CHARACTERIZATION OF *ACIDOVORAX CITRULLI* VIRULENCE ON WATERMELON AND MELON

by

MARIJA ZIVANOVIC

(Under the Direction of Ronald R. Walcott)

ABSTRACT

Acidovorax citrulli is the causal agent of bacterial fruit blotch (BFB) of cucurbits whose population can be distinguished into two groups (I and II). The goal of this research was to further characterize the differences between these groups. We observed no difference in temporal population growth dynamics between AAC00-1 (group II) and M6 (group I) on watermelon and melon. In contrast, in a natural infection assay relative BFB incidence on melon seedlings exposed to group II *A. citrulli* strains was significantly lower (~ 14.5 %) than on melon seedlings exposed to group I strains (~ 85.3 %), or watermelon seedlings exposed to group I and II strains (81.7 % and 82.3 %, respectively). We also investigated the role of the type III effector *AvrBsT* from AAC00-1 by expression in M6 and found decreased virulence of the mutant on watermelon and melon seedlings relative to wild-type AC00-1 and M6 strains.

INDEX WORDS: *Acidovorax citrulli*, bacterial fruit blotch, relative BFB incidence

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B.S., University of Belgrade, Serbia, 2010

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

2014

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May 2014

ACKNOWLEDGEMENTS

I would like to thank everybody who made the completion of my thesis possible: Dr. Ron Walcott, Dr. Tim Denny, Dr. David Langston, Dr. Harald Scherm, Ms. Anita Castro-Sparks, friends, grad students. Apologies to those I did not name specifically.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Brief history, nutritional value, and economic significance of the watermelon crop.

Watermelon (*Citrullus lanatus (Thunb.)* Matsum & Nakai) belongs to the family Cucurbitaceae, subfamily Cucurbitoideae, and it is native to Africa (18). The species has been divided into three subspecies: subsp. *vulgaris* (Schrad. Ex Eckl. et Zeyh.) Fursa, subsp. *lanatus*, and subsp. *mucosospermus* Fursa (18). The most widely cultivated forms with sweet red fruit have been assigned to the subspecies *vulgaris*. The subspecies *lanatus* originated from the Kalahari desert, where it is used as an essential source of food and water and is known locally as tsamma melon. The third subspecies, *mucosospermus*, is grown in West Africa where its soft seeds are a source of edible oil (18).

The edible portion of the watermelon fruit is comprised of approximately 91.45% water and 7.55% total carbohydrates, of which, 6.20% are sugars (Watermelon nutritional facts source: USDA National Nutrient Database for Standard Reference. Release 19. Internet resource: http://www.dietaryfiberfood.com/fruits-vegetables/watermelon-benefits.php). Watermelon is rich in carotenoids, mostly lycopene, and therefore has antioxidant activity. Watermelon seeds are excellent sources of protein (35%) and oil (50%). Dietary fiber makes up 5% percent of the seed, and it has significant amounts of phosphorus, potassium, magnesium, calcium and iron (Watermelon nutritional facts source: USDA National Nutrient Database for Standard Reference. Release 19. Internet resource: http://www.dietaryfiberfood.com/fruits-vegetables/watermelon-benefits.php).

China is the world's leading watermelon producer with approximately 70 million tons produced in 2011. The United States is the fifth largest watermelon producer in the world with approximately 1.8 million tons produced in the same year (Food and Agriculture Organization: FAOStat. United Nations. Internet resource: http://webcache.googleusercontent.com/search?q=cache:_abbaEuHVpUJ:usda01.library.cornell.edu). The economic value of watermelon production in the U.S. in 2012 was $520.8 million and Florida and Georgia were the leading watermelon producing states, followed by California and Texas. In the U.S., fruits are mainly grown for fresh consumption. The growing season is from May through August and the crop is usually ready for harvest 90 days after planting. (Agricultural Marketing Resource Center: Watermelon. Iowa State University. Internet resource: http://www.agmrc.org/commodities__products/vegetables/watermelon/).

Economic value of the melon crop. China is the world's leading producer of melons with approximately 12 million tons produced in 2009, while the U.S. has the fourth biggest melon production (approximately 1 million tons produced in 2009). Additionally, in 2012, the economic value of U.S. melon production was $325.3 million. (United States Department of Agriculture: Economics, Statistics and Market Information System. Internet resource: http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1478). The primary producers of melons are California, Arizona, Texas, Georgia and Florida. The U.S. is also one of the world's leading consumers of melons. (Agricultural Marketing Resource Center: Melon Profile. Internet resource: http://www.agmrc.org/commodities__products/vegetables/melon-profile/).

Bacterial fruit blotch. One economically important threat to cucurbit production is bacterial fruit blotch (BFB) caused by the Gram negative bacterium, *Acidovorax citrulli.* The pathogen was first described in 1978 by Schaad *et al.* and named *Pseudomonas pseudoalcaligenes* subsp. *citrulli* (64). In 1992, Willems *et al.* proposed a reclassification of the bacterium to the genus *Acidovorax* as *A. avenae* subsp. *citrulli* (64,82). Finally, the elevation of *A. avenae* subsp. *citrulli* to the species level, as *A. citrulli*, was proposed by Schaad *et al.* in 2008 (65) based on DNA/DNA hybridization assays; sequence analysis of the 16S rDNA and the 16S-23S rDNA internally transcribed spacer region; amplified fragment length polymorphism analysis; and phenotypic assays.

Bacterial fruit blotch was first reported on watermelon seedlings of several plant introduction (PI) lines in Georgia (USA) in 1965 (67). However, it was not until the late 1980s, when severe outbreaks occurred in watermelon fruit production fields in the Mariana Islands (79), Indiana (44), and Florida (66), that the economic importance of the disease was realized. In some disease outbreaks, yield losses exceeded 90% (54). Since 1989, BFB has continued to occur sporadically throughout the southeastern U.S. and around the world (13).

Geographic distribution and host range. Initially, BFB was considered a threat only to watermelon seedlings, with low damage potential in the field (67). However, after the first outbreak in the Mariana Islands in 1987 (79), when entire fields of watermelon were lost to fruit rot, the destructive potential of this pathogen was acknowledged. Subsequently, frequent BFB outbreaks in commercial watermelon fields occurred in different parts of the U.S. with high yield losses (11,44,66). *A. citrulli* spread worldwide and was reported to occur naturally on various cucurbits, including honeydew (*Cucumis melo* L. var. *inodorus* Naud.), citron melon (*Citrullus lanatus* var. *citroides* (Bailey) Mansf.), cucumber (*Cucumis sativus* L.), pumpkin (*Cucurbita*

pepo L.), and gramma (*Cucurbita moschata* L.) (35,36,43,52,53). Bacterial fruit blotch outbreaks have been reported in major watermelon production regions of the Americas, Asia, the Middle East (Turkey, Iran and Israel), Europe and Australia (3,14,56,58,59,62).

Etiology. *Acidovorax citrulli* is a rod-shaped cell with average dimensions of 0.5 by 1.7 μm that is motile by a single polar flagellum, approximately 5.0 μm long (64). On King's medium B (KMB) (42), *A. citrulli* colonies are visible after 48 h (64) and are non-fluorescent, round with entire edges, translucent, smooth, and slightly convex. Colonies can reach 2-3 mm in diameter after 5 days of growth at 30^{o}C (64). *A. citrulli* is arginine dihydrolase-negative, oxidase-positive, negative for pitting of CVP media, negative for nitrate reduction and levan production, and grows at 42^{o}C. The pathogen is also lipolytic and does not hydrolyze starch, but it causes slight gelatin hydrolysis (66). *A. citrulli* utilizes β-alanine, D-serine, n-propanol, ethanol, citrate, and fructose, but it does not grow on sucrose, or glucose (64). *A. citrulli* strains generally induce a hypersensitive response on tobacco leaves by 24 h after inoculation (66). The GC content of *A. citrulli* ranges from 65-67% (64).

BFB symptoms. Watermelon and melon seedlings and fruit are highly susceptible to *A. citrulli*. Typical BFB symptoms on seedlings consist of water-soaked lesions on cotyledons and hypocotyls, seedling collapse, and death. Lesions on the cotyledons are often adjacent to the veins and become necrotic over time (13). The earliest symptoms of BFB can be detected on the undersides of the cotyledons of 7 to 10-day-old seedlings depending on the level of seedborne inoculum (45). Lesions on mature foliage may be inconspicuous, and include small, dark-brown, necrotic spots, with margins that appear water-soaked (45). The most characteristic BFB symptom, however, is a dark, olive-green blotch on the upper side of the fruit. The blotches rapidly increase in size, and the initial site of infection becomes necrotic. In advanced stages of

lesion development, the epidermis of the rind ruptures, and often a sticky, clear substance oozes from the fruit. Further rotting and collapse of the fruit is normally due to tissue colonization by secondary invading organisms. *A. citrulli* also causes water-soaked pits on the surface of muskmelon fruit (45,58). The lesions on the fruit of honeydew and citron melon are circular, 3-10 mm in diameter, and do not extend into the flesh (35,36). On cucumber leaves, BFB lesions are angular, chlorotic, and water-soaked, and white bacterial ooze may be observed on the undersides of leaf surfaces (53). Lesions are delimited by veins, and over time turn reddish-brown. No symptoms have been reported on cucumber stems or fruit (53). BFB symptoms on pumpkin include necrotic, V-shaped lesions, on the margin of the leaves, that extend inward, toward the mid-rib (43). Round, necrotic spots or cracks may develop on fruit surfaces and tissue surrounding these lesions becomes soft and wrinkled. The rot expands into the flesh of the fruit, and infected fruit may eventually collapse (43).

BFB disease cycle. Infected seed is the primary source of inoculum for most BFB outbreaks. Seed-to-seedling transmission of BFB was demonstrated by Hopkins and Thompson in 2002 for various cucurbits including watermelon, muskmelon, honeydew, acorn squash, butternut squash, yellow squash, zucchini squash, cucumber, and pumpkin. However, not all of these species exhibited BFB fruit symptoms in this study (30). Currently, seedling transplanting, is routinely practiced in commercial watermelon production. Hot and humid conditions in the greenhouse promote disease development and overhead irrigation contributes to rapid BFB spread by splash dispersal of the bacterium (45). Depending on the amount of inoculum present on/in the seed, aggressiveness of the *A. citrulli* strain, environmental conditions, and the host plant, seedling symptoms may vary from severe to asymptomatic (4). It was recently reported

that a single seed containing 10 *A. citrulli* CFU/ml can result in BFB seed-to-seedling transmission under greenhouse conditions (21).

Asymptomatic infected seedlings or those with epiphytic *A. citrulli* populations may be transplanted into the field where they can serve as sources of inoculum for BFB field outbreaks. However, the progress and final impact of BFB in the field depend on the environmental conditions (4). Heavy rainfall and windy conditions during the spring or summer are conducive to BFB development (63). Infected transplants in the field are a source of inoculum for new leaves and plants, and more importantly, undeveloped fruit (45). The pathogen infects fruit by penetrating through stomata (24), or through the infection of female blossoms (75). *A. citrulli* invasion of watermelon fruit decreases 2-3 weeks after anthesis due to the deposition of wax over stomata on the fruit surface (24). Infected fruit are symptomless during the early stages of development, but characteristic water-soaked lesions appear shortly before harvest maturity (13). Infected fruit may rot in the field leaving infected seed in the soil. Infected volunteer watermelon plants that result from these seeds and cucurbit weeds carrying *A. citrulli* can be sources of inoculum for subsequent BFB outbreaks (45).

Biology of seed infection. There are three general pathways by which seed become infected by pathogens: penetration of the ovary pericarp; systemic movement of the pathogen from the mother plant to seed tissues; and invasion of the pistil (75). No evidence was found for systemic migration of *A. citrulli* in watermelon plants (61). According to Rane and Latin, only seeds harvested from symptomatic fruits were infected and transmitted the pathogen to seedlings (61). However, since only asymptomatic fruits are used in commercial seed production, it is unlikely that seeds produced in symptomatic fruits contribute to natural outbreaks involving commercial seed (13). Walcott *et al.* reported that female watermelon flowers inoculated with *A.*

citrulli yielded infected seeds within symptomless fruits (75). Similarly, Bahar *et al.* demonstrated that under conditions that were non-conducive to BFB, seeds extracted from asymptomatic melon fruits were infected with *A. citrulli* (5).

In addition to observing that *A. citrulli* colonized the styles and stigmas of female watermelon flowers, Lessl *et al.* reported a strong positive correlation between the concentration of *A. citrulli* inoculum applied to stigmas and the percent of infected seedlots (47). The authors also observed a strong linear relationship between blossom inoculum level and mean percent of seeds infected within the seedlots that exhibited BFB symptoms (47). Subsequently, Dutta *et al.* demonstrated that pistil invasion results in the deposition of *A. citrulli* cells under the perisperm-endosperm layer, while penetration of the ovary pericarp results in deposition of the bacterium under the seed coat (20). These studies confirmed that watermelon flower infection by *A. citrulli* can result in the production of infected seed within asymptomatic fruit.

Management of BFB. BFB management can be achieved only through integration of a range of measures in seed, transplant, and fruit production (13). *A. citrulli*-infected seed is usually the primary source of inoculum, and therefore the use of pathogen-free seed is crucial for effective BFB management (74). To avoid contamination, seed production fields must be separated from other cucurbit fields (13). Watermelon seed are produced in cool, dry climates, and subjected to visual inspections for BFB symptoms (75). Additionally, rigorous seed health testing is routinely performed. However, standard seed detection assays often display inadequate levels of sensitivity and specificity (77). For PCR-based seed health assays, high quality DNA must be extracted from the target pathogen, which is difficult to achieve due to mixed populations of seedborne microorganisms and PCR inhibitors in seeds (77). Therefore, modifications have been made to PCR-based assays to improve detection sensitivity and

efficiency. These modifications include DNA purification, enrichment of target organisms followed by PCR (BIO-PCR), immunomagnetic separation and PCR (IMS-PCR), and magnetic capture hybridization and PCR (MCH-PCR) (74,76,77). With BIO-PCR seed samples are washed in an appropriate buffer and seed wash samples are plated on semi-selective media. After incubation for 2-3 days, DNA is extracted from colonies and used for PCR with specific primers (77). Immunomagnetic separation involves the use of microscopic magnetic beads (IMBs) coated with antibodies specific to the target pathogen, to separate pathogen cells from heterogeneous cell mixtures (77). Sequestered cells can be recovered on selective media or used for DNA extraction and PCR. IMS-PCR was developed and optimized for detection of *A. citrulli* in watermelon seeds by Walcott and Gitaitis (74,76). MCH-PCR involves the hybridization of single-stranded, biotinylated, oligonucleotide probes, conjugated to streptavidin-coated magnetic beads (SCBs) with single-stranded target DNA from crudely prepared seed sample DNA, followed by a species-specific real-time PCR assay (26). Despite these improvements in seed health testing, PCR-based techniques are still not routinely practiced for detection of *A. citrulli* in commercial seed production and conventional seedling grow-out (SGO) is the industry standard (26,77). Seedling grow out involves growing out 10,000 – 50,000 seeds under greenhouse conditions and visually inspecting the resulting seedlings for BFB symptoms (76). This assay requires substantial greenhouse space, with uniform, disease conducive environmental conditions, and technicians trained to recognize BFB seedling symptoms (76). Subsequent confirmatory assays are often needed to conclusively identify *A. citrulli* (76) which increases the time needed to complete the assay to more than a month. Additionally, Walcott *et al.* reported that SGO only detected 12.5% (1/8) and 37.5% (3/8) of watermelon seedlots (n = 10,000 seeds)

with 0.01 and 0.1% infected seeds, respectively (76) which was deemed inadequate since the infection threshold of 1 infected seed in 30,000 seeds is the industry standard.

A range of antimicrobial seed treatments have been reported to eradicate *A. citrulli* from infected seeds. Sowell and Schaad reported that streptomycin and sodium hypochlorite reduced, but did not eradicate BFB seedling transmission (67). Hydrochloric acid was also reported to prevent BFB seedling transmission, but it had adverse effects on seed germination (31). Peroxyacetic acid was reported to eliminate *A. citrulli* and other pathogens, such as *Didymella bryoniae* and *Fusarium oxysporum*, from watermelon and melon seeds (31) and currently, peroxyacetic acid is routinely used in commercial watermelon seed production. Despite this, BFB outbreaks continue to occur, suggesting that this treatment is not entirely effective (13). Exposure to chlorine gas (68) or acidic electrolyzed water (33) have also been proposed as seed treatments, but are not routinely practiced. Despite this array of chemical seed treatments, additional measures are needed to consistently manage BFB (13).

Copper based compounds like Kocide (copper hydroxide) and Mankocide (mancozeb and copper hydroxide) are the most widely used foliar treatments for BFB and are most efficacious if used as protective measures (13). Biweekly sprays are recommended if no symptoms are observed, and weekly treatments are required to prevent disease spread during epidemics (28). However, phytotoxicity and the development of copper-tolerant *A. citrulli* strains are potential problems associated with the use of copper based compounds (28,78).

Finally, resistance is the most desirable BFB management strategy. Hopkins and Thompson reported five accessions of watermelon from Zimbabwe and Zambia with high levels of BFB resistance (29). In another study, four *Cucumis melo* and one *C. ficifolius* lines out of 332 *Cucumis* spp. accessions exhibited significant levels of BFB resistance (80). Additionally, Bahar

et al. screened commercial melon cultivars and breeding and wild melon lines for BFB resistance or tolerance and observed that plant responses differed depending on the inoculation method (5). They reported that four cultivars exhibited tolerance in seedling transmission assays, while one was tolerant in all assays employed (5). Despite these reports, there are no currently commercially available watermelon or melon cultivars with significant levels of BFB resistance (13).

Genetic diversity of *A. citrulli*. The diversity of *A. citrulli* was initially assessed in an Australian population by O'Brien and Martin (58). Carbon substrate utilization profiles and pathogenicity assays were employed to characterize strains recovered from several cucurbit hosts including watermelon, rockmelon, and honeydew. Carbon substrate utilization profiles separated the strains into two groups, namely, those recovered from rockmelon and honeydew that failed to utilize L-leucine, but utilized 2-amino ethanol, and those recovered from watermelon that utilized L-leucine, but were unable to utilize 2-amino ethanol. In pathogenicity tests all strains exhibited a high level of virulence on watermelon; however, the strains isolated from rockmelon and honeydew were significantly more virulent on rockmelon plants than those from watermelon (58). Subsequently, Walcott *et al.* characterized a population of *A. citrulli* strains recovered from cucurbitaceous hosts in the U.S., China, Taiwan, Thailand, Canada, Australia and Brazil (78) using biochemical (carbon substrate utilization) and genetic analysis (DNA fingerprinting by pulsed-field gel electrophoresis (PFGE) and repetitive extragenic pallindromic (REP) PCR). Two distinct groups, I and II, were established based on these analyses. Eighty-two percent of the group I strains were isolated from non-watermelon hosts. Among the group I strains, the type strain alone utilized L-leucine as a sole carbon source. In contrast, 94% of the group II strains were recovered from watermelon and 96% of these strains utilized L-leucine (78). Group II *A.*

citrulli strains were more virulent on watermelon than melon, while group I strains were moderately virulent on all tested cucurbit hosts (78). In 2005, a similar study done on an *A. citrulli* population from Israel yielded similar results (14). The Israeli strains were distinguished into two distinct clades, one associated with watermelon and the other with non-watermelon cucurbit hosts (14). Furthermore, in 2009, a multilocus sequence typing assay based on seven conserved loci was used to investigate the diversity (23) of an *A. citrulli* population isolated predominantly from China. This population included strains from other countries, including eight watermelon strains from the 1978 BFB outbreaks in the U.S. (64). This study revealed two major clonal complexes (CCs), CC1 and CC2, that corresponded with groups I and II from previous studies. Strains in CC2 were predominantly from watermelon, which coincided with the group II established by Walcott *et al.*, and Burdman *et al.* However, of eight watermelon strains from the 1978 BFB outbreaks in the U.S., six were typed as CC1, and the Chinese strains that were predominant in CC1 originated nearly equally from melon and watermelon (23). This was unexpected because the U.S. group I strains were recovered mostly from non-watermelon hosts. Therefore, *A. citrulli* population structure appears to differ between the U.S. and China (23,78). Additionally, it has recently been shown that two *A. citrulli* strains originating from India (Zum4000 and Zum4001) differ from group I and group II strains in their type III secretion effector repertoire and thus potentially form a separate group (group III) (Eckshtain-Levi *et al.*, Phytopathology, *In review*). More representative strains are required to confirm this putative third group.

