LOCALIZATION OF *ACIDOVORAX CITRULLI* IN WATERMELON SEED AND ITS INFLUENCE ON SURVIVAL AND SEEDLING TRANSMISSION OF BACTERIAL FRUIT BLOTCH OF CUCURBITS

by

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(Under the Direction of Ronald Walcott)

ABSTRACT

Bacterial fruit blotch (BFB) is caused by *Acidovorax citrulli*, a Gram-negative seedborne bacterium that can cause up to 100% fruit yield losses. The goal of this research was to determine the effect of *A. citrulli* invasion pathways on bacterial localization in watermelon seeds and to investigate the effect of bacterial localization on pathogen survival, seed health testing and exudates produced by seeds during germination. Pistil-inoculation of *A. citrulli* resulted in seeds in which the bacterium was localized in the embryo, whereas while pericarp inoculation resulted in bacterial localization under the testa but outside the perisperm-endosperm (PE) layer. In addition, significantly higher percentages of seeds became infested when *A. citrulli* was inoculated via the pistil than via the pericarp by 7 days post inoculation (DPI). We observed that bacterial location in seed is critical for *A. citrulli* survival. *Acidovorax citrulli* survived stress (surface and sub-surface seed treatments) better in pistil-inoculated seeds than in pericarp-inoculated seeds. We also observed that the localization of *A. citrulli* in seed affects seed health

testing. The recovery of *A. citrulli* by seed washing was more effective for pericarp-inoculated lots than for pistil-inoculated lots. Additionally, it was observed that *A. citrulli* infestation affected the release of xylose in germinating watermelon seeds. Finally, we examined the effect of *A. citrulli* seed inoculum load on frequency of BFB seedling transmission and spatio-temporal spread among watermelon seedlings in the greenhouse. The proportion of seedlots that yielded BFB-infected seedlings was considerably higher for lots with one seed inoculated with $\geq 1 \times 10^5$ colony forming units (CFU) (>95%) than for lots with one seed harboring $\leq 1 \times 10^3$ CFU (<16.7%). The temporal and spatial spread of BFB in 128-cell seedling trays increased linearly with *A. citrulli* seed inoculum load.

Keywords: *Acidovorax citrulli*, Perisperm-endosperm layer, Watermelon fruit blotch

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B.Sc., Tamil Nadu Agricultural University, India, 2006

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

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2011

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December 2011

DEDICATION

I would like to dedicate this dissertation to my parents (Mr. Paresh Chandra Dutta and Mrs. Ajanta Dutta) whose love, support and inspiration have helped me throughout my life. My countless thank and love goes to my life partner Mrs. Aparna Petkar Dutta whose love, moral support, and encouragement made everything possible. It was her encouragement and light hearted jokes that made me happy during my tough times.

ACKNOWLEDGEMENT

I would like to thank everyone who helped and guided me throughout my Ph.D. program and encouraged me to become a good seed pathologist. I would first like to pay my sincere thanks to my major advisor Dr. Ron Walcott for his guidance, mentoring and encouragement during my degree program. I really appreciate the amount of trust he showed in my abilities that helped me become a better scientist. I would also like to thank my committee members Dr. Ron Gitaitis, Dr. Harald Scherm, and Dr. David Langston for their continued support and intellectual inputs. I would like to thank Dr. Rock Christiano (Post Doctoral Associate, Department of Plant Pathology, UGA) for his invaluable assistance with statistical analyses. My sincere thank also goes to Drs. William York and Michael Hahn (Professor, Complex Carbohydrate Research Center, UGA) for their input on metabolomics and immunolabeled microscopy. I would also like to acknowledge Drs. Utku Avci and Koushik Mazumdar (Post Doctoral Associate, Complex Carbohydrate Research Center, UGA) for their technical and intellectual assistance on metabolomics and immunolabeled microscopy. My sincere thank goes to Ms. Anita Castro Sparks and Ms. Linda Genzlinger for their technical assistance in the entire project.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Bacterial fruit blotch (BFB), caused by the Gram-negative bacterium *Acidovorax citrulli* (*Acidovorax avenae* subsp. *citrulli* =*Pseudomonas pseudoalcaligenes* subsp. *citrulli*) (Schaad *et al*., 1978; Schaad *et al*., 2008; Willems *et al*., 1992), is an economically important disease of cucurbits (Family *Cucurbitaceae*) worldwide. BFB first became a problem in watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) in the late 1980s. In the United States, it became a matter of concern for fruit growers and commercial seed producers after a severe outbreak in commercial watermelon fields in Florida (Somodi *et al*., 1991) and since then it has been a sporadic threat. BFB can cause up to 50 to 100% yield losses (Latin and Hopkins, 1995; Schaad *et al*., 2003).

Cucurbits provide numerous edible products and seeds that are rich in oil and protein. The flesh is an important source of carbohydrates and water in tropical and semiarid regions of the world (Nayar and More, 1998). In the U.S., cucurbit production has experienced tremendous growth in the last decade (Cantliffe *et al*., 2007). The bulk of cucurbit production occurs in Florida, North Carolina, Michigan, Texas, California, and Georgia. Florida has been the leader in fresh market production of cucumber, squash, and watermelon, while Michigan leads production of processed cucumbers, and California and Arizona lead melon production (Cantliffe *et al*., 2007).

According to the National Agricultural Statistics Service (NASS, USDA), watermelon production in the U.S. (2007) was approximately 4.29 billion pounds valued at $434 million USD. The top five watermelon producing states were Georgia, Florida, Texas, California and Arizona and the per-capita consumption of watermelon was 16.2 pounds (2007).

Watermelon contains about 6% sugar and 90% water by weight and it is a source of vitamin C, but not a significant source of other vitamins and minerals (National Nutrient Database, USDA). Watermelon rinds are also used as a vegetable. In China, the rinds are stir-fried, stewed and pickled. Pickled watermelon rind is also commonly consumed in the southern United States, Russia, Ukraine, Romania, and Bulgaria (Maynard and Dunlap, 1992).

Brief History

Bacterial fruit blotch of watermelon was first reported in 1965 at the USDA Regional Plant Introduction Station at Griffin, Georgia, U.S. (Webb and Goth, 1965). The seedlings of two plant introductions (PI 1714103 and 174104), showed large, irregular water-soaked lesions that gradually turned necrotic on affected cotyledons. Water-soaked lesions also developed on stems and led to necrosis and premature seedling death. The bacterium induced similar symptoms on *Cucurbita moschata* and *Cucumis melo* by artificial inoculation but only caused seedling blight symptoms (Webb and Goth, 1965). The pathogen was found to be seedborne and seed-transmissible with long-term survival in seeds (Webb and Goth, 1965). Severe BFB seedling blight symptoms were observed

on watermelon in 1969 at a research farm in Leesburg, FL (Crall and Schenck, 1969). Subsequently, Schaad *et al*. (1978) identified the causal agent as *Pseudomonas pseudoalcaligenes* subsp. *citrulli* and confirmed the seedborne nature of the bacterium. Sowell and Schaad (1979) documented pathogenicity (both in field and greenhouse experiments), and assessed seed treatment and host resistance in seedlings of watermelon PI lines. They concluded that BFB had a low damage potential on watermelons in the field unless the seeds were heavily infested. Interestingly, *P. pseudoalcaligenes* subsp. *citrulli* was unable to produce a hypersensitive response (HR) on tobacco. The authors suggested a greater risk to greenhouse-grown seedlings because the pathogen could be splash-dispersed with overhead irrigation. *Pseudomonas pseudoalcaligenes* subsp. *citrulli* was reported to cause leaf spots on muskmelon PIs grown at the USDA Plant Introduction Nursery in Griffin, GA (Schaad *et al*., 1978). The pathogen isolated from the PIs produced similar colonies to *P. pseudoalcaligenes* subsp. *citrulli* recovered from watermelon, and the host ranges for both bacteria were similar (Sowell and Schaad, 1979).

The first report of BFB in a commercial watermelon field was documented in the Mariana Islands in 1987 (Wall *et al*., 1987). The disease produced water-soaked lesions on the rind of the fruit that eventually led to fruit rot. The bacterium isolated from the infected fruits was identified as *P. pseudoalcaligenes* subsp. *citrulli*, and this was the first time the bacterium was associated with BFB of watermelon fruits under natural conditions. However, the disease received widespread attention, when an outbreak occurred in commercial watermelon fields in Florida, South Carolina, and Indiana in

1989 (Latin and Rane, 1990, Somodi *et al*., 1991). This outbreak also caused mistrust amongst watermelon seed producers and fruit growers (Walcott, 2008). As the bacterium was seedborne, many U.S. watermelon growers filed costly lawsuits against seed companies, when they experienced BFB. Consequently, seed companies restricted sale of the watermelon seeds in high risk areas (e.g., South Carolina) (Walcott, 2008).

BFB was reported sporadically between 1990 and 1994 in different U.S. watermelon-growing states (Walcott, 2008). In 1995, scientists from the university and seed industry developed a comprehensive integrated management strategy that emphasized pathogen exclusion through seed health testing. Increased seed health testing led to an initial reduction in BFB outbreaks (1995-1998), but after 1999, significant increase in outbreaks on cucurbits other than watermelon were observed (Langston *et al*., 1999; Martin and O' Brien, 1999; Walcott *et al*., 2000). All commercially grown cucurbits now appear to be susceptible. Except in Europe, where it has yet to be reported, sporadic reports of BFB outbreaks continue to occur in seed, transplant, and fruit production systems worldwide (Schaad *et al*., 2003; Walcott, 2008).

BFB etiology and symptomatology

Acidovorax citrulli (Schaad *et al*., 1978; Schaad *et al*., 2008; Willems *et al*., 1992) is a Gram-negative, rod-shaped bacterium with average cell dimensions of 0.5 (L) x 1.7 (W) µm (Schaad *et al*., 1978). It is a member of the Beta-proteobacteria within the Comamonadaceae family (Willems *et al*., 1992), motile by a single polar flagellum, strictly aerobic and grows at 41°C. It produces smooth, round, cream-colored, non-

fluorescent colonies after 48h on King's Medium B (Schaad *et al.*, 1978). Biochemically, the pathogen is oxidase-positive and arginine dehydrogenase-negative. On the basis of physiological characteristics, the bacterium was initially classified as *Pseudomonas pseudoalcaligenes* subsp. *citrulli* (Schaad *et al*., 1978) but subsequent phenotypic analysis, protein studies, and DNA-rRNA and DNA-DNA hybridizations resulted in reclassification as *Acidovorax avenae* subsp. *citrulli* (Willems *et al*., 1992). In 2008, Schaad *et al*. reclassified the bacterium as *A. citrulli* on the basis of 16s rDNA and 16s-23s rDNA internally transcribed sequence data.

Discrepancies between *A. citrulli* strains from the Georgia Agricultural Experiment Station (Schaad *et al*., 1978) and Florida (Somodi *et al*., 1991) outbreaks led to the detailed characterization of *A. citrulli* populations based on DNA fingerprinting, sole carbon source utilization, and fatty acid methyl ester (FAME) analysis (Walcott *et al*., 2000; Walcott *et al*., 2004). Among 64 *A. citrulli* strains collected from watermelon, melon, pumpkin, and squash hosts from the United States, China, Taiwan, Thailand, Canada, Australia, and Israel, two distinct groups were observed. Group I strains were moderately aggressive on melon, watermelon, pumpkin, and squash while the Group II strains were highly aggressive on watermelon (Walcott *et al*., 2004). Burdman *et al*., (2005) also confirmed the segregation of *A. citrulli* into two groups using a smaller population of strains recovered from melon and watermelon in Israel and similar observations were reported by Feng *et al.* (2009) using multilocus sequence typing of strains collected from around the world.

Though *A. citrulli* is seedborne, no visible symptoms are produced on infested watermelon seed. However, the bacterium produces symptoms on seedlings, mature leaves and fruits. Symptoms start as dark water-soaked lesions on the undersides of the cotyledons, which often turn necrotic and progress along the veins of the cotyledons. Lesions can also occur on the hypocotyl as seedlings emerge, and result in seedling blight. On mature leaves, the lesions are light to reddish-brown and progress along the mid-veins. In fruits, symptoms start as small, greasy, water-soaked lesions that gradually enlarge with irregular margins. These lesions do not extend to the flesh, but the rind may eventually crack and produce effervescent ooze. This ooze is often followed by fruit rot. In melons, BFB symptoms start as discrete dark green spots that become sunken in the rind as the fruit matures (O'Brien and Martin, 1999). In melons (e.g., cantaloupe, muskmelon) netting fails to develop over necrotic areas, resulting in smooth sunken spots (O'Brien and Martin, 1999). Melon lesions do not expand on the fruit surface, but penetrate through the pericarp (fruit wall) to cause brown cavities (O'Brien and Martin, 1999). Similar symptoms occur on pumpkin including water-soaked rind lesions with cracks and internal fruit rot (Langston *et al*., 1999).

Host range and geographical distribution

Until the late 1990s, BFB outbreaks were reported primarily on watermelon in the U.S. and Guam. Generally, the disease is restricted to regions with hot and humid climates and has not been reported in cool and dry regions of the U.S. (eg. California). However, since 1999, sporadic BFB outbreaks have occurred in various parts of the world on a range of cucurbitaceous hosts. In the U.S., BFB was reported on several hosts

like honeydew (*Cucumis melo* L.), cantaloupe (*Cucumis melo* var. *cantalupensis*), pumpkin (*Cucurbita pepo)*, citron (*Citrullus lanatus citroides*), and West Indian gherkin (*Cucumis anguria* var. *anguria*) (Isakeit *et al*., 1997; Isakeit *et al*., 1998; Langston *et al*., 1999; Walcott *et al*., 2000). Other natural hosts include squash (*Cucurbita moschata*), gooseberry gourd (*Cucumis myriocarpus*), muskmelon (*Cucumis melo reticulates*) in Australia (Martin and Harlock, 2002; Martin and O' Brien, 1999; O'Brien and Martin, 1999) and watermelon in Mexico, Israel, and Trukey (Asis *et al*., 1999; Burdman *et al*., 2003; Mirik *et al*., 2006).

Epidemiology

Seed production

Infested seed is the primary source of *A. citrulli* inoculum (Rane and Latin, 1992; Hopkins *et al*., 2002), however, there has been little research to understand BFB epidemiology in seed production environments. The role of alternative inoculum sources like weeds, fruit, and foliage debris is also not clearly known in commercial BFB outbreaks. For effective management, it is critical to identify the inoculum sources and mechanisms of seed infestation. In arid and cool environments where seeds are usually produced, bacteria could be introduced through contaminated stock seeds or could be endemic in weeds and local irrigation sources. However, it is expected that low moisture conditions in seed production fields would prevent the development of typical BFB symptoms on foliage and fruits. Despite the lack of symptoms, infested seeds are still

produced, suggesting the need for a better understanding of the epidemiology of BFB in the seed production environment.

To understand the biology of seed infection, Walcott *et al.* (2003) documented the role of blossom invasion in seed infection. The authors observed that pistil-invasion by *A. citrulli* does not result in BFB fruit symptoms, but seeds within the fruit become contaminated. Lessl *et al.* (2007) reported that *A. citrulli* can rapidly colonize watermelon stigmas reaching populations of 10^9 colony forming units (CFU)/ blossom by 96h post-inoculation. They also showed that low levels of *A. citrulli* (10^3 CFU/blossom) applied to stigmas can lead to seed infestation. Subsequently, using a constitutive green fluorescent protein mutant of *A. citrulli,* it was demonstrated that the bacterium can penetrate the stigma and style via the transmitting tract tissues and reach the ovary by 7 days after inoculation (Lessl, 2003). This mechanism of seed infestation provides a possible explanation for the production of contaminated seedlots from fields with no visible BFB symptoms.

Transplant production

The conditions in a transplant house are highly conducive for BFB development (Walcott 2008). Infested seeds are normally the primary source of inoculum, and secondary spread of bacteria is due to the splash-dispersal facilitated by overhead irrigation. *Acidovorax citrulli* cells land on the healthy seedlings, penetrate the cotyledon through stomata and multiply in the intercellular spaces (Walcott, 2008). *Acidovorax citrulli* can also survive as an epiphyte on asymptomatic seedlings that can lead to

outbreaks in the field under suitable conditions. Hence, seedlings from contaminated greenhouses have a high risk of BFB transmission in the field.

Fruit production fields

Infected seeds and seedlings are the most important primary sources of inoculum in commercial fruit production fields. However, there may be other endemic sources of inoculum like debris from infected fruit or foliage tissue, volunteer watermelon seedlings, or cucurbitaceous weeds (Black *et al*., 1994; Latin and Hopkins, 1995; Isakeit *et al*., 1998). In the field, BFB development is heavily dependent on rainfall and relative humidity. Secondary dispersal of *A. citrulli* is by wind-driven rain or over-head irrigation. When *A. citrulli* lands on healthy leaves, it migrates through open stomata into the sub-stomatal intercellular spaces where it multiplies and induces water-soaked lesions. There is currently no evidence of systemic *A. citrulli* movement throughout the plant, and at anthesis, bacteria deposited on the surfaces of immature ovaries migrate through open stomata to initiate fruit infections (Frankle *et al*., 1993). This infection court is accessible for 2-3 weeks after anthesis, and as fruits mature, wax becomes deposited over the stomata preventing further bacterial entry (Frankle *et al*., 1993). Despite this, *A. citrulli* can exist epiphytically on leaf and fruit surfaces and may invade tissues through wounds. Seeds from infected fruits may become buried in the soil where they may serve as an inoculum for subsequent crops. While fruit infection is initiated at anthesis, fruit symptoms do not develop until harvest maturity.

Management of BFB

Resistance: Commercial cultivars with immunity to BFB are currently not available; however, cultivars like Garrisonian, Mountain Hoosier, and Wilhite Wonder were initially reported to display some level of resistance (Goth and Webb, 1981; Sowell and Schaad, 1979). Subsequently, Hopkins *et al.* (1993) reported that the cultivars which were earlier considered as BFB resistant were in fact susceptible. The authors also reported that watermelon cultivars with dark-colored rinds were more resistant than light-colored cultivars. However, under conditions favorable for BFB development, they can also become infected. Furthermore, Hopkins and Thomson (2002) identified two PIs (PI 482279 and PI 494817 from Zimbabwe and Zambia, respectively) out of 1322 accessions tested that displayed resistance under greenhouse and field conditions. Although, the finding was encouraging further breeding is required to introgress the resistance trait into commercial watermelon cultivars.

Bahar *et al.* (2009) assessed the level of tolerance of various commercial melon cultivars and wild lines to BFB, using seed-to-seedling transmission and seedling-inoculation assays under greenhouse conditions. Although the authors did not identify resistant melon cultivars/lines, a commercial cultivar, ADIR339 displayed a high level of tolerance to BFB. In addition, melon cultivars/lines displayed varying levels of BFB tolerance depending on the screening assay used. For instance, cultivar ADIR339 was tolerant in both the assays while BLB-B and EAD-B were tolerant only in seed transmission assays.

Chemical foliar treatments

Chemical management of BFB is limited to copper-based compounds that are effective only under suitable environmental conditions. Traditionally, Kocide was used as preventative treatment against BFB (Hopkins, 1991); however, it can be phytotoxic to watermelon and other cucurbit seedlings. Another potential problem is the development of copper tolerance by *A. citrulli* that limits its long term use as a disease management tool (Hopkins, 1995). There have been other attempts to find effective foliar chemical treatments including antibiotics. For example, protective sprays with streptomycin and kasugamycin to 2-week-old melon seedlings prevented the spread of BFB under greenhouse conditions (Shimizu *et al*., 2008). However, at present antibiotics are not widely used for BFB management.

Seed treatments

Fermentation of freshly harvested seed in watermelon fruit juice is a common practice routinely employed in commercial seed production. The process involves incubation of seeds in fruit juice for 24-48h followed by rinsing and drying. Hopkins *et al.* (1996) demonstrated that fermentation of watermelon seed in fruit juice for 24h followed by rinsing and drying completely eliminated BFB seedling transmission without affecting seed germination. However, fermentation cannot be used for certain watermelon hybrids and other cucurbits as it can adversely affect seed germination (Walcott, 2008).

Many seed treatments have been reported to reduce BFB seedling transmission. Treatments with 0.5-1% CaOCl, or NaOCl for 15-20 min reduced BFB seedling transmission but failed to eliminate the pathogen from the seeds (Hopkin, 1996; Hopkins *et al*., 2003). Hopkins also found that treatment of infested seeds with 1600 µg of peroxyacetic acid per liter of water for 30 min followed by drying at 40°C for 48h completely eliminated BFB seedling transmission (Hopkins *et al*., 2003). Currently, wet seed treatment with peroxyacetic acid is a standard practice by cucurbit seed producers. There have been many other attempts to develop seed treatments for BFB. For example, Feng *et al.* (2009a) evaluated 0.1 % cupric sulfate, acidified 0.1 % cupric sulfate at 50^{o}C for 20 min, acidified cupric acetate (ACA) at 50^{o} C for 20 min, acidified zinc chloride at 50^{o} C for 20 min, NaOCl at 50^{o}C for 20 min, acidic electrolyzed water (AEW) for 30 min, and peroxyacetic acid for 30 min. Although, seed treatments with NaOCl at 50^{o} C for 20 min, peroxyacetic acid for 30 min, and ACA at 50^{o} C for 20 min eradicated the pathogen, seed germination was negatively affected. Among the seed treatments, only AEW eradicated *A. citrulli* from infested seeds without decreasing seed germination or seedling establishment.

Biological control

Because greenhouse studies suggested that female watermelon blossoms might be involved in seed infection by *A. citrulli* (Walcott *et al*., 2003), biological control was explored to prevent female blossom contamination (Fessehaie and Walcott, 2005). When applied to blossoms 5h prior to inoculation with *A. citrulli*, *A. avenae* subsp. *avenae* (AAA 99-2), a pathogen of maize, significantly redcued seed infestation (13.8%) relative

to seeds from blossoms treated with *Pseudomonas fluorescens* A506 (24.1%), Kocide (21.1%), and 0.1M PBS (63%). This work showed that biological control for blossom protection has potential in BFB management, but more work must be done to improve efficacy.

Using a nonpathogenic strain of *A. citrulli* (AAC00-Δ*hrcC*) with a disfunctional type III secretion system, Johnson *et al.* (2011) demonstrated the efficacy of biocontrol seed treatments for BFB. When seeds naturally infested with AAC00-1 were treated with AAC00-1Δ*hrcC*, BFB seedling transmission was reduced by 81.8% relative to 0.1 M PBS treated seeds. In addition, when it was used as a blossom protectant prior to challenge-inoculation with AAC00-1, BFB seedling transmission in the resulting seedlots was significantly reduced (8%) relative to seeds from blossoms protected with 0.1 M PBS (36%). These results indicate that non-pathogenic strains of *A. citrulli* can be effective biocontrol agents and should be included as a component of integrated BFB management.

Exclusion

Seed health testing is the most widely used method for excluding *A. citrulli*. The most widely accepted seed health assay for BFB is the seedling grow out assay that includes growing out 10,000 – 50,000 seeds under greenhouse conditions and observing the seedlings for foci of BFB symptoms. While it is the industry standard, the seedling grow-out assay has many limitations. Although, it is technically simple and based on visual inspection, it is expensive to conduct. It takes 2-3 weeks for completion and requires large amount of environmentally controlled greenhouse space and trained

technicians. Additionally, subsequent laboratory assays (e.g., serological tests, semi-selective media, and polymerase chain reaction (PCR) assays) are required to confirm the identity of bacteria recovered from seedlings. Walcott *et al*., (2006) reported that the seedling grow out assay could detect only 12.5% (1/8) and 37.5% (3/8) of seedlots (n = 10,000 seeds) with 0.01 and 0.1% infested seeds, respectively.

To improve the efficiency of seed health testing, immunomagnetic separation combined with polymerase chain reaction (IMS-PCR) was evaluated (Walcott and Gitaitis, 2000; Walcott *et al*., 2006). This approach was necessary because watermelon seed extracts contain compounds that inhibit PCR, yielding false-negative results (Walcott and Gitaitis, 2000). With IMS-PCR, paramagnetic beads coated with anti- *A. citrulli* antibodies specifically bind target cells. Immunomagnetic beads can then be rinsed to eliminate inhibitory compounds and non-target cells and the captured *A. citrulli* cells are lysed by boiling and the DNA is detected by PCR. Using artificially infested watermelon seedlots (n = 10,000) with 0.01% and 0.1% infestation, detection frequencies of 25% and 87.5% respectively were reported for IMS-PCR as compared to 12.5% and 37.5% for the greenhouse seedling grow-out assay (Walcott *et al*., 2006). While the differences in detection frequency for IMS-PCR and seedling grow-out were not statistically significant, the data demonstrated the potential of IMS-PCR as an effective alternative for seed health testing.

Justification for research and objectives

Despite progress in understanding BFB epidemiology over the last two decades, the disease continues to be a threat to commercial cucurbit production. As infested seeds are the most important source of inoculum, BFB management in the seed production field is critical. Unfortunately, little attention has been paid to epidemiology of BFB in the seed production environment. As a result there are no effective strategies to prevent seed infestation. For effective management, it is important to identify the inoculum sources and mechanisms of dissemination and seed infestation in seed production environment. By understanding various epidemiological factors that promote BFB in the seed production environment, strategies aimed at avoiding or eliminating inoculum can be devised.

Currently, seed health testing and seed treatments are employed routinely for BFB management. However, neither strategy guarantees pathogen-free seeds (Gitaitis and Walcott, 2007; Walcott, 2008). The accuracy and precision of seed health testing for *A. citrulli* is influenced by many factors including sample size, sampling method, pathogen extraction efficiency, and seed inoculum threshold. Inoculum threshold is the level of seed inoculum that will lead to disease development and economic losses when seeds are planted under conducive field conditions (Kuan, 1988). For the exclusion of pathogen via seed health testing, it is critical to determine the seed inoculum threshold experimentally. Inoculum threshold depends on several factors that include pathogen aggressiveness, host susceptibility, and environmental conditions (Gitaitis and Walcott, 2007). As a result, it is difficult to assess and has been determined for only a few bacterial diseases including

black rot of crucifers (*Xanthomonas campestris* pv. *campestris*) and bacterial blight of carrot (*Xanthomonas campestris* pv. *carotae*) (Roberts *et al*., 1999; Umesh *et al*., 1998). To date, an inoculum threshold has not been determined for BFB. Instead, a threshold of one infested seed per 10,000 uninfested seeds has been adopted based on research on black rot of crucifers (Schaad *et al*., 1980). According to Gitaitis and Walcott (2007), a sample size of 30,000 to 50,000 seeds per lot must be tested to guarantee ≥ 95% confidence in detecting one infested seed in 10,000 uninfested seeds. However, this inoculum threshold does not consider seed inoculum density, even though pathogen population influences certain aspects of seed-to-seedling transmission, particularly the incubation period (Roberts *et al*., 1999). Hence, research was required to investigate the effect of *A. citrulli* inoculum density in seed on BFB seedling transmission.

As seeds are the most important source of *A. citrulli* inoculum, seed treatments are routinely used for BFB management. Seed treatments like sodium hypochlorite and 1% HCl significantly reduce seedborne *A. citrulli* populations but generally do not completely eradicate the bacterium (Hopkins *et al*., 1996; Rane and Latin, 1992). Among the many factors that affect seed treatment efficacy, seed anatomy and the location of the pathogen in seed are of great importance (Rane and Latin, 1992; Walcott, 2008). With regards to seed anatomy, cucurbits seeds possess a perisperm-endosperm (PE) layer (a thin membranous envelope that encloses the embryo and endosperm) (Welbaum *et al*., 1995; Welbaum and Bradford, 1990; Welbaum *et al*.,1998) that is composed of a layer of endospermous cells, covered by a thick, non-cellular layer of callose-rich material and a thin suberized outer layer. Since the PE layer completely encloses the embryo, it is

presumed that it might serve as a barrier to seed contamination by phytobacteria. However, no evidence has been provided to support these claims. One factor that influences the location of *A. citrulli* in seeds is the mechanism of seed infestation. It has recently been shown that watermelon seeds may become infested with *A. citrulli* by ingress through the fruit pericarp (Frankle *et al*., 1993) and penetration through the pistil of female blossoms (Walcott *et al*., 2003). In general, *A. citrulli* ingress through the pericarp results in the development of typical BFB fruit symptoms. On the other hand, pistil invasion does not result in fruit symptoms but still yields contaminated seeds. Preliminary data suggest that the pollen germ tube is critical for seed infestation by *A. citrulli* via the pistil pathway (Lessl, 2003). Hence, we hypothesized that the location of *A. citrulli* in seeds is influenced by the mechanism of seed infestation, with the bacterium being deep seated in seeds infested via pistil pathway.

Another goal of this research was to determine the factors that affect long term bacterial survival in seeds. Block and Shepherd (2008) reported that *A. citrulli* can survive for 30 years in watermelon and melon. In another report, *A. citrulli* survived for 63 days in plastic trays containing plant residues in soil stored at 4^{o}C (Latin *et al*., 1995). These reports suggest that *A. citrulli* can survive desiccation; however, to date the exact nature of this survivability has not been studied.

The goals of this research were to investigate the relationship between seed inoculum density and BFB seedling transmission. Additionally this research sought to determine how the *A. citrulli* invasion pathway affects bacterial localization in seeds and,

in turn, how bacterial localization affects pathogen survival, seed health testing and exudates produced by watermelon seeds during germination.

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CHAPTER 2

LOCALIZATION OF *ACIDOVORAX CITRULLI* IN WATERMELON SEEDS IS AFFECTED BY THE PATHWAY OF INVASION

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10/18/2011

Abstract

Watermelon seeds can become infested by *Acidovorax citrulli*, the causal agent of bacterial fruit blotch of cucurbits (BFB) via penetration of the ovary pericarp or by invasion of the pistil. This study investigated the effect of these invasion pathways on *A. citrulli* localization in seeds. Seed samples (n = 20 or 50 seeds/lot) from pistil- and pericarp-inoculated lots were dissected into testa, perisperm-endosperm (PE) layer and embryo tissues and tested for *A. citrulli* by species-specific PCR and by plating on semi-selective media. Less than 8% of the testa samples were *A. citrulli*-positive regardless of the method of seed inoculation. Additionally, the difference in percentages of contaminated testae between the two seedlot types was not significant (P = 0.64). The percentage of *A. citrulli*-positive PE layer samples as determined by real-time PCR assay was significantly greater for seeds from pistil-inoculated lots (97%) than for seeds from pericarp-inoculated lots (80.3 %). The mean percentage of *A. citrulli*-positive embryo samples was significantly greater for seeds from pistil-inoculated lots (94%) than for seeds from pericarp-inoculated lots (~8.75%) (P = 0.0001). Removal of PE layers and testae resulted in a significant reduction in BFB seed-to-seedling transmission for seeds from pericarp-inoculated lots (14.82 %) relative to those from pistil-inoculated lots (72%). Additionally, using immunofluorescense microscopy, *A. citrulli* cells were observed in the PE layers and the cotyledons of pistil-inoculated seeds but only in the PE layers of pericarp-inoculated seeds. These results suggest that pericarp invasion results in superficial contamination of the testae and PE layers while pistil invasion results in the deposition of *A. citrulli* in the embryos of seeds.

Introduction

Bacterial fruit blotch (BFB) is an economically important disease of cucurbits, caused by the Gram-negative, beta-proteobacterium, *Acidovorax citrulli* (=*A. avenae* subsp. *citrulli* formerly *Pseudomonas pseudoalcaligenes* subsp. *citrulli*) (27, 28, 42). Under environmental conditions of high relative humidity and high temperatures, the bacterium causes seedling blight and fruit rot of a range of cucurbitaceous plants including watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), melon (*Cucumis melo* L. var. *cantalupensis* Naudin) and pumpkin (*Cucurbita pepo* L.) (15, 19, 20, 37). In the United States BFB was first observed in commercial watermelon fields in Florida in 1989. Since then, it has emerged as a sporadic threat to cucurbit seed, seedling, and fruit production worldwide (2, 5, 10, 14, 16, 29). Infested seeds are the most important source of primary inoculum for *A. citrulli* and when introduced into transplant houses, they can initiate costly BFB outbreaks (12, 25). Hence, exclusion of *A. citrulli* from cucurbit production systems is critical for effective BFB management (7, 37).

While seed treatments such as peroxyacetic acid, hydrochloric acid, and sodium hypochlorite can reduce seedborne *A. citrulli* inoculum, they fail to eradicate the bacterium (11, 13). To develop more effective seed treatments for BFB, it is important to know the location of *A. citrulli* in naturally infested seeds. The inability of seed treatments to eradicate the bacterium combined with its ability to survive more than 30 years in stored seeds, suggest that *A. citrulli* may be located deep within seed tissues (4, 13). Rane and Latin detected the bacterium in testae and embryos of naturally and

artificially infested seeds and since they did not recover the pathogen from the peduncle of the fruit, they concluded that it did not move systemically from the mother plant to the ovary (25). While, Rane and Latin dissected watermelon seeds in their attempts to determine the location of *A. citrulli*, they did not fully explore the localization patterns of the pathogen in different seed tissues nor did they consider influence of different pathways of seed invasion.

Cucurbit seeds, including muskmelon and watermelon, have a thin, envelope called the perisperm-endosperm (PE) layer that encloses the embryo (24, 26, 40, 41). This envelope is composed of a single layer of endospermous cells covered by a non-cellular layer of callose-rich material and a thinner, waxy outer layer composed of lipids and suberin (24, 40, 41). Welbaum *et al.* suggested that the lipid layer contributes to the semi-permeability of the endosperm envelope in muskmelon (40). Additionally, the PE layer in cucumber, muskmelon, and other cucurbitaceous seeds acts as a primary physical barrier to radicle emergence (24, 40, 41). During germination this barrier becomes weakened by hydrolytic enzymes that facilitate radicle emergence (24). Because the PE layer completely encloses the embryo and is semi-permeable, we hypothesized that it had a role in embryo contamination by *A. citrulli*.

We also hypothesized that location of *A. citrulli* in seeds is influenced the pathway of seed infestation. Watermelon seeds can become infested by bacterial ingress through stomata on the fruit pericarp or by bacterial invasion of the pistil of the female blossom (6). In general, *A. citrulli* ingress through the pericarp leads to typical BFB fruit

symptom development (6). This pathway is accessible for approximately two weeks after anthesis, until a wax layer develops on the fruit pericarp (6). In contrast, pistil penetration by *A. citrulli* leads to seed infestation within asymptomatic fruits (39). Based on these observations, we hypothesized that *A. citrulli* cells become deep-seated in seeds infested via the pistil invasion pathway. Hence, the objective of this work was to investigate the effect of the pathway of infestation on the localization of *A. citrulli* in watermelon seeds.

Materials and methods

Bacterial strains and inoculum preparation. *Acidovorax citrulli* strain AAC00-1 was used in this study and was routinely grown on King's medium B (18) or nutrient agar (Becton-Dickinson, Sparks, MD) for 48h at 28^{o} C. To prepare inoculum, nutrient broth was inoculated with a single colony of AAC00-1 from a 48h agar culture and incubated overnight at 30°C on a rotary shaker (Innova; New Brunswick Scientific Co., Edison, NJ) at 250 rpm. The culture was centrifuged at 6000 × *g* (Allegra^{TM} 25R, Beckman Coulter, Fullerton, CA) for 5 min and the supernatant was decanted. The pellet was resuspended in 0.1 M phosphate buffer saline solution (PBS) and the bacterial concentration was adjusted to an optical density of 0.3 at 600 nm (~1 × 10^{8} CFU/mL) spectrophometrically (Spectronic 20; Bausch and Lomb, Rochester, NY) and diluted to a final concentration of ~1 × 10^{6} CFU/mL in PBS.

