NOVEL BIOCONTROL SEED TREATMENT APPROACH FOR BACTERIAL FRUIT BLOTCH OF WATERMELON

by

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(Under the Direction of Ron Walcott)

ABSTRACT

Bacterial fruit blotch (BFB), caused by the bacterium *Acidovorax citrulli,* is an economically important disease of cucurbitaceous crops worldwide. *A. citrulli* is seed-borne, and infested seeds are the primary source of inoculum for disease epidemics. Efficacy of current seed management practices varies because the pathogen can ingress through female flowers and become deposited deep within the seed, rendering externally applied seed treatments ineffective. The goal of this research was to inoculate watermelon flowers with biological control agents (BCA) to protect watermelon seeds under field conditions. To select candidate BCAs for field studies, we screened ten BCA bacteria from natural watermelon seed microflora, along with a known BCA, *Bacillus mojavensis* RRC101 in a series of *in vitro* assays. We found strains that colonized 24-h watermelon seed exudate and reached higher population levels than *A. citrulli* therein. These strains also reduced BFB incidence by 41.8-49.4% when applied to watermelon seeds artificially inoculated with *A. citrulli*. Whole-genome sequence analysis revealed that two of the candidates that provided the best BFB control were *Bacillus safensis* strains. Furthermore, *B. mojavensis* RRC101 produced *A. citrulli* zones of inhibition on culture media, whereas *B.*

safensis strains did not. When *A. citrulli* was co-inoculated onto watermelon seeds with BCAs, there was a reduction in *A. citrulli* population growth by 2-3 orders of magnitude; however, the growth of the BCAs was not affected. In the field at anthesis, stigmatic papillae were pollinated and treated with 10^8 colony-forming units/flower of *B. mojavensis* RRC101, *B. safensis* strains, or water as a control. Resulting seeds were inoculated with a cocktail of *A. citrulli* strains and tested for BFB seed-to-seedling transmission in a grow-out assay. In two experiments, flowers treated with *B. mojavensis* RRC101 and *B. safensis* BCA#35 produced seeds with reduced BFB seedling transmission (41.2-47.9%) compared with seeds produced from non-treated flowers (81.2%). Findings from this study will help provide a new approach for protection against *A. citrulli*.

Keywords: *Acidovorax citrulli*, biological control agent, bacterial fruit blotch, *Bacillus safensis*, *Bacillus mojavensis*, *Citrullus lanatus*, watermelon.

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DEDICATION

I would like to dedicate this dissertation to my mother (Denise Sutton), and my grandparents (James and Shirley Sutton) whose love, support, and encouragement have guided me throughout my life. It was your selflessness that has allowed me to pursue my biggest aspirations and become the person I am today.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The *Cucurbitaceae* family is comprised of many genera and species that are cultivated as vegetable crops. Economically important members of this family include pumpkin and squash (*Cucurbita* spp.), watermelon (*Citrullus lanatus var. lanatus*), melon (*Cucumis melo*), and cucumber (*Cucumis sativus*) (Paris 2016). Of the above, watermelon is the most widely used species, accounting for approximately half of U.S. per capita consumption, followed by melons at 40%, and honeydew at 10% (Boriss et al. 2006). Watermelon is a xerophytic plant that originated in the Kalahari Desert of Africa (Robinson and Decker-Walters 1997; Schaffer and Paris 2016). The first harvest occurred approximately 5,000 years ago in Egypt, and fruit were often placed in the tombs of kings to nourish them in the afterlife (Paris 2015; Robinson and Decker-Walters 1997). By the 15th and 16th centuries, European colonists and Spanish or African slaves introduced watermelon to the New World, and subsequently the crop was grown throughout the world (Maynard and Maynard 2000).

Watermelon fruit is approximately 92% water, a significant source of lycopene, has no fat or cholesterol, and is an excellent source of vitamins A, B6, and C (Perkins-Veazie et al. 2007). According to the USDA Economic Research Service, in 2017 U.S. the hundredweight count (cwt) for fresh market watermelon production was approximately 39.9 million, and in terms of production, it was among the top three vegetables produced in the U.S. behind onions and head lettuce. According to the National Agricultural Statistics Service (NASS, USDA), the leading U.S. watermelon producing states are Florida, Georgia, California, and Texas; however,

China is the leading producer of watermelon worldwide. Watermelon is an important crop in the state of Georgia. Based on the 2017 Georgia Farmgate Value Report, watermelon was the 19th most valuable crop with a farm gate value of $134.8 million, and the third most valuable vegetable in Georgia behind sweet corn and onion (Wolfe and Stubbs 2017).

Seeds are efficient means of crop propagation, and over time the value of seeds has increased due to advances in technology and enhanced varieties (Afzal et al. 2016; Kozlowski and Gunn 1972). Globally, the seed market reached a value of $66.9 billion in 2018 and is expected to grow to $98.1 billion by 2024 (IMARC 2018). The U.S. seed industry is one of the leading providers of new technologies to improve food and fiber production. According to the International Seed Federation (ISF), the U.S. is the leader in exports and imports of vegetable seeds with 15.9 million metric tons exported (valued at $671 million), and 12,350 metric tons imported (valued at $371 million) as of 2016.

One of the most serious threats to watermelon production is *Acidovorax citrulli* (Schaad et al. 2008; Schaad et al. 1978; Willems et al. 1992), the bacterium causing bacterial fruit blotch (BFB). *A. citrulli* is seed-borne and seed-transmitted and can cause up to 100% yield loss (Bahar and Burdman 2010; Burdman and Walcott 2012). Seeds represent an important source of primary inoculum for BFB outbreaks; hence, the production and use of *A. citrulli*-free seed is critical for effective BFB management (Burdman and Walcott 2012; Rane and Latin 1992). Vegetable seed companies routinely produce watermelon seeds in regions of countries with cool and dry climates to limit seed infection by plant pathogens (Gitaitis and Walcott 2007; Walcott 2008). Despite these efforts, infested watermelon seed continue to be responsible for sporadic BFB outbreaks (Bahar and Burdman 2010; Burdman and Walcott 2012).

No single management practice can control BFB. Since the discovery that copper and streptomycin are effective against some phytobacteria, these chemicals have become commonly used methods of control (Sundin et al. 2016). Despite the efficacy of bactericides, overuse has resulted in the emergence of resistance in populations of some important phytobacteria (McManus et al. 2002). Also, the overuse of chemicals for pest management continues to have negative impacts on human health and the environment. (Aktar et al. 2009; Nicolopoulou-Stamati et al. 2016; Sabarwal et al. 2018). Approximately 453,000 kg of pesticides are used in the U.S. each year and 2.5 billion kg worldwide (Alavanja 2009). Due to the limitations of current management options, alternative approaches have been taken to manage phytobacteria. Options for alternative disease management include biocontrol, antimicrobial compounds, and inducers of systemic acquired resistance (Sundin et al. 2016). Like with most phytopathogenic bacteria, effective chemical control options are limited. Management of BFB poses additional challenges because of the seed-borne nature of the pathogen and the lack of elite resistant watermelon cultivars.

The Watermelon Research and Development Group conducted a survey between 2014 and 2015 to determine the top research and development priorities (Kousik et al. 2016). Results from the survey identified diseases and their management as top priorities. Some of the important priorities included Fusarium wilt, host resistance to gummy stem blight, bacterial fruit blotch, anthracnose, and cucumber green mild mottle virus. While Fusarium wilt and gummy stem blight resistance received 80 and 67% of the votes, respectively, BFB was ranked third by 57% of the respondents and was determined to be a re-emerging serious problem (Kousik et al. 2016).

Based on our current understanding of the biology of seed infection by *A. citrulli*, an effective seed treatment strategy that deposits biological control agents (BCAs) inside watermelon seeds might improve BFB management. Upon seed germination, such BCAs could rapidly colonize seedling tissues and prevent the establishment of *A. citrulli* populations. The focus of this research, therefore, was to improve the delivery of biocontrol treatments by depositing BCAs inside seeds via the stigmas of female watermelon flowers.

History of bacterial fruit blotch

Bacterial fruit blotch was first reported at the Regional Plant Introduction Station in Experiment (Griffin), GA. Initially, seedlings of two plant introduction (PI) lines (PI 174103 and PI 174104) from Turkey were reported as having necrosis on leaf and cotyledon tissues that resulted in seedling death (Webb and Goth 1965). Subsequently, a bacterium was isolated that produced white colonies on potato dextrose agar, and the pathogenicity and seed-borne and seed transmitted nature of the organism was confirmed (Webb and Goth 1965).

In 1969, Crall and Schenk reported large, dark-green, water-soaked lesions on watermelon fruits on a research farm at the Watermelon and Grape Investigations Laboratory in Florida (Crall and Schenck 1969). Additional fruit symptoms included lesions developing from small water-soaked areas and ooze associated with larger lesions (Crall and Schenck 1969). Interestingly, lesions were only noticed when fruit were close to harvest maturity, and did not penetrate deep into fruit tissues (Crall and Schenck 1969). Schaad et al. (1978) isolated and characterized ten non-florescent pseudomonads from water-soaked lesions on watermelon seedlings and identified them as *Pseudomonas pseudoalcaligenes* subsp. *citrulli.* Isolate C-42 was designated as the representative type strain and deposited into the American Type Culture Collection as strain ATCC 29625 (Schaad et al. 1978).

In 1987, 100% yield loss of fruit in watermelon fields was reported in the Mariana Islands (Wall et al. 1990). Symptoms were described as large water-soaked blotches on fruit rinds that started as small lesions that expanded quickly and sometimes covered the entire fruit. Using Koch's postulates, it was determined that a non-fluorescent pseudomonad was the cause of the disease and determinative tests showed that it was similar to *P. pseudoalcaligenes* subsp. *citrulli.* This was the first time an entire watermelon field was lost due to BFB (Wall et al. 1990). In 1989, BFB outbreaks occurred in watermelon fields in Florida with up to 50% loss of marketable fruit (Somodi et al. 1991). Also, in 1989, BFB was observed in Indiana on over 500 ha of watermelon seedlings (Latin and Rane 1990). Additionally, it was reported that 2 weeks before harvest, small dark-green blotches appeared on mature watermelon fruit in areas of the fruit rind that were not in contact with soil (Latin and Rane 1990).

Throughout the 1990s, sporadic BFB outbreaks occurred in Texas, Delaware, Indiana, and Oklahoma (Black et al. 1994; Evans and Mulrooney 1991; Jacobs et al. 1992; Latin and Rane 1990). To date, BFB has become a worldwide problem with outbreaks occurring in America, Asia, Europe, the Middle and Far East and Australia (Black et al. 1994; Burdman et al. 2005; Evans and Mulrooney 1991; Latin and Rane 1990; Mirik et al. 2006; O'Brien and Martin 1999; Palkovics et al. 2008; Ren et al. 2006; Schaad et al. 2003; Somodi et al. 1991).

Acidovorax citrulli physiology and taxonomy

Acidovorax citrulli (Schaad et al. 1978; Willems et al. 1992) is a Gram-negative bacterium that belongs to the family *Comamonadaceae* in the order *Burkholderiales*, class Betaproteobacteria, and the phylum Proteobacteria (Willems et al. 1992). It is a strict aerobe that is motile by a single polar flagellum. Cells are rod-shaped with average dimensions of 0.5 x 1.7 µm. *A. citrulli* colonies are smooth, round, convex, cream-colored to beige, 2 to 3 mm in

diameter and are non-fluorescent on King's B medium after 48 h (Schaad et al. 1978). Biochemically, the bacterium does not reduce nitrate or cause a hypersensitive response (HR) on tobacco. It is positive for lipid hydrolysis, grows at 41℃ but not 4℃, is oxidase-positive, and can utilize citrate, ethanol, fructose, β-alanine, ethanolamine, L-leucine, n-propanol, D-xylose and D-serine as carbon sources (Schaad et al. 1978).

Initially, the BFB pathogen was classified as *Pseudomonas pseudoalcaligenes* subsp. *citrulli* (Schaad et al. 1978). However, phenotypic studies and DNA-DNA hybridization experiments lead to its reclassification as *A. avenae* subsp. *citrulli* (Willems et al. 1992). Schaad et al. (2008) recommended that the bacterium be elevated to species level as *A. citrulli* based on genetic and phenotypic relatedness data that distinguished it from other *A. avenae* subspecies (Schaad et al. 2008).

Genetic diversity of *A. citrulli*

From the time of its discovery, there have been discrepancies regarding the genetic variation within *A. citrulli* populations. The first discrepancy was highlighted when the type strain (ATCC 29265), and strains isolated from outbreaks in Florida differed in their fatty acid and carbon substrate utilization profiles as well as their ability to induce a hypersensitive reaction on tobacco and tomato leaves (Somodi et al. 1991). In 1999, it was discovered that *A. citrulli* could infect rockmelon and honeydew, and it was suggested that the pathogen be considered a threat to all melon crops rather than just watermelon (O'Brien and Martin 1999). In 2004, it was suggested that *A. citrulli* populations could be categorized into two genetically distinct groups. To investigate the genetic diversity of *A. citrulli*, a comprehensive analysis of strains was conducted based on DNA fingerprinting by pulse-field gel electrophoresis (PFGE), repetitive element palindromic PCR (rep-PCR), sole carbon source utilization, and fatty acid methyl ester

(FAME) analysis (Walcott et al. 2004; Walcott et al. 2000a). Among a global population of *A. citrulli* strains, two genetically distinct groups (I and II) were confirmed (Walcott et al. 2004). The DNA fingerprinting data showed that 93% of strains recovered from watermelon belonged to group II. On the other hand, 18% of the strains recovered from watermelon belonged to group I (Walcott et al. 2004). Additionally, between 2000 and 2003, 12 bacterial isolates were recovered from a BFB outbreak on watermelon and melon in Israel and were characterized as *A. citrulli* based on pathogenicity tests, FAME analysis, and substrate utilization profiles (Burdman et al. 2005). Furthermore, DNA fingerprinting by PFGE and rep-PCR separated the 12 isolates into two genetically distinct groups (Burdman et al. 2005).

To further investigate diversity and build a database for reliable identification of *A. citrulli* strains, multilocus sequence typing (MLST) was conducted on 104 strains from China and around the world (Feng et al. 2009a). The MLST analysis clustered all tested strains into two clonal complexes (CC1 and CC2) with strains in the CC1 group recovered from watermelon and melon equally, and strains in the CC2 group primarily recovered from watermelon. The reliability of MLST relative to PFGE was shown in 2013 when 112 *A. citrulli* strains collected in China between 2000 and 2010 and six strains form other countries were characterized. MLST experiments resulted in a higher resolution in detecting genetic diversity within *A. citrulli* populations, and findings confirmed the existence of two distinct *A. citrulli* groups (Yan et al. 2013).

Host range and geographical distribution

The geographical center of origin of *A. citrulli* is unknown, but it is likely that the seed-borne nature of the pathogen facilitated its global dissemination. Since the early 1990s BFB has occurred around the world on a range of cucurbitaceous species. It has been reported to naturally

infect rockmelon (*Cucumis melo reticulates)*, honeydew (*Cucumis melo* var. *inodorus*), citron melon (*Citrullus lanatus* var. *citroides*), prickly paddy melon (*Cucumis myriocarpus*), cucumber (*Cucumis sativus*), pumpkin and squash (*Cucurbita* spp.) (Isakeit et al. 1997; Isakeit et al. 1998; Langston et al. 1999; Martin and Horlock 2002; Martin et al. 1999) .

Outbreaks have been reported in major watermelon production regions of Asia (Xinjiang and Inner Mongolia provinces, Taiwan, Japan) (Cai et al. 2005; Deng et al. 2010; Ren et al. 2006; Shirakawa et al. 2000), the Middle East (Turkey and Israel) (Burdman et al. 2005; Mirik et al. 2006), Europe (Hungary) (Palkovics et al. 2008), Oceania (Australia , Guam, Northern Mariana Islands) (Martin and Horlock 2002; O'Brien and Martin 1999; Wall et al. 1990), South America, Central America, the Caribbean (Brazil, Costa Rica, Nicaragua, Guadeloupe) (Cunty et al. 2019; Macagnan et al. 2003; Muñoz and Monterroso 2002), and North America (California, Florida, Georgia, Texas, Illinois, Delaware, Oregon) (Babadoost and Pataky 2002; Black et al. 1994; Evans and Mulrooney 1991; Hamm et al. 1997; Kumagai et al. 2014; Somodi et al. 1991; Walcott et al. 2000b).

Epidemiology

Infested seeds are the primary source of *A. citrulli* inoculum for BFB outbreaks. The majority of watermelons grown today are started as seedlings and transplanted to ensure high stand counts in the field. Under greenhouse conditions, seedlings are planted in close proximity to each other, and overhead irrigation in combination with hot and humid conditions promote disease development (Latin and Hopkins 1995). In greenhouse environments, a single seed containing 10_1 *A. citrulli* colony-forming units (CFU) can result in BFB seed-to-seedling transmission (Dutta et al. 2012b). Initial BFB symptoms appear as water-soaked lesions on the abaxial side of cotyledons. BFB symptoms may be inconspicuous and may escape visual

detection. Asymptomatic infected seedlings or seedlings that are overlooked by visual inspection may be transplanted into fields, where they can serve as sources of inoculum for BFB outbreaks.

BFB incidence and severity in the field are dependent on rainfall, relative humidity, temperature and windy conditions during the spring and summer (Bahar and Burdman 2010; Jett et al. 2002; Schaad et al. 2003). As plants mature in the field, secondary infections can occur on neighboring healthy plants and serve as inoculum sources for infection of immature fruit (Bahar and Burdman 2010; Burdman and Walcott 2012; Latin and Hopkins 1995). *A. citrulli* can ingress through the stomata of immature watermelon fruit leading to small water-soaked lesions that can cover the upper surface of the fruit (Frankle et al. 1993). Infected fruit that rot in the field can leave infected seeds in the soil. These seeds may germinate to produce infected volunteer watermelon plants that may serve as sources of inoculum for BFB outbreaks (Latin and Hopkins 1995).

BFB symptomatology

A. citrulli can infect all developmental stages of watermelon plants (Fig. 1.1). On seedlings, BFB symptoms start as water-soaked lesions on the abaxial sides of the cotyledons (Fig. 1.1A). Eventually, water-soaked lesions dry and become reddish- to dark-brown lesions along the leaf veins (Fig. 1.1B). At advanced stages of disease development, lesions on cotyledons, hypocotyls and stems may coalesce causing seedling collapse. On mature watermelon leaves, BFB lesions are light- to dark-brown and advance along the veins (Fig. 1.1C). BFB symptoms on cantaloupe leaves are similar to those produced on watermelon. On mature cantaloupe leaves, symptoms appear as tan to dark reddish-brown angular lesions surrounded by yellow halos, and also include large V-shaped lesions that extend from the leaf

margins to the base of the leaf. Symptoms on pumpkin leaves include elongated tan lesions along the leaf veins, and extensive chlorosis can sometimes be present.

On watermelon fruit, symptoms start as small, greasy, water-soaked lesions with irregular margins (Fig. 1.1E). At later stages, lesions develop into cracks in fruit rinds that may produce amber-colored or effervescent ooze (Fig. 1.1F). BFB infection may result in complete fruit rot. In melon fruit, symptoms start as water-soaked spots that become sunken as fruit mature. However, in netted melons (i.e. cantaloupe, muskmelon), the netting does not develop over lesions, resulting in sunken spots that do not expand as the fruit matures (Fig. 1.1G). These lesions penetrate through the fruit pericarp and cause brown cavities in the fruit flesh (Fig. 1.1H). Similar symptoms are reported on pumpkin fruit, including water-soaked rind lesions with cracks and internal fruit tissue rot.

Biology of seed infection

Seeds can become infested with pathogens by three pathways: 1) penetration of the ovary pericarp; 2) systemic movement through the vascular tissues; and 3) invasion of the pistil (Maude 1996). Seeds are the primary source of inoculum for *A. citrulli*. In 1979, 20% of watermelons harvested, from plants produced from infested seeds, resulted in infected seedlings. (Sowell and Schaad 1979). At this point, it was suspected that *A. ctirulli* was seed-transmitted. Similar findings were reported in 1992 when seedlings grown from seeds recovered from naturally infected watermelon fruit resulted in at least 7% BFB incidence (Rane and Latin 1992).

In 2003, Walcott et al. investigated the mechanisms by which watermelon seeds become infested by *A. citrulli*. They reported that female watermelon flowers that were pollinated and inoculated with *A. citrulli* yielded infested seeds within symptomless fruit (Walcott et al. 2003). In that study, 98% of fruit that developed were asymptomatic; however, 44% of the seed lots

assayed by *A. citrulli*-specific PCR assay were positive. Moreover, 27% of the seed lots developed symptomatic seedlings when tested by seedling grow-out assay. These observations suggested that female watermelon flowers are a pathway to seed infection by *A. citrulli*. Later, it was reported that there was a correlation between the concentration of inoculum applied to stigmas of female flowers and the percentage of infected seed lots from fruit that developed from these flowers (Lessl et al. 2007). Furthermore, there was a linear relationship between *A. citrulli* inoculum concentration applied to stigmas and the mean percentage of infested seed lots (Lessl et al. 2007).

Watermelon seeds have a thin semi-permeable layer, the perisperm-endosperm (PE) layer, under the seed coat that encloses the embryo (Ramakrishna and Amritphale 2005; Salanenka et al. 2009; Welbaum et al. 1995). Dutta et al. (2012) showed that *A. citrulli* cells that ingress through the pistil of female flowers accumulate under the PE layer, while cells that ingress by penetration of the fruit ovary pericarp accumulate under the seed coat but outside the PE layer (Dutta et al. 2012a). Additionally, *A. citrulli* can infest seeds as early as 1 day post inoculation through the pistil invasion pathway, and bacterial cells co-localized with the pollen tubes in the stigma, style, and ovary tissues (Dutta et al. 2015).

BFB management

Host resistance

Plant disease resistance is the most desirable option for disease management. Unfortunately, in the case of BFB, there are no commercially available resistant cultivars despite significant efforts. In 1979, watermelon plant introduction (PI) lines were screened for resistance to BFB. One hundred ten out of 740 PIs from the national watermelon collection tested in greenhouse conditions displayed BFB symptoms (Sowell and Schaad 1979). Additionally,

watermelon lines PI295843, PI299378, and Congo had lower BFB incidence than two other commercially available cultivars, Charleston Gray and Jubilee (Sowell and Schaad 1979). In 1981, 38 watermelon cultivars were tested for resistance against the *A. citrulli* strain isolated by Webb and Goth in 1965. Out of 38 cultivars, three (Garrisonian, Mountain Hoosier, and White Wonder) were reported to be the most resistant to BFB (Goth and Webb 1981). Rhodes et al. (1991) suggested that the watermelon PI line 295843 was immune to BFB, and two other lines, USDA WR Congo and USDA Congo, were resistant (Rhodes et al. 1991). These findings were similar to those reported by Sowell and Schaad in 1979 (Sowell and Schaad 1979).

As triploid varieties began to gain popularity, significant differences in BFB tolerance were observed among diploid and triploid watermelon cultivars. In 1994, several commercially available triploid varieties were reported to have reduced BFB incidence by at least 60% on fruit in the field despite being in close proximity to heavily infected diploid fruit with advanced symptoms (Garrett et al. 1995). Rhodes et al. (1996) reported that infection frequencies were higher in diploid cultivars that originated from infested seed than in triploids (Rhodes et al. 1996). Additionally, they observed that triploids had reduced disease severity from secondary infections, and that susceptibility may be associated with a lighter rind color; however, the study was inconclusive due to lack of replication and high variation of infection levels among replicates.

In 1993, 22 watermelon cultivars and two PIs were tested for resistance to BFB in greenhouse conditions. While significant differences in BFB severity were observed among cultivars, no cultivar was immune to BFB (Hopkins et al. 1993). Moreover, cultivars that were more susceptible generally had a lighter rind color (Hopkins et al. 1993). In 2002, Hopkins and Thompson screened 1,344 accessions of *Citrullus* spp. for BFB resistance under field and

greenhouse conditions and found that the most resistant accessions were PI 482279 (Zimbabwe) and PI 494817 (Zambia) (Hopkins and Thompson 2002). Subsequently, in 2008 Hopkins and Levi attempted to integrate BFB resistance from PI 482279 (Zimbabwe) and PI 494817 (Zambia) into Crimson Sweet, but were unsuccessful (Hopkins and Levi 2008).

Carvalho et al. (2013) evaluated different watermelon genotypes in the Northeast Brazilian Cucurbit Germplasm Active Bank at different stages of development for resistance to BFB. Three genotypes, BGCIA 979, BGCIA 34, and Sugar Baby showed highest resistance to BFB at most stages of plant development (Carvalho et al. 2013). In 2015, Ma and Wehner screened 1,699 watermelon cultigens including wild accessions, related species, and elite cultivars for resistance to BFB at the flowering stage in the field (Ma and Wehner 2015). Twenty-three of the most resistant cultigens were from Africa and there was no correlation between rind color and resistance to BFB as suggested by (Hopkins et al. 1993). Furthermore, Carvalho et al. (2013) suggested that Sugar Baby was resistant; however, Ma and Wehner (2015) found it to be susceptible.

With regards to BFB resistance in melon, 30 cultivars of hami melon (*Cucumis melo*), a type of muskmelon, were screened but none were immune (Yang et al. 2008). Bahar *et al.* (2009) used different techniques to inoculate different plant growth stages to evaluate resistance to BFB of various commercial cultivars, breeding, and wild lines of melon. They found that none of the tested cultivars were resistant to BFB; however, the level of tolerance among the cultivars varied in different assays (Bahar et al. 2009). Moreover, Wechter et al. (2011) screened 332 *Cucumis* spp. for BFB resistance using a seed vacuum-infusion assay (Wechter et al. 2011). Similar to the findings of Bahar et al. (2009), they found the majority of the lines to be susceptible to *A. citrulli*. However, they also observed several lines with higher levels of resistance. They selected 16 lines

for additional evaluation by spray-inoculation, and found that *C. melo* PI lines 353814, 381171, 536573, and 614401 had significantly higher levels of resistance than susceptible control cultivars (Wechter et al. 2011). Finally, in a study done in Turkey in 2012, 62 cucurbit genotypes including melons, cucumbers, and watermelon were assessed for their resistance to BFB. Three melon and four cucumber genotypes displayed some level of resistance to BFB (Yilmaz et al. 2012).

Exclusion

Since it has been difficult to develop resistant cultivars, producing pathogen-free seeds is the most effective option for BFB management. Rigorous preventative measures to exclude *A. citrulli* from seed production systems, such as selection of production areas and seed health testing, are routinely used reduce BFB outbreaks. While these approaches have been efficacious, they have disadvantages that allow opportunities for BFB outbreaks to occur. First, when selecting production areas, seed companies seek areas that have cool and dry climates for the duration of the growing season (Lovic and Hopkins 2003). Seeds produced in these areas have low risks of becoming infested with *A. citrulli*; however, outbreaks have occurred in production areas with these climatic conditions, such as California (Kumagai et al. 2014). This approach is difficult because each production area can have unpredictable environmental conditions, making it challenging to predict the risk of BFB outbreaks (Lovic and Hopkins 2003). Additionally, the pathogen may survive as an epiphyte without causing symptoms and asymptomatic fruit may harbor infected seeds (Silva et al. 2006).

Seed health testing is a critical component of BFB management. Seed production companies routinely utilize the seedling grow-out assay that require large subsamples of seed lots (10,000 to 30,000 seeds/lot) for testing (ISF 2018; NSHS 2017). Seed samples are grown in

greenhouse conditions conducive for disease development and visually inspected for BFB symptoms (ISF 2018; NSHS 2017). There are several criteria for conducting seedling grow-out assays that make them cumbersome and expensive, such as the losses that occur from destructive testing, the requirement for large greenhouse spaces and technicians that can identify infected seedlings, being time-consuming, and making sure the greenhouse maintains conditions to consistently promote disease (Walcott 2003). Because seedling grow-out assays can be time-consuming, PCR-based assays have become more popular for seed health testing (Bahar et al. 2008; Park et al. 2008; Song et al. 2003). However, while PCR-based assays can be rapid, PCR inhibitors present in seeds negatively affect the efficiency and accuracy the test. To increase sensitivity and accuracy of PCR-based approaches, techniques such as immunomagnetic separation polymerase chain reaction (IMS-PCR), BIO-PCR and magnetic capture hybridization (MCH) have been developed (Ha et al. 2009; Walcott et al. 2006; Walcott and Gitaitis 2000; Zhao et al. 2009). The efficacy of PCR-based assays is dependent on the threshold of detection and the method of extraction of microbial DNA (Giovanardi et al. 2018; Tian et al. 2016). In 2011, *A. citrulli* enrichment methods including membrane filtration, IMS-PCR, and immune-capture PCR (ICP) were compared, and ICP resulted in the lowest limit of detection (10^2 CFU/ml) and the highest degree of precision (Wang et al. 2011). Despite the precision and sensitivity of these assays, they are still not utilized routinely for seed health testing. Moreover, serological tests such as enzyme-linked immunosorbent assays (ELISA), immuno-isolation (IIS) and immuno-strip tests are typically not utilized due to the low detection sensitivity in seeds and the possibility of false-positive and false-negative results (Feng et al. 2013).

Chemical treatments

To date, there are few commercially available chemical options for managing BFB. The main foliar-applied chemicals are copper-based compounds, such as cupric hydroxide, copper hydrosulfate, or copper oxychloride (ASTA 2009; Hopkins 1991). In 1991. Hopkins reported that when cupric hydroxide alone or in combination with fosetyl-AL was applied to watermelon as a spray treatment, yield loss was reduced by approximately 59% compared with fields that were untreated (Hopkins 1991). Copper has been used as a preventative treatment for BFB; however, due to the phytotoxicity to plants and the development of copper tolerance by *A. citrulli*, these treatments are not sustainable for long-term use (Cervantes and Guiterrez-Corona 1994; Hopkins 1995; Zitter and Rosenberger 2013).