Pathogenicity and virulence factors. A complete genome sequence for AAC00-1, a group II *A. citrulli* strain has been available since 2007 (NCBI accession number NC_008752). The AAC00-1 genome consists of a single circular chromosome of approximately 5.3 Mb, and

putatively encodes 4,858 genes. Additionally, the genome of M6, a group I *A. citrulli* strain, has recently been sequenced (unpublished data). Comparative analysis of these two genomes will provide insight into the genetic factors that determine virulence and host specialization of group I and II *A. citrulli* strains. Pathogenicity and virulence factors investigated in *A. citrulli* thus far include type II and type III secretion systems, type IV pili, polar flagellum, and quorum sensing (13).

Type II secretion system: the type II secretion system (T2SS) is conserved across Gram-negative bacteria. It is a complex (secreton) that spans the inner and outer bacterial membranes, and is comprised of 12 to 16 different proteins (19). The genes that encode these proteins are called general secretion pathway (*gsp*) genes, denoted *gspC* through *gspO* (in certain cases also *gspAB* and *gspN/S*) and are usually organized in large operons. Plant pathogenic bacteria use the T2S for translocation of virulence factors from the bacterial cytoplasm to the extracellular environment (19). This was demonstrated by Hu *et. al* in *Xanthomonas oryzae* pv. *oryzae*-rice (32), and Liu *et al.* in *Ralstonia solanacearum*-tomato pathosystem (49).

In *A. citrulli* AAC00-1, there are two sets of T2S gene clusters. *gsp1* lacks *gspA, B, S* and *N*, and *gsp2* lacks *gspA, B, S* and *F.* After the deletion of both *gspG* copies in AAC00-1, the mutant strain was unable to secrete endoglucanase, which confirmed the existence of a functional T2SS in *A. citrulli* (38). Additionally, this mutant was significantly impaired in its ability to colonize the cotyledons of watermelon seedlings, which suggested that the T2S enzymes may have a role in virulence (38). The *gspG1/G2* deletion mutant of AAC00-1 was also impaired in its ability to colonize germinating watermelon seedlings and induce seed-to-seedling transmission of BFB (38). Three putative cell-wall-degrading enzymes, namely, endoglucanase, xylanase, and pectate lyase, were identified through genome analysis of AAC00-1; however,

only the production and secretion of endoglucanase has been demonstrated to date. Since the AAC00-1 endoglucanase deletion mutant was only slightly impaired in its ability to colonize germinating watermelon seedlings (38) further research is needed to fully understand the role of T2S in seed-to-seedling BFB transmission.

Type IV pili: type IV pili (T4P) are strong and flexible cell surface filaments that are present in all Gram-negative bacteria (17). They are polymeric assemblies of the protein pilin and have various roles, such as surface motility, microcolony and biofilm formation, host-cell adhesion, cell signaling, DNA uptake by natural transformation, and phage attachment. T4P also elicit host immune responses, and their disruption severely reduces the virulence of pathogenic bacteria, highlighting their role in pathogenicity (17). In plant pathogenic bacteria the role of T4P in virulence has mainly been reported for vascular pathogens (13) such as *R. solanacearum* (50), and *Xylella fastidiosa* (55). Proposed mechanisms through which T4P contribute to bacterial colonization and spread in xylem tissue include cell attachment, biofilm formation, and twitching motility (13).

To study the role of T4P in *A. citrulli* virulence, Bahar *et al.* used random transposon mutagenesis of a group I strain M6 and demonstrated the importance of *pilM*, a gene that encodes a protein required for T4P assembly (6). Twitching motility was abolished in the *pilM* mutant, while biofilm formation and virulence in seed transmission and stem inoculation assays of melon seedlings were also reduced (6). Additionally, a knockout mutant of *pilT*, a gene that encodes an ATPase required for T4P retraction, was generated and characterized. As observed for the *pilM* mutant, twitching motility was abolished for the *pilT* mutant of M6; however, the mutant produced T4P and was hyperpiliated relative to the wild-type strain. The *pilT* mutant exhibited a greater reduction in virulence relative to *pilM* in seed-to-seedling transmission and a

similar level of virulence reduction in stem inoculation assays. This suggested that functional T4P and not their mere presence were required for *A. citrulli* virulence on melon seedlings. Additionally, unlike the *pilM* mutant, the hyperpiliated *pilT* mutant did not form a biofilm.

Finally, the importance of T4P for systemic movement inside xylem vessels of melon seedlings was tested using M6 wild-type *A. citrulli* strain and *pilM* and *pilT* M6 mutants. Unlike wild-type M6, T4P mutants of M6 were not recovered from melon xylem tissue. These findings simultaneously demonstrated the systemic movement of M6 in melon seedlings and the role of T4P in this phenotype (6).

Flagella: Flagella are used by many bacteria for motility, adhesion, colonization, and virulence (57). After screening a random transposon mutant library of *A. citrulli* M6, a strain with mutated *fliR* gene encoding a flagellar assembly protein exhibited reduced virulence (7). Similarly, when the *fliC* gene encoding a flagellin subunit was mutated, the resulting strain was impaired in BFB seedling transmission, as well as virulence in foliage and stem inoculation assays. Additionally, the M6 *fliC* mutant exhibited reduced xylem colonization relative to the wild-type, demonstrating the importance of the polar flagellum for the systemic movement of *A. citrulli* M6 in melon and BFB development (7).

Quorum sensing: quorum sensing (QS) is a cell density-dependent intercellular communication that bacteria use to regulate the expression of multiple genes (10). Briefly, bacterial cells produce small molecules called autoinducers and release them into the extracellular space. At threshold cell densities the autoinducers are concentrated enough to be detected by the bacterial cells and this triggers downstream reactions that lead to the regulation of gene expression (10). In Gram-negative bacteria, the autoinducer is typically an acylated homoserine lactone (AHL), synthesized by a *luxI* encoded enzyme. AHLs accumulate both intra-

and extracellularly and after a quorum of cells is achieved, *luxR* encoded proteins bind the accumulated AHL molecules. Subsequently, the LuxR-AHL complexes bind to the promoters of QS-regulated target genes to activate transcription (10). The role of QS in virulence has been studied in many plant pathogenic bacteria including *Agrobacterium tumefaciens*, *P. syringae, R. solanacearum*, *X. campestris*, and *Pectobacterium carotovorum* (25,73). The processes that are QS-regulated include the production of extracellular polysaccharides, cell-wall degrading enzymes, antibiotics, siderophores, pigments, type III secretion, Ti plasmid transfer, motility, biofilm formation, and epiphytic fitness (73).

Homologs of *luxI* and *luxR* genes encoding AHL synthase, and AHL-dependent transcriptional protein, respectively, were found in the genome of *A. citrulli* strain AAC00-1. Subsequently, Tao *et al.* (71) transformed the gene *aiiA*, that encodes the degradation of AHL molecules, into *A. citrulli* strain NJF10 creating the mutant strain, NJF10-*aaiA*. NJF10-*aaiA* displayed significantly reduced AHL accumulation compared to the wild-type NJF10. Additionally, inoculation assays showed that NJF10-*aaiA* exhibited reduced virulence on watermelon fruit (71). Tao *et al.* also reported that N-3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-HSL) was the autoinducer molecule produced by *A. citrulli* (71). Subsequently, Fan *et al.* confirmed 3-oxo-C8-HSL as the quorum sensing signaling molecule, as well as the role of quorum sensing in the virulence of *A. citrulli*. They generated an *aacI* (*luxI* homolog) mutant of *A. citrulli* strain XJL12 and reported its reduced virulence both in watermelon fruit and melon seedlings (22). Johnson *et al.* (39) showed that QS plays a role in seed-to-seedling transmission of BFB using *aacR* (*luxR* homolog) and *aacI* mutants of AAC00-1. Both mutants colonized germinating watermelon seed at wild-type levels, but BFB seed-to-seedling transmission was affected in a cell density-dependent manner. There was no significant difference in BFB seed-to-

seedling transmission between watermelon seed infiltrated with ~ 10^6 CFU/seed of AAC00-1, the *aacR* or *aacI* deletion mutants. However, when seed inoculum was reduced to ~ 10^3 CFU/seed, BFB seed-to-seedling transmission declined to 34.3 % for the *aacI* mutant, which was significantly less than the wild-type (78.6 %). Interestingly, BFB seed-to-seedling transmission for the *aacR* mutant was not significantly different to the wild-type strain (39).

Type III secretion system (T3SS). The most widely studied bacterial secretion system to date is the T3SS. Its role in pathogenesis has been reported in many animal and plant pathogenic bacteria, including *A. citrulli* (1,37,40,41,69,72). Genes encoding the T3SS were discovered as hypersensitive response (HR) and pathogenicity genes (*hrp*) and they were named *hrp* because their mutation typically abolished the ability of the Gram-negative phytobacteria to elicit an HR on non-host plants and cause disease on host plants (48,70). In general, *hrp* genes distinguish pathogenic bacteria from their non-pathogenic relatives. These genes are often part of gene clusters known as pathogenicity islands, that can be acquired by horizontal gene transfer (34). This mechanism enables distantly related pathogens to have closely related *hrp* genes (34). Based on similarities in gene organization and regulation and sequence analysis, *hrp* clusters are divided into two groups. Group I is typified by *Erwinia amylovora* and *P. syringae*, and group II is typified by *R. solanacearum* and *Xanthomonas* species (15). Sequence analysis and cluster organization revealed that *A. citrulli hrp* cluster belongs to group II (4).

The type III secretion system (T3SS) is encoded by approximately 20 genes (34), nine of which are highly conserved both in animal and plant pathogenic bacteria. Therefore, they are named *hrc*, which stands for *hrp* conserved genes (27). Based on sequence analysis and some experimental evidence, *hrc* genes are believed to encode one outer-membrane protein, one outer-

membrane-associated lipoprotein, five inner-membrane proteins, and two cytoplasmic proteins, one of which encodes an ATPase (12).

The role of the T3SS in pathogenicity was confirmed in both *A. citrulli*-watermelon and *A. citrulli*-melon interactions (40,85). A deletion mutant of *hrcC,* a gene that encodes the T3SS outer membrane pore in AAC00-1, lost the ability to induce HR on tobacco leaves and was impaired in its ability to colonize two-week-old watermelon seedlings. However, the mutant strain displayed wild-type ability to colonize watermelon seedlings during the first six days of seed germination (40). Interestingly, the AAC00-1 *gspG1/G2* mutant, with a dysfunctional T2SS, was unable to colonize germinating or two-week-old watermelon seedlings at the wild-type levels (40) which suggests that T3S effectors, unlike T2S proteins, play no role in the colonization of germinating seeds prior to the seedling infection. Additionally, since T3S effector proteins were not critical for *A. citrulli* colonization of germinating watermelon seedlings, it can be inferred that the bacterium behaves like a saprophyte during the initial stages of seed-to-seedling BFB transmission (13).

Further evidence for a functional T3SS in *A. citrulli* was provided by Zhengguang *et al.* using melon as a host. *A. citrulli* mutant strain M543, generated by transposon mutagenesis, was impaired in virulence on melon seedlings (85). The gene disrupted by the Tn5 insertion was a homolog of *hrcR,* which encodes a conserved inner-membrane protein of the T3SS in some bacterial pathogens (85). Mutant strain M543 was also unable to trigger an HR on tobacco leaves (85).

Finally, the importance of a functional T3SS for *A. citrulli* pathogenicity was also demonstrated in *A. citrulli*-cucumber pathosystem. A conserved T3SS gene, *hrcN*, that encodes an ATPase, was identified by transposon mutagenesis of a group I *A. citrulli* strain, FC440 (51).

This gene was shown to be essential for *A. citrulli* pathogenicity, as the *hrcN* mutant lost the ability to cause BFB symptoms on cucumber and to induce HR on tobacco. Although conserved T3SS genes are essential for *A. citrulli* pathogenicity, little is known about *A. citrulli* T3S effectors and their role in pathogenicity, virulence, or host range determination.

Proteins of the type III secretion system. Proteins secreted through the T3SS fall into two categories based on their function – effectors and helpers (16). Effectors are found both in plant and animal pathogens, and can be defined as virulence proteins injected into the cytosol of the host cell through the T3SS. Helpers are extracellular accessory proteins with the primary function of translocating the effectors through the host barriers. Harpins are helper proteins that appear to interact with plant cell walls and membranes. Harpins are secreted more abundantly than effectors, are glycine-rich, and do not contain cysteine. Additionally, they have a heat-stable ability to elicit HR when infiltrated at high concentration into the apoplast of plant leaves (16). While the T3S apparatus (injectisome) is conserved in unrelated pathogens, the secreted proteins differ significantly (34). T3S effectors interfere with host cell signal transduction and other cellular processes and thereby facilitate bacterial pathogenesis (34).

The exact role of most T3S effectors remains unclear. However, some effectors have been shown to contribute to pathogenicity by suppressing host defense responses (1,37,41,69,72). Often the loss of individual effector genes has little or no effect on pathogen virulence (8,81). This could be attributed to multiple factors including effector redundancy, additive effects of individual effectors, or inappropriate testing environments (83,84). One example is the redundancy of *avrBs3* homologs in the virulence of *Xanthomonas campestris* pv. *malvacearum* on cotton. The *avrBs3* family of T3S effectors has ten homologs in *X. campestris* pv. *malvacearum* strain XcmH1005 (84). When seven homologous effectors were inactivated in

XcmH1005 the mutant no longer caused water-soaking on cotton. Interestingly, only five of these genes were needed to restore full water-soaking, while the remaining five appeared to be redundant (84). Bai *et al.* also described the additive effect of effector genes. Genomic copies of three *Xanthomonas oryzae* pv. *oryzae* avirulence (*avr*) genes, *avrXa7*, *avrXa10* and *avrxa5*, and four homologous genes (*aB3.5*, *aB3.6*, *aB4.3* and *aB4.5*) all from the *Xanthomonas avrBs3* gene family were mutated individually or in combination (8). Two double mutants were generated, one lacking *avrXa7* and *avrXa10*, and the other lacking *avrXa7* and *aB3*.6. Triple mutants were also generated that lacked all three *avr* genes, or *avrXa7* and *avrXa10* and one of the homologous genes. Strains with single mutations in *avrXa7*, *avrxa5*, or one of the four homologous genes, caused shorter lesions on the susceptible rice line, IR24. However, the contribution of each gene to lesion length varied, with *avrXa7* contributing the most, and *avrXa10* showing no measurable effect. All triple mutants caused shorter lesions than the *avrXa7* single deletion mutant, or the double mutant lacking *avrXa7* and *avrXa10*. Interestingly, however, the shortest lesions were caused by the other double mutant strain that lacked *avrXa7* and the homologous gene *aB3*.6. (8). This study not only confirmed the additive effect of individual effector genes, but also showed that not all effectors contribute to virulence, and that the contributions of individual effectors are quantitatively different.

Type III secretion effectors have also been reported to influence the host range of pathogens. For example, Lavie *et al.* reported that an alternative codon usage region in the genome of *R. solanacearum* encoded a protein, PopP1, homologous to members of the YopJ/AvrRxv family of T3S effectors that control the interactions between bacteria and their hosts (46). *R. solanacearum* mutant lacking *popP1* became pathogenic on a *Petunia* line that is normally resistant to the wild-type bacterium. Therefore, PopP1 can be considered a typical

avirulence factor that limits the host range of *R. solanacearum* (46). Similarly, Poueymiro *et al.* found that *popP1* and *avrA* limit the host range of *R. solanacearum* strain GMI1000 on at least three tobacco species (*N. tabacum*, *N. benthamiana*, and *N. glutinosa*) (60). Both T3S effectors elicit HR on these tobacco species and simultaneous inactivation of the *avrA* and *popP1* genes allowed GMI1000 to wilt tobacco plants, showing that these two effectors are limiting the host range of GMI1000 (60). Another example is the host specificity determinant in *E. amylovora*, the causal agent of fireblight of rosaceous plants (2). Based on their host range, *E. amylovora* strains can be distinguished into two groups – *Rubus* and *Spiraeoideae*. In the field, *Rubus* strains exclusively infect *Rubus* plants, which include raspberry and blackberry, while *Spiaeoideae* strains have a wide host range including apple and pear. The pathogenicity island that encodes the regulatory components and effector proteins of the T3SS was compared between a *Rubus* strain Ea246 and a *Spiraeoideae* strain Ea273. T3S effector protein Eop1 was identified as unusually divergent between these two strains and selected as a possible host specificity factor. To test this hypothesis, *eop1* deletion mutants were generated from Ea246 and Ea273 and their virulence was compared in immature Bartlett pear fruit (highly susceptible host of *Spiraeoideae* strains) and apple shoot assays. The *eop1* deletion mutant of Ea246 exhibited a gain-of-virulence phenotype on pear, similar to that of wild-type Ea273. Additionally, when *eop1* from Ea273 was expressed in the Ea246 deletion mutant, a similar gain-of-virulence phenotype was observed on pear fruit. Conversely, expression of the *Rubus* strain *eop1* in the Ea273 deletion mutant reduced its virulence both on immature pear fruit and apple shoots. Moreover, *eop1* genes from four additional *Rubus* strains, five *Spiraeoideae* strains, and two *E. pyrifoliae* strains originating from different geographic locations were sequenced and compared. The sequences from *Rubus* strains were nearly identical, as were those from non-*Rubus* strains. In a phylogenetic tree, *eop1*

sequences from the different strains clustered together according to the host. Therefore it was reported that *eop1* from a *Rubus* strain can function as a host specificity determinant. This is a good example of a T3S effector altering the virulence of a bacterial pathogen in a host-specific manner (2). Furthermore, Baltrus *et al.* reported on three T3S effectors contributing to the host range differences between *P. syringae* pathovars *glycinea* and *phaseolicola*. *P. syringe* pv. *phaseolicola* 1448a and *P. syringae* pv. *glycinea* R4 were recovered from naturally infected leaves of French bean and soybean, respectively. After tissue infiltration with 10^4 CFU/ml, each strain reached the highest population densities on its original host and significantly lower population densities on the other plant species (9). At the same time, the loci *hopH1/hopC1* and *hopM1* from *P. syringae* pv. *glycinea* R4 conferred avirulence on French bean. Both copies of *hopH1/hopC1* and *hopM1* were deleted in *P. syringae* pv. *glycinea* R4 to create the mutant strain DAB890. Subsequently, T3S effector genes present in *P. syringae* pv. *phaseolicola* 1448a, but absent in *P. syringae* pv. *glycinea* R4, were expressed in DAB890. Only the expression of *avrB2* increased the growth of DAB890 on French bean. These findings suggest that the presence of T3S effectors *hopC1* and *hopM1* and the absence of *avrB2* potentially contribute to host range differences between pathovars *glycinea* and *phaseolicola* (9).

To date, eleven putative T3S effectors have been identified in the AAC00-1 genome through sequence analysis (Eckshtain-Levi *et al.*, Phytopathology, *In review*). All of these effectors are present in twelve group II *A. citrulli* strains that have been screened so far. Interestingly, of eight group I strains that have been screened, all lacked the effector Aave_2708 (*Xanthomonas euvesicatoria AvrBsT* ortholog), and had truncated versions of Aave_2166 (*X. euvesicatoria AvrBsT* ortholog), and Aave_3062 (*X. oryzae* pv. *oryzicola AvrRxo1* ortholog) (Eckshtain-Levi *et al.*, Phytopathology, *In review*). Furthermore, *X. euvesicatoria AvrBsT* is a

member of the YopJ/AvrRxv protein family that includes proteases and/or acetyltransferases and was reported to suppress the HR elicited by AvrBs1 in resistant pepper plants (69). However, deletion of *AvrBsT* in *X. euvesicatoria* strain 75-3 did not contribute significantly to bacterial virulence on susceptible tomato plants, or alter bacterial growth *in planta* (69).

Hypersensitive response-based resistance to BFB has not been reported in cucurbitaceous hosts and therefore the *AvrBsT* orthologs in *A. citrulli* are not expected to play the same role as those reported for *X. euvesicatoria*. However, the complete absence of *AvrBsT* orthologs in group I *A. citrulli* strains presents an intriguing question with regards to their role in virulence and host range determination amongst different cucurbits.

