium from feeding and the bacterium persists inside the bugs through all nymphal stages (Pair et al. 2004; Wayadande et al. 2005).

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 with eventual death (Bruton et al. 2003). A previous study determined that in areas where the CYVD is prevalent up to 50% of overwintering adults may carry the CYVD bacterium. This same study also found that the bacterium must be inoculated into the squash plants during the first four weeks of growth for the eventual development of CYVD symptoms (Besler 2014). Crop losses from CYVD in organic production in Georgia have ranged from 12 to 93% (Besler and Little 2015).

Information on squash cultivars resistant to squash bugs is limited. Some winter squash cultivars such as green striped cushaw (*C. argyrosperma*), 'Waltham Butternut' (*C. moschata*), and certain other cultivars within *Cucurbita moschata*, *C. pepo* var. *turbinata (*acorn squash), and *C. maxima* (banana squash) are reported to be less attractive and less susceptible to squash bug feeding damage compared to summer squash (Novero et al. 1962; Vogt and Nechols 1993). Resistance to squash bugs has not been quantified in most winter squash, and for the few winter squash cultivars and species that have been tested, durability of resistance is unknown. Varietal resistance to the squash bug vectored disease CYVD has not been tested. Pickleworm, the seasonal larvae of a migrating tropical moth, causes direct economic loss through burrowing into fruits. Some *C. moschata* cultivars appear to get less pickleworm damage most likely due to their thicker skin compared with *C. pepo* cultivars (Brett et al. 1961).

In this study, winter squash cultivars selected for their potential adaption to warm humid climates, such as some cultivars of *C. moschata*, or for their popularity were evaluated in the field for pest and disease resistance. The plants were rated for downy and powdery mildew severity, CYVD incidence, and feeding preference of the squash bugs and pickleworms. Yield and fruit quality were also determined at the end of the growing season. This information will be of great benefit to organic growers, especially in the southeastern United States.

Materials and Methods

Experimental design

Three years (2015 to 2017) of field trials were carried out at the University of Georgia Durham Horticulture Farm in Watkinsville, GA, on USDA certified organic acreage using organic approved practices. A winter cover crop of crimson clover and rye was incorporated into the soil approximately three to four weeks prior to planting each year. Experimental beds were arranged in a randomized complete block design with four replicates of each cultivar (see Figure 5.1). One replicate consisted of five plants with 0.61 m in-row spacing. In-row spacing between replicates was 1.22 m in years one and two, and 3.05 m in year three. Bed centers were spaced 1.83 m apart in year one and was increased to 3.05 m in years two and three to accommodate rampant growth of winter squash vines.

Nature Safe 10:2:8 (Nature Safe, Cold Spring, KY) was broadcast by hand at a rate of 1,221 kg/ha (4.8-6.8 kg/row). The fertilizer was tilled in with a rototiller along bed centers one to two days before planting. A single line of Chapin drip tape (Jain Irrigation Systems, Jalgaon, India) with holes spaced 20.3 cm apart and a flow rate of 1.90 Lpm/30.5 m at a pressure of 0.70 kg/cm^2 was placed down the center of each bed. Plants were irrigated for an hour in the morning

three days a week until plants began vining, after which plants were watered four days per week for 2 h in the morning. Ground cloth (ProGro Supply, Willacoochee, Ga) was placed in the centers between rows. Seeds were directly sown on the following dates in the three seasons: May 21, 2015, May 20, 2016, and May 10, 2017. Following germination, a layer of wheat straw was placed around plants to conserve moisture and suppress weeds.

Seven cultivars were tested in years one and two (Table 5.1), and nine in year three (see Figure 5.2). After the first year, four cultivars that did not perform well and showed little promise for commercial production were dropped and four additional promising cultivars were added. Three *C. moschata* cultivars were grown each of the three years: Waltham Butternut, Seminole and Thai Kang Kob. In total 11 different cultivars of winter squash were grown over the course of the trial. Fruit were harvested span of one week, starting five days after the latest maturation time provided by seed suppliers.

Disease quantification

Downy and Powdery Mildew

To evaluate downy and powdery mildew severity, 15 fully expanded, mature leaves were randomly selected from the fourth through seventh leaves on vines. Disease severity was determined based upon approximate percentage of leaf area covered by lesions. A scale of 0-5 based upon that utilized by Urban and Lebeda (2006) was used with 0 representing no lesions, 1: 1 to 10%, 2: 11 to 24%, 3: 25 to 49%, 4: 50% to 99%, and 5: 100%. Plants were scouted three times a week for the first signs and symptoms of downy mildew and powdery mildew. Thereafter plants were rated weekly, starting when disease severity reached sufficient levels.

Cucurbit Yellow Vine Disease (CYVD)

Squash bug adults were assayed for *S. marcescens.* Adult squash bugs were collected from the UGA Horticulture Research Farm three times during the first year of the trial: June, July, and December of 2015, and once during the second year: June 2016. The thorax, including the foregut and midgut, of each bug was placed in a 1.5 mL Eppendorf tube. The tube was filled with liquid nitrogen and the frozen insect material was ground into a fine powder using a microtube pestle. DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations for the purification of total DNA from insects. The identity of CYVD-strains was confirmed using PCR performed in a Viriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA) and the strain-specific primers A79F (CCAGGATACATCCCATGATGAC) and A79R (CATATTACCTGATGCTCCTC) which target unique CYVD strain sequences (Zhang et al. 2005).

PCR reaction volume was a total of 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 using the following cycle: 5 min at 95°C; 35 cycles of 40 s at 95°C, 60 s at 60°C, 90 s at 72°C, and one final extension cycle of 5 min at 72°C. PCR products were electrophoretically separated in a gel containing 1.0% GenePure agarose (Bioexpress, Kaysville, UT) and ethidium bromide at 100 µg/L.

Plants were scouted three or more times weekly for CYVD symptoms such as wilting or yellowing of the leaves. The phloem tissues in the lower stems of plants that had wilted and yellowed were observed for the browning characteristic of CYVD in the lower stem. A 7-8 cm stem piece from just above the soil line was surface sterilized in 0.83% sodium hypochlorite for 1 min and rinsed in sterilized water. Two 50 mg pieces of vascular tissue were aseptically

extracted and macerated using a microtube pestle in a 1.5 mL microcentrifuge tube containing 500 µL of 0.01 M phosphate buffered saline (PBS), and the suspension was streaked onto duplicate Luria-Bertani (LB) agar plates.

Following incubation for three days at room temperature, bacterial colonies with characteristic morphology of *S. marcescens* were transferred to fresh LB plates. Four day old single bacterial colonies were boiled in 500 µL sterilized distilled water for 5 min and the identity of CYVD-strains was confirmed using PCR and strain- specific primers A79F and A79R as described above.

Insect pest quantification

Numbers of adult squash bugs, nymphs, and egg clutches on stems, leaves, and on the ground beneath the canopy were counted weekly in the inner 1.5 m x 0.6 m area in the center of each replicate (see Figure 5.3). In years one and two, observations were made weekly from two weeks after germination until the fourth week. In year three, squash bug counts started two days after germination. During the first three weeks, counts were performed three times a week. During the fourth week, counts were made twice a week. Cultivars were rated for pickleworm damage by determining incidence of holes from pickleworm burrowing on all viable fruits.

Yield and fruit quality

Harvested fruit were cured in a warm dry area for at least two weeks before processing. Up to three fruits per replicate, depending on availability, were evaluated for Brix and dry matter content in year two and two fruits per replicate were evaluated in year three. For Brix ratings, slices were frozen overnight, crushed, and the sugar content of the resulting juice was measured

with an Atago PAL-1portable refractometer (Atago USA, Inc., Bellevue, WA). To measure dry matter content, 10 to 15 g fruit pieces were weighed, baked at 105ºC for 8 h, and weighed again. In year two and three, selected cultivars were used in blind taste tests. In year two, 25 subjects rated four different cultivars on overall taste on a 1 to 5 scale, with 5 being of highest quality. In year three, 20 subjects evaluated fruits using the same criteria. For yield, harvested fruit from each replicate were weighed and an average replicate fruit weight was calculated for each cultivar.

Statistical analyses

All statistical analyses were run in R Statistical Software (R Foundation for Statistical Computing, Vienna, Austria). Scale numbers were transformed to midpoints of percentage ranges for each scale point prior to analyzing downy and powdery mildew severity. All analyses were run using a one way analysis of variance (ANOVA) accounting for blocking and an $\alpha=0.05$. Multiple comparisons tests were performed using Tukey's HSD. No block effect was observed in the trials.

Results

Disease Quantification

Downy Mildew

In year one, the first symptoms of downy mildew were observed on July 10th. The disease progressed rapidly due to wet conditions and severity was determined at 14 days after first observed symptoms. Downy mildew severity was significantly higher ($P=<0.001$) in the highly susceptible 'Waltham Butternut' ($\bar{x}=84.59$) and the powdery mildew resistant butternut cultivar 'Metro P.M.R' ($\bar{x}=71.50$) when compared to all other cultivars. The lowest severity was

with the *C. moschata* variety 'Thai Kang Kob' ($\bar{x}$=8.30, Figure 5.4). Significant defoliation occurred on both butternut cultivars.