Generation of infested watermelon seedlots. Four *A. citrulli* infested seedlots (two seedlots each by pericarp- and pistil-inoculation) were generated for this study (Table 2.1). Pericarp-inoculated seedlots (1 and 2) were generated under greenhouse conditions

in 2008 and 2009, respectively, as follows. Briefly, 20 watermelon plants (cv. Crimson Sweet) were grown in 15-L plastic pots and at anthesis, 30-40 attached female blossoms were pollinated and simultaneously inoculated by gently rubbing their ovaries with a cotton swab saturated with a cell suspension with ~1×10^6 AAC00-1 CFU/mL. Blossoms were enclosed in plastic bags for 24h and fruits were allowed to develop for 30 days after pollination. Fruits were harvested and stored at 4^o C until seed extraction. For seed extraction, fruits were surface sterilized with either 70% ethanol or 0.5% NaOCl and seeds were manually extracted and air-dried at 25^oC on paper towels. Seeds from different fruits that were treated similarly were pooled and stored at 4^oC until they were used. Pistil-inoculated seedlots (3 and 4) were generated in 2008 and 2009, respectively, by pollinating female watermelon blossoms and simultaneously inoculating their stigmas with AAC00-1 as previously described (23, 40). Briefly, 20-25 watermelon plants (cv. Crimson Sweet) were grown in 15-L plastic pots under greenhouse conditions and at anthesis, stigmas were pollinated and then 10 µL of a suspension containing ~1×10^8 AAC00-1 CFU/mL were transferred to them using a micropipettor. Five blossoms were inoculated per plant, tagged, and allowed to develop for 30 days after pollination. Thirty-five to 40 fruits were harvested at maturity and seeds were extracted as described above and pooled to a produce each seedlot.

Seedlot characteristics. To determine the percentage of *A. citrulli*-infested seeds infestation for each lot, four samples (n = 100 seeds/lot) were planted on blotter paper (Anchor Paper Co., St. Paul, MN) saturated with sterile water in transparent plastic boxes [6 cm (H) ×24 cm (W) × 33.5 cm (L); Tri-State Plastics, Dixon, KY, USA] and

maintained at 30°C and 85% RH with continuous fluorescent light for 14 days. Seeds from the fruits of blossoms that were pistil-inoculated with PBS were used as negative controls. Seed germination percentages and BFB seedling transmission percentages ((number of seedlings with BFB symptoms divided by the total number of seeds germinated) × 100) were recorded at 14 days after planting (DAP). Additionally, mean *A. citrulli* CFU/seed for samples (*n* = 30 seeds) from each lot were determined by ten-fold serial dilution of individually macerated seeds followed by spread-plating onto Nunhem's semi-selective agar (Nunhems Seed Company, Haelen, The Netherlands). After incubation for 5 - 6 days at 28^{o}C, *A. citrulli* colonies were enumerated and mean CFU/seed was determined for each lot.

Localization of *A. citrulli* in infested watermelon seeds. To compare the effect of invasion pathway on *A. citrulli* location, seeds from pericarp- and pistil-inoculated fruits were studied. Seed samples (*n* = 50 seeds) from each lot were dissected into testa, PE layer and embryo tissues and assayed for *A. citrulli*. To separate seed sections, testae were first removed from dry seeds using a sterile scalpel. Each decoated seed was soaked in distilled water for 3h at 25^{o}C in the dark and then the PE layer was removed using sterile forceps (DR Instruments, Palos Hills, IL). Similar sections dissected from ten seeds from fruits of blossoms pistil-inoculated with PBS served as negative controls. To detect *A. citrulli*, each section was placed in a separate microcentrifuge tube containing 1 mL PBS, macerated using a sterile glass rod and vigorously agitated for 30s (Vortex Genie2; Fisher Scientific International). An UltraClean™ Microbial DNA Kit (MO BIO Laboratories, Inc., Carlsbad, CA) was used to extract microbial genomic DNA from seed

tissues according to the manufacturer's instructions. DNA (10-15 ng/µL) was subjected to real-time PCR assay using *A. citrulli*-specific primers as previously described (9). Samples with cycle threshold (Ct) values below 30 were considered to be positive for *A. citrulli*. To ensure that there was no PCR inhibition in samples that yielded negative results, 5 ng/µL of *A. citrulli* genomic DNA was added to DNA preparations from ten control seed samples and subjected to real-time PCR as described (9). The mean percentage of *A. citrulli*-positive seed sections was determined for each seedlot. To confirm real-time PCR assay results, attempts were made to isolate *A. citrulli* from seed sections. Samples (n = 20 seeds/lot) were dissected into testa, PE layer and embryo tissues as described above and the tissues were macerated separately in PBS and 100 µL aliquots were spread onto Nunhems semi-selective medium. Plates were incubated for 5 days at 28^{o} C and the percentage of *A. citrulli*-positive seed sections was compared for seeds inoculated by each method. For statistical analysis the mean percentage of *A. citrulli*-positive seed tissues for each lot (as determined by real-time PCR assay and plating on semi-selective media) were compared by factorial analysis using the Student's t-test ($P < 0.05$) in SAS (version 8.1 for Windows; SAS Institute Inc., Cary, NC).

Effect of seed layer removal on BFB seedling transmission. To further confirm the effect of the pathway of invasion on *A. citrulli* localization in watermelon seeds, we investigated the effect of removing seed layers on BFB seedling transmission. Samples (n = 100 seeds/treatment) from pistil-inoculated and pericarp-inoculated lots were dissected as described above. Treatments in this study included: 1) removal of the testae; 2) removal of the testae and PE layer tissues and 3) intact seeds (positive control). Each seed

was planted in a capped, sterile 10 mL test tube containing cheese cloth saturated with sterile deionized distilled water and maintained at 85% RH. Seeds were incubated at 28°C and 85% RH with continuous fluorescent light, and seed germination and BFB seedling transmission percentages were determined after 14 days. Germination and BFB seedling transmission percentages were compared by factorial analysis using the Student's t-test ($P < 0.05$) in SAS (version 8.1 for Windows; SAS Institute Inc., Cary, NC). To confirm that putative BFB seedling symptoms were caused by *A. citrulli*, bacterial isolation was attempted from at least five symptomatic seedlings from each treatment. Small (2 - 4 mm^2) pieces of symptomatic cotyledon tissue were macerated in 200 µL of sterile PBS and a loopful (~ 20 µL) of macerate was streaked onto Nunhem's medium. After incubation for 5 d at 28 °C, putative *A. citrulli* colonies (round, red colonies with smooth margins) were tested by real-time PCR assay using an *A. citrulli*-specific TaqMan assay (9).

Visualization of *A. citrulli* in pericarp-inoculated and pistil-inoculated seeds. To visually observe the location of *A. citrulli* cells in seed tissues, five seeds from pericarp-inoculated and pistil-inoculated lots were arbitrarily selected and decoated. Seeds were decoated to facilitate fixation and infiltration of resins into tissues. Decoated seeds were fixed for 24h in 1.6% (w/v) paraformaldehyde and 0.2% (w/v) glutaraldehyde in 25 mM sodium phosphate buffer at pH 7.1. Seeds were washed with sodium phosphate buffer (twice for 15 min each), water (twice for 15 min each), and dehydrated using an ascending ethanol series (35, 50, 75, 85, 95, 100, 100, and 100% [v/v] ethanol) for 30 min at each step. The dehydrated tissue was gradually infiltrated with LR White

embedding resin (Ted Pella Inc., Redding, CA) using 33% (v/v) and 66% (v/v) resin in 100% ethanol for 24h each, followed by 100% resin for 24 h three times. For embedding, the resin-infiltrated seeds were transferred to gelatin capsules containing 100% resin and polymerized by incubation at 45^{o} C for 24h followed by 55^{o} C for an additional 24h. Seed sections (250 nm thick) were generated using an ultra-microtome (Leica EM UC6, Leica Microsystems, Austria), and mounted on glass slides (ColorFrost^{TM}Plus; Fisher Scientific, Pittsburgh, PA). For immunolabelling of *A. citrulli*, sections were blocked with 3% (w/v) non-fat dry milk in 0.01 M potassium phosphate, pH 7.1, containing 0.5 M NaCl (KPBS) for 1 h and washed with 10 mM KPBS three times for 5 min each. The primary antibody, polyclonal rabbit anti-AAC (39), was diluted 1: 10 in 3 % BSA in 10 mM KPBS and 10 µl was applied to the seed sections and incubated for 60 to 90 min. Sections were then washed with BSA-KPBS three times for 2 min each, and 10µL of a 1:100 dilution of anti-rabbit IgG conjugated to Alexa-fluor 488 (Invitrogen, Carlsbad, CA) in BSA-KPBS was applied and incubated for 60 to 90 min. Finally, sections were washed with BSA-KPBS and then with distilled water for 5 min each. Before placing the cover slip on the sections, 10 µL of Citifluor antifade mounting medium AF1 (Electron Microscopy Sciences, Hatfield, PA) was applied. Separate seed sections were stained with 0.05% Toluidine blue to visualize the basic cellular arrangement of watermelon seed tissues. The immunolabelled seed sections were observed with an Eclipse 80i microscope (Nikon Instruments Inc., Melville, NY) equipped with epifluorescence optics. A Nikon DS-Ri1 camera was used to capture the images configured with a NIS-Elements

Basic Research software. Images were subsequently assembled using Adobe Photoshop (Adobe Systems McLean, VA).

Results

Seedlot characteristics. Pericarp-inoculated seedlots, 1 and 2, displayed mean seed germination of 78% and 74%, respectively, and mean BFB seedling transmission of 82% and 76.5% respectively (Table 1). The mean *A. citrulli* populations per seed for these lots were 3.5×10^4 and 2.8×10^4 CFU/seed, respectively (Table 1). The pistil-inoculated seedlots, 3 and 4, displayed mean seed germination of 80% and 72%, respectively, and BFB seedling transmission of 74% and 75%, respectively (Table 2.1). *Acidovorax citrulli* populations were 4.5×10^4 and 1.8×10^4 CFU/seed for seeds from lots 3 and 4, respectively (Table 1). No *A. citrulli* colonies were recovered from seeds from the negative control lots that were pistil-inoculated with PBS, and samples from these lots displayed 0% BFB seedling transmission (data not shown).

Localization of *A. citrulli* in watermelon seed. To confirm that DNA extracts from watermelon seed tissues were not contaminated with PCR inhibitors, 5 ng of genomic *A. citrulli* DNA was added to DNA preparations from 10 negative control seeds (harvested from fruits from PBS-inoculated blossoms) and subjected to real-time PCR assay. DNA amplification was observed for 100% of the samples (data not shown) indicating that there was no PCR inhibition. Overall, the tissue colonization results obtained by the real-time PCR assay were not significantly different to those obtained by plating on semi-selective agar media ($P>0.05$). For pericarp-inoculated seedlots, the real-time PCR assay

indicated that 5.3% of the testae samples were contaminated with *A. citrulli* while 6.3% of testae samples from pistil-inoculated lots were positive (Table 2.2). This difference was not statistically significant ($P = 0.64$). With regards to PE layer tissues, 97% of the samples from pistil-inoculated seeds were contaminated with *A. citrulli* as compared to 80.25% for seeds from pericarp-inoculated seeds, as determined by real time PCR assay (Table 2.2). For both seed infestation types, the percentage of *A. citrulli*-positive PE layer samples was significantly greater than the percentage of contaminated testae samples ($P = 0.002$). Additionally, the percentage of *A. citrulli*-positive PE layer samples was significantly greater for seeds from pistil-inoculated (97%) lots than for those from pericarp-inoculated (80.25%) lots ($P = 0.0001$). The percentage of *A. citrulli*-positive embryo samples for seeds from pistil-inoculated lots (94%) was significantly greater than for seeds from pericarp-inoculated lots (8.75%) ($P = 0.0001$). While there was no significant difference ($P = 0.43$) between the percentage of *A. citrulli*-positive embryos (94%) and *A. citrulli*-positive PE layer samples (97%) for pistil-inoculated, for pericarp infested seeds the percentage of infested embryos (8.75%) was significantly lower ($P = 0.0001$) than the percentage of infested PE layers (80.25%).

Effect of seed layer removal on germination and on BFB seedling transmission. Confirmation of the location of *A. citrulli* in seeds was sought by removing seed layers and assaying the seeds for BFB seedling transmission. We assumed that *A. citrulli* cells associated with each tissue would be eliminated as the seed layers were removed, thereby reducing BFB seedling transmission. The mean germination percentages for seeds with only the testae removed (78.5% and 75.7% for pericarp- and pistil-inoculated lots,

respectively) were not significantly different to intact seeds (76.5% and 81.0% for pericarp and pistil-inoculated seeds, respectively) (Fig. 2.1A and B). However, the germination percentages for seeds from which the testae and PE layers were removed (45.5% and 51.0% for pericarp- and pistil-inoculated lots, respectively) were significantly lower than for intact seeds and seeds from which only the testae were removed (Fig. 2.1C). With regards to BFB seedling transmission, intact seeds from pericarp- and pistil-inoculated lots yielded seedlings with 76.3 and 75.0% BFB incidence, respectively (Fig. 2.2A). Testa removal did not have a significant effect on BFB seedling transmission for either seed inoculation method ($P = 0.74$). In contrast, the removal of the testae and PE layers from pericarp-inoculated seeds resulted in a statistically significant reduction in BFB seedling transmission (14.8 %) compared to intact pericarp-inoculated seeds (76.5%) ($P < 0.0001$) and seeds with the testae removed (85%) ($P = 0.003$). This was not the case for pistil-inoculated lots, for which testa and PE layer removal did not have a significant effect ($P = 0.89$) on BFB seedling transmission (72%) relative to intact seeds or seeds with testae removed (Fig. 2.2). Finally, testae and PE layer removal from pericarp-inoculated seeds resulted in a statistically significant ($P = 0.003$) reduction in BFB seedling transmission relative to pistil-inoculated seeds treated in the same manner (Fig. 2.2).

Confirmation of BFB on symptomatic seedlings. All seedlings that displayed putative BFB symptoms were confirmed as infected with *A. citrulli* by bacterial recovery on semi-selective media and subsequent species-specific real-time PCR assays (data not shown).

Visualization of *A. citrulli* in pericarp- and pistil-inoculated seeds. Sections labeled with anti-rabbit IgG conjugated with Alexa-fluor 488 alone did not yield fluorescent signals indicating that there was no non-specific binding of the secondary antibody to watermelon seed tissues (data not shown). Additionally, fluorescence was not observed in seed sections of negative control seeds from PBS-treated blossoms, when labeled with the anti-*A. citrulli* primary antibody. Toluidine blue-stained seed sections (Fig. 2.3A and B) revealed the PE envelope enclosing the cotyledons. In sections of pistil-inoculated seeds, *A. citrulli* cells were observed, not only in the inner cells of the PE envelope, but also in the intercellular spaces of parenchyma tissue of the embryo (Fig. 2.3C). In pericarp-inoculated seeds, *A. citrulli* was observed only in the outer layer of PE envelope (Fig. 2.3D). *A. citrulli* cells were not observed in the embryo of pericarp-inoculated seeds.

Discussion

Seed contamination by phytopathogens can occur systemically through the plant vasculature, via the pistils of flowers or via direct penetration of ovary walls (1). For phytobacteria, the most common pathway of seed infestation is through the pericarp of the ovary. For example, bacterial ingress through stomata on the pods of common bean (*Phaseolus vulgaris* L.) and pea (*Pisum sativum* L.) led to seed infestation by *Xanthomonas axonopodis* pv. *phaseoli* and *Pseudomonas syringae* pv. *pisi*, respectively (32,43). Similar observations were reported for *Clavibacter michiganensis* subsp. *michiganensis* in tomato (*Lycopercicum esculentum* L.) seeds and *Curtobacterium flaccumfaciens* subsp. *flaccumfaciens* in common bean (*Phaseolus vulgaris* L.) seeds

(21,30). Phytobacteria that enter seeds by this pathway generally become deposited in the testa; but they may also become localized in endosperm and cotyledon tissues (7, 34, 35). Pistil invasion is the other pathway of seed infestation reported for phytobacteria. Kauffman and Leben (17) reported that inoculation of soybean blossoms with *P. syringae* subsp. *glycinea* resulted in infested seeds within symptomless pods. Ark *et al.* (3) reported that when walnut pollen, artificially contaminated with *X. juglandis* (causal agent of walnut blight), was transferred to female walnut blossoms the resulting walnuts were internally contaminated with the pathogen. Similarly, van der Wolf *et al.* (36) reported that cauliflower blossom inoculation with *X. campestris* pv. *campestris*-contaminated flies led to internal seed infestation. However, to date there have been no studies of the effect of these invasion pathways on the location of phytobacteria in seeds.

Both pistil and pericarp invasion of watermelon seed have been reported for *A. citrulli* (6, 39). Frankle and Hopkins (6) reported that watermelon fruit became infested with *A. citrulli* when bacteria penetrated open stomata on the pericarp of the ovary. This pathway of ingress was limited to immature fruits (two to three weeks post-anthesis) as stomata on older fruits became covered with wax that prevented bacterial penetration. Walcott *et al.* (39) reported that watermelon seed infestation by *A. citrulli* occurred by pistil invasion and resulted in contaminated seeds within asymptomatic fruits. Lessl *et al.* (23) subsequently reported a positive linear relationship between *A. citrulli* blossom inoculum dose and percentage of infested seedlots. Additionally, using a constitutive green fluorescent protein mutant, they observed that *A. citrulli* rapidly colonized the stigmas of female blossoms, penetrated through the transmitting tract tissues of the style

and entered the ovaries by 7 days post- inoculation (22). Based on these observations, we hypothesized that the pathway of seed infestation may influence the location of *A. citrulli* in seeds.

One interesting finding from this study was that unlike other seedborne phytobacteria, the testa was not a major site of *A. citrulli* accumulation in watermelon seeds. *A. citrulli* was detected in relatively low percentages of testae as compared to embryo and PE layer samples. Additionally, when the testae were removed from pericarp- and pistil-inoculated seeds, BFB seedling transmission percentage was not significantly affected. This indicated that regardless of the invasion pathway, *A. citrulli* cells that accumulate in the testae are not critical for seed-to-seedling transmission of BFB. This was unexpected based on reports on other pathosystems where phytobacteria predominantly survived in the testae (8, 30, 34). One possible explanation for this discrepancy is that in watermelon seed testae, *A. citrulli* cells are exposed to unfavorable conditions that cause populations to decline after harvest. These conditions are influenced by factors like variation in testae anatomy and phytobacterial sensitivity to desiccation that may vary based on pathogen and plant species (31). For example, testae surface features can provide protective sites that help bacteria evade stress during storage. *Xanthomonas campestris* pv. *malvacearum* was reported to survive in cotton seed fibers that remained attached to the testae after delinting (35). Interestingly, we observed that even with seeds from pistil-inoculated lots, a low percentage of testae were contaminated with *A. citrulli*. This was unexpected because we hypothesized that pistil inoculation would deposit the bacterium in the seed embryo. However, Walcott *et al.* (39) previously

observed that fruit pulp tissues also became contaminated with *A. citrulli* by pistil inoculation. Hence, *A. citrulli* cells present in watermelon pulp could subsequently become associated with testae, similar to seeds in pericarp-inoculated fruits.

Another interesting observation from this study was that the watermelon seed PE layer acts as a barrier to embryo invasion by *A. citrulli* via pericarp-inoculation. Irrespective of the pathway of bacterial invasion, > 78% of the PE layer samples was contaminated with *A. citrulli.* However, a significantly higher percentage of embryo samples was contaminated with *A. citrulli* in pistil-inoculated (>90%) than in pericarp-inoculated seeds (<9%). Removal of testae and PE layers significantly reduced BFB seedling transmission in pericarp-inoculated seeds but not in pistil-inoculated seeds. Clearly, removal of the PE layer eliminated *A. citrulli* inoculum from pericarp-inoculated seeds but not from pistil-inoculated seeds, for which 90% of the embryos were contaminated. As expected, germination was significantly reduced (<51%) for seeds from which the testae and PE layers were removed.

Further evidence for the role of the PE layer as a barrier to embryo contamination in pericarp-inoculated seeds was provided by immunofluorescence microscopy. Immunolabeled *A. citrulli* cells were observed in both the inner cell layers of the PE envelope and intercellular spaces of parenchyma tissue of the cotyledons/embryos in pistil-inoculated seeds (Fig. 2.3C). In contrast, *A. citrulli* cells were not observed in the embryos of pericarp-inoculated seeds rather they were localized on the surface of the PE layer (Fig. 2.3D).

A final observation from this research was that pistil-inoculation circumvented the PE layer and deposited *A. citrulli* in the embryos of watermelon seeds. This conclusion was based on the observation that significantly higher percentages of embryos were infested with *A. citrulli* in pistil-inoculated seeds compared to pericarp-inoculated seeds. Additionally, removal of PE layers and testae did not affect BFB transmission for pistil-inoculated seeds, whereas BFB transmission was significantly reduced for pericarp-inoculated seeds. At present, it is unclear how *A. citrulli* cells become deposited in different seed tissues based on the pathway of bacterial ingress. However, bacterial deposition might be influenced by the timing of development of the different seed layers. To date, PE layer development has not been described in detail for watermelon seed but in cucumber seeds the PE layer begins to form by 15 day post anthesis (DPA) and matures by 35-45 DPA (24, 40, 41). At 15 DPA, a lipid layer differentiates in the epidermis of the four- to five-layered nucellus. An immature callose layer appears on the surface of the PE layer by 25 DPA, and by 35 DPA the lipid layer matures and a callose layer is deposited on the endosperm surface (40). At 45 DPA, the nucellar cells degrade to form a mature PE layer. While similar details on watermelon PE layer development are not available, preliminary experiments showed that inoculation of female watermelon blossoms with *A. citrulli* resulted in ovule contamination by *A. citrulli* within 24h (*data not shown*). Since the PE layer is not full developed by this time, it is possible that bacteria can gain access to the seed embryos. In contrast, Frankle and Hopkins (6) showed that the window for *A. citrulli* to migrate through the pericarp of immature

watermelon fruits is open for 14-21 DPA. If the bacterium invades the ovule at this stage, it is likely that the PE layer will serve as a barrier to embryo contamination.

Overall, our results suggest that the location of *A. citrulli* in watermelon seeds is influenced by the pathway of bacterial ingress. In cool, dry conditions that exist in commercial seed production fields, BFB fruit symptoms are not generally observed; however, infested seedlots are still produced (37) suggesting that seed infestation via pistil invasion by *A. citrulli* might be involved. Our data suggest that while the PE layer might block bacterial penetration into the embryo in pericarp-inoculated watermelon seeds, the same is not true for pistil-inoculated seeds. Using the two seed inoculation methods reported in this study, we can begin to investigate how bacterial localization influences *A. citrulli* survival in seeds. Insight gained from these studies may improve the management of BFB and other seedborne phytobacterial diseases.

Acknowledgements

We thank Dr. Rock Christiano for assistance with statistical analysis and Ms. Linda Genzlinger for technical assistance. This research was partially supported by Research Grant No. US-4216-09 from BARD, The United States – Israel Agricultural Research and Development Fund.

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Table 2.1. Characteristics of watermelon seedlots generated for this study.

Seedlot	Mechanism of seed inoculation	Mean *A. citrulli* population per seed (CFU)[a]	Mean seed germination (%)[c]	Mean BFB seedling transmission (%)[d]
1	Pericarp	3.5×10^4 (1.27)[b]	78 (1.63)	82 (3.62)
2	Pericarp	2.8×10^4 (2.74)	74 (2.64)	76.5 (1.68)
3	Pistil	4.5×10^4 (3.83)	80 (3.26)	74 (2.52)
4	Pistil	1.8×10^4 (2.56)	72 (0.81)	75 (4.62)

[a] Mean *A. citrulli* population per seed recovered by crushing individual seed in 0.1 M PBS followed by dilution plating on Nunhem's semi-selective medium. Number denotes the mean *A. citrulli* populations (CFU/seed) from 30 individual seeds.

[b] Numbers in parentheses denotes standard error of the means.

[c] Mean percentages of seed germination for four replicates of seed sample (n = 100) from each lot. Seeds were planted in a transparent, closed, plastic box with saturated blotter paper and incubated for 14 days at 28^{o}C and 80% R.H.

[d] Mean percentages of BFB seedling transmission for four replicates of seed sample (n = 100) from each lot. Seeds were planted in a transparent, closed, plastic box with saturated blotter paper and incubated for 14 days at 28^{o}C and 80% R.H. BFB seedling transmission

was determined as number of seedlings showing typical BFB symptoms on cotyledons divided by total number of seeds germinated × 100.

Table 2.2. Comparisons of *A. citrulli*-positive testae, PE layer and embryo samples from pericarp-infested and pistil-infested seedlots by real-time PCR and plating on semi-selective media.

Seed sections	Pistil-infested (%)		Pericarp-infested (%)	
	Plating[1]	Real-time PCR[2]	Plating	Real-time PCR
Testae	7.5 c	6.25 C	2.5 c	5.25 C
PE layers	77.5 b	97 A	95.0 a	80.25 B
Embryos	90 ab	94 AB	6.5 c	8.75 C

[1] Mean percentage of *A. citrulli*-positive seed sections by plating on semi-selective media. Fifty seed sections seedlot were dissected individually into testa, PE layer, and embryo samples and assayed for the presence of *A. citrulli* by crushing individual seed sections in 0.1M PBS followed by spread plating on semi-selective medium. Fifty individual seeds per seedlot of each infection type were used in an experiment and the experiment was repeated twice. Means with similar lower case letters are not significantly different according to factorial analysis by T-test ($P < 0.05$).

[2] Mean percentage of seed section tested positive for the bacterium by real-time PCR. Fifty seed sections were dissected individually into testa, PE layer, and embryo samples and assayed for the presence of *A. citrulli* by total microbial DNA extraction from individual sections followed by real-time PCR with *A. citrulli* specific primers. Fifty

individual seeds per seedlot of each infection type were used in an experiment and the experiment was repeated twice. A threshold of 30 fluorescence units was used and the cycle at which fluorescence exceeded the threshold was considered as the cycle threshold (CT) value. Samples with CT values below 30 were considered to be positive for *A. citrulli*. Means with similar upper case letters are not significantly different according to factorial analysis by T-test ($P < 0.05$).

Figure 2.1. The effect of removal of seed layers on seed germination. Bars represent mean percentage of seed germination for pericarp-infested (symptomatic fruit) (lot 1 and 2) and pistil infested (asymptomatic fruit) (lot 3 and 4) seeds in two independent experiments. Error bars represent the standard deviation of the mean. **Figure 2.1 A** shows mean seed germination for seeds with intact testae and PE layers (control). **Figures 2.1 B and 2.1 C** show mean seed germination for seeds with testae alone removed and with both testae and PE layers removed. Means with similar upper and lower case letters are not significantly different according to factorial analysis by T-test ($P < 0.05$).

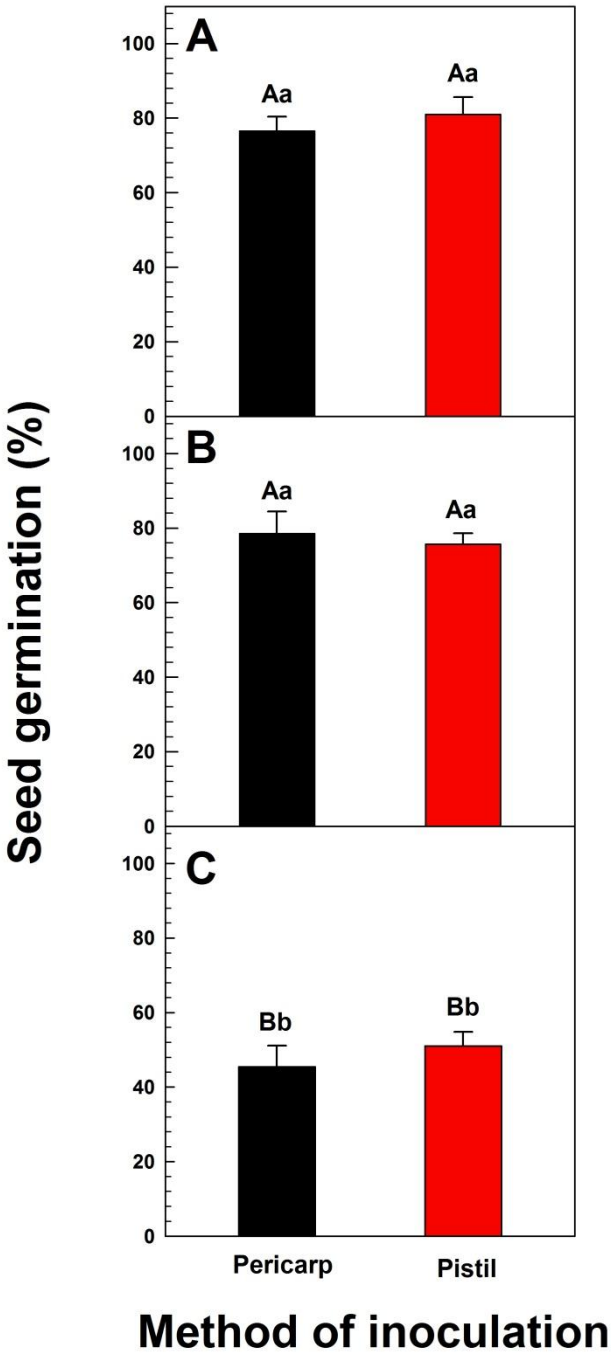

A
Aa
Aa
B
Aa
Aa
C
Bb
Bb
100
80
60
40
20
0
Seed germination (%)
Pericarp
Pistil
Method of inoculation

Figure 2.2. The effect of removal of seed layers on BFB seedling transmission. Bars represent mean BFB seedling transmission percentage for seeds infested by pericarp invasion (symptomatic fruit) (lot 1 and 2) and pistil invasion (asymptomatic fruit) (lot 3 and 4) in two independent experiments. Error bars represent the standard deviation of the mean. **Figure 2.2 A** shows BFB seedling transmission for seeds with intact testae and PE layers (control). **Figures 2.2 B and 2.2 C** show BFB seedling transmission for seeds with testae alone removed and with both testae and PE layers removed. Means with similar upper and lower case letters are not significantly different according to factorial analysis by T-test ($P < 0.05$).

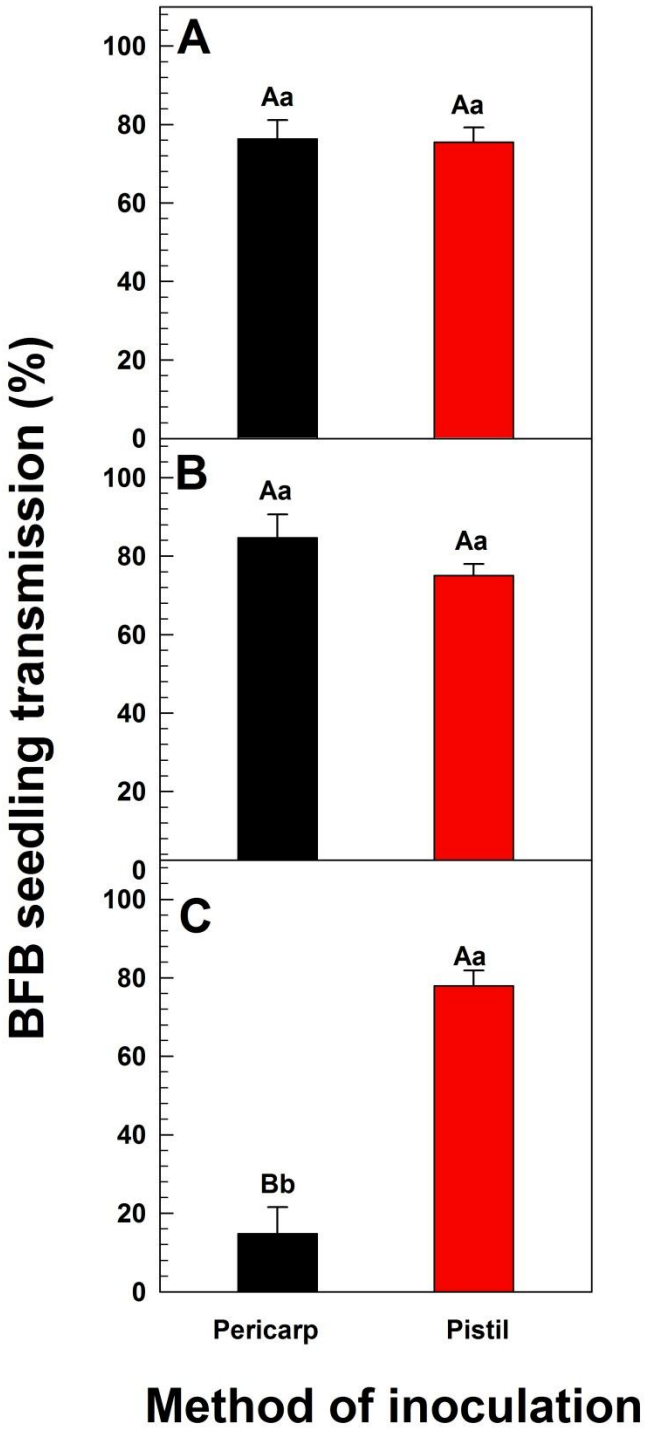
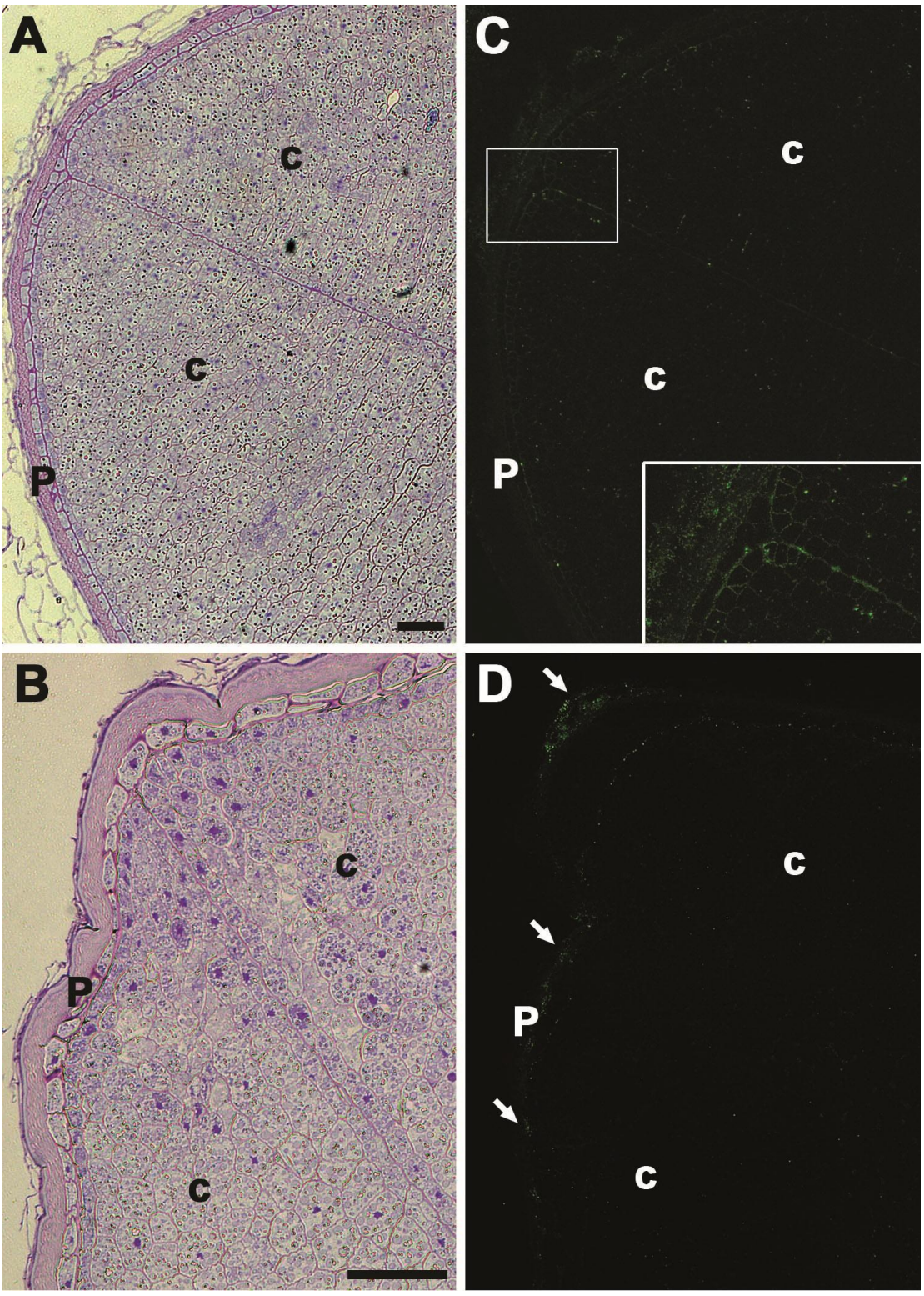

A
B
C
Aa
Aa
Aa
Aa
Aa
Bb
100
80
60
40
20
0
BFB seedling transmission (%)
Pericarp
Pistil
Method of inoculation

Figure 2.3. Immunolocalization of *A. citrulli* cells in pistil-infested and pericarp-infested seed sections by light microscope **(A-D).** Toluidine blue stained cross-sections of a decoated pistil- (A) and pericarp-infested (B) watermelon seeds showed with overall anatomy. The perisperm-endosperm envelope and cotyledons were denoted by P and c, respectively. *A. citrulli* cells immunolocalized in pistil-infested (C) and pericarp-infested (D) seeds. The lower right corner image in C corresponds to a magnified image of the highlighted box in C, which illustrated that *A. citrulli* cells were observed in pericarp-endosperm envelope (P) and in the intercellular spaces of parenchyma tissue of the cotyledons (c). White arrows in D show the localization of *A. citrulli* cells limited to the PE layer. Bar (100µm) in panel A is also applicable for panel B. Bar (100 µm) in panel C is applicable for panel D.