Project objectives and hypotheses

The overall goal of this study was to further characterize the differences in virulence phenotype between group I and II *A. citrulli* strains on different cucurbit hosts and investigate the factors that contribute to these differences. Specific objectives of the study were:

1) To compare temporal population growth dynamics of representative group I and group II *A. citrulli* strains on watermelon and melon seedlings after artificial inoculation.
2) To compare the relative incidence of BFB on watermelon and melon seedlings exposed to inoculum of group I and group II *A. citrulli* strains under natural infection conditions.
3) To investigate the effect of introducing two *AvrBsT* orthologs from a group II strain into a group I *A. citrulli* strain on virulence and relative BFB incidence.

Hypotheses:

1) Population growth dynamics of group I and group II *A. citrulli* strains differ on watermelon and melon seedlings.
2) Group I *A. citrulli* strains cause higher incidence of BFB on melon seedlings than group II strains.
3) Ectopic expression of *AvrBsT* orthologs from AAC00-1 will reduce virulence and relative BFB incidence of M6 on melon seedlings.

Literature cited

1. Abramovitch, R. B., and Martin, G. B. 2005. AvrPtoB: a bacterial type III effector that both elicits and suppresses programmed cell death associated with plant immunity. FEMS Microbiol. Lett. 245:1-8.
2. Asselin, J. E., Bonasera, J. M., Kim, J. F., Oh, C. S., and Beer, S. V. 2011. Eop1 from a *Rubus* strain of *Erwinia amylovora* functions as a host-range limiting factor. Phytopathology 101:935-944.
3. Assis, S., Mariano, R., Silva-Hanlin, D., and Duarte, V. 1999. Bacterial fruit blotch caused by *Acidovorax avenae* subsp. *citrulli* in melon in the state of Rio Grande do Norte, Brazil. Fitopatologia Brasileira 24.
4. Bahar, O., and Burdman, S. 2010. Bacterial fruit blotch: A threat to the cucurbit industry. Isr. J. Plant Sci. 58:19-31.
5. Bahar, O., Kritzman, G., and Burdman, S. 2009. Bacterial fruit blotch of melon: screens for disease tolerance and role of seed transmission in pathogenicity. Eur. J. Plant Pathol. 123:71-83.
6. Bahar, O., Goffer, T., and Burdman, S. 2009. Type IV pili are required for virulence, twitching motility, and biofilm formation of *Acidovorax avenae* subsp. *citrulli*. Mol. Plant-Microbe Interact. 22:909-920.
7. Bahar, O., Levi, N., and Burdman, S. 2011. The cucurbit pathogenic bacterium *Acidovorax citrulli* requires a polar flagellum for full virulence before and after host-tissue penetration. Mol. Plant-Microbe Interact. 24:1040-1050.
8. Bai, J., Choi, S. H., Ponciano, G., Leung, H., and Leach, J. E. 2000. *Xanthomonas oryzae* pv. *oryzae* avirulence genes contribute differently and specifically to pathogen aggressiveness. Mol. Plant-Microbe Interact. 13:1322-1329.
9. Baltrus, D. A., Nishimura, M. T., Dougherty, K. M., Biswas, S., Mukhtar, M. S., Vicente, J., Holub, E. B., and Dangl, J. L. 2012. The molecular basis of host specialization in bean pathovars of *Pseudomonas syringae*. Mol. Plant-Microbe Interact. 25:877-888.
10. Bassler, B. L. 2002. Small talk: cell-to-cell communication in bacteria. Cell 109:421-424.
11. Black, M., Isakeit, T., Barnes, L., Kucharek, T., Hoover, R., and Hodge, N. 1994. First report of bacterial fruit blotch of watermelon in Texas. Plant Dis. 78.
12. Bogdanove, A. J., Beer, S. V., Bonas, U., Boucher, C. A., Collmer, A., Coplin, D. L., Cornelis, G. R., Huang, H. C., Hutcheson, S. W., and Panopoulos, N. J. 1996. Unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria. Mol. microbiol. 20:681-683.
13. Burdman, S., and Walcott, R. 2012. *Acidovorax citrulli*: generating basic and applied knowledge to tackle a global threat to the cucurbit industry. Mol. Plant Pathol. 13:805-815.
14. Burdman, S., Kots, N., Kritzman, G., and Kopelowitz, J. 2005. Molecular, physiological, and host-range characterization of *Acidovorax avenae* subsp *citrulli* isolates from watermelon and melon in Israel. Plant Dis. 89:1339-1347.
15. Büttner, D., and Bonas, U. 2002. Getting across—bacterial type III effector proteins on their way to the plant cell. EMBO J. 21:5313-5322.
16. Collmer, A., Lindeberg, M., Petnicki-Ocwieja, T., Schneider, D. J., and Alfano, J. R. 2002. Genomic mining type III secretion system effectors in *Pseudomonas syringae* yields new picks for all TTSS prospectors. Trends Microbiol. 10:462-469.

17. Craig, L., Pique, M. E., and Tainer, J. A. 2004. Type IV pilus structure and bacterial pathogenicity. Nat. Rev. Microbiol. 2:363-378.
18. Dane, F., and Liu, J. 2007. Diversity and origin of cultivated and citron type watermelon (*Citrullus lanatus*). Genetic Resources and Crop Evolution 54:1255-1265.
19. Douzi, B., Filloux, A., and Voulhoux, R. 2012. On the path to uncover the bacterial type II secretion system. Philosophical Transactions of the Royal Society B: Biological Sciences 367:1059-1072.
20. Dutta, B., Avci, U., Hahn, M., and Walcott, R. 2012. Location of *Acidovorax citrulli* in infested watermelon seeds is influenced by the pathway of bacterial invasion. Phytopathology 102:461-468.
21. Dutta, B., Scherm, H., Gitaitis, R., and Walcott, R. 2012. *Acidovorax citrulli* seed inoculum load affects seedling transmission and spread of bacterial fruit blotch of watermelon under greenhouse conditions. Plant Dis. 96:705-711.
22. Fan, J. Q., Qian, G. L., Chen, T., Zhao, Y. Q., Liu, F. Q., Walcott, R. R., and Hu, B. S. 2011. The acyl-homoserine lactone (AHL)-type quorum sensing system affects growth rate, swimming motility and virulence in *Acidovorax avenae* subsp *citrulli*. World J. Microb. Biot. 27:1155-1166.
23. Feng, J. J., Schuenzel, E. L., Li, J. Q., and Schaad, N. W. 2009. Multilocus sequence typing reveals two evolutionary lineages of *Acidovorax avenae* subsp *citrulli*. Phytopathology 99:913-920.
24. Frankle, W., and Hopkins, D. 1993. Ingress of the watermelon fruit blotch bacterium into fruit. Plant Dis. 77:1090-1092.
25. Fray, R. G. 2002. Altering plant–microbe interaction through artificially manipulating bacterial quorum sensing. Ann. Bot-London 89:245-253.
26. Ha, Y., Fessehaie, A., Ling, K., Wechter, W., Keinath, A., and Walcott, R. 2009. Simultaneous detection of *Acidovorax avenae* subsp. *citrulli* and *Didymella bryoniae* in cucurbit seedlots using magnetic capture hybridization and real-time polymerase chain reaction. Phytopathology 99:666-678.
27. He, S. Y., Nomura, K., and Whittam, T. S. 2004. Type III protein secretion mechanism in mammalian and plant pathogens. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1694:181-206.
28. Hopkins, D. 1995. Copper-containing fungicides reduce the spread of bacterial fruit blotch of watermelon in the greenhouse. Phytopathology 85:510.
29. Hopkins, D., and Thompson, C. 2002. Evaluation of *Citrullus* sp. germ plasm for resistance to *Acidovorax avenae* subsp. *citrulli*. Plant Dis. 86:61-64.
30. Hopkins, D., and Thompson, C. 2002. Seed transmission of *Acidovorax avenae* subsp. *citrulli* in cucurbits. HortScience 37:924-926.
31. Hopkins, D., Thompson, C., Hilgren, J., and Lovic, B. 2003. Wet seed treatment with peroxyacetic acid for the control of bacterial fruit blotch and other seedborne diseases of watermelon. Plant Dis. 87:1495-1499.
32. Hu, J., Qian, W., and He, C. 2007. The *Xanthomonas oryzae* pv. *oryzae eglXoB* endoglucanase gene is required for virulence to rice. FEMS Microbiol. Lett. 269:273-279.
33. Huang, Y.-R., Hung, Y.-C., Hsu, S.-Y., Huang, Y.-W., and Hwang, D.-F. 2008. Application of electrolyzed water in the food industry. Food Control 19:329-345.

34. Hueck, C. J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. R. 62:379-433.
35. Isakeit, T., Black, M., and Jones, J. 1998. Natural infection of citronmelon with *Acidovorax avenae* subsp. *citrulli*. Plant Dis. 82:351-351.
36. Isakeit, T., Black, M., Barnes, L., and Jones, J. 1997. First report of infection of honeydew with *Acidovorax avenae* subsp. *citrulli*. Plant Dis. 81:694-694.
37. Jamir, Y., Guo, M., Oh, H. S., Petnicki-Ocwieja, T., Chen, S., Tang, X., Dickman, M. B., Collmer, A., and R Alfano, J. 2004. Identification of *Pseudomonas syringae* type III effectors that can suppress programmed cell death in plants and yeast. Plant J. 37:554-565.
38. Johnson, K. L. 2010. Elucidation of the molecular host-pathogen interactions that influence seed-to-seedling transmission of *Acidovorax citrulli*, University of Georgia.
39. Johnson, K. L., and Walcott, R. R. 2013. Quorum sensing contributes to seed-to-seedling transmission of *Acidovorax citrulli* on watermelon. J. Phytopathol.
40. 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.
41. Kearney, B., and Staskawicz, B. J. 1990. Widespread distribution and fitness contribution of *Xanthomonas campestris* avirulence gene *avrBs2*.
42. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301.
43. Langston Jr, D., Walcott, R., Gitaitis, R., and Sanders Jr, F. 1999. First report of a fruit rot of pumpkin caused by *Acidivorax avenae* subsp. *citrulli* in Georgia. Plant Dis. 83:199-199.
44. Latin, R. X., and Rane, K. K. 1990. Bacterial fruit blotch of watermelon in Indiana. Plant Dis. 74:331-331.
45. Latin, R. X., and Hopkins, D. L. 1995. Bacterial fruit blotch of watermelon - The hypothetical exam question becomes reality. Plant Dis. 79:761-765.
46. Lavie, M., Shillington, E., Eguiluz, C., Grimsley, N., and Boucher, C. 2002. PopP1, a new member of the YopJ/AvrRxv family of type III effector proteins, acts as a host-specificity factor and modulates aggressiveness of *Ralstonia solanacearum*. Mol. Plant-Microbe Interact. 15:1058-1068.
47. Lessl, J., Fessehaie, A., and Walcott, R. 2007. Colonization of female watermelon blossoms by *Acidovorax avenae* ssp. *citrulli* and the relationship between blossom inoculum dosage and seed infestation. J. Phytopathol. 155:114-121.
48. Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv." *phaseolicola*" controls pathogenicity of bean plants and hypersensitivity of nonhost plants. J. Bacteriol. 168:512-522.
49. Liu, H., Zhang, S., Schell, M. A., and Denny, T. P. 2005. Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence. Mol. Plant-Microbe Interact. 18:1296-1305.
50. Liu, H., Kang, Y., Genin, S., Schell, M. A., and Denny, T. P. 2001. Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system. Microbiology 147:3215-3229.
51. Liu, J., Luo, S., Zhang, Q., Wang, Q., Chen, J., Guo, A., and Shan, W. 2012. Tn5 transposon mutagenesis in *Acidovorax citrulli* for identification of genes required for pathogenicity on cucumber. Plant Pathol. 61:364-374.

52. Martin, H., and Horlock, C. 2002. First report of *Acidovorax avenae* subsp. *citrulli* as a pathogen of gramma in Australia. Plant Dis. 86:1406-1406.
53. Martin, H., O'Brien, R., and Abbott, D. 1999. First report of *Acidovorax avenae* subsp. *citrulli* as a pathogen of cucumber. Plant Dis. 83:965-965.
54. Maynard, D. N., and Hopkins, D. L. 1999. Watermelon fruit disorders. HortTechnology 9:155-161.
55. Meng, Y., Li, Y., Galvani, C. D., Hao, G., Turner, J. N., Burr, T. J., and Hoch, H. 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. J. Bacteriol. 187:5560-5567.
56. Mirik, M., Aysan, Y., and Sahin, F. 2006. Occurrence of bacterial fruit blotch of watermelon caused by *Acidovorax avenae* subsp. *citrulli* in the Eastern Mediterranean region of Turkey. Plant Dis. 90:829-829.
57. Moens, S., and Vanderleyden, J. 1996. Functions of bacterial flagella. Crit. Rev. Microbiol. 22:67-100.
58. O'Brien, R. G., and Martin, H. L. 1999. Bacterial blotch of melons caused by strains of *Acidovorax avenae* subsp *citrulli*. Aust. J. Exp. Agr. 39:479-485.
59. Palkovics, L., Petróczy, M., Kertész, B., Németh, J., Barsony, C., Mike, Z., and Hevesi, M. 2008. First report of bacterial fruit blotch of watermelon caused by *Acidovorax avenae* subsp. *citrulli* in Hungary. Plant Dis. 92:834-834.
60. Poueymiro, M., Cunnac, S., Barberis, P., Deslandes, L., Peeters, N., Cazale-Noel, A.-C., Boucher, C., and Genin, S. 2009. Two type III secretion system effectors from *Ralstonia solanacearum* GMI1000 determine host-range specificity on tobacco. Mol. Plant-Microbe Interact. 22:538-550.
61. Rane, K. K., and Latin, R. X. 1992. Bacterial fruit blotch of watermelon - association of the pathogen with seed. Plant Dis. 76:509-512.
62. Ren, Y., Li, H., Li, G., Wang, Q., and Li, J. 2006. First report of *Acidovorax avenae* subsp. *citrulli* infecting edible seed watermelon (*Citrullus lanatus* var. *lanatus*) in China. Plant Dis. 90:1112-1112.
63. Schaad, N., Postnikova, E., and Randhawa, P. 2003. Emergence of *Acidovorax avenae* subsp. *citrulli* as a crop-threatening disease of watermelon and melon. Pages 573-581 in: Presentations 6th Int. Conf. *Pseudomonas syringae* Pathovars and Related Pathogens N. W. Schaad, ed. Kluwer Press, Maratea, Italy.
64. Schaad, N. W., Sowell, G., Goth, R. W., Colwell, R. R., and Webb, R. E. 1978. *Pseudomonas pseudoalcaligenes* subsp. *citrulli.* nov. Int. J. Syst. Bacteriol. 28:117-125.
65. Schaad, N. W., Postnikova, E., Sechler, A., Claflin, L. E., Vidaver, A. K., Jones, J. B., Agarkova, I., Ignatov, A., Dickstein, E., and Ramundo, B. A. 2008. Reclassification of subspecies of *Acidovorax avenae* as *A. avenae* (Manns 1905) emend., *A. cattleyae* (Pavarino, 1911) comb. nov., *A. citrulli* Schaad *et al*., 1978) comb. nov., and proposal of *A. oryzae* sp. nov. Syst. Appl. Microbiol. 31:434-446.
66. Somodi, G. C., Jones, J. B., Hopkins, D. L., Stall, R. E., Kucharek, T. A., Hodge, N. C., and Watterson, J. C. 1991. Occurrence of a bacterial watermelon fruit blotch in Florida. Plant Dis. 75:1053-1056.
67. Sowell, G., and Schaad, N. W. 1979. *Pseudomonas pseudoalcaligenes* subsp. *citrulli* on watermelon - seed transmission and resistance of plant introductions. Plant Dis. Rep. 63:437-441.

68. Stephens, D., Schneider, R., Walcott, R., and Johnson, C. 2008. A procedure, based on exposure to chlorine gas, for disinfesting watermelon seeds. Pages S150-S151. in: Phytopathology. Amer. Phytopathological Soc. 3340 Pilot Knob Road, St. Paul, MN 55121 USA.
69. Szczesny, R., Büttner, D., Escolar, L., Schulze, S., Seiferth, A., and Bonas, U. 2010. Suppression of the AvrBs1-specific hypersensitive response by the YopJ effector homolog AvrBsT from *Xanthomonas* depends on a SNF1-related kinase. New Phytol. 187:1058-1074.
70. Tang, X., Xiao, Y., and Zhou, J. M. 2006. Regulation of the type III secretion system in phytopathogenic bacteria. Mol. Plant-Microbe Interact. 19:1159-1166.
71. Tao, C., Guo-Liang, Q., Xiao-Li, Y., Jun-Yi, M., Bai-Shi, H., and Feng-Quan, L. 2009. Detection of a quorum sensing signal molecule of Acidovorax avenae subsp. citrulli and its regulation of pathogenicity. Chinese Journal of Agricultural Biotechnology 6:49-53.
72. Vinatzer, B. A., Teitzel, G. M., Lee, M. W., Jelenska, J., Hotton, S., Fairfax, K., Jenrette, J., and Greenberg, J. T. 2006. The type III effector repertoire of *Pseudomonas syringae* pv. *syringae* B728a and its role in survival and disease on host and non-host plants. Mol. Microbiol. 62:26-44.
73. von Bodman, S. B., Bauer, W. D., and Coplin, D. L. 2003. Quorum sensing in plant-pathogenic bacteria. Annu. Rev. Phytopathol. 41:455-482.
74. Walcott, R., and Gitaitis, R. 2000. Detection of *Acidovorax avenae* subsp. *citrulli* in watermelon seed using immunomagnetic separation and the polymerase chain reaction. Plant Dis. 84:470-474.
75. Walcott, R., Gitaitis, R., and Castro, A. 2003. Role of blossoms in watermelon seed infestation by *Acidovorax avenae* subsp. *citrulli*. Phytopathology 93:528-534.
76. Walcott, R., Castro, A., Fessehaie, A., and Ling, K. 2006. Progress towards a commercial PCR-based seed assay for Acidovorax avenae subsp. citrulli. Seed Sci. Technol. 34:101-116.
77. Walcott, R. R. 2003. Detection of seedborne pathogens. HortTechnology 13:40-47.
78. Walcott, R. R., Fessehaie, A., and Castro, A. C. 2004. Differences in pathogenicity between two genetically distinct groups of *Acidovorax avenae* subsp *citrulli* on cucurbit hosts. J. Phytopathol. 152:277-285.
79. Wall, G., and Santos, V. 1988. A new bacterial disease of watermelon in the Mariana islands. Phytopathology 78:1-9.
80. Wechter, W. P., Levi, A., Ling, K.-S., Kousik, C., and Block, C. C. 2011. Identification of resistance to *Acidovorax avenae* subsp. *citrulli* among melon (*Cucumis* spp.) plant introductions. HortScience 46:207-212.
81. White, F. F., Yang, B., and Johnson, L. B. 2000. Prospects for understanding avirulence gene function. Curr. Opin. Plant. Biol. 3:291-298.
82. Willems, A., Goor, M., Thielemans, S., Gillis, M., Kersters, K., and Deley, J. 1992. Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov., comb. nov., *Acidovorax avenae* subsp. *citrulli*, *Acidovorax avenae* subsp. *cattleyae*, and *Acidovorax konjaci*. Int. J. Syst. Bacteriol. 42:107-119.
83. Yang, B., and White, F. F. 2004. Diverse members of the AvrBs3/PthA family of type III effectors are major virulence determinants in bacterial blight disease of rice. Mol. Plant-Microbe Interact. 17:1192-1200.

84. Yang, Y., Yuan, Q., and Gabriel, D. 1996. Watersoaking function (s) of XcmH1005 are redundantly encoded by members of the *Xanthomonas* avr/pth gene family. Mol. Plant-Microbe Interact. 9.
85. Zhengguang, R., Lei, H., Zhiguo, S., and Liqun, Z. 2009. Screening of the pathogenicity mutants of *Acidovorax avenae* subsp. *citrulli* and cloning of the *hrcR* gene. Acta Phytopathologica Sinica 39:501-506.