When applied in transplant houses, peroxyacetic acid at 80 µg/ml and ionized copper at 1.0 and 1.5 µg/ml applied through the daily irrigation water was more effective at reducing the spread of *A. citrulli* than cupric hydroxide (Hopkins et al. 2009). Also, Hopkins et al. (2009) demonstrated that combining ionized copper or peroxyacetic acid in irrigation water with a weekly foliar application of acibenzolar-S-methyl (ASM) was also effective in reducing the spread of *A. citrulli* and recommended a combination of applying these chemicals with other management practices (Hopkins et al. 2009). Cabral et al. (2010) studied the effect of ASM, mannano-oligosaccharides (MOS), and citrus bioflavonoids (BFC) for their ability to control BFB on two hybrid melons, yellow melon (hybrid AF4945) and Pele de Sapo melon (hybrid Nilo) (Cabral et al. 2010). They reported that 10 days after plant emergence, applications of BFC and ASM reduced disease incidence between 60 and 88% (Cabral et al. 2010).

There have been other attempts to find effective chemical controls for BFB, such as the use of antibiotics and essential oils. For example, spraying 2-week-old melon seedlings at the 3-4

leaf stage with copper, kasugamycin-copper, and organocopper 3-5 days before inoculation in greenhouse conditions prevented the spread of BFB (Shimizu et al. 2008). Additionally, when watermelon seeds were soaked in kasugamycin alone, or in combination with 72% streptomycin or 72% embamycin, the BFB incidence was lowered (He et al. 2011). Plant extracts such as essential oils can have antimicrobial activity (Deans and Ritchie 1987; Nazzaro et al. 2013). The antimicrobial activity of essential oils against *A. citrulli* was reported by Mengulluoglu and Soylu (2012). In their study, the authors used disc diffusion assays to test the antimicrobial activity of several essential oils against *A. citrulli* and found that thyme and origanum oils produced the largest zones of inhibition between 9.4 and 20.7 mm (Mengulluoglu and Soylu 2012). Despite the promise of essential oils, drawbacks include not being able to produce large quantities of effective oils, their stability, potency and testing having been limited to *in vivo* efficacy (Bajpai et al. 2011; Chouhan et al. 2017).

Seed treatments

Hopkins et al. (1996) demonstrated that fermentation in watermelon juice, treatment with 1% HCL or $CaOCl_2$ followed by washing and drying seeds reduced the level of BFB seed transmission (Hopkins et al. 1996). Despite being effective, fermentation is not routinely used because it can cause seed discoloration and have negative effects on germination in some hybrids (Walcott 2008; Welbaum 2005). Rane and Latin (1992) reported that treatment of infested seeds with 0.525% NaOCl for 20 min or 1.8% HCl for 5 min reduced BFB seedling transmission, but did not eradicate *A. citrulli* from seeds (Rane and Latin 1992). The most effective treatment for *A. citrulli* was to ferment seeds for 24-48 h followed by treatment with 1% HCl or 1% $CaOCl_2$ followed by washing and drying. However, this method may not be applicable for all cucurbit seeds (Hopkins et al. 1996; Walcott 2008). Treating seeds with 1600 µg/l of peroxyacetic acid

for 30 min followed by drying at 40℃ with low humidity for 48 h eliminated seed transmission of BFB from artificially inoculated seeds. However, *A. citrulli* can be localized deep within the seeds, where it is difficult for chemical treatments to penetrate (Hopkins et al. 2003). Feng et al. (2009) demonstrated that seed treatment with NaOCl at 50℃ for 20 min, peroxyacetic acid for 30 min, or acidified cupric acetate at 50℃ for 20 min eradicated *A. citrulli*. However, seed germination and emergence were negatively affected (Feng et al. 2009b). They also reported that treating seeds with acidic electrolyzed water for 30 min eradicated *A. citrulli* without negative effects on seed germination and seedling establishment.

Other approaches, such as thermotherapy, have been used for treating seeds for *A. citrulli* management. In 1989, Wall reported that incubating seeds at 50-55℃ for 10-30 min eliminated *A. citrulli* without affecting seed health (Wall 1989). However, there was a discrepancy regarding the effectiveness of the approach since these results could not be reproduced subsequently (Rane and Latin 1992; Walcott 2008). In 2006, Hopkins and Thompson reported that treating seeds at 55°C for 2 h and at 60°C for 1 h eliminated seed transmission (Hopkins and Thompson 2006), even though the 60°C treatment negatively impacted seed germination. Additionally, thermotherapy is difficult to employ commercially because all infested seeds are not uniformly exposed to the effective temperature. Therefore, it cannot ensure pathogen eradication on a commercial scale (Walcott 2008). Dry heat was also evaluated to decontaminate melon, cucumber, squash, and gourd seeds infected with *A. citrulli* (Kubota et al. 2012). The authors reported that seeds exposed to 85℃ dry heat for 3-5 days eliminated *A. citrulli* without affecting seed health.

Biological control

Problems associated with utilization of chemicals for management of plant diseases have increased interest in finding sustainable solutions for integrated disease management (Aktar et al. 2009; Dewaard et al. 1993; Zhan et al. 2014). Identifying and utilizing biocontrol agents (BCAs) is a desirable option for managing plant diseases. Broadly, biological control is the use of beneficial and antagonistic microbes to suppress plant disease (Köhl et al. 2019; Pal and McSpadden Gardener 2006). Biological control seed treatments have been suggested as an effective and sustainable approach for managing seed-borne diseases (Spadaro et al. 2017; Taylor and Harman 1990) (El-Mougy and Abdel-Kader 2008; Mancini and Romanazzi 2014; Matarese et al. 2012; Song et al. 2017).

Since *A. citrulli* can utilize the pistil invasion pathway to infect seeds, the efficacy of *Pseudomonas fluorescens* (A506), *Acidovorax avenae* subsp. *avenae* (AAA 99-2), and an unidentified Gram-positive bacterium recovered from watermelon seed (WS-1) were evaluated as flower treatments (Fessehaie and Walcott 2005). When applied to female watermelon flowers 5 h prior to inoculation with *A. citrulli*, AAA 99-2 yielded seed lots with 13.8% infested seeds, which was significantly lower than for seeds from flowers treated with *P. fluorescens* A506 (21.1%), Kocide (21.1%), 0.1M PBS (63%), and *WS-1* (48.3%) (Fessehaie and Walcott 2005). Despite these promising results, there was a need to find more appropriate BCA candidates since AAA99-2 was pathogenic to maize. As a result, a non-pathogenic type III secretion mutant of *A. citrulli* (AAC00-1*ΔhrcC)* was evaluated as a BCA (Johnson et al. 2011; Walcott et al. 2003). Johnson et al. (2011) demonstrated that AAC00-1*ΔhrcC* could colonize germinating watermelon seeds at wild-type *A. citrulli* levels, reduce BFB seedling transmission when applied as a seed treatment, and reduce seed-to-seedling transmission of BFB when applied as a flower protectant

(Johnson et al. 2011). Despite this, the AAC00-1*ΔhrcC* mutant is genetically modified and unsuitable as commercial application.

In 2006, 96 epiphytic and 69 endophytic bacterial strains were isolated from asymptomatic melon plants and screened for their efficacy against BFB (Oliveira et al. 2006). Seeds inoculated with *A. citrulli* and subsequently treated with *Bacillus* spp. showed reduced BFB incidence and severity (Oliveira et al. 2006). Santos et al. (2006) reported that *Bacillus* spp. produced compounds in fermented broths of *B. subtilis* R14, *B. megaterium* pv. *cerealis* RAB7, *B. pumilus* C116 and *Bacillus* sp. MEN2, with and without bacterial cells that displayed antimicrobial activity against *A. citrulli* in *in vitro* assays (Santos et al. 2006). Additionally, they reported that the *B. megaterium* pv. *cerealis* strain RAB7 reduced BFB incidence by 89% and slowed the rate of disease development by almost 3 days.

After 2006, more *Bacillus* spp. and other microbes with antagonistic activity against *A. citrulli* were reported. For example, Cai et al. (2009) recovered 95 bacterial strains from spinach and cucurbit crops, five of which were identified as *Bacillus* spp. with antagonistic activity against *A. citrulli*. Medeiros et al. (2009) found that *Bacillus* sp. RAB9 slowed BFB disease development by 47% when applied to infected seeds (Medeiros et al. 2009). Additionally, they reported that when *Paenibacillus lentimorbus* MEN2 was sprayed on melon seedlings, BFB development was reduced by 88%, severity was reduced by 81%, and incidence was reduced by 77%. In 2015, a strain *of Bacillus amyloliquefaciens* was reported to promote plant growth and control BFB by increasing the expression of the defense-related gene *PR1* and inducing hydrogen peroxide accumulation in watermelon (Jiang et al. 2015). Also, Fan et al. (2017) demonstrated that *Bacillus subtilis* 9407 reduced BFB incidence by 62% under greenhouse conditions. The mode of action for this strain included competition and surfactin-mediated

antibacterial activity (Fan et al. 2017).

Other microbes besides *Bacillus* spp. have been identified as potential BCAs against BFB. In 2006 several *Streptomyces* isolates were reported to produce zones of inhibition against A. *citrulli in vitro* (Yaeram et al. 2006). This suggested that *Streptomyces* spp. produce antimicrobial compounds that have activity against *A. citrulli.* Epiphytic yeasts have also been screened for use as BCAs against BFB. Wang et al. (2009) reported that a yeast strain, *Pichia anomala* 0732-1, reduced BFB incidence and severity to similar levels as streptomycin sulfate and HCl when applied as a seed or seedling treatment. Another study reported two rhizosphere-associated bacteria, *Paenibacillus polymyxa* SN-22 and *Sinomonas atrocyanea* NSB-27, that reduced BFB severity in greenhouse conditions when applied as a soil treatment (Adhikari et al. 2017). Despite these reports, further optimization of parameters related to biocontrol seed treatments for *A. citrulli*, and an understanding of the mode of action could improve the efficacy of biocontrol.

Research objectives

Although biological control has been studied widely for suppressing plant diseases, there have been limited studies on biocontrol of BFB. Additionally, there are limited BFB management strategies that involve biological control seed treatments. We propose to utilize the pistil invasion pathway to introduce BCAs into watermelon seeds and thereby limit seedling transmission of BFB. This approach could yield an environmentally sound and sustainable management strategy by which seed producers can reduce BFB outbreaks in watermelon production systems.

The specific objectives of this study are to:

1) Select and characterize candidate BCAs that are antagonistic against *A. citrulli*.

2) Identify these candidate BCAs.
3) Evaluate the efficacy of candidate BCAs as flower treatments under field conditions to internally protect watermelon seeds against *A. citrulli*.

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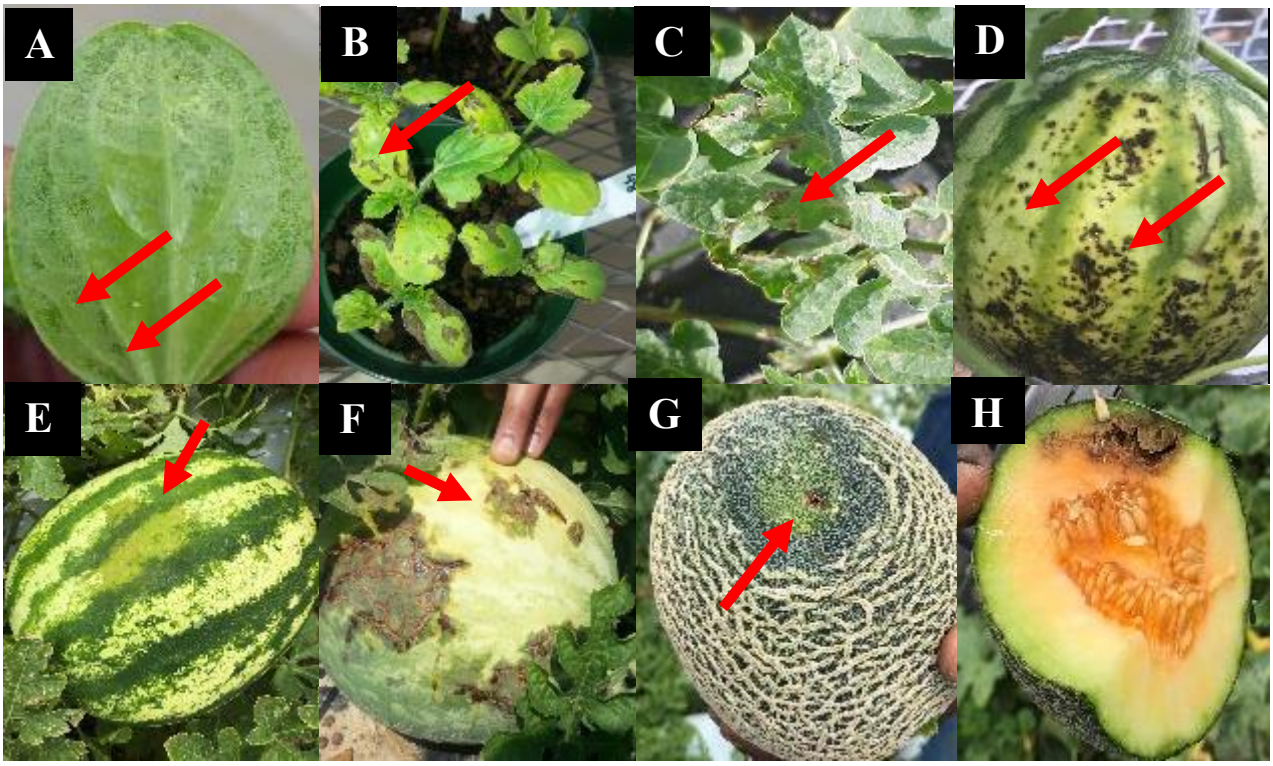

Figure 1.1. Bacterial fruit blotch symptoms on different developmental stages of watermelon and melon. A, 1-week-old cotyledon displaying water soaking; B, 2-week-old seedlings displaying advanced lesions; C, mature leaf displaying advanced lesions; D, 2-week-old fruit displaying water-soaking and advanced lesions; E, mature fruit displaying initial water-soaking symptoms; F, mature fruit displaying advanced stages of disease development with water-soaking, fruit cracking, and lesions with irregular margins; G, young infected melon fruit displaying sunken spots and restriction of netting around infected area; H, immature melon fruit displaying advanced disease symptoms where the lesion has penetrated through the fruit causing a large brown cavity to form.

CHAPTER 2

SELECTION OF BIOCONTROL AGENTS WITH ANTAGONISTIC ACTIVITY AGAINST *ACIDOVORAX CITRULLI*

Sutton, S. and Walcott, R. to be submitted to Plant Disease

ABSTRACT

Bacterial fruit blotch (BFB), caused by the bacterium *Acidovorax citrulli*, is a major seed-borne and seed-transmitted pathogen of cucurbits worldwide. Currently, a peroxyacetic acid/hydrogen peroxide mix is the most commonly used commercial seed treatment for BFB management. Despite this, BFB outbreaks continue to occur suggesting that additional management strategies are needed. This study aimed to identify watermelon seed microflora that are antagonistic to *A. citrulli* in order to develop potential biocontrol agents (BCAs). Eleven candidate BCAs were isolated from watermelon seedlings germinated for 48 h. These strains colonized 24-h watermelon seed exudate and reached higher population levels in the exudate than *A. citrulli*. Nine of eleven strains also reduced BFB incidence by 41.8-49.4% when applied to watermelon seeds artificially inoculated with *A. citrulli*. Candidates that provided the best BFB control (5.2 and 7.3% transmission, respectively, relative to the control transmission of 54.6%) were strains 24 (BCA#24) and 35 (BCA#35). In later studies, fatty acid-methyl ester analysis, and whole genome sequence analysis via two *in silico* platforms, the average nucleotide identity calculator and genome-to-genome distance calculator, revealed that BCA#24 and #35 had 96% or higher sequence identity to *Bacillus safensis*. A known BCA, *Bacillus mojavensis* RRC101 also reduced BFB seedling transmission to 7.5% and produced *A. citrulli* zones of inhibition on Luria-Bertani and potato dextrose agar media. In contrast, BCA#24 and BCA#35 did not produce such inhibition zones. When *A. citrulli* was co-inoculated onto watermelon seeds with BCAs, there was a reduction in *A. citrulli* population growth by 2-3 orders of magnitude, whereas the growth of the BCA was not affected.

Keywords: *Acidovorax citrulli*, *Bacillus safensis*, *Bacillus mojavensis*, biological control agents, seed microflora, *Citrillus lanatus*, bacterial fruit blotch.

INTRODUCTION

Acidovorax citrulli (Schaad et al. 2008; Schaad et al. 1978) is a Gram-negative bacterium that causes bacterial fruit blotch (BFB) of cucurbits. Since 1989, when BFB first caused economic damage in commercial watermelon fields in Florida, successive outbreaks have occurred across the southeastern and midwestern U.S. (Black et al. 1994; Evans and Mulrooney 1991; Jacobs et al. 1992; Latin and Rane 1990; Somodi et al. 1991). BFB has since become a sporadic, but economically important disease worldwide and has occurred in Asia (China, Taiwan, Japan) (Cai et al. 2005; Deng et al. 2010; Ren et al. 2006; Shirakawa et al. 2000), the Middle East (Turkey and Israel) (Burdman et al. 2005; Mirik et al. 2006), Europe (Hungary) (Palkovics et al. 2008), Oceania (Australia, Northern Mariana Islands) (Martin and Horlock 2002; O'Brien and Martin 1999; Wall et al. 1990), South America, Central America, and the Caribbean (Brazil, Costa Rica, Nicaragua, Guadeloupe) (Cunty et al. 2019; Macagnan et al. 2003; Muñoz and Monterroso 2002) and on a range of cucurbits including melon (*Cucumis melo reticulates)*, honeydew (*Cucumis melo* var. *inodorus*.), citron melon (*Citrullus lanatus* var. *citroides*), prickly paddy melon (*Cucumis myriocarpus*), cucumber (*Cucumis sativus*), pumpkin and squash (*Cucurbita* spp.) (Isakeit et al. 1997; Isakeit et al. 1998; Langston et al. 1999; Martin and Horlock 2002; Martin et al. 1999).

Seeds represent an important source of inoculum of *A. citrulli*, and the bacterium can survive for up to 30 years in infected seeds (Block and Shepherd 2008; Latin and Hopkins 1995). Long-term survival can be explained by the fact that the bacterium can ingress through the pistil of the female flowers and localize in embryos of watermelon seeds (Dutta et al. 2012; Dutta et al. 2016). Producing *A. citrulli*-free seeds is critical for managing BFB (Rane and Latin 1992).

Several chemical seed treatments including chlorine dioxide, sodium hypochlorite, peroxyacetic acid, hydrogen peroxide, acetified cupric acetate, calcium hypochlorite, hydrochloric acid, and acetic acid have been employed to manage BFB; however, none are 100% effective (Chao et al. 2010; Feng et al. 2009; Hopkins et al. 1996; Hopkins and Thompson 2006; Hopkins et al. 2003). To date, the most effective approach for managing BFB is producing pathogen-free seeds. This can be achieved by producing seeds in regions with cool, dry climates, combined with visual field inspections and rigorous seed health testing (Lovic and Hopkins 2003). Despite these efforts, BFB is still difficult to manage (Walcott 2003; Walcott et al. 2006; Walcott and Gitaitis 2000).

One desirable approach for BFB management is biological control. Generally, biological control of plant pathogens involves utilization of organisms that inhibit pathogen growth and reduce disease (Nega 2014). There is a growing interest in using biological control agents (BCAs) for controlling a broad spectrum of plant diseases (Chowdhury et al. 2015; Daguerre et al. 2016; Daranas et al. 2019). Most of the literature on biocontrol of plant diseases has focused on fungal pathogens, with limited attention to diseases caused by bacteria (Gerbore et al. 2014; Nega 2014; Pal and McSpadden Gardener 2006). The potential advantages of using BCAs include increased plant health, multiple modes of action, minimal impact on microflora diversity, sustainability, reduced toxicity, and enhanced plant resistance to pathogens and other environmental stressors (Gerbore et al. 2014; Hossain et al. 2015; Jensen et al. 2004; Mao et al. 1998; Nega 2014; Pal and McSpadden Gardener 2006). The most common genera of microorganisms associated with biocontrol of plant diseases include *Bacillus* spp., *Pseudomonas* spp., *Streptomyces* spp., and *Trichoderma* spp. (Ongena and Jacques 2008; Pérez et al. 2015; Vurukonda et al. 2018; Weller 2005). The *Bacillus* genus includes some of the most commonly

studied BCAs, and *Bacillus* spp. have been reported to be antagonistic against *A. citrulli* (Adhikari et al. 2017; Fan et al. 2017; Jiang et al. 2015; Medeiros et al. 2009). Several modes of action contribute to biological control of plant diseases, such as hyper-parasitism/predation, antibiosis, lytic enzymes, physical/chemical interference, competition, and induction of host resistance (Jamalizadeh et al. 2011; Nega 2014; Pal and McSpadden Gardener 2006). *Bacillus* spp. have been found to limit plant disease development by antibiosis, competition, and induction of plant host resistance (Chowdhury et al. 2015; Fira et al. 2018; Pal and McSpadden Gardener 2006).

Seed treatment with BCAs has been reported to reduce disease and improve seed health. Gupta et al. (2002) demonstrated that treating seeds with *Pseudomonas aeruginosa* GRC2, a strain isolated from the potato rhizosphere, reduced charcoal rot of peanut by more than 70%, increased germination by 10-40% compared with non-treated seeds or seeds inoculated with *Macrophomina phaseolina* and increased overall plant health (Gupta et al. 2002). Bio-fungicides and plant extracts have also resulted in increased plant health and disease reduction when used as seed treatments. Applying a *Trichoderma*-based fungicide or plant extracts from garlic, ginger, kaligera, neem, and mehedi to wheat seeds resulted in a higher grain yield in wheat, reduced disease severity and reduced mycelial growth of *Bipolaris sorokiniana* (Hossain et al. 2015). Fessehaie and Walcott (2005) reported that naturally infested watermelon seeds treated with *A. avenae* (AAA99-2) (Schaad et al. 2008) reduced BFB seed-to-seedling transmission by 97% (Fessehaie and Walcott 2005). However, *A. avenae* AAA9-2 was pathogenic on maize, and hence unsuitable for commercial application. More recently, Johnson et al. (2011) generated a nonpathogenic strain of *A. citrulli* (AAC00-1*ΔhrcC*) by deleting the *hrcC* gene that encodes the type III outer membrane pore. When AAC00-1*ΔhrcC* was applied to watermelon seeds naturally

infected with pathogenic *A. citrulli*, the BFB seed-to-seedling transmission percentage was significantly reduced, relative to the negative control. However, AAC00-1*ΔhrcC* is a genetically modified strain, and thus unsuitable for commercial application as a seed treatment. Despite advances in seed treatments, effective biocontrol seed treatments are not available for BFB management. For successful application, BCAs should be selected based on their ability to survive in the environment in which they will be deployed.

The aims of this study, therefore, were to select *A. citrulli* BCAs naturally adapted to the watermelon seed environment and to characterize the mode of action of these microorganisms. We screened 11 bacterial candidates and selected the top three that were antagonistic to *A. citrulli* through a series of *in vitro* assays. Two of the top three candidates selected were isolated from watermelon seed microflora and identified in later studies as strains of *Bacillus safensis* based on fatty acid methyl-ester analysis and whole genome analysis via two *in silico* platforms, the average nucleotide identity calculator (ANI) and genome-to-genome distance calculator (GGDC).

MATERIALS AND METHODS

Isolation of candidate BCAs from watermelon seedlings

Watermelon seeds (n = 7) of an unknown cultivar were placed in sterilized Petri dishes lined with sterilized blotter paper (Hoffman Manufacturing Inc., Corvallis, OR) and saturated with sterilized distilled deionized water (sddH_2O). Seeds were allowed to germinate at 28°C and 85% relative humidity (RH) with continuous fluorescent light for 48 h. Individual seeds were crushed in microcentrifuge tubes with 1 ml of 0.1 M phosphate buffered saline (PBS) and ten-fold serial dilutions of the seed macerate were spread onto nutrient agar plates (Beckton–

Dickinson, Sparks, MD). Plates were incubated at 28°C for 48 h and ten colonies were selected and stored at -20℃ until further use.

Bacterial isolates and culture

Bacteria isolated from seed macerate from watermelon seeds that were germinated for 48 h or a bacterium with known biological control characteristics were used in this study (Table 2.1). Bacterial strains were routinely grown on Luria-Bertani (LB) agar (VWR Life Science, Radnor, PA) for 24 h at 28 or 37°C. As needed, rifampin (50 μg/ml) was added to LB media. To prepare inoculum, LB broth was inoculated with a single colony of each isolate and incubated overnight at 28°C on a rotary shaker (Innova, New Brunswick Scientific Co., Edison, NJ) at 120 min^{-1}. Five milliliters of broth cultures were centrifuged at 10,000 min^{-1} for 5 min. The supernatant was decanted, and the pellet was suspended in sddH_2O. Bacterial concentrations were estimated spectrophotometrically with an optical density OD_{600} 0.3, corresponding to ~ 1 x 10^8 colony-forming units (CFU)/ml, and adjusted to the desired concentration by ten-fold serial dilutions.

A. citrulli strains used in this study are listed in Table 2.1. Strains were routinely grown on King's medium B (KMB) (King et al. 1954) for 48 h at 28°C, and, as needed, gentamycin (50 μg/ml) was added. To prepare *A. citrulli* inoculum, LB broth was inoculated with a single colony of each strain and incubated overnight at 28°C on a rotary shaker (Innova) at 120 min^{-1}. Cultures were centrifuged at 10,000 $min^{-1}$for 5 min and the pellet was re-suspended in sddH2O. Bacterial concentrations were determined spectrophotometrically with an optical density OD_{600} = 0.3 (~10^8 CFU/ml) and adjusted to the desired final concentrations with sddH2O.

Preparation of watermelon seed exudate

Watermelon seed exudate (SE) was used to simulate the germinating watermelon seed spermosphere. Seed exudate was collected from germinating watermelon seeds as follows. Twenty-six grams of seeds were added to 100 ml of sddH_2O in a sterilized flask and incubated at 28℃ on a rotary shaker (Innova) at 120 min^{-1} for 24 h. Seed exudate debris was removed by centrifugation at 10,000 min^{-1} for 5 min, and the supernatant was transferred to a sterilized 250-ml beaker. The pH of the SE was adjusted to 7.0 and the SE was passed through a 0.2-μm pore rapid-flow sterile disposable filter (Thermo Fisher Scientific, Waltham, MA) and stored at 4℃ for immediate use or -20℃ for long-term storage.

BCA growth in watermelon SE

Bacteria isolated from watermelon seeds were screened for their ability to colonize exudates from 24-h germinating watermelon seeds using a Bioscreen C automated microbial growth curve analysis system (Oy Growth Curves AB Ltd, Helsinki, Finland). Honeycomb 2 plates (Thermo Fisher Scientific) were filled with 360 µl of 24-h SE plus 40 µl of a 10^5 CFU/ml suspension of each BCA, AAC00-1 and AAC213-60. Wells loaded with SE alone served as negative controls. Plates were then incubated in the Bioscreen C machine for 24 h at 28℃ with continuous shaking, and OD readings were recorded at 30-min intervals. One plate represented one experiment and each sample was replicated in 10 wells per plate. The experiment was conducted two times and the area under the population growth curve (AUPGC) was calculated using the trapezoidal rule (Simko and Piepho 2012). Analysis of variance (ANOVA) was applied to AUPGC values recorded for two biological replications to assess differences in AUPGC between candidate BCAs and *A. citrulli* strains, and mean separation was conducted using Tukey's test.

Biocontrol seed treatment assays

Watermelon seeds (cv. Crimson Sweet) (Johnny's Selected Seeds, Fairfield, ME) (n = 40 seeds) were inoculated with a bacterial suspension of *A. citrulli* strains from two groups. Strains from group I have been generally isolated from non-watermelon cucurbits, while strains from group II are closely associated with watermelon. A cocktail of inoculum was generated containing 10_6 CFU/ml of a mixture of group I *(*AAC213-60*)* (Silva et al. 2016) and II (AAC00-1) (Walcott et al. 2000) strains by vacuum-infiltration explained below similar to experiments described by Dutta *et al*. (2016) (Dutta et al. 2016). Seeds were placed in sterilized beakers containing 50 ml of the bacterial suspension, and beakers placed in a Nalgene vacuum chamber (Thermo Fisher Scientific) and attached to a vacuum line. Cell suspensions were infiltrated into seeds by applying a vacuum to the chamber in four 10-min intervals. Seeds were removed from the cell suspensions and air-dried at room temperature for 24 h. Subsequently, seeds were vacuum-infiltrated with 10_8 CFU/ml of each candidate BCA, as described above, and air-dried at room temperature for 24 h. Inoculated seeds were placed into test tubes (Pyrex), one seed/tube, with plastic caps and a cotton ball saturated with sddH20 and allowed to germinate and grow for 14 days at 100% RH and 30°C. Percent BFB incidence and germination were recorded for the seedlings. This experiment was conducted three times. ANOVA was conducted on *A. citrulli* disease incidence for three biological replications to determine the effect of candidate BCAs on BFB seed-to-seedling transmission, and treatment means were separated using Tukey's test.

Elucidation of the MOA of BCAs against *A. citrulli*

Antibiosis. An *in vitro* zone of inhibition (ZOI) assay was used to investigate if production of secondary metabolites contributed to biocontrol activity against *A. citrulli. A.* citrulli inoculum was prepared as described above. Bacterial concentrations were determined spectrophotometrically (OD_{600} = 0.3, ≈10_8 CFU/ml) and 100 µl of a 10_8 CFU/ml suspension of

each *A. citrulli* strain was spread onto potato dextrose agar (PDA) (Thermo Fisher Scientific), nutrient agar (NA) (Difco, Becton Dickinson and Co., Sparks, MD) LB (VWR Chemicals, Solon, OH), or KMB agar plates with sterilized cotton tip applicators (World Wide Life Sciences Division, Bristol, PA). Plates were then allowed to air-dry at room temperature for 2 min. Cells were harvested from plate cultures containing BCAs and deposited onto plates that had been previously inoculated with *A. citrulli* strain AAC00-1 or AAC213-60. Each candidate BCA was spotted four times on an individual plate and AAC00-1Δ*hrcC* (non-pathogenic T3SS mutant) (Johnson et al. 2011) was used as a negative control. Plates were incubated at room temperature for 72 h and the diameters of the ZOI were measured. Experiments were conducted three times.