A
C
B
D
c
P

CHAPTER 3

COMPARISON OF THE PATHWAYS OF WATERMELON SEED INVASION BY *ACIDOVORAX CITRULLI*

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Abstract

Ingress of *Acidovorax citrulli* into watermelon seeds by pistil- and pericarp-invasion was compared and the role of pollen germ tubes in pistil invasion was examined. Under greenhouse conditions, female watermelon blossoms were hand pollinated and inoculated by applying 10^6 *A. citrulli* CFU/ blossom to stigmas or ovary pericarps. Seeds (n = 20/ovary fruit) were collected at 0, 1, 2, 3, 4, 7, and 14 days post inoculation (DPI) and assayed individually for *A. citrulli* by plating on semi-selective agar plates. Bacterial ingress was also microscopically observed for pericarp and pistil-inoculated blossoms at 0, 1, 2, 3, and 4 DPI. A significantly higher percentage of seeds was infested with *A. citrulli* when inoculated via the pistil than via the pericarp at all sampling points except at 14 DPI ($P \leq 0.006$). Immunofluorescence microscopy showed that with pistil-inoculation, *A. citrulli* colonized the stigma, style and ovary by 1 DPI and that bacterial cells colocalized with pollen germ tubes in these tissues. Scanning electron micrographs of germinating pollen germ tubes on the stigma of pistil-inoculated blossoms showed *A. citrulli* in the outer layers of pollen tubes at 1 DPI. In contrast, with ovary pericarp-inoculation, *A. citrulli* cells penetrated exocarp and mesocarp tissues of the pericarp by 1 DPI but did not reach endocarp tissues until 4 DPI. These results suggest that pistil inoculation results in faster colonization of watermelon seeds by *A. citrulli* than pericarp inoculation. The data also suggest a role of pollen germ tubes in *A. citrulli* ingress into watermelon seeds with pistil-invasion.

Introduction

Bacterial fruit blotch (BFB) is an important disease of cucurbits caused by the Gram-negative bacterium *Acidovorax citrulli* (=*A. avenae subsp. citrulli* (formerly *Pseudomonas pseudoalcaligenes subsp. citrulli*) (27,28,36). Infested watermelon seeds are the most important source of primary inoculum for BFB outbreaks (12,25), hence, effective disease management requires pathogen exclusion. Current BFB management guidelines recommend seed production in regions with cool and dry climates with no history of BFB, visual inspection of seed production fields, fermentation and peroxyacetic acid seed treatments and seed health testing (13,34). Despite these efforts, BFB outbreaks due to seedborne inoculum still occur sporadically indicating a need for better disease management during seed production (9, 14, 20, 29, 32, 34). This in turn requires a better understanding of the process by which *A. citrulli* infests watermelon seeds.

Acidovorax citrulli can infest seeds by penetration of stomata on the ovary pericarp or by pistil invasion (8, 23, 35). Under field conditions, pericarp invasion can lead to the development of fruit symptoms including irregular water soaked lesions (8, 34). This pathway is accessible for 2-3 weeks after anthesis, after which a waxy layer covers the stomata and prevents further bacterial ingress. While the role of pericarp invasion in disease development on fruits has been studied, seed contamination via this pathway has not been elucidated.

Watermelon pistil invasion by *A. citrulli* does not result in BFB symptom development on fruit, but seeds within the fruits become contaminated (22,23,35). Lessl

et al. reported that *A. citrulli* rapidly colonized watermelon stigmas and reached populations of 10^9 colony forming units (CFU)/ blossom by 96h post-inoculation (22). They also showed that low levels of *A. citrulli* (10^3 CFU/blossom) applied to stigmas resulted in seed infestation. Finally, using a constitutive green fluorescent protein mutant of *A. citrulli*, they demonstrated that *A. citrulli* can penetrate the stigma and style via the transmitting tract tissues and reach the ovary by 7 days post inoculation (22). However, the exact timing of this series of events relative to ovule development and seed maturation has not been studied.

We hypothesized that time of ovule infestation by *A. citrulli* is influenced the pathway of bacterial ingress. Since, the bacterium was observed in the transmitting tract tissues during pistil-invasion (22), we speculated that *A. citrulli* can co-migrate with the pollen germ tubes and infect watermelon seeds during the early stages of development. With respect to pericarp-invasion, as *A. citrulli* ingress occurs via stomata on the pericarp (8), bacterial ingress and subsequent seed infestation may take longer to occur. Hence, the overall goal of this research was to gain a better understanding of the process of seed infestation by *A. citrulli* by pistil- and pericarp-invasion. More specifically, the objectives were to further elucidate the ingress of *A. citrulli* into seeds via pistil and pericarp invasion and to determine the role of pollen germ tubes in pistil-invasion.

Materials and methods

Inoculum preparation. *Acidovorax citrulli* strain AAC00-1 was used throughout this study. This strain was routinely grown on King's medium B (16) or nutrient agar (Becton-Dickinson, Sparks, MD) for 48h at 28^{o} C. To prepare inoculum, nutrient broth was inoculated with a single colony of AAC00-1 from a 48h agar culture and incubated overnight at 28°C on a rotary shaker (Innova; New Brunswick Scientific Co., Edison, NJ) at 250 rpm. After incubation, the culture was centrifuged at 6000 × *g* (Allegra^{TM} 25R, Beckman Coulter, Fullerton, CA) for 5 min and the supernatant was decanted.
The resulting pellet was resuspended in 3 mL of 0.1 M phosphate buffered saline solution (PBS) and the bacterial concentration was adjusted to an optical density of 0.3 at 600 nm (≈1 x 10^{8} colony-forming units (CFU)/mL) using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY). The final concentration was adjusted to 10^{6} CFU/mL by ten-fold serial dilution with PBS.

Effect of *A. citrulli* invasion pathway on the rate of seed infestation. Stigmas and ovaries of female watermelon blossoms were inoculated with *A. citrulli* as follows. Under greenhouse conditions of 28^{o}C and 65% R.H with 12-14h of natural sunlight, 60 watermelon plants (cv. Crimson Sweet) (30 plants/inoculation method) were grown in 15-L plastic pots, and at anthesis 90-100 female blossoms were pollinated by hand (22,23,35). For pericarp inoculation, the ovaries of thirty pollinated blossoms were inoculated by rubbing a cell suspension containing ~1 × 10^{6} AAC00-1 CFU/mL uniformly onto the pericarp using a cotton swab. Blossoms were incubated at 100% relative humidity (RH) in plastic bags for 24h and then returned to ambient greenhouse

conditions. For pistil inoculation, immediately after pollination, 10 µL of a suspension containing ~ 1×10^8 AAC00-1 CFU/mL (~1×10^6 CFU/blossom) was applied to the stigmas of 30 pollinated blossoms using a micropipettor (Gilson S.A.S., Villiers-le-Bel, France). Thirty blossoms pistil-inoculated with PBS served as negative controls. Samples of ovaries from pistil- and pericarp-inoculated blossoms were collected at 0, 1, 2, 3, 4, 5, 7 and 14 days post inoculation (DPI). Ovaries were dissected longitudinally using a sterile scalpel and 20 arbitrarily selected seeds were assayed for *A. citrulli* individually as follows. Each ovule was crushed in 200 µL PBS and the macerate was spread onto Nunhem's semi-selective medium (Nunhems Seed Company, Haelen, The Netherlands). Plates were incubated for 5-6 days at 28^o C and the percentages of *A. citrulli*-positive seeds were compared for pistil- and pericarp-inoculated ovaries. Three ovaries were sampled at each time point for each treatment and the experiment was repeated twice. The mean percentage of *A. citrulli*-positive seeds produced by pistil and pericarp inoculation at each sampling time was compared by repeated measure analysis ($P < 0.05$) using SAS (version 9.2 for Windows; SAS Institute Inc., Cary, NC).

***Acidovorax citrulli* ingress through the pistil of watermelon blossoms.** To visually observe the invasion of *A. citrulli* cells through the pistil, female watermelon blossoms were pollinated and stigmas were inoculated as described above. Blossoms were collected at 0, 1, 2, 3 and 4 DPI and prepared for immunofluorescence microscopy as follows. Stigma, style and ovary tissue samples from three arbitrarily selected blossoms/time point were fixed for 24h in 1.6% (w/v) paraformaldehyde and 0.2% (w/v) glutaraldehyde in 25 mM sodium phosphate buffer (pH 7.1) at 4 ^{o}C. The fixed samples

were washed with 25 mM phosphate buffer (three times for 15 min each), water (two times for 15 min each), and dehydrated using the following ascending ethanol series: 35, 50, 75, 85, 95, 100, 100, and 100% [v/v] for 30 min at each step. The dehydrated tissues were gradually infiltrated with LR White embedding resin (Ted Pella Inc., Redding, CA) using 33 (v/v) and 66% (v/v) resin in 100% ethanol for 24h each at 4^{o}C, followed by three repeated incubation steps with 100% resin at 4^{o}C for 24h each. The resin-infiltrated samples were transferred to gelatin capsules containing fresh 100% LR White resin (Electron Microscopy Sciences, Hatfield, PA) and polymerized at 45^{o} C for 24h and then 55^{o} C for additional 24h. Sections (250 nm) of stigma, style and ovary tissues were cut using an ultra-microtome (Leica EM UC6, Leica Microsystems, Austria) and mounted on glass slides (ColorFrost^{TM}Plus; Fisher Scientific, Pittsburgh, PA). Three blossoms pistil-inoculated with PBS and collected at 0, 1, 2, 3, and 4 DPI served as negative controls. For immunofluorescence microscopy, 12 arbitrarily selected sections of stigma, style, and ovary tissues from each blossom/time point were used. Two sections/sample were stained with 0.05% Toluidine blue to visualize the basic cellular arrangement and the remaining sections were immunolabeled as follows. Sections were blocked with 3% (w/v) non-fat dry milk in 0.01 M potassium phosphate, pH 7.1, containing 0.5 M NaCl (KPBS) for 15 min and washed with 10 mM KPBS three times for 5 min each. The primary antibody (polyclonal rabbit anti-AAC) was diluted 1:9 in 3% BSA in 10 mM KPBS (BSA-KPBS) and 10 µL aliquots were applied to the sections and incubated for 60 min at 24^{o}C. Sections were then washed with BSA-KPBS three times for 5 min each. Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen, Carlsbad, CA) was diluted 1:99 in

BSA-KPBS and 10 µl aliquots were applied to the sections, which were then incubated in the dark for 60 min at 24^{o}C. After washes in 10 mM KPBS (three times for 5 min each) and distilled water (two times for 5 minutes each), 10 µL of Citifluor antifade mounting medium AF1 (Electron Microscopy Sciences, Hatfield, PA) was applied to the sections, which were then covered with a cover slip. Sections were observed using a fluorescence microscope (Nikon Eclipse 80i) with filter (wavelength 450-650nm) attached to a Nikon DS-Ri1 camera for image capture. Representative images of immunolabeled stigma, style and ovary sections from each time point were assembled using Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

Colocalization of *A. citrulli* with pollen germ tubes in pistil-inoculated blossoms. Using female blossoms collected at 1 DPI, longitudinal sections of stigmas and styles and cross sections of ovary tissues were prepared as described above. Separate tissue sections were immunolabeled with polyclonal rabbit anti-*A. citrulli* and mouse- anti-callose (Biosupplies Co. Inc, Philadelphia, PA) (six sections for each antibody). Additionally, three sections/tissue sample were stained with 0.05% Toluidine blue. Immunolabeling for *A. citrulli* was conducted as described above. The pollen tubes were immunolabeled using a mouse-anti-callose (primary antibody) and its corresponding secondary antibody, Alexa Fluor 568 goat anti-mouse (Invitrogen, Carlsbad, CA). Immunolabeled sections were prepared and observed using a fluorescence microscope, as described above. Immunofluorescence micrographs for each sample were assembled using Adobe Photoshop (Adobe Systems Inc.). Additionally, *A. citrulli* in the stigmas of pistil-inoculated blossoms were observed using transmission electron microscopy (TEM). At 1

DPI, stigma samples from three inoculated blossoms were arbitrarily selected and processed (fixation, dehydration and resin infiltration) as described above. Six 80-nm thick sections of stigma tissue were collected on formvar-coated grids (Electron Microscopy Sciences, Hatfield, PA) and incubated with polyclonal rabbit anti-*A. citrulli* diluted 1 : 9 in 3 % BSA and 10 mM BSA-KPBS followed by washing (three times for 5 min each) in 10mM BSA-KPBS. After washing, sections were incubated in goat anti-rabbit colloidal gold (18 nm, Electron Microscopy Sciences, Hatfield, PA) for 60 min followed by another regime of washing in 10mM BSA-KPBS (three times for 5 min each). Immunolabeled sections were then stained with uranyl acetate (2% w/v, aq) for 5 min, washed again by ten dips in three changes of distilled water, dried by wicking and stained for 2 min with Reynold's lead citrate (26). Sections were again washed by five dips in three changes of distilled water and dried by wicking. Micrographs of the sections were recorded on silver film in a TEM Zeiss 902 (Carl Zeiss SMT, Oberkochen, Germany) and the negatives were scanned with an Epson 3200 scanner (Epson America, Inc ., Long Beach, CA) and assembled using Adobe Photoshop. At least 3 replications of all samples were made.

***Acidovorax citrulli* ingress through the pericarp of watermelon ovaries.** Three ovaries, pericarp-inoculated with *A. citrulli* were collected at 0, 1, 2, 3, and 4 DPI. Samples of three ovaries inoculated with PBS served as negative controls. Ovaries were dissected transversely using a scalpel and from one of the sections, ~1 mm-thick free-hand cross-sections were made. Each transverse section was further divided into four quadrants (from periphery to the center of the ovary), with each section including

exocarp, mesocarp, and endocarp tissues. From three of the four quadrants, longitudinal sections (~1 mm thick) were taken from the exocarp, mesocarp and endocarp tissues. Sections were fixed, dehydrated, and infiltrated with resin as described above. After resin-infiltration, ultra-thin sections (250 nm) were cut using an ultra-microtome (Leica EM UC6) and 12 arbitrarily selected sections/sample were mounted on glass slides. Ten sections/sample were immunolabeled with polyclonal rabbit anti-*A. citrulli* (primary) and Alexa Fluor 488 conjugated goat anti-rabbit (secondary) as described above. Two additional sections/tissue sample were stained with 0.05% Toluidine blue. Sections were observed using a fluorescence microscope (Nikon Eclipse 80i) attached to a Nikon DS-Ri1 camera for image capture. Representative images of immunolabeled sections of exocarp, mesocarp and endocarp tissues at each time point were assembled using Adobe Photoshop.

Results

Effect of *A. citrulli* invasion pathway on rate of ovule infestation. As expected, *A. citrulli* was not detected in seeds harvested from watermelon ovaries whose stigmas were inoculated with PBS. There was a significant difference between the percentages of *A. citrulli*-infested seeds that developed in ovaries that were pericarp and pistil-inoculated ($P = 0.005$). With the exception of samples collected at 14 DPI, the interaction between percentage *A. citrulli* seed infestation and method of inoculation was significant for all incubation periods ($P \leq 0.006$). Eleven point four percent of the seeds from pistil inoculated blossoms were infested with *A. citrulli* by 1 DPI (Fig. 3.1) as compared to 0%

of the seeds from pericarp-inoculated blossoms (P=0.02). By 2 DPI, a significantly higher percentage of the seeds from pistil-inoculated ovaries (20.6%) were contaminated with *A. citrulli* as compared to seeds from pericarp-inoculated ovaries (1.63%) (Fig. 3.1). By 7 DPI, the percentage of *A. citrulli*-positive seeds from pistil-inoculated ovaries (23.3%) was significantly greater than for pericarp-inoculated ovaries (4.13%). Finally, by 14 DPI, the mean percentages of *A. citrulli*-infested seeds in pistil- and pericarp-inoculated ovaries were 31.3% and 28.3%, respectively, however; this difference was not significant (P=0.49).

Elucidation of the pathway of *A. citrulli* ingress through the watermelon pistil. Sections stained with toluidine blue showed the basic cellular organization of stigma, style and ovary tissues (Fig. 3.2A, D, and G). Negative control sections labeled with secondary antibody (Alexa Fluor 488 goat anti-rabbit) alone did not produce fluorescent signals, confirming that there was no background labeling (data not shown). At 0 DPI, *A. citrulli* cells were observed only in the stigma tissue but not in style and ovary of inoculated blossom. At 1 DPI, *A. citrulli* cells were observed in the intercellular spaces of papillae on the surfaces of stigmatic lobes (Fig. 3.2B). At this time, bacteria penetrated the style and colonized the tissues adjoining the stylar canal (transmitting tissue) (Fig. 3.2E). However, *A. citrulli* cells were not observed in the center of the stylar canal. During the same period, *A. citrulli* cells penetrated into the intercellular spaces of parenchyma cells of the ovary (Fig. 3.2H). At 2, 3 and 4 DPI, *A. citrulli* colonized stigma, style and ovary tissues (Fig. 3.2C, F, and I); however; in the ovary tissues the intensity of

the *A. citrulli* signal decreased by 4 DPI. *Acidovorax citrulli* cells were observed in the transmitting tissues of the style by 2-4 DPI (Fig. 3.2E and F).

Colocalization of *A. citrulli* with pollen germ tubes in pistil-inoculated blossoms. At 1 DPI, pollen germ tubes were observed in the stigma producing red fluorescent signals (Fig. 3.3A). In these germ-tubes, *A. citrulli* cells (green fluorescent signals) were observed at the peripheries of the pollen tube (Fig. 3.3 D). In addition to the pollen germ tubes, *A. citrulli* cells were observed in the intercellular spaces of papillary cells on stigmas (Fig. 3.3D). At this time, pollen germ tubes penetrated stigma tissues and reached the style (Fig. 3.3B). In the style, *A. citrulli* cells were observed at the edges of pollen germ tubes (Fig. 3.3E). Additionally, bacterial cells were observed in the intercellular spaces of stylar tissue (Fig. 3.3E). During this time pollen germ tubes penetrated further into the ovary, specifically in transmitting tissue (Fig. 3.3C). Even though *A. citrulli* cells were observed in the intercellular spaces throughout the ovary; the intensity of labeling was greater around the pollen tubes, suggesting higher *A. citrulli* populations in these tissues (Fig. 3.3F).

Using transmission electron microscopy, germ tubes were observed emerging from the pollen grains on the stigma (Fig. 3.4A and B). The pollen germ tube appeared cylindrical with a diameter of 15-17µm. During pollen tube germination part of electron dense protoplasm from the pollen grain flowed into the pollen germ tube (Fig. 3.4A and B). The pollen tube wall appeared to be bilayered with a 4-6 µm thick outer layer and a 7-9 µm thick inner layer. *Acidovorax citrulli* cells were associate with the outer exine layers of the pollen grains (Fig. 3.4A) and adjacent to the outer layers of pollen tubes

(Fig. 3.4B). As expected, *A. citrulli* cells were not observed in the micrographs of PBS-inoculated blossoms (data not shown).

Invasion of *A. citrulli* through the pericarp of watermelon ovaries. Sections stained with Toluidine blue showed the basic cellular organization of exocarp, mesocarp and endocarp tissues (Fig. 3.5A, D, and G, respectively). The exocarp consisted of 2-3 cell thick chlorenchymatous epidermis tissue and several layers of parenchymatous cortex. The mesocarp tissue was multi-layered and consisted of thin-walled parenchymatous cells that were devoid of chlorophyll. In the endocarp tissue, the basic structure of the developing seed was observed (Fig. 3.5G). Ovary tissues stained with Alexa Fluor 488-conjugated goat anti-rabbit antibody did not produce fluorescent signals, indicating that there was no background labeling (data not shown). *Acidovorax citrulli* cells were observed only in the exocarp but not in the mesocarp and endocarp of inoculated blossom at 0 DPI. At 1 DPI, *A. citrulli* cells penetrated the epidermis of exocarp and colonized the intercellular spaces of cortex. During the same period, *A. citrulli* cells penetrated further into the mesocarp and colonized the intercellular spaces of the parenchyma tissue (Fig. 3.5B and E). By 4 DPI, *A. citrulli* cells did not penetrate further into the endocarp (Fig. 3.5H). Even at 4DPI, *A. citrulli* cells were visualized primarily in the intercellular spaces of exocarp and mesocarp tissues, but not in the endocarp of the ovary (Fig. 3.5 C, F, and I).

Discussion

A better understanding of the seed infection is important for improving the management of seedborne diseases. In the current study, we observed that *A. citrulli* colonization of watermelon seeds occurred faster by pistil invasion than by pericarp invasion. Bacterial ingress and subsequent seed infestation through the pistil occurred as early as 1 DPI. Interestingly, while *A. citrulli* was recovered from a low percentage of seeds (1.63%) at 2 DPI with pericarp-inoculation, they could not be visualized in endocarp tissues at 4 DPI. This discrepancy could be explained by populations of *A. citrulli* at 4 DPI that were too low to generate a visible fluorescent signal in the endocarp. In contrast, bacterial cells were visualized in pistil-inoculated ovary tissues at 1 DPI and a 11.4% of seeds were infested.

The intensity of the *A. citrulli* fluorescent signal in pistil-inoculated ovaries decreased at 4 DPI suggesting a decline in bacterial population. Although, the exact reason for this phenomenon is unknown, it is possible that conditions in the developing watermelon fruit including variation in concentrations of different sugar (5,6) limit bacterial growth. For example, sugars like fructose and glucose were predominant in immature fruit (12-20 days after anthesis) of all tested cultivars, however; their concentrations varied widely (5,6). In the case of watermelon cultivar Charleston Gray, fructose and glucose levels peaked at 24 DPA whereas in cultivar Dixielee fructose level was highest at 32 DPA. While sucrose was not detected until 24 DPA in Charleston Gray, it was detected as early as 12 DPA in Sugarlee (5). Bacterial inhibition under high sucrose concentrations was reported for several bacterial species including *Pseudomonas*

aeruginosa, *Klebsiella pneumonia*, and *Staphylococcus aureus* (3). High sugar concentrations create a hypertonic environment that can result in the plasmolysis of bacterial cell. Although, variations in sugar concentrations in Crimson Sweet have not been studied, it is possible that such variations may occur and this might explain why internal fruit rot is not observed with pistil inoculation with *A. citrulli* (35).

With pistil inoculation, *A. citrulli* was observed in the transmitting tract tissues at the periphery of the stylar canal. This pathway of ingress coincided with the pathway of ingress of the pollen tubes. *Acidovorax citrulli* cells colocalized with pollen tubes in stigma, style and ovary tissues and this may explain how *A. citrulli* migrates into the ovary to infest seeds. During angiosperm reproduction, pollen tubes elongate from the stigma through the transmitting tract tissue to the ovary to deliver male gametes for fertilization (2,7,30). The transmitting tissues form a differentiated tract between the stigma and ovary that guides pollen tubes to the ovule (7). Apart from its role in guidance, transmitting tract tissue also provides nutrients for growth and pollen tube development (7,30). Further support for the the role of pollen tubes in the transmission of *A. citrulli* was provided by Ha *et al.* (*Personal communication*). In greenhouse studies, without pollination, stigma inoculation with *A. citrulli* did not result in seed infestation. In contrast, stigma inoculation of pollinated blossoms resulted in *A. citrulli*-infested seeds. The fact that seed infestation occurred 24h after stigma inoculation also suggests the involvement of pollen tubes in seed infection by *A. citrulli*. It is common for pollen tubes to penetrate floral tissues, including watermelon flowers in 24h (30,31,39). We observed that without pollination, stigma inoculation with *A. citrulli* did not result in

colonization of the ovary by 24h after inoculation (Supplementary fig. 3.1B and D). However, *A. citrulli* cells colonized the stigma and style of inoculated, non-pollinated blossoms during this same period (Supplementary fig. 3.1 F). These observations suggest that *A. citrulli* cells alone cannot reach ovary by 24h post inoculation.

The ability of phytobacteria to ingress through blossoms and infest seeds has been reported for other plant diseases. For example, inoculation of soybean (*Glycine max* L.) blossoms with *P. syringae* subsp. *glycinea* resulted in infested seeds within symptomless pods (15). Similarly, cauliflower (*Brassica campestris* var *capitata* L.) blossoms when inoculated with *X. campestris* pv. *campestris*-contaminated flies led to internal seed infestation (33). Finally, Ark *et al.* (1) inoculated walnut stigmas with *X. arboricola* pv. *juglandis*-contaminated pollen and observed internal contamination of walnuts. While *X. arboricola* pv. *juglandis* inoculum is primarily dispersed by wind-driven rain (19), dissemination of infested pollen from diseased catkins can also transmit the bacterium to pistillate flowers (1).

Overall, observations from this study suggest that pollen tubes may be involved in *A. citrulli* ingress into watermelon seeds. Pollen tubes have been reported to exhibit directional growth in response to physical and chemical cues (lipids, proteins, and organic acids) from the ovary (2, 10, 11,37,38). For example, *cis*-triacylglycerides have been shown to guide pollen tubes into the stigmatic papillae of tobacco (37). In the style of tobacco flowers, the transmitting tissue specific-glycoprotein forms a sugar gradient in the transmitting tract extracellular matrix that promotes pollen tube growth (38). Additionally, pollen tubes of lily (*Lilium longiflorum*) showed *in vitro* directional growth

towards chemocyanin (a copper-containing protein) that was isolated from its stylar transmitting canal (17). Genetic studies in *Arabdiopsis thaliana* showed the involvement of gamma-amino butyric acid in pollen tube guidance to the micropyle (24). However, at present, the chemical cues that guide watermelon pollen tubes are unknown.

To our knowledge, this is the first study to explore the pistil- and pericarp-invasion pathways of watermelon seeds by *A. citrulli*. The observations suggest that pollen tubes may be involved in bacterial ingress into seeds during pistil-invasion. At present, however, the nature of the interaction between pollen tubes and *A. citrulli* cells remains to be determined. Further studies of this interaction may provide better understanding of *A. citrulli*-pollen tube interactions involved in watermelon seed infestation.

Acknowledgements: We thank Dr. Rock Christiano for assistance with statistical analysis and Ms. Anita Castro-Sparks for technical assistance.

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Figure 3.1. Percentage of infested seeds that developed over time with pericarp and pistil inoculation of watermelon blossoms with *Acidovorax citrulli*. Twenty seeds were sampled at 0, 1, 2, 3, 4, 5, 6, 7, and 14 days post inoculation and assayed for *A. citrilli* on semi-selective medium. Each data point represents the mean percentage of *A. citrulli*-positive seeds (three immature fruits/time point/treatment in two independent experiments) and lines indicate the standard errors of the means.

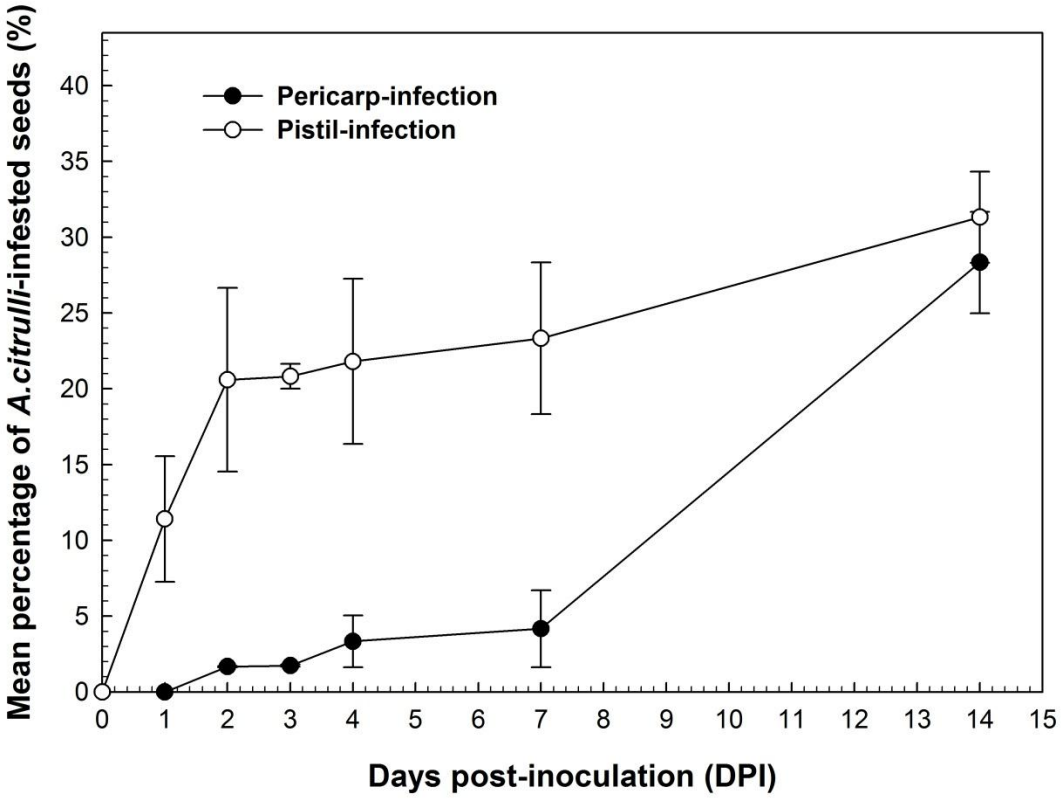

Pericarp-infection
Pistil-infection
Mean percentage of A.citrulli-infested seeds (%)
Days post-inoculation (DPI)
0
5
10
15
20
25
30
35
40
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Figure 3.2. Visualization of ingress of *Acidovorax citrulli* cells into pollinated pistil-inoculated watermelon blossoms by immunofluorescence microscopy. Stigmas of female blossoms were pollinated and inoculated with 10^6 CFU/blossom of *A. citrulli*. Transverse sections (250 nm) were prepared from stigma, style and ovary tissues of blossoms and at 1, 2, 3, and 4 days post inoculation. Panels A-C represent sections from stigma tissues; panels D-F represent sections from style tissue, and panels G-I represent sections from ovary tissue. SL and SC represent stigmatic lobe and stylar canal. The scale bar (50 μm) in panel A is also applicable for the panels B and C. Bar (50 μm) in panel D is also applicable for the panels E and F. The scale bar (50 μm) in panel G is applicable for the panels H and I.

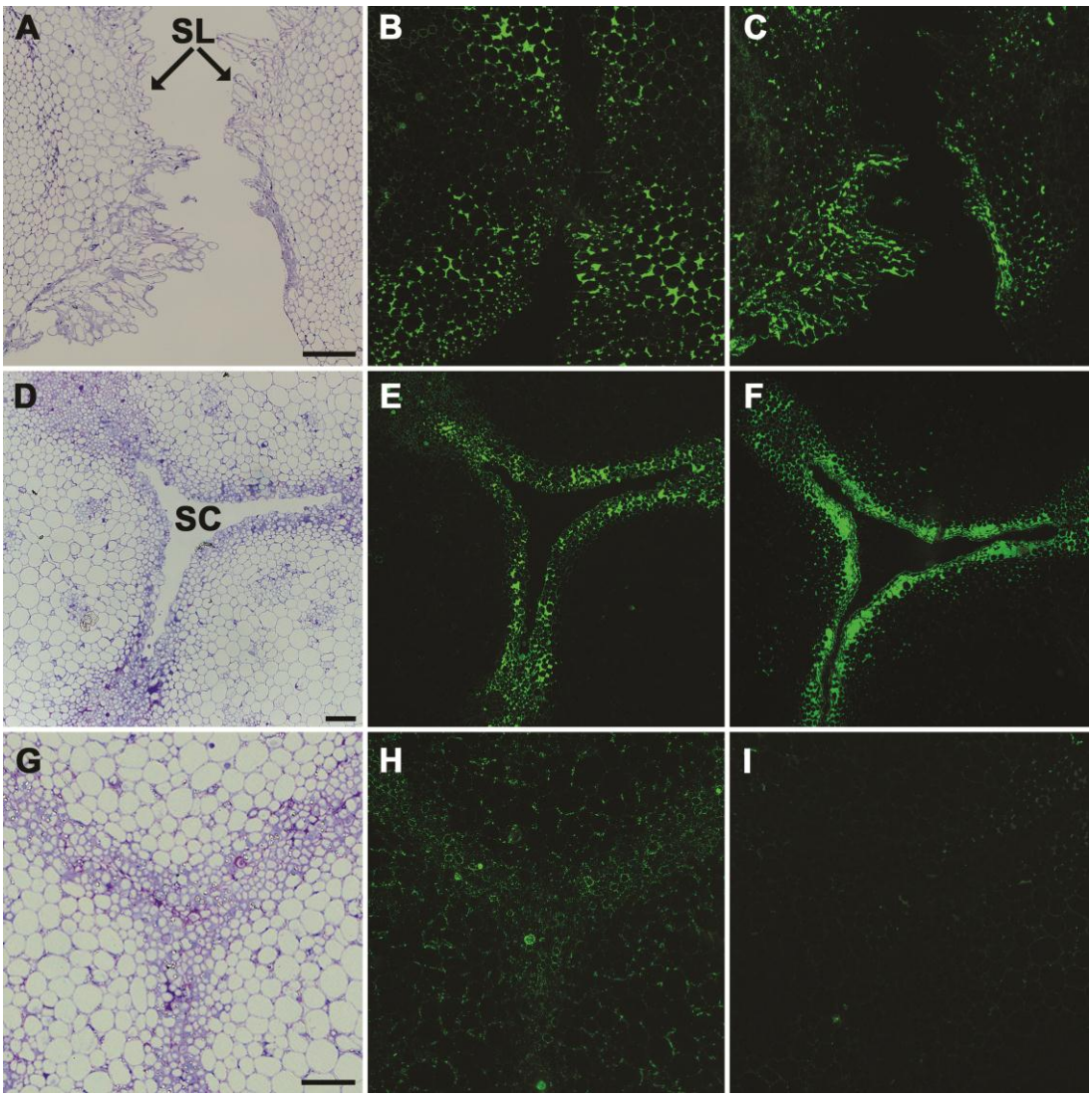

A
SL
B
C
D
SC
E
F
G
H
I

Figure 3.3. Colocalization of *Acidovorax citrulli* and pollen germ tubes in pistil-inoculated watermelon blossoms. Immunolabeling of *A. citrulli* cells and pollen germs tubes was performed with polyclonal rabbit anti-*A. citrulli* and callose-specific antibodies (mouse-anti-callose, Biosupplies Co. Inc, Philadelphia, PA), respectively. Transverse sections (250 nm) of stigma and style tissues and longitudinal sections of ovary tissue were prepared. Panels A and D represent stigma; panels B and E represent style tissue, and G-I represent ovary tissue sections. Bar (50 μm) in panel A is also applicable for panel D. Bar (50 μm) in panel B is applicable for panel E. Bar (50 μm) in panel C is also applicable for panel E.