CHAPTER 2

CHARACTERIZATION OF *ACIDOVORAX CITRULLI* VIRULENCE ON WATERMELON AND MELON

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Abstract

Bacterial fruit blotch (BFB) of cucurbits is caused by the Gram negative bacterium *Acidovorax citrulli* whose populations can be distinguished into two genetically distinct groups, I and II. Strains in group I have been reported to be moderately to highly virulent accross different cucurbit hosts, while group II strains exhibit high virulence on watermelons, but low virulence on other cucurbits. Additionally, group I strains naturally infect a wider range of cucurbits, while group II strains have been observed to naturally infect watermelon. Our goal was to further characterize the differences in virulence and host range between representative group I and II *A. citrulli* strains on diffferent cucurbit hosts. We observed no difference in temporal population growth dynamics between representative group I (M6) and group II (AAC00-1) *A. citrulli* strains on watermelon and melon seedlings after inoculation by infiltration and spraying. We also investigated the relative BFB incidence on watermelon and melon seedlings exposed to three group I and three group II *A. citrulli* strains during natural spread and infecion under growth chamber conditions. After five days of exposure, the mean relative BFB incidence on melon seedlings exposed to group II *A. citrulli* strains was 14.5% which was significantly lower than for melon seedlings exposed to group I strains (~ 85.3 %), or watermelon seedlings exposed to group I and II strains (81.7 % and 82.3 %, respectively). Bioinformatics data indicated that *AvrBsT* type III secretion effectors are present in twelve group II strains, but absent in eight group I strains, so we investigated the role of this effector in virulence. Ectopic expression of two *AvrBsT* orthologs from AAC00-1 in M6 reduced its virulence on watermelon and melon seedlings compared to the wild-type AAC00-1 and M6 strains and reduced its ability to infect both hosts naturally.

Introduction

Bacterial fruit blotch of cucurbits (BFB) is caused by the Gram-negative bacterium, *Acidovorax citrulli* (formerly *A. avenae* subsp. *citrulli*) (33). Even though the disease was discovered in mid-1960s (26), it only gained importance in the late 1980s when it caused up to 100 % yield losses in commercial watermelon fruit production fields in several states in the U.S. (20,23,27,31). At present, BFB occurs sporadically, but due to its destructive potential, it poses a serious threat to watermelon and melon production worldwide (7).

The *A. citrulli* population can be distinguished into two groups (I and II), based on biochemical (carbon substrate utilization) and genetic analysis (pulse-field gel electrophoresis (PFGE), repetitive-PCR (rep-PCR), multilocus sequence typing) (8,12,30). The two groups of *A. citrulli* strains also differ in virulence and natural occurrence on watermelon and non-watermelon hosts. Group I strains exhibit a moderate to high level of virulence on a range of cucurbits, while group II strains have been reported as highly virulent on watermelon, but weakly virulent on other cucurbitaceous hosts (8,24,30). Additionally, group I strains occur naturally on a range of cucurbit hosts including melon and pumpkin, while group II strains are predominantly recovered from watermelon. To date, the difference in virulence between group I and II strains has been assessed based on disease severity on different hosts using visual rating scales (8,13,24,30). Although disease severity rating scales are routinely used for assessing host plant resistance to BFB (4,13,32), there have been no attempts to develop less variable assessments of *A. citrulli* aggressiveness. Several studies have used tissue colonization assays to determine the effects of specific genes on *A. citrulli* virulence (16-18), however there have been no attempts to compare the virulence of group I and group II strains using these assays.

Despite reports on differences in virulence of group I and II *A. citrulli* strains on different cucurbit hosts, little is known about the factors that influence this phenotype. One mechanism that might explain these differences is the arsenal of type III secretion (T3SS) system effectors possessed by group I and II *A. citrulli* strains. The type III secretion system is the most widely studied bacterial secretion system to date, and its role in pathogenesis has been reported in many animal and plant pathogenic bacteria, including *A. citrulli* (1,15,18,19,22,28,29,37). Additionally, T3S effector proteins have been reported as virulence factors (5,36), as well as factors that influence host range in some plant pathogenic bacteria (3,6,21,25). However, at present, little is known about the role that these effectors play in the *A. citrulli*-cucurbit pathosystem. Several researchers demonstrated that *A. citrulli* has a functional T3SS (18,22,37); however, the repertoire and functions of the *A. citrulli* effector proteins are largely unknown.

Through analysis of the complete *A. citrulli* genome sequence (NCBI accession number NC_008752), 11 putative T3SS effectors were identified in AAC00-1, a group II strain. Using these sequences, PCR primers were designed and the sequences of these effector genes were determined for 12 group II and eight group I *A. citrulli* strains (Eckshtain-Levi *et al.*, Phytopathology, *In review*). Out of 12 group II strains, all possessed the 11 effector genes. In contrast, out of eight group I strains, none possessed Aave_2708 (*AvrBsT Xanthomonas euvesicatioria* otholog), while Aave_2166 (*AvrBsT Xanthomonas euvesicatoria* ortholog) possessed a 123 bp sequence deletion from 72 bp upstream to 51 bp downstream of the predicted start codon (Eckshtain-Levi *et al.*, Phytopathology, *In review*). Aave_2708 and Aave_2166 from AAC00-1 share 99 % and 61 % sequence identity, respectively, with their *AvrBsT* orthologs in *X. euvesicatoria* (Eckshtain-Levi *et al.*, Phytopathology, *In review*). Based on this observation we hypothesized that this variation in T3S effector repertoire might influence virulence of group I

and II *A. citrulli* strains on different cucurbit hosts. Hence the objectives of this research were: 1) to compare population growth dynamics between representative group I and group II *A. citrulli* strains on watermelon and melon seedlings after artificial inoculation; 2) to compare the relative incidence of BFB on watermelon and melon seedlings exposed separately to inoculum of group I and II *A. citrulli* strains under natural infection conditions and 3) to investigate the effect that introducing two *AvrBsT* orthologs from a group II strain into a group I *A. citrulli* strain has on virulence and relative BFB incidence.

Materials and methods

Host species and cultivars. Two-week-old seedlings of the watermelon cultivar Crimson Sweet and the melon cultivar Joaquin Gold were established in 250 ml plastic pots, or 36- or 48-cell polystyrene flats (Speedling Inc., Sun City, FL) filled with pasteurized potting mix (90% composted pine bark and 10% vermiculite) under greenhouse or growth chamber (Conviron E15, Conviron, Winnipeg, Canada) conditions (28^{o}C day and 21^{o}C night temperature, and 12 h/day fluorescent and incandescent light).

Bacterial strains, growth conditions and inoculum preparation. The *A. citrulli* and *Escherichia coli* strains used in this study are listed in Table 2.1. *A. citrulli* strains were routinely grown on nutrient agar (NA), (Difco^{TM}, Becton, Dickinson and Co. Sparks, MD) for 48 h, or in nutrient broth (NB), (EMD Chemicals Inc., Gibbstown, NJ) overnight, with appropriate antibiotics at 28-30^{o}C. *Escherichia coli* strains were routinely grown on Luria-Bertani (LB) agar (Difco^{TM}), or in LB broth (Fisher BioReagents, Fisher Scientific, Fair Lawn, NJ), with appropriate antibiotics for 24-48 h at 37^{o}C. When used, antibiotics were incorporated into the media at the following concentrations: kanamycin (Km) 50-100 µg/ml, ampicillin (Ap) 100

µg/ml, spectinomycin (Sp) 100 µg/ml, and trimethoprim (Tp) 100 µg/ml. *A. citrulli* inoculum was prepared by adding cells from an overnight NB culture to sterile distilled water. The cell suspension was adjusted to ~ 1 x 10^8 CFU/ml spectrophotometrically (~ 0.3 optical density at 600 nm) (Spectronic 20; Bausch and Lomb, Rochester, NY) and 10-fold serial dilutions were conducted to reach the desired final concentrations.

Temporal population dynamics of representative group I and II *A. citrulli* strains in watermelon and melon cotyledons after tissue infiltration. Cell suspensions (~ 1 x 10^4 CFU/ml) of M6 and AAC00-1 were infiltrated separately, using a syringe without a needle, into the intercellular spaces of the cotyledons of two-week-old watermelon and melon seedlings after poking a hole in the central leaf vein on the abaxial side of the cotyledons. Four watermelon and four melon seedlings were used for each treatment and sterile distilled water was used as negative control. After infiltration, cotyledons were allowed to air dry for 1 h and the plants were incubated in a growth chamber (Percival, Perry, IA) at 28^{o}C and 75-80% RH with 12 h/day of fluorescent light. Five leaf disks (5 mm in diameter) per treatment, and 2 leaf disks for the negative control, were collected from a single cotyledon on each plant at 1, 2, 3 and 4 days post inoculation (dpi). Each leaf disk was macerated in 500 µl of sterile distilled water and after centrifugation for 3 min at 16,100 x g the pellet was used for microbial DNA extraction using the Ultra Clean Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions except that the cells were lysed thermally at 65^{o}C for 10 min with vortexing every 2-3 min. *A. citrulli* populations in leaf tissues were quantified by TaqMan real-time PCR (qPCR) using proprietary *A. citrulli*-specific primers and probe (UGA3979) at the concentrations of 0.3 and 0.2 µM per reaction, respectively. PCR amplification was conducted using the iQ Supermix reagents (BioRad Laboratories, Berkley,

CA) according to manufacturer's instructions and the following thermal profile: 95^{o}C for 180 sec, 40 cycles of 95^{o}C for 20 sec, 55^{o}C for 30 sec, and 72^{o}C for 30 sec. Cycle threshold (Ct) data below 33 were collected for each sample and converted into CFU/disk based on a standard curve (Fig. 2.1). The experiment was conducted twice and bacterial populations were plotted over time to generate population growth curves using Sigma Plot 10.0 (Systat Software Inc., San Jose, CA).

Temporal population dynamics of *A. citrulli* strains on watermelon and melon seedlings after spray-inoculation. Temporal population dynamics of *A. citrulli* strains, AAC00-1 and M6 were monitored on two-week-old watermelon and melon seedlings after spray-inoculation as described previously (30). Suspensions of each strain (~ 1 x 10^4 CFU/ml) were sprayed onto seedlings until run-off. The plants were placed in plastic bags and incubated in the growth chamber for 24 h at 30^{o}C and approximately 100 % RH. Subsequently, the bags were removed and the seedlings were incubated in a growth chamber at 30^{o}C, and 70-80 % RH with 12 h/day of fluorescent light for 7 days (8 days total incubation time). Twelve leaf disks (5 mm in diameter) were collected from three individual plants (four disks/cotyledon) at 4, 6, and 8 dpi. Microbial DNA was prepared as described above and *A. citrulli* DNA was measured by qPCR. The experiment was conducted twice and bacterial populations were plotted over time to generate population growth curves using Sigma Plot 10.0. Additionally, mean AAC00-1 and M6 populations at each time point were used to generate area under population growth curves data on melon seedlings using Sigma Plot 10.0. Bacterial fruit blotch symptoms on watermelon and melon seedlings were visually rated using a 0-5 scale (2). Severity ratings were compared using the GLIMMIX procedure in SAS (version SAS 9.2 T&R for Windows; SAS Institute Inc, Cary, NC).

Comparison of natural infection of watermelon and melon seedlings by *A. citrulli* strains. Six two-week-old melon and watermelon seedlings were spray-inoculated with cell suspensions (~ 1 x 10^6 CFU/ml) of group I and group II *A. citrulli* strains, respectively. The seedlings were placed in plastic bags and incubated at 20-25^{o}C and approximately 100 % RH for 24 h. Subsequently, the bags were removed and the plants were placed in the centers and corners of separate 48-cell planting trays containing alternately positioned, uninoculated two-week-old watermelon and melon seedlings. Each planting tray contained 20 watermelon and 20 melon plants. Plants were incubated in a mist chamber at 20-25^{o}C and > 90% RH, with 6-8 h/day of fluorescent and incandescent light, for up to 7 days. Seedlings were sprayed twice a day with a stream of sterile water to promote splash dispersal of bacterial cells. The proportion of watermelon and melon seedlings showing at least one lesion on the cotyledons or leaves was recorded each day and the experiment was conducted twice. Relative BFB incidence at 5 days of exposure, relative area under the disease progress curve (AUDPC) data, and the time required for 20 % disease development were compared with and without group-host interaction using the GLM procedure in SAS.

Generation of M6-AvrBsT2. PCR primers listed in Table 2.2 were designed to amplify the target genes, Aave_2166 and Aave_2708, and ~ 1.2 kb of their upstream regions that putatively included the native promoters. PCR assays were performed with MasterAmp Tfl DNA Polymerase (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's instructions, and the primers were added at the concentration of 0.5 µM per reaction. PCR was conducted using an Eppendorf Mastercycler Personal (Eppendorf, Hamburg, Germany) and the following thermal profile: 95^{o}C for 5 min, 30 cycles of 95^{o}C for 30 sec, 60^{o}C for 40 sec, and 72^{o}C for 2 min and 30 sec, and one cycle of 72^{o}C for 15 min. PCR amplicons were gel purified

using a Qiagen gel extraction kit (Qiagen Sciences, Maryland, USA), eluted with water, and using a TOPO TA Cloning Kit for Sequencing (Invitrogen Life Technologies, Grand Island, NY) each PCR amplicon was cloned separately into the vector, pCR4, and subsequently transformed into *E. coli* DH5α, according to the manufacturer's instructions. The recombinant plasmids, pCR2166 and pCR2708, were extracted using an AccuPrep Plasmid Mini Extraction Kit (USA Bioneer, Inc., Alameda, CA, USA). The target genes were then digested using the appropriate restriction enzymes in the PCR primer sites and pUFR043 was digested with *Kpn*I (NEB BioLabs, Inc., Ipswich, MA) and *Sal*I (NEB BioLabs, Inc.). All three molecules were ligated using T4 DNA Ligase (NEB BioLabs, Inc.) and the ligation product, designated as pUFR2166,2708 was electroporated into *E. coli* S17-1 λ pir, as described in Appendix A. The recombinant plasmid was then introduced into M6 through tri-parental mating with pRK2073 as a helper as previously described (10), to generate the mutant strain M6-AvrBsT2. *In vitro* growth of M6-AvrBsT2 (Appendix B) and the retention of the recombinant plasmid in M6 were characterized.

pUFR2166,2708 plasmid retention in M6. The intercellular spaces of two-week-old watermelon seedlings were infiltrated with ~1 x 10^4 CFU/ml of M6-AvrBsT2 bacterial suspension using a syringe without a needle. The cotyledons were allowed to air dry for 1 h and the plants were subsequently incubated at 28^{o}C and RH 70-80% with 12 h/day of light by fluorescent light bulbs. Single leaf disks, 5 mm in diameter, were collected from single cotyledons of three different plants at 3, 4 and 5 days post inoculation (dpi), and macerated in separate microcentrifuge tubes containing 500 µl of sterile water. Appropriate ten-fold serial dilutions were made with the macerates in sterile water and replica-plated onto NA and NA amended with Km. Colonies were enumerated after 48 h at 28^{o}C and 70-80 % RH and the

proportion of colonies on Km amended plates relative to the non-amended plates was determined.

Role of *AvrBsT* in the natural infection of watermelon and melon seedlings by *A. citrulli*. Six two-week-old seedlings, three watermelons and three melons, were spray-inoculated until run-off, separately, with cell suspensions of M6-AvrBsT2 and M6 (~ 1 x 10^6 CFU/ml) which were then used for comparing natural infection as described above, except that the trays had 15 seedlings of each species. The experiment was conducted three times. Relative BFB incidence at 5 days of exposure, relative area under the disease progress curve (AUDPC) data, and time required for 20 % disease incidence development were compared with and without strain-host interaction using the GLIMMIX procedure in SAS.

Role of *AvrBsT* in *A. citrulli* virulence on watermelon and melon seedlings. Cell suspensions of *A. citrulli* strains, M6-AvrBsT2, M6 and AAC00-1, containing ~ 1 x 10^6 CFU/ml, were generated and used to spray-inoculate five two-week-old watermelon and five melon seedlings until run-off. After inoculation, the plants were incubated in plastic bags for 48 h at 20-25^{o}C and approximately 100 % RH. Subsequently, the bags were removed and the seedlings were incubated for 3 additional days at 28^{o}C during the day, and 21^{o}C during the night, with a 12 h/day of fluorescent and incandescent light. BFB severity of each seedling was visually rated five days after inoculation, as described above (2). The average BFB severity rating for each host/strain combination was compared using the GLIMMIX procedure in SAS.

Results

Temporal population dynamics of representative group I and II *A. citrulli* strains in watermelon and melon seedlings after cotyledon infiltration. No reliable differences were

observed in the population growth dynamics of *A. citrulli* strains AAC00-1 and M6 in watermelon and melon seedlings. In one experiment, both strains exhibited similar growth trends on both hosts (Figure 2.2 A). The fastest growth was observed between 1 and 2 dpi, when the population densities of both strains on melon, and AAC00-1 on watermelon, increased from ~ 5 x 10^6 to ~ 5 x 10^8 CFU/disk. The maximum population density of M6 on watermelon cotyledons was ~ 1 x 10^8 CFU/disk, which was reached at 2 dpi. At 4 dpi the AAC00-1 populations on both hosts, and M6 populations on melon were similar (~ 5 x 10^8 CFU/disk), while the M6 population on watermelon decreased to ~ 5 x 10^7 CFU/disk.

In the second experiment, AAC00-1 populations in watermelon cotyledons were lower than in all other host/strain combinations (Fig. 2.2 B); they peaked at ~ 5 x 10^7 CFU/disk at 3 dpi and declined to ~ 1 x 10^7 CFU/disk at 4 dpi. AAC00-1 and M6 populations reached their highest densities (~ 5 x 10^8 CFU/disk) in melon cotyledons at 3-4 dpi. Populations of M6 in watermelon seedlings increased to ~ 5 x 10^8 CFU/disk by 2 dpi.

By 3 dpi, both AAC00-1 and M6 caused collapse of watermelon and melon cotyledons (Fig. 2.3). *A. citrulli* DNA was not detected in negative control watermelon and melon cotyledons infiltrated with sterile water (data not shown).

Temporal population dynamics of *A. citrulli* strains on watermelon and melon seedlings after spray-inoculation. There were no consistent differences in population dynamics between AAC00-1 and M6 after spray-inoculation onto watermelon and melon seedlings (Fig. 2.4). In one experiment, at 4 dpi, population densities of AAC00-1 were higher on watermelon (~ 8 x 10^5 CFU/disk), than on melon seedlings (~ 5 x 10^5 CFU/ disk), but lower than the population densities of M6 on both hosts (~ 5 x 10^6 CFU/disk). At 6 dpi, population densities of AAC00-1 on both hosts, and M6 on melon were similar (~ 1 x 10^6 CFU/disk), while the population density

of M6 on watermelon was slightly higher (~ 5 x 10^6 CFU/disk). Finally, at 8 dpi, AAC00-1 and M6 had higher population densities on watermelon (~ 8 x 10^6 and ~ 1 x 10^7 CFU/disk, respectively) than on melon (~ 8 x 10^5 and ~ 4 x 10^6 CFU/disk, respectively).

In the second experiment, AAC00-1 and M6 population densities on melon seedlings ranged from ~ 1 x 10^5 at 4 dpi to ~ 7 x 10^5 CFU/disk at 8 dpi (Fig. 2.4 B). The population densities of both strains were consistently higher on watermelon than on melon seedlings, except at 6 dpi when *A. citrulli* M6 cells were not detected. At 4 dpi, population densities of AAC00-1 and M6 on watermelon seedlings were ~ 8 x 10^5 and ~ 5 x 10^5 CFU/disk, respectively. At 8 dpi the population density of M6 was higher (~ 1 x 10^7 CFU/disk) than that of AAC00-1 (~ 2 x 10^6 CFU/disk) on watermelon cotyledons. Area under the disease progress curve data for AAC00-1 and M6 on melon seedlings largely overlapped in both experiments indicating no significant difference in the population growth dynamics of the two strains on melon (Fig. 2.5).

After spray inoculation, BFB symptoms on watermelon seedlings developed as localized necrotic lesions with varying degree of chlorosis (Fig. 2.6 A, C). At each time point, the watermelon symptoms ranged from asymptomatic cotyledons to some degree of necrosis, with full collapse of some cotyledons by 8 dpi, regardless of the strain. In contrast, on melon cotyledons, AAC00-1 generally did not cause BFB symptoms (Fig. 2.6 B). In a few instances, cotyledons developed slight marginal necrosis with narrow chlorotic edges. M6 primarily caused chlorosis on the cotyledons of melon seedlings, along with necrotic lesions of different sizes (Fig. 2.6 D). By 8 dpi some of the melon cotyledons inoculated with M6 were necrotic and collapsed completely. Similar to watermelon, there was a range of disease severity on melon cotyledons at every time point. By visual assessment (0-5 scale) at 8 dpi there was no significant

difference in disease severity between watermelon and melon seedlings after spray-inoculation with AAC00-1 and M6.