In year two, drier conditions led to a delay in development of downy mildew, with first symptoms appearing 15 days later than in year one on July 25th. Downy mildew severity was lower overall in year two for cultivars repeated from year one (Figure 5.5). All cultivars rated had significantly lower downy mildew severity at seven (P=0.01), 14 (P=<0.001), and 21 days (P=<0.001) than the highly susceptible 'Waltham Butternut' ($\bar{x}$=80.57 at 21 days; see Figure 5.6). 'Thai Kang Kob' again exhibiting the lowest downy mildew severity at all rating dates ($\bar{x}$=3.94 at 21 days).

In year three, weather conditions were again wet and downy mildew was first observed earlier than in previous seasons, with initial symptoms seen on June 23rd. Disease severity increased slowly (Figure 5.7), leading to a delay in the first rating date. Only one replicate of 'Thai Kang Kob' and 'North Georgia Candy Roaster' survived to the first rating date. 'Thai Kang Kob' plants failed to establish and died early in the planting season while 'North Georgia Candy Roaster' succumbed to fusarium crown rot (*Fusarium solani* f.sp. *cucurbitae).* CYVD killed three or more plants in two or more replicates in 'Waltham Butternut' and 'Delicata Zeppelin' and few plants survived long enough to be affected by downy mildew.

Powdery Mildew

In year one, a rainy season inhibited development of powdery mildew, and no signs were seen on any cultivar throughout the season. In year two, powdery mildew was first observed on July 25th, with severity increasing on all cultivars over the 21 day rating period (Table 5.2). By the 14 day rating, 'Waltham Butternut' was defoliated by downy mildew and did not have enough mature foliage to rate for powdery mildew. No significant differences in powdery

mildew severity was seen at 7 days (*P*= 0.08), 14 days (*P*=0.27), or 21 days (*P*=0.12) and severity was low in all cultivars, with 'Thai Kang Kob', 'Seminole', and 'Crowning' not developing any signs of powdery mildew.

In year three, signs of powdery mildew developed on June 23rd, with severity increasing slowly over the next 28 days. Ratings could not be carried out on most replicates of 'Waltham Butternut', 'Delicata Zeppelin', 'North Georgia Candy Roaster', or 'Thai Kang Kob' due to either poor establishment or death from other diseases, and these cultivars were not included in statistical analyses. A significant difference among cultivars in powdery mildew severity (Table 5.3) was seen at 21 days (*P*= 0.01) and 28 days (*P*=<0.001). Highest severity was in 'Tan Cheese' and 'Mrs. Amerson's' on both rating dates. 'Crowning' again displayed no disease signs with low severity in 'Seminole' at 21 ($\bar{x}$=0.28) and 28 days ($\bar{x}$=0.46).

Cucurbit Yellow Vine Disease

In year one, 75% (n=6) of squash bug adults in June, 75% (n=16) in July, and 50% (n=30) in December were found to carry *S. marcescens* CYVD strains. In June of year two, 75% (n=20) of adults carried the bacterium. In year one squash bug numbers at the beginning of the season were very low and no plants developed symptoms associated with CYVD. In year two, squash bug populations increased over the season and at least some plants within each cultivar, except for 'Waltham x Seminole F5', developed CYVD with an average incidence within cultivars of 0 to 25% (Figure 5.9). No significant difference was seen among cultivars (*P*= 0.51). In year three, there was a significantly higher incidence of CYVD in 'Delicata Zeppelin' (see Figure 5.8) and 'Waltham Butternut' compared to other cultivars (*P*=<0.001). 'Thai Kang Kob' and 'North Georgia Candy Roaster' were not included in the statistical analysis due to lack of sufficient plants.

Insect pest quantification

In year two, no difference in cultivar feeding preference was seen among adult squash bugs during the first four weeks after planting (*P*= 0.40; Table 5.4). Nymphs exhibited no feeding preference during the first four weeks after planting (*P*=0.74). Adults showed no preference for where they laid eggs during the first four weeks after planting (*P*=0.28). A heat map was constructed to show replicates with higher adult squash bug numbers and where CYVD occurred (Figure 5.10). No strong aggregation was seen in adult squash bugs. With the exception of one infected plant on the far end of the field, CYVD incidence was concentrated in the center of the field.

In year three, adults showed a preference for feeding on 'North Georgia Candy Roaster' over several other cultivars (*P*=<0.001; Table 5.5). Nymphs were only seen on 'Waltham Butternut' during the first four weeks ($\overline{x}$=0.25; *P*=0.46). Significantly more eggs (*P*=0.01) were counted on 'Tan Cheese' ($\overline{x}$=19.25) than 'Mrs. Amerson's' ($\overline{x}$=2.75) or 'Seminole' ($\overline{x}$=2.50). A heat map was constructed to show replicates with higher adult squash bug numbers and where CYVD occurred (Figure 5.11). Higher adult squash bug numbers typically occurred along the edges of the field. CYVD also occurred largely along the edge of the field, with CYVD again mostly clustered.

For pickleworm ratings, only a small sample size was available for 'Crowning', 'Thai Kang Kob', and 'Tan Cheese' in year two due to availability of few mature fruits. The least pickleworm damage was observed in the cultivars 'Mrs. Amerson's' ($\overline{x}$=7.4), 'Waltham Butternut' ($\overline{x}$=18.5), and 'Waltham x Seminole F5' ($\overline{x}$=39.1; Table 5.6).

More fruits were evaluated for pickleworm damage in year three, and pickleworm was lower on average for all cultivars (Table 5.6). The least number of damaged fruits was seen in

‘Mrs. Amerson’s’ ($\overline{x}$=1.6), ‘Seminole x Waltham F6’ ($\overline{x}$=5.5), and ‘Seminole’ ($\overline{x}$=7.2). Insufficient fruits were available of ‘Thai Kang Kob’, ‘Waltham Butternut’, ‘North Georgia Candy Roaster’, and ‘Delicata Zeppelin’ for pickleworm counts.

Yield and fruit quality

In year one, replication integrity was lost and yield could not be assessed due to rampant uncontrollable growth of the vines. Yield was high but most fruit rotted in the field due to the wet conditions. In year two, with the exception of ‘Waltham Butternut’, fruit did not reach full maturity before plants collapsed and no yield data was taken. Fruit of the appropriate size, weight, and color to indicate maturity was only available for testing for some cultivars. Number of fruit tested for each cultivar ranged from one to twelve depending on availability of fruit. Most fruit were not mature and Brix values were below the 10°Bx standard on average for all cultivars, although ‘Waltham Butternut’ was close at 9.55°Bx (Table 5.8). Only ‘Waltham Butternut’ met the 10% standard for dry matter and was the only cultivar rated highly for taste.

In year three, yield was high at above 10 fruit/replicate for all cultivars except ‘Mrs. Amerson’s’ which only had an average of 7.5 fruit/replicate (Table 5.7). Average fruit weight was slightly below expected based upon cultivar descriptions provided by seed sources, with the exception of ‘Tan Cheese’. No information on expected fruit size was available for ‘Seminole x Waltham F6’. Dry matter content was above 10% for all cultivars except ‘Tan Cheese’ ($\overline{x}$=9.02; Table 5.8). Brix was above 10°Bx on average for ‘Seminole x Waltham F6’ and ‘Crowning’, and was very low in ‘Tan Cheese’ ($\overline{x}$=6.19) and ‘Seminole’ ($\overline{x}$=5.55).

Discussion

In this study, greater disease resistance appears to be present in *C. moschata* cultivars compared to *C. pepo*, with the exception of the butternut cultivars 'Waltham' and 'Metro P.M.R'. Several cultivars were found to be well-adapted to southeastern conditions and offered improved control of downy mildew and CYVD compared to the commonly grown cultivar 'Waltham Butternut'. Similar results were reported in studies that investigated field resistance to pickleworm, downy mildew, and powdery mildew, in which most cultivars of *C. moschata* were less affected than most cultivars of *C. pepo* (Dilbeck et al. 1974; Lebeda and Křístková 1993, 1994)

Downy mildew was the most damaging foliar disease evaluated during the three years of the study, and had completely or nearly defoliated susceptible cultivars such as 'Waltham Butternut', 'Metro P.M.R', and *C. pepo* 'Delicata Zeppelin' within three weeks after symptoms were first seen. Several of the *C. moschata* cultivars evaluated, such as 'Thai Kang Kob', 'Crowning', and 'Seminole', were developed in tropical or subtropical climates where downy and powdery mildew are present for much or all of the year, and appear to have the highest levels of resistance to these pathogens.

The downy mildew epidemics differed in starting date and disease progression in each year of the study. Factors typically influencing downy mildew development and progression include temperature, sun exposure, humidity, and amount of rainfall (Lebeda and Cohen 2011; Ojiambo et al. 2015). Downy mildew arrived sooner and severity was higher in years one and three compared to year two. The lower number of rainy days (17) in year two, compared to years one and three (23 for both), provided less optimal conditions for downy mildew development

(Georgia Automated Environmental Network) as sun exposure can reduce sporangia dispersal and germination (Kanetis et al. 2010).

The first observation of symptoms of downy mildew occurred more than two weeks earlier in year 3 (June 23rd) than in year 1 (July 10th) although disease progression was slower in year three when compared to year one. As some seeds had to be sown again due to poor germination, plants were 6 to 12 days younger at the start of the epidemic in year three than in year one. Age-related resistance is well documented in plants (Develey-Riviere and Galiana 2007; Kollar 1996), but interaction between downy mildew on foliage and plant age has not been studied in either squash or grape downy mildew systems. Older squash plants, like those in year one, may have been diverting more resources into fruit production and maturation than host defenses, which may explain why the epidemic progressed more rapidly in year one, despite similar climactic conditions in year three.