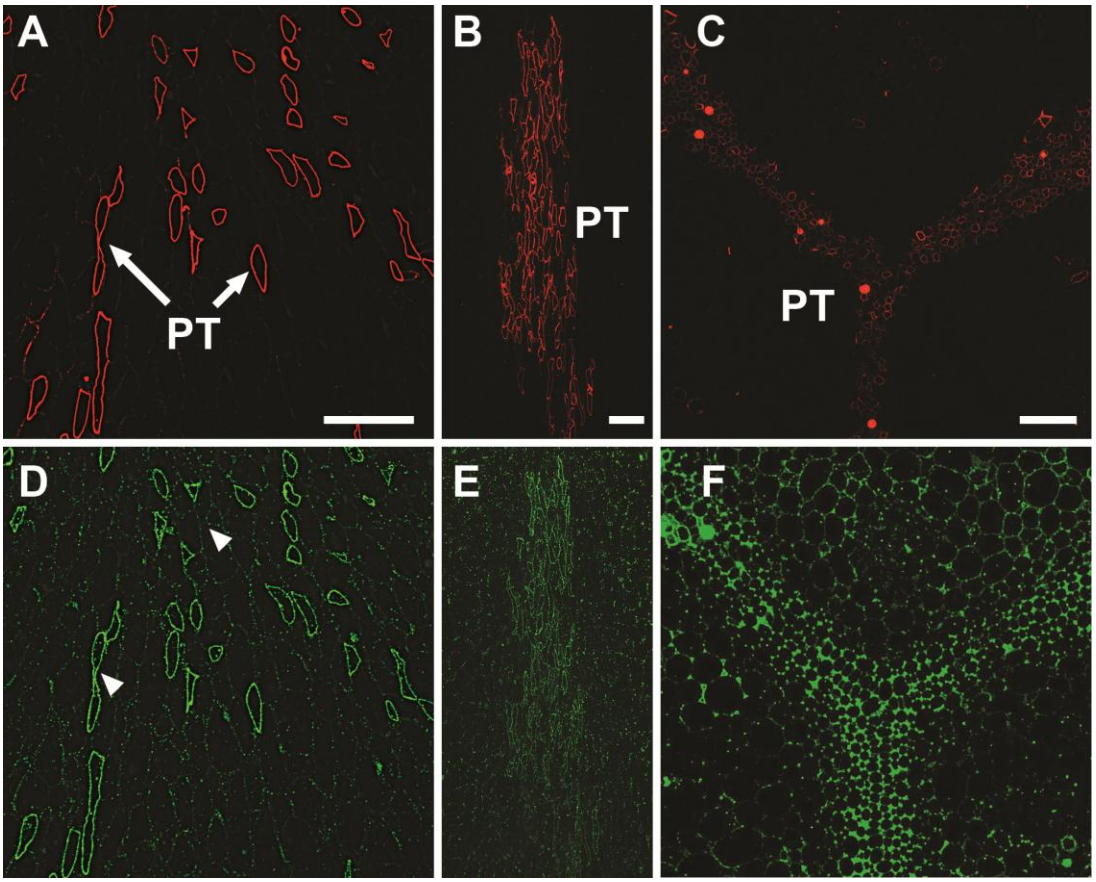

A
PT
B
PT
C
PT
D
E
F

Figure 3.4. Transmission electron micrographs of a watermelon stigma one day after pollination and inoculation with *Acidovorax citrulli.* Sections were stained with a polyclonal rabbit anti-*A. citrulli* antibody and labeled with a goat anti-rabbit antibody conjugated with gold. Sections (80nm) were immunolabled and observed with transmission electron microscopy (Zeiss 902). The black arrow indicates immunogold labeled bacteria. The rectangle in panel A represents a magnified image of immunogold labeled bacterium. Panels A and B represent localization of *A. citrulli* with pollen grains (PG) and pollen germ tubes (PT) in the stigma of the watermelon blossom. Bars in the panel represent 2.5 nm and bars in the rectangle represent 500 nm.

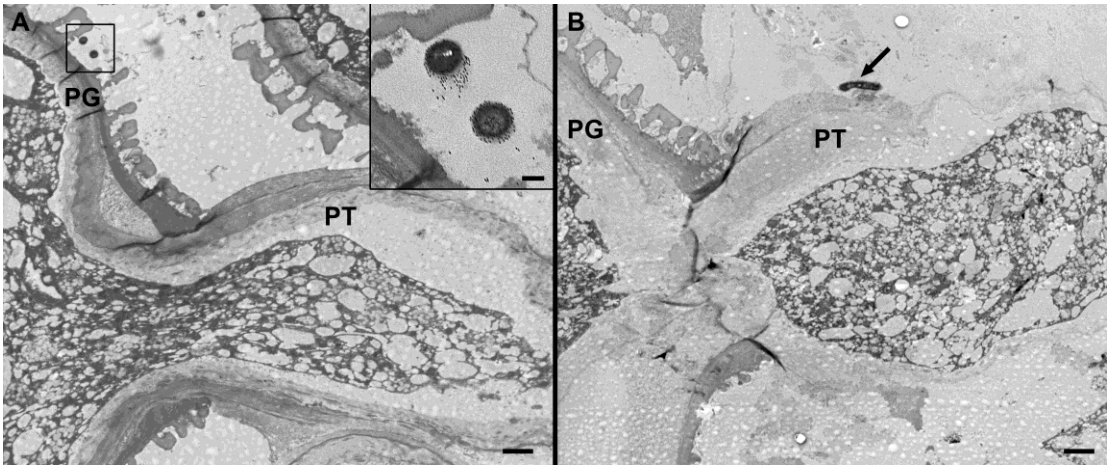
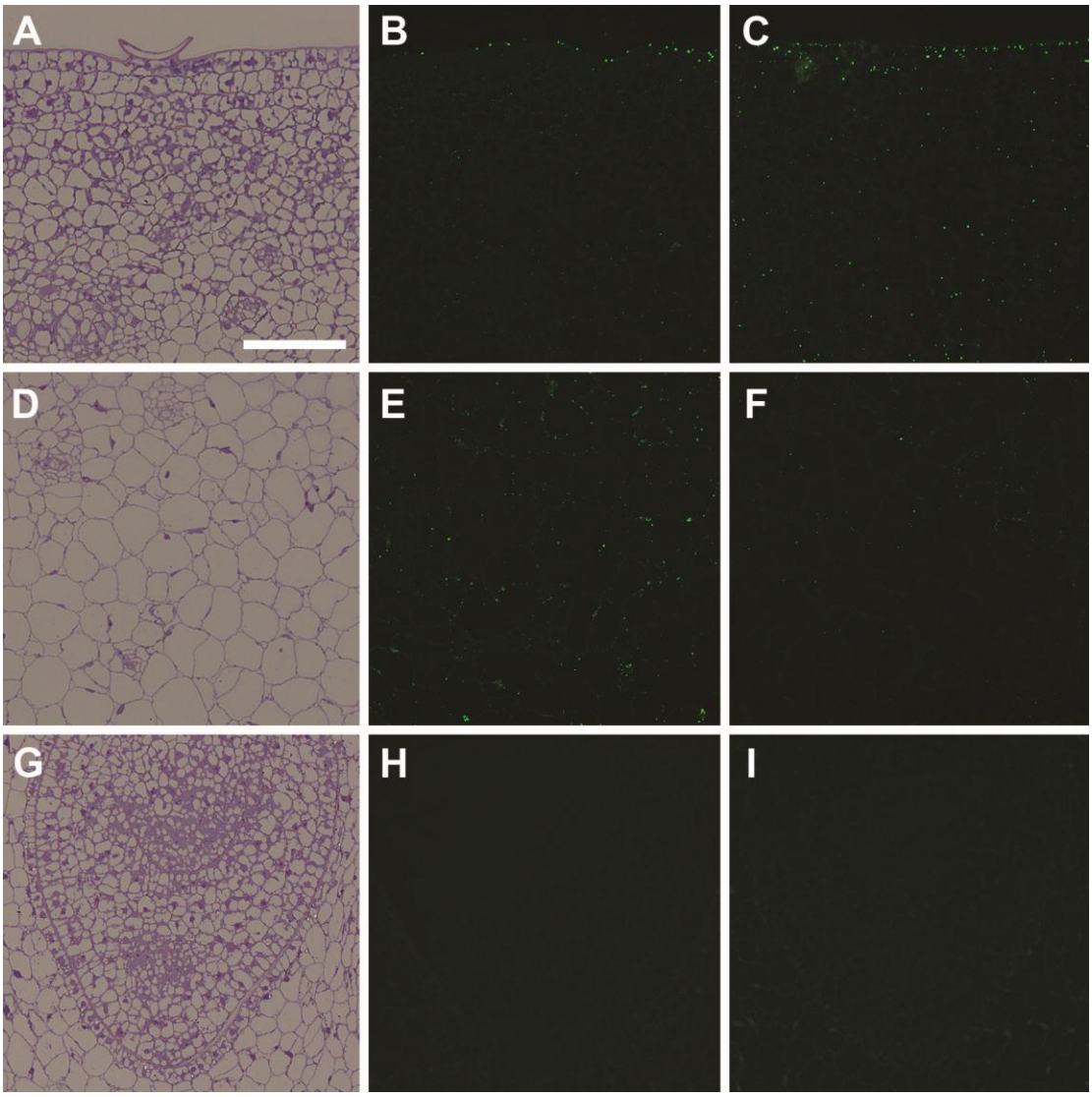
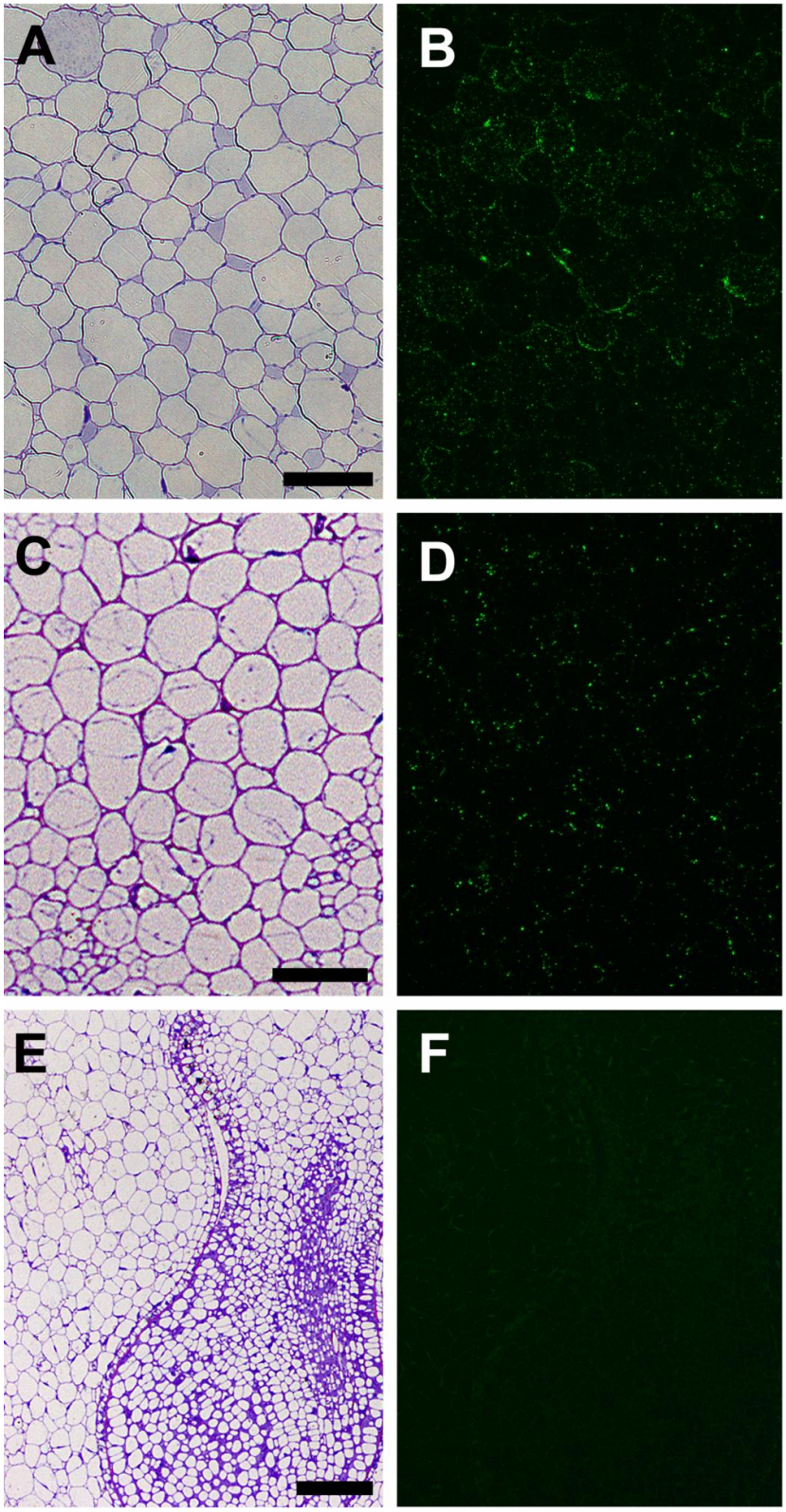

A
PG
PT
B
PG
PT

Figure 3.5. Visualization of *Acidovorax citrulli* ingress into the ovaries of the pericarp-inoculated watermelon blossoms by immunofluorescence microscopy. Pericarps of ovaries were inoculated with 10^6 CFU/mL of *A. citrulli* and longitudinal sections (250 nm) were prepared from epicarp, mesocarp, and endocarp tissues at 1, 2, 3 and 4 days post inoculation. Panels A-C represent epicarp sections; panels D-F represent mesocarp sections and G-I represent endocarp sections. Bar (50 μm) in panel A is also applicable for the panels B, E, F, G, H, and I.


A
B
C
D
E
F
G
H
I

Supplementary figure 3.1. Visualization of *Acidovorax citrulli* ingress into non-pollinated, pistil-inoculated watermelon blossoms by immunofluorescence microscopy. Stigmas of female blossoms were inoculated with 10^6 CFU/blossom of *A. citrulli* and transverse sections (250 nm) were prepared from stigma, style and ovary tissues 1 day post-inoculation. Panels A and B represent sections from stigma tissues; panels D and E represent sections from style tissue, and panels G and I represent sections from ovary tissue. Bar (50 μm) in panel A is also applicable for panel B. Bar (50 μm) in panel D is also applicable for panel E. Bar (50 μm) in panel G is applicable for panel H.


A
B
C
D
E
F

CHAPTER 4

ACIDOVORAX CITRULLI SURVIVAL IS INFLUENCED BY ITS LOCATION IN WATERMELON SEEDS

Dutta B., Stephens, D.J., Schneider, R. W., and Walcott, R.R. 2011. To be submitted to *Phytopathology*

Abstract

The ability of *Acidovorax citrulli* to survive on host and non-host seeds was compared to other phytobacteria including *Xanthomonas campestris* pv. *campestris*, *Pantoea stewartii* subsp. *stewartii* and *Ralstonia solanacearum*. When seeds were inoculated with bacterial suspensions containing ~ 1 x 10^8 colony forming units (CFU) separately, *A. citrulli* and *X. campestris* pv. *campestris* populations declined to ~1 x 10^3 CFU/g of seed for after 12 weeks of storage at 4^oC and 50% R.H. Irrespective of seed type, *R. solanacearum* populations declined to ~1 x 10^5 CFU/ g and *P. stewartii* subsp. *stewartii* CFU were not recovered after 12 weeks of storage.The effect of *A. citrulli* localization in seed was also explored as a survival factor. Watermelon seedlots infested with *A. citrulli* by pistil (deep-seated infection) and pericarp (superficial infection) -inoculation were subjected to treatments with peroxyacetic acid (1600µg/mL for 20 min) or Cl_2 gas (750 ppm of chlorine gas for 9h). Following these treatments, a significantly higher reduction in BFB seedling transmission percentage was observed for pericarp-inoculated lots (≥89.5%) than for pistil-inoculated lots (≤76.5%) (P<0.05). Additionally, with the same treatment, a significantly higher reduction of infested seeds was observed for pericarp- (≥74.4%) than for pistil-inoculated lots (≤50.7%) (P<0.04). These results suggest that greater number of *A. citrulli* CFU survived in pistil-inoculated seeds than in pericarp-inoculated seeds indicating that bacterial localization in seeds is an important survival factor. Further confirmation was achieved, when the survival of *P. stewartii* subsp. *stewartii* populations was better in pistil- than in vacuum-infiltrated watermelon seeds.

Introduction

Bacterial fruit blotch of cucurbits (BFB) is a serious threat to watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) production worldwide (6,14,23,28). This disease is caused by the Gram-negative bacterium, *Acidovorax citrulli* (=*A. avenae* subsp. *citrulli* formerly *Pseudomonas pseudoalcaligenes* subsp. *citrulli*), which is a member of the β-Proteobacteria sub-family (21,24,30). Because infested seeds are the most important source of primary inoculum for BFB epidemics (9,20), pathogen exclusion aided by seed health testing is an important component of BFB management (8,28). Seed treatments with hydrochloric acid, sodium hypochlorite and peroxyacetic acid are also commonly used for BFB management (10,20); however, they do not completely eradicate the pathogen (20). The inability of externally-applied seed treatments to eradicate *A. citrulli* suggests that the bacterium resides under the seed coat. However, it was recently reported that the location of *A. citrulli* in watermelon seeds is influenced by the pathway of bacterial invasion (7). More specifically, ingress through the fruit pericarp resulted in the localization of *A. citrulli* cells under the testa (7) while ingress via the pistil of the female blossoms resulted in bacterial localization in the embryo.

In general, the longevity of a seedborne pathogen is influenced by factors including seed storage conditions; intrinsic characteristics of the pathogen e.g., tolerance to desiccation; and the location of bacteria in seeds (25). *Acidovorax citrulli* was reported to survive in, and be transmitted from watermelon and melon (*Cucumis melo* L. var. *cantalupensis* Naudin) seeds that were stored for more than 30 years (5). However, the pathogen survived for only 63 days on the surface of untreated Speedling polystyrene

trays (TSC Polyform, Minneapolis, MN) containing potting soil and seedling root debris (3). While these reports suggest that *A. citrulli* can survive desiccation; to date the factors that influence its longevity have not been studied. Hence, the goal of this research was to investigate the factors that influence *A. citrulli* survival in watermelon seeds. Specific objectives of this research were 1) to investigate the effect of host and non-host seed type on *A. citrulli* survival and 2) to determine the effect of *A. citrulli* location in seed on bacterial survival.

Materials and methods

Bacterial strains. The bacterial strains used in this study are listed in Table 4.1. *Acidovorax citrulli* (AAC00-1) was routinely cultured on King's medium B (11), *X. campestris* pv. *campestris* (XCC00-1) was grown on yeast dextrose chalk (YDC) (22) amended with 100 µg/mL of streptomycin, *P. stewartii* subsp. *stewartii* (DS-203) was grown on Luria Bertani (LB) (22) agar amended with 50 µg/mL of nalidixic acid, and *R. solanacearum* AW1 was grown on LB agar. Inoculum for each bacterial strain was prepared by transferring a single colony from a 24h culture into nutrient broth followed by overnight incubation at 28 °C on a rotary shaker (Innova; New Brunswick Scientific Co., Edison, NJ) at 250 rpm. Subsequently, cultures were centrifuged at 6000 × *g* (Allegra^{TM} 25R centrifuge, Beckman Coulter, Fullerton, CA) for 5 min and the supernatant was decanted. The resulting pellets were resuspended in 0.1 M phosphate buffered saline (PBS) and the bacterial concentrations were adjusted to an optical densities of 0.3, 0.5, 0.3 and 0.1 for *A. citrulli*, *P. stewartii* subsp. *stewartii*, *X. campestris*

pv. *campestris*, and *R. solanacearum*, respectively at 600 nm (~1 x 10^8 CFU/ml) (Spectronic 20; Bausch and Lomb, Rochester, NY).

Survival of phytobacteria on filter paper. The ability of each bacterial strain to survive on filter paper was investigated. Bacterial suspensions containing ~ 1 x 10^8 CFU/mL of each bacterium were generated as described above and strips of sterile filter paper (~10 cm × 4 cm) (Whatman Inc., Sanford, ME) were incubated in each suspension for 10 min followed by air-drying at ~25^{o}C for 12h. Each filter paper strip was placed in a Petri dish with the lid partially open and the dish was incubated in a desiccator cabinet (Secador 4.0, Scienceware, Pequannock, NJ) at 50% relative humidity (RH). The desired RH was achieved by placing a 1:1 mixture of terpentine oil (Pine Chemicals Pvt. Ltd., Singapore) and water in the desiccator and incubating at 4^o C. Strips of filter paper saturated with PBS served as negative controls. After 0, 1, 2, 3, 4, 8, and 12 weeks of incubation, filter paper samples (~ 4 mm^2) were taken and bacterial populations were enumerated by ten-fold serial dilution-plating on respective semi-selective media as described above. For each treatment, three replicates were sampled at each time point and the experiment was repeated twice. Bacterial colonies were enumerated after 36 to 48h of incubation depending on the species, and the replicates of each treatment per time point for two experiments were compared using student's t-test at $P<0.05$ in SAS (version 9.2 for Windows; SAS Institute Inc., Cary, NC). If there was no significant effect of replication and experiment, data from replicates from the two experiments were pooled to determine mean bacterial populations. Repeated measure analysis was used to determine the effect of storage time on mean bacterial population and differences in the abilities of bacterial

species to survive on filter paper at $P<0.05$ in SAS (version 9.2 for Windows; SAS Institute Inc., Cary, NC).

Survival of phytobacteria on host and non-host seeds. The four phytobacteria selected for this study were inoculated separately onto host and non-host seeds by vacuum infiltration. The host seed species used for *A. citrulli*, *P. stewartii* subsp. *stewartii*, *X. campestris* pv. *campestris*, and *R. solanacearum* were watermelon (cv. Crimson sweet), corn (cv. Tom Tom), cabbage (cv. Saratoga), and tomato (cv. Bonnie Best), respectively. Seeds (n = 25 g) were placed in a sterile 250 ml side-arm flask containing 20 mL of each bacterial cell suspension (~1 x 10^8 CFU/mL), and the flask was attached to a vacuum line. Cell suspensions were infiltrated into the seeds (in bulk) by applying vacuum to the flask for 1h. Seeds were subsequently removed from the cell suspension and air-dried at ~25^{o}C for 24h. Treated seeds were stored in open Petri plates at 4^{o}C and 50% RH and after 0, 1, 2, 3, 4, 8, and 12 weeks of incubation, samples (n = 1 g of seed) were macerated in PBS, ten-fold serial diluted and spread plated (100 µL of seed extract) onto respective semi-selective media. After 36 to 48h of incubation at 28 ^{o}C, bacterial colonies were enumerated. For each treatment three seed samples were tested per time point and the experiment was repeated twice. Student's t- test was used to compare the replicates of each treatment per point for two experiments at $P<0.05$. If the effect of replication and experiment was not statistically significant, replicates from two experiments were pooled to determine mean bacterial populations. Repeated measures analysis was used to determine the effect of host and non-host seeds on bacterial survival at $P<0.05$.

Effect of *A. citrulli* location in seeds on its survival. Pistil- and pericarp-inoculated seedlots infested with *A. citrulli* strain AAC00-1 were generated as described previously (15, 27). Four seedlots (two each by pistil- and pericarp-inoculation) infested with *A. citrulli* were generated for this study (Table 4.2). The two pericarp-inoculated lots were recovered from artificially inoculated fruits with BFB symptoms and the two pistil-inoculated lots were recovered from symptomless fruits that developed from female blossoms whose stigmas were inoculated with *A. citrulli.* For the pericarp-inoculated seedlots, 25 watermelon plants (cv. Crimson Sweet) were grown in pots under greenhouse conditions. At anthesis 30-40 female blossoms were hand-pollinated and the pericarps of the ovaries were uniformly inoculated using a cotton swab saturated with inoculum (1×10^6 *A. citrulli* CFU/mL). Blossoms were incubated at 100% RH for 24h and fruits were allowed to develop for 30 days post-pollination under greenhouse conditions. Fruits were harvested and stored at 4^{o}C until seed extraction. Seeds were manually extracted from each fruit, air-dried overnight at 25^{o}C, on paper towels and stored in paper bags at 4^{o}C until they were used. Pistil-inoculated seedlots were generated by pollinating female watermelon blossoms and inoculating the stigmas with AAC00-1 as previously described (27). Briefly, 25 watermelon plants (cv. Crimson Sweet) were established in pots under greenhouse conditions and at anthesis, female blossoms were pollinated manually followed by inoculating the stigma with 10 µL of a bacterial suspension containing ~ 1×10^8 AAC00-1 CFU/mL. Five blossoms were inoculated and tagged per plant and allowed to grow for 30 days after pollination. Seeds were extracted and stored as described above.

Seedlot characteristics. To determine the percentage of *A. citrulli*-infested seeds in each lot, four samples (n = 100 seeds/lot) were planted in transparent plastic boxes [6 cm (H) ×24 cm (W) × 33.5 cm (L); Tri-State Plastics, Dixon, KY,] on blotter paper (Anchor Paper Co., St. Paul, MN) and maintained at 28°C and 85% RH for 14 days. Seeds from PBS-inoculated blossoms were used as negative control. Seed germination and BFB seedling transmission percentages (number of seedlings with symptoms divided by the total number of seeds germinated × 100) were determined 14 days after planting (DAP).

Seed treatments with antimicrobials. Since it was impractical to conduct a multiyear survival study with naturally infested seeds, seed aging was simulated by chemically stressing the seeds to determine the effect of *A. citrulli* location in seed on bacterial survival. Pericarp- and pistil-inoculated seed were treated with peroxyacetic acid (Tsunami 100, Ecolab, McDonough, GA) or chlorine gas. For the seed treatment with peroxyacetic acid, seeds [n = 20 grams/lot (~ 450 seeds)] were placed in a 250 mL side-arm flask with 100 mL of peroxyacetic acid (1600 µg/mL). The flask was sealed with a rubber cork and then a vacuum was drawn for 20 min. The peroxyacetic acid was decanted, seeds were rinsed with sterile water for 10 min and air-dried for 48h at 40^{o}C in a seed dryer (Grainman Machinery Manufacturing Corp., Miami, FL). Pericarp-and pistil-inoculated seeds (n = 20 grams) from the corresponding lots that were vacuum infiltrated with PBS served as positive controls. Seeds (n = 20 grams) from PBS-inoculated blossoms, vacuum infiltrated with peroxyacetic acid was used as a negative control.

For chlorine gas treatment, seeds (*n* = 20 grams/lot) were placed in a glass chamber (50 cm ×25 cm ×30 cm) fitted with plastic test tube racks, a 400 mL- mixing vat, a pH probe, wet and dry bulb thermometers, a fan and a glass top with a sample septum. Chlorine gas was produced by mixing 100 mL of 6.0% NaOCl and 100 mL of 0.6 M monobasic potassium phosphate (KH_2PO_4) buffer in the mixing vat followed by sealing glass chamber as described previously (24). Chlorine concentrations inside the chamber were monitored with a Kitagawa gas detector system (Matheson Tri-gas, Newark, CA). Seeds were placed inside the glass chamber for 9h and the concentration of chlorine gas was maintained at 750 ppm. Seeds from PBS-inoculated blossoms exposed to chlorine gas as described above served as negative control. Untreated pericarp- and pistil-inoculated seeds from corresponding lots were used as positive controls.

For the two seed treatments, three replicates of seeds of each inoculation type (pericarp or pistil) were used in a single experiment and the experiment was repeated twice. To determine the effect of the seed treatments on *A. citrulli* survival, the percent reduction in BFB seedling transmission [(BFB transmission percentage for the positive control lot – BFB transmission percentage for the treated lot) / BFB transmission percentage for the positive control lot × 100] and percent reduction in infested seeds [(percentage of infested seeds for the positive control lot – percentage of infested seeds after seed treatment)/percentage of seeds for the positive control lot × 100] for each seed sample were determined separately. Mean percent reductions of BFB transmission and infested seeds were calculated for seed samples generated by each inoculation method.

To determine the effect of the seed treatments on BFB seedling transmission percentage peroxyacetic acid and chlorine gas treated seed samples (n = 100 seeds/lot) were planted in transparent plastic boxes on blotter paper and maintained at 28°C and 85% RH for 14 days as described above. At 14 days after planting, seed germination and BFB transmission percentages were recorded. Seed samples from positive and negative control lots were also tested and percent reduction in BFB transmission was determined as described above.

To determine the population of *A. citrulli* in seeds, samples (n = 20 seeds/replicate/lot) from each treatment were macerated individually in 1 mL of PBS and spread onto Nunhem's agar plates. After 6 days of incubation, the percentage of *A. citrulli* infested seeds for each seed sample was recorded. The percentage of infested seeds in positive and negative control lots was determined as described above and percent reduction in infested seeds was calculated. The effect of seed treatment on percent reduction in BFB seedling transmission percentage and percent reduction in infested seeds was determined using analysis of variance in the General Linear Model of the SAS (version 9.2 for Windows; SAS Institute Inc., Cary, NC). Mean separation was done using Least Significance Difference ($P < 0.05$). Seed germination data from negative control lots were used to determine the effect of seed treatments on seed physiology using student's t-test at $P<0.05$ in SAS (version 9.2 for Windows; SAS Institute Inc., Cary, NC).

Survival of *P. stewartii* subsp. *stewartii* in watermelon seeds. The effect of pathogen location on survival of *P. stewartii* subsp. *stewartii* in seeds was investigated to determine if a weak survivor can survive better when localized in the embryo. Since *P. stewartii* subsp. *stewartii* was reported to survive for few months in seed and infected seeds do not play a significant role in the transmission of Stewart's wilt disease (3,4). Hence, it is considered to be a weak surviving seedborne bacterium, however; its primary mode of survival is in the gut of corn flea beetle (3,4,17).

Watermelon seeds were pistil-inoculated with *P. stewartii* subsp. *stewartii* DS-203 as described for *A. citrulli* above. Seeds from fruits that developed from PBS-inoculated blossoms were used as negative control. To confirm that pistil-inoculation deposited *P. stewartii* subsp. *stewartii* in the embryos of watermelon seeds, samples (n = 50 seeds) from a pistil-inoculated lot were dissected into seed coat (testa), perisperm-endosperm (PE) layer, and endosperm as described previously (7). Sections were assayed for *P. stewartii* subsp. *stewartii* by species-specific PCR and plating. For real-time PCR, forward (ES-F: TGCGAAGCGAGGACACACGTA and reverse primers (ES-R: GACACCTTTCACCTG TCACCAA) along with SYBR green (Applied Biosystems, Foster City, CA) were used to amplify the target sequence in the bacterium. Total genomic DNA from each seed section (≈5 ng/µL) was used as template, and amplification was performed using a commercially available SYBR green mastermix (Applied Biosystems, Foster City, CA) and a real-time PCR machine (Smart Cycler II; Cepheid, Sunnyvale, CA). The following thermal profile was used for amplification: denaturation at 95°C for 180 s, followed by 35 cycles of denaturation at 95°C for 15 s,

and annealing and elongation at 55°C for 40 s. Samples with cycle threshold (Ct) values below 30 were considered to be positive for *P. stewartii* subsp. *stewartii*. The mean percentage of *P. stewartii* subsp. *stewartii*-infested seed sections for each lot was compared by factorial analysis ($P < 0.05$) in SAS (version 8.1 for Windows; SAS Institute Inc., Cary, NC). For seed inoculation by vacuum infiltration, 30 g of watermelon seeds (cv. Crimson Sweet) were placed in a suspension containing ~1×10^6 *P. stewartii* subsp. *stewartii* CFU/mL and a vacuum was applied for 1h. Seeds were subsequently air-dried at ~25^o C for 24 h and samples (n = 30 g of seeds/lot) were incubated under condition of 4^o C and 50% RH. Samples (n = 1 g of seed/treatment/sample time) were taken after 0, 1, 2, and 3 weeks of incubation, macerated in PBS, ten-fold serially diluted and spread plated on LB agar amended with 50 µg/mL of nalidixic acid. Bacterial colonies were enumerated 48h after incubation. Three replicates per treatment/time point were used and the experiment was repeated twice. The replicates for each treatment/time point in two experiments were compared using student's t-test and if the effect of replicates and experiments was not significant at $P<0.05$, they were pooled and mean bacterial populations were determined. Repeated measures analysis was used to determine the effect of pathogen location on bacterial survival at $P<0.05$.

Results

Bacterial survival on filter paper. As expected, in two independent experiments, bacteria were not recovered from filter paper strips inoculated with sterile PBS (negative control). There was a significant difference in the abilities of different bacterial species to survive on filter paper at 4^o C and 50% RH (P=0.013). At 0 weeks post inoculation

(WPI), mean populations of *A. citulli*, *R. solanacearum*, *X. campestris* pv. *campestris*, and *P. stewartii* subsp. *stewartii* ranged from ~10^7 to 10^8 CFU/mm^2**.** By 2 WPI, mean *P. stewartii* subsp. *stewartii* population declined to 1.02×10^4 CFU/mm^2 while populations of the other bacteria ranged from 2.13×10^6 to 3.63×10^6 CFU/mm^2. At 4 WPI, the mean *P. stewartii* subsp. *stewartii* CFU population decreased to 1.54×10^3 CFU/mm^2 and by 8 WPI, it was 1.34×10^2 CFU/mm^2. By 12 WPI *P. stewartii* subsp. *stewartii* was not recovered from the filter paper strips. In contrast, by 4 WPI, mean populations of *X. campestris* pv. *campestris*, *A. citrulli*, and *R. solanacearum* declined to a range of $9.33 \times 10^4 – 9.54 \times 10^5$ CFU/mm^2 and by 8 WPI, mean populations for *X. campestris* pv. *campestris* and *A. citrulli* declined to $2.95 \times 10^4 – 3.23 \times 10^4$ CFU/mm^2 while the mean *R. solanacearum* population was ~2.95×10^5 CFU/mm^2. At 12 WPI, mean *X. campestris* pv. *campestris* and *A. citrulli* populations declined to ~$2.29 \times 10^2 – 3.09 \times 10^2$ CFU/mm^2 while *R. solanacearum* populations were ~ 1.12×10^5 CFU/mm^2 (Fig. 4.1).

Bacterial survival on host and non-host seeds. The ability of bacteria to survive on host and non-host seed species after 12 weeks varied significantly ($P<0.0001$). At 12 WPI, the mean *R. solanacearum* population was significantly higher on cabbage seeds (~ 1.31×10^5 CFU/g) than on other seed types. During the same period, *A. citrulli* and *X. campestris* pv. *campestris* populations were significantly higher on cabbage seeds than on other seed species, with mean CFUs of 3.09×10^3 and 1.90×10^3, respectively (Fig. 4.2). Irrespective of seed type, *P. stewartii* subsp. *stewartii* was not recovered by 12 WPI. On watermelon seed, by 12 WPI, the mean population of *R. solanacearum* (3.63×10^4) was significantly higher than the other bacteria ($P<0.0002$). During the same period, no

significant difference in the populations for *A. citrulli* (1.65×10^3) and *X. campestris* pv. *campestris* (3.63×10^2) was observed ($P = 0.26$). On both tomato and corn seeds, mean *R. solanacearum* populations at 12 WPI were 1.90×10^3 and 3.32×10^4 CFU/g, respectively, which were significantly higher than other bacterial species. On these seeds, there was no significant difference in the *A. citrulli* and *X. campestris* pv. *campestris* populations ($P = 0.988$). The mean population estimates for *A. citrulli* and *X. campestris* pv. *campestris* on tomato and corn seeds were1.69×10^2, 2.81×10^2 CFU/g and 2.95×10^2 , 2.88×10^2 CFU/g, respectively (Fig. 4.2).