Comparison of natural infection of watermelon and melon seedlings by *A. citrulli* strains. To assess the natural infection of watermelon and melon seedlings by group I and II *A. citrulli* strains, we compared temporal changes in relative BFB incidence on the two hosts while exposed to M6 and AAC00-1 under growth chamber conditions that promoted plant-to-plant pathogen movement. We also compared relative AUDPC and time required for 20 % disease development for each strain. In the first experiment, the relative BFB incidence on watermelon seedlings was the same (100 %) after 5 days of exposure to M6 and AAC00-1 (Fig. 2.7 A). BFB incidence on melon seedlings exposed to M6 was also similar (90 %) to that observed on watermelon exposed to M6 and AAC00-1. On the other hand, exposure to AAC00-1 resulted in lower BFB incidence (35 %) on melon seedlings than in all other host/strain combinations. In the second experiment symptoms appeared more slowly than in the first experiment and fewer plants became diseased. Similar levels of BFB incidence were observed on watermelon seedlings exposed to AAC00-1 (47.37 %) and M6 (50 %), as well as on the melon seedlings exposed to M6 (55 %) for five days (Fig. 2.7 B). BFB incidence on melon seedlings exposed to AAC00-1 was again lower (5.26 %) than in the other host/strain combinations after 5 days of exposure. Bacterial fruit blotch symptoms on watermelon and melon seedlings exposed to AAC00-1 and M6 for 5 days are shown in Fig. 2.8. To confirm that this difference in ability of group I and group II strains to naturally infect melon seedlings was not strain specific, we repeated the experiment using two additional group II (AAC94-21 and AAC206-102) and two additional group I strains (AU9 and AAC98-17). In the first experiment, the mean BFB incidences on watermelon seedlings exposed to group I and II strains and on melon seedlings exposed to group

I strains were between 80 and 100 % (Fig. 2.9 A). The lowest BFB incidence was recorded on melon seedlings exposed to group II strains AAC94-21 (13.33 %) and AAC206-102 (16.67 %).

In the second experiment, after 5 days of exposure, the BFB incidences for group I and II strains on watermelon and group I strains on melon were between 74 and 100 % (Fig. 2.9 B). Again, the lowest BFB incidences were observed on melon seedlings exposed to group II strains AAC94-21 and AAC206-102 (6.67 % and 0 %, respectively) (Fig. 2.9 B).

The mean BFB incidence on melon seedlings exposed for 5 days to three group II *A. citrulli* strains was significantly lower (~ 14.5 %) than on melon seedlings exposed to three group I (~ 85.3 %), or watermelon seedlings exposed to three group I and three group II strains (81.7 % and 82.3 %, respectively) ($P < 0.001$ and $P = 0.004$, in the first and second experiments, respectively). Additionally, there was a significant difference between the effects of group I and II strains on BFB incidence ($P < 0.0001$ and $P = 0.01$ in the first and second experiment, respectively), as well as a significant host effect ($P = 0.0002$ and $P = 0.02$ in the first and second experiment, respectively). There was also a significant group-host interaction related to BFB incidence ($P = 0.0001$ and $P = 0.008$ in the first and second experiment, respectively).

There was no statistically significant difference in relative AUDPC between group I and II strains on watermelon and melon seedlings in the first experiment (data not shown). However, in the second experiment, the relative AUDPC for group II strains on melon seedlings (2.87) was significantly lower ($P = 0.002$) than for melon seedlings exposed to group I strains (26.32), or watermelon seedlings exposed to strains of both group I and II (29.10 and 35.48, respectively). Additionally, there was a significant difference in the effect of the host ($P = 0.002$) on relative AUDPC in the second experiment and a significant group-host interaction ($p = 0.005$). There was

no significant difference between groups or hosts with regards to time required to reach 20 % BFB incidence in either experiment (data not shown).

Role of *AvrBsT* in the natural infection of watermelon and melon seedlings by *A. citrulli*. There was no difference between M6 and M6-AvrBsT2 in *in vitro* growth in nutrient broth (Appendix B, Fig. 2.10). Additionally, more than 90 % of the M6-AvrBsT2 colonies retained the pUFR2166,2708 plasmid for up to 5 dpi *in planta* (data not shown). In the first and second experiments, the relative BFB incidence on both hosts after 5 days of exposure to M6-AvrBsT2 was similar to that of M6 (Fig. 2.11 A and B). In the first experiment after 4 days of exposure, the relative BFB incidences on watermelon and melon seedlings exposed to M6 were higher (74 and 87 %, respectively) than on both hosts exposed to M6-AvrBsT2 (40 and 47 %, respectively). The relative BFB incidences on watermelon seedlings after 5 days of exposure to M6-AvrBsT2 and M6 were 86.67 % and 93.33 %, respectively, while the relative BFB incidence on melon seedlings exposed to both strains was 86.67 % (Fig. 2.11 A). In the second experiment, the relative BFB incidence on watermelon and melon seedlings exposed to M6 and on melon seedlings exposed to M6-AvrBsT2 was 100 %, while the relative BFB incidence on watermelon seedlings exposed to M6-AvrBsT2 was 93.33 % after 5 days of exposure (Fig. 2.11 B). In the third experiment, by five days of exposure to M6-AvrBsT2 the relative BFB incidences on watermelon and melon seedlings were 73.3 % and 80 %, respectively, whereas the relative BFB incidence on both watermelon and melon seedlings exposed to M6 was 93.33 % (Fig. 2.11 C).

In the three experiments M6 had a significantly higher ($P = 0.007$) mean relative AUDPC on watermelon and melon seedlings (26.48 and 26.97, respectively), than M6-AvrBsT2 (20.78 and 22.22, respectively). There were no significant differences between M6 and M6-AvrBsT2 in

relative BFB incidence after 5 days of exposure on watermelon and melon seedlings and time required to cause 20 % incidence (data not shown).

Role of *AvrBsT* in *A. citrulli* virulence on watermelon and melon seedlings. To assess the role of *AvrBsT* in *A. citrulli* virulence, the severity of BFB symptoms on watermelon and melon seedlings spray-inoculated with M6-AvrBsT2 was compared to AAC00-1 and M6 by visual assessment at 5 dpi. Overall, M6-AvrBsT2 was significantly ($P = 0.02$) less virulent than AAC00-1 and M6 on both hosts. Mean BFB severity rating was significantly lower on watermelon ($P = 0.03$) than on melon seedlings at 5 dpi, but the strain-host interaction was not significant.

In the first two experiments, M6-AvrBsT2 and M6 were more virulent on melon than on watermelon seedlings (Fig. 2.12 A and B). However, in the first experiment, BFB symptoms on melon seedlings inoculated with M6-AvrBsT2 were more severe (mean rating = 3.4) than on melon seedlings inoculated with M6 (mean rating = 2.8), whereas disease severity ratings for watermelon seedlings inoculated with M6-AvrBsT2 and M6 were similar (mean ratings = 1.3 and 1.4, respectively). In the second experiment, BFB severity induced by M6 was greater than that induced by M6-AvrBsT2 on both hosts (Fig. 2.12 B). Average BFB severity induced by AAC00-1 on watermelon and melon seedlings was 1 and 0.9, respectively, in the first experiment, and 4.5 on both hosts in the second experiment (Fig.2.12 A and B). In the third experiment, maximum BFB disease severity was lower for every strain-host combination than in the first two experiments. M6-AvrBsT2 and AAC00-1 resulted in greater BFB severity ratings on watermelon than on the melon seedlings (Fig. 2.12 C) and BFB severity caused by AAC00-1 was greater than that caused by M6-AvrBsT2 on both hosts. M6 caused the same BFB severity on watermelon and melon seedlings (0.7) in the third experiment (Fig. 2.12 C).

Discussion

Previous studies demonstrated that the population of *A. citrulli* can be distinguished into two groups (I and II) (8,12,24,30) based on molecular and biochemical analysis and visual assessment of disease severity on various cucurbit hosts. More recently, it was shown that these groups also differ in their repertoire of T3S effectors (Eckshtain-Levi *et al.*, Phytopathology, *In review*). According to these studies, group I *A. citrulli* strains are moderately to highly aggressive on all tested cucurbits, whereas group II strains are highly aggressive on watermelon, but mildly aggressive on other cucurbits. Additionally, group I strains have been mainly recovered from a range of cucurbits, whereas group II strains are usually recovered from watermelons (30). To date most of the virulence assays used to screen for BFB resistance have relied on visual assessment of BFB severity based on different rating scales (2,14,24). However, these assays suffer from lack of reproducibility across experiments, between researchers, and between laboratories. Efforts to alleviate this variability include the use of multiple independent evaluators that are trained to score disease severity prior to data collection; however, this does not allow disease severity data to be compared across independent studies. For these reasons, we sought to determine if quantification of *A. citrulli* colonization of cucurbit seedling tissue could reflect the differences in virulence between group I and II strains based on visual symptom assessment (8,24,30). We observed that after inoculation by cotyledon infiltration there was no difference between the population growth dynamics of AAC00-1 and M6 on watermelon and melon seedlings. Both strains reached similar population densities in both hosts (~ 5×10^8 CFU/disk) and caused collapse of watermelon and melon cotyledons. This suggests that once in the plant apoplast, the differences in the arsenals of virulence factors possessed by the two strains do not affect tissue colonization.

In contrast to our observations, Baltrus *et al.* reported that three closely related pathovars of *Pseudomonas syringe*: *P. syringe* pv. *phaseolicola* 1448a and 1644R, and *P. syringae* pv. *glycinea* R4, recovered from naturally infected leaves of French bean, mung bean, and soybean, respectively, reached the highest population densities on their original hosts and significantly lower population densities on the non-host species after tissue infiltration with 10^4 CFU/ml (6). The authors further reported that the presence of two genes encoding T3S effectors (*hopC1* and *hopM1*) and the absence of another (*avrB2*) potentially contribute to host range differences between pathovars *glycinea* and *phaseolicola* (6). Similarly, we hypothesized that differences in the range of T3S effectors possessed by M6 and AAC00-1 would explain the difference in virulence on different cucurbits. We also predicted that this difference in virulence would manifest itself in a differential ability of the strains to colonize the cotyledons of the different hosts after tissue infiltration. However, our data suggest that differences between M6 and AAC00-1 do not manifest themselves in the plant apoplast.

In our tissue infiltration assays, we observed significant variation in *A. citrulli* population densities on watermelon and melon seedlings between experiments. This variation could be attributed to differences in leaf disk collection and processing for DNA extraction; individual plant responses to inoculation, or experimental error. Nevertheless, because of the lack of significant differences in population growth of AAC00-1 and M6 in watermelon and melon cotyledons we concluded that quantifying *A. citrulli* populations after tissue infiltration is inadequate for determining the differences in virulence between group I and II strains.

Since infiltration of bacterial cells into the plant apoplast does not reflect the natural infection process, we further hypothesized that quantifying *A. citrulli* population dynamics after spray-inoculation would better reflect differences in the virulence of group I and II strains on

different cucurbit hosts. However, similar to the tissue infiltration assay, we observed no consistent differences between population growth dynamics of M6 and AAC00-1 on the watermelon and melon seedlings after spray-inoculation. Overall our data indicate that quantifying the population dynamics of group I and II *A. citrulli* strains during tissue colonization is inadequate for assessing differences in virulence. Hence, despite its shortcomings, at present, visual assessment of disease severity remains the best approach for evaluating the virulence of *A. citrulli* strains.

Unlike inoculation by infiltration, spray-inoculation resulted in highly variable BFB symptom development on both hosts inoculated with M6 and watermelon seedlings inoculated with AAC00-1. On the other hand, we consistently observed low BFB severity on melon cotyledons sprayed with AAC00-1. Hence, we visually rated BFB severity on seedlings spray-inoculated with AAC00-1 and M6 using a previously established 0-5 scale (2). Unexpectedly, we observed no significant difference between the virulence of AAC00-1 and M6 on the two hosts. It is possible that this observation was due to the lack of sensitivity of the 0-5 rating scale that we employed. This scale was chosen because it is adapted for assessing symptoms on the cotyledons; however, it does not discriminate between finer differences in disease severity e.g., a rating of 1 covers 0 – 25 % cotyledons covered by BFB lesions which could mean a single lesion a few millimeters in diameter, or 50 % of one cotyledon could be necrotic. It is likely that a more discriminatory visual rating scale e.g. the 1-9 scale (Hopkins and Thompson) (13), or measuring relative lesion size might have allowed the distinction between virulence of AAC00-1 and M6 on melon hosts with spray inoculation.

After spray-inoculation, the population densities of AAC00-1 and M6 on melon cotyledons were similar, even though higher disease severity was observed with M6 than with

AAC00-1. Although the differences in BFB severity caused by AAC00-1 and M6 on melon seedlings were not statistically significant, the visual differences were quite striking (Figure 2.6). This could be explained by a reduced ability of AAC00-1 to penetrate into the apoplast of the melon cotyledons at low inoculum levels. In this case it is possible that the AAC00-1 populations detected in melon cotyledon disks were mostly epiphytic, which might explain the lack of symptom development. Therefore, it would be informative to quantify epiphytic population dynamics of M6 and AAC00-1 on both hosts after spray inoculation by enumerating viable bacterial cells washed from the surface of cotyledons and dilution plated on semi-selective agar media.

The inability of AAC00-1 to survive as an epiphyte on melon cotyledons might also explain the discrepancy in BFB symptom expression. Wilson and Lindow observed reduced epiphytic fitness of *Pseudomonas syringae* on bean leaves after spray inoculation with low concentrations of bacterial cells (34). After primary leaves of bean plants were spray-inoculated with *P. syringae* strain MF714R at concentrations ranging from 10^5 to 10^9 CFU/ml, the authors showed that mortality rates and proportional decline in bacterial populations decreased at higher inoculum levels (34). Therefore, it is conceivable that spray-inoculation of melon seedlings with 10^4 CFU/ml of AAC00-1 results in high mortality of the pathogen on the plant surface and low disease severity which is an additional reason to compare epiphytic survival of AAC00-1 and M6 on melon seedlings. Investigation of the physiological and morphological differences in melon and watermelon cotyledons also might provide some insight into the differences in pathogen survival.

Another explanation for the difference in BFB severity induced by group I and II *A. citrulli* strains after spray-inoculation, despite similar population levels, is that AAC00-1

possesses cell density-dependent virulence factors. These virulence factors may be essential for successful infection of melon seedlings but are expressed only at high population levels during the epiphytic stage of the disease.

Ercolani and Crosse (11) investigated the effect of different inoculum concentrations on the colonization rate of host and non-host plants by *Pseudomonas* species after spray-inoculation. *P. syringae* pv. *phaseolicola* and *P. syrngae* pv. *morsprunorum* recovered from naturally infected bean and cherry leaves, respectively, were used to inoculate bean and cherry plants. The authors found that the greatest difference between growth rates of each bacterium on the two plant species was at lower inoculum levels (10^3-10^5 CFU/ml) (11). These findings suggest that it would be informative to investigate the differences in virulence and host range between group I and II strains using low inoculum concentrations.

Despite the lack of statistical significance, we observed very mild BFB symptoms on melon plants spray-inoculated with 10^4 CFU/ml of AAC00-1. Since spray-inoculation is more natural than infiltration, we suspected that it would provide more information about the differences in virulence between group I and II strains. Furthermore, based on observations that group II strains naturally occur on watermelon whereas group I strains naturally infect other cucurbits (30), we hypothesized that in a natural infection assay BFB incidence would be lower on melon seedlings exposed to AAC00-1 than in all other strain-host combinations. As hypothesized, the relative BFB incidence on melon seedlings at 5 days of exposure to AAC00-1 was significantly lower than on melon seedlings exposed to M6, or watermelon seedlings exposed to either strain. Additionally, in the second experiment, group II strains had significantly lower relative AUDPC values on melon seedlings than with other group-host combinations. The lack of statistically significant differences in AUDPC data in the first experiment could be

explained by variations in environmental conditions. Four out of six tested strains exhibited slower symptom development in the first experiment, which was likely due to lower temperatures in the growth chamber. This difference in disease dynamics between experiments was reflected in relative AUDPC values.

From a practical standpoint, it would be interesting to investigate the natural infection of watermelon and melon fruits exposed to group I and II *A. citrulli* strains. The current study was limited to cucurbit seedlings and conclusions may not directly be applicable to fruit, because seedling infection and fruit infection occur separately in BFB epidemics. Knowing the potential of an *A. citrulli* strain to naturally infect different cucurbit fruits would inform growers of the risks associated with producing different cucurbit fruits in close proximity. Additionally, it would be informative to repeat the natural infection assays under field conditions.

Of all of the assays attempted, the natural infection assay demonstrated the highest degree of fine scale differentiation in virulence between group I and II strains. Our findings confirmed previous observations of reduced virulence and incidence of group II strains on melon. Therefore, this relatively simple assay is recommended to assist the characterization of new *A. citrulli* strains.

Because Eckshtain-Levi *et al.* reported the consistent absence of the T3S effector, *AvrBsT*, in group I *A. citrulli* strains, we hypothesized that ectopic expression of *AvrBsT* in M6 would reduce its virulence on melon seedlings. Additionally, we hypothesized that *AvrBsT* would reduce relative BFB incidence caused by M6 on melon seedlings in the natural infection assay. To understand the role of *AvrBsT* in *A. citrulli* virulence, we expressed two orthologs of the gene from AAC00-1 in M6. Potential pitfalls of this approach include the introduction of genetic point mutations during PCR that might render the effectors inactive; absence of complete

promoters of the genes; and one or both effectors not being translated or translocated. *AvrBsT* effectors were reported to have an enzymatic function that depends on the catalytic triad in *X. euvesicatoria* (28). On examination of the sequences of the amplified *AvrBsT* genes and their upstream regions, we did not find mutations in the amino acids that correspond to the catalytic triad. Furthermore, we found no mutations introducing stop codons into the PCR amplicons of the two effector genes. The possibility that the upstream regions of the genes did not include their native promoters and hence prevented the transcription of the effectors remains to be investigated by RT-PCR. Since expression of the two *AvrBsT* orthologs altered the phenotype of M6-AvrBsT2 relative to M6, we can assume that transcription, as well as translation and translocation of the effectors occurred; however, the phenotype of the mutant strain could have been due to the presence of the vector.

We observed no difference between relative BFB incidence on watermelon and melon seedlings induced by M6 mutant strain in the natural infection assay. Interestingly, M6-AvrBsT2 had lower relative AUDPC values and was less virulent than M6 and AAC00-1on both hosts. In the natural infection assay, there was substantial variation in the dynamics of disease development across experiments. Similarly, we observed substantial variation in virulence of both the mutant and the wild-type strains in spray-inoculation assays. These variations could be attributed to variations in temperature and humidity between experiments. Overall, adding two *AvrBsT* orthologs from AAC00-1 decreased the ability of M6 to naturally infect watermelon and melon seedlings and reduced its virulence on both hosts relative to AAC0-1 and M6.

In general, the deletion of individual effector genes often has little or no effect on virulence due to effector gene redundancy, additive effects of individual effectors, or an inappropriate testing environment (35). In some *Xanthomonas* species redundancy and additive

effects of effector genes were reported within families of effector proteins (5,36). One example of this is the report of Yang *et al.* on the homologous effector genes of *Xanthomonas campestris* pv. *malvacearum*, where five out of ten *avrBs3* homologs were sufficient for full virulence on cotton, while the remaining five appeared to be redundant (36). Additionally, Bai *et al.* reported on the additive effect of the effector genes from the *avrBs3* family in *Xanthomonas oryzae* pv. *oryzae*, further indicating that all effectors do not contribute to virulence (5). Since we expressed two *AvrBsT* orthologs from AAC00-1 in M6, which had no other *AvrBsT* copies (Eckshtain-Levi *et al.*, Phytopathology, *In review*), we can eliminate effector redundancy based on homology. However, Collmer *et al.* demonstrated that T3S effectors are redundant in their functions, regardless of genetic similarity (9). In *P. syringae* pv. *tomato* DC3000, all 28 effector proteins were deleted to produce a polymutant strain, DC3000D28E, that was symptomless on *Nicotiana benthamiana*. By reintegration of effector genes into DC3000D28E, the authors demonstrated that eight effectors were sufficient to restore almost wild-type virulence. They speculated that different combinations of effector genes could have achieved the same result (9). From these findings it is possible that genetically different effectors present in M6 with similar biological functions to *AvrBsT* orthologs may have masked the *AvrBsT* phenotype in M6. It is also possible that the two *AvrBsT* orthologs that we selected may not have been the most effective copies of the gene. Recently, a third copy of *AvrBsT* was discovered in the AAC00-1 genome, but it was not included in our study (Minsavage, personal communication). Bai *et al.* reported that of seven homologous genes in *AvrBs3* family, *avrXa7* contributed the most to the virulence of *X. oryzae* pv. *oryzae* on susceptible rice plants (5). Considering those findings, it is possible that the third *AvrBsT* ortholog in AAC00-1contributes the most to the difference in virulence between group I and II *A. citrulli* strains on melon seedlings.