Competition. To determine if competition played a role in the biocontrol of *A. citrulli* on germinating watermelon seeds by candidate BCAs, co-inoculation/competition assays were conducted. In order to reduce contamination by seed microflora, seed coats were removed from watermelon seeds cv. Crimson Sweet (n = 24 seeds/treatment). After de-coating, seeds were placed on a cotton ball saturated with sddH_2O in a well of a 24-well flat-bottom cell culture plate with a lid (Stem Cell Technologies, Vancouver, CA). Treatments included a constitutive green fluorescent protein (GFP) mutant of *A. citrulli* AAC00-1GFP in order to readily identify *A. citrulli* colonies, the known BCA *B. mojavensis* RC112 (Bacon et al. 2012), *B. safensis* strain 35 (isolated in the present study), and each *Bacillus* spp. co-inoculated with AAC00-1GFP. A micropipette was used to deliver 5 µl of each bacterial suspension containing ~ 10_4 CFU to seeds, and samples were incubated with constant fluorescent light at 30°C and 100% RH for 3 days. Each day, four seeds were sampled individually and crushed in 1 ml of sddH2O. Three 10-µl aliquots of suspension from each seed macerate were spotted on LB agar amended with gentamycin to select for *A. citrulli*, or rifampin to select for BCAs. Colonies were counted to

determine the mean CFU/seed for each treatment. ANOVA was used to determine the effect of BCAs on the growth of *A. citrulli* populations on germinating watermelon seeds, and mean separation tests were conducted using Tukey's test.

Induced systemic resistance. BCAs were evaluated for their ability to trigger induced systemic resistance (ISR) in germinating melon seedlings. For each treatment, 20 melon (cv. Joaquin Gold) seeds were vacuum-infiltrated for 40 min with bacterial suspensions of *B. mojavensis* RRC101 or *B. safensis* strain 35 containing 10_8 CFU/ml. Vacuum infiltration was interrupted at 10-min intervals. As a positive control, a 45-µM solution of the plant defense activator methyl jasmonate, (MeJA) (Sigma Aldrich, St. Louis, MO) was vacuum-infiltrated into melon seeds. Melon seeds vacuum-infiltrated with sddH_2O served as negative controls. Treated seeds were placed on paper towels saturated with sddH_2O in 108-cm_3 clear plastic boxes (Uline, Pleasant Prairie, WI) and incubated at 28°C with 100% RH and constant fluorescent light for 6 days. Three seeds were sampled individually per treatment at 3 and 6 days after germination.

For RNA extraction, melon seeds (n = 3) were de-coated and RNA was extracted from ~90 mg of tissue using the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA) according to manufacturer's instructions. Total RNA concentration was determined using a ND-1000 spectrophotometer (Thermo Fisher Scientific), and 200 ng/µl of RNA was used for cDNA synthesis with a TAKARA Prime Script First Strand cDNA Synthesis Kit (Clonetech Laboratories, Inc., Mountain View CA). qRT-PCR was used to measure expression of the plant defense-related gene lipoxygenase 2 (*LOX2*) as previously described (García-Gutiérrez et al. 2013). Three microliters of cDNA from each sample were amplified in 17 µl of PCR master mix containing 10 µl of iQ™ supermix (2x) (Bio-Rad Life Science Research, Hercules, CA), 1 µl of each primer (10 µM), 1µl (10 µM) of each TaqMan probe, and 4 µl of nuclease-free water.

Expression levels were normalized to the endogenous control actin (*ACT1*) and compared by calculating the fold-change using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). There were three replications for each treatment per experiment, and experiments were conducted three times. ANOVA was used to determine if there was a significant difference in the expression of *LOX2* in BCA-treated seeds vs non-treated seeds, and mean separation was conducted by Tukey's test.

RESULTS

Selection of BCA candidates

Eleven BCA candidates were selected based on their ability to colonize 24-h watermelon SE and reduce seed-to-seedling transmission of BFB. All bacterial strains grew in 24-h SE (Fig. 2.1). Tukey's mean separation analysis revealed that nine of the BCA candidates grew to a higher OD than did *A. citrulli* in SE (Fig. 2.2). In seedling grow-out assays, seeds treated with BCA candidates that displayed significantly lower BFB seed-to-seedling transmission percentages ranged from 5.2-12.8% compared with non-treated seeds at 54.6% (Fig. 2.3). BFB incidence for seeds treated with BCA candidates 24, 40, 35, 54, 96, 36, 92, 30 and *B. mojavensis* RRC101 was significantly ($P = 0.013$) lower than for seeds treated with sddH2O (Fig. 2.3). Seed treatments with *B. mojavenis* RRC101, BCA#35, and BCA#24 resulted in the lowest mean BFB incidence levels (7.5, 7.3, and 5.2%, respectively) compared with the control (54.6% incidence) and were selected for further studies (Fig. 2.3).

In vitro antagonistic effects of BCAs against *A. citrulli*

To investigate the antibacterial activity of BCAs against *A. citrulli*, ZOI plate assays were conducted. Each BCA candidate was spotted four times on individual LB and PDA plates that

were previously inoculated with *A. citrulli,* and this experiment was conducted three times. The *A. citrulli* ZOIs generated by each BCA were measured on LB and PDA plates (Table. 2.2). Zones of inhibition were not produced by BCA#24, BCA#35, *B. safensis* FO-36b or AAC00-1*Δhrc*C (Fig. 2.4 B, D, E, F, H, J, K, and L). In contrast ZOI were produced by *B. mojavensis* and *B. pumilus* SAF-032 on PDA and LB media (Fig. 2.4 A, G, and I). On average, the ZOIs produced by *B. mojavensis* RRC101 on LB agar and PDA were 4-5 mm and 4-7 mm in diameter, respectively. *B. pumilus SAF-032* did not produce ZOI on PDA; however, on average, it produced ZOI that were 3-4 mm in diameter on LB agar.

Watermelon seed competition assays

The ability of BCAs to colonize germinating watermelon seeds and affect *A. citrulli* colonization was determined through seed co-inoculation/competition assays. Individual watermelon seeds were inoculated with AAC00-1GFP, *B. mojavensis* RRC112, or BCA#35, and bacterial populations were estimated each day for 3 days. All strains reached population levels ranging from 10^5-10^{12} CFU/seed by 3 days after planting (dap) (Fig. 2.5). *B. mojavensis* RRC112 and BCA#35 reached population levels ranging from 10^5-10^7 CFU/seed 3 dap. According to Tukey's means separation test, AAC00-1GFP grew to higher populations on germinating watermelon seeds than the BCA candidates, reaching 10^{12} CFU/seed. *A. citrulli* AAC00-1GFP did not affect the growth of *B. mojavensis* RRC112 nor BCA#35 on germinating watermelon seeds. *B. mojavensis* BCA#35 (Fig. 2.6) and RRC112 (Fig. 2.7) populations reached 10^6-10^7 CFU/seed by 3 dap. When *A. citrulli* was co-inoculated onto seeds with BCAs, there was a reduction in AAC00-1GFP population growth by 2-3 orders of magnitude starting at 2 dap (Fig. 2.8).

The role of ISR in biocontrol of BFB on melon seedlings

To determine if BCA candidates induced ISR, melon seeds were treated with *B. mojavensis* RRC101, BCA#35, or MeJA. Only seeds treated with MeJA resulted in significantly different expression of *LOX2* 3 days ($P = 0.0005$) and 6 days ($P = 0.0087$) after treatment. On day 3, a 28-fold increase in *LOX2* expression was observed for seeds treated with MeJA, and on day 6, expression decreased 22-fold (Fig. 2.9). Seeds treated with BCAs did not display a significant difference in *LOX2* expression compared with water-treated seeds (Fig. 2.9). On day 3, seeds treated with BCA#35 and *B. mojavensis* RRC101 resulted in a 2-3-fold increase in *LOX2* expression, respectively. By day 6, *LOX2* expression in BCA#35 and *B. mojavensis* RRC101 treated seeds decreased 1-2-fold, respectively (Fig. 2.9).

DISCUSSION

We hypothesized that watermelon seed microflora includes bacteria that are antagonistic to *A. citrulli*. Seeds carry unique microbial communities that may be beneficial to plant health (Barret et al. 2015; Hashem et al. 2019; Pitzschke 2016). In this study, we identified *Bacillus* spp. among the watermelon seed microflora. *Bacillus* spp. are ubiquitous in nature and commonly associated with soil, water, and food products (Amin et al. 2015; Pandey and Palni 1997). They are considered strong candidates for biocontrol because they can form spores, are easy to produce *in vitro*, and can retain viability for extended periods of time (Gotor et al. 2019; Petras and Casida 1985; Young et al. 1995).

Screening for bacterial growth in 24-h watermelon seed exudate (SE) is a rapid way to identify BCAs that may be well adapted as biocontrol seed treatments. Once seeds imbibe water, germination begins and exudates are released producing a chemical matrix (spermosphere) around seeds (Schiltz et al. 2015). Screening SE can be a suitable strategy for identifying

efficacious plant growth-promoting bacteria (PGPB). By screening bean SE, a specific organic acid responsible for enhancing growth of *Bacillus amyloliqufaciens* strain ALB629 was identified (Martins et al. 2017). When bean seeds were treated with *B. amyloliqufaciens* strain ALB629 and amended with malic acid, seedlings supported a higher bacterial population and demonstrated increased drought tolerance and growth (Martins et al. 2017). In the current study, we selected 11 bacterial strains based on their ability to colonize watermelon SE. To further identify BCAs, we applied cell suspensions to artificially inoculated *A. citrulli* watermelon seeds and tested them by seedling grow-out assays. Nine of the 11 strains reduced the mean BFB incidence to 5.2-12.8% compared with non-treated seeds at 54.6%. The top three strains were *B. mojavensis* RRC101, BCA#24 and BCA#35 (Table 2.1). While *Bacillus* spp. have been previously reported to have biocontrol activity against *A. citrulli*, this is the first report of antagonistic activity for *B. safensis* and *B. mojavensis* (Adhikari et al. 2017; Fan et al. 2017; Jiang et al. 2015). Bio-priming, the application of bacteria to seeds, has been effective against faba bean root rot pathogens, *Fusarium verticillioides* in maize, and pre-emergence damping-off of sweet corn (Callan et al. 1990; El-Mougy and Abdel-Kader 2008; Mahmood et al. 2016; Nayaka et al. 2010; Taylor and Harman 1990).

Little is known about the modes of action for biological control against *A. citrulli*. Hence, we investigated if candidate BCAs inhibited *A. citrulli* by producing antimicrobial compounds, reducing populations on germinating watermelon seeds by direct competition, or inducing systemic resistance in host seedlings. We found that *B. mojavensis* RRC101 and *B. safensis* produced ZOI against *A. citrulli*. The specific compounds responsible for this inhibition are unknown; however, *Bacillus* spp. produce an arsenal of lipopeptides that inhibit a broad range of plant pathogens (Ongena and Jacques 2008). Lipopeptides inhibit pathogen growth *in*

vitro, but this does not always translate to *in planta* efficacy. Fan et al. (2017) reported that surfactin was the active compound that inhibited *A. citrulli* growth based on *in vitro* antagonistic assays (Fan et al. 2017). *Bacillus subtilis* 9407 mutants incapable of producing surfactin displayed reduced antibacterial activity against *A. citrulli* (Fan et al. 2017). Interestingly, we did not observe similar results with *in vitro* assays with surfactin or utilization of *B. mojavensis* NRRL B-14708 (Bacon et al. 2018), a bacterial strain that over-produces surfactin. This could be due to differences in the *A. citrulli* strains used or the specific *Bacillus* species. Antagonistic activity can be dependent on the specific surfactin produced when *Bacillus* spp. interact with plant tissues (Malfanova et al. 2012). The *Bacillus* genus contains over 70 species, and *B. mojavensis* RRC101 is in the *B. subtilis* complex. *B. mojavensis* RRC101 produces surfactin isomers C-13, C-14, and C-15, which are similar to those produced by *B. subtilis* (Bacon et al. 2018; Fan et al. 2017). Furthermore, *Bacillus* spp. produce varying quantities and isoforms of surfactin (Liu et al. 2015; Peypoux et al. 1999; Wen et al. 2011). Further investigations of chemical structures and quantity of the compound produced by strains used in this study are needed to elucidate if they are responsible antagonistic activity against *A. citrulli*. BCA#24 and BCA#35 did not produce ZOI, but still reduced BFB incidence on watermelon seedlings. These findings suggest that antibiosis may contribute to the biocontrol ability of *B. mojanensis* RRC101 but may not be involved in *B. safensis* control of *A. citrulli*.

Competition for nutrients or space can be an effective mode of action against plant pathogens when sufficient populations of the antagonist are present and can outcompete *A. citrulli* for limited resources (Spadaro and Droby 2016). To determine if candidate BCAs directly compete with and limit *A. citrulli* colonization of germinating watermelon seeds, an *in vitro* seedling assay was conducted. Unexpectedly, when inoculated independently onto watermelon

seeds, *A. citrulli* grew to higher populations than candidate BCAs. Because *Bacillus* spp. can colonize plant tissues rapidly (Shafi et al. 2017), we expected that the candidate BCAs would reach higher levels than *A. citrulli*. Additionally, by removing seed coats, seed dormancy is broken, plant embryo growth and greening are triggered, and stored nutrients become more rapidly available (Finch-Savage and Leubner-Metzger 2006; Lee et al. 2010). Since watermelon is the host plant for *A. citrulli*, it is possible that the rapid availability of nutrients enhanced the ability of the bacterium to switch from epiphytic growth to pathogenic growth. This switch in growth could explain why *A. citrulli* populations increased rapidly by 3 dap and symptoms were observed. Despite not colonizing seeds as well as *A. citrulli*, candidate BCAs reached populations of 10^6-10^7 CFU/seed when they were inoculated independently or with *A. citrulli* onto germinating watermelon seeds. Similar populations of *Bacillus* spp. were reported to be effective for reducing disease incidence. Khedher et al. (2015) demonstrated that treating potato tuber slices with a cell suspension of *B. subtilis* V26 containing 10^6 or 10^9 spores/ml decreased black scurf incidence caused by *Rhizoctonia solani* (Khedher et al. 2015). Interestingly, when co-inoculated with candidate BCAs, *A. citrulli* populations were reduced by 2-3 orders of magnitude by 2 and 3 days after inoculation. BCA#35 reduced *A. citrulli* populations by 3 orders, and this may be a component of biological control. Despite these findings, it is unclear whether *A. citrulli* populations were reduced due to competition. Biocontrol activity due to competition can occur by rapid BCA colonization leading to the utilization of substrates. For example, *Enterobacter cloacae* suppresses *Pythium ultimum* by outcompeting it for plant-derived unsaturated long-chain fatty acids (vanDijk and Nelson 2000). *Pseudomonas* spp. also provided biological control activity based on competition for iron (Pal and McSpadden Gardener 2006; Weller 2005). Similar to other *Bacillus* spp., *B. safensis* can colonize a wide range of habitats, has some plant

growth-promoting traits, secretes a variety of lipopeptides and can survive extreme conditions (Lateef et al. 2015). *B. safensis* has some biocontrol activity against fungi based on chitinase production (Jacobson 2000; Mayer and Kronstad 2017).

Systemic acquired resistance is an innate plant response that results in broad-spectrum resistance to successive infections (Ryals et al. 1994). Induced systemic resistance (ISR) is induced by rhizobacteria or other abiotic stressors (Ramamoorthy et al. 2001). *Bacillus* spp. have been shown to induce ISR in crops such as tomato, bell pepper, muskmelon, watermelon, sugar beet, tobacco, and cucumber (Kloepper et al. 2007; Shafi et al. 2017). Moreover, they activate ISR in plants through jasmonic acid and ethylene (JA/ET) pathways (Beneduzi et al. 2012; vanLoon et al. 1996). Lipoxygenases are a family of enzymes that have diverse and important functions in plant cells and are associated with roles in signaling during plant stress or plant-microbe interactions (Kuhn and Thiele 1999; Sucharitha et al. 2010). There are many isoforms of lipoxygenase, and 18 have been identified in melon (Zhang et al. 2014). In this study, we found that applying *B. mojavensis* RRC101 and BCA#35 to germinating melon seeds did not result in result in ISR. While there was a slight increase of *LOX2* expression in germinating melon seeds treated with *B. mojavensis* RRC101 or BCA#35, expression levels were not significantly higher than seeds treated with water. A similar response was reported by García-Gutiérrez et al. (2013) where melon plants treated with *B. subtilis* UMAF6639 resulted in a 1-3-fold increase in expression of *LOX2* after challenge with powdery mildew (García-Gutiérrez et al. 2013). Also, Buzi et al. (2004) demonstrated that melon seeds treated with MeJA showed increased lipoxygenase activity, which resulted in resistance to infection by *Didymella bryoniae* and *Sclerotinia sclerotiorum*. Findings from their study revealed that seed treatments can induce ISR in seedlings and lead to disease reduction. Unfortunately, we did not observe the same

results as Buzi et al. (2004) with regards to BFB. These findings suggest that further investigation is needed to understand the mechanisms by which BCAs applied as seed treatments reduce BFB incidence.

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Table 2.1. Bacterial strains used in this study

Bacterium	Relevant characteristics	Source
Acidovorax citrulli		
AAC00-1	Group II strain	Walcott, R., University of Georgia
AAC00-1*Δhrc*C	*hrcC* deletion mutant	Johnson, K., University of Georgia
A00-1GFP	GFP mutant of AAC00-1	Walcott, R., University of Georgia
AAC213-60	Group I strain	Walcott, R., University of Georgia
AAC213-60GFP	GFP mutant of AAC213-60	Zhao, M., University of Georgia
Bacillus mojavensis		
RRC 101		Bacon, C., USDA-ARS
RRC 112	Rifampicin-resistant mutant of RRC 101	Bacon, C., USDA-ARS
Bacillus safensis strain #24	Isolate from watermelon seed	This study
Bacillus safensis strain #35	Isolate from watermelon seed	This study
Bacillus safensis FO-36b	Type strain of *Bacillus safensis*	Kasthuri, J., Jet Propulsion Laboratory, Biotechnology and Planetary Protection Group, NASA
Bacillus pumilus SAFR-032	Type strain of *Bacillus pumilus*	Kasthuri, J., Jet Propulsion Laboratory, Biotechnology and Planetary Protection Group, NASA

Table 2.2. Effect of different antagonistic bacteria on the growth of *Acidovorax citrulli* on different microbiological media.

	Medium			
	Luria-Bertani		Potato Dextrose Agar	
Bacterial strain	AAC00-1	AAC213-60	AAC00-1	AAC213-60
AAC00-1*Δ hrcC*	-	-	-	-
B. safensis strain #24	-	-	-	-
B. safensis strain #35	-	-	-	-
B. safensis FO-36b	-	-	-	-
B. pumilus SAFR-032	++	+	-	-
B. mojavensis RC101	++	++	++	++

Zone of inhibition in dual culture assay; -, 0 mm; +, 1-3 mm; ++, 4-7 mm

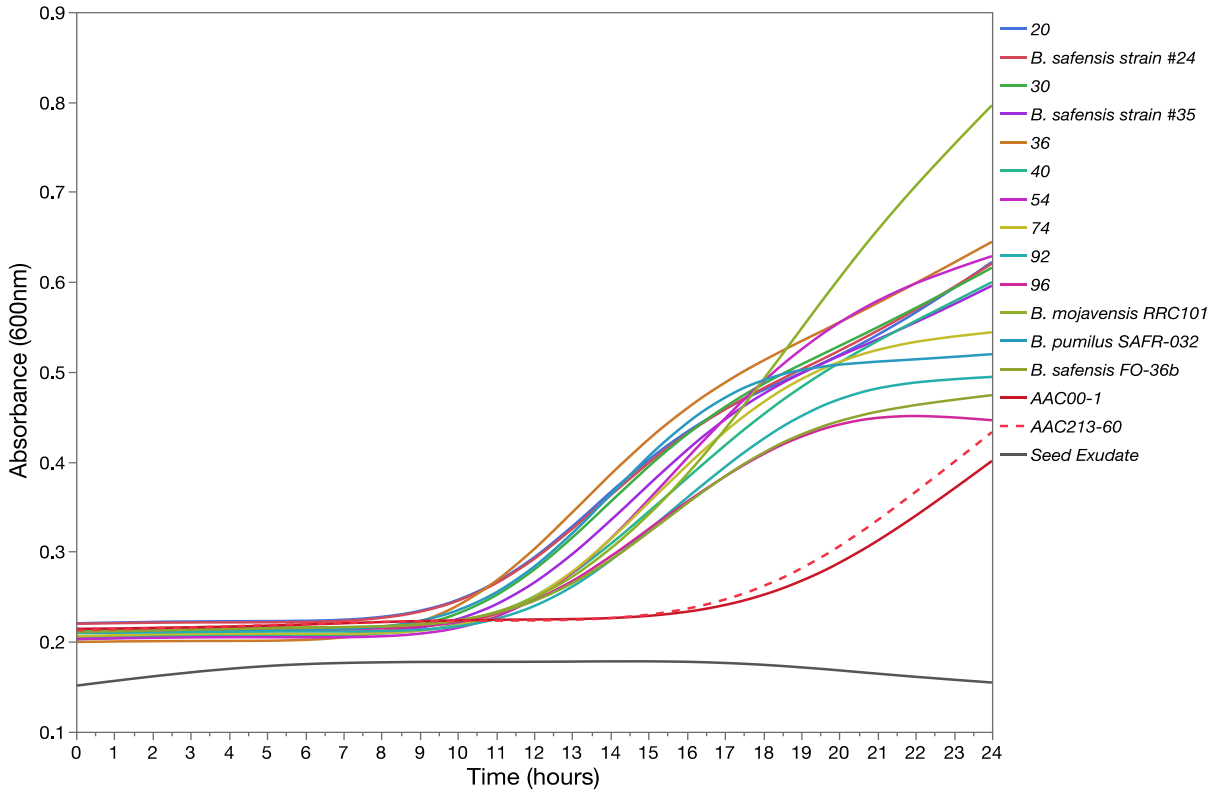

Figure 2.1. Growth of candidate biocontrol bacteria from watermelon seeds in 24-h watermelon seed exudate (SE). Bacterial cells were placed in SE and optical density (OD_{600nm}) was measured at 30-min intervals for 24 h using the Bioscreen C automated microbial growth monitoring system. Curves represent mean bacterial OD values for two independent experiments with ten technical replicates per bacterial strain. The two red lines represent growth of a group I (red dotted line) and group II (red solid line) strain of *Acidovorax citrulli*. The gray line represents the negative control SE and other colors represent different candidate strains.

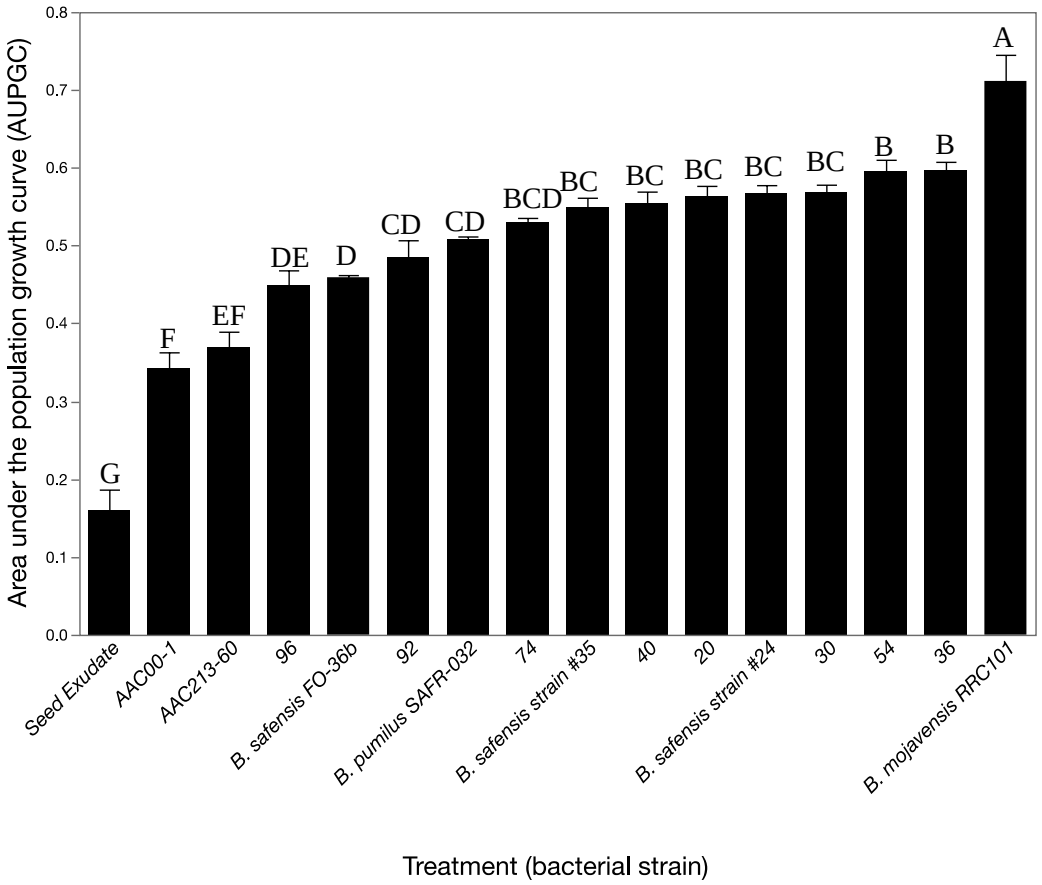

Figure 2.2. Area under the population growth curve (AUPGC) data calculated for biocontrol agent candidates and *Acidovorax citrulli* in 24-h watermelon seed exudate. Bars represent the mean AUPGC for two independent experiments with ten technical replications per treatment for each experiment, and lines indicate the standard errors of the means. Treatments with the same letters are not significantly different according to Tukey's mean separation test ($P < 0.05$).