Overall, the difference in survival between bacterial species was statistically significant ($P<0.0001$). Significantly higher populations of *R. solanacearum* ($\sim 10^4$ - 10^5 CFU/g) were observed on all seed species by 12 WPI (Fig. 4.2) than other bacteria tested. During this same period, there was no significant difference between the *A. citrulli* and *X. campestris* pv. *campestris* populations ($\sim 10^2$-10^3 CFU/g) ($P \leq 0.271$) (Fig. 4.2) and *P. stewartii* subsp. *stewartii* was not recovered from seeds by 12 WPI.

Effect of *A. citrulli* location in seeds on its survival. The two pericarp-inoculated seedlots displayed mean seed germination percentages of 77.5% and 68%, respectively and mean BFB seedling transmission percentages of 61.3% and 64.7%, respectively (Table 4.2). The mean seed germination percentages for the two pistil-inoculated seedlots were 74% and 85%, respectively, and they displayed BFB seedling transmission percentages of 56.8% and 63.5%, respectively (Table 4.2). As expected, peroxyacetic acid did not affect the germination of the control seeds ($P = 0.34$) and these seeds did not transmit BFB to resulting seedlings. Seed treatment with peroxyacetic acid resulted in a

statistically significant reduction in BFB seedling transmission percentage ($P = 0.0004$); however, no significant differences in reduction in BFB transmission were observed between lots of the same infection type ($P = 0.43$). The mean percent reduction in BFB transmission for seeds treated with peroxyacetic acid was significantly higher for pericarp-inoculated seed (89.5%) than for pistil-inoculated seeds (61.1%) (Fig. 4.3). Peroxyacetic acid also resulted in a significant reduction in the mean percentage of *A. citrulli*-infested seeds for pericarp-inoculated lots (74.8%) than for pistil-inoculated lots (9.63%) ($P = 0.0001$) (Fig. 4.4). As expected, bacterial colonies were not recovered from negative control seeds.

Chlorine gas seed treatments did not affect watermelon seed germination ($P = 0.67$) but significantly reduced BFB seedling transmission for both pericarp-and pistil-inoculated seedlots ($P = 0.0004$). With chlorine gas treatment, the percent reduction in BFB seedling transmission for pericarp-inoculated seeds was 99.1% (Fig. 4.3) which was significantly higher than for pistil-inoculated seeds (76.5%) ($P = 0.047$) (Fig. 4.3). Chlorine gas also resulted in a significant reduction in the mean percentage of *A. citrulli*-infested seeds in pericarp-inoculated lots (80.8%) than for pistil-inoculated lots (54.02%) ($P = 0.05$) (Fig. 4.4).

Survival of *P. stewartii* subsp. *stewartii* in watermelon seeds. In pistil-inoculated watermelon seeds, greater than 63% of the embryo and PE layer samples were positive for *P. stewartii* subsp. *stewartii* as compared to <8% of the testa samples. The difference between *P. stewartii* subsp. *stewartii* populations in vacuum-infiltrated and pistil-inoculated watermelon seeds was significant ($P<0.003$). At 0 WPI, the mean *P. stewartii*

subsp. *stewartii* populations recovered from vacuum infiltrated and pistil-inoculated watermelon seeds were 3.46×10^6 and 1.32×10^3 CFU/g, respectively (Fig. 4.5). By 3 WPI, populations of *P. stewartii* subsp. *stewartii* were not recovered from vacuum-infiltrated seeds while mean populations from pistil-inoculated seeds were 4.07×10^3 CFU/g (Fig. 4.5).

Discussion

Factors including storage conditions, sensitivity to desiccation, and location of bacteria in seeds contribute to the survival of bacteria in seeds (1, 15, 25). With regards to storage conditions, low temperature and low moisture allow seedborne bacteria to remain viable for longer periods (1, 25). For example, *Pseudomonas syringae* pv. *phaseolicola* and *X. campestris* pv. *manihotis* survived longer in bean and cassava seeds, respectively, when stored at 5 - 7^{o}C and 50 - 60% RH(19, 27). Bacterial fruit blotch seedling transmission was reported for watermelon and melon seeds after 34-38 years in storage at 5^{o}C and 50% RH (5); however, the factors that influence this longevity have not been previously studied. The current study revealed that *A. citrulli* cells were not intrinsically tolerant to desiccation. In fact, superficial (vacuum infiltration) seed inoculation did not enhance *A. citrulli* survival on watermelon seeds relative to other bacteria. In all instances, *A. citrulli* populations declined from 10^7-10^8 CFU/g to 10^3-10^2 CFU/g of seeds after 12 weeks of storage. Interestingly, the trend of *A. citrulli* population decline in watermelon seeds was similar to that observed for non-host seed species. *Pantoea stewartii* subsp. *stewartii* was a weak survivor as it was undetectable after 12 weeks of storage on filter paper and host, and non-host seeds. This may explain why seedborne

inoculum is not important in Stewart's wilt epidemiology (3,4). In fact, based on previous reports, rates of seed-to-seedling transmission of *P. stewartii* subsp. *stewartii* in sweet corn hybrids were as low as 0.038-0.14% (3, 4, 17). In comparison, *R. solanacearum* was a good survivor as the highest bacterial populations were recovered after 12 weeks of storage on filer paper and host and non-host seeds. This was expected as *R. solanacearum* is a persistent, soil borne bacterium (2,18). Regardless of the substrate, the population dynamics of *A. citrulli* and *X. campestris* pv. *campestris* were not significantly different ($P>0.05$). Based on our observations, we conclude that the ability of *A. citrulli* to survive for more than 30 years in melon and watermelon seeds is not due to its inherent tolerance to desiccation, nor its superficial association with watermelon seeds.

Since *A. citrulli* can become localized to different regions of watermelon seeds depending on the method of inoculation (7), we hypothesized that bacterial survival might be enhanced by location in the seed. Because of the practical limitations of conducting a multiyear survival study with naturally infested seeds, we used surface and subsurface antimicrobial treatments to determine if the bacterium's location in the seed might confer longevity. The selected treatments consist of two different forms of seed treatments with varying level of effectiveness. Peroxyacetic acid is an industry standard chemical treatment for *A. citrulli*, which is effective on surface and sub-surface populations of *A. citrulli* (10). In contrast, chlorine gas is a gaseous treatment that can penetrate seed coat as well as internal seed tissue (26). However, the depth of penetration by gaseous chlorine is unknown. Using these treatments, it was expected that *A. citrulli* in pericarp-inoculated seeds would be disinfested effectively. Contrastingly, *A. citrulli* in

pistil-inoculated seeds would not be affected by peroxyacetic acid treatment, however; treatment with chlorine gas would efficiently disinfest internal populations of *A. citrulli*. Peroxyacetic acid resulted in a significantly higher reduction in the percentage of *A. citrulli*-infested seeds and BFB seedling transmission in pericarp-inoculated seeds than in pistil-inoculated seeds. Chlorine gas also resulted in significantly higher reduction in percentage of infested seeds and BFB seedling transmission for pericarp-inoculated than in pistil-inoculated seeds. This indicates that *A. citrulli* is protected from stress in the embryo of watermelon seed as compared to when it is just under the seed coat. Hence, we conclude that *A. citrulli* location in watermelon seeds is an important factor in pathogen survival.

To further confirm that location was a factor in bacterial survival in seeds a weak survivor, *P. stewartii* subsp. *stewartii*, was pistil-inoculated into watermelon seeds and its ability to survive was compared to that of bacteria on superficially contaminated seeds. External contamination of watermelon seeds with *P. stewartii* subsp. *stewartii* was performed using vacuum–infiltration, as natural pericarp invasion was not possible. Previously, Walcott *et al.* (29) reported that *Pantoea ananatis* (causal agent of center rot of onion) could colonize watermelon blossoms and subsequently infest seeds. Based on this observation, we speculated that pistil inoculation would also deposit *P. stewartii* subsp. *stewartii* into the embryo. While *P. stewartii* subsp. *stewartii* could not be recovered from vacuum infiltrated seeds after 3 weeks of storage, bacterial populations in pistil-inoculated seeds ranged from 4.07×10^3 CFU/ g of seeds. These data suggested that

enhanced survival of *P. stewartii* subsp. *stewartii* in watermelon seeds was due to its location in the embryo.

Based on data collected in this study, we conclude that relative to *R. solanacearum*, *A. citrulli* is sensitive to desiccation. However, it is unlikely that tolerance to desiccation alone explains the pathogen's longevity in stored watermelon seeds. Alternatively, our data suggest the location of the bacterium in seed is an important factor of long-term *A. citrulli* survival in seed.

Acknowledgements

We thank Dr. Rock Christiano for assistance with statistical analysis and Ms. Linda Genzlinger, Ms. Isabela Ontaneda, and Ms. Anita Castro-Sparks for technical assistance.

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Table 4.1. Identity, origin, and source of bacterial strains used in this study.

Bacterium	Strain	Host	Inoculum source	Source
Acidovorax citrulli	AAC00-1	Watermelon	Seed	R. Walcott, University of Georgia
Xanthomonas campestris pv. *campestris*	XCC00-1	Cabbage	Seed	R. Walcott, University of Georgia
Pantoea stewartii subsp. *stewartii*	DS-203	Corn	Seed and insect	D. Coplin, Ohio State University
Ralstonia solanacearum	AW-1	Tomato	Soil and host debris	T. Denny, University of Georgia

Table 4.2. Characteristics of watermelon seedlots generated for this study.

Seedlot	Inoculation method	Mean seed germination (%)[a]	Mean BFB seedling transmission (%)[b]
1	Pericarp	77.5 (2.67)[c]	61.3 (1.42)[c]
2	Pericarp	68.0 (1.34)	64.7 (2.54)
3	Pistil	74.0 (3.26)	56.8 (3.87)
4	Pistil	85.0 (1.84)	63.5 (2.17)

[a] Mean seed germination percentages for four replicates of seed samples (n = 100) from each lot. Seeds were planted in transparent, closed, plastic boxes with saturated blotter paper and incubated for 14 days at 28^{o}C and 80% R.H.

[b] Mean bacterial fruit blotch (BFB) seedling transmission percentages for four replicates of seed samples (n = 100) from each lot. Seeds were planted in transparent, closed, plastic boxes with saturated blotter paper and incubated for 14 days at 28^{o}C and 80% R.H. BFB seedling transmission percentages were recorded as number of seedlings showing typical BFB symptoms divided by total number of seeds germinated × 100.

[c] Numbers in parentheses denote standard error of the mean.

Figure 4.1. Survival of phytobacteria on filter paper at 4^{o} C and 50% R.H. Filter paper strips were inoculated with 10^{8}CFU/mL of each bacteria, and stored at 4^{o} C and 50% R.H for 12 weeks. Each data point represents mean CFU/mm^{2} as estimated by dilution plating on respective semi-selective medium. Bars represent standard errors of the means (n = 6).

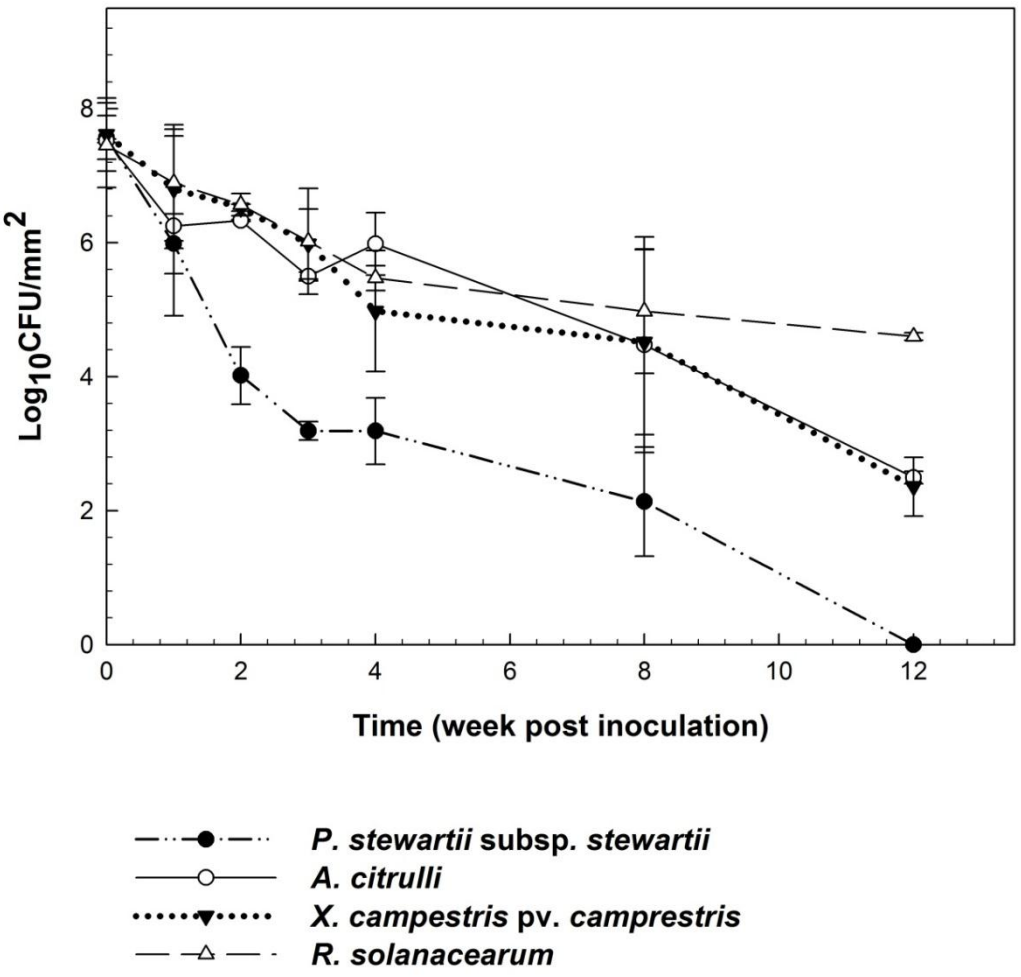

Log10CFU/mm2
0
2
4
6
8
0
2
4
6
8
10
12
Time (week post inoculation)
P. stewartii subsp. stewartii
A. citrulli
X. campestris pv. camprestris
R. solanacearum

Figure 4.2. Survival of *Acidovorax citrulli*, *Pantoea stewartii* subsp. *stewartii*, *Xanthomonas campestris* pv. *campestris* and *Ralstoinia solanacearum* on A) watermelon, B), tomato, C) cabbage and D) maize seeds. Each seed species (watermelon, tomato, cabbage, and corn) was inoculated with ~10^8 CFU/g of each bacterium dried to their original weight and stored at 4^o C and 50% R.H for 12 weeks. Each data point represents mean CFU/g of seed estimated by dilution plating of 1 g of seeds at each sample time on respective semi-selective medium. Bars represent standard errors the means for three replicates per treatment per experiment and the experiment was repeated twice (n = 6).

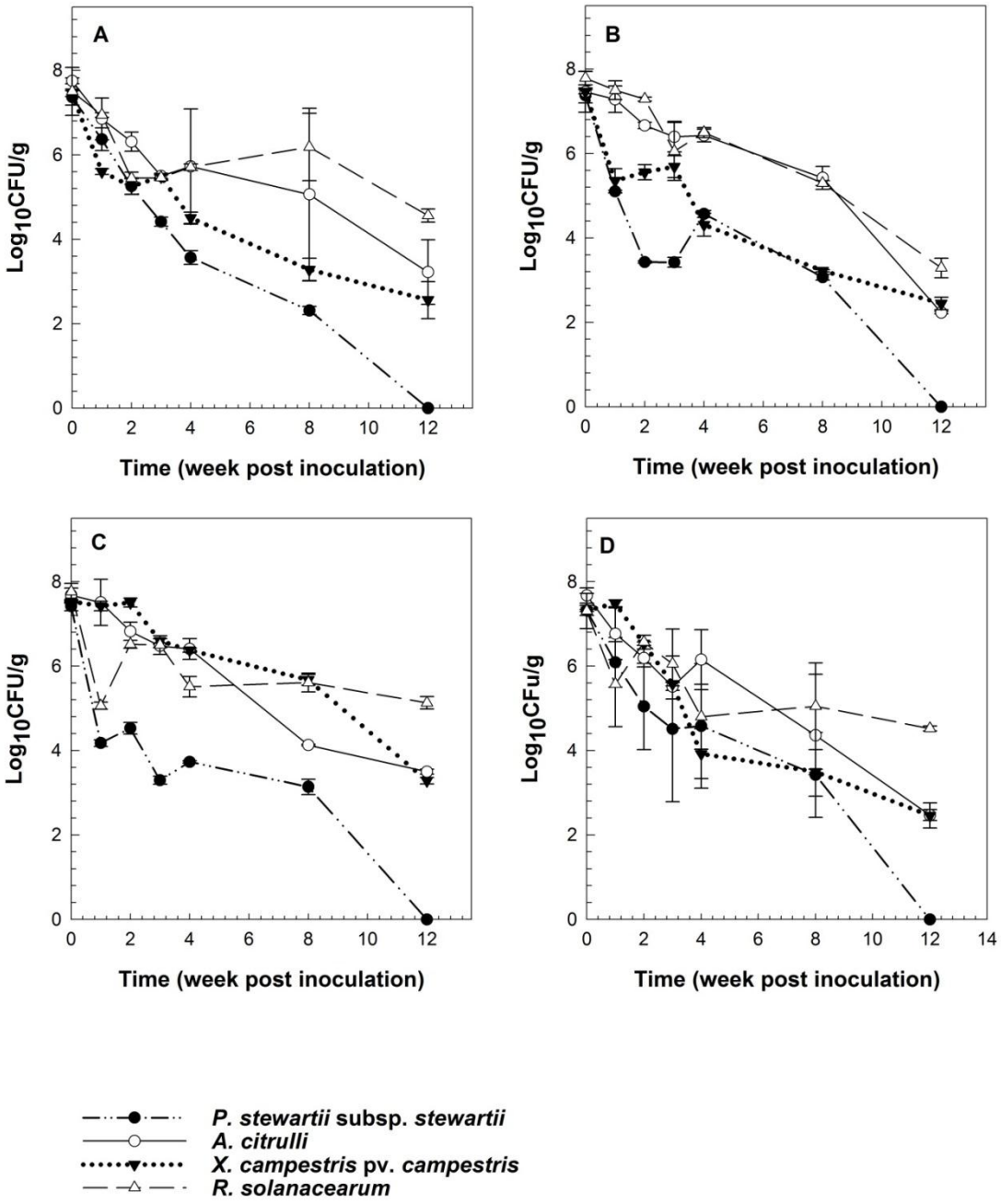

A
B
C
D
Log10CFU/g
Log10CFu/g
Time (week post inoculation)
P. stewartii subsp. stewartii
A. citrulli
X. campestris pv. campestris
R. solanacearum

Figure 4.3. Reduction in bacterial fruit blotch seedling transmission after surface and sub-surface antimicrobial seed treatments of watermelon seeds pericarp and pistil-inoculated with *Acidovorax citrulli*. One gram of seed from two seedlots of each infection type were subjected to surface treatment (1 % Tsunami 100 for 1h followed by overnight drying) and sub-surface treatment (750 ppm of chlorine gas for 9h). Error bars represent the standard errors of the means with three replicates per treatment in two independent experiments. Means with similar upper and lower case letters are not significantly different according to student's t-test ($P < 0.05$) ($n = 12$).

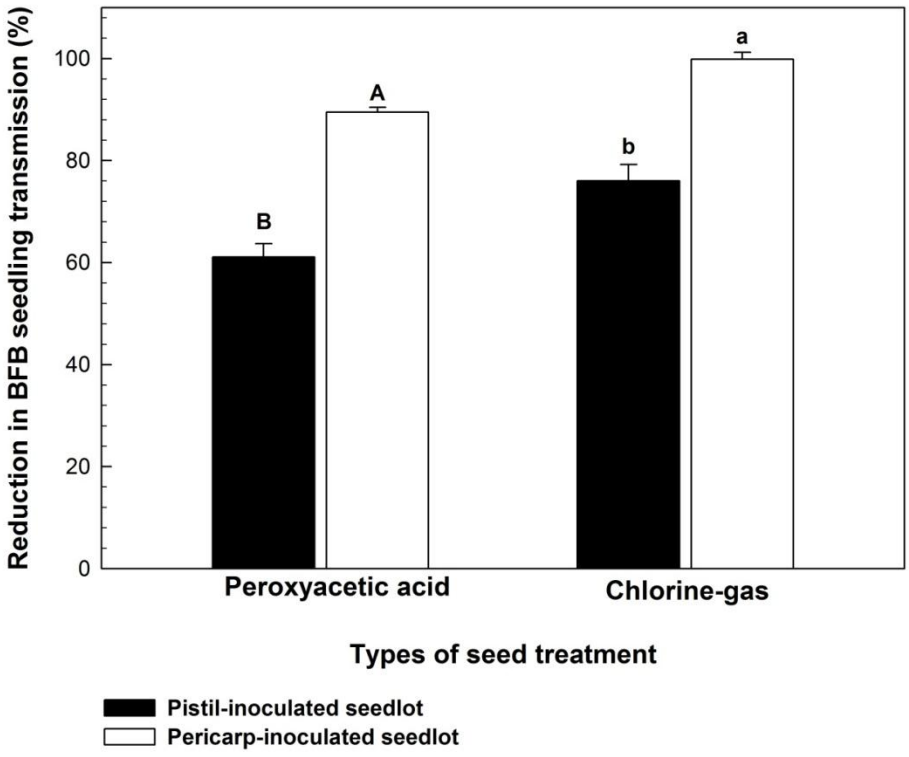

Reduction in BFB seedling transmission (%)
100
80
60
40
20
0
A
B
a
b
Peroxyacetic acid
Chlorine-gas
Types of seed treatment
Pistil-inoculated seedlot
Pericarp-inoculated seedlot

Figure 4.4. Reduction in the percentage of *Acidovorax citrulli*-infested seeds after surface and sub-surface seed treatment of pericarp and pistil inoculated watermelon seeds. One gram of seed from two seedlots of each infection type were subjected to surface treatment (1 % Tsunami 100 for 1h followed by overnight drying) and sub-surface treatment (750 ppm of chlorine gas for 9h). Error bars represent the standard errors of the means with three replicates per treatment in two independent experiments. Means with similar upper and lower case letters are not significantly different according to student's t-test ($P < 0.05$) ($n = 12$).

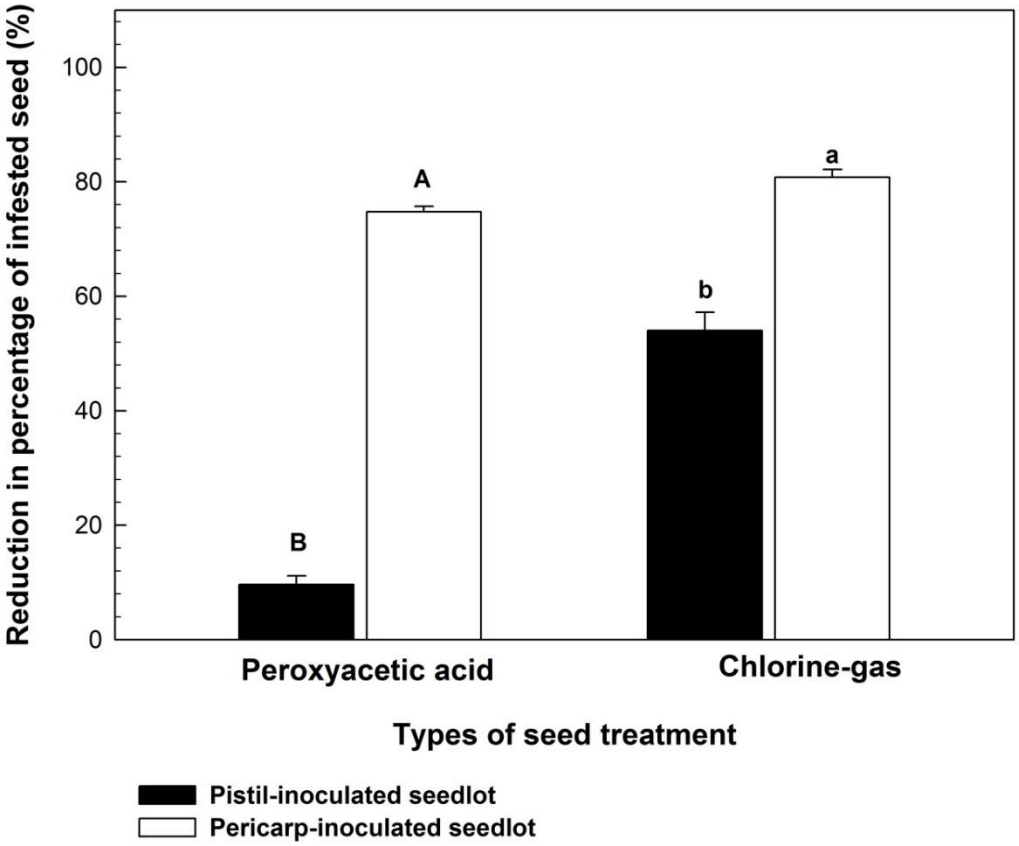
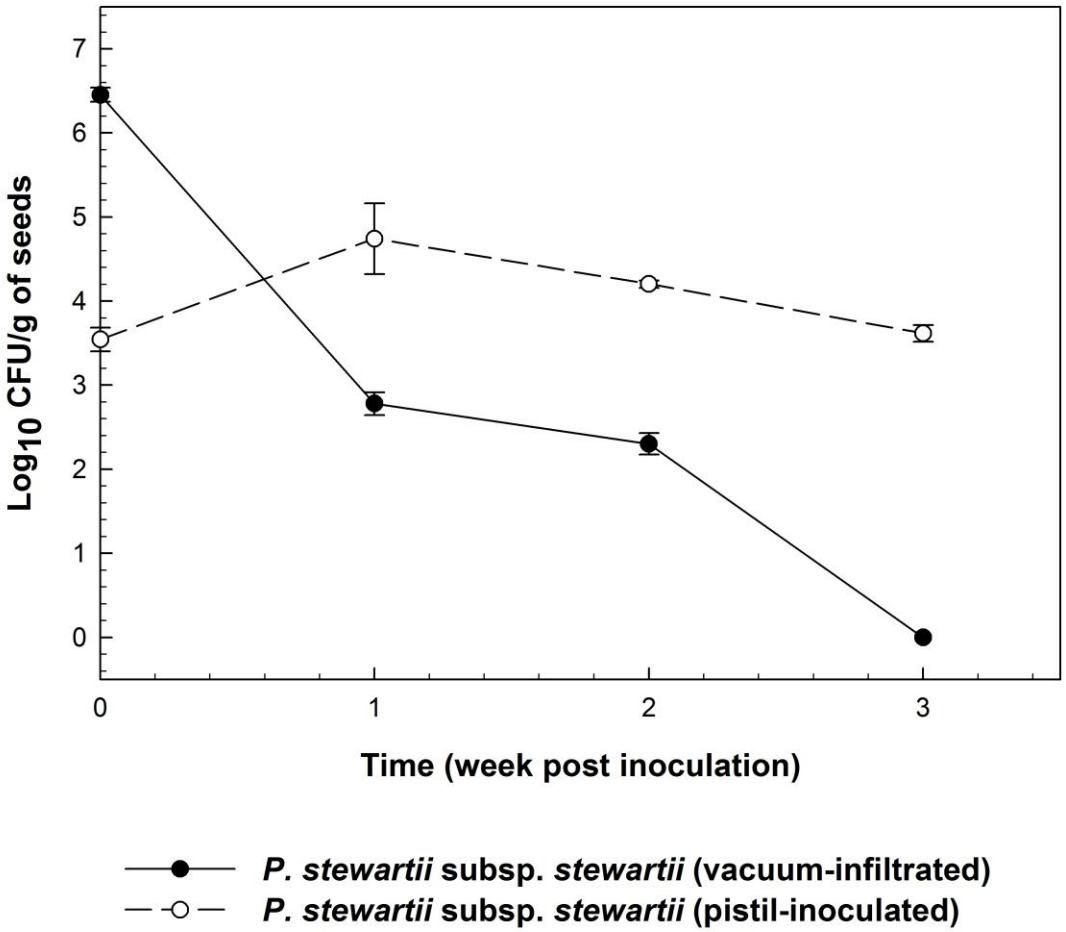

Reduction in percentage of infested seed (%)
100
80
60
40
20
0
A
B
a
b
Peroxyacetic acid
Chlorine-gas
Types of seed treatment
Pistil-inoculated seedlot
Pericarp-inoculated seedlot

Figure 4.5. Survival of *Pantoea stewartii* subsp. *stewartii* in vacuum and pistil-inoculated watermelon seeds. Vacuum inoculated seeds were inoculated with 10^6CFU/g of tested bacteria and dried to their original weight. Pistil-inoculated seeds were extracted from fruits from blossoms whose stigmas were inoculated with bacterial suspension of 10^6 CFU/blossom. Seed sample (30 g) from both vacuum- and pistil-inoculated lot was stored at 4^o C and 50% R.H. Each data point represents mean Log_{10}CFU/g of seed estimated by dilution plating of 1 g of seeds at each time point on semi-selective medium. Bars represent standard errors of the means for three replicates and the experiment was repeated twice (n = 6).


Log10 CFU/g of seeds
0
1
2
3
4
5
6
7
0
1
2
3
Time (week post inoculation)
P. stewartii subsp. stewartii (vacuum-infiltrated)
P. stewartii subsp. stewartii (pistil-inoculated)

CHAPTER 5

EFFECT OF *ACIDOVORAX CITRULLI* LOCATION IN WATERMELON SEEDS ON DETECTION BY SEED HEALTH TESTING

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Abstract

This study investigated the influence of the location of *Acidovorax citrulli* in watermelon seeds on pathogen extraction and detection. In two independent experiments, fifty infested watermelon seedlots were generated by inoculating watermelon stigmas (pistil inoculation) or ovary pericarps (pericarp inoculation) with 1×10^8 *A. citrulli* CFU/mL. *Acidovorax citrulli* cells were extracted from watermelon seed samples (n = 120 seeds/lot/inoculation method) by maceration or washing with 0.1M phosphate buffered saline. Total microbial genomic DNA was purified from samples after each extraction technique and subjected to real-time PCR with *A. citrulli*-specific primers. To confirm real-time PCR assay results, bacterial isolations were attempted from seed macerates and seed washes. Seed washing facilitated detection of *A. citrulli* from 80.0% and 82.5 % of pericarp-inoculated seedlots by PCR and plating, respectively as compared to 12.0% and 14.0% for pistil-inoculated seedlots. In contrast, seed maceration facilitated the detection of *A. citrulli* from ≥94% of the seedlots regardless of seed inoculation method. Based on a seedling grow-out assay, 100% of the seedlots were infested with *A. citrulli* as they transmitted BFB to resulting seedlings. Additionally, bacterial populations from individual pericarp-and pistil inoculated lots were enumerated from seed macerates and seed washes by both quantitative –real time PCR and plating on semi-selective medium. The frequency distribution of *A. citrulli* populations ($\log_{10}$ CFU/g) in seed macerate and seed wash was not significantly different for pericarp inoculated lots (P = 0.07) whereas for pistil-inoculated lots, the difference between frequency distributions was significant (P = 0.0001). These results indicate that the ability to extract *A. citrulli* from watermelon

seeds was influenced by the location of the pathogen. Additionally, seed maceration is more effective for extracting *A. citrulli* from seeds regardless of the type of seed infection.

Introduction

Bacterial fruit blotch (BFB) of cucurbits, caused by the Gram negative seedborne bacterium, *Acidovorax citrulli* (=*A. avenae* subsp. *citrulli* formerly *Pseudomonas pseudoalcaligenes* subsp. *citrulli*) (15,17,21) is a serious threat to watermelon production worldwide (16,18). Infested seeds are the most important primary source of *A. citrulli* inoculum (8, 14) and once the bacterium is introduced into transplant houses, high plant populations, overhead irrigation, and hot and humid conditions increase the risk of BFB development (16,18). Under such favorable environmental conditions, BFB can cause 100% yield loss (1,10,16,18).

Because of the high risk of BFB outbreaks in transplant houses, strategies that seek to exclude the pathogen are most effective for disease management (5,16,18). These primarily involve seed health testing and seed treatments (5,8); however, neither approach guarantees pathogen-free seeds (18). Currently, the most widely employed seed health test for *A. citrulli* is the seedling grow-out assay (SGO) with seed sample sizes ranging from 10,000 to 30,000 seeds/lot (5,18). While it is technically simple, SGO is expensive to conduct because it requires large areas of greenhouse space and trained technicians to recognize BFB symptoms. To provide more cost-effective and efficient seed health assays, several molecular-based techniques such as direct polymerase chain

reaction (PCR), immunomagnetic separation and PCR, Bio-PCR, and magnetic capture hybridization and PCR (MCH-PCR) (6,19,22) have been explored. While they have great potential, a major shortfall of all of these techniques has been their robustness/repeatability, which is heavily dependent on the efficiency of *A. citrulli* extraction from seeds.

Previously, we demonstrated that the location of *A. citrulli* in watermelon seeds varies depending on the method of seed inoculation (2). More specifically, inoculation of the pistils of female watermelon blossoms resulted in bacterial deposition in the embryo of seeds while inoculation of the fruit pericarp resulted in bacterial cell deposition under the seed coat. Based on this observation, we hypothesized that location of *A. citrulli* in watermelon seed affect the efficiency of bacterial extraction, which in turn affects pathogen detection. Hence, the objective of this work was to determine the effect of *A. citrulli* location in watermelon seeds on pathogen extraction by maceration and washing.

Materials and methods

Inoculum preparation. *Acidovorax citrulli* strain AAC00-1, which was isolated from a natural BFB outbreak in watermelon in Georgia in 1992 was used in this study (20). The bacterium was routinely grown on King's medium B (KMB) (11) or nutrient agar (NA) (Beckton-Dickinson, Sparks, MD) for 48h at 28°C. To prepare inoculum, a single AAC00-1 colony from 48h KMB or NA cultures was transferred to nutrient broth and incubated overnight at 28°C on a rotary shaker (Innova; New Brunswick Scientific Co., Edison, NJ) at 250 rpm. After overnight incubation, the cell culture was centrifuged at

5000 × g for 5 min (Allegra 25R, Beckman Coulter, Fullerton, CA), the supernatant was decanted, and the pellet was resuspended in 1 mL of 0.1M phosphate buffered saline (PBS). Bacterial concentration was determined using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY) (optical density at 600 nm = 0.3; ~1 $\times 10^8$ CFU/mL) and adjusted to the desired final concentration of ~ 1 × 10^6 CFU/mL with sterile PBS.

Generation of *A. citrulli*-infested watermelon seedlots. Twenty-five watermelon seedlots were inoculated with *A. citrulli* by pistil and pericarp inoculation under greenhouse conditions as previously described (4, 13, 20). For pericarp-inoculation, 25 watermelon plants (cv. Crimson Sweet) were grown in 15-L plastic pots under greenhouse conditions at 24^{o}C and 65% RH with 12-14h of natural sunlight. At anthesis, 60-70 female blossoms were hand-pollinated with pollen from adjacent male blossoms and simultaneously ovaries were inoculated by gently rubbing their surfaces with a cotton swab saturated with a cell suspension of 1 × 10^6 AAC00-1 CFU/mL. Blossoms were incubated at 100% RH in plastic bags for 24h and fruits were allowed to develop for 30 d. Fruits were harvested and stored at 4^{o}C until seed extraction. Fruits were surface-sterilized by wiping with either 70% ethanol or 0.5% NaOCl followed by manual seed extraction. Seeds were then air-dried at 25^{o}C on paper towels and seeds from each fruit were maintained as a separate lot in paper bags at 5^{o}C until used.