Some innate powdery mildew resistance appears to be present in *C. moschata* cultivars. Powdery mildew was not severe on any of the *C. moschata* cultivars tested, regardless of resistance levels to downy mildew, including on the downy mildew susceptible 'Waltham Butternut'. Previous trials with 'Waltham Butternut' by McGrath and Shishkoff (1999) also showed low powdery mildew severity (6.7%). While a negative correlation between downy and powdery mildew resistance was reported in summer squash cultivars of *C. pepo* (Lebeda and Kristkova 2000), this did not appear to be the case for most of the *C. moschata* cultivars in this study. Three cultivars: 'Seminole', 'Thai Kang Kob', and 'Crowning' had exceptionally high levels of resistance to both powdery and downy mildew.

Germination of powdery mildew conidia is inhibited by prolonged periods of free moisture on plant surfaces, making the disease less problematic in rainy years. The pathogen

prefers dry, but humid conditions, low light, dense plant canopies, and temperatures between 20 to 27°C (De Vicente et al. 2009; McCreight 2017). Year one was wet, with 23 rainy days between June 1st and August 1st, (Georgia Automated Environmental Network) and no powdery mildew was observed. Conditions were drier and powdery mildew severity was higher in year two with only 17 rainy days from June 1st to August 1st. Similar disease levels as year two were observed in year three which had 23 rainy days (Georgia Automated Environmental Network). The development of powdery mildew in years two and three may have been helped by the training of vines into dense plantings, which was not done in year one. Dense foliage provides ideal conditions for the powdery mildew pathogen with less UV exposure and higher humidity (De Vicente et al. 2009; McCreight 2017).

While vine training was necessary in this study to effectively evaluate pests and diseases, producers would likely achieve better yield by allowing vines to grow undisturbed. In year two, plant growth was controlled throughout the growing season and fruit yield was low when compared to years one and three. In year three, growth of vines were only controlled for the first half of the season. Moving vines to new positions, even when performed carefully, can damage the vines, foliage, and/or female flowers and may have contributed to a reduced yield in this study.

CYVD was only observed in the last two years as few adult squash bugs were observed during the critical four week window of infection in year one. While only year three showed any statistically significant differences in CYVD incidence, four cultivars: 'Tan Cheese', 'Crowning', 'Mrs. Amerson's', and 'Seminole' did not have more than 20% of plants with CYVD in both seasons. Of these, 'Mrs. Amerson's' only had 5% CYVD incidence in both seasons. Although several more seasons of testing would be needed for confirmation, these

cultivars may offer sources of CYVD resistance. ‘Delicata Zeppelin’ and ‘Waltham Butternut’, which are moderately to highly susceptible to downy mildew, both appear to be susceptible to CYVD with ‘Waltham Butternut’ showing the highest incidence in years two and three.

No correlation was seen between adult squash bug counts and CYVD incidence. On several occasions in both years two and three, CYVD was recorded, but no squash bugs were seen on these plants during the critical infection period during the first four weeks of growth, even though the count frequency was increased to three times weekly in year three. Therefore, a threshold population of squash bugs needed for transmission could not be determined. Looking at the heat maps, CYVD incidence did not appear to be randomly distributed across the field but occurred in clusters both years. Besler previously showed that anywhere from 5-50% of overwintering adults carried the bacterium (Besler 2014). However, 75% of recently emerged overwintering adults in both years of this study were found to carry the bacterium. The clustering of diseased plants could be influenced by movement patterns of the squash bugs across the field as they seek out preferred sites for feeding and egg laying, infecting plants as they move.

While squash bug numbers had no obvious effect on CYVD incidence, there were observed feeding preferences during the first four weeks after planting. Squash bugs in all life stages were most commonly found on the more vigorous cultivars such as ‘Seminole’, ‘Mrs. Amerson’s’, Crowning’, and ‘Tan Cheese’. Squash bugs are known to prefer larger and denser plants (Fargo et al. 1988; Palumbo et al. 1991). Two cultivars from this study, ‘North Georgia Candy Roaster’, a *C. maxima* cultivar, and ‘Tan Cheese’, a *C. moschata* cultivar, would be ideal candidates to test as trap crops for squash bugs due to their vigorous early season growth and squash bug preference. Trap cropping has been shown to be an effective method of squash bug control (Dogramaci et al. 2004; Pair 1997). Adler and Hazzard (2009) also showed ‘Blue

Hubbard', a *C. maxima* cultivar, was an effective trap crop for cucumber beetles, but did not evaluate squash bugs.

Overall, squash bug counts were lower in year three than in year two during the full eight week rating period (data not shown), despite more frequent ratings. Lower squash bug numbers in year three may be due to two factors: vine training and weather. In year three, when vines were only trained for part of the season, no exponential growth was observed in squash bug numbers. However, in year two, squash canopies became increasingly dense as the vine training continued throughout the season, providing ideal conditions for squash bugs (Fargo et al. 1988; Palumbo et al. 1991). The amount of rain received likely impacted squash bug populations as well. Greater precipitation, cooler average daily temperatures, and greater number of rainy days in year three likely impacted reproduction and survival of nymphs as previously seen by Olson et al. in a wet, cool year (1996).

Pickleworm damage has been shown to be variable between years (McCreight 2017). Pickleworm damage was not quantified in the first season, but damage appeared to be high in the two *C. pepo* cultivars 'Delicata Zeppelin' and 'Thelma Sander's Sweet Potato'. Due to their thinner skin, *C. pepo* cultivars are generally more susceptible to pickleworm infestations than *C. moschata* or *C. maxima* (Dilbeck et al. 1974; Elsey 1985). Even among *C. moschata* cultivars, there was variation in susceptibility. In year two, not enough fruits were available of most cultivars to make strong comparisons, but in general, thinner-skinned fruit such as those of 'Crowning' had more damage in both years.

In addition to disease resistance, fruit quality and yield are important considerations for grower profitability. Among the tropical and heirloom cultivars, 'Tan Cheese', while not exceptional in taste, yields well for a large-fruited variety and is an exceptionally vigorous plant

with good downy mildew resistance. The smaller fruiting ‘Seminole’ yields well and has exceptional resistance to downy and powdery mildew. ‘Crowning’ yielded well with resistance to downy and powdery mildew, although this cultivar may be more susceptible to pickleworm damage. ‘Mrs. Amerson’s’ has excellent disease resistance, but the fruit and seed cavity are large and the flesh has a low dry matter content resulting in a watery texture. ‘Mrs. Amerson’s’ may be better suited for soups and sweetened pies. Low seed germination was also common with this cultivar so more seeds are needed to achieve a good stand.

The remaining cultivars were not as attractive for fresh market production but may be good for home consumption. ‘Choctaw Sweet Potato’ performed very well as far as yield and downy mildew resistance, but fruit size was too large to be readily marketable. ‘Thai Kang Kob’, though exceptionally disease resistant with flavorful and unusual fruits, was very low yielding, and may be better for breeding improved resistant lines than production. The *C. pepo* cultivars ‘Thelma Sander’s Sweet Potato’ and ‘Delicata Zeppelin’ did not perform well in this study but may produce well in drier years.

C. moschata winter squash cultivars appear to be better adapted to the semi-tropical conditions of the southeast and several are good candidates for organic production in Georgia. The exception is ‘Waltham Butternut’, a *C. moschata* cultivar which may have some *C. maxima* background. ‘Waltham Butternut’ may perform well in a dry year in the southeast when disease and pest pressures are lower, but this cultivar is highly susceptible to multiple diseases in a wet year. In this study, early defoliation from downy mildew and death from CYVD resulted in only a few low quality fruits in year three. Breeding plants for target characteristics often leads to inbreeding depression, which can introduce susceptibility to breeding lines that were previously resistant (Kariyat et al. 2012; Schierup and Christiansen 1996). During selection for fruit

quality, shorter time to maturity, and smaller plants, ‘Waltham Butternut’ and ‘Metro P.M.R.’ became more susceptible to downy mildew and perhaps also CYVD.

To meet the demand for butternuts in the market, ‘Seminole x Waltham’ may be a good replacement. The cross was made in Virginia under organic growing conditions and this cultivar has the productivity, plant vigor, and disease resistance of ‘Seminole’, with equal to or better taste than ‘Waltham Butternut’. The fruits are somewhat larger than a traditional butternut, which may not appeal to all consumers, although further selection may reduce fruit size.

On-farm selection and improvement of cultivars that have adaption to local pest and disease pressures may facilitate the selection of appropriate cultivars for organic production in the southeast (Murphy et al. 2007; Sperling et al. 1993). Over time, selection can result in high disease resistance and yield as can be seen in ‘Seminole’, a highly resistant heirloom variety cultivated for centuries in Florida. ‘Seminole x Waltham’ is an excellent example of how controlled crossing and selection under organic conditions can result in an exceptional cultivar.

Literature Cited

Adam, K. L. 2006. Squash bug and squash vine borer: Organic controls. ATTRA-National Sustainable Agriculture Information Service.

Adler, L. S., and Hazzard, R. V. 2009. Comparison of perimeter trap crop varieties: Effects on herbivory, pollination, and yield in butternut squash. Environ. Entomol. 38:207-215.

Alston, D. G., and Barnhill, J. V. 2008. Squash bug. in: Utah Pest Fact Sheet Utah State University Extension, Logan, UT.

Besler, K. R. 2014. Epidemiology and management of cucurbit yellow vine disease, and characterization of the causal agent *Serratia marcescens*. University of Georgia, Athens, GA.