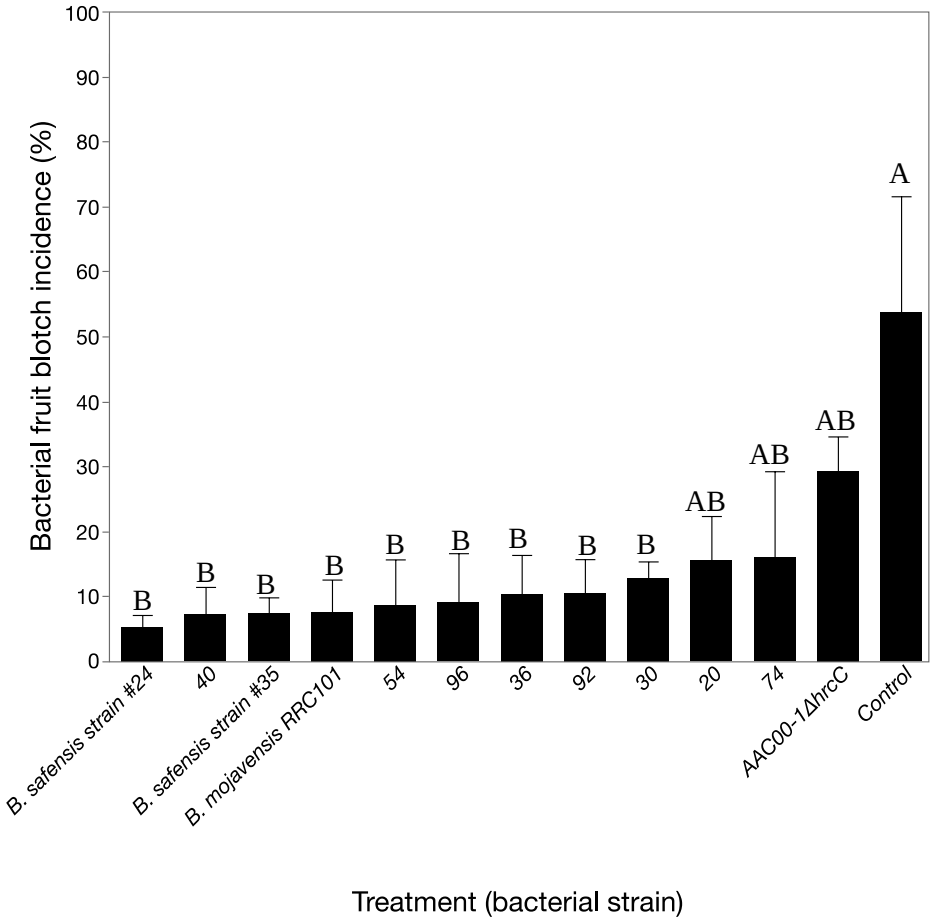

Figure 2.3. Efficacy of seed treatments with biocontrol agents for reducing seed-to-seedling transmission of bacterial fruit blotch (BFB). Watermelon seeds were vacuum-infiltrated with 10_6 colony-forming units (CFU)/ml of a cocktail of *Acidovorax citrulli* inoculum and dried for 24 h. Subsequently, seeds were vacuum-infiltrated with 10_8 CFU/ml of each biocontrol agent candidate strain and dried for 24 h. Seeds were placed in individual tubes and allowed to germinate for 14 days and then assessed for disease incidence. Bars represent mean BFB incidence on watermelon seedlings for three independent experiments with 40 seedlings per treatment. Lines represent the standard errors of the means. Mean values with the same letters are not significantly different according to Tukey's mean separation test ($P < 0.05$)

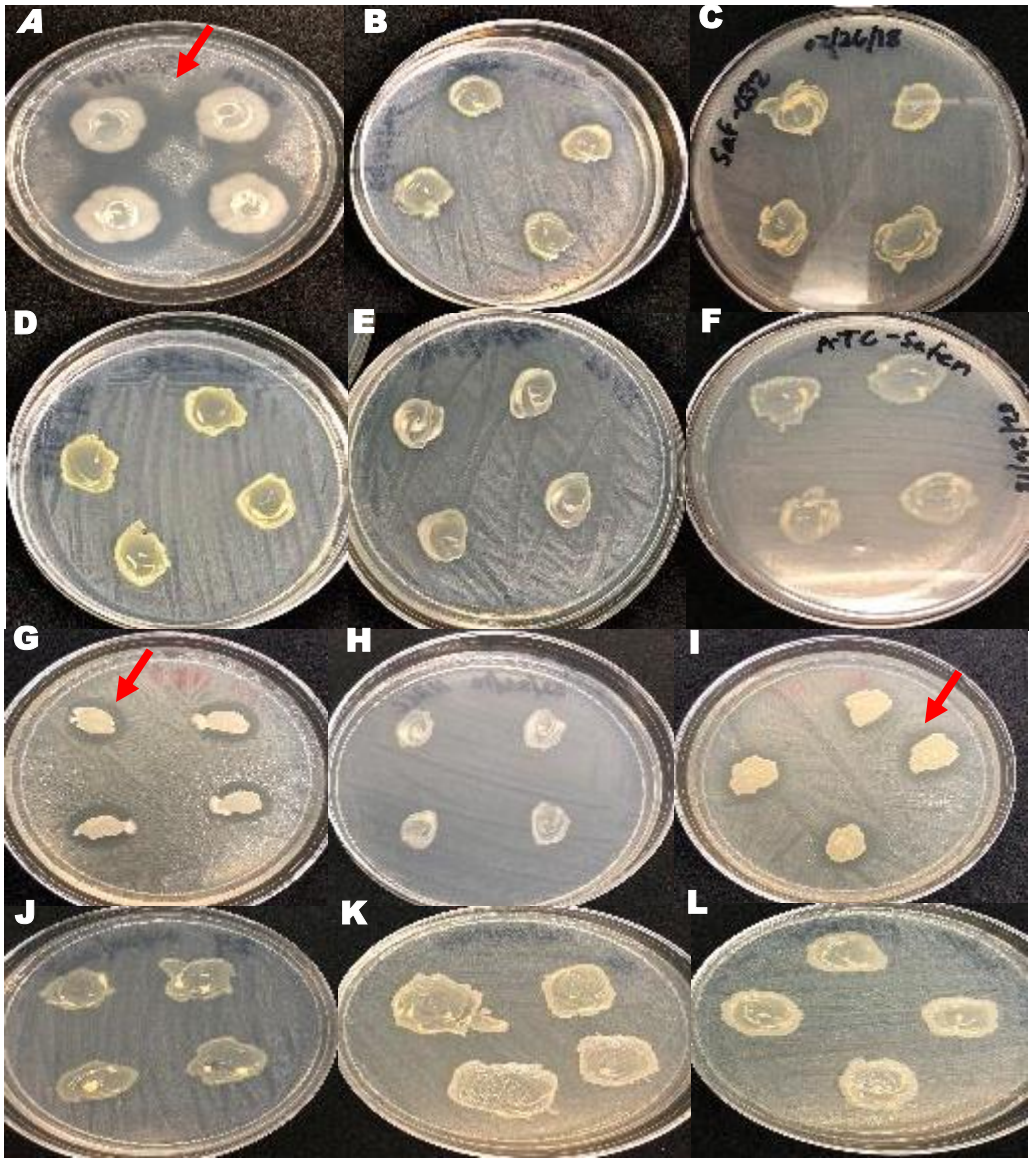

Figure 2.4. *In vitro* antagonistic activity of biocontrol agents (BCA) against *Acidovorax citrulli* on potato dextrose agar (**A-F**) and Luria-Bertani (**G-L**) plates. Zones of inhibition (indicated by red arrows) in a lawn of *A. citrulli* with each BCA candidate spotted in four sections. **A**: *Bacillus mojavensis* RRC101; **B**: AAC00-1*Δhrc*C; **C**: *B. pumilus* strain*;* **D**: *B. safensis* strain *#24*; **E**: *B. safensis* strain *#35*; **F**: *B. safensis* strain*;* **G**: *B. mojavensis* RRC101; **H**:AAC00-1*Δhrc*C; **I**: *B. pumilus* strain*;* **J**: *B. safensis* strain *#24*; **K**: *B. safensis* strain *#35*; **L**: *B. safensis* strain.

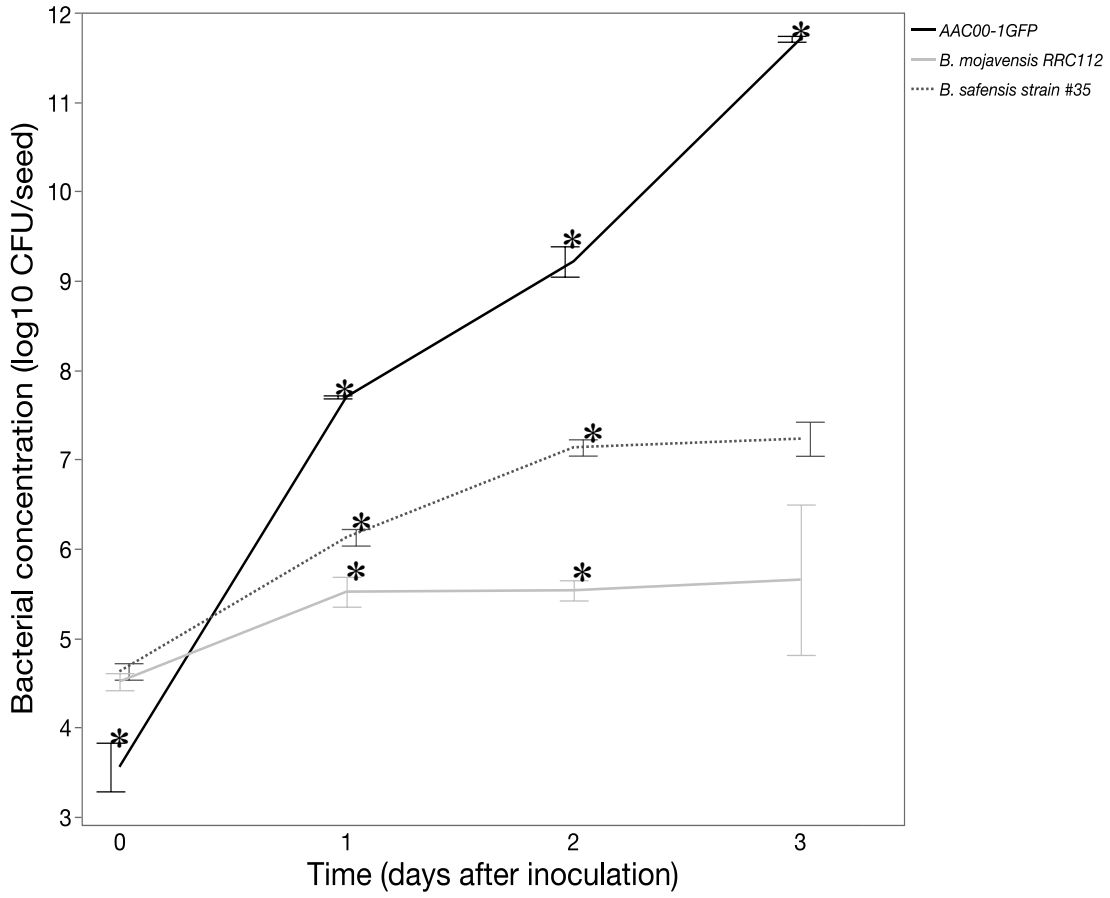

Figure 2.5. *In vitro* colonization of germinating watermelon seeds (cv. Crimson Sweet) by *Acidovorax citrulli* AAC00-1GFP, *Bacillus mojavensis* RRC112 (a rifampicin mutant of RRC101), and *B. safensis* strain #35 (strain isolated from germinating watermelon seeds). De-coated watermelon seeds were inoculated with a 5-µl drop containing ~10_4 colony-forming units (CFU) of *B. mojavensis* RRC101, *B. safensis* strain #35 or AAC00-1GFP individually and allowed to germinate for 3 days. Bacterial cells were enumerated each day for each strain. The experiment was conducted twice, and each data point represents the mean of the two experiments (n = 4 seeds/treatment). Lines represent the standard errors of the means. Treatments with asterisks are significantly different at a given time point according to Tukey's mean separation test ($P < 0.05$).

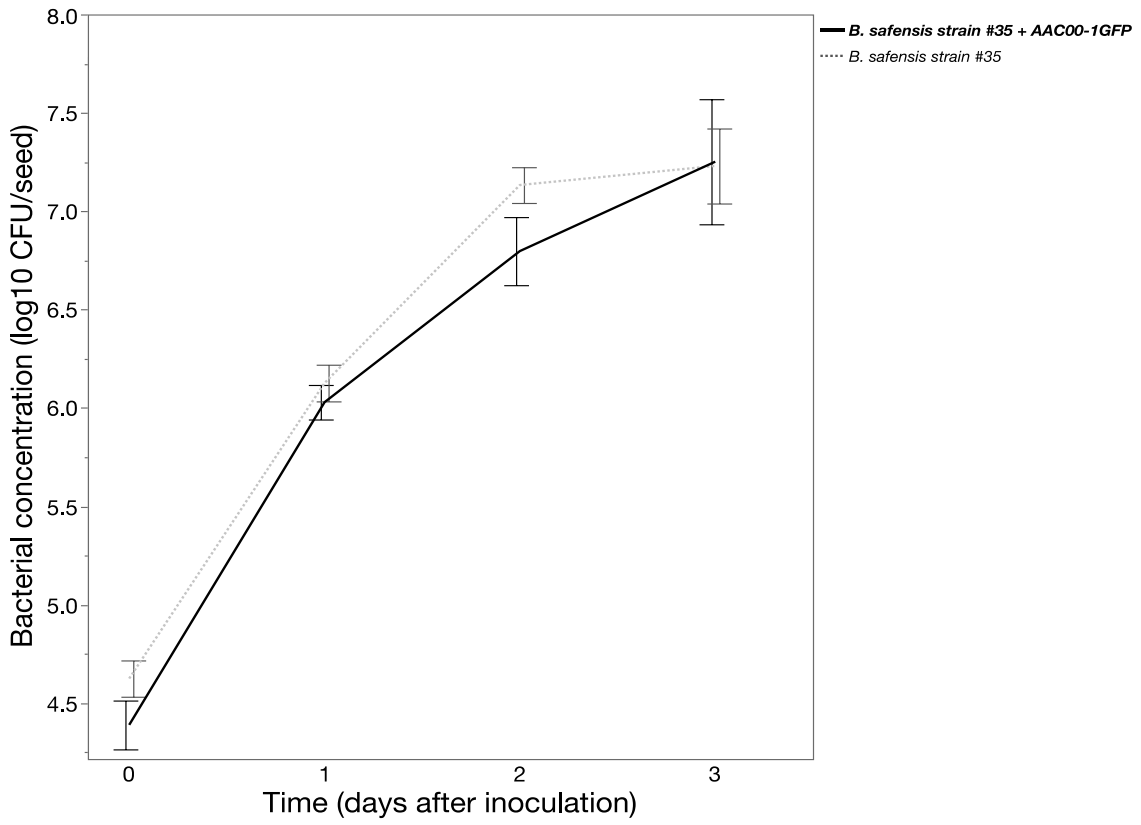

Figure 2.6. *In vitro* colonization of germinating watermelon seeds (cv. Crimson Sweet) by *Acidovorax citrulli* AAC00-1GFP and *Bacillus safensis* strain #35 (isolated from germinating watermelon seeds) co-inoculated with AAC00-1GFP. De-coated watermelon seeds were inoculated with a 5-µl drop containing ~10_4 colony-forming units (CFU) of *B. safensis* strain #35 individually, or *B. safensis* strain #35 co-inoculated with *A. citrulli* and allowed to germinate for 3 days. Bacterial cells were enumerated each day for each strain. The experiment was conducted twice, and each data point represents the mean of the two experiments (n = 4 seeds/treatment). Lines represent the standard errors of the means.

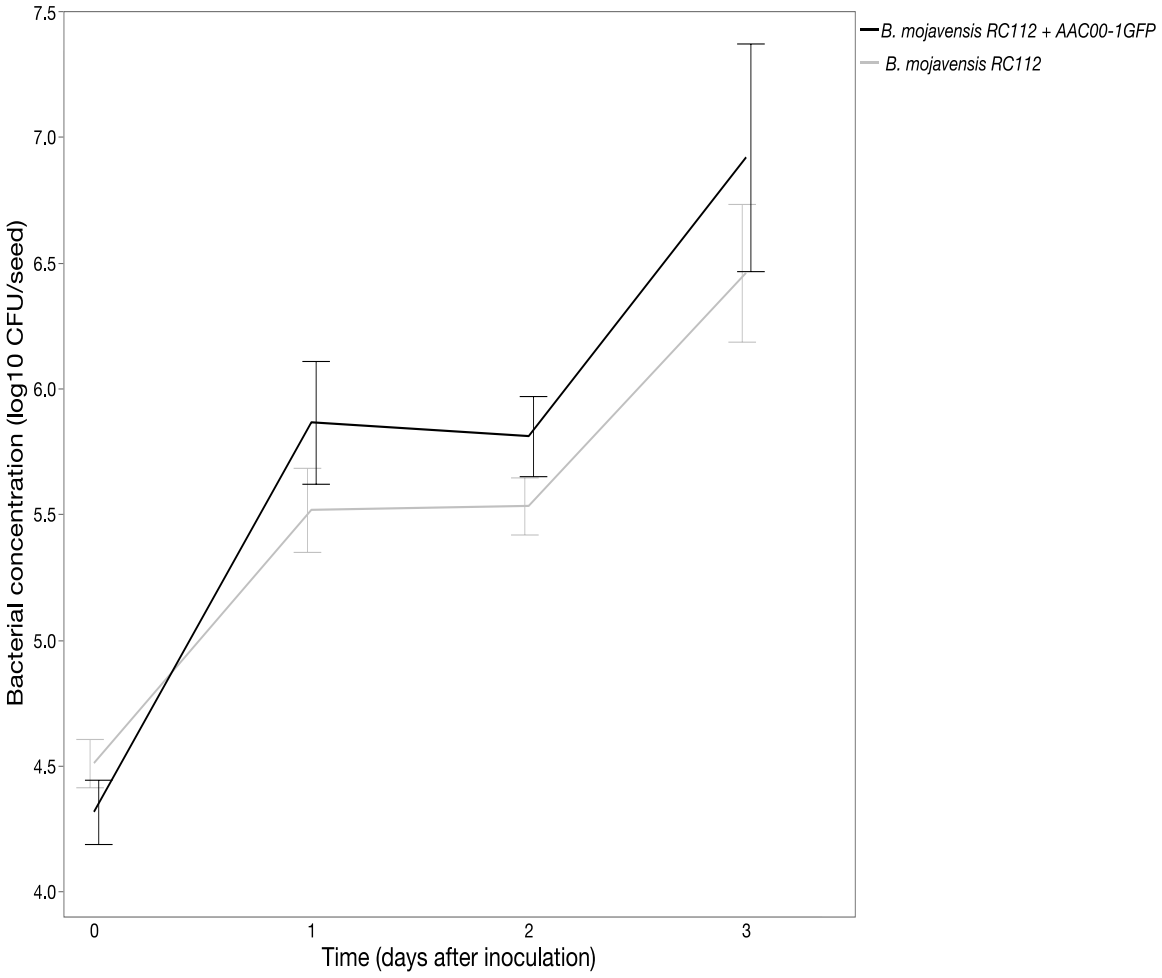

Figure 2.7. *In vitro* colonization of germinating watermelon seeds (cv. Crimson Sweet) by *Acidovorax citrulli* AAC00-1GFP and *Bacillus mojavensis* RRC112 (a rifampicin mutant of RRC101) co-inoculated with AAC00-1GFP. De-coated watermelon seeds were inoculated with a 5-µl drop containing ~10_4 colony-forming units (CFU) of *B. mojavensis* RRC112 individually, or *B. mojavensis* RRC112 co-inoculated with AAC00-1GFP and allowed to germinate for 3 days. Bacterial cells were enumerated each day for each strain. The experiment was conducted twice, and each data point represents the mean of the two experiments (n = 4 seeds/treatment). Lines represent the standard errors of the means.

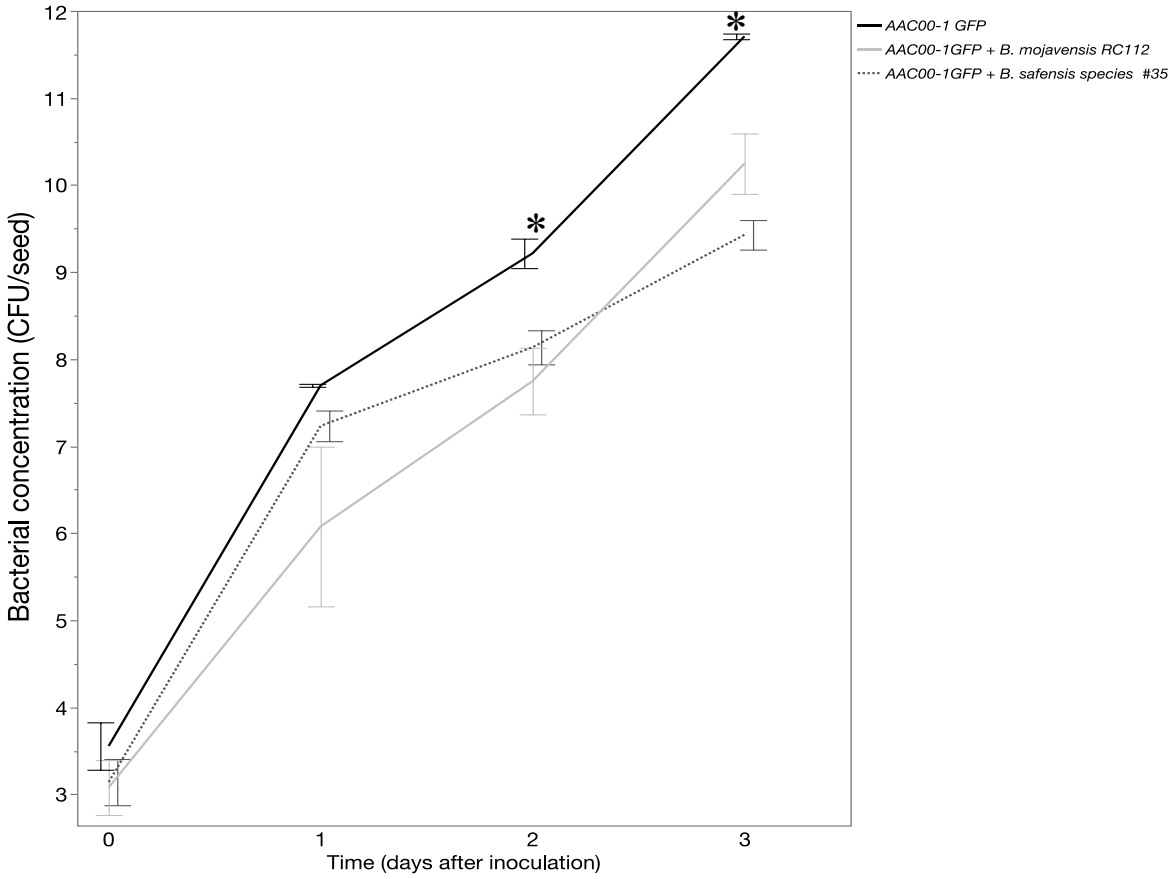

Figure 2.8. *In vitro* colonization of germinating watermelon seeds (cv. Crimson Sweet) by *Acidovorax citrulli* AAC00-1GFP independently, AAC00-1GFP co-inoculated with *Bacillus mojavensis* RRC112 (a rifampicin mutant of RRC101), or AAC00-1GFP co-inoculated with *B. safensis* strain #35 (strain isolated from germinating watermelon seeds). De-coated watermelon seeds were inoculated with a 5-µl drop containing ~10_4 colony-forming units (CFU) of AAC00-1GFP individually, or each BCA co-inoculated with AAC00-1GFP and allowed to germinate for 3 days. Bacterial cells were enumerated each day for AAC00-1GFP. The experiment was conducted twice, and each data point represents the mean of the two experiments (n = 4 seeds/treatment). Lines represent the standard errors of the means. Treatments with asterisks are significantly different at a given time point according to Tukey's mean separation test ($P < 0.05$).

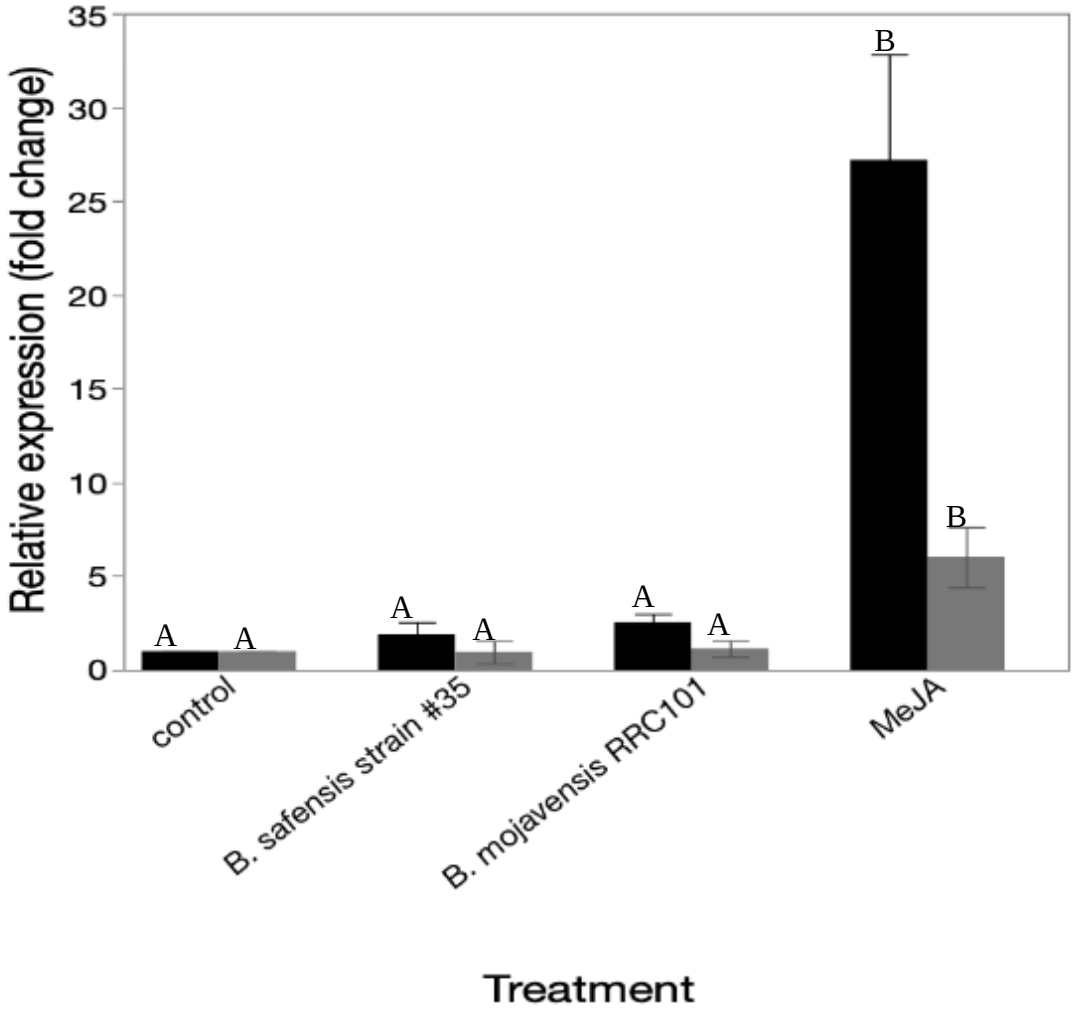

Figure 2.9. Expression of *LOX2* in germinating melon seedlings following seed treatment with water (control), 45 µM methyl jasmonate (MeJA), *Bacillus safensis* strain #35, *B. mojavensis* RRC101 at 3 (black) and 6 (gray) days after germination. Data are mean values from two independent experiments, with three seedlings per treatment. Lines represent the standard errors of the means. Mean values with the same letters are not significantly different according to Tukey's mean separation test ($P < 0.05$).

CHAPTER 3

NOVEL *BACILLUS SAFENSIS* STRAINS FROM THE WATERMELON SPERMOSPHERE ANTAGONISTIC TO *ACIDOVORAX CITRULLI*

Sutton, S. and Walcott, R. to be submitted to International Journal of Systematic and Evolutionary Microbiology

ABSTRACT

The spermosphere is enriched with bacterial that may aid in seed survival and germination and that may protect seedlings from invading plant pathogens. We isolated several bacterial strains from 24-h germinating watermelon seeds that were antagonistic toward *Acidovorax citrulli*, the bacterium causing bacterial fruit blotch, *in vitro*. Three strains were selected for further characterization and identification based on reduced disease incidence when challenged with *A. citrulli*, ability to colonize watermelon seed exudate, and ability to reduce *A. citrulli* populations on germinating watermelon seeds. Phylogenetic analysis of 16S rRNA gene sequences placed the strains within the genus *Bacillus* with 96% sequence identity. Analysis of an additional housekeeping gene, *purH*, provided a higher resolution among the strains and revealed that the closest neighbors were *B. safensis* or *B. pumilus*. Phenotypic analysis (carbon substrate usage and chemical sensitivity assays) showed minor similarities between the watermelon seed strains (BCA#24 and BCA#35) and *B. safensis* and *B. pumilus*; however, the fatty acid composition of the strains was identical to *B. safensis*. Additionally, two *in silico* platforms were utilized to determine the average nucleotide identity similarity between genomes as well as DNA-DNA hybridization analogous values. The average nucleotide identity of the two watermelon strains was 99% identical to *B. safensis* and 90% to *B. pumilus* indicating that the strains are more closely related to *B. safensis*. Further validation was carried out by comparing the watermelon seed strains to other *Bacillus* species using a genome-to-genome distance calculator. This analysis showed a 96% relatedness of watermelon seed isolates with *B. safensis* FO-36bT. On the basis of phylogenic analysis of 16S rRNA and *purH* gene sequences, fatty acid composition, and whole-genome sequence comparison analysis, we propose that the novel

biocontrol strains (BCA#24 and BCA#35) isolated from watermelon seeds belong to the species *B. safensis*.

Keywords: *Bacillus safensis*, *Bacillus mojavensis*, *Bacillus pumilus*, watermelon seed microflora, *Acidovorax citrulli*, biological control agents, bacterial fruit blotch.

INTRODUCTION

Watermelon is the most widely used cucurbit species and accounts for approximately 50% of U.S. per capita consumption, followed by cantaloupe at 40%, and honeydew at 10% (Boriss et al. 2006). It is an important crop in the state of Georgia, and based on the 2017 Georgia Farmgate Value Report, it was the 19th most valuable crop with a farm gate value of $134.8 million, and the third most valuable vegetable in Georgia behind sweet corn and onion (Wolfe and Stubbs 2017). One of the most destructive phytopathogens of watermelon is the Gram-negative bacterium *Acidovorax citrulli* (Schaad et al. 2008; Schaad et al. 1978; Willems et al. 1992), the causal agent of bacterial fruit blotch (BFB).

A. citrulli is seed-borne and seed-transmitted and can cause up to 100% yield loss (Bahar and Burdman 2010; Burdman and Walcott 2012). Seeds represent an important source of inoculum for *A. citrulli*, and the bacterium can survive for up to 30 years in infested seeds (Block and Shepherd 2008; Latin and Hopkins 1995). Previous research in our lab has demonstrated that long-term survival can be achieved when the bacterium contaminates watermelon seeds by ingressing through the pistil of the female flower and localizing in embryo tissues (Dutta et al. 2012). To date the most effective approach for managing BFB is producing pathogen-free seeds and excluding the pathogen from production systems. This can be achieved by producing seeds in regions with cool, dry climates, combined with rigorous seed health testing and visual field inspections (Lovic and Hopkins 2003). Despite these efforts, limitations still exist for managing BFB (Walcott 2003; Walcott et al. 2006; Walcott and Gitaitis 2000).

Isolation, identification, and utilization of biocontrol agents (BCAs) for managing a broad-spectrum plant diseases is an environmentally sound management approach that is increasing in popularity (Chowdhury et al. 2015; Daguerre et al. 2016; Daranas et al. 2019). For

successful application, BCAs should be selected based on their ability to survive in the environment where they will be utilized. Since the primary inoculum source for *A. citrulli* is seed, our lab previously screened germinating watermelon seeds for BCA candidates. We successfully isolated 11 bacterial BCA candidates that could colonize watermelon seed exudate (derived from seeds upon incubation for 24 h) to higher population compared with *A. citrulli*. The BCAs reduced BFB incidence in watermelon seedlings under *in vitro* conditions. Seeds treated with *B. mojavenis* RRC101 or BCA strains (BCA#24, and BCA#35) resulted in significantly lower mean BFB incidence (*B. mojavensis*: 7,5%, BCA#24: 7.3%, and BCA#35: 5.2%) compared with the non-BCA treated control (54.6% incidence), when challenged with *A. citrulli*. Lastly, it was observed that BCA strains (BCA#24 and BCA#35), and *B. mojavensis* RRC101 reduced *A. citrulli* populations on germinating watermelon seeds between 2-3 $\log_{10}$ orders at 2 days post-inoculation. Based on the above findings, we hypothesized that these strains (BCA#24 and BCA#35) could serve as BCA seed treatments for management of *A. citrulli*. Hence, we sought to identify the bacterial candidates through phylogenetic, biochemical, and whole-genome comparison analysis.