Pistil-inoculated watermelon seedlots were generated as previously described (13,20). Briefly, 25 watermelon plants (cv. Crimson Sweet) were grown in 15-L plastic pots under greenhouse conditions (described above), and at anthesis, female blossoms were hand-pollinated. Immediately after pollination 10 µL of a suspension containing ~ 1

$\times 10^8$ AAC00-1 CFU/mL (= 1×10^6 AAC00-1 CFU/blossom) were inoculated onto the stigmas of female blossoms. Five blossoms/plant were inoculated, tagged, and allowed to develop for 30 d. Fruits were harvested and seeds were extracted as described above. Seeds harvested from fruits (ten/inoculation type) generated from blossoms inoculated with 0.1M PBS using each inoculation technique served as negative controls.

Confirmation of *A. citrulli* localization in seeds. To confirm the location of *A. citrulli* cells in pericarp- and pistil-inoculated seeds, samples (*n* = 10 seeds/lot) from five lots generated by each method were dissected into testa, perisperm endosperm layer (PE) layer and embryo as described previously (2). Each section was macerated in separate microcentrifuge tubes in 1 ml PBS, and 100 µL aliquots of macerate was spread onto Nunhem's medium (Nunhems Seed Company, Helen, Netherlands). This experiment was repeated twice and the mean percentage of *A. citrulli*-positive seed sections for seed inoculated by each method was determined.

***Acidovorax citrulli* detection in watermelon seeds.** Each watermelon seedlot was divided into three sub-lots of equal weight (5 g = ~ 120 seeds) and tested for *A. citrulli* by seed washing followed by plating/PCR; seed maceration followed by plating/PCR and SGO as follows.

***Acidovorax citrulli* extraction by seed washing.** To extract *A. citrulli* by washing, seeds (*n* = 120 seeds/lot/inoculation method) were placed in a 50 mL polypropylene screw cap tube (Corning Inc., Corning, NY) containing 10 mL PBS and shaken at 250 rpm (New Brunswick Sc. Co., New Brunswick, NJ) for 60 min at ~ 25^o C. Five milliliters

of seed wash was transferred using a micropipette to a 50 mL polypropylene screw cap tube and centrifuged at 8000 × g (Allegra™ 25R centrifuge, Beckman Coulter Inc., Brea, CA) for 5 min. The supernatant was decanted and the pellet was resuspended in 1 mL PBS by vigorous agitation (Fisher Vortex Genie2; Fisher Scientific Pittsburgh, PA) at 100 rpm for 5 sec. The seed extract was divided into two fractions: one 900 µL and the other 100 µL, and DNA was extracted from 900 µL aliquot using an UltraClean™ Microbial DNA Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. DNA (10 to 15 ng/µL) was subjected to real-time PCR using *A. citrulli*-specific primers as previously described (6). Samples of seed extracts from ten negative control lots were also processed as described above. In order to test for PCR inhibition in samples that yielded negative results, 5 ng/µL of *A. citrulli* genomic DNA was added to seed extracts from five negative control lots followed by real-time PCR as described above (6). The 100 µL aliquot of seed extract was used to isolate *A. citrulli* CFU by ten-fold serial dilutions in PBS followed by spread plating of 100 µL aliquots onto Nunhem's semi-selective agar. Plates were incubated for 5 days at 28°C and *A. citrulli* colonies (round, red colonies with smooth margins) were enumerated. Seed extracts from ten negative control lots (n =120 seeds) of each inoculation method were also plated as described above. The mean percentages of *A. citrulli*-positive lots as determined by seed washing for seeds generated by the two inoculation methods were recorded.

***Acidovorax citrulli* extraction by seed maceration.** Seeds (*n* = 120seeds/lot/inoculation method) from the second sublot were placed in an extraction bag (Bioreba, Switzerland) and macerated using a semi-automated Homex 6 homogenizer (Bioreba). Seed macerate was suspended in 10 mL PBS and transferred to 50 mL polypropylene screw cap tubes (Corning Inc). Macerates were agitated vigorously at 100 rpm (Fisher Vortex Genie2; Fisher Scientific International) for 30 sec and centrifuged at 8000 × g (Allegra^{TM} 25R centrifuge, Beckman Coulter Inc.) for 5 min. Subsequently, the supernatant was decanted and the pellet was suspended in 1 mL PBS. As described above, 900 µL of seed extract was used for DNA purification and the real-time PCR assay using *A. citrulli*-specific primers and the remaining 100 µL was ten-fold serially diluted in PBS and spread (100 µL/diluent) onto Nunhem's agar. Samples (*n* = 120 seeds) from ten negative control lots of each inoculation method were processed as described above. The mean percentages of *A. citrulli*-positive lots as determined by seed maceration for seeds generated by the two inoculation methods were recorded.

Seedling grow-out assay. The third seed sub-sample (*n* = 120 seeds/lot/inoculation method) was tested for *A. citrulli* by a modified seedling grow out as follows. Each seed was placed in a separate capped 10 ml test tube containing cheese cloth (Lina International Inc., Jamaica, NY) saturated with sterile deionized distilled water. Seeds were incubated for 14 d at 28^{o}C and 80% RH with continuous fluorescent light. Seed germination and BFB seedling transmission percentages (number of seedlings with BFB symptoms divided by the total number of germinated seeds × 100) were recorded for each lot 18 d after planting. Seed samples (*n* = 120 seeds) from ten negative control lots of each

inoculation method were evaluated by seedling grow-out as described above. To confirm that putative BFB seedling symptoms were caused by *A. citrulli*, the bacterium was recovered from at least three symptomatic seedlings from each sublot. Small (~4 mm^2) pieces of symptomatic cotyledon tissue were macerated in 200 µL of sterile PBS and ~20 µL aliquots of macerate were streaked onto Nunhem's medium and incubated for 5 d at 28^oC. *Acidovorax citrulli* colonies were tested by real-time PCR assay using an *A. citrulli*-specific TaqMan assay as previously described (6). Seedlots showing at least one symptomatic seedling were considered positive and the percentage of positive lots was recorded.

Data analysis. The proportions *A. citrulli*-infested seedlots as determined by PCR assay and plating across all treatments were analyzed using chi-square test in SAS (version 9.2 for Windows; SAS Institute Inc., Cary, NC). Chi-square statistics for stratified data [2 (seed inoculation method) × 2 (pathogen extraction technique) × 2 (detection outcome by PCR/plating)] were calculated. For chi-square test, the total number of positive and negative detections by each extraction method was calculated. Additionally, the odds ratio, an estimate of strength of association between two binary data, was also calculated. Odds ratio for positive detection was calculated separately for the two seed inoculation methods as the number of lots that were positive by maceration × number of lots that were negative by washing / number of lots that were positive by washing × number of lots that were negative by maceration. Overall association between the explanatory (seed health asay) and response variables (detection of *A. citrulli*-positive lots) was determined by Mantel-Haenszel and Breslow-Day tests in SAS. Additionally, a paired t-test was

conducted to determine if *A. citrulli* populations detected by quantitative real time PCR assay and plating were significantly different at P = 0.05. *Acidovorax citrulli* populations recovered from individual lots by washing and maceration were compared using frequency distribution. Kolmogorov–Smirnov (KS) test in SAS NPAR1WAY procedure was used to compare the frequency distribution of *A. citrulli* populations recovered from washed and macerated seeds from pericarp- and pistil-inoculated lots.

Results

Confirmation of the location of *A. citrulli* in seeds after pericarp-and pistil-inoculation. For pericarp-inoculated seedlots, the mean percentages of *A. citrulli*-positive testa and embryo samples were 10.0 % and 12.0%, respectively. For similar seedlots, *A. citrulli* was detected in 83.0% of the PE layer samples. In contrast, for pistil-inoculated lots, *A. citrulli* was recovered in 13.0% and 73.0% of the testa and PE layer samples, respectively. For the same lots, *A. citrulli* was detected in 77.0% of the embryo samples.

Detection of *A. citrulli*. As expected, *A. citrulli* was not detected in seed wash or seed macerates from negative control watermelon lots. However, based on the SGO, 100% of the seedlots were infested with the pathogen. The BFB seedling transmission percentages observed for pericarp-and pistil-inoculated lots ranged from 6.4 to 79.6% and 6.0 to 74.2%, respectively (data not shown). Seedlings displaying putative BFB symptoms were confirmed by *A. citrulli* isolation followed by real-time PCR assay (data not shown).

Acidovorax citrulli was detected in the seed wash of 80.0% and 82.5% of pericarp-inoculated lots by PCR and plating (Fig. 5.1A and B), respectively. In contrast, *A. citrulli*

was detected in 14 and 12% of seed wash of pistil-inoculated lots by PCR and plating, respectively. When seed samples from the pericarp- and pistil-inoculated lots were macerated, *A. citrulli* was detected in 94.0 and 100% of the lots, respectively by PCR and plating. Detection of *A. citrulli* by PCR and plating across all treatments (inoculation method and extraction technique) was not significant ($P \geq 0.715$) by the chi-square test; hence, PCR data were used for further statistical analyses. For pericarp-inoculated seeds, the odds ratio of a positive detection from seed macerate to seed wash, calculated from the contingency table, was 3.92 (95% confidence interval: 1.008 to 15.211), indicating 3.92 times better detection from seed macerate than from seed wash for pericarp-inoculated lots. For pistil-inoculated seeds, the corresponding odds ratio was 307.1 (95% CI: 36.33 to 2596.4). The odds ratio was significantly greater for pistil-inoculated lots than for pericarp-inoculated lots according to the Breslow-Day test for homogeneity ($P < 0.0001$), indicating that the ability to extract *A. citrulli* cells from watermelon seeds inoculated by pistil and pericarp methods differed for washing and maceration techniques.

The paired t-test showed no significant difference in *A. citrulli* quantification ($\log_{10}$CFU/g) between plating and the PCR assay ($P = 0.898$). Hence, *A. citrulli* cells recovered by plating were used for further statistical analysis. There was no significant difference ($P=0.90$) between the mean numbers of *A. citrulli* CFU recovered from pericarp-inoculated seeds by washing (3.62×10^8 CFU/g) and maceration (3.22×10^8 CFU/g). However, washing yielded 100-fold less *A. citrulli* CFU (8.2×10^3 CFU/g) than maceration (3.4×10^5 CFU/g) for pistil-inoculated seeds. The frequency distributions of

A. citrulli populations recovered by washing and maceration of individual lots were not significantly different for pericarp-inoculated seeds ($P = 0.07$) (Fig. 5.2 A and B), but for pistil-inoculated seeds, the difference was significant ($P<0.001$) (Fig. 5.2 C and D) according to the Kolmogorov-Smirnov test.

Discussion

Development of cost-effective and sensitive detection assays is critical for seed-health testing and management of seedborne diseases. However, a major limiting factor is that pathogen detection sensitivity is highly dependent on pathogen extraction efficiency. While pathogen extraction efficiency from seeds has been shown to be influenced by factors like duration and temperature of extraction, formulation of extraction buffer, and extraction technique (e.g., soaking, washing, maceration or vacuum extraction) (5), little attention has been given to the effect of pathogen location within seeds on extraction efficiency.

Results from the current study showed that the ability to extract *A. citrulli* depends on the location of the pathogen in watermelon seeds. *Acidovorax citrulli* recovery by washing was less effective for pistil-inoculated seeds than for pericarp-inoculated seeds. In contrast, maceration yielded a higher percentage of *A. citrulli*-positive lots (≥94% for both) for seeds generated by both pistil- and pericarp-inoculation. Maceration also resulted in 3.92 fold greater detection of *A. citrulli* from pericarp-inoculated seeds than washing. Even more dramatic results were observed with seed maceration of pistil-inoculated seeds. Maceration improved the detection of *A. citrulli* in pistil inoculated lots

by 301 times relative to seed washing. These results suggest that maceration was more effective in extracting *A. citrulli* from infested seedlots, regardless of pathogen location. However, washing was effective in extracting *A. citrulli* from pericarp inoculated seedlots. This is likely due to the fact that most of the *A. citrulli* inoculum in pericarp-inoculated seed resides under the testa, on the surface of the PE layer.

Observations from this study were supported by previous studies that showed improved yield of phytobacterial cells by seed maceration (3, 7). For example, Fatmi and Schaad (3) reported higher recovery of *Clavibacter michiganensis* subsp. *michganensis* (causal agent of bacterial canker of tomato) cells by seed maceration than by washing. In a similar study, Hadas *et al.* (7) reported that tomato seed maceration alone or in combination with seed soaking improved detection of *C. michiganensis* subsp. *michiganensis*.

The differential localization of *A. citrulli* in watermelon seeds did not affect BFB seed-to-seedling transmission. Regardless of the seed inoculation technique, 100% of the lots transmitted BFB to seedlings in seedling grow out assays. However, BFB seedling transmission percentages for individual seedlots varied from 6.4 to 79.6% and 6 to 74.2% for pericarp- and pistil-inoculated lots, respectively. While this observation suggests that the SGO is reliable for seed health testing, the high degree of variability in transmission percentages/lot suggests otherwise. In fact, the efficacy of SGO depends on many factors such as sample size, sampling method, inoculum density, pathogen aggressiveness, host susceptibility, and environmental conditions (5) that can ultimately affect the outcome of

the result. Hence, assays that rely directly on the presence of the pathogen are likely to be more robust.

The recovery of *A. citrulli* CFU from pericarp- and pistil-inoculated seed lots was influenced by the method of bacterial extraction. The mean *A. citrulli* CFUs recovered from pericarp-inoculated lots by washing and maceration did not differ significantly. This could be explained by the presence of *A. citrulli* cells on the surface of the PE layers could be released from seeds efficiently by agitation. In contrast, mean *A. citrulli* CFUs recovered from pistil-inoculated seedlots by washing and maceration were significantly different. Washing failed to extract *A. citrulli* cells from a high proportion (12%) of pistil-inoculated seeds, whereas maceration resulted in bacterial recovery from 100% of the lots. These results clearly indicate that washing was not effective for extracting *A. citrulli* from pistil-inoculated lots and that maceration was critical to release cells for subsequent detection. This observation can be explained by the fact that in pistil-inoculated seedlots, *A. citrulli* inoculum resides in the embryo tissues, where it is trapped under the PE layer, and not easily liberated from seeds by washing.

Findings from the current study suggest that for accurate seed health testing, watermelon seeds must be macerated to liberate *A. citrulli* from pistil-inoculated seedlots. We are fully aware of the technical and logistical challenges associated with processing large watermelon seed samples by maceration i.e., difficulty in extracting DNA from seed flour and release of PCR inhibitory compounds. However, since the consequences of false-negative results in *A. citrulli* seed heath testing can be significant, sampling

schemes must be developed to facilitate the commercial application of this approach to enhance detection and BFB management by pathogen exclusion.

Acknowledgements

We thank Dr. Harald Scherm and Dr. Rock Christiano for their assistance with statistical analyses.

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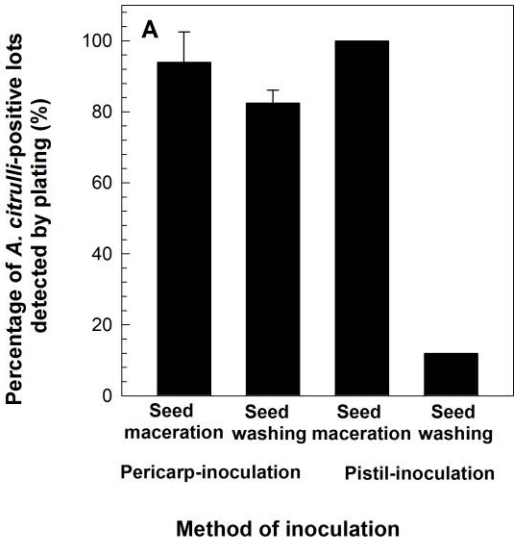
Figure 5.1. Percentage of *Acidovorax citrulli*-infested watermelon seed samples from pericarp- and pistil-inoculated lots as determined by washing and maceration followed by A) plating and B) real-time PCR assay using a *A. citrulli*-specific TaqMan assay. Seed samples (*n* = 120 seeds/lot/inoculation method) were tested for *A. citrulli* by seed washing for 60 min in 0.1 M phosphate buffered saline or seed maceration followed by plating/PCR. Error bars represent standard error of the means of 25 seedlots generated by pistil and pericarp- inoculation in two independent experiments under greenhouse conditions.


A
Percentage of A. citrulli-positive lots detected by plating (%)
100
80
60
40
20
0
Seed maceration
Seed washing
Seed maceration
Seed washing
Pericarp-inoculation
Pistil-inoculation
Method of inoculation

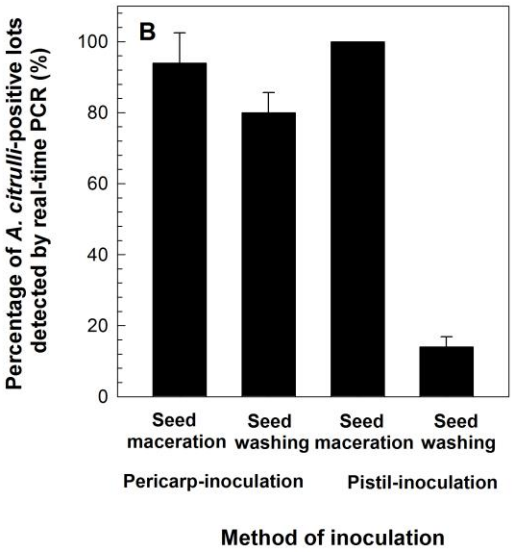
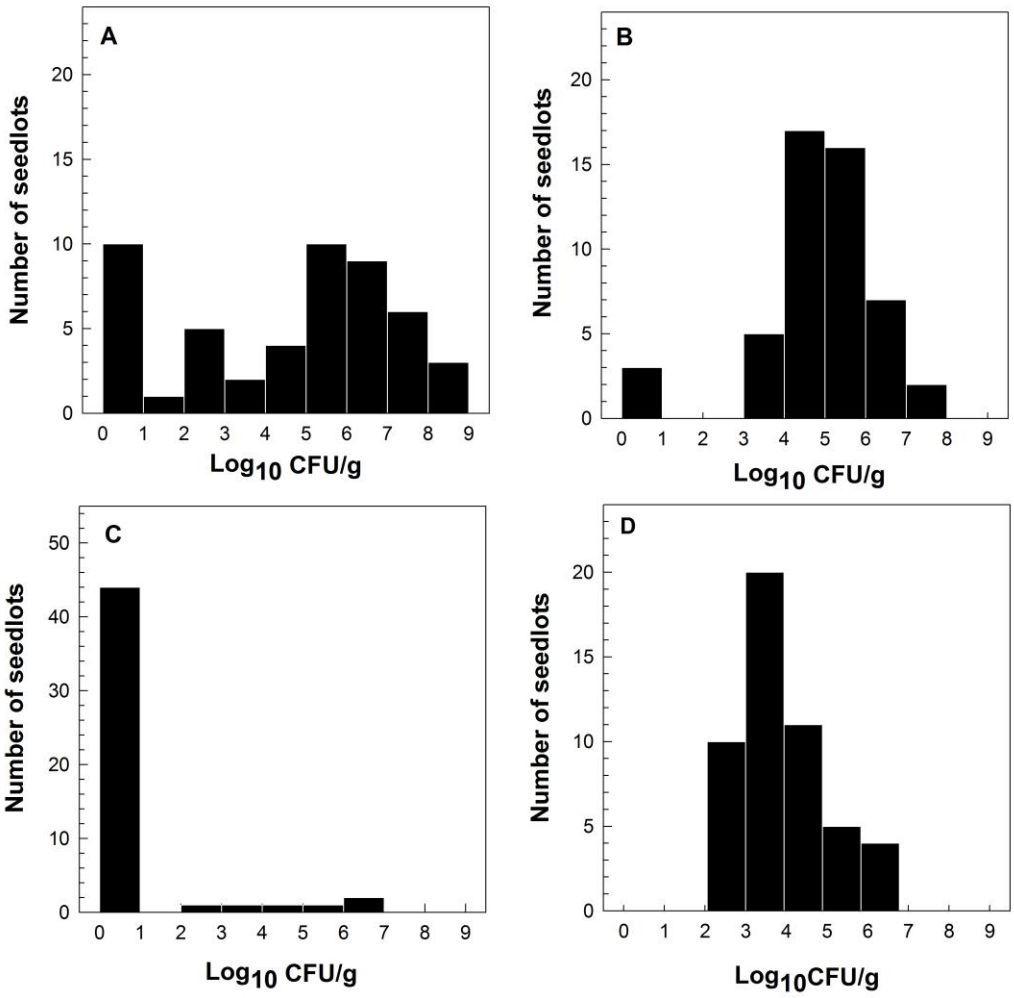

B
Percentage of A. citrulli-positive lots detected by real-time PCR (%)
100
80
60
40
20
0
Seed maceration
Seed washing
Seed maceration
Seed washing
Pericarp-inoculation
Pistil-inoculation
Method of inoculation

Figure 5.2. Frequency distributions of *A. citrulli* populations (Log_{10} CFU/g) by plating from A) washing of pericarp-inoculated watermelon seeds; B) maceration of pericarp-inoculated watermelon seeds; C) washing of pistil-inoculated watermelon seeds and D) maceration of pistil-inoculated watermelon seeds. Seed samples (*n* = 120 seeds/ lot/inoculation method) were washed in 0.1 M PBS for 60 min or macerated followed by ten-fold serial dilutions in 1 mL phosphate buffered saline and spread-plating of 100 µL aliquots onto Nunhem's agar. Plates were incubated for 5d at 28^{o}C, and *A. citrulli* colonies (round, red colonies with smooth margins) were enumerated. Bars represent the numbers of seedlots with varying levels of *A. citrulli* populations (Log_{10} CFU/g) recovered from 50 pericarp- and pistil-inoculated lots by washing and maceration.


A
B
C
D
Number of seedlots
Log10 CFU/g
Log10CFU/g

CHAPTER 6

EFFECT OF *ACIDOVORAX CITRULLI* ON XYLOSE EXUDATION FROM GERMINATING WATERMELON SEEDS

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Abstract

In this study, the effect of *A. citrulli* on exudation of sugars from watermelon seeds during germination was investigated. Metabolic analysis of seed exudates from infested and uninfested seeds at 0, 3, 5, and 7 days after planting (DAP) was performed using gas chromatography-electrospray-mass spectrometry. Apart from xylose, the concentrations of galactose, arabinose, mannose and rhamnose decreased consistently during the first 7 DAP for both noninfested and *A. citrulli*-infested seeds. By 7 DAP, significantly higher concentrations of xylose were detected in the exudates of uninfested seeds than in infested seeds. Furthermore, when *A. citrulli*-infested seeds were disinfested with chlorine gas, xylose concentration in seed exudates increased significantly. These data suggest that *A. citrulli* may decrease the release of xylose from watermelon seeds during germination.

Introduction

Bacterial fruit blotch (BFB) is an economically important seedborne disease of cucurbits caused by the Gram-negative bacterium, *Acidovorax citrulli* (=*A. avenae subsp. citrulli*, formerly *Pseudomonas pseudoalcaligenes* subsp. *citrulli*) (18, 20, 24). Seeds are the most important primary source of inoculum for *A. citrulli* (10, 15) and under transplant house conditions conditions; they can initiate severe BFB outbreaks (22). Hence, BFB management relies heavily on pathogen exclusion by seed health testing and seed treatments with antimicrobial compounds (7, 11, 22). However, at present, neither of these strategies can ensure pathogen free seeds. To limit the impact of seedborne *A.*

citrulli inoculum, a better understanding of host-pathogen interactions is needed during the early stages of seed germination.

Seed colonization by phytobacteria is a critical first step in successful seedling disease transmission. For seed colonization, bacteria utilize nutrients in seed exudates released during germination (14). These seed exudates are rich in simple sugars, amino acids, organic acids, fatty acids and lipids (1, 8, 14 16). Identifying the sugars exuded from watermelon seeds during germination may give insight into the factors that influence BFB seed-to-seedling transmission Hence, the goal of this investigation was to determine the effect of seedborne *A. citrulli* on the release of metabolites from germinating watermelon seeds.

Materials and methods

Bacterial strains and inoculum preparation. *Acidovorax citrulli* strain AAC00-1 was used in this study. This strain was routinely grown on King's medium B (12) or nutrient agar (Becton-Dickinson, Sparks, MD) for 48h at 28^{o} C. To prepare inoculum, nutrient broth was inoculated with a single colony of the bacterial strain from a 48h agar culture and incubated overnight at 30°C on a rotary shaker (Innova; New Brunswick Scientific Co., Edison, NJ) at 250 rpm. The culture was centrifuged at 6000 × *g* (Allegra^{TM} 25R, Beckman Coulter, Fullerton, CA) for 5 min and the supernatant was decanted. The pellet was resuspended in 3 mL of 0.1 M phosphate-buffered saline solution (PBS) and the bacterial concentration was adjusted to an optical density of 0.3 at 600 nm (≈10^{8} CFU/

mL) using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY) and diluted in PBS to a final concentration of 10^6 CFU/mL.

Generation of infested watermelon seedlots. Two *A. citrulli*-infested seedlots (one each by pericarp- and pistil-inoculation) were generated for this study to determine the effect of location of bacterial cells on seed exudates. Pericarp-inoculated lots were generated by inoculating watermelon fruits with AAC00-1 under greenhouse conditions as previously described (chapter 2). Pistil-inoculated lots were generated by pollinating female watermelon blossoms and simultaneously inoculating the stigmas with AAC00-1 as described previously (23). Five blossoms were inoculated per plant, tagged, and allowed to develop for 30 days after inoculation. Forty eight fruits were harvested at maturity and seeds were extracted and pooled to form a seedlots as described above. Seeds from fruits whose stigmas were inoculated with 0.1M PBS served as a negative control lot.

Seedlot characteristics. To determine *A. citrulli* infestation (%) for each seedlot, four replicates of samples (n = 100 seeds/lot) were planted on blotter paper (Anchor Paper Co., St. Paul, MN) saturated with sterile water in transparent plastic boxes [6 cm (H) ×24 cm (W) × 33.5 cm (L); Tri-State Plastics, Dixon, KY, USA] and maintained at 30°C and 85% RH with continuous fluorescent light for 14 d. Samples (n = 100 seeds) from fruits pistil-inoculated with PBS- were used as negative controls. Seed germination and BFB seedling transmission percentages were recorded at 14 d after planting (DAP).

Effect of *A. citrulli* on exudation of monosaccharides from germinating watermelon seeds. Identification and quantification of monosaccharides from the exudates of germinated watermelon seeds were performed using gas chromatography-electron impact-mass spectrometry (GC-EI-MS). Seed exudates were collected from three replicated samples (n = 0.5 g ~15 seeds/lot) from pericarp- and pistil-inoculated lots and analyzed as described below. Seed exudates from negative control lots were also analyzed and the experiment was repeated twice.

Collection of seed exudates: Seed exudates were collected from watermelon seeds as described previously (9). Briefly, seed samples (n = 0.5 g ~15 seeds/lot) were placed in 50 ml polypropylene screw cap tubes (Corning Inc., Corning, NY) with 10 mL of sterile distilled water. Seeds were incubated at room temperature (~25^{o}C) for 0, 3, 5, and 7 days in a rotary shaker (Roch Mechatronics Inc., Red Deer, Alberta, Canada) at 100 rpm. Five milliliters of seed exudate were collected from each treatment at each sampling time and centrifuged at 1000 × g for 2 min to precipitate debris. The supernatant was decanted, transferred to a sterile 50 mL polypropylene screw cap tube and immediately lyophilized (VirTris, Gardener, NY) to prevent sugar degradation. After lyophilization, 3 mg samples were used for further analysis.

Analysis of neutral sugar residues: Carbohydrates include neutral sugars, amino sugars, sugar alcohols, and sugar acids. Of these constituents, neutral sugars are the major components in the dissolved total saccharide pool and therefore have received the most attention in analyses of seed exudate samples. The neutral monosaccharide composition in seed exudates was determined as an alditol acetate derivative by gas chromatography

as previously described (5). This procedure involved hydrolysis of polysaccharides to their constituent monosaccharides with 2 M trifluoroacetic acid (TFA) at 121°C for 2h and then reducing the neutral monosaccharides with sodium boroduetero hydride to the their corresponding alditols. Alditols were acetylated using 5 M acetic anhydride to alditol acetate and identified and quantified by gas chromatography and mass spectrometry (25). Repeated measure analysis was used to determine the effect of *A. citrulli* on monosaccharide composition at $P<0.05$ in SAS over 0, 3, 5, and 7 DAP (version 9.2 for Windows; SAS Institute Inc., Cary, NC).

GC-EI-MS analysis: Derivatized monosaccharides were analyzed with a Hewlett-Packard chromatograph (5890) coupled to a mass spectrometer (American Laboratory Trading, East Lyme, CT). One microliter samples of derivatized monosaccharides were introduced via the splitless injection mode onto an SP 2330 column (30 m × 0.25 mm, 0.25 µm film thickness, Supelco) using helium as the carrier gas. The alditol acetate and partially methylated alditol acetate derivatives were separated using the following temperature gradient: 80^{o}C for 2 min, 80–170 ^{o}C at 30^{o}C/min, 170–240^{o}C at 4^{o}C/min, and 240^{o}C held for 20 min. Samples were ionized by electron impact at 70 eV.

Effect of *A. citrulli* disinfestation on the temporal release of xylose from germinating watermelon seeds. To determine if *A. citrulli* affected the release of xylose from germinating watermelon seeds, pericarp- and pistil-inoculated seeds were disinfested using a chlorine gas treatment as previously described (21). Briefly, seeds (n = 20g (~450 seeds)/lot) were treated with chlorine gas in an enclosed glass chamber (50 cm × 25 cm × 30 cm) fitted with a circulation fan, pH probe, and wet/dry bulb thermometers.

Chlorine gas was produced by mixing equal volumes of commercial bleach (6.0% NaOCl) and 0.6M KH_2PO_4 in a 400 ml mixing vat inside the chamber. The Cl_2 gas concentration was maintained at 750 ppm and seeds were treated for 9 h. Seeds from PBS-inoculated blossoms served as negative controls and were treated with Cl_2 gas as a negative control. Non-treated pericarp- and pistil-inoculated seeds served as positive controls. After treatment, seeds were stored at 4ºC in paper bags until further use. To determine the efficacy of chlorine gas treatment, samples (n = 20 seeds/lot) were macerated in 1 mL of PBS, and 100 µL of macerate was spread onto Nunhem's agar medium. After 6 days of incubation, the percentage of seeds infested with *A. citrulli* was recorded. Similarly, 20 untreated pericarp- and pistil-inoculated seeds were tested for *A. citrulli*, as described above. The percent reduction in *A. citrulli* infested seeds after Cl_2 gas treatment for pericarp- and pistil-inoculated lots was determined as percentage of infested seeds for the positive control lot – percentage of infested seeds after seed treatment)/percentage of seeds for the positive control lot × 100.

Monosaccharide composition analysis was performed using three replicated seed samples (n = 0.5 g (~15 seeds)/lot) from Cl_2 gas-treated seedlots as described above. Mean concentrations of xylose in the seed exudates from all treatments were compared at each time point using repeated measure analysis in SAS (version 9.2 for Windows; SAS Institute Inc., Cary, NC).

Results

Seedlot characteristics. The pericarp-inoculated lot displayed mean seed germination and BFB seedling transmission percentages of 78.0% and 68.0%, respectively. The pistil-inoculated lot displayed mean seed germination and BFB seedling transmission percentages of 72.0% and 75.5%, respectively.

Effect of *A. citrulli* on exudation of monosaccharides from germinating watermelon seeds. Seed exudates from *A. citrulli*-infested and uninfested watermelon seeds contained fucose, arabinose, ribose, rhamnose, xylose, galactose, glucose, and mannose. The concentrations (mole percentage) of arabinose, galactose, xylose, mannose, and rhamnose in seed exudates were quantified because these substrates are utilized by *A. citrulli* (AAC00-1). There were significant differences ($P<0.001$) in arabinose secreted from uninfested and *A. citrulli*-infested seeds. By 7 DAP a significantly higher concentration of arabinose was detected in the exudates from pistil-inoculated seeds (38.9%) than from pericarp-inoculated seeds (30.3%) ($P<0.0001$). There was also a significant difference in the arabinose concentration between the exudates from pericarp-inoculated seeds (30.3%) and uninfested seeds (32.3%) ($P=0.025$). At 1 DAP, the mean concentration of arabinose in pistil-inoculated seed exudates (16.5%) was significantly higher than exudates from pericarp-inoculated (6.9%) or uninfested seeds (8.9%) (Fig. 6.1). By 3 DAP, mean arabinose concentration remained significantly higher in exudates from the pistil-inoculated seed (15.1%) than from pericarp-inoculated (12.2%) and uninfested seeds (5.7%) (Fig. 6.1). However, by 7 DAP, the mean concentration of arabinose in exudates

from pistil-inoculated seed (4.6%) was significantly less than in the exudates from pericarp-inoculated (6.7%) and uninfested seeds (8.4%) (Fig. 6.1).

There were significant differences in mannose and rhamnose in the exudates of *A. citrull*-infested and uninfested seeds ($P<0.001$). At day 0, the concentration of mannose was higher in the exudates from uninfested seeds (31.3%) than those from pistil- (2.4%) and pericarp-inoculated seed (26.4%) ($P<0.001$) (Fig. 6.1). By 7 DAP, the concentration of mannose in exudates from uninfested seeds (1.7%) decreased significantly relative to exudates from pericarp-inoculated seeds (3.4%) (Fig. 6.1). However, there was no significant difference between the mannose concentrations in seed exudates from uninfested (1.7%) and pistil-inoculated seeds (2.1%) ($P=0.541$).

A significantly higher concentration of rhamnose was observed in exudates from pericarp-inoculated seed exudates (8.5%) than in the exudates of pistil-inoculated (0.1%) and uninfested (1.9%) seeds at 0 DAP (Fig. 6.1). However, there was no difference in rhamnose secreted by uninfested seeds and pistil inoculated seeds ($P=0.26$). By 7 DAP, the concentration of rhamnose was significantly less ($P<0.003$) in exudates from pistil-inoculated seed (0.5%) than in the exudates from pericarp-inoculated (9.6%) and uninfested seeds (7.4%) (Fig. 6.1).

Significant differences were observed in galactose concentration in exudates from *A. citrulli*-infested and uninfested seed exudates ($P=0.013$) by 7 DAP. However, the interaction between time and treatment was not significant ($P=0.104$). The concentration of galactose in pericarp-inoculated seed exudates increased from 11.5% at 0 DAP to

40.0% at 3 DAP then decreased to 15.9% by 7 DAP. For pistil-inoculated seeds, the galactose concentration in exudates increased from 3.2% at 0 DAP to 8.0% at 7 DAP. For exudates from uninfested seed the galactose concentration, declined from 18.0% at 0 DAP to 10.2% at 7 DAP, however this decline was not statistically significant (P=0.78).

The difference between xylose concentrations in the exudates from *A. citrulli*-infested and uninfested seeds was statistically significant (P<0.0001). At 0 DAP, significantly higher concentrations of xylose were in exudates from uninfested seed (14.7%) than in exudates from pericarp (6.4%) and pistil-inoculated seeds (3.5%) (Fig. 6.1). By 3 DAP, significantly higher concentrations of xylose were present in exudates from uninfested seed (44.2%) than in exudates from pericarp-inoculated (16.2%) and pistil-inoculated (5.9%) seeds (Fig. 6.1). At 3, 5, and 7 DAP, significantly higher xylose concentrations were present in exudates from pericarp-inoculated seeds than in exudates from pistil-inoculated seed. Throughout the study, the concentration of xylose was significantly higher in the exudates of pericarp-inoculated seeds than in the exudates from pistil-inoculated seeds (Fig. 6.1).

