Biological watermelon (*Citrullus lanatus* L.) seed treatments for control of *Acidovorax citrulli*

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Major Project/Report submitted to the faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Agriculture and Life Sciences

In

Plant Sciences and Pest Management

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May 15, 2020

Blacksburg, VA

Keywords: organic agriculture, cucurbits, watermelon, melon, bacterial fruit blotch, antimicrobial, antibacterial, catechin, green tea, seed treatment, *Acidovorax citrulli*

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ABSTRACT

Acidovorax citrulli is a seedborne pathogen responsible for bacterial fruit blotch (BFB), an economically important disease in melon and watermelon throughout the world. BFB is highly virulent and in affected fields can cause yield reduction of up to 95%, which has resulted in over $100,000 in losses to melon growers in some cases. The efficacy of green tea as an antimicrobial seed treatment against *A. citrulli* was tested. Watermelon seeds were treated with green tea after inoculation with transgenic *A. citrulli* expressing green fluorescent protein (GFP). Forty five percent of watermelon seedlings inoculated with a high level (OD600:1.0, ~8 x 10^8 cells/ml) of *A. citrulli* displayed GFP in their cotyledons. When these seeds were treated with green tea, only 11.2% displayed GFP in their cotyledons. None of the treated watermelon seedlings inoculated with a low level (OD600:0.001, ~8 x 10^5 cells/ml) of *A. citrulli* displayed GFP in their cotyledons. Green tea treatments effectively controlled the disease when administered as a liquid to infected watermelon seeds. Green tea has potential as an effective commercial treatment for pericarp infected seeds that could also be used by growers participating in the National Organic Program.

Introduction

Acidovorax avenae subsp. *citrulli* is the causal agent of seedling blotch and bacterial fruit blotch (BFB) diseases of cucurbit crops including watermelon, melon, squash, cucumber, and pumpkin. *A. citrulli* is a biotrophic gram-negative bacterium, order of *Burkholderiales*, family *Comamonadaceae* (Burdman and Walcott, 2012). It is economically important worldwide in melon and watermelon crops, where it is most virulent.

In 2004, Walcott et al. used repetitive extragenic palindromic (REP)- polymerase chain reaction (PCR) to confirm the existence of two genetically distinct groups of *A. citrulli* strains. Group I strains are moderately aggressive on a variety of cucurbit hosts such as melon, squash, and cucumber, whereas group II strains are highly aggressive on watermelon and only moderately aggressive on non-watermelon cucurbit hosts (Walcott et al., 2004). Group I strains have been generally isolated from non-watermelon cucurbit hosts whereas Group II strains are closely associated with watermelon.

BFB was first observed by Webb and Goth in 1965 at the US Department of Agriculture Plant Introduction Station in Griffin, GA. They reported an, "unidentified, seed-borne phytobacterium" isolated from necrotic watermelon cotyledons imported from Turkey (Webb and Goth, 1965). Four years later, Crall and Schneck reported BFB symptoms on watermelon at the University of Florida experimental station in Leesburg, FL. However, the destructive potential of the disease was not recognized until a major BFB outbreak occurred in 1987 on the Mariana Islands (Wall and Santos, 1988). In 1989, the first major BFB outbreaks in United States commercial melon fields were observed in Florida, South Carolina, North Carolina, Maryland, Delaware, and Indiana. Losses were calculated at more than 90% of total fruit yield (Somodi et al., 1991; Latin and Rane, 1990). From 1992 – 1994, BFB in Georgia caused

thousands of hectares of crop losses. Seeds were identified as the primary inoculum source and as a result of major outbreaks, melon and watermelon growers filed lawsuits against their seed suppliers. Many small seed companies went out of business (Latin and Hopkins, 1995).

Between the emergence of the disease and the year 2020, BFB also has been found worldwide in South America, Asia, Europe, Africa and Australia (Amadi et al., 2009; Black et al., 1994; Burdman et al., 2005; Evans and Mulrooney, 1991; Holeva et al., 2010; Latin and Rane, 1990; Mirik et al., 2006; Schaad et al., 2003; Somodi et al., 1991; Walcott et al., 2004). Additionally, there is evidence that the bacteria can spread via non-host plants. For example, *A. citrulli* was isolated from tomato seeds and eggplant seedlings imported into Israel (Assouline et al., 1997) prior to BFB outbreaks in both melon and watermelon crops (Burdman et al., 2005). In fields where the disease occurs, individual growers have lost over $100,000 (Hopkins, University of Florida, 1997, as cited in CABI).