Overall, our findings confirmed the difference in virulence between group I and II *A. citrulli* strains on watermelons and melons. Because this difference does not manifest itself in the plant apoplast, we suspect that the critical virulence factors are active during the epiphytic phase of infection. We found that *AvrBsT* alone did not explain the differences in virulence and host range between AAC00-1 and M6 and further studies are necessary. We strongly suggest that these additional studies be focused on the epiphytic phase of the disease.

Literature cited

1. Abramovitch, R. B., and Martin, G. B. 2005. AvrPtoB: a bacterial type III effector that both elicits and suppresses programmed cell death associated with plant immunity. FEMS Microbiol. Lett. 245:1-8.
2. Araújo, D. V., Mariano, R., and Michereff, S. J. 2005. Métodos de inoculação de *Acidovorax avenae* subsp. *citrulli* em melão. Summa Phytopathologica 31.
3. Asselin, J. E., Bonasera, J. M., Kim, J. F., Oh, C. S., and Beer, S. V. 2011. Eop1 from a *Rubus* strain of *Erwinia amylovora* functions as a host-range limiting factor. Phytopathology 101:935-944.
4. Bahar, O., Kritzman, G., and Burdman, S. 2009. Bacterial fruit blotch of melon: screens for disease tolerance and role of seed transmission in pathogenicity. Eur. J. Plant Pathol. 123:71-83.
5. Bai, J., Choi, S. H., Ponciano, G., Leung, H., and Leach, J. E. 2000. *Xanthomonas oryzae* pv. *oryzae* avirulence genes contribute differently and specifically to pathogen aggressiveness. Mol. Plant-Microbe Interact. 13:1322-1329.
6. Baltrus, D. A., Nishimura, M. T., Dougherty, K. M., Biswas, S., Mukhtar, M. S., Vicente, J., Holub, E. B., and Dangl, J. L. 2012. The molecular basis of host specialization in bean pathovars of *Pseudomonas syringae*. Mol. Plant-Microbe Interact. 25:877-888.
7. Burdman, S., and Walcott, R. 2012. *Acidovorax citrulli*: generating basic and applied knowledge to tackle a global threat to the cucurbit industry. Mol. Plant Pathol. 13:805-815.
8. Burdman, S., Kots, N., Kritzman, G., and Kopelowitz, J. 2005. Molecular, physiological, and host-range characterization of *Acidovorax avenae* subsp *citrulli* isolates from watermelon and melon in Israel. Plant Dis. 89:1339-1347.
9. Cunnac, S., Chakravarthy, S., Kvitko, B. H., Russell, A. B., Martin, G. B., and Collmer, A. 2011. Genetic disassembly and combinatorial reassembly identify a minimal functional repertoire of type III effectors in *Pseudomonas syringae*. Proceedings of the National Academy of Sciences 108:2975-2980.
10. De Feyter, R., and Gabriel, D. W. 1991. Use of cloned DNA methylase genes to increase the frequency of transfer of foreign genes into *Xanthomonas campestris* pv. *malvacearum*. J. Bacteriol. 173:6421-6427.
11. Ercolani, G. L., and Crosse, J. E. 1966. The growth of *Pseudomonas phaseolicola* and related plant pathogens *in vivo*. J. Gen. Microbiol. 45:429-439.
12. Feng, J. J., Schuenzel, E. L., Li, J. Q., and Schaad, N. W. 2009. Multilocus sequence typing reveals two evolutionary lineages of *Acidovorax avenae* subsp *citrulli*. Phytopathology 99:913-920.
13. Hopkins, D., and Thompson, C. 2002. Evaluation of *Citrullus* sp. germ plasm for resistance to *Acidovorax avenae* subsp. *citrulli*. Plant Dis. 86:61-64.
14. Hopkins, D., and Thompson, C. 2002. Seed transmission of *Acidovorax avenae* subsp. *citrulli* in cucurbits. HortScience 37:924-926.
15. Jamir, Y., Guo, M., Oh, H. S., Petnicki-Ocwieja, T., Chen, S., Tang, X., Dickman, M. B., Collmer, A., and R Alfano, J. 2004. Identification of *Pseudomonas syringae* type III effectors that can suppress programmed cell death in plants and yeast. Plant J. 37:554-565.

16. Johnson, K. L. 2010. Elucidation of the molecular host-pathogen interactions that influence seed-to-seedling transmission of *Acidovorax citrulli*, University of Georgia.
17. Johnson, K. L., and Walcott, R. R. 2013. Quorum sensing contributes to seed-to-seedling transmission of *Acidovorax citrulli* on watermelon. J. Phytopathol.
18. 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.
19. Kearney, B., and Staskawicz, B. J. 1990. Widespread distribution and fitness contribution of *Xanthomonas campestris* avirulence gene *avrBs2*.
20. Latin, R. X., and Rane, K. K. 1990. Bacterial fruit blotch of watermelon in Indiana. Plant Dis. 74:331-331.
21. Lavie, M., Shillington, E., Eguiluz, C., Grimsley, N., and Boucher, C. 2002. PopP1, a new member of the YopJ/AvrRxv family of type III effector proteins, acts as a host-specificity factor and modulates aggressiveness of *Ralstonia solanacearum*. Mol. Plant-Microbe Interact. 15:1058-1068.
22. Liu, J., Luo, S., Zhang, Q., Wang, Q., Chen, J., Guo, A., and Shan, W. 2012. Tn5 transposon mutagenesis in *Acidovorax citrulli* for identification of genes required for pathogenicity on cucumber. Plant Pathol. 61:364-374.
23. Maynard, D. N., and Hopkins, D. L. 1999. Watermelon fruit disorders. HortTechnology 9:155-161.
24. O'Brien, R. G., and Martin, H. L. 1999. Bacterial blotch of melons caused by strains of *Acidovorax avenae* subsp *citrulli*. Aust. J. Exp. Agr. 39:479-485.
25. Poueymiro, M., Cunnac, S., Barberis, P., Deslandes, L., Peeters, N., Cazale-Noel, A.-C., Boucher, C., and Genin, S. 2009. Two type III secretion system effectors from *Ralstonia solanacearum* GMI1000 determine host-range specificity on tobacco. Mol. Plant-Microbe Interact. 22:538-550.
26. Schaad, N. W., Sowell, G., Goth, R. W., Colwell, R. R., and Webb, R. E. 1978. *Pseudomonas pseudoalcaligenes* subsp. *citrulli*. nov. Int. J. Syst. Bacteriol. 28:117-125.
27. Somodi, G. C., Jones, J. B., Hopkins, D. L., Stall, R. E., Kucharek, T. A., Hodge, N. C., and Watterson, J. C. 1991. Occurrence of a bacterial watermelon fruit blotch in Florida. Plant Dis. 75:1053-1056.
28. Szczesny, R., Büttner, D., Escolar, L., Schulze, S., Seiferth, A., and Bonas, U. 2010. Suppression of the AvrBs1-specific hypersensitive response by the YopJ effector homolog AvrBsT from *Xanthomonas* depends on a SNF1-related kinase. New Phytol. 187:1058-1074.
29. Vinatzer, B. A., Teitzel, G. M., Lee, M. W., Jelenska, J., Hotton, S., Fairfax, K., Jenrette, J., and Greenberg, J. T. 2006. The type III effector repertoire of *Pseudomonas syringae* pv. *syringae* B728a and its role in survival and disease on host and non-host plants. Mol. Microbiol. 62:26-44.
30. Walcott, R. R., Fessehaie, A., and Castro, A. C. 2004. Differences in pathogenicity between two genetically distinct groups of *Acidovorax avenae* subsp *citrulli* on cucurbit hosts. J. Phytopathol. 152:277-285.
31. Wall, G., and Santos, V. 1988. A new bacterial disease of watermelon in the Mariana islands. Phytopathology 78:1-9.

32. Wechter, W. P., Levi, A., Ling, K.-S., Kousik, C., and Block, C. C. 2011. Identification of resistance to *Acidovorax avenae* subsp. *citrulli* among melon (*Cucumis* spp.) plant introductions. HortScience 46:207-212.
33. Willems, A., Goor, M., Thielemans, S., Gillis, M., Kersters, K., and Deley, J. 1992. Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov., comb. nov., *Acidovorax avenae* subsp. *citrulli*, *Acidovorax avenae* subsp. *cattleyae*, and *Acidovorax konjaci*. Int. J. Syst. Bacteriol. 42:107-119.
34. Wilson, M., and Lindow, S. E. 1994. Inoculum density-dependent mortality and colonization of the phyllosphere by *Pseudomonas syringae*. App. Environ. Microb. 60:2232-2237.
35. Yang, B., and White, F. F. 2004. Diverse members of the AvrBs3/PthA family of type III effectors are major virulence determinants in bacterial blight disease of rice. Mol. Plant-Microbe Interact. 17:1192-1200.
36. Yang, Y., Yuan, Q., and Gabriel, D. 1996. Watersoaking function (s) of XcmH1005 are redundantly encoded by members of the *Xanthomonas* avr/pth gene family. Mol. Plant-Microbe Interact. 9.
37. Zhengguang, R., Lei, H., Zhiguo, S., and Liqun, Z. 2009. Screening of the pathogenicity mutants of *Acidovorax avenae* subsp. *citrulli* and cloning of the *hrcR* gene. Acta Phytopathologica Sinica 39:501-506.

CHAPTER 3

APPENDICES

Appendix A

Preparation of S17-1 λ pir competent cells for electroporation. For electroporation, S17-1 λ pir competent cells were prepared as follows. Briefly, a colony from a 24-h culture of S17-1 λ pir was used to inoculate a 10 ml tube of LB broth, which was then incubated with agitation at 200 rpm for 14-18 h, at 37°C. The overnight culture was used to inoculate 100 ml of LB broth to an OD_{600} ~ 0.1 in a side arm flask, and incubated for approximately 3 h (~ 0.5 OD_{600}). The cells were then pelleted four times by centrifugation at 9802.6 x g for 10 min at 4°C and each time they were resuspended in a smaller volume of liquid: 100 ml sterile Mili-Q water (at 4°C), 50 ml Milli-Q water, 25 ml 10 % glycerol (prepared in Milli-Q water, kept at 4°C), and 1 ml 10 % glycerol, respectively.

S17-1 λ pir electro-transformation with pUFR2166,2708. Two to five microliters of pUFR2166,2708 plasmid DNA and 50-125 µl of S17-1 λ pir electro-competent cells were combined in an electroporation cuvette. Gene Pulser apparatus (Bio-Rad, Richmond, CA, USA), set to 25 µF and 12.5 kV, and the Pulse Controller (Bio-Rad, Richmond, CA, USA) set to 200 Ω, were used for electro-transformation. The desired time constant was 4-5 msec.

Appendix B

In vitro growth of M6-AvrBsT2. A BioScreen C (Growth curves, USA) was used to continuously measure *in vitro* growth of the populations of M6-AvrBsT2 and M6 strain in

nutrient broth. Two hundred microliters of M6 and M6-AvrBsT2 bacterial suspensions prepared in NB, containing ~ 1 x 10^4 CFU/ml, were loaded into a honeycomb plate. *In vitro* growth was measured for 20 replicates of each strain and 10 replicates of an uninoculated NB as negative control. The samples were incubated for 36 h with the following conditions: continuous shaking with medium amplitude and normal speed (200 rpm), 30^{o}C, and a measurement interval of 30 min. The experiment was conducted twice and mean OD values were used to generate growth curves using Sigma Plot 10.0 (Systat Software Inc., San Jose, CA).

Tables

Table 2.1. Bacterial strains and plasmids used in this study.

Strains and plasmids	Relevant characteristics	Source
Bacteria		
Acidovorax citrulli		
AAC00-1	Group II, wild-type, Ap^R	(27)
AAC94-21	Group II, wild-type	(27)
AAC206-102	Group II, wild-type	(13)
M6	Group I, wild-type, Ap^R	(7)
AU9	Group I, wild-type	(27)
AAC98-17	Group I, wild-type	(27)
M6-AvrBsT2	*avrBsT* orthologs with their promoter regions in pUFR043	This study
Escherichia coli		
DH5α	F – Φ80*lacZ*ΔM15 Δ *(lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ – *thi*-1 *gyr*A96 *rel*A1	Invitrogen
S17-1 λ pir		Dr. Denny, UGA
HB101		Dr. Denny, UGA
Plasmids		
pCR4	Km^R	Invitrogen
pCR2166,2708	*avrBsT* orthologs with their promoter regions in pCR4	This study
pUFR043	Km^R	Dr. Jones, UFL
pRK2073	Sp^R, Tp^R	Dr. Denny, UGA
pUFR2166,2708	*AvrBsT* orthologs with their promoter regions in pUFR043	This study

Km^R, Ap^R, Sp^R, and Tp^R = resistant to kanamycin, ampicillin, spectinomycin, and trimethoprim, respectively

Table 2.2. Sequences of oligonucleotide primers used to PCR amplify the *AvrBsT* orthologs, (Aave_2166 and Aave_2708) with their native promoters in AAC00-1. Underlined sequences represent the introduced restriction sites *Kpn*I (A), *Spe*I (B), and *Sal*I (C), respectively, for cloning of the PCR amplicons into the vector pUFR043 and subsequent transformation into *Acidovorax citrulli* strain M6.

Primer	**Sequence (5' -> 3')**
P1 (A) 2166 pUFR	ATGGTACCACATCGCCCGCCGTTATTC
P2 (B) 2166 pUFR	TAACTAGTCTGCCGCCTTTGTTCGTCC
P1 (B) 2708 pUFR	TAACTAGTGGTGATGGATGGGCGGAC
P2 (C) 2708 pUFR	ATGTCGACCTGGAGGCGGATGCTAAGCTG

Figures

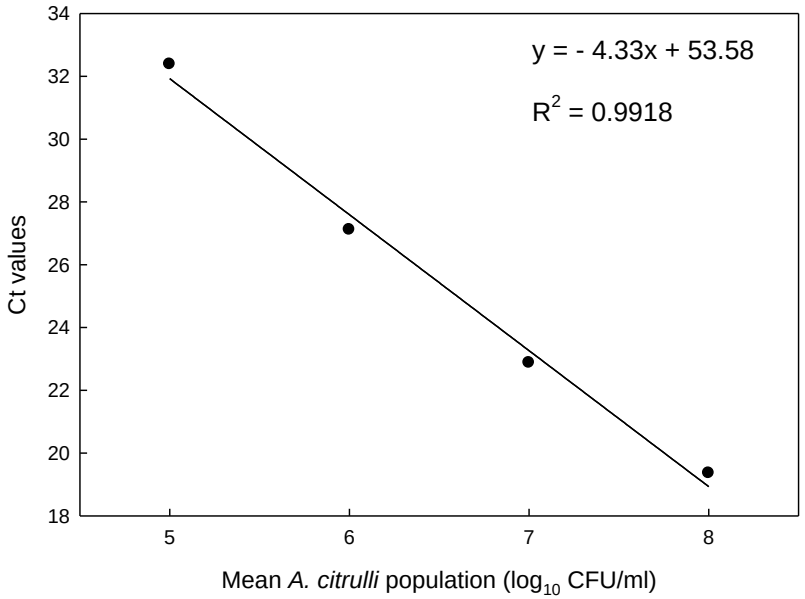
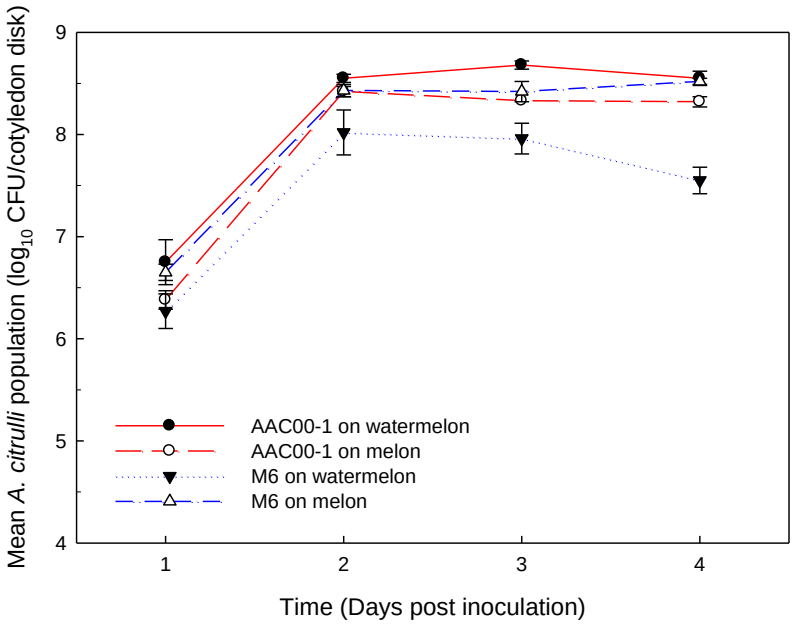


Figure 2.1. Standard curve generated for converting cycle threshold (Ct) values obtained in quantitative real-time PCR to the concentration of bacterial cells (log_{10} CFU/cotyledon disk). Cotyledon disks (5 mm in diameter) from two-week-old watermelon seedlings were collected and macerated in 450 µl of sterile water and spiked with 50 µl of bacterial suspensions to obtain final concentrations ranging from 10^5 to 10^8 CFU/ml. Total microbial DNA was extracted and qPCR conducted using an *A. citrulli*-specific TaqMan assay to obtain the Ct values. The experiment was conducted three times and each datum point represents mean Ct value of the four experiments. Linear regression was conducted and the equation for the regression line was used to calculate log_{10} CFU/cotyledon disk.

A



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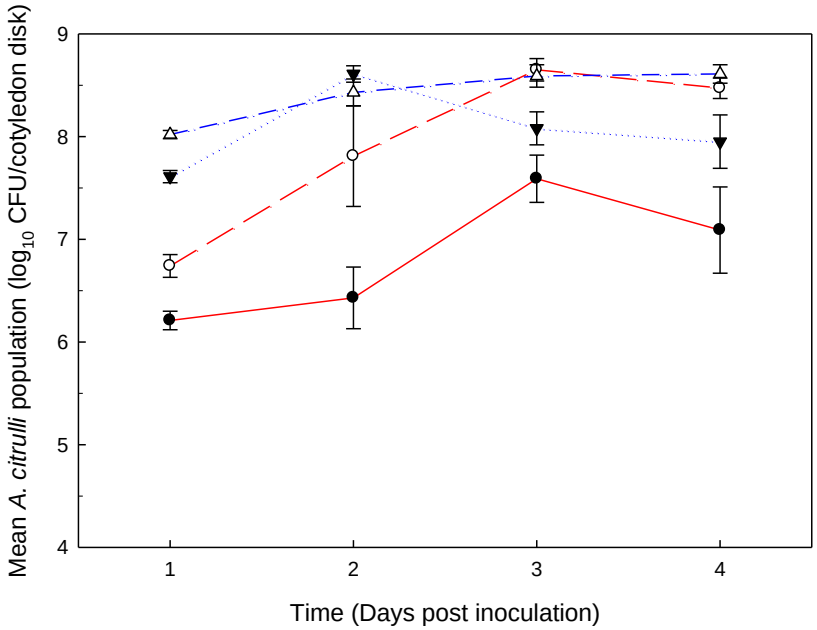


Figure 2.2. Temporal population dynamics of *Acidovorax citrulli* strains M6 and AAC00-1 in watermelon and melon cotyledons after inoculation by infiltration. Intercellular spaces of the cotyledons of two-week-old watermelon and melon seedlings were infiltrated with cell suspensions containing ~ 1 x 10^4 CFU/ml of each strain. Plants were incubated at 30^{o}C, and 70-80 % RH, with a 12 h light period and 5 cotyledon disks were collected from a single plant at 1, 2, 3, and 4 days after inoculation. Leaf disks were macerated and used for total microbial DNA extraction followed by quantitative real-time PCR using an *A. citrulli*-specific TaqMan assay. The experiment was conducted twice and data for each experiment (1 and 2) are presented in figures A and B. Data points represent means of the $\log_{10}$ *A. citrulli* CFU/cotyledon disk and error bars show the standard errors of the means.