Since the concentration of xylose increased consistently during seed germination and there was a consistent difference in its concentration between *A. citrulli*-infested and uninfested seeds, it was selected as a marker for further analysis.

Effect of *A. citrulli* disinfestation on the concentration of xylose released from germinating watermelon seeds. *Acidovorax citrulli*-infested seeds were completely disinfested using Cl_2 gas treatment as viable bacterial colonies were not recovered from infested seeds after treatment (data not shown). However, *A. citrulli* cells were recovered from 67.5% and 72.5% of untreated pericarp- and pistil-inoculated seeds, respectively. The effect of *A. citrulli* on the xylose concentration in watermelon seed exudates by 7 DAP was significant ($P<0.001$). By 7 DAP, mean xylose concentrations in the exudates from untreated pericarp-inoculated seeds (22.9%) was significantly higher than in the exudates from untreated pistil-inoculated seeds (15.4%) ($P<0.001$) (Fig. 6.2). In contrast, during the same period, there was no significant difference between the xylose concentration in the exudates of treated pericarp-(28.5%) and pistil-inoculated seeds (34.7%) ($P=0.193$) (Fig. 6.2). Overall, significantly higher concentrations of xylose were observed in the exudates of *A. citrulli*-infested watermelon seeds after decontamination with chlorine gas ($P<0.001$).

Discussion

The composition of seed exudates during germination can influence the metabolic activity and population dynamics of seed-borne pathogens (14). While the effect of sugars in seed exudates on microbial colonization has been studied extensively (9, 16, 17), limited studies have been conducted on the effect of phytobacterial seed infection on seed exudates. In the current study, we observed that galactose, rhamnose, arabinose and mannose decreased rapidly within 7 DAP in exudates from uninfested and *A. citrulli*-infested watermelon seeds. Hence, we speculated that these sugars were utilized by the

germinating seedling during the germination process. In contrast, the concentration of xylose in seed exudates of uninfested and *A. citrulli*-infested seeds increased consistently during the same period. Although, a constant increase in the concentration of xylose was observed for *A. citrulli* infested seeds, overall exudation of xylose was significantly reduced for infested seeds as compared to uninfested seeds. It is possible that xylose secreted from germinating seeds could be utilized to support the growth of *A. citrulli* populations.

To confirm the trends in xylose secretion from watermelon seeds during germination, *A. citrulli*-infested seeds were disinfested using chlorine gas and their xylose profiles were compared to those of uninfested seeds. Chlorine treatment disinfested the *A. citrulli*-infested seeds as expected and did not significantly affect the xylose release profiles of treated seeds (P=0.184). By 7 DAP, xylose concentration was significantly higher for decontaminated *A. citrulli*-infested seeds than for *A. citrulli*-infested seeds suggesting that *A. citrulli* seed infection may influence the release of xylose. The xylose concentration in the exudates of decontaminated seeds was lower than that of uninfested seeds, suggesting the possibility of incomplete decontamination of *A. citrulli*-infested seeds or inability of plating assay to detect bacterial populations below detection threshold. However, the exact reason for this observation was unclear.

This is the first report on the effect of *A. citrulli* on the temporal release of xylose in germinating watermelon seed exudates. Since there is a strong relationship between seed exudate production and spermosphere microfloral growth (9, 14), it would be informative to explore underlying biochemical processes that govern these interactions as they may

provide greater insight into the biology *A. citrulli* seed colonization and BFB seedling transmission.

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Figure 6.1. Quantification of monosaccharides A) xylose, B) galactose, C) rhamnose, D) arabinose and E) mannose in exudates of uninfested and *A. citrulli*-infested seeds during 7 days after planting by gas chromatography-electron impact-mass spectrometry. Seeds (n = 0.5 g) were incubated in deionized water and exudates (5 mL) were collected at 0, 3, 5, and 7 days after planting followed by freeze drying. Each data point represents the mean of three replicates in two independent experiments.

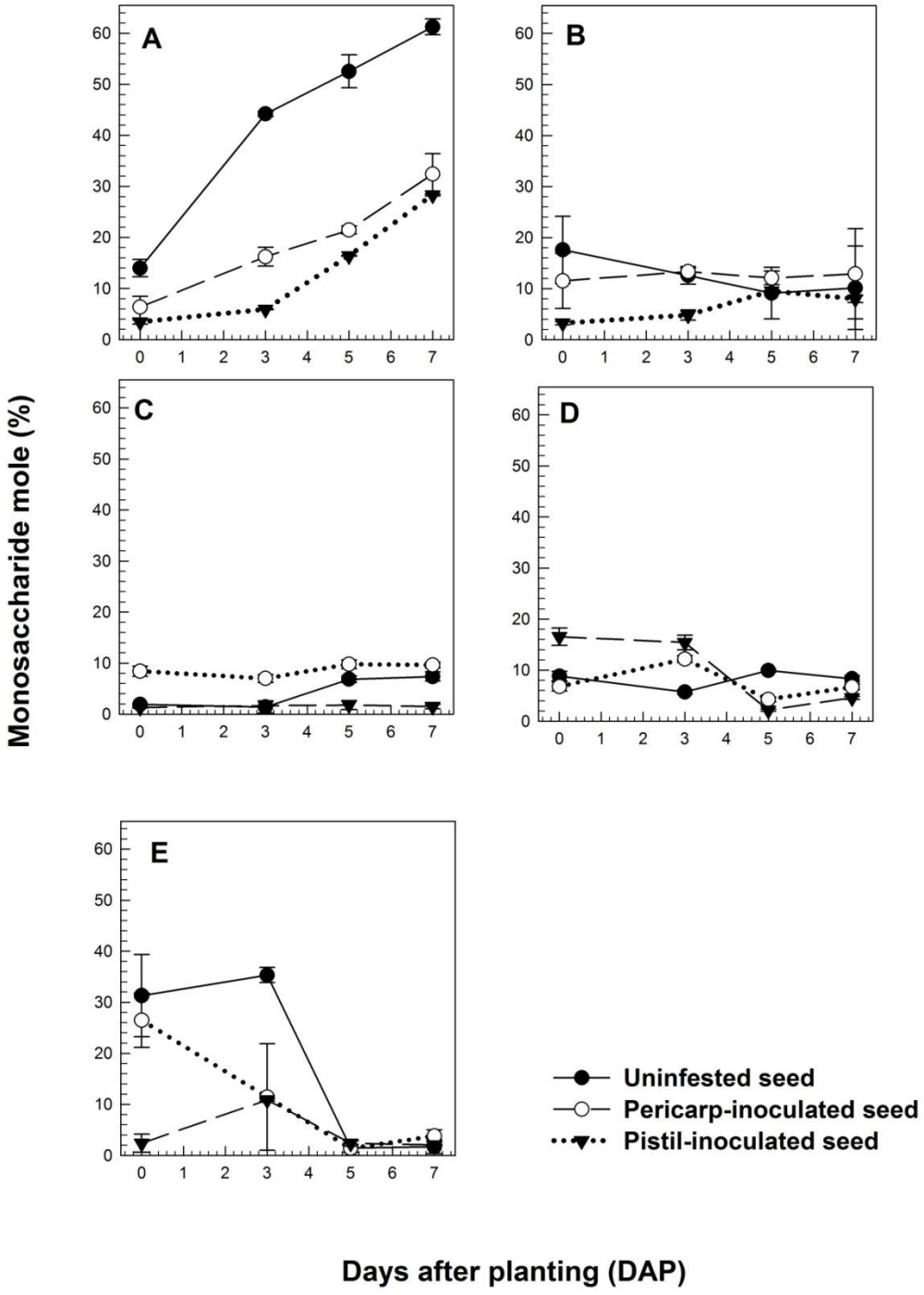
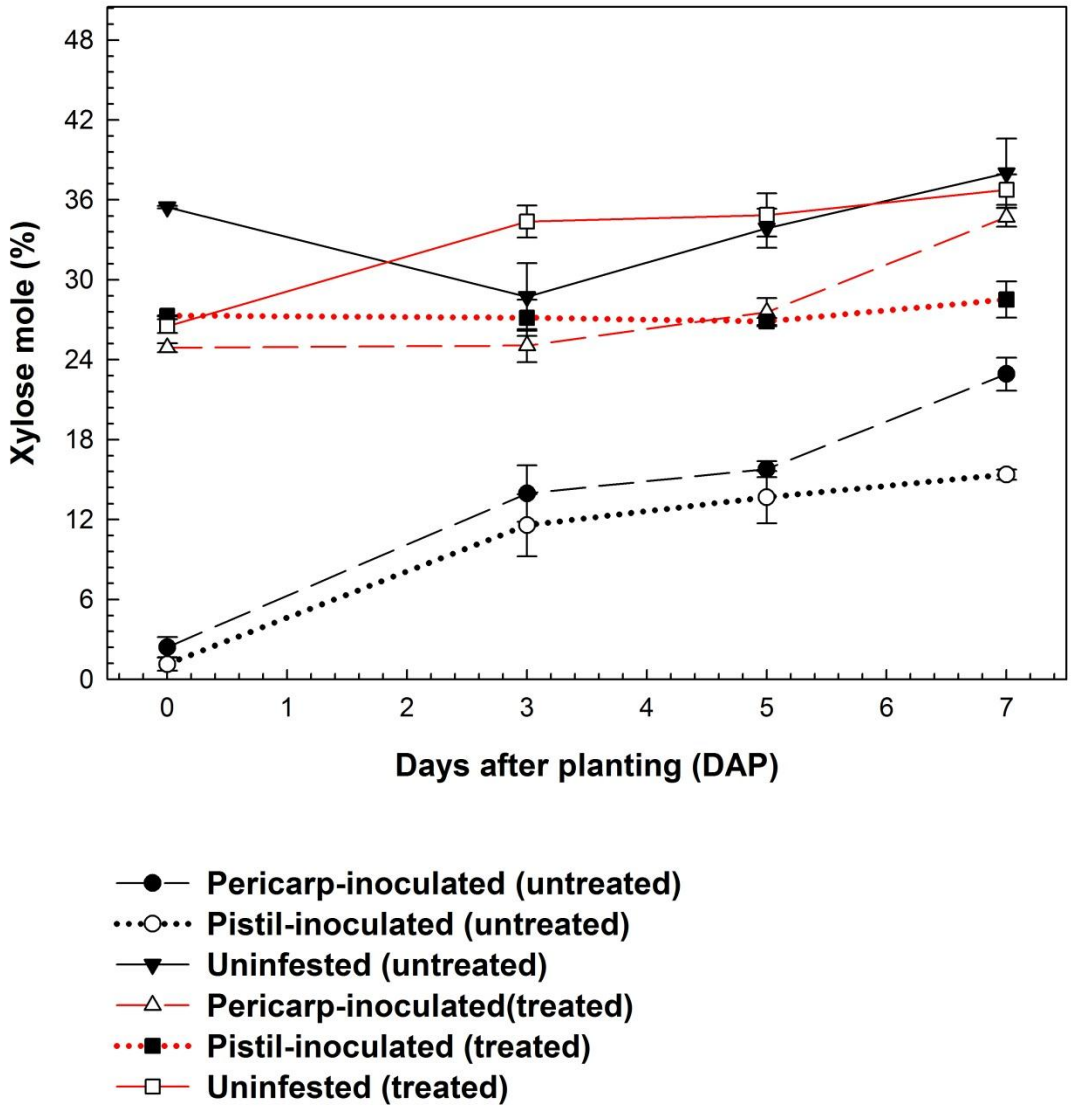

A
B
C
D
E
Monosaccharide mole (%)
Days after planting (DAP)
Uninfested seed
Pericarp-inoculated seed
Pistil-inoculated seed

Figure 6.2. Quantification of xylose in exudates of germinating uninfested and *A. citrulli*-infested watermelon seeds after microbial decontamination with chlorine gas. *Acidovorax citrulli*-infested seed samples (*n* = 20 g of seed) (pericarp and pistil-inoculated) were decontaminated with chlorine gas for 9h at 750 ppm and xylose concentration was determined for samples (*n* = 0.5 g) by incubation at 0, 3, 5, and 7 days after planting. Monosaccharide composition analysis was done by gas chromatography-electron impact-mass spectrometry. Each data point represents the mean of three replicates in two independent experiments.


Xylose mole (%)
48
42
36
30
24
18
12
6
0
0
1
2
3
4
5
6
7
Days after planting (DAP)
Pericarp-inoculated (untreated)
Pistil-inoculated (untreated)
Uninfested (untreated)
Pericarp-inoculated(treated)
Pistil-inoculated (treated)
Uninfested (treated)

CHAPTER 7

ACIDOVORAX CITRULLI SEED INOCULUM LOAD AFFECTS SEEDLING TRANSMISSION AND SPREAD OF BACTERIAL FRUIT BLOTCH OF WATERMELON UNDER GREENHOUSE CONDITIONS

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Abstract

Infested seeds are typically the primary source of inoculum for bacterial fruit blotch (BFB) of cucurbits. An inoculum threshold of one infested seed/10,000 seeds is widely used in seed health testing for *Acidovorax citrulli*. However, the influence of seed inoculum load on BFB seedling transmission has not been elucidated. In this study, watermelon seedlots (128 seeds/lot) containing one seed inoculated with *A. citrulli* at levels ranging from 1×10^1 to 1×10^7 colony forming units (CFU) were used to investigate the effect of seed inoculum load on seedling transmission and spatio-temporal spread of BFB under greenhouse conditions. The relationship between *A. citrulli* seed inoculum load and frequency of BFB seedling transmission followed a sigmoidal pattern ($R^2 = 0.986$, $P = 0.0047$). One-hundred and 96.6% of seedlots containing one seed with 1×10^7 and 1×10^5 CFU *A. citrulli*, respectively, transmitted the pathogen to seedlings; in contrast, the proportion of seedlots that yielded BFB-infected seedlings was even lower for lots with one seed infested with 1×10^3 (46.6%) and 1×10^1 CFU *A. citrulli* (16.7%). The relationship between *A. citrulli* seed inoculum load and frequency of pathogen detection in seedlots using immunomagnetic separation (IMS) combined with a real-time PCR assay also followed a sigmoidal pattern ($R^2 = 0.997$, $P = 0.0034$). Whereas 100% of samples from seedlots (10,000 seeds/lot) with one seed containing $\geq 1 \times 10^5$ CFU tested positive for *A. citrulli* , 75% of samples from lots with one seed containing 1×10^3 CFU tested positive for the pathogen, and only 16.7% of samples with one seed containing 10 CFU tested positive. Since disease transmission was observed for lots with just one seed containing 10 *A. citrulli*, zero tolerance for seedborne *A. citrulli* is recommended for

effective BFB management. The seedling transmission experiments also revealed that temporal spread of BFB in 128-cell seedling trays increased linearly with *A. citrulli* inoculum load ($r^2 = 0.976$, $P = 0.0037$). Additionally, the frequency of spatial spread of BFB from an inoculated seedling in the center of a planting tray to adjacent healthy seedlings over one-, two-, or three-cell distances was greater for lots with one seed infested with at least 1×10^5 CFU than for lots with one seed infested at lower inoculum loads (1×10^1 and 1×10^3 CFU/seed).

Introduction

Bacterial fruit blotch (BFB) is an economically important disease of cucurbits, including watermelon (*Citrullus lanatus*) (29,38). The disease is caused by the Gram negative bacterium *Acidovorax citrulli* (formerly *Acidovorax avenae* subsp. *citrulli* = *Pseudomonas pseudoalcaligenes* subsp. *citrulli*) (26,30,40). In the United States, BFB was first observed in commercial watermelon fields in Florida in 1989 (33). Since then, the disease has caused significant economic losses to watermelon seed, transplant, and fruit production worldwide (2-4,10,13,14,17,19,33). Epidemiologically, infested seeds are the most important source of primary inoculum for BFB (17,22,39). Once the pathogen is introduced into transplant houses via infested seed, high humidity, high temperature, and overhead irrigation increase the risk of BFB epidemic development. Hence, strategies to exclude *A. citrulli* from seeds and transplants are critical for minimizing the threat of BFB.

Currently, seed health testing and seed treatments are employed routinely for BFB management (1,11-13). However, neither strategy guarantees pathogen-free seeds. The accuracy and precision of seed health testing for *A. citrulli* is influenced by many factors including sample size, sampling method, and pathogen extraction efficiency (18,20,21). The effectiveness of seed health testing is also influenced by the seed inoculum threshold, which is the level of seed inoculum that will lead to disease development and economic losses when seeds are planted under conducive field conditions. Inoculum threshold is also defined as the level of seedborne inoculum that leads to an unacceptable risk of disease (25,28). For disease management based on pathogen exclusion via seed health testing, it is critical to determine the seed inoculum threshold experimentally. In practice, the factors that influence the inoculum threshold are numerous and include pathogen aggressiveness, host susceptibility, and environmental conditions (7). As a result, inoculum thresholds are difficult to establish and have been determined for only a few bacterial diseases including black rot of crucifers (caused by *Xanthomonas campestris* pv. *campestris*) and bacterial blight of carrot (caused by *Xanthomonas hortorum* pv. *carotae*) (27,35). To date, an inoculum threshold has not been determined for BFB. Instead, a threshold of one infested seed/10,000 seeds has been adopted based on research on black rot of crucifers (27). According to Gitaitis and Walcott (7), a sample of 30,000 to 50,000 seeds/lot must be tested to guarantee 95 and 99% confidence, respectively, at detecting one infested seed in a sample of 10,000 seeds. However, this inoculum threshold does not consider seed inoculum load (inoculum concentration in

CFU/seed), even though pathogen population can influence aspects of seed-to-seedling transmission, particularly the incubation period (6,15,24).

Despite acceptance as the industry standard, the seedling grow-out assay for *A. citrulli* seed health testing has many limitations (1). Although the assay is technically simple and based on visual inspection of seedlings, the assay is expensive because of greenhouse space and technical labor requirements. The effectiveness of the seedling grow-out assay is dependent on the rate of seed-to-seedling transmission of the pathogen, which depends on environmental conditions in the greenhouse. The assay can take up to 3 weeks to complete and requires significant greenhouse space. Additionally, the assay requires trained technicians to recognize variations in BFB seedling symptoms depending on host-strain interactions. Finally, laboratory assays [e.g., serological tests and polymerase chain reaction (PCR) assays] are required to confirm the identity of bacteria recovered from seedlings. Walcott et al. (36) reported that the greenhouse seedling grow-out assay detected only 12.5 and 37.5% of seedlots (10,000 seeds/lot) with 0.01 and 0.1% infested seeds, respectively. In comparison, immunomagnetic separation combined with the PCR assay (IMS-PCR) yielded detection frequencies of 25.2 and 87.5% for inoculated watermelon seedlots (10,000 seeds/lot) with 0.01 and 0.1% *A. citrulli*-infested seeds, respectively (37). Although the differences in detection frequencies for the IMS-PCR assay and seedling grow-out assay were not statistically significant, the data demonstrated the potential of the IMS-PCR assay as an effective alternative to the grow-out assay for *A. citrulli* seed health testing.

The goal of this research was to determine the effect of seed inoculum load on seedling transmission and the spread of BFB in watermelon seedlings in the greenhouse. Since an effective seed health assay must display a pathogen detection threshold that is lower than the seed inoculum threshold for a given disease (16,25), and the BFB inoculum threshold had not been previously determined, we also sought to determine this parameter for *A. citrulli* under greenhouse conditions.

Materials and methods

Bacterial strain and inoculum preparation. *A. citrulli* strain AAC00-1 (36), used in this study, was recovered from a natural BFB outbreak in watermelon crops in Georgia in 1992. The strain was stored in 15% glycerol at -80^{o}C, and cultured on nutrient agar for 48 h at 28°C as needed. To prepare inoculum, 3 ml nutrient broth in a test tube was inoculated with a single colony of AAC00-1 from a 48h culture and incubated overnight at 30°C on a rotary shaker (Innova; New Brunswick Scientific, Edison, NJ) at 250 rpm. After 24h, the culture was centrifuged at 4,000 × g for 5 min (Allegra 25R, Beckman Coulter, Fullerton, CA), the supernatant was decanted, and the pellet was resuspended in 1 mL 0.1M phosphate buffered saline (PBS). Bacterial concentration was estimated using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY) and adjusted to the desired concentration (optical density of 0.3 at 600 nm = ~1 ×10^{8} CFU/mL) with sterile PBS. The bacterial suspension was concentrated 10-fold by centrifugation, and 10-fold serial dilutions were generated from suspensions containing 1 × 10^{3}, 1 × 10^{5}, 1 × 10^{7}, and 1 × 10^{9} CFU/mL.

Seed inoculation. Watermelon seeds of cv. Crimson Sweet (Hollar Seeds, Rocky Ford, CO) were inoculated individually to achieve final levels of AAC00-1 inoculum of 1×10^1, 1×10^3, 1×10^5, and 1×10^7 CFU/seed. Each seed was first incised at the micropylar end with a sterile scalpel with a #3 blade (DR Instruments, Palos Hills, IL). A sterile teasing needle (DR Instruments) was then used to make a shallow hole through the testa at the chalazal end of each seed. With a Pasteur pipette attached to a vacuum line, ~10 µL bacterial suspension (1×10^3, 1×10^5, 1×10^7, and 1×10^9 CFU/ml) was aspirated into each seed as follows. Using a vacuum, each seed was attached to the Pasteur pipette at the puncture wound at the chalazal end. Ten microliters of inoculum was deposited on a sterile Petri dish lid, and the vacuum was used to draw the cell suspension into the seed via the wound at the micropylar end. Seeds inoculated with PBS were used as a negative control treatment. Inoculated seeds were air-dried at room temperature overnight. Before seed inoculation, the inoculum concentration in each bacterial suspension was verified by spread-plating one 100 µL aliquot for each 10-fold serial dilution onto proprietary Nunhem's semi-selective agar medium (Nunhems Seed Company, Haelen, Netherlands). Furthermore, immediately following inoculation, the *A. citrulli* populations present in each seed were confirmed. Five inoculated seeds were crushed individually in 1 mL sterile PBS, and one 100-µL aliquot of each 10-fold serial dilution of each seed macerate was spread-plated onto Nunhem's agar medium. After incubation at 28°C for 4 to 5 days, *A. citrulli* colonies were enumerated and CFU/seed estimated. The efficiency of the seed inoculation protocol was estimated as: ($\log_{10}$ *A. citrulli* CFU recovered/seed/$\log_{10}$ *A. citrulli* CFU applied/seed) × 100 (23).

Effect of *A. citrulli* seed inoculum load on BFB seedling transmission and spread of *A. citrulli*. Humidity chambers were constructed in a greenhouse to generate conditions conducive for BFB development. Each humidity chamber (4.5 m × 1.0 m × 1.0 m) was supported by a frame of PVC pipes and covered with transparent plastic (6 Mil clear polyethylene sheeting, Norkan, Warren, MI). Inside each chamber, high relative humidity (RH) was maintained using a humidifier (Trion 707U, Indian Trail, NC), and temperature and RH were monitored using data loggers (WatchDog 250, Spectrum Technologies, East Plainfield, IL). Throughout the study the mean environmental conditions in the chambers were 25.4 to 28^{o}C and 76.2 to 80% RH.

To investigate the effect of seed inoculum load on seedling transmission, each inoculated seed was placed at the center of a 128-cell tray (Speedling, Sun City, FL) surrounded by noninoculated seeds. Each seedlot was limited to 128 seeds because of space limitations in the humidity chambers. Additionally, this sample size allowed multiple replicates of each treatment to be tested that would not have been possible with larger seedlots. Trays were filled with fine-grade composted pine bark mixed with vermiculite in a 3:1 ratio. A randomized complete block design was employed with the experimental unit being one seedlot in a 128-cell tray. In each of three independent experiments, treatments (the five levels of *A. citrulli* inoculum/seed noted above) were arranged in 10 replicate blocks with trays arranged contiguously. Manual watering (rather than automated overhead irrigation) was used to reduce the risk of cross-contamination among treatments from splash dispersal of bacteria. This was done by carefully directing a gentle stream of water from a hose at the base of the plants instead of onto the foliage.

A seedlot was considered to have transmitted the disease when the inoculated seedling in a tray developed typical BFB symptoms (water soaking on the abaxial surface of cotyledons). The frequency of BFB seedling transmission for each treatment was recorded every 3 days for 18 days after planting. The mean frequency of BFB seedling transmission was calculated as: (number of seedlots with at least one seedling with BFB symptoms)/(total number of seedlots planted) × 100, for each treatment. The relationship between *A. citrulli* seed inoculum load and frequency of BFB seedling transmission was determined by regression analysis using SigmaPlot Version 10.0 (Systat, San Jose, CA). The regression analysis was based on means from the three experiments after a preliminary analysis of variance (PROC GLM in SAS Version 9.1; SAS Institute, Cary, NC) showed no significant effect of the experiment factor at $P = 0.05$. To determine the effect of *A. citrulli* seed inoculum load on temporal spread of BFB among seedlings, the average rate of BFB development was calculated as: (number of symptomatic seedlings)/(total number of seedlings)/(18 days) for each treatment on the final day of the experiment. Linear regression analysis was used to determine the relationship between *A. citrulli* seed inoculum load and mean rate of BFB development.

Effect of *A. citrulli* seed inoculum load on BFB spatial spread. In the BFB transmission experiment described above, the location of each symptomatic seedling within each 128-cell tray was recorded at 3-day intervals for 18 days after inoculation to determine the effect of *A. citrulli* seed inoculum load on spatial spread of BFB among seedlings. Spread was quantified as the cumulative number of across-cell transmission events of BFB symptoms from the seedling that developed from an inoculated seed to

adjacent seedlings over one-, two-, or three-cell distances. Linear regression analysis was used to determine the relationship between *A. citrulli* seed inoculum load and the mean cumulative number of across-cell transmission events for each seed inoculum level. The regression analysis was based on means from the three experiments after a preliminary analysis of variance showed no significant effect of the experiment factor at $P = 0.05$.

Confirmation of BFB on symptomatic seedlings. To confirm that the symptoms observed on seedlings were caused by *A. citrulli*, bacterial isolations were performed from at least five seedlings from each treatment. Small (2 to 4-mm^2) pieces of symptomatic tissue were macerated in 200 µL sterile PBS, streaked onto Nunhem's agar, and incubated for 5 days at 28^{o}C. After incubation, round, red colonies with smooth margins were putatively identified as *A. citrulli*. For further confirmation, genomic DNA was extracted from 2 or 3 colonies/ isolation and tested with a TaqMan real-time PCR assay using *A. citrulli*-specific primers (9).

Risk of BFB epidemic development from asymptomatic seedlings in the field. In the above-mentioned greenhouse transmission experiments, some of the seedlings that developed from seeds inoculated with 0, 1×10^1, and 1×10^3 CFU did not develop BFB symptoms during the 18-day experiment. To determine whether asymptomatic watermelon seedlings exposed to *A. citrulli* BFB in the greenhouse posed a risk for disease development in the field, asymptomatic seedlings from each of these three inoculation treatments were transplanted into field plots at the Black Shank Farm at the University of Georgia Tifton Campus in July 2008 (with seedlings from the second run of

the greenhouse experiment) and April 2009 (using seedlings from the third experiment). The plants were then monitored for disease development. For each of the three inoculum levels, five replicates of five asymptomatic seedlings (that developed from inoculated seed at the center of the tray, along with four adjacent seedlings) were utilized, giving a total of 15 five-plant plots/year. Seedlings were transplanted into raised, plastic-covered beds with drip irrigation and a plant spacing of one seedling/4 m^2. To prevent bacterial spread between plots, a 6 m-long section of bed was left fallow on both sides of each plot. Routine fungicide applications were made to control fungal diseases and downy mildew. To manage gummy stem blight caused by *Didymella bryoniae,* plants were sprayed every 5 days with chlorothalonil (Syngenta Crop Protection, Greensboro, NC) (3.52 L/ha) + difenoconazole (Syngenta Crop Protection) (525 mL/ha) + cyprodinil (Syngenta Crop Protection) (525mL/ha). At vine run, azoxystrobin (Syngenta Crop Protection) (1.2 L/ha) was applied to manage belly rot. At fruit set, flupicolide (Valent Agricultural Products, Walnut Creek, CA) (300 mL/ha) and mancozeb (Limin Chemical Co. Ltd., Jiangsu, China) (210 mL/ha) were sprayed biweekly to manage downy mildew. For fertilization, 182 kg calcium nitrate was side-dressed/ha. In the two field trials, plots were observed visually for BFB incidence (both foliar and fruit symptoms) at 20 to 25-day intervals for 70 days after transplanting, and again at harvest (85 days after transplanting).

Effect of seed inoculum load on detection of *A. citrulli* by IMS real-time PCR assay. Seedlots were generated by mixing one watermelon seed (cv. Crimson Sweet) inoculated with different concentrations of *A. citrulli* (1×10^1, 1×10^3, 1×10^5, or 1×10^7 *A. citrulli*

CFU, as described above) with 10,000 noninfested seeds. Seeds inoculated with PBS and mixed with 10,000 noninfested seeds served as negative control lots. Each seedlot was tested by IMS real-time PCR assay as described previously (35,36). Briefly, seeds were shaken in 1 liter PBS buffer for 1h in a sterile 2 liter side-arm flask attached to a vacuum line. A vacuum was drawn and interrupted abruptly at 15 min intervals to aid extraction of bacteria from under the testae. During the extraction process, seeds were agitated continuously using a magnetic stir bar and a stirring plate. After extraction, the seed wash was passed through two layers of cheesecloth to remove seeds, filtered through one layer of Whatman #1 filter paper, and centrifuged at 13,800 × *g* for 15 min. The supernatant was decanted and the pellet resuspended in 6 mL PBS-bovine serum albumin (PBS-BSA). For IMS, 2.5×10^7 immunomagnetic beads (IMBs) coated with 40 µg anti-AAC antibody/10^8 IMBs were used for each sample. Immunomagnetic separation was conducted on each sample for 1h at 4^oC with constant agitation. After rinsing three times with PBS-BSA and once with deionized distilled water, IMBs were resuspended in 30 µL sterile deionized water. Captured cells were lysed by boiling for 10 min to release genomic DNA (36,37) and a 5 µL sample of eluted DNA was tested by real-time PCR assay using a species-specific TaqMan assay, as described previously (9). Cycle threshold (Ct) values were recorded for each sample. Four replications of each treatment were assayed, and each experiment was repeated four times. The mean frequency of *A. citrulli* detection was determined as: (number of positive detections for each treatment)/(total number of seedlots tested for each treatment) × 100. Regression analysis was conducted to determine the relationship between *A. citrulli* seed inoculum load and

detection of infested seedlots. The regression analysis was based on means from the four experiments after a preliminary analysis of variance showed no significant effect of the experiment factor at $P = 0.05$.

***Acidovorax citrulli* seed inoculum threshold.** To estimate the *A. citrulli* seed inoculum threshold (seed inoculum level that could be detected reliably by IMS real-time PCR assay and that displayed the lowest frequency of BFB seedling transmission), the frequency of detection of *A. citrulli*-infested lots was plotted against the frequency of BFB seedling transmission for different seed inoculum loads, analogous to a receiver operating characteristic (ROC) curve (39). This was done using mean values from the three experiments. The point closest to the theoretical optimum of no transmission and 100% detection was determined visually.

Results

Efficiency of seed inoculation method. The efficiency of the seed inoculation method calculated for inoculum levels of 1×10^3, 1×10^5, and 1×10^7 CFU/seed was 93.5 ± 2.5, 92.0 ± 0.8, *and* 98.0 ± 3.1% (mean ± standard error), respectively (Table 7.1). In contrast, *A. citrulli* was not recovered from seed inoculated with 1×10^1 CFU *A. citrulli* by plating the seed macerate on a semi-selective agar medium. Hence, the inoculation efficiency for this treatment was 0%.

Effect of *A. citrulli* seed inoculum load on frequency of BFB transmission and temporal disease dynamics. The mean frequencies of BFB seedling transmission for seed lots (128 seeds/lot) with one seed inoculated with 1×10^1, 1×10^3, 1×10^5, and $1 \times$

10^7 CFU were 16.7, 46.6, 96.6, and 100%, respectively (Fig. 7.1). Linear and non-linear regression curves were fitted, and the relationship between *A. citrulli* seed inoculum load and frequency of BFB transmission was described best by a two-parameter sigmoidal model (R^2 = 0.986; P = 0.0047). In contrast, a linear regression model fitted to this relationship was not statistically significant (P = 0.067). As expected, control seedlots with 0 CFU did not yield symptomatic seedlings. A subset of seedlings associated with each treatment that led to putative BFB symptoms was confirmed to be infected with *A. citrulli* by pathogen isolation followed by real-time PCR assays (*data not shown*).

The relationship between *A. citrulli* seed inoculum load and mean rate of BFB development over time was linear (r^2 = 0.976; P = 0.0037) (Fig. 7.2). BFB symptoms did not develop on seedlings from negative control lots comprised of one seed treated with PBS instead of *A. citrulli* and planted with 127 noninfested seeds. The mean rate of BFB development for lots with one infested seed containing 1×10^1, 1×10^3, 1×10^5, or 1×10^7 CFU was 0.0004, 0.0012, 0.0020, and 0.0023/day, respectively (Fig. 7.2).

Effect of *A. citrulli* seed inoculum load on BFB spatial spread among seedlings. The relationship between *A. citrulli* seed inoculum load and secondary BFB spread among seedlings (over one-, two-, and three cell-distances) was linear ($r^2 \geq 0.91$; $P \leq 0.005$). As expected, BFB seedling transmission and secondary spread were not observed for the negative control treatments in any repeat of the experiment. As *A. citrulli* seed inoculum load increased, so too did BFB spatial spread in terms of cumulative transmission over one-, two-, and three-cell distances from the seedling that developed from the inoculated seed (Fig. 7.3). With regards to BFB seedling-to-seedling transmission across a one-cell

distance, the higher the *A. citrulli* seed inoculum load the greater the number of disease transmission events. The relationship between *A. citrulli* seed inoculum load and BFB transmission across a one-cell distance was linear (r^2 = 0.954; P < 0.005) (Fig. 7.3A). A positive linear relationship was also observed between *A. citrulli* seed inoculum load and BFB transmission events across two cell (r^2 = 0.982; P < 0.003) (Fig. 7.3B) and three-cell distances (r^2 = 0.916; P < 0.001) (Fig. 7.3C). However, the slope of the line describing the relationship between BFB transmission and *A. citrulli* seed inoculum load was greatest for transmission events across a one-cell distance (7.78) followed by transmission across two-cell distances (3.49) and subsequently three-cell distances (1.66) (Fig. 7.3). As expected, shorter across-cell BFB seedling-to-seedling transmission events were more common than disease transmission across multiple-cell distances.

Risk of BFB transmission from asymptomatic seedlings exposed to *A. citrulli* in *the* greenhouse and transplanted into *the* field. In both field trials, BFB epidemic did not develop from asymptomatic seedlings that were exposed to *A. citrulli* in the greenhouse. Additionally, *A. citrulli* was not recovered from plant tissue samples collected from the field plots in either year. During 2008 and 2009, Tifton, GA received a mean rainfall of 140.2 cm with 111 rainy days. The mean average temperature during this period was 26.4^{o}C. Although not excessively rainy, the field conditions were deemed conducive for BFB development as a natural BFB outbreak occurred in nearby Colquitt Co. in 2009.

Effect of *A. citrulli* seed inoculum load on detection by IMS-real time PCR assay. As expected, *A. citrulli* was not detected in negative control seedlots throughout this study. However, the mean detection frequencies for lots (10,000 seeds/lot) with one seed

containing 1×10^1, 1×10^3, 1×10^5, and 1×10^7 *A. citrulli* CFU by IMS real-time PCR assay were 18.8, 75.0, 100, and 100%, respectively (Fig. 7.4). The mean Ct values for IMS real-time PCR assays conducted on seed samples with seed inoculated with 1×10^1, 1×10^3, 1×10^5, and 1×10^7 CFU were 34.01, 32.84, 31.72, and 28.94 respectively (Table 7.2). Linear and non-linear regression models were fitted, and the relationship of inoculum load/infested seed vs. frequency of pathogen detection by IMS-PCR assay was described best by a two-parameter sigmoidal model ($R^2 = 0.997$; $P = 0.0034$). In contrast, a linear regression model fitted to this relationship was not statistically significant ($P = 0.100$).