MATERIALS AND METHODS

Bacterial isolates and culture

Bacterial strains in this study included *B. safensis* FO36bT (type strain), *B. pumilus* SAF-032 (reference strain), BCA#24 and BCA#35, and *B. mojavensis* RRC101 (Table 2.1). Strains were routinely grown on Luria-Bertani (LB) agar (VWR Life Science, Radnor, PA) for 24 h at 37°C.

Phylogenic analysis

Total microbial genomic DNA for strains (BCA#24, BCA#35 and *B. mojavensis* RRC101) were isolated using an E.Z.N.A. Bacterial DNA Kit (Omega Bio-tek, Inc, Norcross, GA) according to manufacturer's instructions. Two microliters of bacterial DNA (5 ng/μL) were amplified in 50 μL of a PCR master mix containing universal primers 16S rRNA primer pair (27F-5'- AGAGTTTGATCCTGGCTCAG-3') and (1492R-5'- GGTTACCTTGTTACGACTT-3') (Chen et al. 2015; Lane 1991). For sequencing, PCR amplicons were purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-tek) and sequenced (Georgia Genomics and Bioinformatics Core, Athens, GA). Additionally, sequence analysis of the housekeeping gene, *purH,* was performed. To retrieve *purH* sequence data for BCA#24 and BCA#35, contigs generated from whole-genome sequencing in later experiments with each BCA were uploaded into Geneious prime 2019.1.3 sequence analysis software. Sequence data for *purH* was extracted from contigs of BCA#24 and BCA#35. Phylogenic analysis of gene sequence data was conducted using neighbor joining (NJ), maximum likelihood (ML), and maximum parsimony methods with phylogeny.fr software (http://www.phylogeny.fr/index.cgi). MUSCLE 3.8.31 was used to align sequences in combination with Gblocks 0.91b to eliminate poorly aligned positions and divergent regions. Additionally, to construct phylogenic trees, the PHYML 3.1/3.0 aLRT and TreeDyn198.3 options were selected. The reliability of the branches was assessed from 1,000 bootstrap replicates. To determine the genus of our strains, the basic local alignment search tool (BLAST) was used.

Biochemical characteristics

Biochemical tests were performed on *B. safensis* FO-36bT along with *B. pumilus* reference strain SAFR-032 (Tirumalai et al. 2018) and the two BCA strains (BCA#24 and

BCA#35). BIOLOG GNIII (BIOLOG, Hayward, CA) microplates were used to determine the carbon substrate utilization patterns and chemical sensitivity for the above strains according to manufacturer's instructions. Microplates were incubated for 24 h at 37℃ and evaluated manually after 24 h.

Fatty acid methyl-ester (FAME) analysis for phenotypic comparison

Whole-cell FAME composition was determined for *B. safensis* FO-36bT, *B. pumilus* SAFR-032, BCA#24 and BCA#35. Strains were cultured on tryptic soy broth agar for 24 h at 28℃, and whole-cell fatty acids were saponified, methylated, and extracted as previously described by Miller and Berger (1985) (Miller and Berger 1985). FAME analysis was conducted using the Microbial Identification System, Sherlock version 3.10 (MIDI).

Whole-genome sequencing

For whole-genome sequencing, total microbial genomic DNA (30 ng/µL) was isolated from bacterial strains BCA#24 and BCA#35 using an E.Z.N.A. Bacterial DNA Kit (Omega Bio-tek) according to manufacturer's instructions and sequenced by MicrobesNG (MicrobesNG, Edgbaston, Birmingham). DNA was quantified in triplicates with the Quantit dsDNA HS assay in an Ependorf AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, CA) following the manufacturer's protocol with the following modifications: 2 ng of DNA was used as input, and PCR elongation time was increased to 1 min. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq using a 250-bp paired end protocol according to MicrobesNG procedures section. Reads were adapter-trimmed using Trimmomatic

0.30 with a sliding window quality cutoff of Q15. De novo assembly was performed on samples using SPAdes version 3.7, and contigs were annotated using Prokka 1.11.

Contigs for strains BCA#24 and BCA#35 received from MicrobesNG were deposited in the genome-to-genome distance calculator (GGDC, https://ggdc.dsmz.de/ggdc.php) and the average nucleotide identity (ANI) calculator (http://enve-omics.ce.gatech.edu/ani/) for a whole genome-based taxonomic analysis. Strains utilized for comparison include *B. cytotoxicus* NVH 391-98 **(**NC_009674.1), *B. altitudinis* GQYP101 (NZ_CP040514), *B. subtilis* subsp. *subtilis* strain 168 (NC_000964), *B. pumilus* ATC7061T (ABRX01000001), *B. safensis* BRM1 (CP018100), and *B. safensis* FO-36bT (NZ_CP010405). On the GGDC platform, all pairwise comparisons among the set of genomes were conducted using genome blast distance phylogeny (GBDP) distances and intergenomic distances inferred under the algorithm "trimming" and distance formula according to (Meier-Kolthoff et al. 2013). One hundred distance replicates were calculated for each genome assessed. Digital DDH (DNA-DNA Hybridization) values and confidence intervals were calculated using the recommended settings of GGDC 2.1 according to (Meier-Kolthoff et al. 2013).

RESULTS

Phylogenic analysis

A phylogenetic tree based on the 16S rRNA locus (Fig. 3.1) showed that BCA#24 and BCA#35 clustered with several *Bacillus* spp. including *B. pumilus, B. safensis, B. invictate*, and *B. altitudinis*. Both BCA strains were grouped in a distinct cluster separate from *B. mojavensis* and *B. subtilis*. The sequence of BCA#24 was 96% identical to several members of the *Bacillus* genus; however, the top match was *B. safensis*. Similarly, BCA strain #35 was 93% identical to

several members of the *Bacillus* genus; and the top match was also *B. safensis*. A phylogenetic tree based on the *purH* gene (Fig. 3.2) showed that the BCA strains clustered with members of the genus *Bacillus*, and that the nearest neighbors were *B. safensis* or *B. pumilus*. Analysis of the *purH* gene provided higher resolution among the strains and based on BLAST analysis, both BCA#24 and BCA#35 displayed 99% sequence identity with *B. safensis*.

Biochemical characteristics

Metabolic profiles for *B. safensis* FO-36bT (type strain), *B. pumilus* SAF-032 (reference strain), and two BCA strains (BCA#24 and BCA#35) were determined based on the utilization pattern of 71 carbon sources and sensitivity to 23 chemicals. Of the 71 carbon sources tested, the two BCA strains were similar to *B. safensis* FO-36bT but not to *B. pumilus* SAF-032, by utilization of D-saccharic acid and sensitivity to tetrazolium violet, and lack of sensitivity to 1% sodium lactate. The BCA strains were similar to *B. pumilus* SAF-032 by showing lack of sensitivity to D-serine. L-alanine, glycerol, L-aspartic acid, citric acid, L-glutamic acid, mucic acid, D-aspartic acid quinic acid, and L-malic acid were utilized by all four strains. Additionally, the four strains were not sensitive to 1% NaCl, aztreonam, pH 6, lithium chloride, sodium butyrate, pH 5, and potassium tellurite, but were sensitive to troleandomycin, linocomycin, vancomycin, fusidic acid, minocycline, and tetrazolium blue. For the rest of the substrates, the BCA strains displayed a different utilization pattern compared with *B. safensis* FO-36bT (type strain) and *B. pumilus* SAF-032. The carbon source utilization and chemical sensitivity for test strains are listed in Tables 3.2 and 3.3.

Fatty acid methyl-ester (FAME) analysis for phenotypic comparison

BCA#24, BCA#35 and *B. safensis* FO-36bT had similar FAME profiles. The most abundant fatty acids identified were $C_{15:0}$ iso (35.4- 41.6%), $C_{15:0}$ anteiso (24.9-26.6%), $C_{17:0}$ iso (11.6-13.8%) and $C_{17:0}$ anteiso (7.5-8.5%) (Table 3.4).

Whole-genome sequencing

The target depth of coverage was 30x for BCA #24 and BCA#35. BCA#24 was 3,679,742 bp long with 47 contigs and a GC content of 46.55%. BCA#35 was 3,676,568 bp long with 42 contigs and a GC content of 49.29%. When contigs were compared with *B. cytotoxicus* NVH 391-98 **(**NC_009674.1), *B. altitudinis* GQYP101(NZ_CP040514), *B. subtilis* subsp. *subtilis* strain 168 (NC_000964), *B. safensis* BRM1 (CP018100), *B. safensis* FO-36bT (NZ_CP010405), and *B. pumilus* ATC7061T (ABRX01000001) using the average nucleotide index calculator (ANI), strains were most closely related to *B. safensis* by 99% (Table 3.5). Moreover, contigs were added to the genome-to-genome distance calculator and compared with strains of *Bacillus* spp. including *B. cytotoxicus* NVH 391-98 **(**NC_009674.1), *B. altitudinis* GQYP101(NZ_CP040514), *B. subtilis* subsp. *subtilis* str. 168 (NC_000964), *B. safensis* BRM1 (CP018100), *B. safensis* FO-36bT (NZ_CP010405), and *B. pumilus* ATC7061T (ABRX01000001), and similarly, strains were most related to *B. safensis* FO-36bT by 96% (Table 3.6).

DISCUSSION

In this study, we identified two BCA strains from germinating watermelon seeds as *B. safensis*. *Bacillus* comprises a group of bacteria that are Gram-positive, rod-shaped, and endospore-forming microorganisms (DE Vos et al. 2009). These organisms are ubiquitous in nature, in part due to their ability to form endospores that are resistant to heat, desiccation, lack

of nutrients, and exposure to UV (Paszczynski and Burbank 2015). 16s rRNA gene sequencing is one of the most common approaches to study bacterial phylogeny and taxonomy because this gene is present in all bacteria (Janda and Abbott 2007). Based on 16S rRNA sequencing, *Bacillus* is divided into five groups including *B. cereus*, *B. megaterium*, *B. subtilis*, *B. circulans* and *B. brevis* (Liu et al. 2013). BCA#24 and BCA#35 were identified as *Bacillus* spp. with 96% identity to several species in the *Bacillus* genus. Due to the high sequence similarity of the 16S rRNA sequences between the BCA strains and other *Bacillus* spp., two main clusters formed that separated the *Bacillus subtilis* complex from the *Bacillus pumilus* complex. Similar to our findings, a phylogenetic analysis of the *Bacillus subtilis* complex by Rooney et al. (2009) resulted in *B. pumilus* forming a distinct clade (Rooney et al. 2009). Because there were two distinct clades, we determined that our strains grouped with members of the *B. pumilus* complex and not the *B. subtilis* complex. Although 16S rRNA gene sequencing has been regarded as a useful tool, it has low power to identify bacteria to the species level and poor discriminatory power for some genera of microorganisms such as *Bacillus* (Liu et al. 2013).

To discriminate among *Bacillus* spp., a polyphasic approach including phenotypic characteristics, biochemical test, FAME analysis, and sequencing of two housekeeping genes, have been used to identify bacterial species (Slabbinck et al. 2008; Tourasse et al. 2011). We sequenced an additional housekeeping gene, phosphoribosylamino imidazolecarboxamide formyltransferase (*purH)*. NCBI BLAST results revealed that BCA#24 and BCA#35 had 99% and 93% sequence identity with *B. safensis* FO-36bT and *B. pumilus* SAFR-032, respectively. Further evaluation of the strains revealed that the carbon source utilization profiles were different for BCA#24 and BCA#35 when compared to *B. safensis* FO-36bT (type strain) and *B. pumilus* SAF-032. However, the profiles for the chemical sensitivity tests were similar for all the strains

tested. Despite achieving rapid results with the BIOLOG system, there are several drawbacks. When utilizing BIOLOG, criteria such as fermenting vs. non-fermenting bacteria, positive vs. moderate utilization of carbon sources, false-positives, and manual vs. computer analysis of microplates can interfere with interpretation of the biochemical properties of bacterial strains (Baillie et al. 1995; Holmes et al. 1994). Moreover, strains with different modes of metabolism can interfere with correct identification, and information obtained from metabolic assays should not be used as a single method to identify *Bacillus* spp. (Baillie et al. 1995; Holmes et al. 1994; Pires and Seldin 1997).

FAME is another tool that can provide qualitative information for bacterial taxonomy. Over 300 fatty acids have been described in bacteria and differences in their chain length, position of double bonds, and their binding of functional groups, has enhanced their usefulness as taxonomic markers (Dawyndt et al. 2006). FAME analysis has potential for differentiating species within the *Bacillus* genus; however, it was suggested that while FAME analysis could identify some taxa to the species level, that may not be possible for all strains (Kampfer 1994). FAME analysis of BCA#24 and BCA#35 revealed that the most abundant fatty acids were $C_{15:0}$ iso, $C_{15:0}$ anteiso, $C_{17:0}$ iso and $C_{17:0}$ anteiso, which was similar to the profile obtained for *B. safensis* FO-36bT. In 2006, Satomi et al. isolated 13 strains of spore-forming, Gram-positive bacteria from spacecraft surfaces (Mars Odyssey Orbiter) and assembly facility surfaces at the Jet Propulsion Laboratory in California and the Kennedy Space Center in Florida (Satomi et al. 2006). In their study, a polyphasic approach was used to identify *B. safensis* FO-36bT, and based on FAME analysis, the major fatty acids of the isolates were $C_{15:0}$ iso (50.4–56.7 %), $C_{15:0}$ anteiso (23.3– 25.2 %), $C_{17:0}$ iso (4.52–6.93 %) and $C_{17:0}$ anteiso (3.71– 4.69 %), which was similar to our findings.

We sequenced the genomes and used *in silico*-based identification methods for genome-to-genome comparison of BCA#24 and #35. DNA-DNA hybridization has been the standard approach for classification of prokaryotes by comparing the DNA relatedness of two genomes based on their degree of DNA reassociation (Rosselló-Móra et al. 2011). Drawbacks of doing wet lab procedures, such as DNA-DNA hybridization (DDH), including inaccurate results, inability to produce incremental databases, and the cumbersome task of performing the procedure, has motivated scientists to move towards alternative *in silico* methods that can produce equivalent results (Cho and Tiedje 2001; Rosselló-Móra et al. 2011). Goris et al. (2007) proposed ANI as an alternative to DDH. They reported that a thorough measure of genetic and evolutionary distance could be determined by comparing the conserved DNA present in two sequenced strains (Goris et al. 2007). Additionally, they suggested that the cut-off point of 70% DDH relatedness values for species delineation is correlated to 95% ANI and 69% conserved DNA. In this study, the DNA-DNA relatedness based on ANI exhibited 99% similarity to *B. safensis* FO-36b^T indicating close relatedness of BCA strains with *B. safensis*. These results were similar to phylogenic analysis of the *purH* gene, which also resulted in *B. safensis* FO-36b^T being one of the closest neighbors.

To further verify our BCA strains as *B. safensis*, we utilized a genome-to-genome distance calculator (GGDC), which is an additional *in silico* method that allows genome-to-genome comparisons (Meier-Kolthoff et al. 2013). Advantages of the GGDC program over ANI include higher correlations with wet-lab procedures used to obtain DDH values, better use of statistical models that improve on the linear models used with ANI, and utilization of confidence intervals to support decisions regarding relatedness of compared strains. Based on the GGDC program, BCA strains in this study were most closely related to *B. safensis* FO-36b^T with a DDH

value of 96% and a confidence interval between 94 and 97% for both strains. These findings were similar to the phylogenic analysis of the *purH* gene and the ANI results. Based on results of the polyphasic approach taken in this study, we identify BCA#24 and BCA#35 isolated from watermelon seeds as *B. safensis*.

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Table 3.1. Bacterial strains used in this study.

Bacillus spp.	Relevant characteristics	Source
B. mojavensis RRC 101		Bacon, C., USDA-ARS, Athens, GA
B. safensis strain #24	Isolated from watermelon seed	This study
B. safensis strain #35	Isolated from watermelon seed	This study
B. safensis FO-36bT	ATCC type strain	Kasthuri, J., Jet Propulsion Laboratory and Biotechnology, Planetary Protection Group, NASA
B. pumilus SAFR-032	ATCC type strain	Kasthuri, J., Jet Propulsion Laboratory and Biotechnology, Planetary Protection Group, NASA

Table 3.2. Carbon source utilization of *Bacillus safensis* (strains BCA#24 and BCA#35), *B. safensis* FO36bT (type strain) and *B. pumilus* SAF-032 (reference strain).

Carbon Source	BCA#24	BCA#35	*B. safensis* FO36bT	*B. pumilus* SAF-032
Pectin	-	-	+	/
D-Mannitol	/	/	+	+
Glycyl-L-Proline	+	-	-	-
Methyl Pyruvate	-	-	/	+
D-Raffinose	/	-	-	+
Gelatin	+	/	-	+
γ-Amino-Butryric Acid	+	/	-	+
D-Melibiose	-	-	/	/
D-Fructose	/	-	+	+
L-Alanine	+	+	+	+
L-Galactonic Acid Lactone	-	-	/	+
D-Trehalose	/	/	+	+
β-Methyl-D-Glucoside	/	/	+	+
D-Galactose	-	-	+	/
myo-Inositol	-	-	/	/
L-Arginine	+	/	+	+
D-Gluconic Acid	+	/	+	/

*Score sheet adopted from the GEN III MicroPlate instruction manual: (+) = wells with a noticeably more purple color in reference to the negative well, (/) = wells with a faint color that were considered borderline, and (-) = wells that visually resembled the negative well and did not have a purple color. Diagnostic traits were determined from 71 carbon source utilization wells.

Table 3.3. Chemical sensitivities of *Bacillus safensis* (strains BCA#24 and BCA#35), *B. safensis* FO36bT (type strain) and *B. pumilus* SAF-032 (reference strain).

Chemical	BCA#24	BCA#35	*B. safensis* FO36bT	*B. pumilus* SAF-032
1% NaCl	+	+	+	+
1% Sodium Lactate	+	+	+	-
Nalidixic Acid	-	-	+	+
Aztreonam	+	+	+	+
pH 6	+	+	+	+
4% NaCl	-	/	+	+
Fusidic Acid	+	-	-	-
Rifamycin SV	+	-	+	+
Guanidine HCl	+	/	/	+
Lithium Chloride	+	+	+	+
Sodium Butyrate	+	+	+	+
pH 5	+	+	+	+
8% NaCl	-	-	+	+
D-Serine	+	+	-	+
Troleandomycin	-	-	-	-
Linocomycin	-	-	-	-
Vancomycin	-	-	-	-
Nalidixic Acid	-	-	+	+
Fusidic Acid	-	-	-	-
Tetrazolium Violet	-	-	-	/
Minocycline	-	-	-	-
Tetrazolium Blue	-	-	-	-

*Score sheet adopted from the GEN III MicroPlate instruction manual: (+) = wells with a noticeably more purple color in reference to the negative well, indicating the strain was not sensitive to the chemical (/) = wells with a faint color that were considered borderline, and (-) = wells that visually resembled the negative well and did not have a purple color, indicating the strain was sensitive to the chemical. Diagnostic traits were determined from a total of 23 chemical sensitivity assays.

Table 3.4. Cellular fatty acid profiles of *Bacillus safensis*, *B. pumilus*, and strains isolated from 24-h germinating watermelon seeds (*B. safensis* BCA#24 and BCA#35).

Fatty acid	*B. safensis* FO-36bT	BCA#24	BCA#35	*B. pumilus* SAF-032
13:0 iso	-	-	-	0.55
14:00	-	-	-	0.67
14:0 iso	0.69	0.75	0.75	0.96
15:0 iso	41.62	35.42	36.97	29.57
15:0 anteiso	24.96	25.16	26.57	35.74
16:0 iso	2.62	2.87	3.12	2.83
16:1 w11c	0.54	1.32	1.29	-
16:0	5.79	7.48	6.88	10.34
17:1 iso w10c	0.80	1.38	1.70	-
17:0 iso	13.77	11.63	12.28	8.24
17:0 anteiso	7.53	7.89	8.47	7.84
18:1 w9c	0.59	2.94	0.94	1.49
18:1 w7c	-	1.61	-	-
18:0	1.09	1.53	1.03	1.76
Summed feature 8:	-	1.61	-	-

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 8 comprises C18: 1w7c and/or C18: 1w6c.

Table 3.5**.** Average nucleotide identity (ANI) of *Bacillus safensis* strains BCA#24 and BCA#35 compared with the genomes of closely related *Bacillus* spp.

Query strain	Subject taxon	Strain	Accession#	ANI (%)
BCA#24	*B. cytotoxicus*	NVH 391-98	NC_009674.1	77.25
	B. altitudinis	GQYP101	NZ_CP040514	87.82
	B. subtilis subsp. *subtilis*	str. 168	NC_000964	77.62
	B. pumilus	ATC7061T	ABRX01000001	90.63
	B. safensis	BRM1	CP018100	99.02*
	B. safensis	FO-36bT	NZ_CP010405	99*
BCA#35	*B. cytotoxicus*	NVH 391-98	NC_009674.1	77.35
	B. altitudinis	GQYP101	NZ_CP040514	87.87
	B. subtilis subsp. *subtilis*	str. 168	NC_000964	77.57
	B. pumilus	ATC7061T	ABRX01000001	90.54
	B. safensis	BRM1	CP018100	99.01*
	B. safensis	FO-36bT	NZ_CP010405	98.99*
	BCA#24			100*

*indicates the strain the query strain was most similar to.

Table 3.6**.** Analogous DNA-DNA hybridization (DDH) percentages determined based on the genome-to-genome distance calculations (GGDC) of *Bacillus safensis* strains BCA#24 and BCA#35 in relation to the genomes of closely related *Bacillus* spp.

Query	Subject taxon	Strain	Accession#	GGDC (DDH %)
BCA#24	*B. cytotoxicus*	NVH 391-98	NC_009674.1	12.90
	B. altitudinis	GQYP101	NZ_CP040514	85.40
	B. subtilis subsp. *subtilis*	str. 168	NC_000964	15.20
	B. pumilus	ATC7061T	ABRX01000001	24.20
	B. safensis	BRM1	CP018100	95.70*
	B. safensis	FO-36bT	NZ_CP010405	96.10*
BCA#35	*B. cytotoxicus*	NVH 391-98	NC_009674.1	12.9
	B. altitudinis	GQYP101	NZ_CP040514	88.40
	B. subtilis subsp. subtilis	str. 168	NC_000964	15.20
	B. pumilus	ATC7061T	ABRX01000001	24.20
	B. safensis	BRM1	CP018100	95.70*
	B. safensis	FO-36bT	NZ_CP010405	96.10*

*indicates the strain the query strain was most similar to.

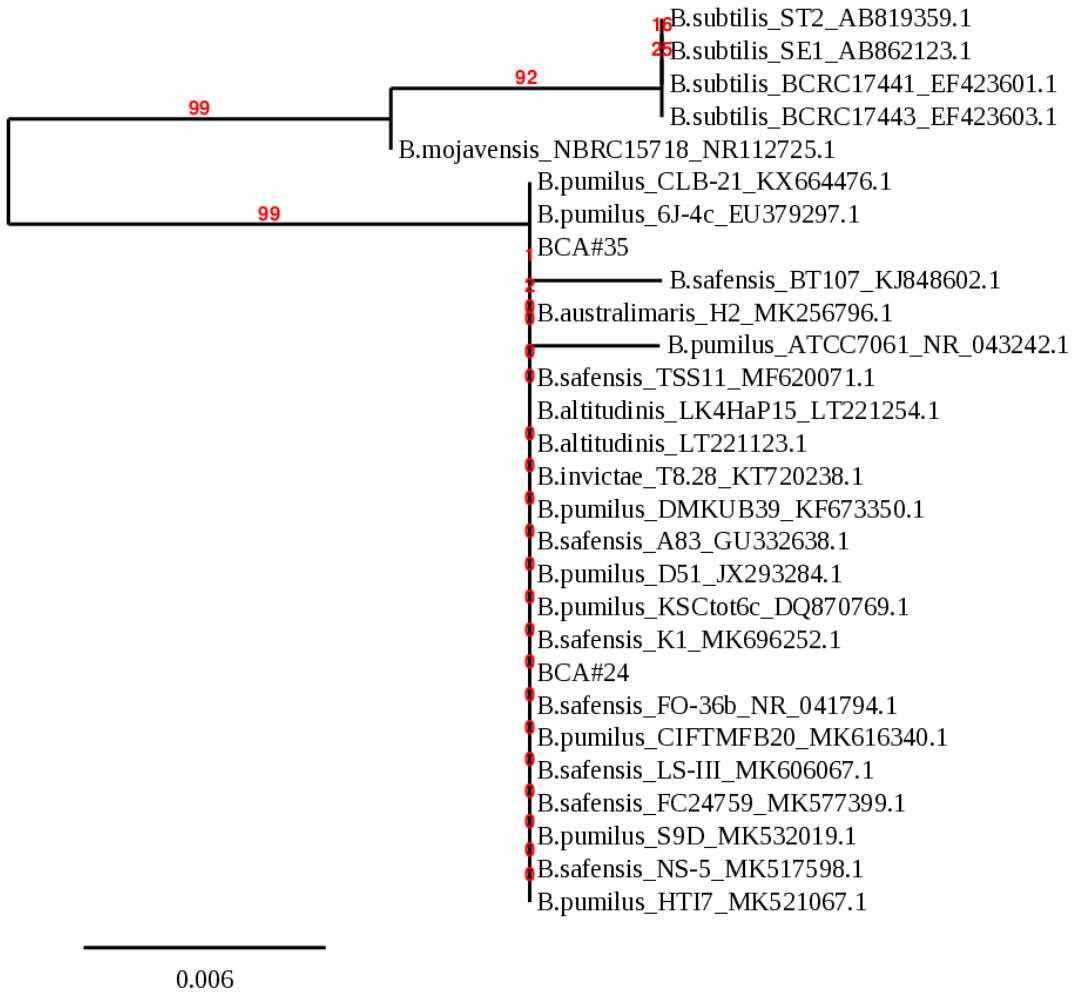

Figure 3.1. Maximum likelihood phylogenetic tree based on the 16S rRNA gene sequences for *Bacillus* spp. Bootstrap values are shown at the nodes based on 1,000 replications. Sequences were obtained from NCBI GenBank and aligned using phylogeny.fr: web service to analyze phylogenic relationships. Bar 0.006 represents substitutions per nucleotide position. The accession numbers are listed adjacent to respective bacterial strains.

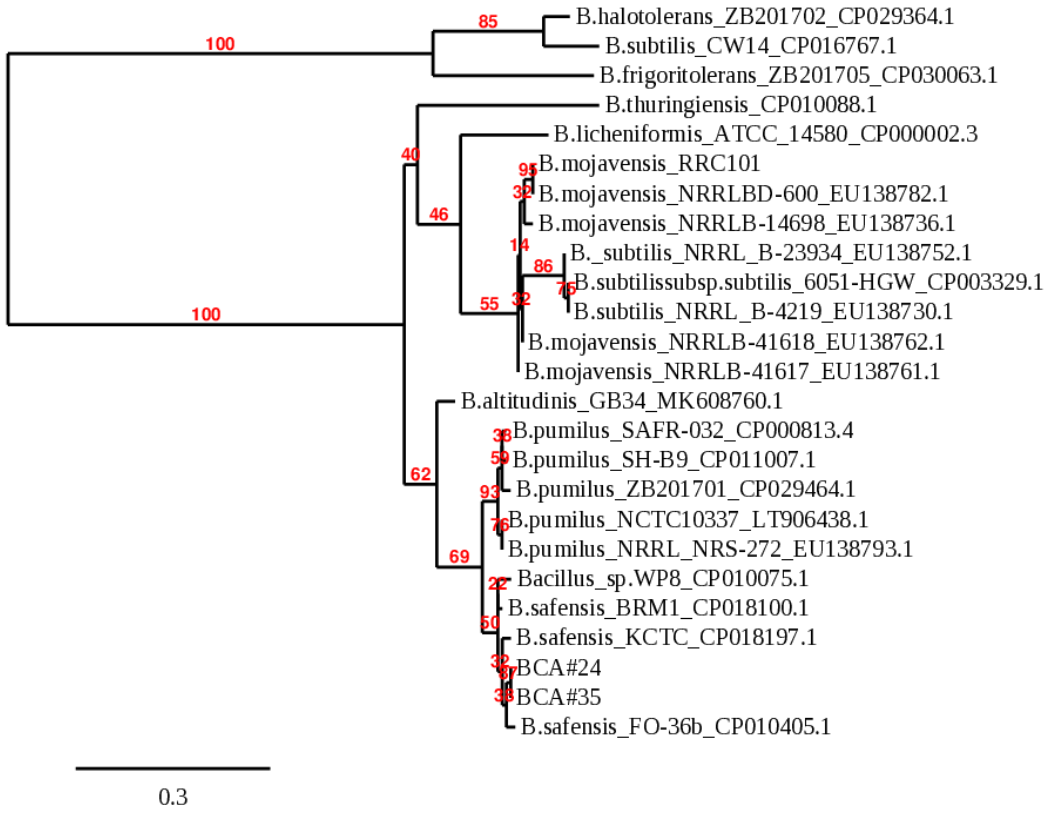

Figure 3.2. Maximum likelihood phylogenetic tree based on the *purH* gene sequences for *Bacillus* spp. Bootstrap values are shown at the nodes based on 1,000 replications. Strains were obtained from NCBI GenBank and aligned using phylogeny.fr: web service to analyze their phylogenic relationships. Bar 0.3 represents substitutions per nucleotide position. The accession numbers are listed adjacent to the respective bacterial strains.