A. citrulli is primarily distributed via contaminated seeds. The bacteria can survive on dried seeds for several years (Latin and Hopkins, 1995). Although the disease can cause symptoms on all tested cucurbit crops, watermelon, cantaloupe, and honeydew are the most susceptible (Isakeit et. al., 1997). Initial symptoms of the disease are water-soaked lesions on the cotyledons and leaves (Webb and Goth, 1965). Extensive water-soaked lesions cause collapse and death of affected seedlings. In the middle stages of plant growth, the leaf lesions may become infrequent and inconspicuous. The symptom most associated with BFB in melon is a dark, water-soaked lesion on the surface of infected fruit, starting a few millimeters in diameter and quickly growing to several centimeters. In late stages of the disease, the lesions cause necrotic cracks in the rind, resulting in secondary infection from fruit rot (Latin and Hopkins, 1995). *A. citrulli* spreads easily through a field and in greenhouses when plants are closely

spaced. The bacteria can either reside in the embryo (embryo infection) or the seed coat (pericarp infection). Infected stigmas in melon blossoms can transmit the bacteria through the style, infecting embryos in developing seeds (Walcott et al., 2000; Dutta et al., 2012). The seed coat can also be infected via bacteria residing in the pericarp tissue of affected fruit. In pericarp infected seeds, the endosperm/perisperm layer which encloses the embryo prevents BFB bacteria from infecting the embryo via the infected fruit tissue. Embryo infection is less common than pericarp infection. Walcott et al. (2003) studied the role of watermelon blossom inoculation with *A. citrulli* on seed infection. Inoculated watermelon plants were asymptomatic, bacteria were present in 44% of the resulting seeds and viable bacteria were found in 31% of the seed lots. Twenty-seven percent of the seed lots yielded seedlings that displayed BFB symptoms when grown at 30°C and 90% relative humidity (RH) (Walcott et al., 2003). Alternatively, only seed coats became infected from the fruit tissue, referred to as pericarp infection. Thus, cucurbit seeds may be embryo infected, pericarp infected, or both (Dutta et al., 2012).

When infected seeds are sown directly in the field, BFB symptoms can occur 6 – 10 days after germination. However, symptom development depends on high temperature and high RH, as well as inoculum load. Because of this, outbreaks in the field via infected seeds can be sporadic. Many melon and watermelon producers, especially seedless watermelon producers, establish crops from transplants using 3- to 4-week-old greenhouse grown seedlings to increase production efficiency. These greenhouse facilities operate at high temperatures, maintain high RH, have dense seedling populations, and use overhead irrigation, all optimal conditions for BFB development and spread (Burdman and Walcott, 2012). Even if greenhouse-grown seedlings do not display BFB symptoms, infected seedlings can lead to BFB outbreaks in the field when conducive environmental conditions occur. Preplant seed disinfection treatments and chemical

applications so far have limited effectiveness to reduce losses associated with BFB. There is currently no known plant genetic resistance to BFB (Burdman and Walcott, 2012). Although BFB outbreaks in the field are sporadic, its high potential for damage makes BFB a significant threat to the global melon and watermelon industry.

Purpose

The purpose of this study was to evaluate the efficacy of organic biological seed treatments to control *A. citrulli* on watermelon. Several studies have examined whether biological or chemical seed treatments could combat *A. citrulli* but no effective treatment has yet been identified (Burdman and Walcott, 2012). Peroxyacetic acid is sometimes used by the seed treatment industry as a preplant treatment against BFB (Hopkins et al., 2003), but there are many naturally occurring antimicrobial biological compounds that need to be tested (e.g., Cowan et al., 1999). The goal of this study was to determine whether treatments with green tea, a natural compound with known antimicrobial properties, can be used as a seed treatment to lower the pathogenicity of BFB on watermelon and melon seeds. It is hoped that information learned from this study will enable seed producers and seed treatment companies to incorporate biological seed treatments into their production regiments to control *A. citrulli* with little hazard to workers and minimal cost for melon and watermelon producers.