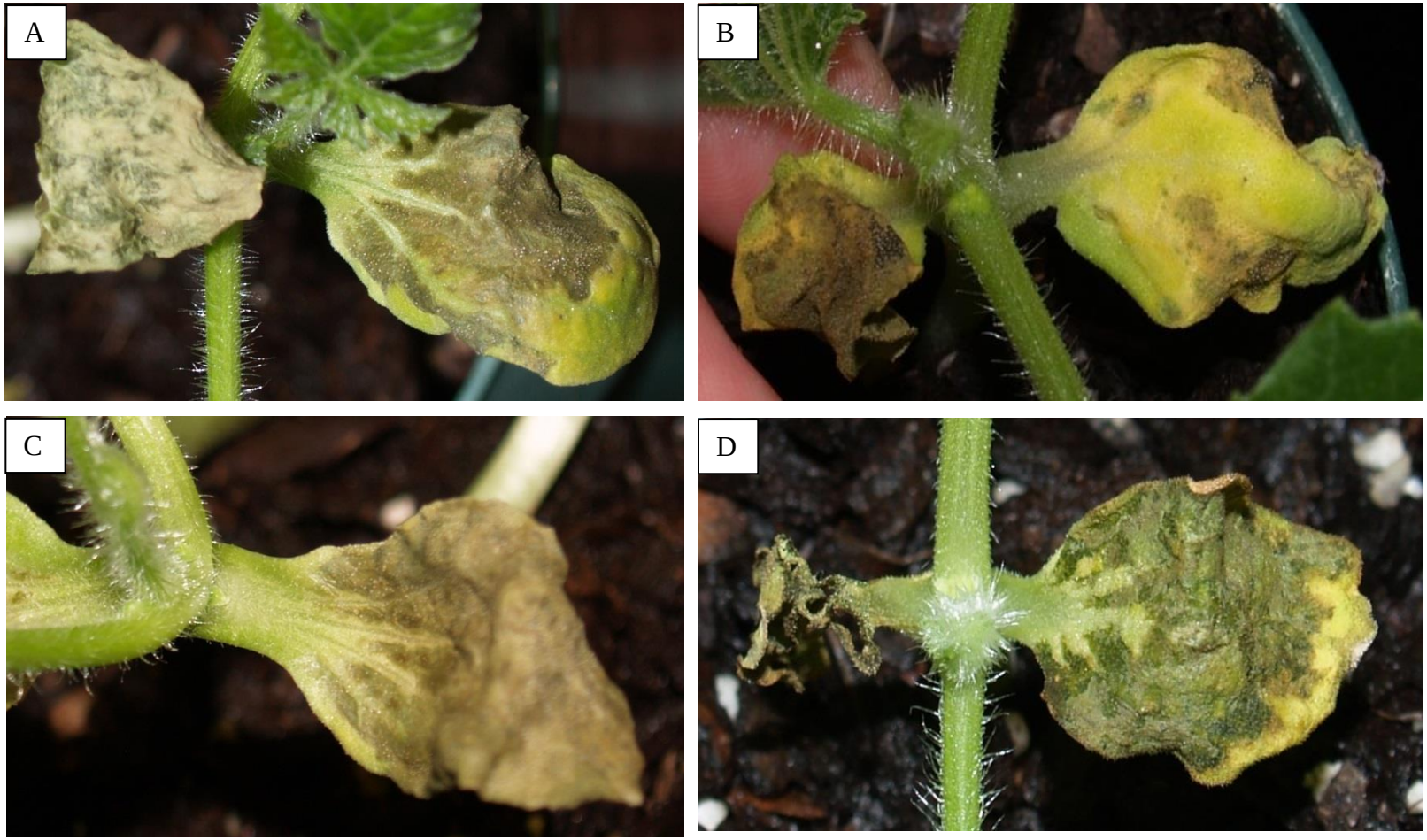
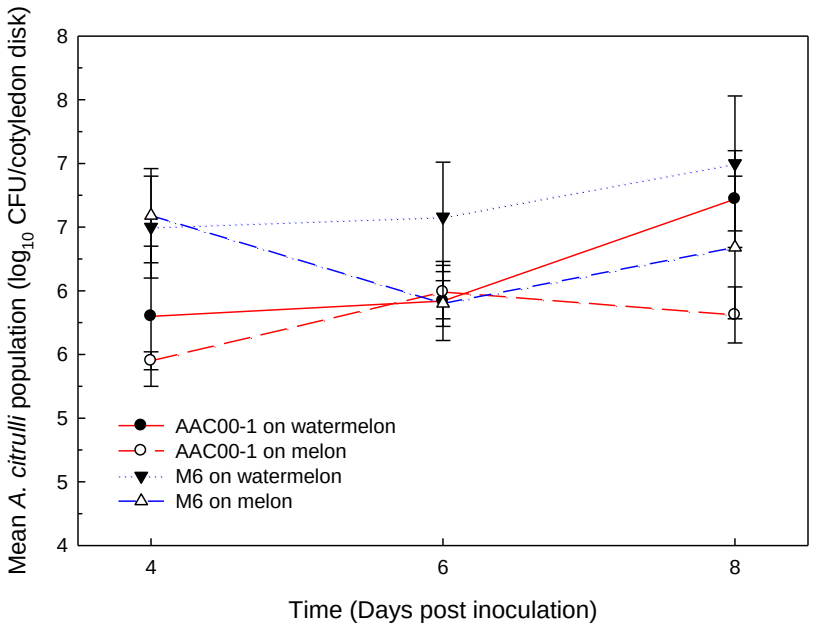


Figure 2.3. Bacterial fruit blotch symptoms on two-week-old watermelon and melon seedlings 3 days after inoculation by infiltration with ~ 1 x 10^4 CFU/ml of group I (M6) and II (AAC00-1) *Acidovorax citrulli* strains. Figure A shows a watermelon seedling infiltrated with AAC00-1; figure B shows a melon seedling infiltrated with AAC00-1; figure C shows a watermelon seedling infiltrated with M6, and figure D shows a melon seedling infiltrated with M6.

A



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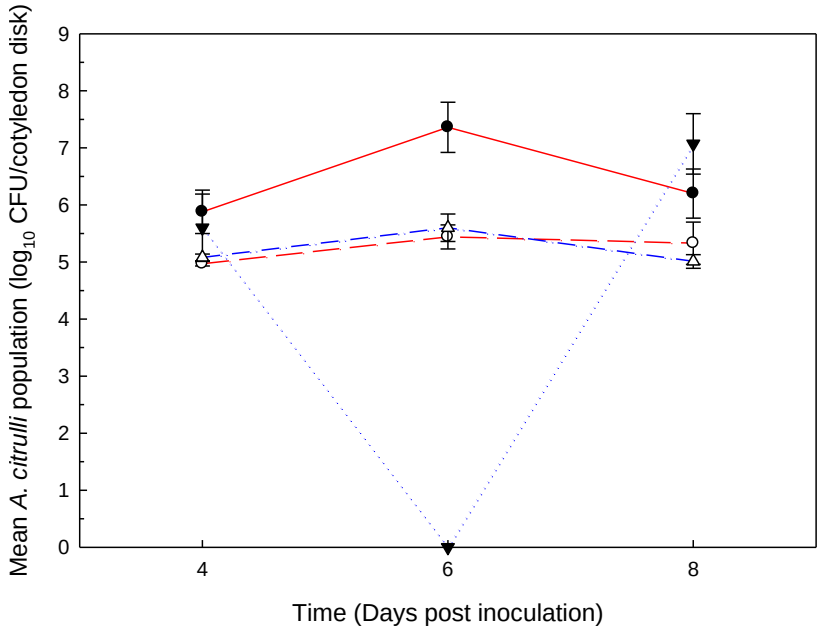


Figure 2.4. Temporal population dynamics of *Acidovorax citrulli* strains M6 and AAC00-1 on watermelon and melon seedlings after spray-inoculation. Two-week-old watermelon and melon seedlings were spray-inoculated until run-off with cell suspensions containing ~ 1 x 10^4 CFU/ml of each strain. Plants were incubated at 30^{o}C, and 70-80 % RH, with a 12 h light period, and 4 cotyledon disks were collected from three plants at 4, 6, and 8 days after inoculation. Leaf disks were macerated and used for total microbial DNA extraction followed by quantitative real-time PCR using an *A. citrulli*-specific TaqMan assay. The experiment was conducted twice and data for each experiment (1 and 2) are presented in figures A and B. Data points represent means of the $\log_{10}$ *A. citrulli* CFU/cotyledon disk and error bars represent the standard errors of the means.

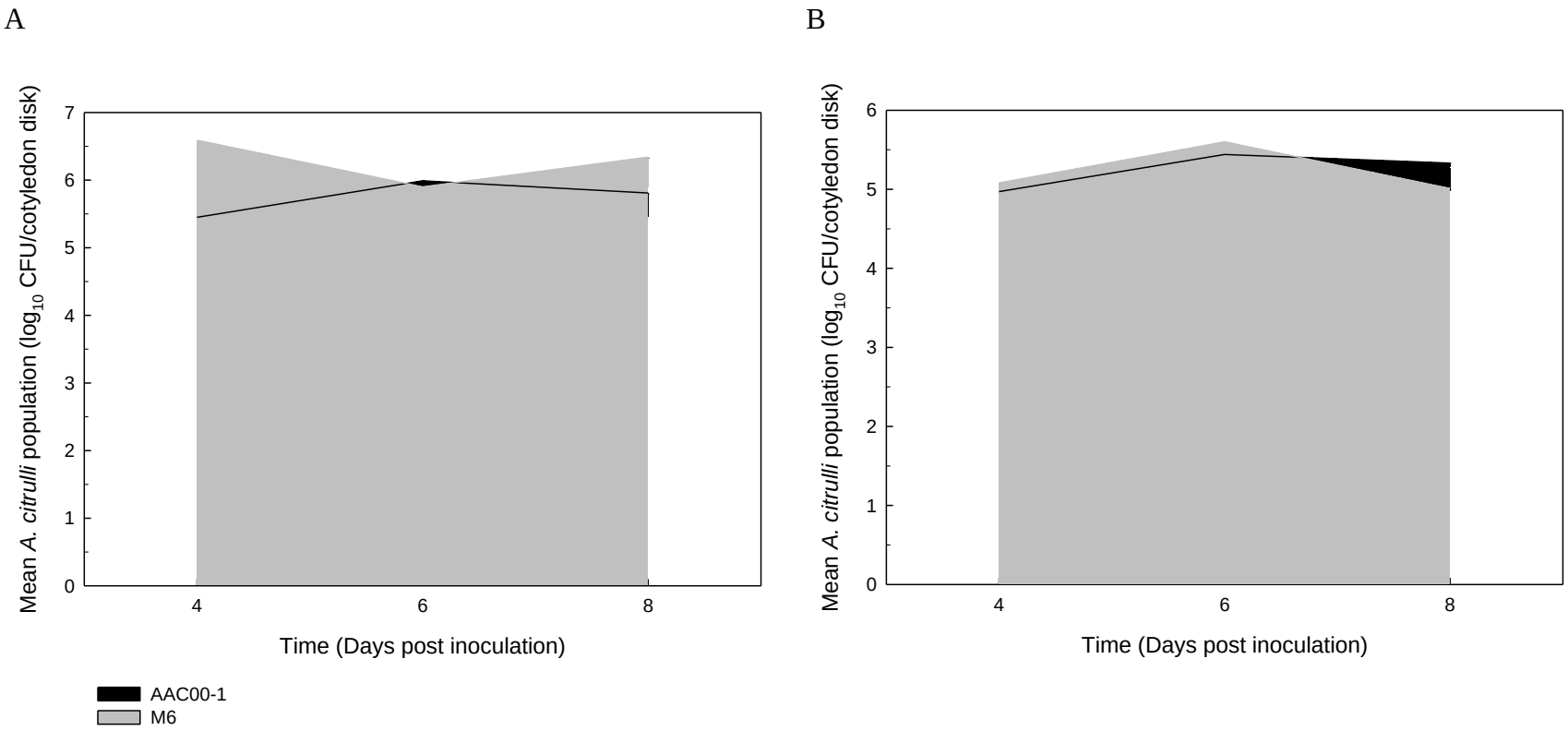


Figure 2.5. Area under population growth curves of *Acidovorax citrulli* strains M6 and AAC00-1 after spray-inoculation onto melon seedlings. Two-week-old melon seedlings were spray-inoculated until run-off with cell suspensions containing ~ 1 x 10^4 CFU/ml of each strain. Plants were incubated at 30^{o}C, and 70-80 % RH, with a 12 h light period, and 4 cotyledon disks were collected from three plants at 4, 6, and 8 days after inoculation. Leaf disks were macerated and used for total microbial DNA extraction followed by quantitative real-time PCR using an *A. citrulli*-specific TaqMan assay. The experiment was conducted twice and data for each experiment (1 and 2) are presented in figures A and B. Lines at the top connect mean *A. citrulli* populations (log_{10} CFU/cotyledon disk) at each time point.

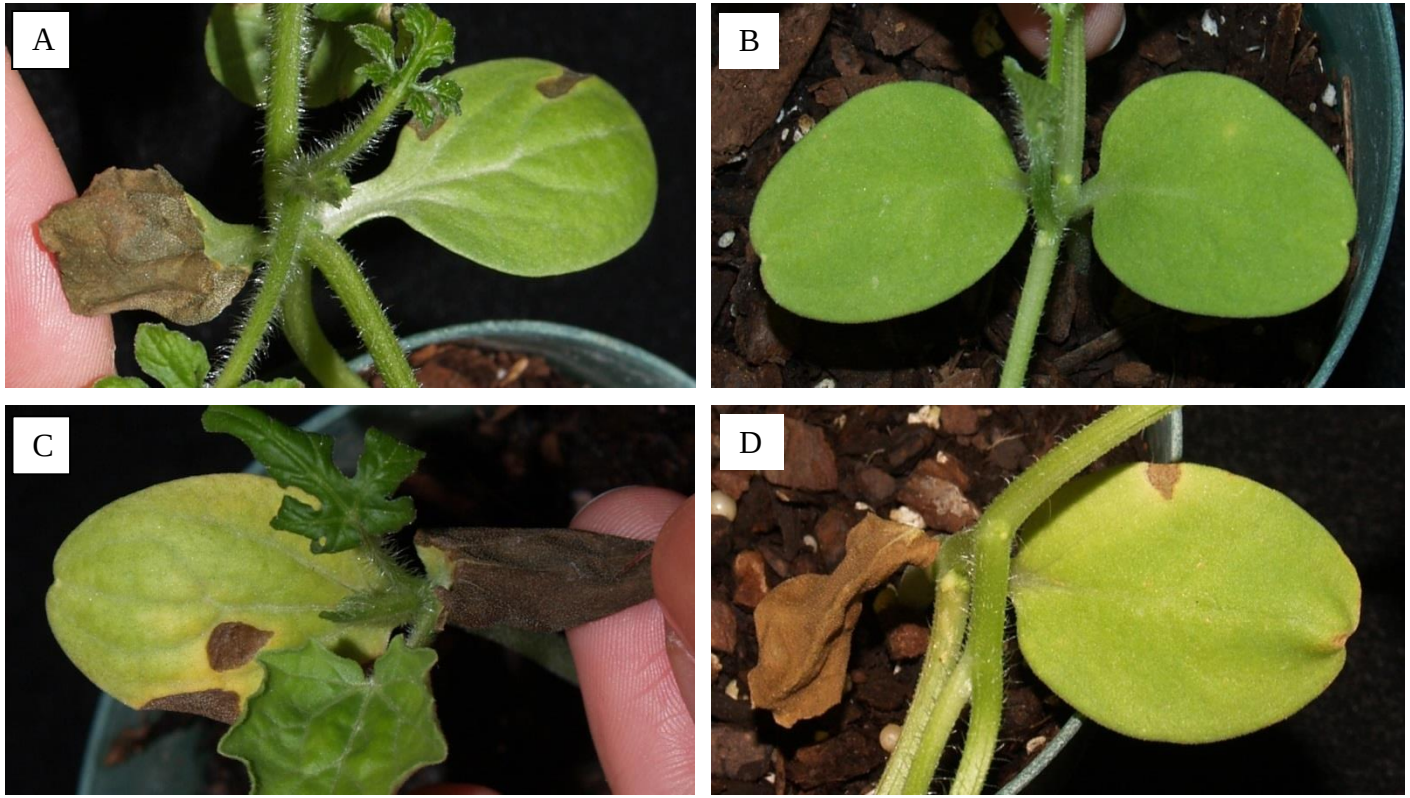
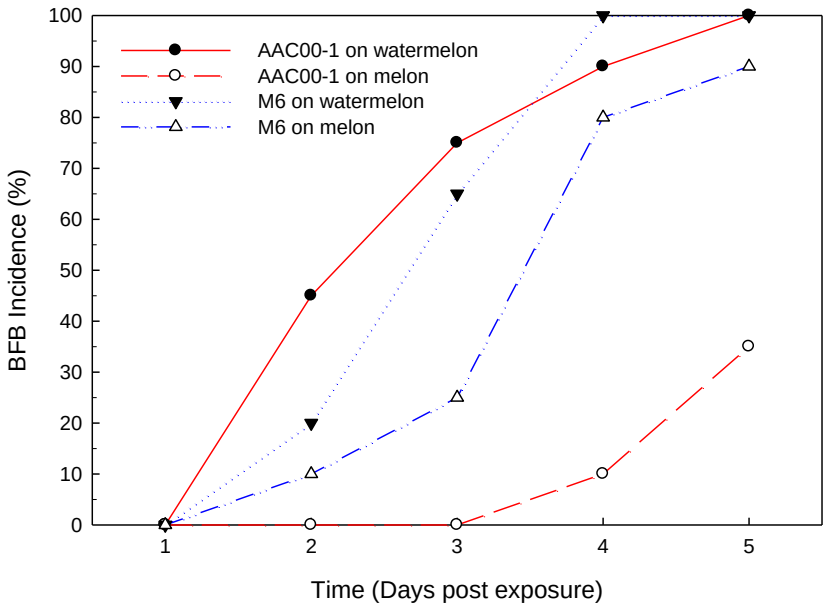


Figure 2.6. Bacterial fruit blotch symptoms that developed on watermelon and melon seedlings spray-inoculated with *Acidovorax citrulli* strains M6 and AAC00-1. Two-week-old seedlings were spray inoculated with suspensions of each strain containing ~ 1 x 10^4 CFU/ml until run-off and incubated for 8 days at 30^{o}C, and 70-80 % RH, with a 12 h light period. Figure A shows a watermelon seedling inoculated with AAC00-1; figure B shows a melon seedling inoculated with AAC00-1; figure C shows a watermelon seedling inoculated with M6, and figure D shows a melon seedling inoculated with M6.

A



B

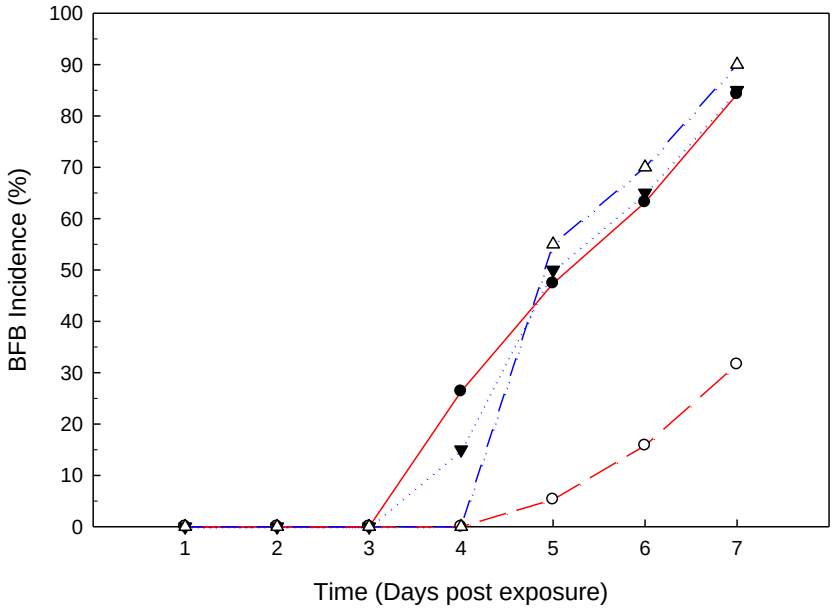


Figure 2.7. Temporal changes in bacterial fruit blotch (BFB) incidence on a mixed population of watermelon and melon seedlings exposed to group I (M6) and group II (AAC00-1) *Acidovorax citrulli* strains under conditions that facilitated natural spread of bacterial cells and infection. Two-week-old watermelon and melon seedlings infected with *A. citrulli* strains AAC00-1 and M6, respectively, were placed separately in the center and corners of seedling trays with mixed populations of uninoculated two-week-old watermelon and melon seedlings. Seedlings were incubated at > 90 % RH and 20-25^{o}C with 6-8 h light/day and irrigated daily using a spray bottle to facilitate natural pathogen spread. BFB incidence was recorded for 5-7 days after exposure and the experiment was conducted twice; figures A and B represent the results of the two independent experiments.