***Acidovorax citrulli* seed inoculum threshold.** The plot of detection frequency for *A. citrulli*-infested seedlots vs. frequency of BFB seedling transmission revealed that among the inoculum loads tested, the point closest to the theoretical optimum of 0% transmission and 100% detection frequency was a seed inoculum load of ~10^3 CFU/seed (Fig. 7.5). For this seed inoculum load, the frequency of *A. citrulli* detection by IMS real-time PCR assay was 75.0% and the frequency of BFB seedling transmission was 46.6%. At the next lowest seed inoculum load of ~10^1 CFU/seed, BFB seedling transmission occurred at 16.7% frequency but *A. citrulli* could only be detected in 18.8% of the assays.

Discussion

Overall this study showed that under greenhouse conditions, one watermelon seed infested with *A. citrulli* and mixed with 127 noninfested seeds could initiate a BFB epidemic in >96% of the seedlots when the bacterial population on the infested seed was $\geq 1 \times 10^5$ CFU. Lots with one seed containing 1×10^3 CFU also transmitted the disease,

but the frequency of transmission was reduced to < 50%. Furthermore, although the data suggest that there is a reduced risk of BFB seedling transmission when a single seed contains ≤ 10 *A. citrulli* CFU, zero tolerance may still be the best approach for BFB management in commercial transplant houses because, even for the lowest inoculum load tested, the frequency of seedling transmission still approached 20%.

Initially, attempts to determine the effect of individual seed inoculum load on BFB seedling transmission were made with large seed samples (10,000 seeds/lot). However, due to greenhouse space constraints, only four replicates of each treatment could be evaluated in an experiment and there was significant cross-contamination among treatments. To avoid this problem, an experimental design was adopted whereby the experimental unit was a 128-cell seedling tray, in which one seed with a specific level of inoculum was planted per treatment. This design enabled more replicates of each treatment to be included/experiment. To generate infested seedlots, seeds were inoculated individually by aspirating *A. citrulli* cell suspensions into each seed, followed by mixing one inoculated seed with 127 noninfested seeds. This technique enabled reproducible generation of seeds with specific concentrations of bacterial cells. The efficiency of inoculation was >90% for inoculum levels of 1×10^3, 1×10^5, and 1×10^7 *A. citrulli* CFU/seed. However, the inoculation efficiency fell to 0% as inoculum load decreased to 10 CFU/seed. This may have been due to the dilution effect that made it impossible to recover CFU from seeds with low inoculum loads, but it is also possible that a proportion of the pathogen cells died or became non-viable (31) after inoculation, making recovery

on solid agar medium impossible. This is even more likely considering that after inoculation, seeds were air-dried at room temperature for 12 h before planting.

The ability of an IMS real-time PCR assay to detect *A. citrulli* in watermelon seedlots with one seed with varying levels of inoculum was tested. In a previous study, IMS-PCR was used to detect *A. citrulli* in 10,000-seed watermelon lots with 0.1 and 0.01% infested seeds (37). However, the effect of seed inoculum load on pathogen detection was not considered. While the IMS real-time PCR assay is not currently a standard BFB seed health assay, it has potential for commercial use. In this study, we observed a sigmoidal relationship between inoculum load/infested seed and frequency of pathogen detection by *the* IMS-PCR assay. For seedlots with the highest risk of BFB seedling transmission evaluated (lots with one seed infested with 10^7 or 10^5 *A. citrulli* CFU), the IMS real-time PCR assay detected the bacterium in 100% of the samples. Lots with one seed containing 10^3 CFU of *A. citrulli* displayed lower frequency of BFB transmission (46.6%) but still a relatively high level of pathogen detection (75.0%) by the IMS real-time PCR assay. At 10 CFU/seed, the lowest seed inoculum load tested, the frequencies of both BFB seedling transmission and pathogen detection dropped below 20%, indicating that detection by grow-out assay as well as IMS combined with real-time PCR assay becomes unreliable at low inoculum loads.

Another aim of this study was to investigate the effect of *A. citrulli* seed inoculum load on temporal spread of BFB among watermelon seedlings in the greenhouse. The results indicated that under greenhouse conditions without typical automated overhead irrigation, BFB developed faster from lots with one seed infested with $\geq 1 \times 10^5$ CFU than

from lots with one seed infested with $\leq 1 \times 10^3$ CFU. One possible explanation for this observation is the effect of seed inoculum load on incubation period. Gitaitis and Nilakhe (6) demonstrated the effect of inoculum load on incubation period for bacterial blight of cowpea by infiltrating 10-fold serial dilutions of seed wash from *Xanthomonas axonopodis* pv. *vignicola*-infested seed samples into primary cowpea leaves. Seedlings were observed for symptom development and the inoculum dilution endpoint (highest dilution that led to symptom expression) was calculated. An incubation period of 5 to 20 days was required for symptom development in seed samples with 10^{-8} and 10^{-3} dilution endpoints. They also observed the highest disease incidence in the field from seedlots with the highest dilution endpoints.

A fourth goal of this study was to examine the influence of *A. citrulli* seed inoculum load on BFB spatial spread under greenhouse conditions. These data may be useful for developing strategies for managing BFB in transplant production facilities. Currently, when BFB occurs in commercial transplant houses, it is recommended that all exposed seedlings (seedlings growing in a greenhouse where an outbreak has occurred) be destroyed (17). In many cases, however, growers cannot afford to destroy exposed seedlings as this may result in missed markets and significant economic losses. Alternatively, the growers often eliminate seedlings proximal to infection foci and continue to cultivate the remaining seedlings. Currently, there are no empirical data on the spatio-temporal spread of *A. citrulli* under transplant house conditions, nor how spread may be influenced by seed inoculum load. In this study, increased *A. citrulli* seed inoculum load led to an increase in spatial spread of BFB. Lots with one seed with high

inoculum loads (1×10^7 or 1×10^5 *A. citrulli* CFU) resulted in a higher frequency of disease spread compared to lots with lower levels of inoculum (1×10^3 or 1×10^1 CFU). This could be explained by earlier disease onset and spread among seedlings in treatments with high inoculum density, providing increased likelihood of greater disease spread. Additionally, when asymptomatic seedlings that developed from seeds inoculated with 0, 1×10^1, and 1×10^3 CFU were planted in the field, no BFB symptoms developed. Although these seedlings were exposed to *A. citrulli* in the greenhouse, these observations suggest a low likelihood the seedlings could initiate BFB epidemics under field conditions.

This is the first report of the effect of *A. citrulli* seed inoculum load on spatial spread of BFB under greenhouse conditions. Roberts et al. (24) described secondary spread of *X. campestris* pv. *campestris* from inoculated seeds to non-inoculated seedlings when planted in a greenhouse under overhead irrigation. Their results suggested that inoculum dose/seed and the watering regime (type and frequency of irrigation) affected both the proportion of plants with symptoms and the proportion of contaminated but asymptomatic plants. Seeds harboring 3.6×10^3 CFU bacteria displayed significantly higher numbers of diseased plants than seeds harboring 1.5×10^2 CFU. During the first 2 weeks after planting in their study, only the inoculum load/seed affected the proportion of symptomatic plants. However, by 3 weeks after planting overhead irrigation rather than inoculum load/seed, significantly influenced the proportion of symptomatic seedlings and secondary spread.

This study also evaluated the appropriateness of developing an *A. citrulli* seed inoculum threshold. Empirically-determined seed inoculum thresholds have been successfully used to manage plant diseases (5,8,25); however, thresholds have not been determined for many vegetable bacterial diseases, including BFB. To establish a seed inoculum threshold, correlation analyses should be made between levels of seed infestation and disease incidence in the field or greenhouse (16). From a practical perspective, all levels of seedborne inoculum may lead to some degree of disease transmission under conducive environmental conditions. Hence, in this study the seed inoculum threshold for *A. citrulli* was considered to be the infestation level that resulted in *low* BFB incidence in the greenhouse while still having a high probability of pathogen detection by the IMS- real-time PCR assay. A plot of the frequency of pathogen detection *from* an infested seedlot vs. the frequency of seedling transmission revealed that ~10^3 CFU/seed was closest to this hypothetical inoculum threshold as this inoculum load was detected in 75.0% of the samples tested and resulted in transmission of BFB in ~46.6% of the seedlots tested. Practically, however, this threshold would be of no use because of the high disease transmission rate. Furthermore, seedlots with as little as one seed infested with 10 *A. citrulli* CFU transmitted BFB to seedlings in 16.7 % of the seed samples, suggesting that for effective BFB management, zero tolerance might be appropriate.

To our knowledge, this is the first study of the effect of individual *A. citrulli* seed inoculum load on BFB seedling transmission and spread under greenhouse conditions. Data generated in this study should aid in BFB management in transplant production

facilities and improve sample size determination for seed health testing. At present, results from BFB seed health assays are qualitative; however, based on this study, the risk of disease transmission is significantly influenced by seed inoculum load. This study may also have implications for interpretation of standard greenhouse seedling grow-out assay results (1). For example, seedlings from seedlots with low *A. citrulli* populations may not develop BFB symptoms within the standard 18 day test period. This may lead to false-negative results with important economic consequences. Although this study demonstrated that seed inoculum load has an important effect on BFB seedling transmission, it is impossible to determine the inoculum load of individual seeds in a lot. At best, a quantitative, real-time PCR assay could enable estimation of pathogen population/seed sample, which may be of some practical use for predicting seedling disease transmission, even though such an assay does not take into account distribution of inoculum within a seedlot. Benefits may be gained from exploring the relationship between quantitative real-time PCR assay data, distribution of *A. citrulli* in naturally infested seedlots, and the risk of BFB seedling transmission.

Acknowledgements

This project was supported by the American Seed Trade Association Vegetable Permanent Research Fund. We acknowledge Eurofins STA for material support and labor. We thank Linda Genzlinger and Anita Castro Sparks for their valuable technical contributions, and Dr. Rock Christiano for assistance with statistical analyses.

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Table 7.1. Efficiency of the watermelon seed inoculation protocol with different levels of *Acidovorax citrulli* inoculum used to assess seed transmission of the pathogen under greenhouse conditions

Mean $\log_{10}$(CFU *A. citrulli*/mL) inoculated into each seed[a]	**Mean $\log_{10}$(CFU *A. citrulli*/seed) recovered**[b]	**Efficiency of recovery (%)**[c]
7.51 ± 0.58	7.36 ± 0.38	98.0 ± 3.05
5.65 ± 0.67	5.20 ± 0.82	92.0 ± 0.76
3.70 ± 0.45	3.46 ± 0.75	93.5 ± 2.06
1 ± 0.47	0	0
0	0	…

[a] Values represent mean $\log_{10}$CFU *A. citrulli*/mL ± standard error of five replicates from each inoculum concentration determined spectrophotometrically. CFU = colony forming units.

[b] Values represent mean $\log_{10}$CFU *A. citrulli*/seed ± standard error of five replicates from each inoculated seed determined by dilution-plating onto semi-selective medium.

[c] [($\log_{10}$ CFU/seed of *A. citrulli* recovered)/($\log_{10}$ CFU/mL of *A. citrulli* inoculated) × 100].

Table 7.2. Mean cycle threshold (Ct) values generated by an immunomagnetic separation (IMS) and real-time PCR assay for inoculated watermelon seedlots (10,000 seeds/lot) containing one seed with different levels of *Acidovorax citrulli* inoculum

Inoculum load (*A. citrulli* CFU/seed)	**Mean Ct[a]**
10^7	28.94 ± 0.32
10^5	31.72 ± 0.17
10^3	32.84 ± 0.92
10^1	34.01 ± 11.30
0	0
Positive control (10^3 CFU/ml of *A. citrulli* cell suspension)	27.82 ± 0.62

[a] Mean ± standard error of the cycle threshold values of four replicate samples across four independent IMS-real time PCR assay experiments (n = 16).

Figure 7.1. Non-linear regression analysis of *Acidovorax citrulli* inoculum load/seed vs. bacterial fruit blotch (BFB) seedling transmission frequency over 18 days. Single seeds were inoculated with *A. citrulli* at concentrations ranging from 0 to 1×10^7 CFU. Each seed was planted in the center of a 128-cell tray with 127 noninfested seeds. Each data point represents the mean frequency of BFB seedling transmission for 10 replicates across three independent experiments ($n = 30$).

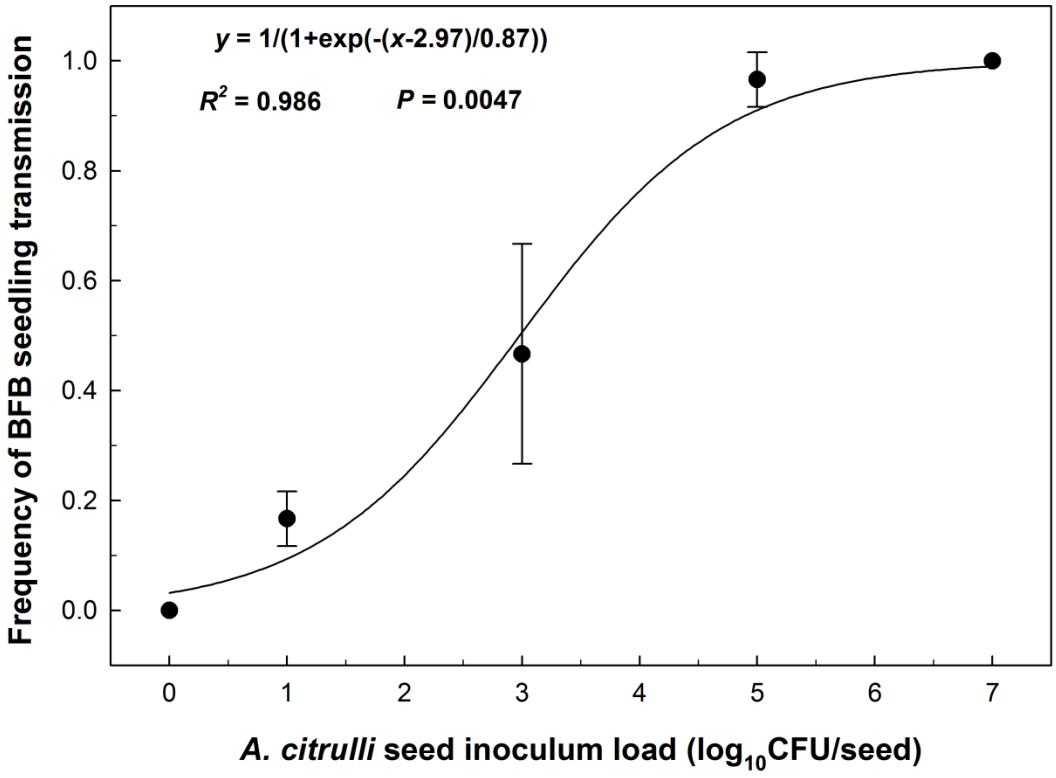

y = 1/(1+exp(-(x-2.97)/0.87))
R2 = 0.986
P = 0.0047
Frequency of BFB seedling transmission
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
7
A. citrulli seed inoculum load (log10CFU/seed)

Figure 7.2. Linear regression analysis of the temporal rate of spread of bacterial fruit blotch (BFB) vs. *Acidovorax citrulli* inoculum load/seed under greenhouse conditions. Temporal spread was determined as final disease incidence (number of symptomatic seedlings/total number of seedlings)/18 days. Each data point is the mean rate of BFB progress for 10 replicates across three independent experiments (n = 30).

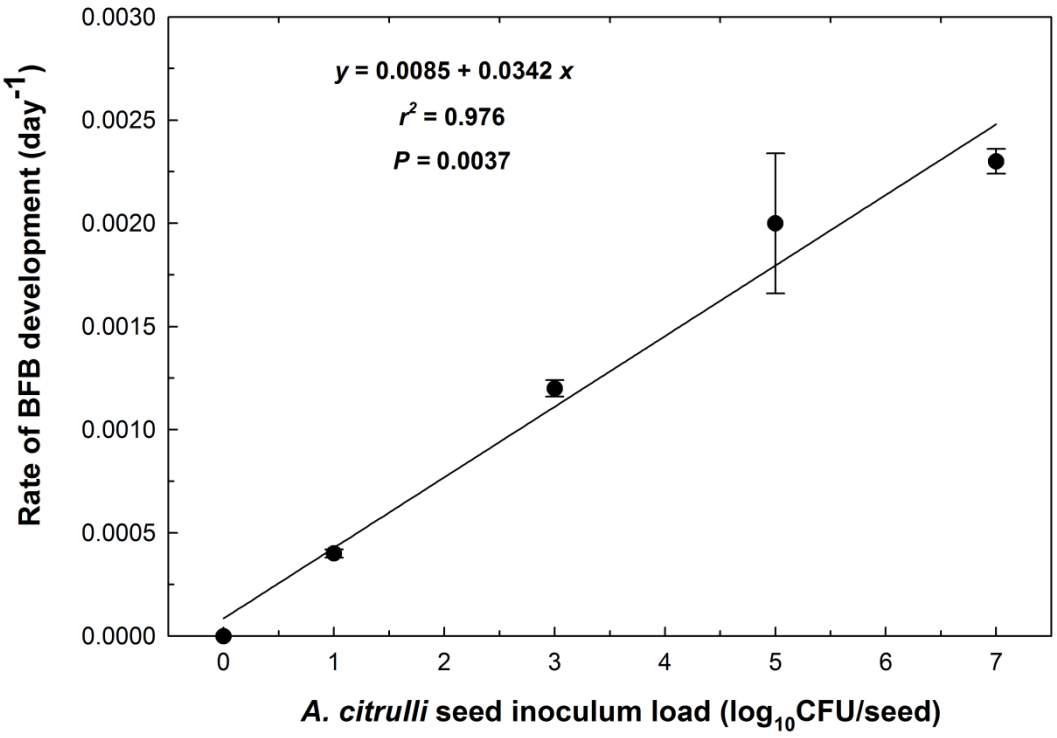

y = 0.0085 + 0.0342 x
r2 = 0.976
P = 0.0037
Rate of BFB development (day-1)
A. citrulli seed inoculum load (log10CFU/seed)
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
0
1
2
3
4
5
6
7

Figure 7.3. Linear regression of spatial spread of bacterial fruit blotch (BFB) under greenhouse conditions vs. *Acidovorax citrulli* inoculum load/seed over 18 days. An *A. citrulli*-inoculated watermelon seed was placed at the center of a 128-cell tray and secondary spread was determined in terms of the cumulative number of cells across which the disease spread (BFB symptoms observed) from a seedling that developed from the inoculated seed to adjacent seedlings over one-cell (A), two-cell (B) and three-cell (C) distances. Each data point is the mean of 10 replicates across three independent experiments (n = 30).

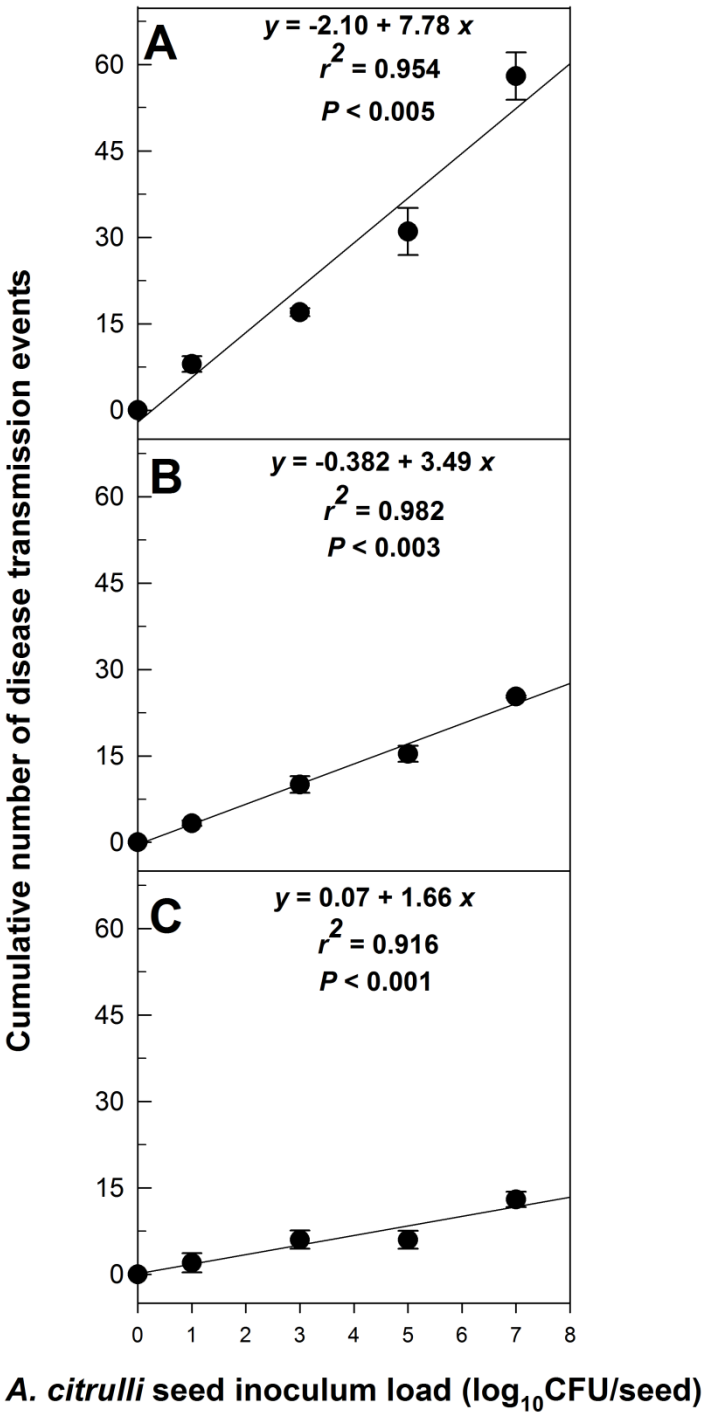

A
y = -2.10 + 7.78 x
r2 = 0.954
P < 0.005
B
y = -0.382 + 3.49 x
r2 = 0.982
P < 0.003
C
y = 0.07 + 1.66 x
r2 = 0.916
P < 0.001
Cumulative number of disease transmission events
A. citrulli seed inoculum load (log10CFU/seed)

Figure 7.4. Non-linear regression analysis of *Acidovorax citrulli* detection frequency by immunomagnetic separation (IMS) and real-time PCR assay vs. inoculum load of *A. citrulli*/seed for inoculated watermelon seedlots (10,000 seeds) spiked with one seed infested at a range of colony-forming units (CFU). Each data point represents the mean frequency of *A. citrulli* detection for four replicates in each of four independent experiments (n = 16).

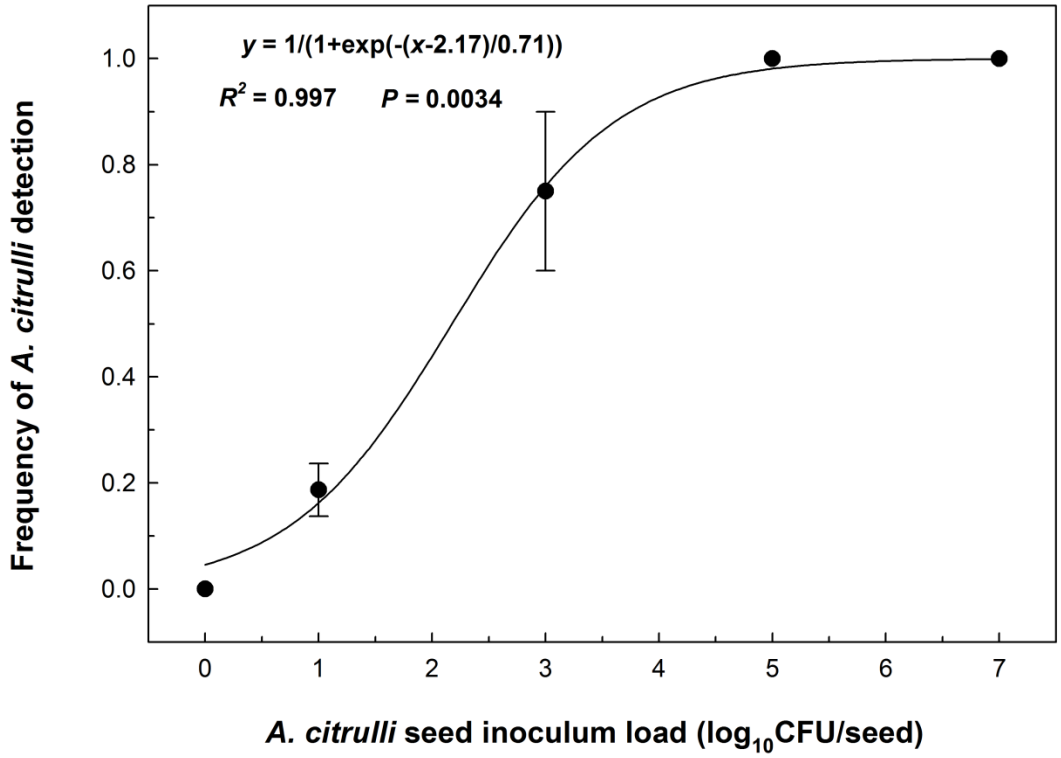
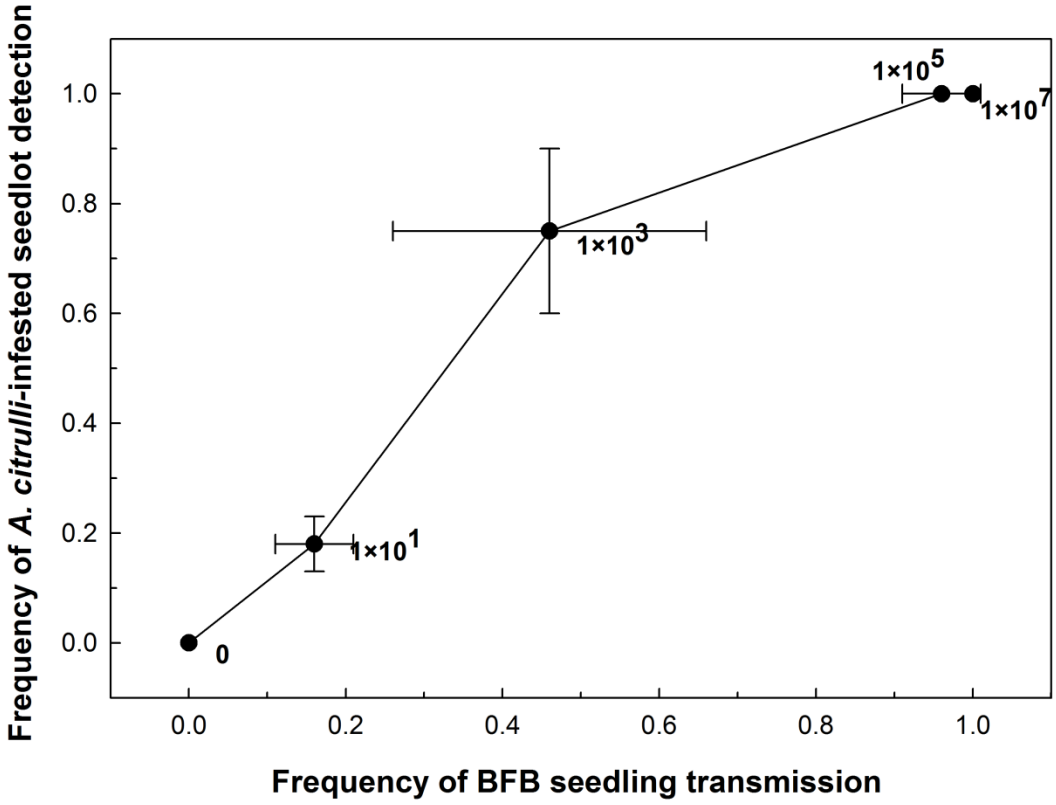

y = 1/(1+exp(-(x-2.17)/0.71))
R2 = 0.997 P = 0.0034
Frequency of A. citrulli detection
A. citrulli seed inoculum load (log10CFU/seed)
0.0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
5
6
7

Figure 7.5. Plot of the frequency of *Acidovorax citrulli* detection by an immunomagnetic separation (IMS) real-time PCR assay vs. bacterial fruit blotch (BFB) seedling transmission on watermelon for varying levels of *A. citrulli* seed inoculum load/seed. The number shown at each data point represents the concentration of *A. citrulli* inoculated/seed. Lines represent the standard errors of the mean frequencies.


Frequency of A. citrulli-infested seedlot detection
Frequency of BFB seedling transmission
0.0
0.2
0.4
0.6
0.8
1.0
1×10^5
1×10^7
1×10^3
1×10^1
0

CHAPTER 8

SUMMARY

Annually, the U.S produces approximately 1.95 billion kilograms of watermelon valued at $434 million (National Agricultural Statistical Service, USDA). Watermelon production is constantly threatened by pests and pathogens that result in millions of dollars in losses annually. One such disease threat is bacterial fruit blotch of cucurbits (BFB) that is caused by the Gram negative bacterium *Acidovorax citrulli* (Schaad *et al*., 2008) formerly *Acidovorax avenae* subsp. *citrulli* (Willems *et. al.*, 1992) = *Pseudomonas pseudoalcaligenes* subsp. *citrulli)* (Schaad *et. al*., 1978). *Acidovorax citrulli* can affect all parts of cucurbit plants causing typical water-soaked lesions on cotyledons and reddish-brown lesions along veins of true leaves (Latin and Hopkins, 1995). On fruit, *A. citrulli* causes water-soaked lesions with irregular margins that gradually crack, leading to fruit rot (Somodi *et al.*, 1991). Infested seeds are an important primary source of *A. citrulli* inoculum (Rane and Latin 1992; Hopkins and Thomson, 2002) and under favorable conditions, seedborne bacteria can spread rapidly in transplant houses and fruit production fields and cause severe economic losses (Latin and Hopkins, 1995; Schaad *et al.* 2003; Walcott, 2008). Despite continuous attempts by vegetable seed companies, it has been practically impossible to produce *A. citrulli*-free commercial seedlots (Walcott, 2008). To improve BFB management, a better understanding of the mechanism(s) of seed

infestation by *A. citrulli* in commercial seed production is needed. Unfortunately, there is limited information available on the processes by which bacteria become associated with and survive on seeds; and ultimately become transmitted to seedlings. Therefore, the overarching goal of this research was to gain better insight into these processes and use this knowledge to limit seed infection by phytobacteria. More specifically, the objectives of this research were to 1) determine the effect of mechanism of invasion on location of *A. citrulli* in watermelon seeds, 2) determine the effect of mechanism of invasion on timing of ovule infestation, 3) determine the effect of *A. citrulli* localization in seeds on pathogen survival, 4) determine the effect of *A. citrulli* localization in seeds on detection by seed health testing, 5) determine the effect of *A. citrulli* localization in seeds on metabolites produced in germinating seeds, and 6) determine the effect of *A. citrulli* seed inoculum load on seedling transmission and spread of BFB under greenhouse conditions.

The results of this work showed that localization of *A. citrulli* in infested seeds was influenced by the mechanism of infestation. Contrary to previous assumptions, the seed coat was not the major site of *A. citrulli* accumulation in naturally infested watermelon seeds. Rather, for seeds from symptomatic fruits (pericarp invasion), *A. citrulli* was located on the surface of the PE layer and to a lesser extent in the seed coat and the embryo. For seeds from asymptomatic fruit (pistil invasion), *A. citrulli* accumulated predominantly in the embryo and to a lesser extent in the seed coat. In commercial seed production fields, foliar and fruit BFB symptoms are not usually observed, yet infested seeds are still produced. This suggests that seed infestation via pistil invasion may partially account for *A. citrulli* infestation of commercial seed. Data from this study also

provided a possible explanation for the ability of *A. citrulli* to survive for more than 30 years in stored seeds, as bacteria under the PE layer may be protected from desiccation.

Acidovorax citrulli seed invasion pathways affected the timing of ovule infestation in watermelon fruit. Significantly higher percentages of ovules become infested when *A. citrulli* was inoculated via the pistil than via the pericarp during the early phases of seed development. Immunolocalization studies revealed that during pistil-invasion, *A. citrulli* colonized stigma, style and ovary tissues within one day post-inoculation. Additionally, the bacterium was also observed to co-localize with the pollen germ tubes in these tissues. In contrast, during pericarp-invasion, *A. citrulli* colonized the exocarp and mesocarp of fruit tissues but failed to colonize the endocarp. These results suggest that watermelon ovules become infested with *A. citrulli* earlier via pistil-invasion than by pericarp invasion.

In studying the effect of the location of *A. citrulli* in seeds on bacterial survival, we observed that *A. citrulli* was sensitive to desiccation and that localization of *A. citrulli* in seed is important for survival. Watermelon seedlots generated via pistil and via pericarp inoculation were treated with peroxyacetic acid (1600 µg/mL for 30 min followed by air-drying at 40°C for 48h) and chlorine gas (750 ppm for 9h) and tested for *A. citrulli* survival. Results showed that *A. citrulli* can survive stress better in pistil-inoculated seeds than in pericarp-inoculated seeds. In addition, a bacterium with weak survival ability, *Pantoea stewartii* subsp. *stewartii* survived better in pistil-inoculated than in vacuum infiltrated watermelon seed. These results suggest that bacterial localization is an important determinant of pathogen survival in seed.

Our results suggest that the ability to extract *A. citrulli* from watermelon seeds was influenced by the location of the pathogen. In pistil-inoculated seedlots, there was a low likelihood that the pathogen would be extracted by washing. In contrast, extraction of *A. citrulli* from the pericarp-inoculated lots was effective by washing. Regardless of the location of the bacterium, seed maceration was more effective for *A. citrulli* extraction.

We observed that *A. citrulli* infestation affected the release of xylose in germinating watermelon seeds. Apart from xylose, concentration of monosaccharides including galactose, rhamnose, mannose and arabinose, decreased consistently over 7 DAP in the exudates of both uninfested and *A. citrulli*-infested seeds. Xylose was detected in significantly higher concentration from uninfested seeds than from *A. citrulli*-infested seeds by 7 DAP. Interestingly, when *A. citrulli*-infested seeds were disinfested using chlorine gas, significantly higher concentration of xylose were detected in seed exudates of uninfested seeds as compared to exudates from *A. citrulli*-infested seeds.

The influence of seed inoculum load on BFB seedling transmission and the spatio-temporal spread of BFB under greenhouse conditions were studied. More than 95% of the seedlots containing one seed with $\geq 1 \times 10^5$ *A. citrulli* CFU, transmitted BFB to resulting seedlings. The proportion of seedlots that yielded BFB-infected seedlings was lower for lots with one seed inoculated with $\leq 1 \times 10^3$ (46.6%). While lots (n = 10,000 seeds) with one seed containing $\geq 1 \times 10^5$ CFU were detected in 100% of attempts, lots with one seed containing 1×10^3 CFU were positive in 75.0% of attempts. Lots with one seed containing 10 CFU were positive in only 16.7% of attempts. Seedling transmission experiments also revealed that temporal spread of BFB (rate of disease development)

increased linearly with *A. citrulli* inoculum load. Additionally, we observed that increased *A. citrulli* seed inoculum load led to a corresponding increase in the spatial spread of BFB. Lots with one seed with high inoculum load (1×10^7 or 1×10^5 *A. citrulli* CFU) resulted in a higher frequency of spread events compared to lots with lower levels of inoculum (1×10^3 or 1×10^1 CFU).

Overall, the results from this research gave an insight on the processes by which bacteria become associated with and survive on seeds; and ultimately how they get transmitted to the seedlings. The knowledge gained from this investigation will also add to our basic understanding of seed infection by *A. citrulli* that may be important for developing environmentally sound and effective strategies for managing BFB.

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