Besler, K. R., and Little, E. L. 2015. First report of cucurbit yellow vine disease caused by *Serratia marcescens* in Georgia. Plant Dis. 99:1175-1175.

Brett, C. H., McCombs, C. L., and Daugherty, D. M. 1961. Resistance of squash varieties to the pickleworm and the value of resistance to insecticidal control. J. Econ. Entomol. 54:1191-1197.

Bruton, B., Mitchell, F., Fletcher, J., Pair, S., Wayadande, A., Melcher, U., Brady, J., Bextine, B., and Popham, T. 2003. *Serratia marcescens*, a phloem-colonizing, squash bug-transmitted bacterium: Causal agent of cucurbit yellow vine disease. Plant Dis. 87:937-944.

Cohen, Y., Van den Langenberg, K. M., Wehner, T. C., Ojiambo, P. S., Hausbeck, M., Quesada-Ocampo, L. M., Lebeda, A., Sierotzki, H., and Gisi, U. 2015. Resurgence of *Pseudoperonospora cubensis*: the causal agent of cucurbit downy mildew. Phytopathology 105:998-1012.

De Vicente, A., Fernández-Ortuño, D., López-Ruiz, F., Pérez-García, A., Romero, D., and Torés Montosa, J. A. 2009. The powdery mildew fungus *Podosphaera fusca* (synonym *Podosphaera xanthii*), a constant threat to cucurbits: Pathogen profile. Mol. Plant Pathol.

Decker, K. B., and Yeargan, K. V. 2008. Seasonal phenology and natural enemies of the squash bug (Hemiptera: Coreidae) in Kentucky. Environ. Entomol. 37:670-678.

Develey-Riviere, M. P., and Galiana, E. 2007. Resistance to pathogens and host developmental stage: a multifaceted relationship within the plant kingdom. New Phytol. 175:405-416.

Dilbeck, J. D., Todd, J. W., and Canerday, T. D. 1974. Pickleworm resistance in *Cucurbita*. Fla. Entomol. 57:27-32.

Dogramaci, M., Shrefler, J. W., Roberts, B. W., Pair, S., and Edelson, J. V. 2004. Comparison of management strategies for squash bugs (Hemiptera: Coreidae) in watermelon. J. Econ. Entomol. 97:1999-2005.

Doughty, H. B., Wilson, J. M., Schultz, P. B., and Kuhar, T. P. 2016. Squash bug (Hemiptera: Coreidae): Biology and management in cucurbitaceous crops. J. Integr. Pest Manag. 7:1-8.

Elsey, K. D. 1985. Resistance mechanisms in *Cucurbita moschata* to pickleworm and melonworm (Lepidoptera: Pyralidae). J. Econ. Entomol. 78:1048-1051.

Fargo, W. S., and Bonjour, E. L. 1988. Developmental rate of the squash bug, *Anasa tristis* (Heteroptera: Coreidae) at constant temperatures. Environ. Entomol. 17:926-929.

Fargo, W. S., Rensner, P. E., Bonjour, E. L., and Wagner, T. L. 1988. Population dynamics in the squash bug (Heteroptera: Coreidae)-squash plant (Cucurbitales: Cucurbitaceae) system in Oklahoma. J. Econ. Entomol. 81:1073-1079.

Kanetis, L., Holmes, G. J., and Ojiambo, P. S. 2010. Survival of *Pseudoperonospora cubensis* sporangia exposed to solar radiation. Plant Pathol. 59:313-323.

Kariyat, R. R., De Moraes, C. M., Stephenson, A. G., and Mescher, M. C. 2012. Inbreeding increases susceptibility to powdery mildew (*Oidium neolycopersici*) infestation in horsenettle (*Solanum carolinense* L). Plant Signal Behav. 7:803-806.

Kollar, A. 1996. Evidence for loss of ontogenetic resistance of apple leaves against *Venturia inaequalis*. Eur. J. Plant Pathol. 102:773-778.

Lebeda, A., and Křístková, E. 1993. Resistance of *Cucurbita pepo* and *Cucurbita moschata* varieties to cucurbit downy mildew (*Pseudoperonspora cubensis*). Plant Var. Seeds 6:109-114.

Lebeda, A., and Křístková, E. 1994. Field resistance of *Cucurbita* species to powdery mildew (*Erysiphe cichoracearum*). J. Plant Dis. Protect. 101:598-603.

Lebeda, A., and Kristkova, E. 2000. Interactions between morphotypes of *Cucurbita pepo* and obligate biotrophs (*Pseudoperonospora cubensis*, *Erysiphe cichoracearum* and *Sphaerotheca fuliginea*). Acta Hortic. 510:219-225.

Lebeda, A., and Cohen, Y. 2011. Cucurbit downy mildew (*Pseudoperonospora cubensis*)—biology, ecology, epidemiology, host-pathogen interaction and control. Eur. J. Plant Pathol. 129:157-192.

McCreight, J. D. 2017. Compendium of cucurbit diseases and pests. Pages 1-9 in: Compendium of cucurbit diseases and pests. A. P. Keinath, W. M. Wintermantel and Z. T.A., eds. APS Press, St. Paul, Minn.

McGrath, M. T. 2004. Diseases of cucurbits and their management. Pages 455-510 in: Diseases of fruits and vegetables volume I: Diagnosis and management. S. A. M. H. Naqvi, ed. Springer Netherlands, Dordrecht.

McGrath, M. T., and Shishkoff, N. 1999. Evaluation of biocompatible products for managing cucurbit powdery mildew. Crop Protect. 18:471-478.

McGrath, M. T., and Shishkoff, N. 2003. First report of the cucurbit powdery mildew fungus (*Podosphaera xanthii*) resistant to strobilurin fungicides in the United States. Plant Dis. 87:1007-1007.

Murphy, K., Garland-Campbell, K., Lyon, S., and S. Jones, S. 2007. Evidence of varietal adaptation to organic farming systems. Field Crops Res. 102:172–177.

Nechols, J. 1987. Voltinism, seasonal reproduction, and diapause in the squash bug (Heteroptera: Coreidae) in Kansas. Environ. Entomol. 16:269-273.

Network, G. A. E. Georgia Weather. College of Agricultural and Environmental Sciences, University of Georgia, Griffin, GA.

Novero, E. S., Painter, R. H., and Hall, C. V. 1962. Interrelations of the squash bug, *Anasa tristis*, and six varieties of squash (*Cucurbita* spp.). J. Econ. Entomol. 55:912-919.

Ojiambo, P. S., Gent, D. H., Quesada-Ocampo, L. M., Hausbeck, M. K., and Holmes, G. J. 2015. Epidemiology and population biology of *Pseudoperonospora cubensis*: a model system for management of downy mildews. Annu. Rev. Phytopathol. 53:223-246.

Olson, D. L., Nechols, J. R., and Schuhle, B. W. 1996. Comparative evaluation of population effect and economic potential of biological suppression tactics versus chemical control for squash bug (Heteroptera: Coreidae) management on pumpkins. J. Econ. Entomol. 89:631-639.

Pair, S. D. 1997. Evaluation of systemically treated squash trap plants and attracticidal baits for early-season control of striped and spotted cucumber beetles (Coleoptera: Chrysomelidae) and squash bug (Hemiptera: Coreidae) in cucurbit crops. J. Econ. Entomol. 90:1307-1314.

Pair, S. D., Bruton, B. D., Mitchell, F., Fletcher, J., Wayadande, A., and Melcher, U. 2004. Overwintering squash bugs harbor and transmit the causal agent of cucurbit yellow vine disease. J. Econ. Entomol. 97:74-78.

Palumbo, J. C., Fargo, W. S., and Bonjour, E. L. 1991. Colonization and seasonal abundance of squash bugs (Heteroptera: Coreidae) on summer squash with varied planting dates in Oklahoma. J. Econ. Entomol. 84:224-229.

Schierup, M. H., and Christiansen, F. B. 1996. Inbreeding depression and outbreeding depression in plants. Heredity 77:461-468.

Sperling, L., Loevinsohn, M., and Ntabomvura, B. 1993. Rethinking the farmer's role in plant breeding: Local bean experts and on-station selection in Rwanda. Exp. Agric. 29:509-519.

Urban, J., and Lebeda, A. 2006. Fungicide resistance in cucurbit downy mildew – methodological, biological and population aspects. Ann. Appl. Biol. 149:63-75.

USDA. 2014. 2012 USDA census of agriculture. United States Department of Agriculture, Washington, DC.

Vogt, E. A., and Nechols, J. R. 1993. Responses of the squash bug (Hemiptera: Coreidae) and its egg parasitoid, *Gryon pennsylvanicum* (Hymenoptera: Scelionidae) to three *Cucurbita* cultivars. Environ. Entomol. 22:238-245.

Wadley, F. M. 1920. The squash bug. J. Econ. Entomol. 13:416-425.

Wayadande, A., Bruton, B., Fletcher, J., Pair, S., and Mitchell, F. 2005. Retention of cucurbit yellow vine disease bacterium *Serratia marcescens* through transstadial molt of vector *Anasa tristis* (Hemiptera: Coreidae). Ann. Entomol. Soc. Am. 98:770-774.

Zhang, Q., Melcher, U., Zhou, L., Najar, F. Z., Roe, B. A., and Fletcher, J. 2005. Genomic comparison of plant pathogenic and nonpathogenic *Serratia marcescens* strains by suppressive subtractive hybridization. Appl. Environ. Microbiol. 71:7716-7723.