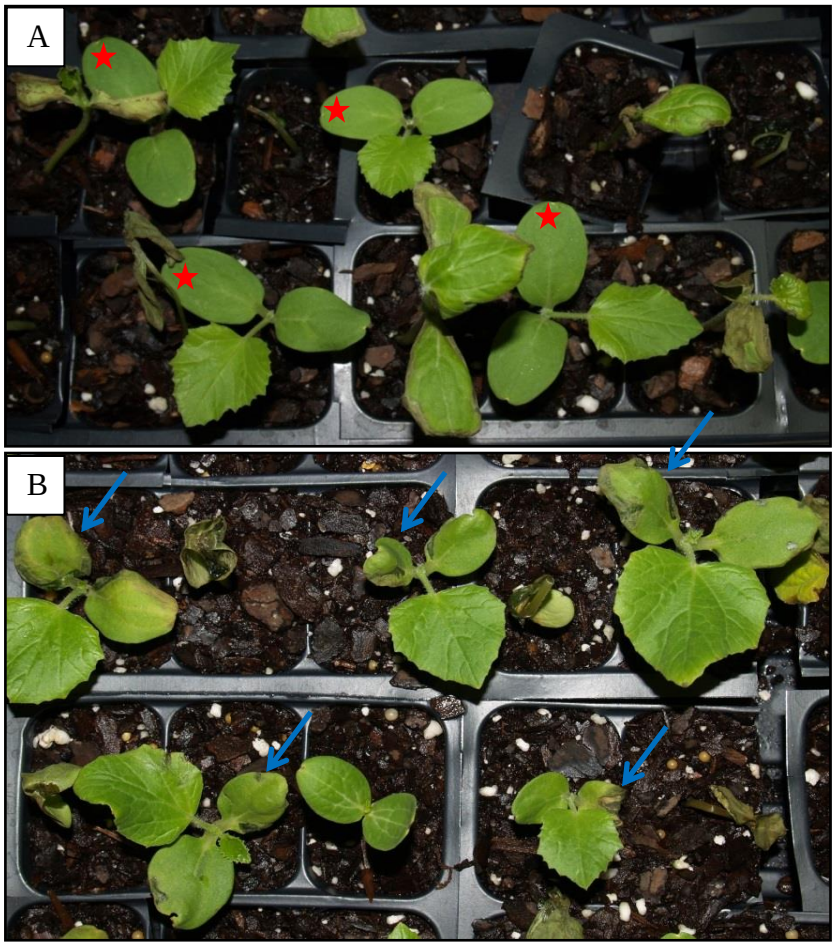
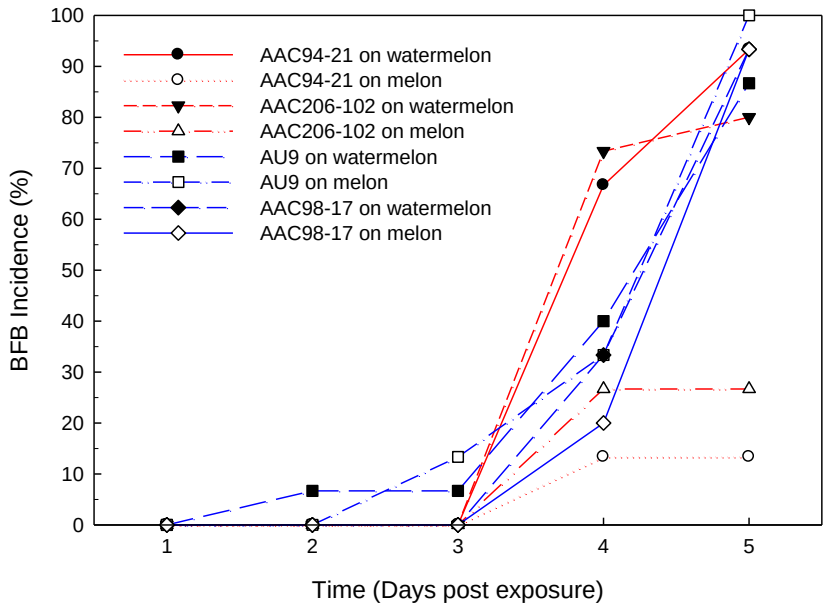


Figure 2.8. Bacterial fruit blotch symptoms on mixed populations of two-week-old watermelon and melon seedlings exposed to inoculum of group II (AAC00-1) (A) and group I (M6) (B) *Acidovorax citrulli* strains. Seedlings were incubated at > 90 % RH and 20-25^{o}C with 6-8 h light/day and irrigated daily using a spray bottle to facilitate the dispersal of inoculum. Image was taken 5 days after seedlings were exposed to *A. citrulli* inoculum. Stars indicate asymptomatic melon seedlings exposed to AAC00-1 and arrows indicate melon seedlings exposed to M6 that exhibit BFB symptoms.

A



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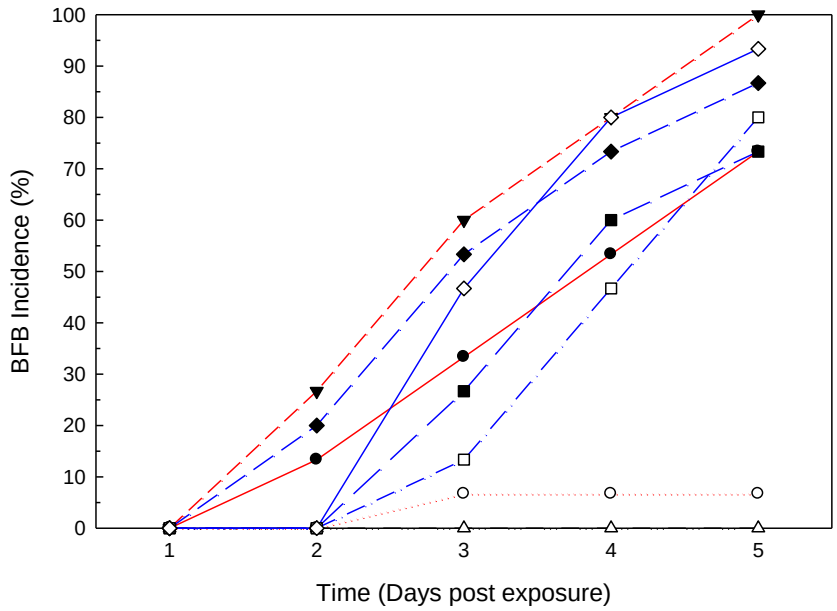
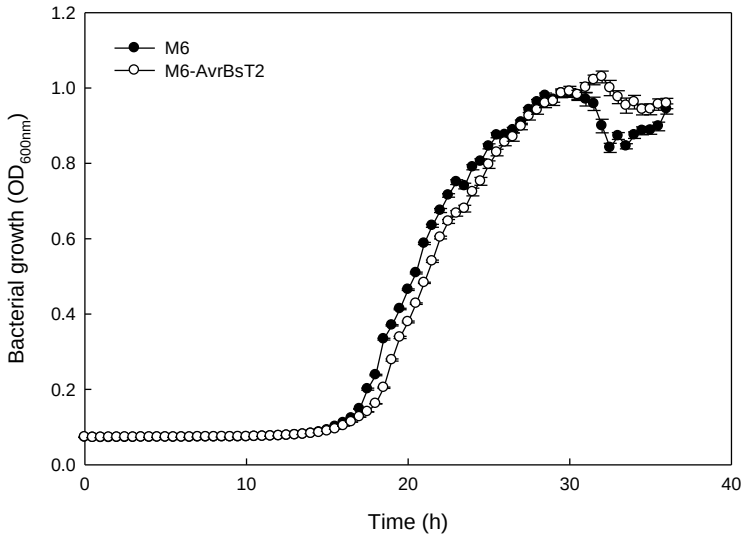


Figure 2.9. Temporal changes in relative bacterial fruit blotch (BFB) incidence in a mixed population of watermelon and melon seedlings exposed to representative group I (AU9, AAC98-17) and group II (AAC94-21, AAC206-102) *Acidovorax citrulli* strains. Two-week-old melon and watermelon seedlings infected with group I and II *A. citrulli* strains, respectively, were placed separately in the center and corners of seedling trays with mixed populations of uninoculated two-week-old watermelon and melon seedlings. Seedlings were incubated at > 90 % RH and 20-25^{o}C with 6-8 h light/day and irrigated daily using a spray bottle to facilitate natural pathogen spread. BFB incidence was recorded each day for 5 days and the experiment was conducted twice. Figure A and B represent the results of experiments 1 and 2, respectively.

A



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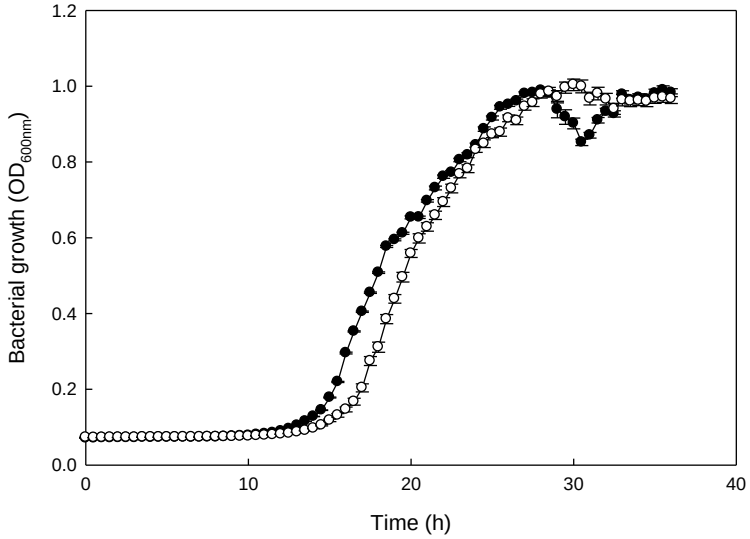

Figure 2.10. Comparison of *in vitro* growth of *Acidovorax citrulli* strain M6 and the mutant strain M6-AvrBsT2, which was transformed with two *AvrBsT* ortholog genes from AAC00-1. Each strain was inoculated into 200 µl of nutrient broth at a starting concentration of ~ 1 x 10^4 CFU/ml. Cultures were incubated in a Bioscreen C automated microbiology growth curve analysis system (Growth Curves, USA) at 30^{o}C with continuous agitation at 200 rpm for 36 h. Optical density data were collected at 600nm every 30 min. Each strain was replicated 20 times and the experiment was conducted twice (Figures A and B). Each datum point represents the mean OD_{600} reading for 20 replicates.

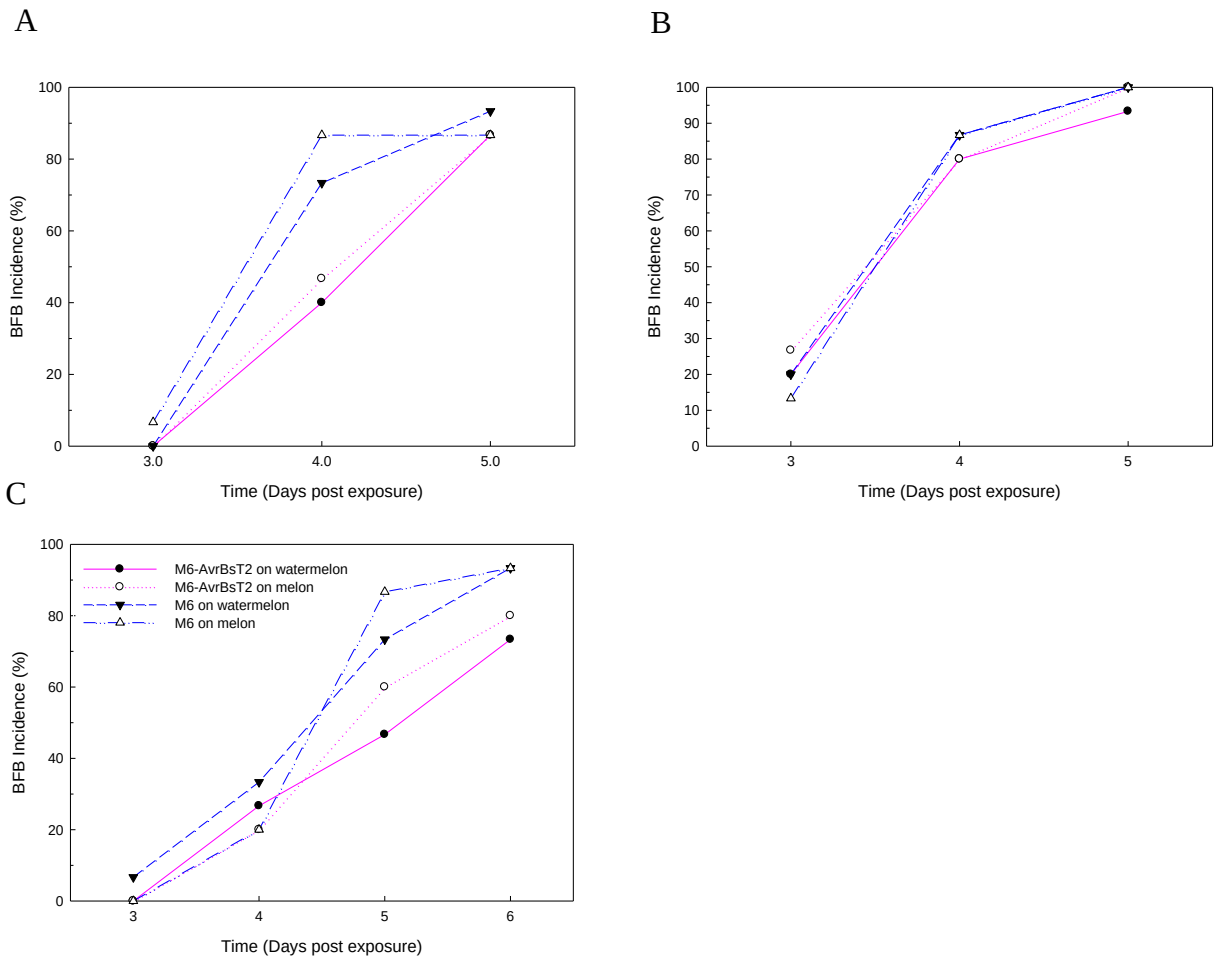

Figure 2.11. Comparison of relative bacterial fruit blotch (BFB) incidence on watermelon and melon seedlings exposed to *Acidovorax citrulli* strains M6-AvrBsT2 and wild-type M6 in a natural infection assay. Two-week-old watermelon and melon seedlings infected with M6-AvrBsT2 and M6 were placed separately in the center and corners of seedling trays with mixed populations of uninoculated two-week-old watermelon and melon seedlings and incubated at > 90 % RH and 20-25^{o}C with 6-8 h light/day and spray irrigated to facilitate natural pathogen spread. BFB incidence was recorded for 5-6 days after exposure and the experiment was conducted three times. Figures A, B, and C represent the results of three independent experiments.

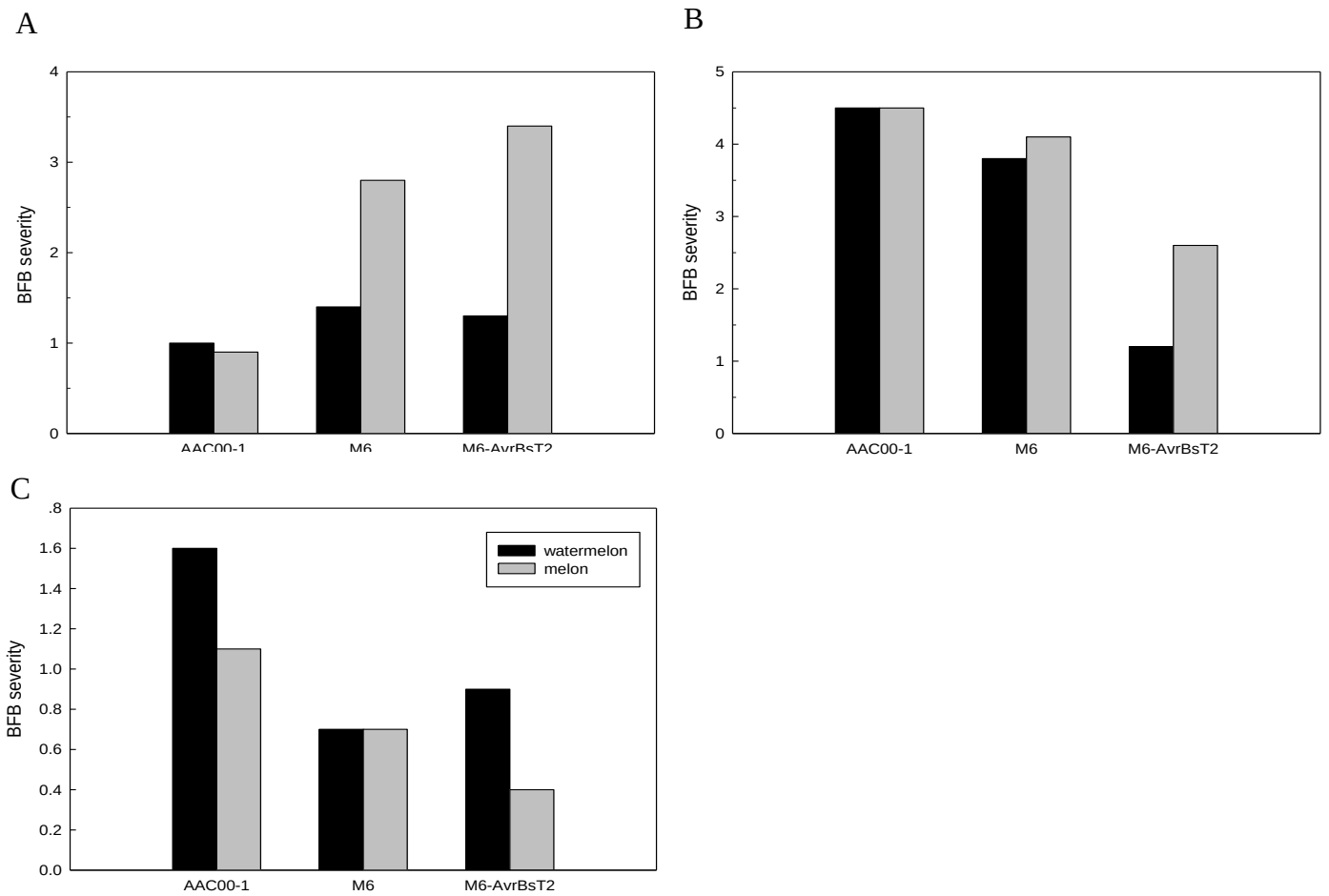

Figure 2.12. Comparison of bacterial fruit blotch severity induced by *Acidovorax citrulli* strains M6, M6-AvrBsT2, and AAC00-1, on watermelon and melon seedlings at 5 days post inoculation. Two-week-old watermelon and melon seedlings were sprayed until run-off with cell suspensions containing ~ 1 x 10^6 CFU/ml of each strain. Plants were incubated for 2 days at 20-25^{o}C and approximately 100 % RH, and then at 28^{o}C during the day, and 21^{o}C during the night, with a 12 h light period for 3 additional days. Each seedling was visually rated for BFB severity using a 0-5 scale by two independent evaluators. Each treatment was replicated on five plants and the experiment was conducted three times (figures A, B, and C represent experiments 1, 2, and 3, respectively). Each bar represents the mean BFB severity rating for five plants.