CHAPTER 4

EFFICACY OF FLOWER-APPLIED TREATMENTS TO INOCULATE WATERMELON SEEDS WITH BIOLOGICAL CONTROL BACTERIA

Sutton, S. and Walcott, R. to be submitted to Plant Disease

ABSTRACT

Acidovorax citrulli is a Gram-negative bacterium that causes bacterial fruit blotch (BFB) of cucurbits. Despite implementation of disease management practices, periodic outbreaks continue to occur worldwide. *A. citrulli* is seed-borne and seed-transmitted, and several seed treatments have been utilized to manage outbreaks. However, the bacterium can localize within the endosperm of seeds, where it is protected from externally applied treatments. Biological control is a desirable approach for BFB management. Because seed-borne *A. citrulli* can be protected from externally applied chemicals, we propose using the pistil pathway of watermelon flowers to inoculate seed with biocontrol agents (BCAs). In the current study, the efficacy of flower treatments to internally inoculate watermelon seeds with BCAs under field conditions was evaluated. Watermelon field plots were established and at anthesis, stigmatic papillae were pollinated and treated with 10_8 colony-forming units (CFU)/flower of *Bacillus mojavensis* RRC101, *B. safensis* (BCA#24 and BCA#35) or water as a control. Watermelon seeds were harvested from mature fruit, inoculated with a cocktail of *A. citrulli* strains (10_7 CFU/ml) and tested for BFB seed-to-seedling transmission in a grow-out assay. In two experiments, flowers treated with *B. mojavensis* RRC101 and *B. safensis* BCA#35 produced seeds that displayed reduced BFB seedling transmission (41.2-47.9%) compared with seeds produced from non-treated flowers (81.2%). Findings from this research suggest that watermelon flowers can be used to introduce BCA into seeds to protect them against *A. citrulli*. However, further optimization is required to improve the efficacy of this approach.

Keywords: Bacterial fruit blotch, flower treatments, biological control, *Acidovorax citrulli*, *Bacillus mojavensis*, *Bacillus safensis*, seed treatment.

INTRODUCTION

Bacterial fruit blotch (BFB), caused by *Acidovorax citrulli* (Schaad et al. 2008; Schaad et al. 1978) is a major concern for cucurbit producers worldwide. Since the first report of BFB in commercial watermelon production fields in Florida, the disease has been a persistent threat to cucurbit seed production worldwide (Bahar and Burdman 2010; Somodi et al. 1991). The frequency of BFB outbreaks in Georgia has declined compared with the early 1990s; however, the pathogen has been re-emerging and was ranked as one of the top three research priorities for watermelon producers (Kousik et al. 2016). *A. citrulli* is a destructive pathogen under conducive conditions and can spread rapidly through transplant houses and fields (Bahar and Burdman 2010). BFB symptoms typically start as water-soaked lesions on cotyledons that appear 6 to 10 days after germination (Burdman and Walcott 2012; Webb and Goth 1965). Water-soaked lesions may be inconspicuous and overlooked by visual inspection, and as a result, infected seedlings may be transplanted into the field. These infected seedlings can serve as primary inoculum sources and can imitate epidemics (Walcott 2008).

Infected seeds are the most important primary source of inoculum for *A. citrulli* and the bacterium has been reported to survive for at least 30 years in stored watermelon ad melon seeds (Block and Shepherd 2008; Latin and Hopkins 1995; Rane and Latin 1992). Seeds can become internally contaminated when *A. citrulli* cells penetrate the ovary pericarp or invade the pistil of the female flower (Dutta et al. 2012a). Infested seeds that result from *A. citrulli* pistil invasion make BFB management particularly challenging because bacterial cells localized within the endosperm are protected from externally applied seed treatments (Dutta et al. 2012a; Dutta et al. 2012b). Seed treatments including seed fermentation, sodium hypochlorite, chlorine dioxide, hydrochloric acid, streptomycin sulfate, mercury chloride, thermotherapy and peroxyacetic acid

have been utilized to manage BFB; however, none have been 100% effective (Chao et al. 2010; Feng et al. 2009; Hopkins et al. 1996; Hopkins and Thompson 2006; Hopkins et al. 2003). To manage BFB seed producers rely on producing *A. citrulli*-free seeds in regions where environmental conditions are not conducive for BFB development, visual field inspections and rigorous seed health testing (Lovic and Hopkins 2003). To date, there is no management approach that is 100% efficacious for eliminating BFB from watermelon seeds; hence, new strategies are constantly being sought.

To improve BFB management, we propose applying biocontrol agents (BCAs) to female watermelon flowers in order to internally inoculate and protect seeds. The objective of the current study was to evaluate the ability of BCAs applied as watermelon flower treatments to protect against seedborne *A. citrulli*.

MATERIALS AND METHODS

Bacterial culture and inoculum preparation

Candidate BCAs that were isolated from 48-h germinated watermelon seeds or obtained from collaborators are listed in Table 4.1. Strains were routinely grown on Luria-Bertani (LB) agar (VWR Life Science, Radnor, PA) for 24 h at 37°C. To prepare BCA inoculum, LB broth was inoculated with a single colony of each strain and incubated overnight at 28°C on a rotary shaker (Innova; New Brunswick Scientific Co., Edison, NJ) at 120 min^{-1}. Five milliliters of broth culture were then centrifuged at 10,000 min^{-1} for 5 min. The supernatant was decanted, and the pellet was suspended in sterilized distilled deionized water (sddH_2O). Bacterial concentrations were estimated spectrophotometrically with an optical density OD_{600} nm = 0.3 (~ 1 x 10^8 CFU/ml) and adjusted to the desired concentration by ten-fold serial dilutions. *A. citrulli* strains

used in this study Table 4.1 were routinely grown on King's medium B (KMB) (King et al. 1954) for 48 h at 28°C and inoculum was prepared as described above.

Watermelon flower inoculation with BCAs

Experiments were conducted in the summers of 2015 and 2018 where watermelon field plots were established at the Durham Horticulture Farm, Athens, GA and the UGA Black Shank Farm, Tifton, GA. Field plots were grown on plastic mulch with drip irrigation, and fertilization, and pest management was conducted according to the UGA Extension guidelines for watermelon (Boyhan et al. 2000; Dutta 2018). Watermelon transplants (cv. Crimson Sweet) were generated in the greenhouse for 3 weeks. During the fourth week, transplants were transferred outside the greenhouse for 1 week to reduce transplant shock during transplanting. During the month of May, seedlings were transplanted to field plots. Experiments were organized in a randomized complete block design with three blocks and four treatments per block. Each block contained four rows with ten plants per row. The four treatments were: 1) sddH_2O, 2) *B. mojavensis* RRC101, 3) *B. safensis* BCA#24, and 4) *B. safensis* BCA#35. Randomizer.org was used to randomly assign treatments in each block. At anthesis (during late June through mid-July), female watermelon flowers were hand-pollinated, and a micropipette was used to deliver 10 µl of cell suspension containing 10^8 CFU/flower or sddH_2O to the stigmatic papillae of female flowers. Flowers were allowed to develop for 35-40 days and then fruit were harvested and stored at 4°C until seeds were extracted. To extract seeds, fruit and all tools were surface sterilized with 0.5% NaOCl before each fruit was processed. Fermentation was not employed for seed extraction and seeds were rinsed, air-dried for 24-48 h at room temperature and stored in paper envelopes at room temperature until they were tested. Seeds from each fruit were maintained as separate lots.

Field experiments were conducted two times in Tifton, GA (summer 2015 and 2018), and one time in Athens, GA (summer 2018).

Efficacy of BCA-inoculated seeds in reducing BFB seed-to-seedling transmission

Samples (n = 40 seeds) from each seed lot were inoculated with cell suspensions of *A. citrulli* (10_7 CFU/ml of a mixture of AAC00-1 and AAC213-60) as follows. Seeds were vacuum-infiltrated with the cell suspensions in four 10-min intervals and then air-dried at room temperature for 24 h. Inoculated seeds were placed into capped Pyrex glass tubes (one seed/tube) containing a cotton ball saturated with sddH_2O. Seeds were allowed to germinate and grow for 14 days at 100% RH and 30°C with continuous fluorescent light. Germination percentages were recorded as: (number of seedlings with a protruded radicle and visible cotyledons/number of seeds planted) x 100. BFB incidence and seed germination percentages were recorded. BFB incidence was determined as: (number of seedlings showing typical water-soaked lesions/total number of germinated seedlings) x 100. Analysis of variance (ANOVA) was used to determine the effect of flower treatment with BCAs on germination and BFB seed-to-seedling transmission. Treatment means were separated using Tukey's test.

Correlation of BCA bacteria in seeds to BFB incidence

To determine the presence of BCA bacteria in seeds, 52 seed lots from flowers treated with *B. safensis* BCA#24, *B. safensis* BCA#35, *B. mojavensis* RRC101 or sddH_2O were tested by qPCR assay as described below. The 52 seed lots included at least four lots per treatment. Seed samples were enriched for BCA bacteria before microbial DNA extraction as follows. Samples (n = 25 seeds) from each lot were crushed in Bioreba universal extraction bags (BIOREBA AG, Kanton Reinach, Switerland) and 10 ml of sddH_2O were added to each bag followed by incubation at room temperature for 5 min. Two milliliters of seed macerate were

added to 3 ml of LB broth followed by overnight shaking at 28˚C on a rotary shaker (Innova; New Brunswick Scientific Co., Edison, NJ). DNA was extracted from 2 ml of enriched seed crush using an UltraClean™ Microbial DNA Kit (MO BIO Laboratories, Inc., Carlsbad, CA.

To develop species-specific PCR assays, sequences of the *purH* and *gyrB* genes from *B. mojavensis* RRC101, BCA#24, BCA#35, *B. safensis* FO-36bT, *B. pumilus* SAF-032, and *B. subtilis* ATCC6051 were aligned using Geneious 2019.1.3 sequence analysis software. Two PCR primer sets per gene were generated for *B. mojavensis* and *B. safensis* (Table 4.2). To test for specificity, 1 ml of 10_8 CFU/ml bacterial cell suspensions from *B. mojavensis* RRC101, BCA#24 and BCA#35, *B. safensis* FO-36bT, *B. pumilus* SAFR-032, AAC00-1, AAC213-60, *B. subtilis* ATCC6051, and *C. michiganensis* were incubated at 95℃ for 10 min to lyse bacterial cells. Subsequently, 2.5 µl of each cell suspension was used in a qPCR assay using Applied Biosystems Power SYBR Green PCR Master Mix (Applied Biosystems) on a StepOne Real-Time PCR System (Applied Biosystems). The reaction included 12.5 µl of SYBR Green PCR master mix, 1 µl of forward and reverse primers (10nM) each, 2.5 µl of template DNA and 8 µl of H_2O. The PCR amplification was performed on a StepOne Real-Time PCR System with the following thermal profile for the *purH* primer pairs: 95℃ for 10 min, 40 cycles of 95℃ for 15 s, 65℃ for 60 s, and a melt curve analysis. The thermal profile for the *gyrB* primers sets was as mentioned above with the adjustment of the primer annealing temperature set to 60℃. To test for primer sensitivity, 271 ng of *B. mojavensis* RRC 101 and 153 ng of BCA#24 DNA were serially diluted by seven orders, and each dilution was used in PCR assay as described above. Cycle threshold (Ct) values greater than 33 were considered negative for BCA bacteria. To determine whether BFB transmission was related to the concentration of BCAs in seeds, correlation analysis was conducted for BFB incidence data and Ct values for each seed lot.

RESULTS

Effect of BCA flower treatment on seed germination

Seed germination percentages were 86% or higher for all seed lots. In the two experiments conducted in Tifton, GA, the effect of flower inoculation on germination was statistically significant ($P = 0.03$). Flowers inoculated with *B. mojavensis* RRC101 yielded seed lots with an average germination percentage of 88.2%, which was significantly lower than that observed for seed lots from flowers treated with *B. safensis* BCA#24 (98.6%). Flowers inoculated with water or *B. safensis* BCA#35 yielded seeds with germination percentages of 96.2% and 90.1%, respectively, and were not significantly different from seed lots from other treatments (Fig. 4.1). For the experiment conducted in Athens, GA, the flower treatments did not have a significant effect on germination percentage ($P = 0.81$), and all treatments resulted in germination percentages of at least 86.3% (Fig. 4.2).

Efficacy of BCA-inoculated seeds in reducing BFB seed-to-seedling transmission

According to a two-way ANOVA, there was a significant interaction between location and treatment ($P = 0.0318$); therefore, seed lots generated in Athens, GA were analyzed separately from lots generated in Tifton, GA. In two independent field experiments conducted at Tifton in 2015 and 2018, a total of 14 negative control, 13 BCA#24, 10 BCA#35, and 14 B. *mojavensis* RRC101-inoculated watermelon seed lots were generated, and each treatment had a minimum of two seedlots per experiment. Overall, the effect of flower treatment on BFB seedling transmission was significant ($P = 0.003$). BFB seedling transmission for seeds from watermelon flowers treated with *B. safensis* BCA#35 (47.9%) and *B. mojavensis* RRC101

(41.2%) were significantly lower than for seeds from flowers treated with water (81.2%) and B. *safensis* BCA#24 (56.4%) (Fig. 4.3).

In the experiment conducted in Athens in 2018, 38 watermelon seed lots were generated including 12 negative control, 7 *B. safensis* BCA#24, 9 *B. safensis* BCA#35, and 10 B. *mojavensis* RRC101-inoculated seed lots. Each treatment had a minimum of 7 seed lots. In this experiment, the effect of treatment was not significant ($P = 0.71$) and all lots, regardless of treatment resulted in mean BFB incidences of 60.7% or higher (Fig. 4.4).

Correlation of BCA bacteria in seeds with BFB seed transmission

Primers designed for detection of BCAs were found to be specific for *B. safensis* and *B. mojavensis* RRC101 when screened against bacteria used in this study (Tables 4.3. and 4.4). However, the PCR assays were limited in terms of sensitivity, displaying a limit of detection (LOD) of 10^5-10^6 CFU/ml (Tables 4.5 and 4.6). The qPCR MOV*gyrb*2 primer set developed for the amplification of *B. mojavensis* RRC101 was not specific (data not shown) and amplified *C. michiganensis*, *B. safensis*, and *B. pumilus*. Therefore, the *purH* primer sets were used for detection of *B. mojavensis* RRC101 instead of *gyrB* primers.

In experiments conducted in Tifton, flowers treated with *B. safensis* BCA#35 resulted in the highest percentage of positive seed lots for this BCA (100%) (Fig. 4.5). In comparison, seed lots from flowers treated with *B. safensis* BCA#24 and *B. mojavensis* RRC101, or water resulted in lots that were 85, 65, and 75% positive respectively (Fig. 4.5). In experiments conducted in Athens, seeds from flowers treated with water were 20% positive for BCA bacteria, and seeds from flower treatments with BCA bacteria were all 50% positive for BCAs (Fig. 4.6). A correlation analysis was conducted to investigate the relationship between BCA concentration in seeds and seedling BFB incidence based on seedling grow-out assays. Overall, we observed low

correlations between BFB incidence and BCA concentration in seeds. R_2 values for the relationships between the concentration of *B. safensis* BCA#24, BCA#35, and *B. mojavensis* RRC101 and BFB incidence were 0.02, 0.28, and 0.13, respectively.

DISCUSSION

Flowers represent unique opportunities to deposit beneficial microorganisms inside seeds of host or non-host plants (Ark 1994; Donati et al. 2018; Dutta et al. 2012a; Dutta et al. 2014; Kauffman and Leben 1974; van der Wolf and van der Zouwen 2010). Studies have demonstrated the biocontrol of *Erwinia amylovora*, *A. cirtulli*, *Monilinia vaccinii-corymbosi*, *Dothiorella aromatica* and *Phomopsis perseae* by applying antagonistic BCAs to flowers (Dedej et al. 2004; Demoz and Korsten 2006; Fessehaie and Walcott 2005; Johnson et al. 2011; Roselló et al. 2013). While there has been some success in reducing disease incidence through BCA applications to flowers, there have been no efforts to assess the ability to internally colonize watermelon seeds with BCA. Lessl et al. reported that increasing the concentration of bacteria applied to watermelon flowers increased the level of infested seeds (Lessl et al. 2007). However, they did not consider the distribution of inoculum in seeds within seed lots. The goal of generating seeds internally inoculated for biological control is to deposit BCAs in high concentrations into high percentages of seeds. One of the major challenges with utilizing floral tissues for deposition of BCA bacteria into seeds is being able to generate high proportions of seeds with high levels of inoculum. To overcome this challenge, a better understanding of the relationship between inoculum concentration applied to flowers and the percentage of inoculated seeds in resulting lots is an essential factor to consider.

Although it was suggested that the percentage of infested seeds per lot increased with increasing watermelon flower inoculum concentration, Lessl et al. (2007) reported a discrepancy in the strength of the linear relationship. They recommended using qPCR assays to allow better quantitative interpretation of results. Before initiating our field flower inoculation studies, we empirically determined that 10_8 CFU/flower was the optimal concentration for seed inoculations (data not shown).

With regards to the ability to inoculate watermelon flowers with BCAs and thereby protect seeds from *A. citrulli*, the two experiments conducted in Tifton were not significantly different, so the data were combined. Flower treatments with *B. mojavensis* RRC101 and BCA#35 resulted in seed lots that displayed reduced BFB incidences of 41.2 and 47.9% respectively when challenged with 10_7 CFU/ml of *A. citrulli*. These BFB seedling transmission percentages were significantly lower than for seeds from flowers treated with water (81.2%). Unexpectedly, BCA flower treatments in Athens did not result in reduced BFB seed-to-seedling transmission, and all treatments had mean BFB incidence levels of 60.7% or higher. It is not uncommon to observe variable results in biocontrol efficacy under field conditions (Axel et al. 2012; Dorn et al. 2007). One of the disadvantages of using BCAs under field conditions is that the environment can have a large impact on efficacy, and this may have contributed to the of lack of reproducibility that we observed (Kn et al. 2012). The efficacy of BCAs under natural conditions can be affected by variations in temperature and humidity, lack of survival of BCAs, and variable production of secondary metabolites (Mark et al. 2006).

To determine the presence of BCA bacteria in the seed lots generated under field conditions, qPCR assays were utilized. Due to the reduction in BFB incidence we observed, we wanted to determine the level of BCA bacteria in seeds and additionally correlate the presence of

BCA bacteria with disease reduction. However, this approach was problematic because it was difficult to quantify BCA bacteria in seeds. We designed PCR primers specific to *B. safensis* and *B. mojavensis* but the limits of detection (LOD) for these assays were relatively high, between $10^5$and 10^6 CFU/ml. Based on experiments conducted to optimize parameters for flower inoculation (Appendix A), it was determined that 30-60% of seeds that developed from flower inoculation contained $10^2 – 10^4$ CFU of the BCA. It has been reported that in naturally infected seed lots, the majority of infested seeds contain 10-10^2 CFU (Dutta et al. 2013). It is possible, then, that our inability to consistently detect BCAs by qPCR was due to BCAs that were below the qPCR LOD. Technical difficulties detecting bacteria in seeds such as PCR inhibitors in plants and seeds, live vs. dead cell detection, and designing specific, sensitive primers and probes are common with this type of research (Mazzaglia et al. 2014; Palacio-Bielsa et al. 2009). Because low levels of detectable BCA bacteria were an issue, we used enrichment in LB broth to enhance detection. After enrichment, we observed that 50-100% of the lots generated were positive for BCA bacteria. Unexpectedly, 20 and 75% of the control lots were also positive for BCA bacteria in Athens and Tifton. Since we did not extensively test the specificity of our BCA-specific primers using a large number of bacterial species, it is possible that the qPCR assay detected non-target *Bacillus* spp. However, since we observed a significant reduction in BFB seedling transmission percentage for seeds from flowers inoculated with BCA bacteria in Tifton, it is likely that BCAs were responsible for this effect.

Overall, watermelon flower inoculation with BCAs significantly reduced BFB seed-to-seedling transmission. However, we observed a high level of variability in the efficacy of the treatment. It is important to note that we challenged the BCA inoculated seeds with high concentrations of *A. citrulli* (10^7 CFU/ml), which is higher than what is normally observed for

naturally infected seed lots. The limited quantity of seed lots and seeds prevented us from retesting the seeds using lower *A. citrulli* concentrations. However, it would be interesting to re-evaluate the efficacy of BCA flower treatment with lower *A. citrulli* seed inoculum levels.

Finally, we were unable to correlate the presence of BCA bacteria in seeds with seedling BFB reduction. We hypothesized that BFB seedling transmission would be strongly and negatively correlated with high levels of BCA bacteria, but this was not the case. Since our results were highly variable, we conclude that there is a need for further optimization of the methods by which we generate and evaluate the efficacy of BCA seed priming by watermelon flower inoculation.

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Table 4.1. Strains used in this study.

Bacterium	Relevant characteristics	Source	Reference
Acidovorax citrulli			
AAC00-1	Group II strain	Walcott, R., UGA	Fessehaie and Walcott 2005
AAC213-60	Group I strain	Walcott, R., UGA	Silva et al., 2016
Bacillus spp.			
B. mojavensis RRC101	Isolated from maize kernels,	Bacon, C., USDA-ARS	Bacon and Hinton 2002
B. safensis #24 (BCA#24)	Isolated from watermelon seed	Sutton, S., UGA	This study
B. safensis #35 (BCA#35)	Isolated from watermelon seed	Sutton, S., UGA	This study

Table 4.2. Primers used in this study to detect *Bacillus mojavensis* and *Bacillus safensis.*

Primer name	Sequence 5'-3'	Function
B.m-F	CTCGCAGTTCGCGGCAATGA	*purH* gene amplification
B.m-R	GAACAGGGCTGTAATCCGCT	*purH* gene amplification
24-F	TAGCAGTGAGAGAAAACGAT	*purH* gene amplification
24-R	ATGACTGTATCATAGTCACG	*purH* gene amplification
SafengyrB3-F	TGCAGCAAGGAAAACGTGTG	*gyrB* gene amplification
SafengyrB3R	TCCCAAAGCTGAGTCGCATT	*gyrB* gene amplification
MovgyrB2-F	AAGGCGGTACTCATGAAGCC	*gyrB* gene amplification
MovgyrB2-R	CGTGCCTCTGAGTTGCCTAA	*gyrB* gene amplification

Table 4.3. Specificity of *gyrB* primers for *Bacillus safensis*.

Bacterial strain	Ct Value
Acidivorax citrulli AAC-00-1	-
BCA#24	27.07
BCA#35	27.5
Bacillus safensis FO-36b	23.36
B. pumilus SAF-032	-
B. mojavensis RRC101	-
B. subtilis ATC6051	-
Clavibacter michiganensis	-
B. mojavensis RRC101 field lot	-
Negative control	-

Table 4.4. Specificity of *purH* primers for *Bacillus mojavensis*.

Bacterial strain	Ct value
Acidovorax citrulli AAC-00-1	-
Bacillus safensis BCA#24	-
B. safensis BCA#35	-
B. safensis FO-36b	-
B. pumilus SAF-032	-
B. mojavensis RRC101	33
B. subtilis ATC6051	22.23
C. michiganensis	-
B. mojavensis RRC101	-
Negative control	-

Table 4.5. Cycle threshold values for serial dilutions of bacterial cell cultures from *Bacillus safensis* BCA#24 or *B. mojavensis* RRC101 with *gyrB*- or *purH* -based primers.

Primer Tested	Serial dilution of BCA#24 Log_{10} CFU/ml	Average Ct value
safen*gyrb*3	8	26
	7	29
	6	33
	5	-
	4	-
	3	-
	2	-
B.m. *purH*	Serial dilution of *B. mojavensis* RRC101	
	8	24
	7	28
	6	33
	5	-
	4	-
	3	-
	2	-

Table 4.6. Cycle threshold values for colony forming units (CFU) based on serial dilutions of microbial DNA of *Bacillus mojavensis* RRC101 or *B. safensis* BCA#24 using *purH* primers.

DNA concentration	Ct Value	Estimated Log_{10} CFU
Sample *B. mojavensis* RRC101		
271 ng	24.15	9
27.1 ng	20.8	8
2.71 ng	23.1	7
0.271ng	29	6
27.1pg	31.9	5
2.71pg	36	4
0.271pg	37.59	3
0.0271pg	0	2
BCA#24		
153 ng	29.3	9
15.3 ng	25	8
1.53 ng	30	7
0.153ng	33	6
15.3pg	37.04	5
1.53pg	37.45	4
0.153pg	0	3
0.0153pg	0	2

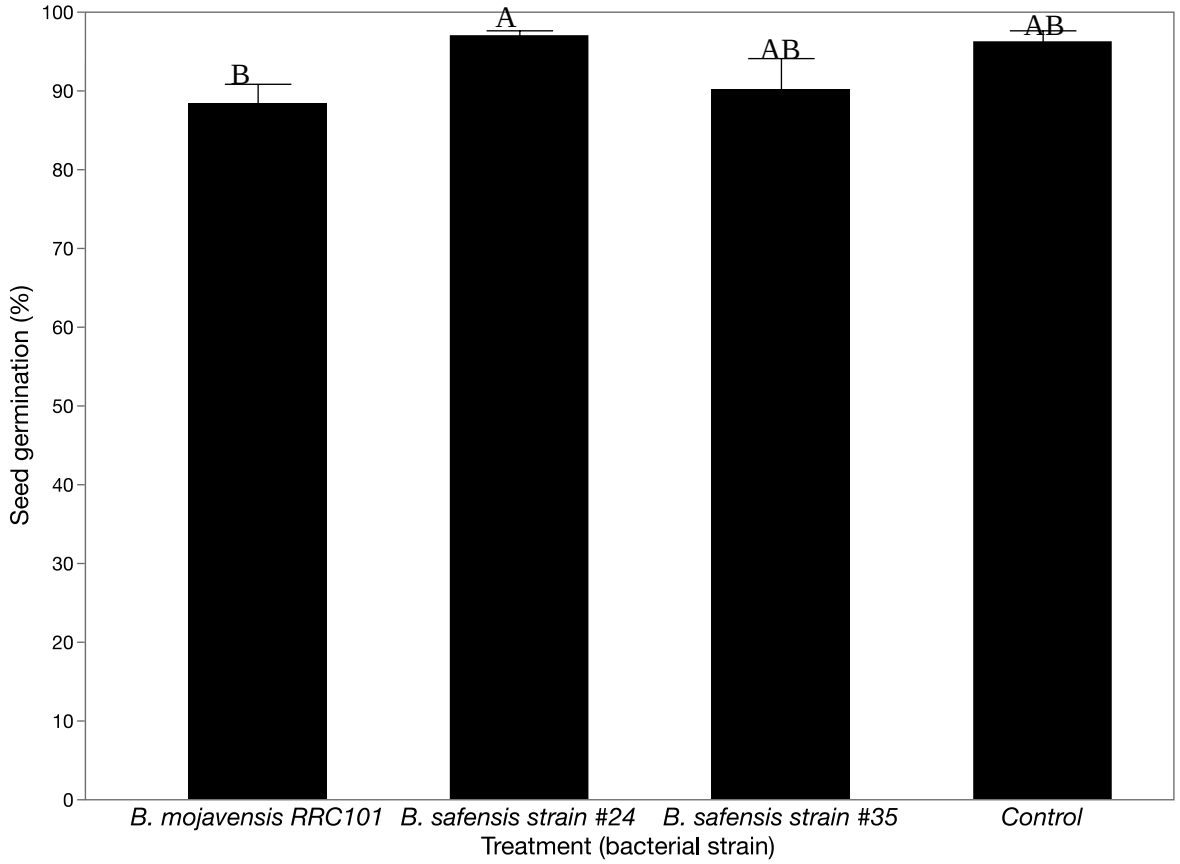

Figure 4.1. Effect of treatment of female watermelon flowers with *Bacillus mojavensis* RRC101, *B. safensis* strain #24, *B. safensis* strain #35, or water as a negative control on germination for seed lots generated in Tifton, GA. Bars represent the mean percentage of germination for two independent experiments and lines represent the standard errors of the mean. Treatments with the same letters are not significantly different according to Turkey's test ($P = 0.05$).

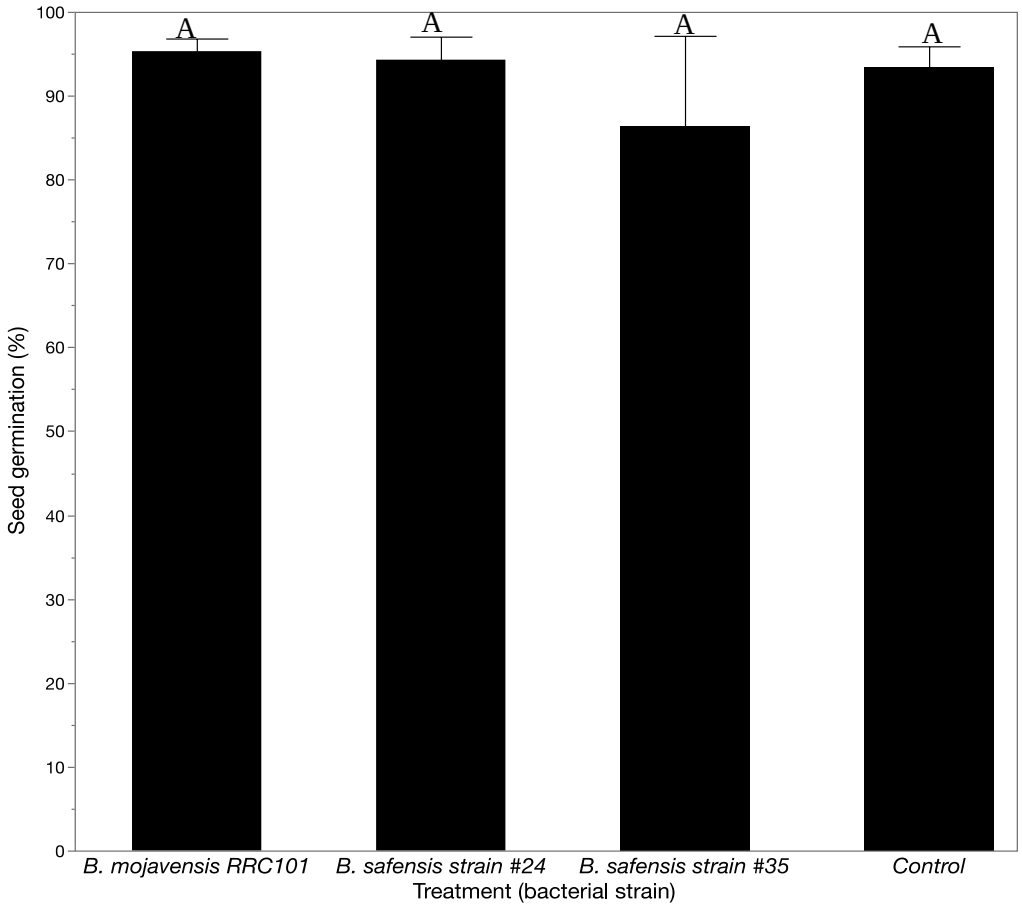

Figure 4.2. Effect of treatment of female watermelon flowers with *Bacillus mojavensis* RRC101, *B. safensis* strain #24, *B. safensis* strain #35, or water as a negative control on germination for seed lots generated in Athens, GA. Bars represent the mean percentage of germination for one experiment and lines represent the standard errors of the mean. No significant differences were observed according to Turkey's test.