Tables

Table 5.1. Winter squash cultivar seed sources and years planted. All seeds met requirements for certified organic production.

Cultivar	Years Grown
Cultivars from Commonwealth Seeds (formally Twin Oaks Farm)	
Choctaw Sweet Potato (S.P.)	2015
Crowning (Chinese Tropical Pumpkin)	2016, 2017
Seminole x Waltham (F5, F6)	2016, 2017
Seminole	2015, 2016, 2017
Thai Kang Kob	2015, 2016, 2017
Cultivars from Southern Exposure Seed Exchange	
Thelma Sander's Sweet Potato (S.P.)*	2015
North Georgia Candy Roaster**	2017
Delicata Zeppelin*	2015, 2017
Mrs. Amerson’s	2016, 2017
Tan Cheese	2016, 2017
Waltham Butternut	2015, 2016, 2017
Cultivars from Johnny’s Seeds	
Metro P.M.R	2015
All cultivars *Cucurbita moschata* except for cultivars marked with * *C. pepo* or ** *C. maxima*	

Table 5.2. Year two powdery mildew disease severity ratings of winter squash cultivars. Fifteen leaves were selected from the fourth through seventh leaves on vines and were rated on a scale of 0-5 with 0 representing no lesions, 1: 1 to 10%, 2: 11 to 24%, 3: 25 to 49%, 4: 50 to 99%, and 5: 100%. Scale numbers from the four replicates were converted to midpoint percentages for analyses. Treatments with the same letters indicate no significant difference (α=0.05) in powdery mildew severity between treatments based upon a one-way ANOVA and Tukey's HSD.

Cultivar	**Year Two Average Powdery Mildew Severity**		
	Days After Symptom Appearance		
	7	**14**	**21**
Waltham Butternut	3.79a	ND	ND
Mrs. Amerson's	1.28a	3.47a	8.41a
Tan Cheese	1.41a	2.19a	3.48a
Waltham x Seminole F5	0.46a	0.55a	1.78a
Thai Kang Kob	0a	0a	0a
Seminole	0a	0a	0a
Crowning	0a	0a	0a

ND=No data recorded. Waltham Butternut was only rated once due to early defoliation.

Table 5.3. Year three powdery mildew disease severity ratings of winter squash cultivars. Fifteen leaves were selected from the fourth through seventh leaves on vines and were rated on a scale of 0-5 with 0 representing no lesions, 1: 1 to 10%, 2: 11 to 24%, 3: 25 to 49%, 4: 50 to 99%, and 5: 100%. Scale numbers from the four replicates were converted to midpoint percentages for analyses. Treatments with the same letters indicate no significant difference (α=0.05) in powdery mildew severity between treatments based upon a one-way ANOVA and Tukey's HSD.

Cultivar	Year Three Average Powdery Mildew Severity	
	Days After Symptom Appearance	
	21	28
Mrs. Amerson's	4.08ab	6.38a
Tan Cheese	5.13a	6.37a
Seminole x Waltham F6	2.85abc	3.93a
Seminole	0.28bc	0.46b
Crowning	0c	0b
Thai Kang Kob	ND	ND
Waltham Butternut	ND	ND
North Georgia Candy Roaster	ND	ND
Delicata Zeppelin	ND	ND

ND=No data recorded. Not enough plants survived to maturity.

Table 5.4. Squash bug numbers during the first four weeks following planting in year two. Counts began at two weeks after germination and ended after two weeks. Adults, nymphs, and eggs were counted in a 1.5 m x 0.6 m block in the center of each replicate weekly. Treatments with the same letters indicate no significant difference (α=0.05) in squash bug counts between treatments based upon a one-way ANOVA and Tukey's HSD.

Squash Bug Population During First Four Weeks					
Adults		**Nymphs**		**Eggs**	
Cultivar	**Count**	**Cultivar**	**Count**	**Cultivar**	**Count**
Seminole	4.75a	Seminole	47.75a	Tan Cheese	13.00a
Crowning	3.25a	Mrs. Amerson's	39.50a	Crowning	10.75a
Tan Cheese	3.00a	Crowning	34.00a	Seminole	7.75a
Mrs. Amerson's	2.25a	Tan Cheese	25.25a	Mrs. Amerson's	4.50a
Waltham Butternut	2.00a	Thai Kang Kob	22.50a	Waltham Butternut	4.50a
Seminole x Waltham F5	0.75a	Seminole x Waltham F5	16.00a	Seminole x Waltham F5	4.25a
Thai Kang Kob	0.50a	Waltham Butternut	7.75a	Thai Kang Kob	1.50a

Table 5.5. Squash bug numbers during the first four weeks following planting in year three. Counts began two days after germination. Adults, nymphs, and eggs were counted in a center 1.5 m x 0.6 m block in each replicate three times weekly for the first three weeks and twice in the fourth week. Treatments with the same letters indicate no significant difference (α=0.05) in squash bug counts between treatments based upon a one-way ANOVA and Tukey's HSD.

Squash Bug Population During First Four Weeks					
Adults		**Nymphs**		**Eggs**	
Cultivar	**Counts**	**Cultivar**	**Counts**	**Cultivar**	**Counts**
Candy Roaster	11.75a	Waltham Butternut	0.25a	Tan Cheese	19.25a
Tan Cheese	6.00ab	Thai Kang Kob	0a	Seminole x Waltham F6	16.00ab
Seminole x Waltham F6	6.00ab	Mrs. Amerson's	0a	Candy Roaster	14.50ab
Waltham Butternut	4.50b	Seminole	0a	Thai Kang Kob	8.25ab
Seminole	4.00b	Seminole x Waltham F6	0a	Waltham Butternut	6.50ab
Crowning	3.75b	Crowning	0a	Delicata Zeppelin	6.25ab
Thai Kang Kob	3.50b	Tan Cheese	0a	Crowning	5.25ab
Delicata Zeppelin	1.75b	Delicata Zeppelin	0a	Mrs. Amerson's	2.75b
Mrs. Amerson's	0.75b	Candy Roaster	0a	Seminole	2.50b

Table 5.6. Pickleworm damage on fruits in years two and three. Pickleworm burrows were counted on harvested fruit. Harvest began 125 days after seed germination and five days after the latest maturation time provided by seed suppliers. Fruit were collected over the span of one week. Pickleworm ratings were performed seven days after the last day of harvest. Percentage affected was based on number of fruit containing at least one hole.

Cultivar	Number of Fruit	Fruit Affected (%)
Year Two		
Crowning	12	100.0
Thai Kang Kob	10	90.0
Seminole	26	80.8
Tan Cheese	15	60.0
Seminole x Waltham F5	46	39.1
Waltham Butternut	81	18.5
Mrs. Amerson's	21	7.4
Year Three		
Crowning	81	13.9
Tan Cheese	57	12.4
Seminole	145	7.2
Seminole x Waltham F6	68	5.5
Mrs. Amerson's	30	1.6

Table 5.7. Year three winter squash yield. Squash fruits from each replicate of each cultivar were counted and weighed. The average yield weight, number of fruits produced, and average fruit weight of each cultivar was determined. Expected fruit weight is based upon cultivar descriptions provided by sources of seed.

Year Three Yield Data				
Cultivar	**Average Yield**		**Average Fruit Weight (kg)**	**Expected Fruit Weight (kg)**
	Weight (kg)	**Fruit Number**		
Mrs. Amerson's	16.6	7.5	2.21	2.27-4.08
Seminole	28.7	36.3	0.79	1.36
Seminole x Waltham F6	35.7	27.0	1.32	ND
Crowning	35.7	22.7	1.57	1.81
Tan Cheese	40.3	11.8	3.42	2.72-5.44

ND = No data on expected fruit weight provided by seed source

Table 5.8. Fruit quality in years two and three. Brix ratings were taken using a refractometer (Atago PAL-1) with the juice extracted from frozen squash pieces. Dry matter was determined by weighing out 10-15 g of internal squash fruit flesh, baking at 105°C for 8 h and reweighing for a dry weight. Taste preferences were determined using a blind taste test (n=25). Brix and dry matter values above 10% or 10°Bx are considered acceptable for market.

Cultivar	Brix (°Bx)	Dry Matter (%)	Taste
Year Two			
Waltham Butternut	9.55	10.87	4.46 of 5
Seminole x Waltham F5	7.62	9.63	2.85 of 5
Mrs. Amerson's	6.85	7.95	3.08 of 5
Tan Cheese	6.90	6.99	2.50 of 5
Year Three			
Seminole x Waltham F6	10.97	11.51	3.92 of 5
Crowning	10.77	13.48	3.32 of 5
Mrs. Amerson's	9.20	11.89	3.00 of 5
Tan Cheese	6.19	9.02	2.95 of 5
Seminole	5.55	10.73	2.74 of 5

Figures

Figure 5.1. Winter squash field plots at UGA Horticulture Farm. Randomized complete block design with four replications was used to evaluate seven cultivars in years 1 and 2, and nine in year 3. Wider spacing of plants years two and three facilitated evaluation of plants and harvested fruit.

Figure 5.2. Examples of winter squash cultivars planted in year three. Front, left to right: Seminole, Seminole x Waltham F6, Waltham butternut, candy roaster, and Mrs. Amerson's. Back, left to right: Tan Cheese, two Crowning (aka Chinese tropical pumpkin) fruit showing variation in shape. All cultivars are *Cucurbita moschata* except 'Candy Roaster' which is *C. maxima*.