Review of Literature

Several studies have looked for effective treatments for *A. citrulli*. Currently, it is standard practice for cucurbit seed producers to use a wet seed treatment of peroxyacetic acid to

reduce the incidence of disease. Although this treatment can reduce disease transmission, it does not eradicate the pathogen (Hopkins et al., 2003). Feng et al. (2009) attempted to develop a more robust seed treatment for BFB by testing the efficacy of cupric sulfate, acidified cupric sulfate, acidified cupric acetate, acidified zinc chloride, sodium hypochlorite, acidic electrolyzed water, and peroxyacetic acid. Seed treatments with sodium hypochlorite, peroxyacetic acid, and acidified cupric acetate reduced transmission of the bacteria, but seed germination was negatively impacted by these treatments. Only the acidic electrolyzed water eradicated the bacteria without decreasing germination or seedling health. However, commercial use of acidic electrolyzed water as a seed treatment is hindered by its short shelf life and a rigorous, expensive production process (Huang et al., 2008; Wang, 2015).

In recent years, the global agricultural industry has experienced a massive shift towards organic agriculture. As consumers become more aware of the environmental and human health hazards associated with chemical pesticide treatments, the demand for organic produce has increased dramatically. For example, as of 2017, 69.8 million hectares were under organic agricultural management worldwide (Lernoud and Willer, 2019). Since organic growers are looking for viable, cost effective, accessible, organic treatments for many pest and disease problems, understanding more about organic antimicrobial treatments has never been more important. In fact, many natural compounds have strong antimicrobial properties: Cowan (1999) compiled a list of 103 plants that have proven antimicrobial properties including garlic, grapefruit, eucalyptus, licorice, willow, and green tea.

Several studies have tested the ability of natural products to abrogate *A. citrulli* infection. One study tested the efficacy of essential oils derived from plants in the Lamiaceae family to combat BFB by using a paper disc diffusion assay (Mengulluoglu and Soylu, 2012). In this

assay, agar plates were inoculated with 200 µl of *A. citrulli* bacterial suspension and filter paper discs containing 10 µl of the tested essential oil was placed on the center of the agar surface. Essential oils from thyme (*T. spicata*) showed the highest antibacterial activity against *A. citrulli* with 20.7mm zones of inhibition and a minimum inhibitory concentration (MIC) of 6.0 mg/ml. A similar study by Choi et al. (2016) examined the antimicrobial effects of 32 essential oils for their efficacy against *A. citrulli*, including mint, thyme, black pepper, and lemon. They found that cinnamon oil exhibited the greatest antimicrobial activity and that two major components of cinnamon oil, benzaldehyde and cinnamaldehyde, performed best, with MICs of 0.1 and 0.01 (v/v), respectively.

Studies on other pathogens suggest that *Camellia sinensis* (green and black tea) may reduce BFB infection. *C. sinensis* contains catechin flavonoid compounds shown to be antagonistic towards a variety of bacteria such as *Shigella* (Vijaya et al., 1995)*, Vibrio cholerae* (Toda et al., 1992)*,* and *Streptococcus mutans* (Ooshima et al., 1993), as well as certain viruses (Keating and O'Kennedy, 1997). Because green tea is not fermented it contains more catechin compounds, about 25-35% of the dry weight, than black tea (Lovato et al., 2019; Abdel-Rahman et al., 2011; Zaveri, 2006; Wanasundara et al., 1995). One type of green tea polyphenol catechin, epigallocatechin gallate (EGCG), exhibits the highest bactericidal activity (Gopal et al., 2016), by damaging *Escherichia coli* membranes (Maeyama et al., 2005), inhibiting biofilm formation in *Staphococcus* and *Eikenella corrodens* (Blanco et al., 2005; Matsunaga et al., 2010), and inhibiting *Porphyromonas gingivalis* growth (Sakanaka et al., 1996).

Several studies have demonstrated the effectiveness of green tea extracts against *Pseudomonas* infections. Extracts were efficient in combating *Pseudomonas aeruginosa* ATCC 27853 and multi-drug resistant *Pseudomonas aeruginosa (*MDR-P. *aeruginosa)* in the paper disc