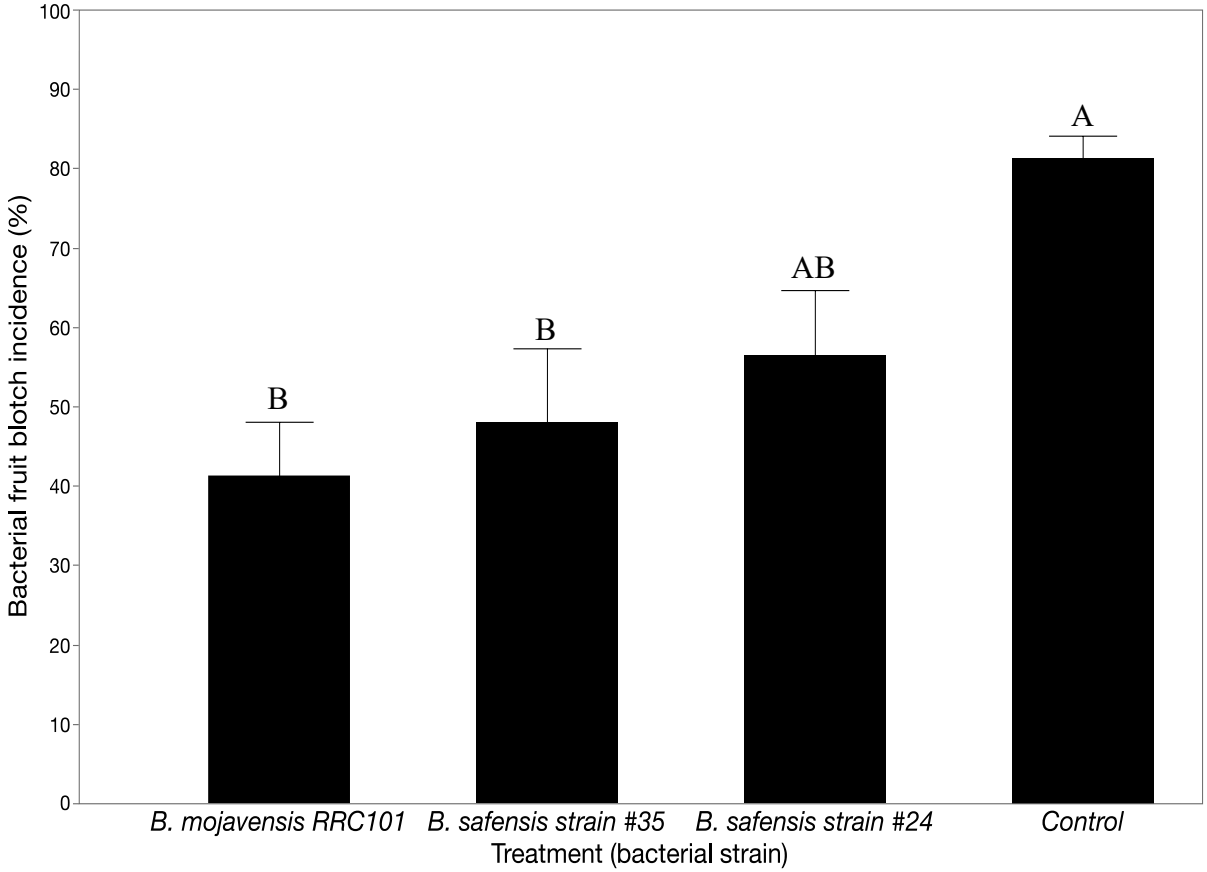

Figure 4.3. Effect of treatment of female watermelon flowers in Tifton, GA, with *Bacillus mojavensis* RRC101, *B. safensis* strain #24, *B. safensis* strain #35, or water as a negative control on bacterial fruit blotch (BFB) seed-to-seedling transmission after the resulting seeds were challenged with *Acidovorax citrulli*. Seeds (n=40) were vacuum-infiltrated with a cell suspension containing 10_7 colony-forming units/ml of an *A. citrulli* cocktail and germinated in sterilized test tubes at 30℃ and 100% relative humidity for 14 days. Bars represent the mean percentage disease incidence and lines represent the standard errors of the mean. Treatments with the same letters are not significantly different according to Turkey's test (P = 0.05).

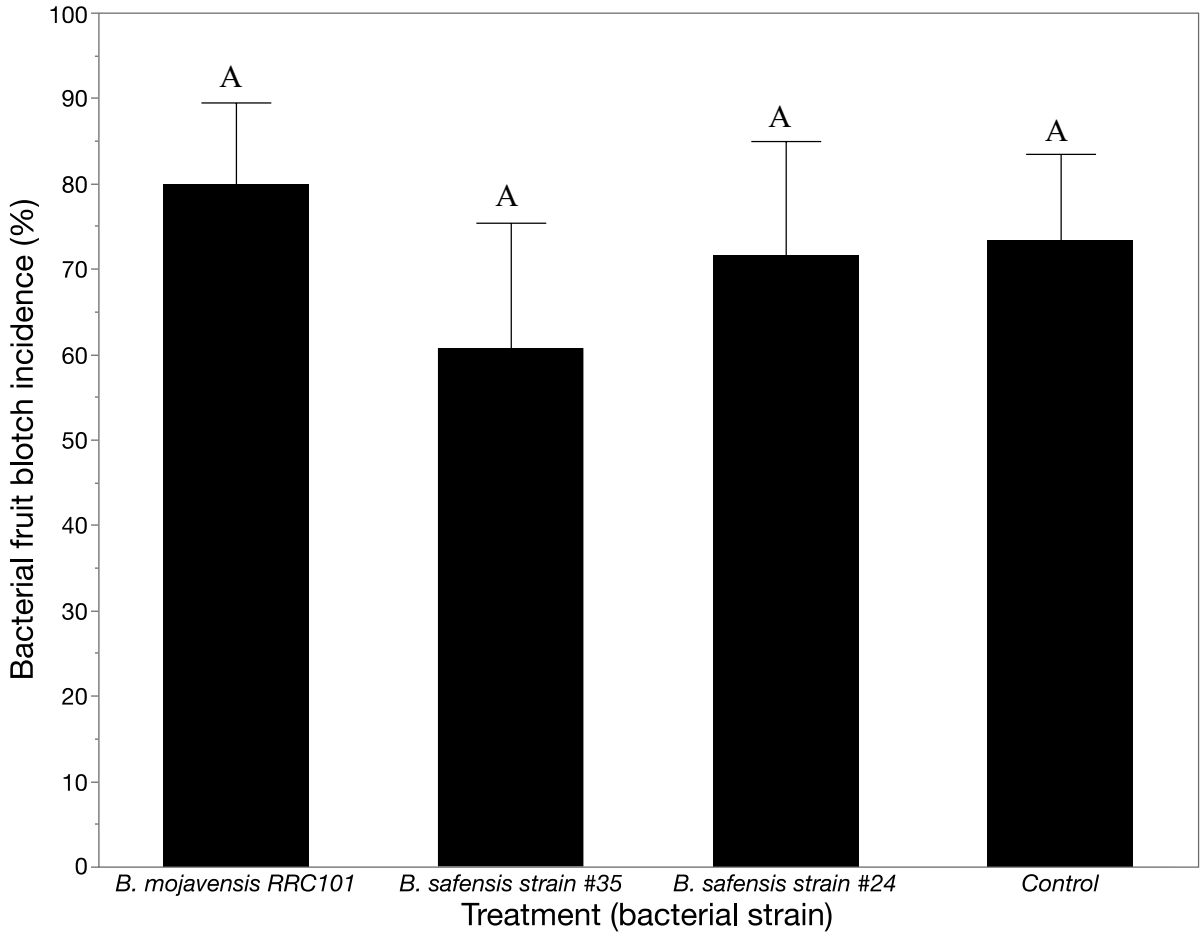

Figure 4.4. Effect of treatment of female watermelon flowers with *Bacillus mojavensis* RRC101, *B. safensis* strain #24, *B. safensis* strain #35, or water as a negative control on bacterial fruit blotch (BFB) seed-to-seedling transmission after the resulting seeds were challenged with *Acidovorax citrulli* for seed lots generated in Athens, GA. Seeds (*n*=40) were vacuum-infiltrated with a cell suspension containing 10_7 colony-forming units/ml of an *A. citrulli* cocktail and germinated in sterile test tubes at 30°C and 100% relative humidity for 14 days. Bars represent the mean BFB incidence and lines represent the standard errors of the mean. No significant differences were observed according to Turkey's test.

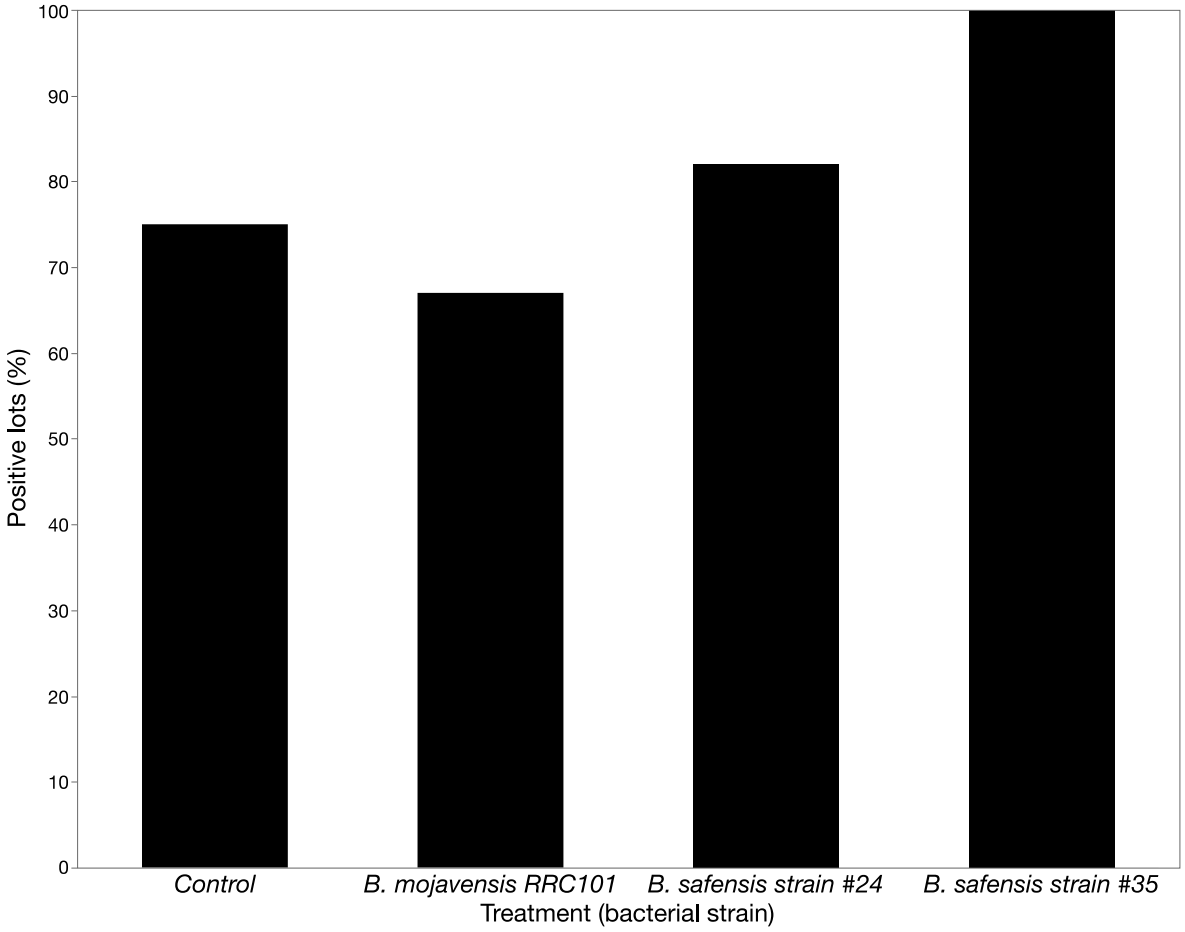

Figure 4.5. Percentage of seed lots positive with biological control agent (BCA) bacteria for watermelon seed lots from Tifton, GA. Twenty-five seeds from at least four lots for each treatment were crushed in sterilized water. Subsequently, 2-ml aliquots of seed crush were placed in 3 ml of LB broth and left in an incubator/shaker overnight to enrich for BCA bacteria. Species-specific primers were used in qPCR assays to determine the presence of BCA bacteria in seed lots. Bars represent mean percentage of lots positive for BCA bacteria.

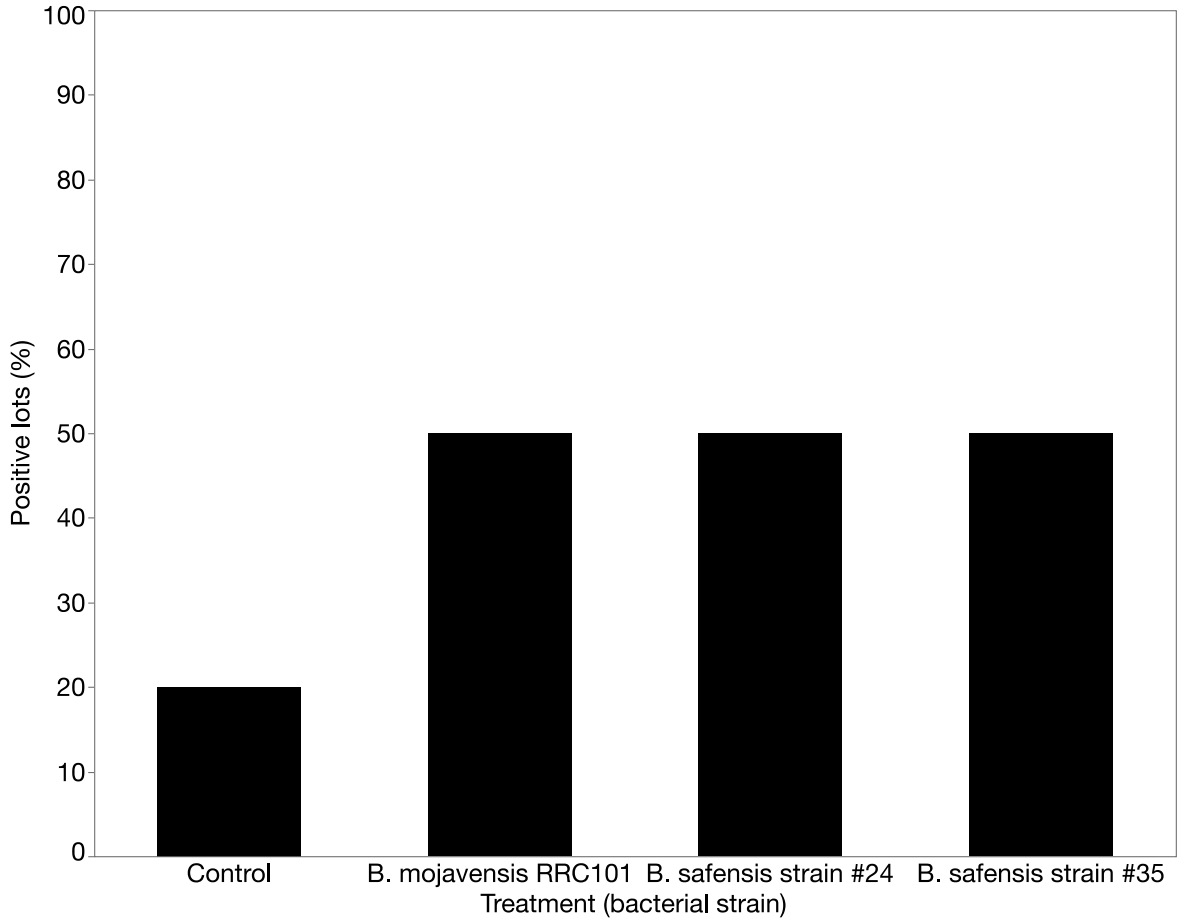

Figure 4.6. Percentage of seed lots positive with biological control agent (BCA) bacteria for watermelon lots from Athens, GA. Twenty-five seeds from at least four lots from each treatment were crushed in sterile water. Subsequently, 2-ml aliquots of seed crush were placed in 3 ml of LB broth and left in an incubator/shaker overnight to enrich for BCA bacteria. Species-specific primers were used in qPCR assays to determine the presence of BCA bacteria in seed lots. Bars represent mean percentage of lots positive for BCA bacteria.

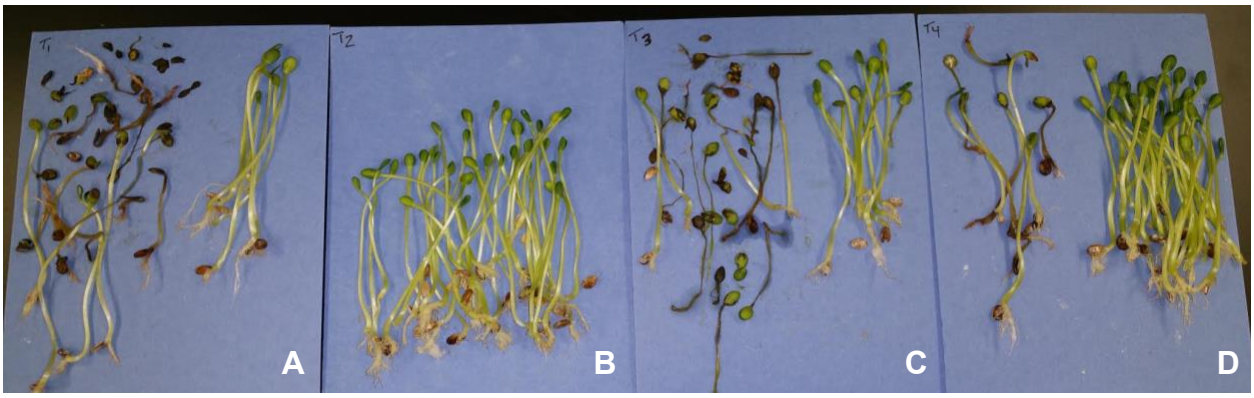

Figure 4.7. Efficacy of biological control agent (BCA)-inoculated seeds to reduce bacterial fruit blotch seed-to-seedling transmission. Image represents an example of seedlings recovered from tubes from a seedling grow-out assay where seedlings produced from seeds (*n*=40) developed from flowers incoculated with BCAs or water as a control were challenged with a cell suspension of a mixture of *Acidovorax citrulli* AAC00-1/AAC213-60 (10_7 colony-forming units/ml) by vacuum-infiltration. Seedlings produced from seeds whereby the flowers were inoculated with **A)** water **B)** *Bacillus mojavensis* RRC101 **C)** *B. safensis* strain #24 and **D)** *B. safensis* strain #35 and subsequently challenged with *A. citrulli*.

CHAPTER 5

CONCLUSIONS

Watermelon is a an economically important vegetable crop in the U.S. that was valued at $596 million in 2017 (NASS, USDA). The crop is under constant disease threat that results in substantial economic losses. For example, in 2016 in the state of Georgia alone, there was a 10% reduction in crop value based on $14.2 million worth of damage and $6 million dollars were spent on disease control (Little et al. 2016). One economically important disease that affects watermelon is bacterial fruit blotch (BFB) caused by *Acidovorax citrulli* (Schaad et al. 2008). According to the Watermelon Research Board, BFB is ranked as the third most important research priority and is determined to be a re-emerging serious problem (Kousik et al. 2016).

Seeds are the primary source of inoculum for *A. citrulli* and can become infested when bacterial cells ingress through the ovary pericarp or through the pistil of the female flower (Frankle et al. 1993; Rane and Latin 1992; Walcott et al. 2003). Interestingly, the latter pathway can lead to seed infection without the development of BFB fruit symptoms (Walcott et al. 2003). Since *A. citrulli* can localize in the embryo of seeds, it is protected from externally applied seed treatments (Dutta et al. 2012a; Dutta et al. 2012b). Despite continuous attempts to manage BFB, there has been no breakthrough in producing pathogen-free seeds (Walcott 2008).

Biological control has become an essential component of managing plant diseases in part due to concerns about environmental and human health issues associated with chemical treatments in agriculture (Tracy 2014). Several examples of biological control of BFB suggest it may be an effective part of an integrated disease management strategy. To date, most reports of

biological control of BFB have been based on *in vitro* screenings, greenhouse studies, protecting watermelon flowers, or externally applied seed treatment applications (Adhikari et al. 2017; Fessehaie and Walcott 2005; Jiang et al. 2015; Johnson et al. 2011; Medeiros et al. 2009; Sumer and Aysan 2017; Wang et al. 2008). There have been limited studies focused on internally supplying seeds with biological control agents (BCAs) as a management strategy for BFB. Therefore, the goal of this research was to evaluate the ability of watermelon flower inoculation with BCAs to protect seeds against *A. citrulli.* The specific objectives were to 1) select and characterize candidate BCAs antagonistic against *A. citrulli*, 2) identify candidate BCAs, and 3) evaluate the efficacy of candidate BCAs as flower treatments under field conditions.

Using a non-pathogenic strain of *A. citrulli*, we determined that the optimal level of BCA needed to inoculate watermelon flowers to maximize seed inoculation was 10_8 CFU/flower, at least for this specific taxon. By screening watermelon seeds germinated in moist chambers for 48 h, we identified bacteria antagonistic to *A. citrulli* under *in vitro* conditions. Additionally, we acquired a *Bacillus mojavensis* strain with known biological control activity from a culture collection. To screen candidate BCAs, we generated seed exudate (SE) by soaking watermelon seeds in water for 24 h and used it evaluate the ability of the BCAs to rapidly colonize germinating seeds. Using an automated microbial growth system, we measured the population dynamics of 11 candidate BCAs in SE. All candidate BCAs, except one, grew to an optical density (OD) (between 0.3 and 0.8) that was higher than that of *A. citrulli* in a 24-h period. To further select BCAs, we evaluated their ability to limit BFB seedling transmission by vacuum-infiltrating them individually into watermelon seeds, followed by inoculation with *A. citrulli* (10_6

CFU/ml). Based on these criteria, we identified two candidates BCA#24 and BCA#35 that resulted in the lowest BFB seedling transmission (5.2 and 7.3%, respectively).

Based on 16S rRNA gene sequence analysis, we identified BCA#24 and BCA#35 as a *Bacillus* sp. with 96% sequence identity. Furthermore, based on *purH* gene sequence analysis, we found that BCA#24 and BCA#35 had 99 and 93% sequence identity with *B. safensis* and *B. pumilus*, respectively. FAME analysis of the BCA strains revealed that the most abundant fatty acids identified were $C_{15:\,0}$ iso (35.4- 41.6%), $C_{15:\,0}$ anteiso (24.9-26.6%), $C_{17:\,0}$ iso (11.6-13.8%) and $C_{17:\,0}$ anteiso (7.53-8.47%), which were similar to the profile for the *B. safensis* type strain FO-36b^{T}. Two *in-silico*-based analyses, average nucleotide index calculator (ANI) and genome-to-genome distance calculator (GGDC) were utilized to confirm the identities of the BCAs. For both strains, DNA-DNA analysis based on ANI showed 99% similarity to *B. safensis* FO-36b^{T}. Similarly, GGDC revealed that strains had 96% sequence similarity with *B. safensis* FO-36b^{T}. Based on these data, we concluded our BCA bacteria were *Bacillus safensis*.

We sought to elucidate the mode of action of our *B. safensis* and *B. mojavensis* isolates against *A. citrulli*. More specifically, we investigated if the BCAs produced antimicrobial compounds, had a competitive advantage, or induced systemic resistance (ISR), which are commonly associated with biological control activity of *Bacillus* spp. *B. mojavensis* RRC101 produced a distinct *A. citrulli* zone of inhibition (ZOI), which suggested that antimicrobial compounds were a component of biocontrol against *A. citrulli*. In contrast, *B. safensis* did not produce a ZOI. To determine if competition was a component of biocontrol activity, we inoculated de-coated germinating watermelon seeds with candidate BCAs individually and in combination with *A. citrulli*. Interestingly, when BCAs were co-inoculated onto germinating watermelon seeds with *A. citrulli*, the *A. citrulli* population growth was reduced by 2-3 orders of

magnitude 2 days after planting. However, when each strain was inoculated independently onto germinating seeds, the *A. citrulli* populations increased to significantly higher populations than the BCAs. Finally, to determine whether *Bacillus* spp. induced ISR, we artificially inoculated melon seeds and measured *LOX2* expression at 3 and 6 days after planting. Our results indicated that there was no consistent upregulation of *LOX2* in melon seedlings from seeds treated with *B. safensis* or *B. mojavensis*.

Finally, we investigated the efficacy of watermelon seed treatments with BCAs by flower inoculation under field conditions. Sixty-three watermelon seed lots colonized with BCA bacteria were generated by flower inoculation in the field. After enrichment of seed lots for BCA bacteria, more than 50% of the lots tested positive for BCA bacteria by qPCR analysis. Samples of each seed lot were challenged with 10_7 CFU/ml of *A. citrulli* to determine if BCA-colonized seeds reduce BFB seedling transmission. We observed a 41.2 and 47.9% reduction in BFB seedling transmission for seeds from flowers treated with *B. mojavensis* RRC101 and *B. safensis* strain #35 respectively for experiments conducted in Tifton, GA. These treatments were significantly different from seeds resulting from flowers treated with water (81.2%). However, in one experiment conducted in Athens, GA, there was no significant reduction in BFB incidence.

The efficacy of biological control has always been a concern for disease management. However, there have been some success stories. For example *Bacillus thuringiensis* has been used for 25 years to control insects, and *Bacillus cereus* UW85 was used as a seed treatment for damping off caused by *Phytophthora* in soybean (Emmert and Handelsman 1999). To the best of our knowledge, this is the first-time seeds were internally inoculated under field conditions with *Bacillus* spp. While we observed significant reduction of BFB seed transmission, the

interactions that contribute to biocontrol of BFB may involve multiple complex modes of action (Emmert and Handelsman 1999). Overall, findings from this research have provided insight into the challenges of internally inoculating watermelon seeds with BCA.

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APPENDIX A

OPTIMIZATION OF FLOWER INOCULATION FOR DELIVERY OF BIOLOGICAL CONTROL AGENTS INTO WATERMELON SEEDS

The goal of this experiment was to optimize flower inoculations for introducing biological control agents (BCAs) into watermelon seeds. In the greenhouse, flowers were hand-pollinated, treated with varying levels of a non-pathogenic strain of *Acidovorax citrulli* (AAC00-1*ΔhrcC*) (Johnson et al. 2011) and allowed to develop into mature fruit for 35-40 days. Resulting seeds were harvested and tested for the presence and concentration of AAC00-1*ΔhrcC* by qPCR using *A. citrulli*-specific primers similar to qPCR assays utilized by Johnson et al. (2011). We hypothesized that increasing the concentration of inoculum on the flower would increase the percentage of seeds inoculated with AAC00-1*ΔhrcC*, as well as increase the concentration of inoculum per seed.

Bacterial culture and inoculum preparation

AAC00-1*ΔhrcC* was routinely grown on King's medium B (KMB) for 48 h at 28°C. To prepare inoculum, Luria-Bertani (LB) broth (VWR Life Science, Radnor, PA) was inoculated with a single colony of AAC00-1*ΔhrcC* and incubated overnight at 28°C on a rotary shaker (Innova, New Brunswick Scientific Co., Edison, NJ) at 120 min^{-1}. After incubation, 5-ml broth cultures were centrifuged at 10,000 min^{-1} for 5 min. The supernatant was decanted, and the pellet was suspended in sddH_2O. Bacterial concentrations were estimated spectrophotometrically with an optical density OD_{600} nm = 0.3 (~ 1 x 10^8 CFU/ml) and adjusted to the desired concentration by serial dilutions.

Greenhouse flower inoculation

Watermelon plants (cv. Crimson Sweet) were established in 11.4-liter pots under standard greenhouse conditions. At anthesis, female watermelon stigmas were hand-pollinated and subsequently inoculated with AAC00-1Δ*hrcC*. A micropipette was used to deliver 10 µl of cell suspension to each stigma with bacterial concentrations including 10^7, 10^8 and 10^9 CFU/flower on ten different flowers, each on a separate plant. As negative controls, flowers were treated with water. Flowers were allowed to develop into mature fruit (35-40 days after pollination), and the fruit were harvested and stored at 4°C until seed extraction. Fruit were surface sterilized with 0.5% NaOCl and seeds were extracted manually, without fermentation. Seeds from each fruit were maintained as a separate lot, air-dried for 24-48 h at room temperature, then stored at room temperature. Each experiment was conducted twice. After seeds were harvested, an UltraClean Microbial DNA Kit (MO BIO Laboratories, Inc., Carlsbad, CA) was used to extract total microbial DNA according to the manufacturer's instructions. Subsequently, an *A. citrulli*-specific qPCR assay was used to determine the percentage of inoculated lots, the percentage of inoculated seeds, the concentration per lot, the concentration per seed, and the distribution of inoculum within lots similar to methods used by Dutta *et al.* 2013 and Johnson et al. 2011(Dutta et al. 2013; Johnson et al. 2011).

Real-time PCR.