Figure 5.3. Squash insect pests. **A.** Adults and instar nymphs of the squash bug (*Anasa tristis)* feeding on butternut squash. Squash bugs progress through five instars over six to eight weeks. **B.** Pickleworm (*Diaphania nitidalis*) damage on butternut squash. Fruit is unmarketable when the larvae of this moth pest burrow into fruit.

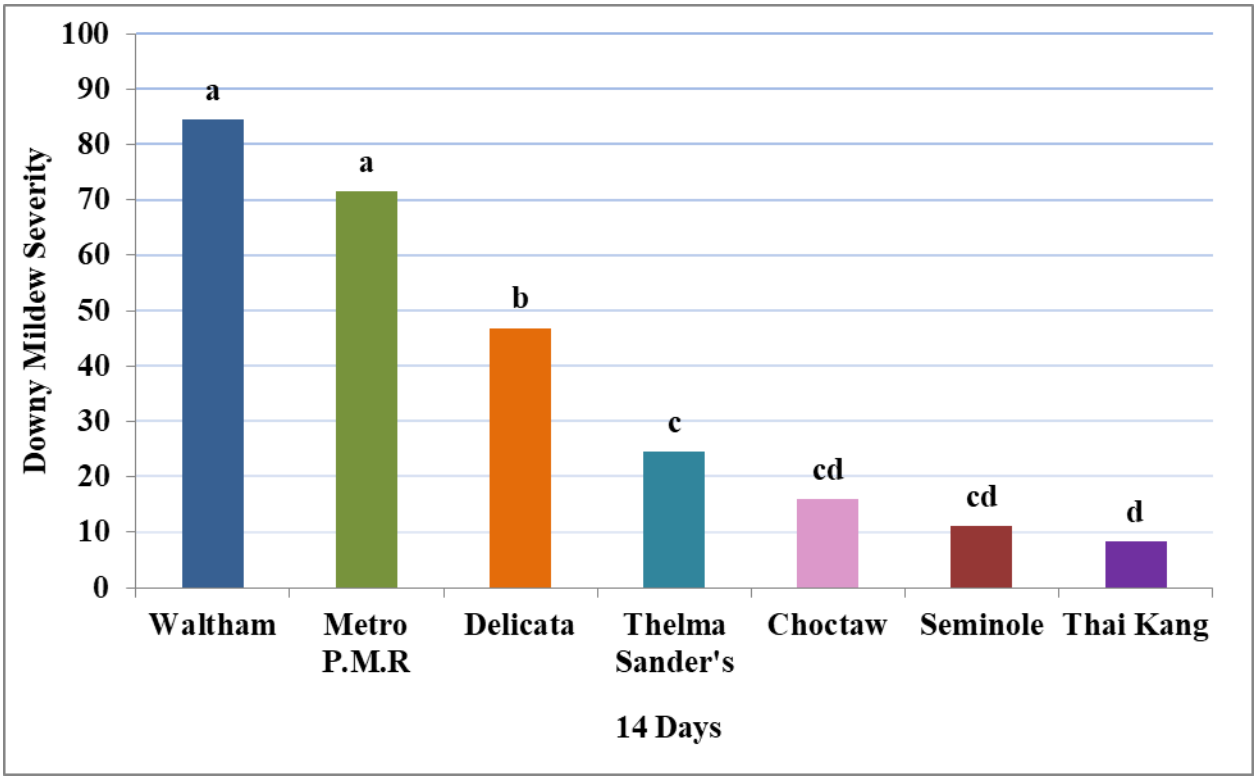


Figure 5.4. Downy mildew disease severity ratings in year one of winter squash cultivars. Fourteen days after first disease symptoms were seen in the field, fifteen leaves were randomly selected from the fourth through seventh leaves on each plant and were rated on a scale of 0-5 with 0 representing no lesions, 1: 1 to 10%, 2: 11 to 24%, 3: 25 to 49%, 4: 50 to 99%, and 5: 100%. Scale numbers of the four replicates were converted to midpoint percentages for analyses. Treatments with the same letters indicate no significant difference (α=0.05) in downy mildew severity between treatments based upon a one-way ANOVA and Tukey's HSD.

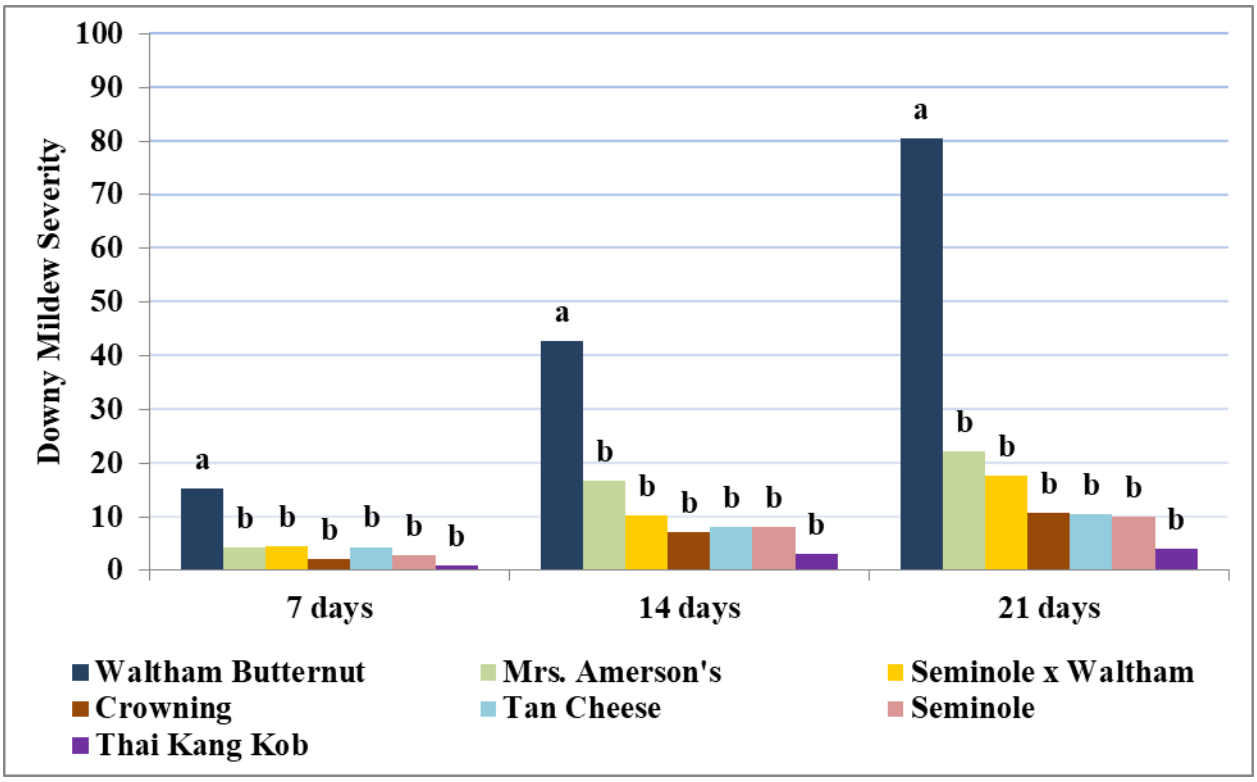


Figure 5.5. Downy mildew disease severity ratings in year two of winter squash cultivars. At seven, 14 and 21 days after the symptoms appeared in the planting, 15 leaves were randomly selected from the fourth through seventh leaves on vines and were rated on a scale of 0-5 with 0 representing no lesions, 1: 1 to 10%, 2: 11 to 24%, 3: 25 to 49%, 4: 50 to 99%, and 5: 100%. Scale numbers of the four replicates were converted to midpoint percentages for analyses. Treatments with the same letters indicate no significant difference (α=0.05) in downy mildew severity between treatments based upon a one-way ANOVA and Tukey's HSD.