Five microliters of DNA from each sample were used as template and amplified in 25 µl of PCR master mix containing 10 mM Tris-HCL (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$ and 0.2 mM of each nucleotide (dATP, dCTP, dGTP and dTTP). 10 µM of each primer BOXAACF and BOXAACR2 and 10 µM of probe AACPROBE (Ha et al. 2009) per sample. DNA was amplified in a Cepheid Smart Cycler (Sunnyvale, CA.) To quantify results, a

standard curve was generated using 10-fold serial dilutions of AAC00-1Δ*hrcC*, and the standard curve was based on four independent replicates. The curve was constructed by plotting mean C*t* values against $\log_{10}$ cells of AAC00-1Δ*hrcC* cell dilutions.

Results

Effect of inoculum concentration applied to flowers on percentage of inoculated seed lots

Four treatments were applied to flowers in the greenhouse including 1) 10^7 CFU/flower, 2) 10^8 CFU/flower, 3) 10^9 CFU/flower, or 4) water as a control. For each treatment (n = 4 seed lots), 1 g of seed from each lot was used to extract microbial DNA. Lots from flowers treated with 10^7 CFU resulted in the lowest percentage of inoculated lots with 13%. Flowers treated with 10^8 and 10^9 CFU resulted in a higher percentage of inoculated seeds lots with 89 and 50%, respectively (Fig. A1); however, there were no significant differences among treatments.

Effect of inoculum concentration applied to flowers on bacterial concentrations in seeds

For each treatment (n= 4 seed lots), 1 g of seed from each lot was used to extract microbial DNA. Seed lots from flowers treated with 10^7 CFU/flower resulted in the lowest CFU/g with $<10^1$ CFU/g of seed. Flowers treated with 10^9 CFU/flower resulted in seeds with 10^1 CFU/g, which was not significantly different from seeds from flowers treated with 10^7 CFU/flower. Flowers treated with 10^8 CFU/flower resulted in the highest colonization with 10^3 CFU/g, which was significantly higher than all other treatments according to a Wilcoxon Rank-Sum Test (P = 0.0046) (Fig. A2).

Effect of inoculum concentration applied to flowers on percentage of inoculated seeds

In one trial, for each treatment (n = 5 seed lots), microbial DNA was extracted from 20 individual seeds per lot. There was no significant difference among treatments, and each

treatment resulted in lots with 70-79% of inoculated seeds (Fig. A3). In a second experiment, for each treatment (n = 4 seed lots), there was no significant difference among treatments, and each treatment resulted in lots with 54-93% of inoculated seeds (Fig. A4).

Effect of inoculum concentration applied to flowers on bacterial concentration per seed

In the first trial, for each treatment (n = 5 seed lots), microbial DNA was extracted from 20 individual seeds per lot, and there was a significant difference among treatments according to the Wilcoxon Rank-Sum Test (P = 0.017). Flowers treated with 10^7 CFU or 10^9 CFU resulted in lots with seeds containing 10^2 CFU/seed and were not significantly different from each other. Flowers treated with 10^7 CFU resulted in seed lots containing the highest concentrations with 10^3 CFU/seed, which was significantly different (Fig. A5). In a second experiment, for each treatment (n = 4 seed lots) there was a significant difference among treatments according to the Wilcoxon Rank-Sum Test (P = 0.0001). Flowers treated with 10^7 CFU or 10^9 CFU resulted in seed lots with seeds containing 10^2 CFU/seed and were not significantly different from each other. Flowers treated with 10^8 CFU resulted in seed lots containing the highest concentrations with 10^3 CFU/seed, which was significantly greater than that for seeds from all other treatments (Fig. A6).

Effect of inoculum concentration applied to flowers on distribution of *A. citrulli* inoculum in seeds

In one experiment, 10^3 CFU/seed occurred in 39-43% of seeds (Fig. A7). The concentration with the second highest percentage of occurrence was 10^4 CFU/seed occurring in 20-28% of seeds (Fig. A7). Flowers treated with 10^7 CFU resulted in the best distribution of inoculum in seeds with 21% of seeds containing no inoculum, 3% containing 10^2 CFU, 42% containing 10^3 CFU, 28% containing 10^4 CFU, and 6% containing 10^5 CFU. In a second

experiment, the concentration with the highest percentage of occurrence in seeds for all treatments was 10^2 CFU/seed occurring in 20-40% of seeds (Fig. A8). The concentration with the second highest percentage of occurrences was 10^3 CFU/seed occurring in 19-40% of seeds. Flowers treated with 10^8 CFU resulted in the best distribution of inoculum in seeds with 7.5% of seeds containing no inoculum, 40% containing 10^2 CFU, 40% containing 10^3 CFU, 10% containing 10^4 CFU, and 1.25% containing 10^5 CFU, and 1.25% containing 10^7 CFU (Fig. A8).

Conclusions

Increasing the inoculum load on flowers from 10^7 CFU to 10^8 CFU increased the percentage of colonized seed lots, the percentage of colonized seeds, and the concentration of *A. citrulli* per lot and per seed. However, the level of infected lots and seeds decreased when flowers were treated with 10^9 CFU. Eighty-nine percent of lots produced from flowers treated with 10^8 CFU were infested. Additionally, seed lots produced from flowers treated with 10^8 CFU yielded 10^3 CFU/g of seed, which was significantly different from all other treatments.

In two experiments, flowers treated with 10^8 CFU produced the best distribution of inoculum within seed lots. Overall, flowers treated with 10^8 CFU had the lowest percentage of seeds with no inoculum compared with other treatments. Based on these findings, the optimal level of flower inoculum for seed colonization was determined to be 10^8 CFU/flower.

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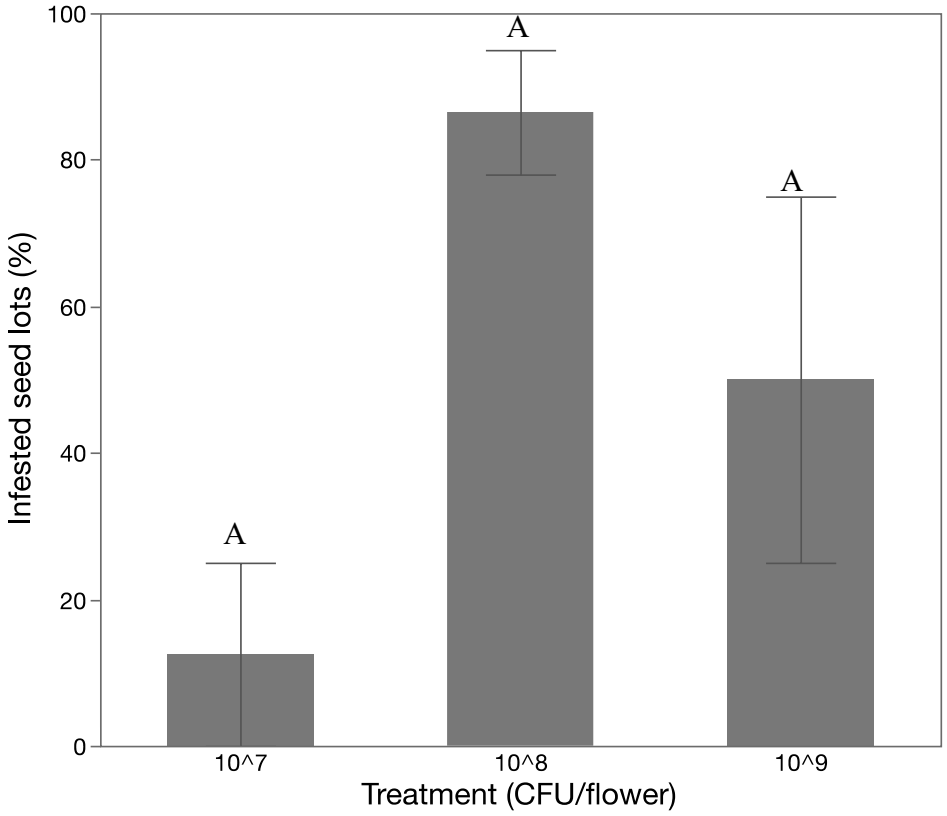

Figure A1. Effect of *Acidovorax citrulli* AAC00-1*ΔhrcC* concentration applied to female watermelon flowers on percentage of inoculated seed lots for experiment 1**.** Bars represent mean percentage of positive inoculated lots (n = 4) with AAC00-1*ΔhrcC* detected by qPCR for two independent experiments. Error bars represent the standard errors of the mean. No significant differences were observed among treatments based on the Wilcoxon-Rank sum test at P = 0.05.

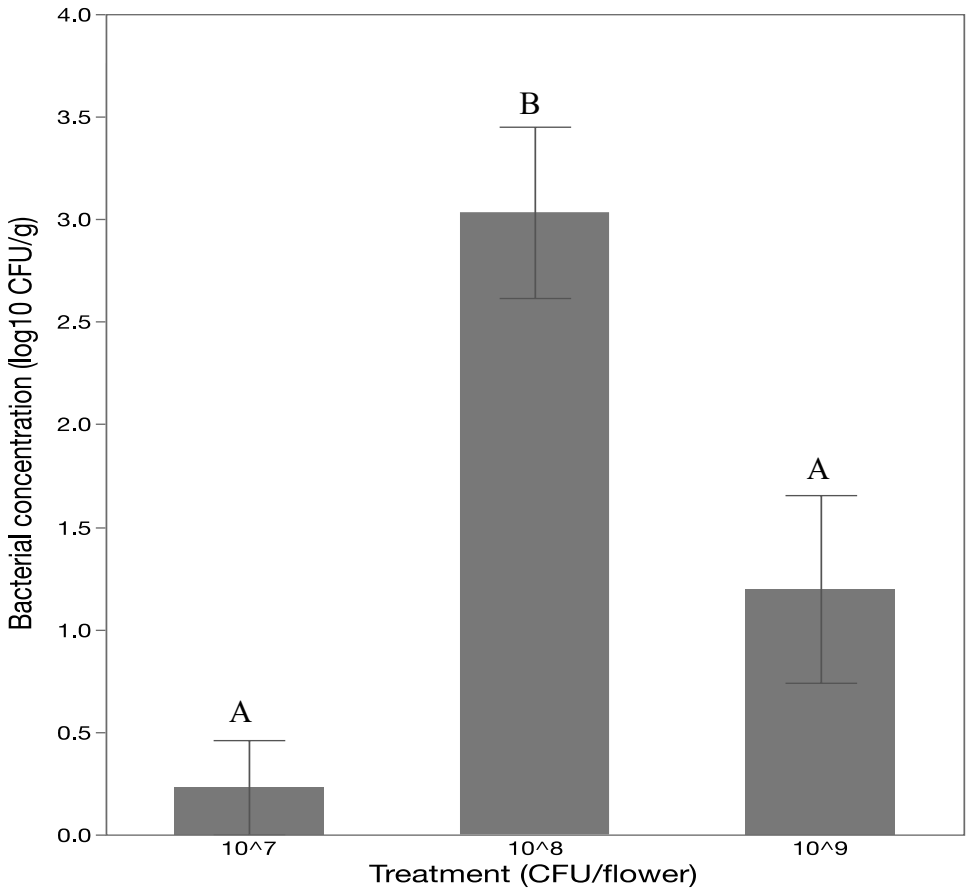

Figure A2. Effect of *Acidovorax citrulli* AAC00-1*ΔhrcC* concentration applied to female watermelon flowers on bacterial concentration in watermelon seed lots for experiment 2**.** Bars represent mean concentration (n = 4) of inoculated lots with AAC00-1*ΔhrcC* detected by qPCR for two independent experiments. Error bars represent the standard errors of the mean. Mean values with the same letters are not significantly different according to the Wilcoxon Rank-Sum Test ($P < 0.05$).

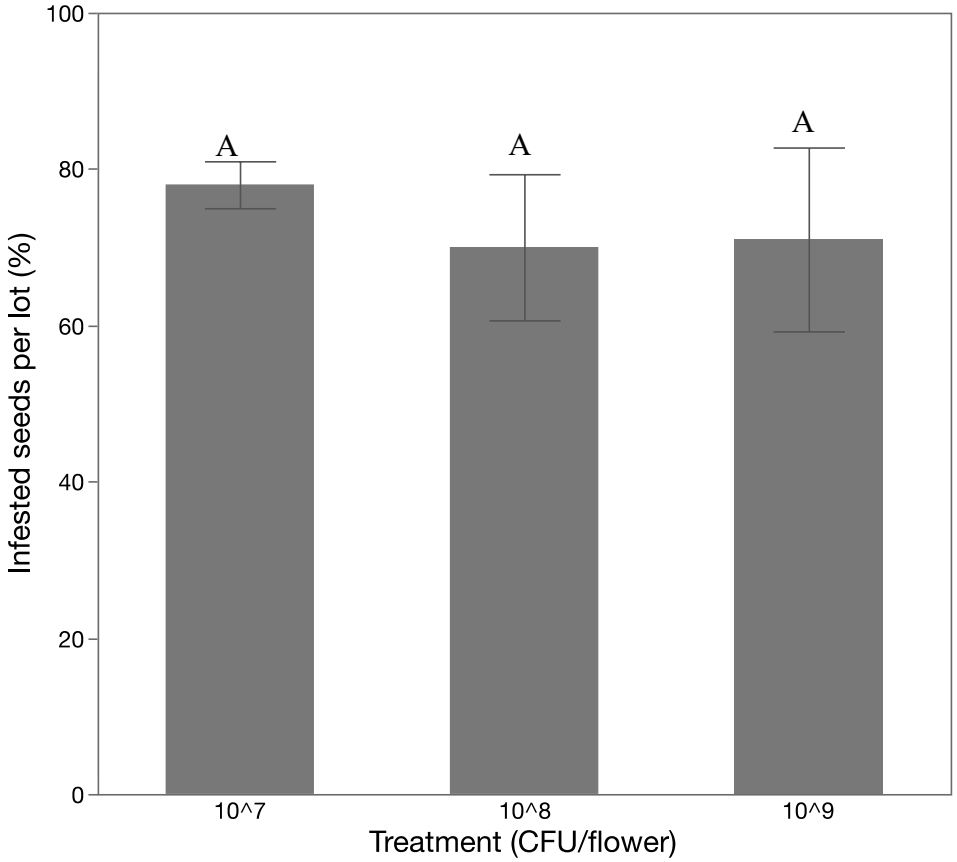

Figure A3. Effect of *Acidovorax citrulli* AAC00-1*ΔhrcC* concentration applied to female watermelon flowers on percentage of inoculated seeds for experiment 1**.** Bars represent mean percentage for 20 seeds per lot (n = 5) of inoculated seeds with AAC00-1*ΔhrcC* detected by qPCR. Error bars represent the standard errors of the mean. No significant differences were observed among treatments based on the Wilcoxon Rank-Sum Test at P = 0.05.

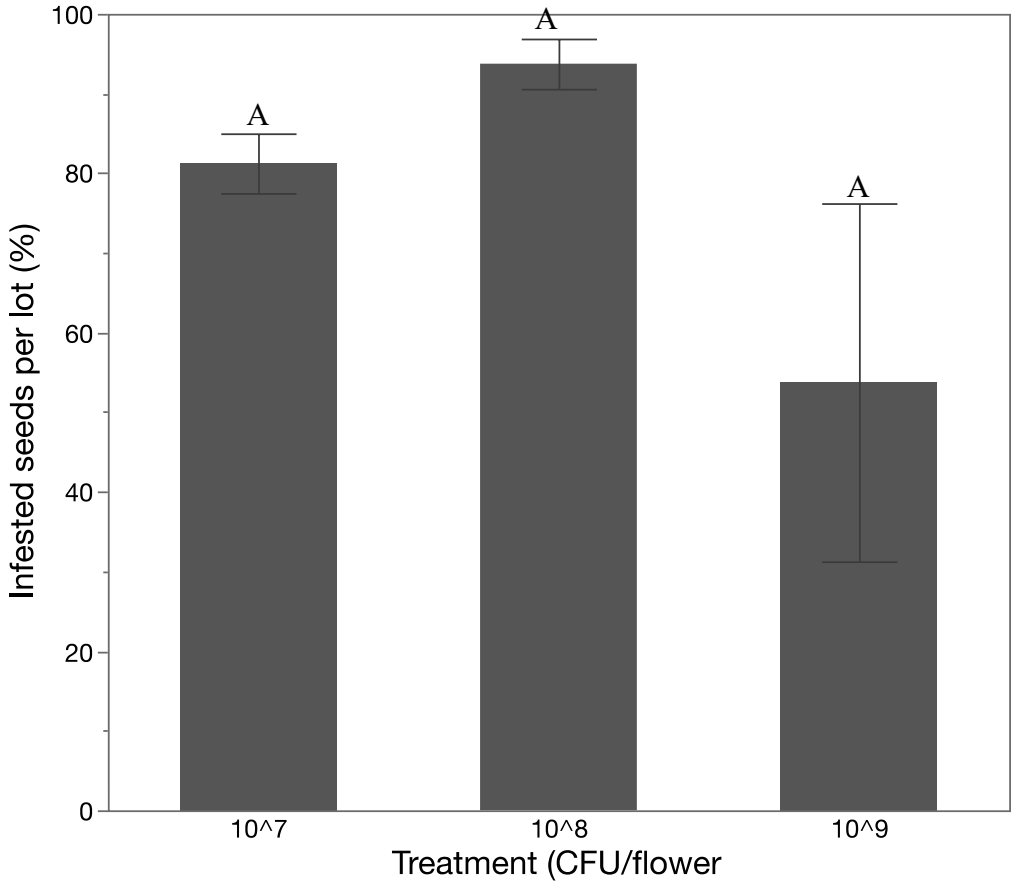

Figure A4. Effect of *Acidovorax citrulli* AAC00-1*ΔhrcC* concentration applied to female watermelon flowers on percentage of inoculated seeds for experiment 2. Bars represent mean percentage from 20 seeds per lot (n = 4) of inoculated seeds with AAC00-1*ΔhrcC* detected by qPCR. Error bars represent the standard errors of the mean. No significant differences were observed among treatments based on the Wilcoxon Rank-Sum Test at P = 0.05.

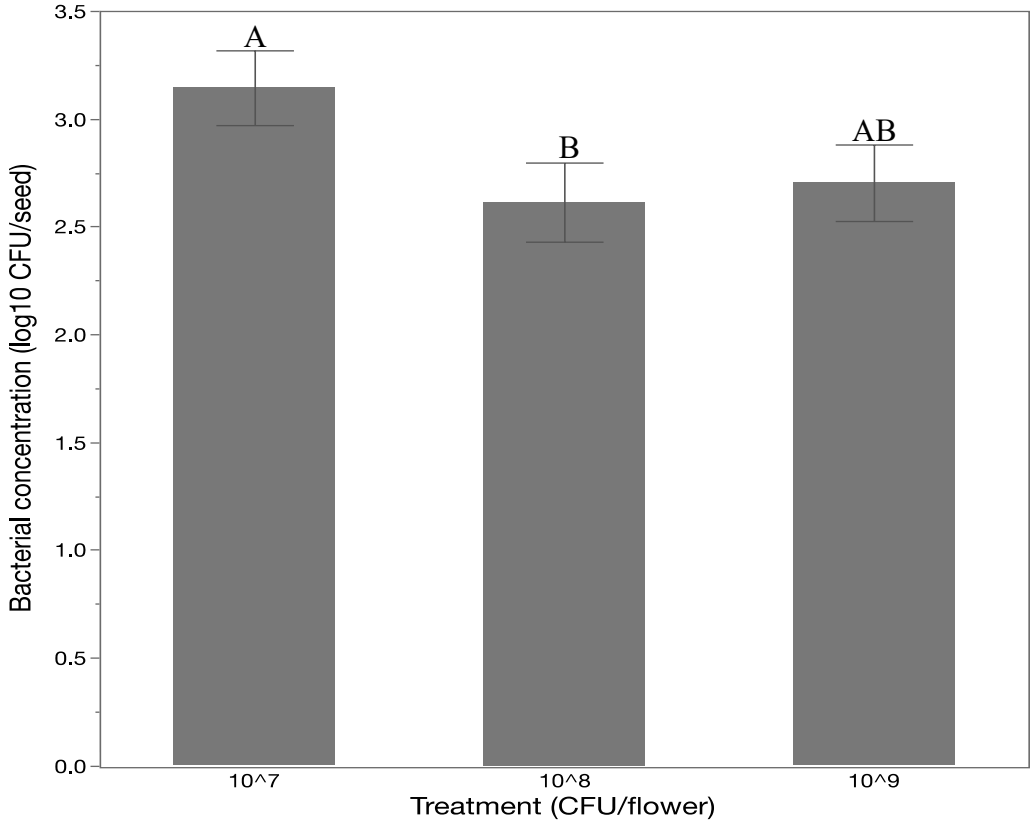

Figure A5. Effect of *Acidovorax citrulli* AAC00-1*ΔhrcC* concentration applied to female watermelon flowers on bacterial concentration in watermelon seeds for experiment 1**.** Bars represent mean concentration from 20 seeds per lot (n = 5) of AAC00-1*ΔhrcC* detected by qPCR. Error bars represent the standard errors of the mean. Mean values with the same letters are not significantly different according to the Wilcoxon Rank-Sum Test ($P < 0.05$).

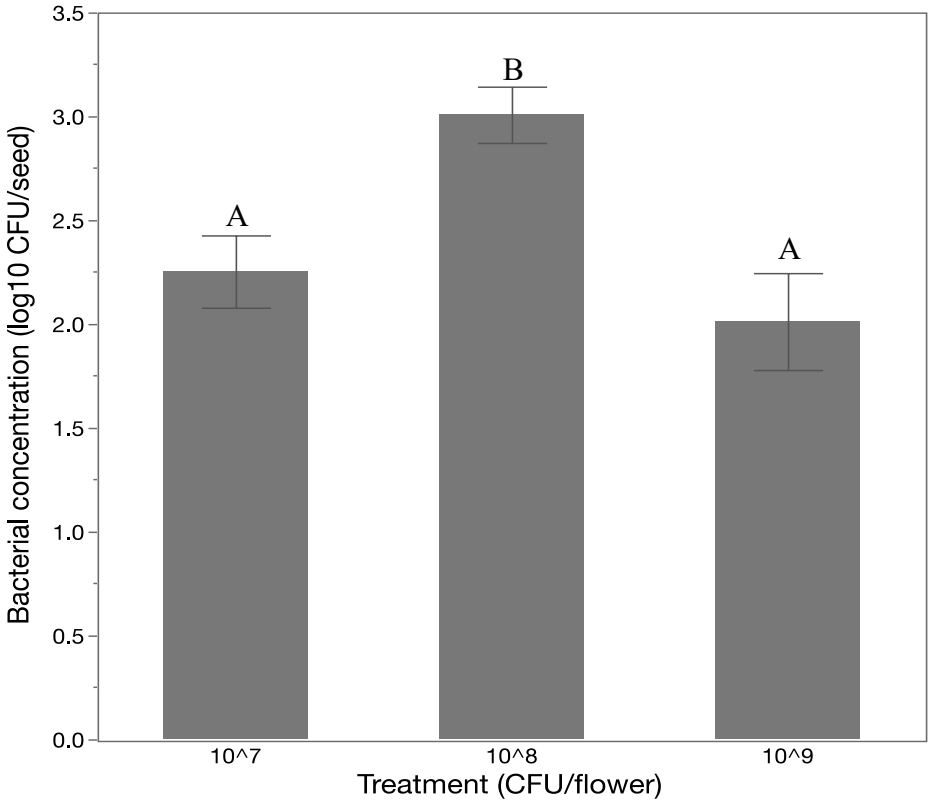

Figure A6. Effect of *Acidovorax citrulli* AAC00-1*ΔhrcC* concentration applied to female watermelon flowers on bacterial concentration in watermelon seed lots for experiment 2**.** Bars represent mean concentration from 20 seeds per lot (n = 4) of AAC00-1*ΔhrcC* detected by qPCR. Error bars represent the standard errors of the mean. Mean values with the same letters are not significantly different according to the Wilcoxon Rank-Sum Test ($P < 0.05$).

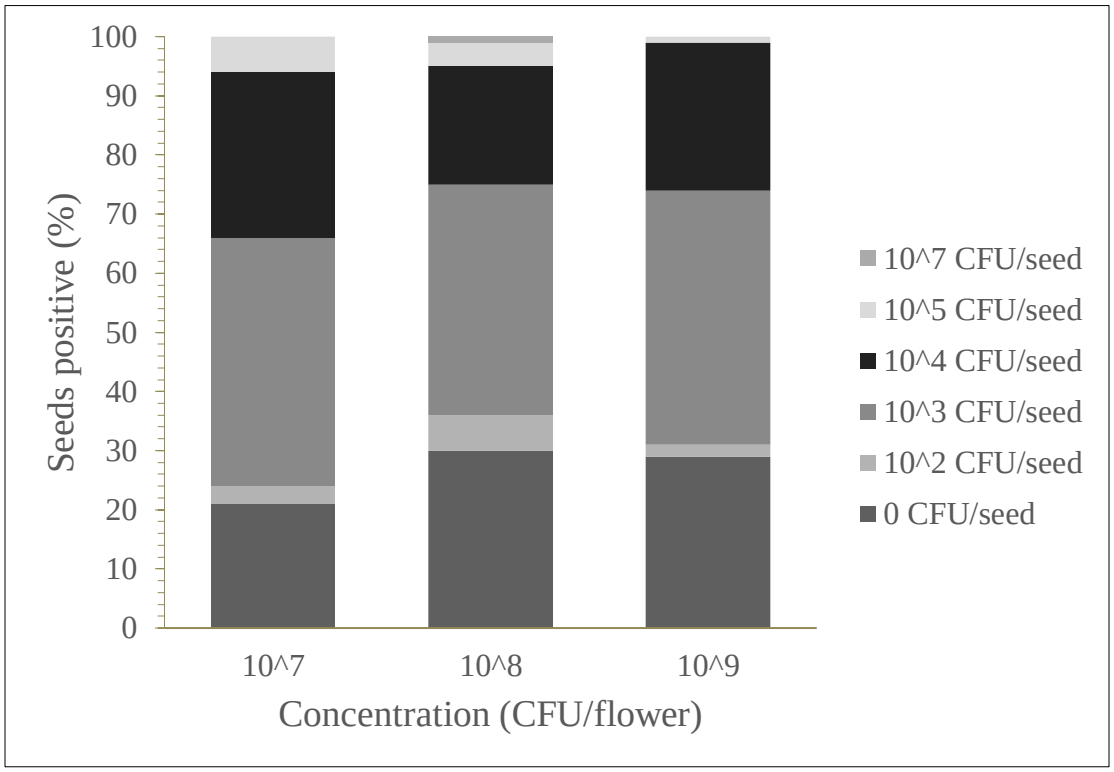

Figure A7. Distribution of *Acidovorax citrulli* AAC00-1*ΔhrcC* in watermelon seed lots for experiment 1. DNA was extracted from 20 seeds per lot for five independent lots, and the frequency and concentration of AAC00-1*ΔhrcC* in watermelon seeds was determined by qPCR. Bars represent the percentage of occurrence for bacterial concentrations in seeds for each treatment.

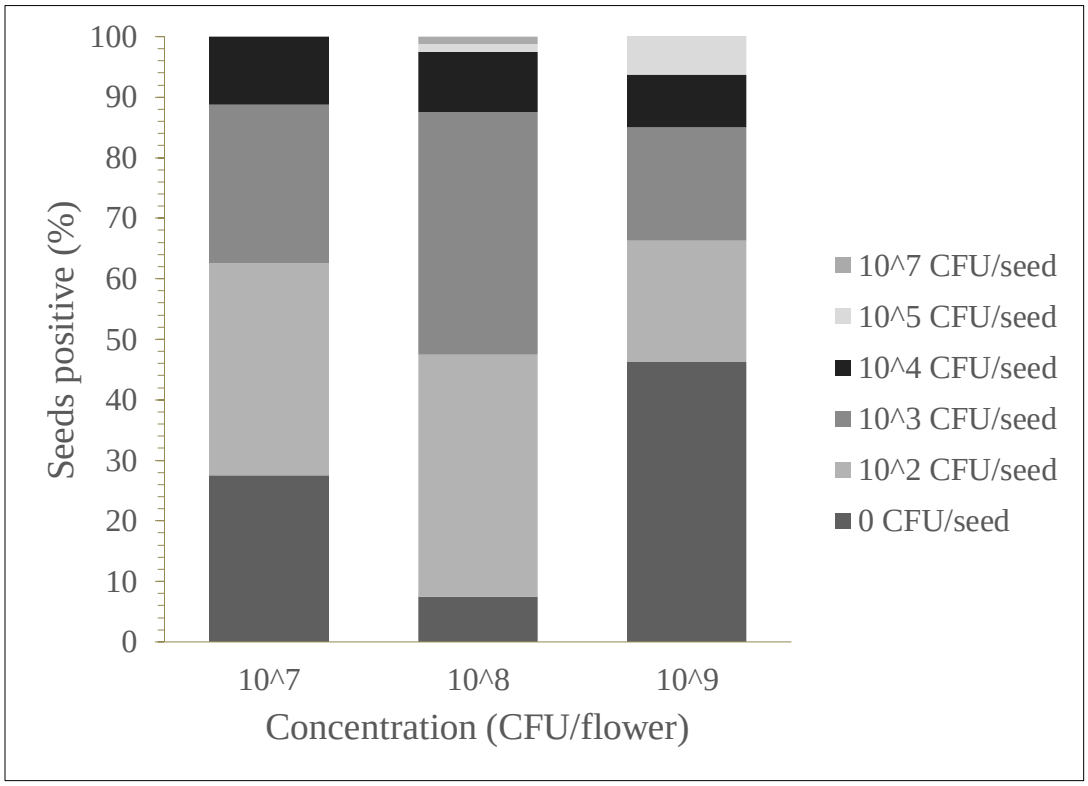

Figure A8. Distribution of *Acidovorax citrulli* AAC00-1*ΔhrcC* in watermelon seed lots for experiment 2. DNA was extracted from 20 seeds per lot for four independent lots, and the frequency and concentration of AAC00-1*ΔhrcC* in watermelon seeds was determined by qPCR. Bars represent the percentage of occurrence for bacterial concentrations in seeds for each treatment.