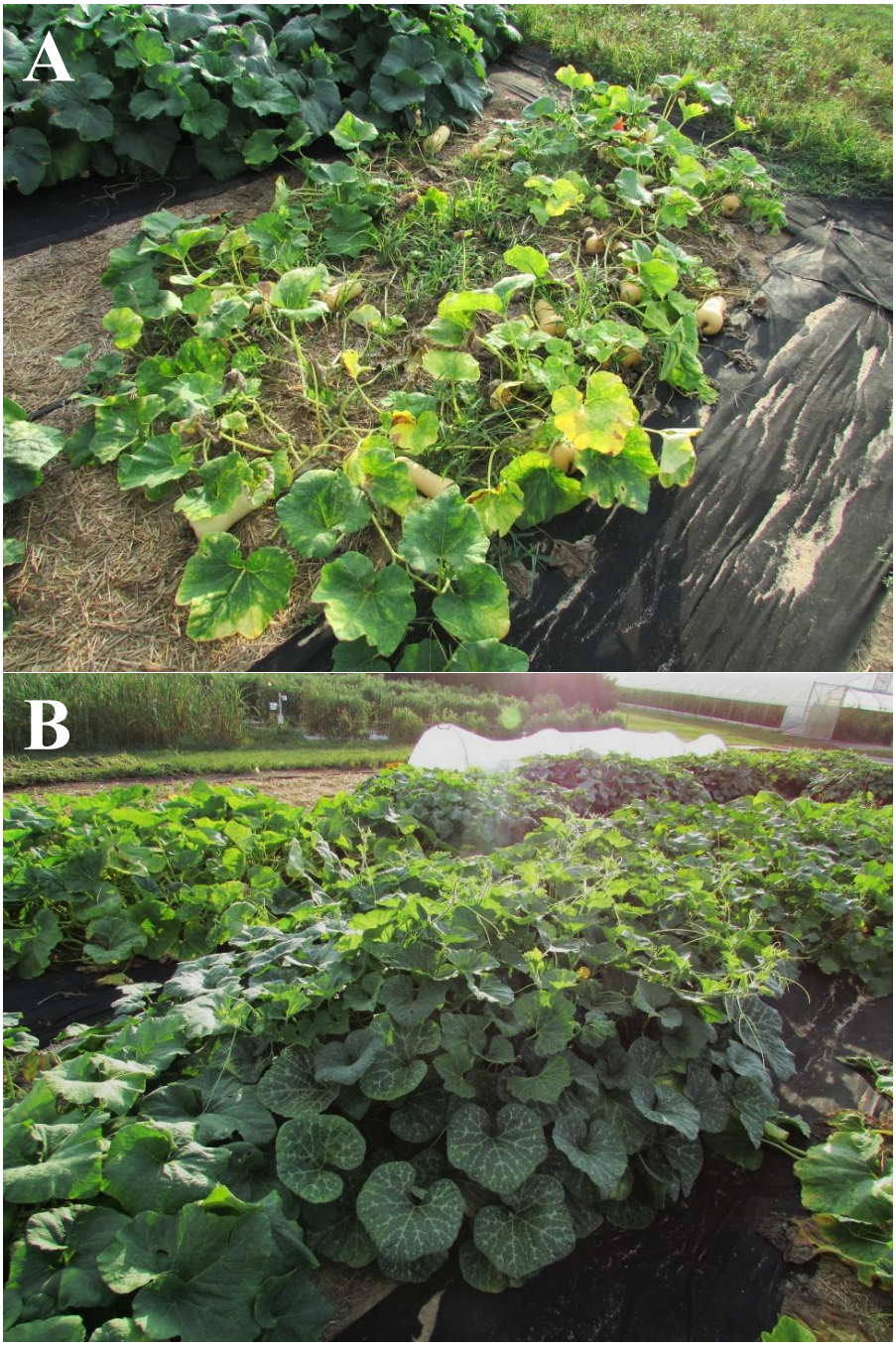


Figure 5.6. Downy Mildew on (A) susceptible ‘Waltham Butternut’ and (B) resistant ‘Crowning’ in year two, 14 days after symptoms first appeared.

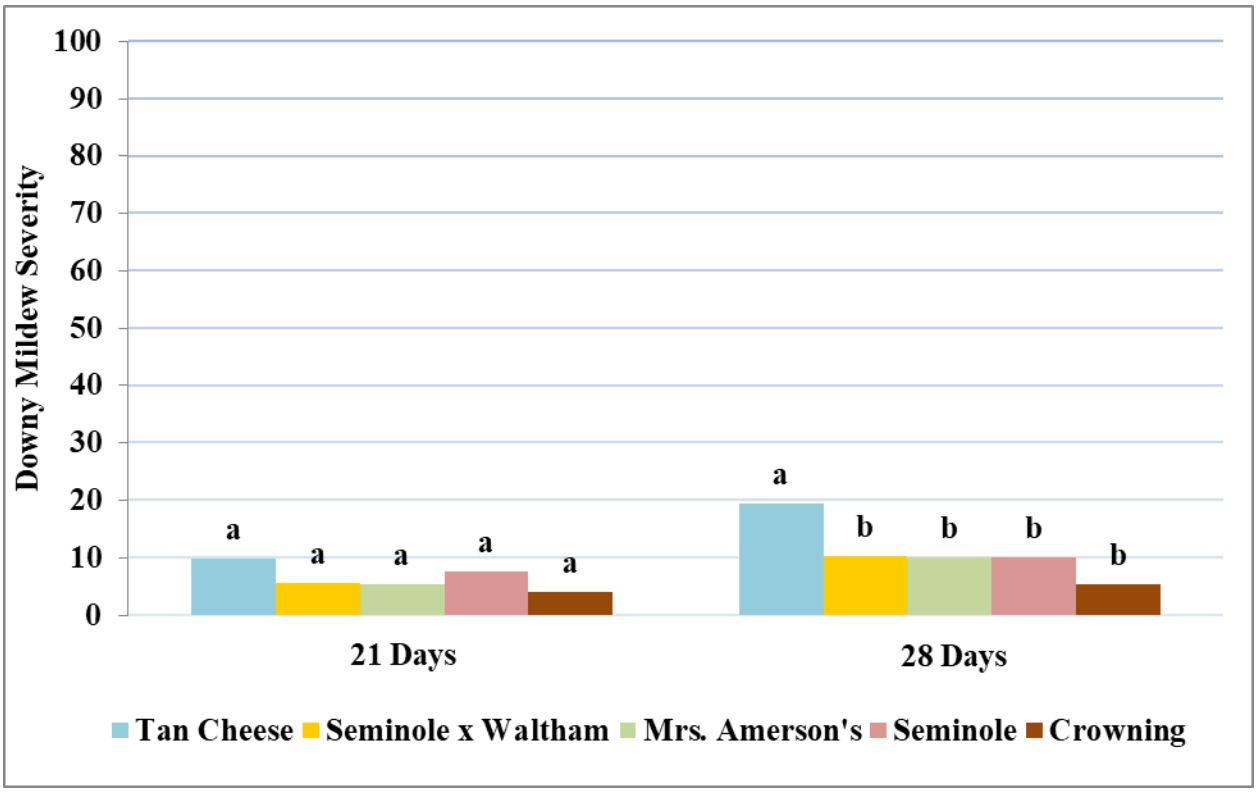
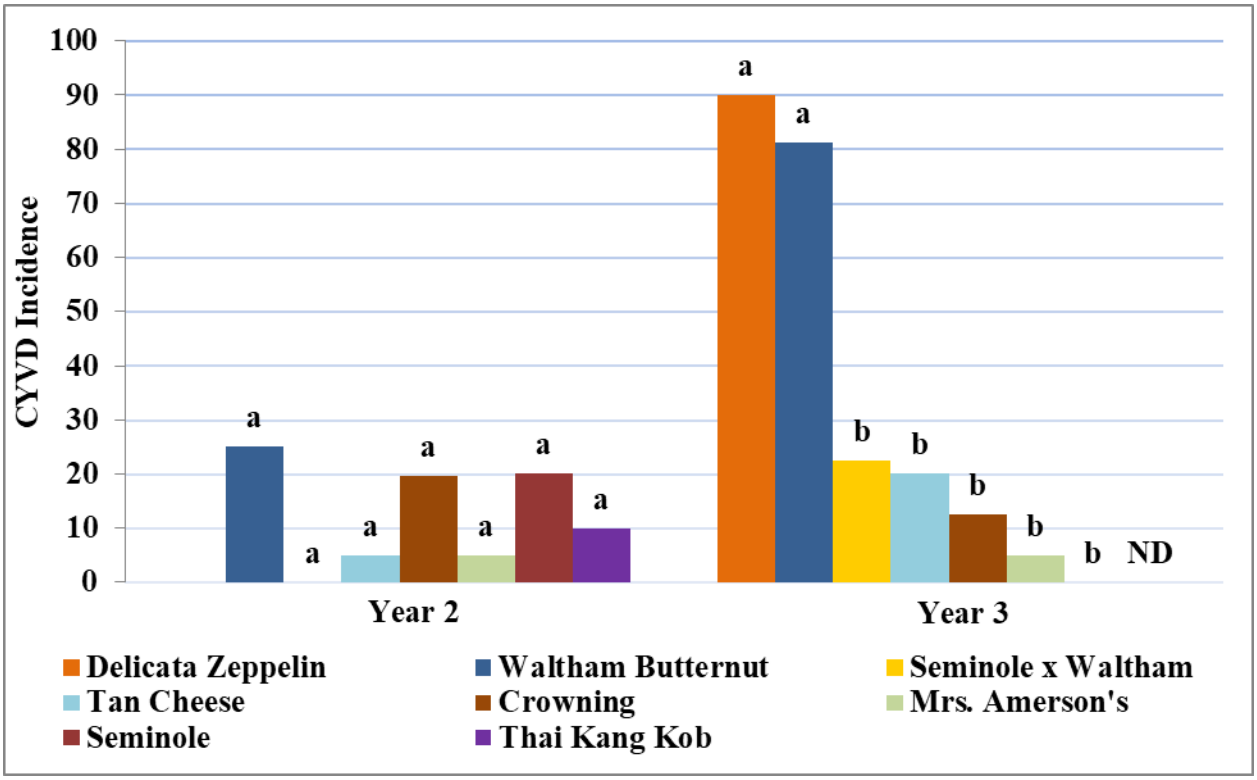


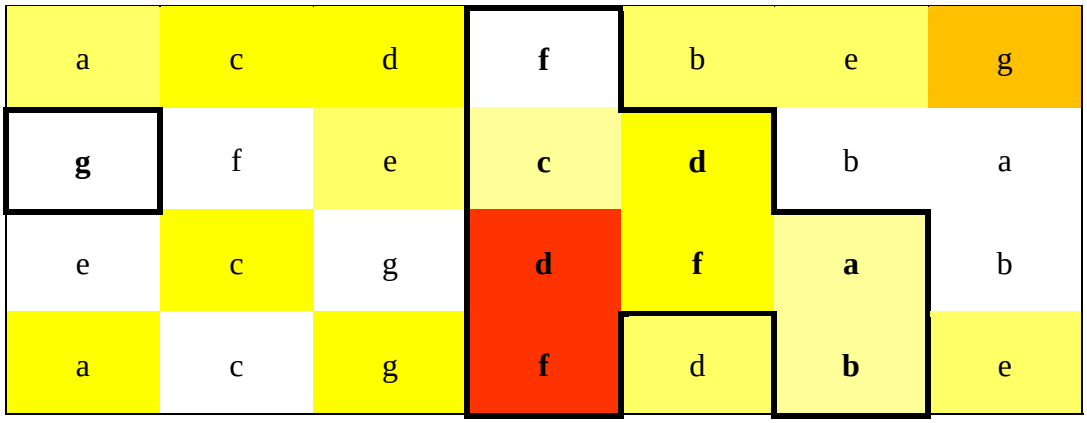
Figure 5.7. Downy mildew disease severity ratings in year three of winter squash cultivars. Fifteen leaves were selected from the fourth through seventh leaves on vines and were rated on a scale of 0-5 with 0 representing no lesions, 1: 1 to 10%, 2: 11 to 24%, 3: 25 to 49%, 4: 50 to 99%, and 5: 100%. Scale numbers of the four replicates were converted to midpoint percentages for analyses. Treatments with the same letters indicate no significant difference (α=0.05) in downy mildew severity between treatments based upon a one-way ANOVA and Tukey's HSD.

Figure 5.8. Incidence of cucurbit yellow vine disease (CYVD) in the third year of winter squash trials on ‘Delicata Zeppelin’ (front center, *C. pepo*). ‘Delicata Zeppelin’ and ‘Waltham Butternut’ (*C. moschata*, not shown) had significantly more CYVD in year three than all other cultivars evaluated.



ND=No data recorded

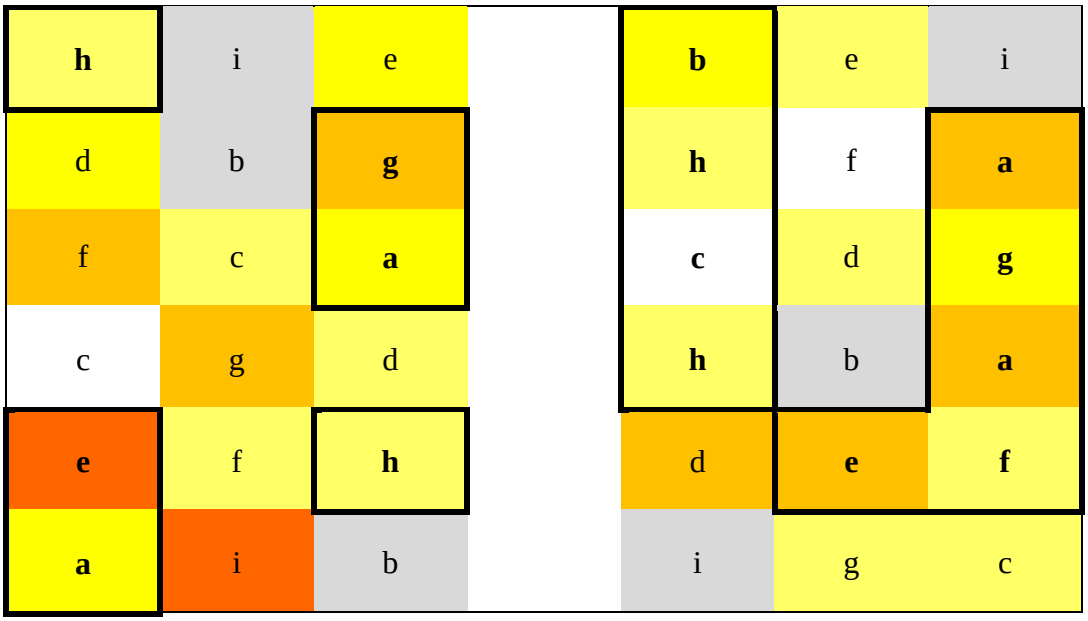
Figure 5.9. Incidence of cucurbit yellow vine disease (CYVD) in years two and three. Incidence was determined based on symptoms of yellowing and collapse in the field and confirmed using PCR and strain-specific primers. A one-way ANOVA was run to determine differences and Tukey's HSD was run for separation of means. Treatments with the same letters indicate no significant difference (α=0.05) in CYVD incidence between treatments based upon a one-way ANOVA and Tukey's HSD.



Adult Squash Bugs	Color Code
0	
1-2+	
3-5+	
6-9+	
10+	
CYVD Occurrence	

Letter Code	Cultivar
a	Waltham Butternut
b	Thai Kang Kob
c	Mrs. Amerson's
d	Seminole
e	Seminole x Waltham
f	Tan Cheese

Figure 5.10. Year two heat map of adult squash bugs during the first four weeks after planting and location of CYVD within the field. Adult squash bugs were counted weekly in the central 1.5 m x 0.6 m area of each replicate. High adult squash bug numbers are represented by orange and red blocks. Light yellow and yellow blocks indicate proportionately low to medium populations of adults. Letters within blocks correspond to cultivar. Contiguous regions outlined with emboldened lines and text indicates replicates where CYVD occurred.



Adult Squash Bugs	Color Code
No Data	
0	
1-2+	
3-5+	
6-9+	
10+	
CYVD Occurrence	

Letter Code	Cultivar
a	Waltham Butternut
b	Thai Kang Kob
c	Mrs. Amerson's
d	Seminole
e	Seminole x Waltham
f	Crowning
g	Tan Cheese
h	Delicata Zeppelin
i	Candy Roaster

Figure 5.11. Year three heat map of adult squash bugs during the first four weeks after planting and location of CYVD within the field. Adult squash bugs were counted three times weekly for the first three weeks and twice in the fourth week in the central 1.5 m x 0.5 m area of each replicate. High adult squash bug numbers are represented by orange and red blocks. Light yellow and yellow blocks indicate proportionately low to medium populations of adults. Letters within blocks correspond to cultivar. Contiguous regions outlined with emboldened text and lines indicate replicates where CYVD occurred.

CHAPTER 6

CONCLUSIONS

The first study compared CYVD strains of *S. marcescens* to the closely-related nonpathogenic rice endophyte, R01-A, using whole genome sequences. Several unique sequences in the CYVD strains may have possible virulence functions such as polysaccharide biosynthesis, homoserine lactone production, and siderophores. The type II, III, and IV secretion systems that are found in many other plant pathogenic bacteria could not be found in the CYVD strains and R01-A. R01-A and CYVD strains possess some components of a type VI system which contribute to virulence in some plant pathogenic bacterium, although the T6SS shared between the strains may not be functional. Further analysis is needed to determine the mechanisms of pathogenicity and virulence in CYVD strains. CYVD strains were found to have a reduced genome compared to R01-A and other *S. marcescens* strains which may be the result of adaptation to a more specialized niche. Comparisons of average nucleotide identity between *S. marcescens* strains revealed little genetic difference between CYVD strains and that some strains belong to a different *Serratia* species. Two regions shared between CYVD strain SB03 and ATCC13880 were identified and may contribute to the similarities seen using MLSA and rep-PCR. Targeting of more variable regions of the genomes for comparison may reveal more subtle differences between *S. marcescens* strains.

The second study compared the use of squash bug nymphs to needle inoculation to experimentally reproduce CYVD symptoms in squash. Uptake of CYVD strain P1 by second instar nymphs was fairly consistent and second instar nymphs were found to be capable of

transmitting CYVD. Incidence of phloem browning achieved using nymphs was comparable to needle inoculated plants and was higher than previous studies using squash bug adults. Needle inoculation and inoculation using nymphs did not produce full CYVD symptoms and could be further optimized.

The third study evaluated non-pathogenic bacterial strains as biocontrol agents against squash diseases. Three nonpathogenic, pigmented *S. marcescens* strains were antagonistic to CYVD strains in plate assays. Root dips of pumpkin plants with a solution of W11 did not result in consistent colonization of roots or stems, although W11 was present in some stems indicating possible dispersal through vascular tissues. No significant reduction in endophytic CYVD strain populations was seen in plants exposed to strain W11. In field trials using squash plants, seed soaks and soil drenches of W11 and *B. mojavensis* RRC101 resulted in no significant difference from controls in downy and powdery mildew severity and CYVD incidence, indicating that either the biocontrol strains are not effective in this system or the application methods need improvement.

The fourth study evaluated winter squash cultivars for pest and disease resistance, yield, and fruit quality. With the exception of butternut cultivars which may have some *C. maxima* ancestry, *C. moschata* cultivars had good resistance to downy mildew and powdery mildew. CYVD occurred in two out of three years, with no significant difference between cultivars in the second year. 'Waltham Butternut' and the *C. pepo* cultivar 'Delicata Zeppelin' had a significantly higher incidence of CYVD in year three while the remaining *C. moschata* had relatively low CYVD incidence in both years. Squash bugs had no significant preference in year two, but preferred the *C. maxima* cultivar 'North Georgia Candy Roaster' over most cultivars in year three. Presence of squash bugs during the infective period was not correlated with CYVD

incidence. Yields were excellent for most *C. moschata* cultivars in year three and fruit quality was very good in ‘Seminole x Waltham’ and the tropical pumpkin ‘Crowning’. Based on these results, some of the *C. moschata* are the preferred cultivars for consistent yields under organic conditions in the southeast and several of these cultivars offer promising sources of disease resistance for cultivar improvement.