diffusion assay, with inhibition zones of 17.550 ± 0.393 mm and 17.670 ± 0.398, respectively, and a MIC of 400 µg/ml for both (Radji et al., 2013). Green tea extracts reduced the proteolytic activity, elastase activity, swarming motility, and biofilm formation in *P. aeruginosa* (Yin et al., 2015). The same study showed that green tea polyphenols (GTP) decreased the pathogenicity of *P. aeruginosa* on free-living, soil-based *Caenorhabditis elegans* nematodes. Survival percentage positively correlated with GTP concentration: untreated *C. elegans* infected with *P. aeruginosa* had a 20% survival percentage, infected *C. elegans* treated with 0.159 mg/ml GTP had a 40% survival percentage, and infected *C. elegans* treated with 3.125 mg/ml GTP had a 63.3% survival percentage. These studies indicate that GTP is antagonistic towards *P. aeruginosa*, which is especially pertinent to this study because of the similarities between *Pseudomonas* and *Acidovorax*. When *A. citrulli* was first discovered, it was classified as *Pseudomonas pseudoalcaligenes* ssp. *citrulli* (Schaad et al., 1978). Later, several phytopathogenic *Pseudomonas* species, including *A. citrulli,* were reclassified as the genus *Acidovorax* due to phenotypic and rRNA similarities to existing members of that genus (Williams et al., 1992). Thus, it is hypothesized that green tea treatment may be effective against *A. citrulli* infection.

Materials and Methods

The studies of natural compounds reviewed above and the materials used for this study were provided and explained by Ph.D. candidate Merve Kiremit who is studying the transmission, pathogenicity, and control of BFB in the Welbaum Lab, at Virginia Tech. All experiments were conducted *in vitro* and the research with *A. citrulli* was approved by USDA permit number P526P-19-04186. The goal of this study was to develop a model system and methodology to test the efficacy of organic biological seed treatments after inoculation with *A.*

citrulli in order to control seed transmission of BFB. The study focused on the use of green tea as a natural antimicrobial compound.

Genetic tagging of *A. citrulli* and seed inoculation

The M6 strain of *A. citrulli* was chosen for this experiment because, as a Group I strain, it can infect a broad range of hosts, including cantaloupe and watermelon (Walcott et al., 2000). In the Walcott Lab at the University of Georgia Department of Plant Pathology, the M6 strain *A. citrulli* were transfected with a BAC containing the *Aequorea victoria* gene encoding Green Fluorescent Protein (GFP), so bacteria could easily be identified in melon seedlings. A kanamycin resistance gene was included in the BAC to select for transfected bacteria. Next, Merve Kiremit, Ph.D. candidate in the Virginia Tech Welbaum Lab, cultured the transfected M6 *A. citrulli* in Luria's Broth (LB) supplemented with 50 ppm of kanamycin to select for only GFP-tagged bacteria. Bacteria were resuspended in sterile 10 mM $MgCl_2$ buffered saline and diluted to OD600:1.0 (~8 x 10^8 cells/ml) or OD600:0.001 (~8 x 10^5 cells/ml).

Cantaloupe (*Cucumis melo* 'Hales Best Jumbo') and watermelon (*Citrullus lanatus* 'Crimson Sweet') (Eden Brothers Seed) seeds were infected via either vacuum infiltration (to penetrate the seed coat) or soaking infiltration (to infect the seed coat) methods. Both methods were meant to simulate pericarp infection, the vacuum infiltration method was designed to allow for deeper penetration into the seed coat. In the vacuum infiltration method, seeds and inoculant solution were placed into a vacuum flask. Oxygen was removed from the flask by a vacuum air pump and seeds were allowed to soak in the bacterial suspension solution at room temperature until oxygen bubbles stopped appearing from the surface of the seeds. In the soaking infiltration method, seeds were incubated in 50 ml Falcon test tubes with 10 ml bacterial suspension and agitated for 2 hours at room temperature. After the seeds were inoculated by either method, they

were dried in a desiccator with charged sodium silicate desiccator beads (Fisher Scientific) for four days until the RH in the desiccator dropped to 40%. Control seeds were prepared with the same method using only sterile 10mM $MgCl_2$. The vacuum infiltration method was anticipated to be more effective because it replaces the air trapped in the seed coat with the bacterial solution, thus allowing for deeper penetration into the seed.

The inoculated seeds, along with uninoculated control seeds from the same seed lot, were shipped to the Moody Laboratory at the George Washington University, Washington D.C. The lab is a BSL1 facility approved by the Institutional Biosafety Committee for this project (Protocol number IBC-19-009). A USDA APHIS permit to handle *A. citrulli* was obtained (Permit number P526P-19-04186). Seeds were treated with dry Captan 50WP fungicide to inhibit fungal growth that could interfere with bacterial colonization.

Seed Germination

Because this was the first study of tea extracts on artificially inoculated cucurbit seeds, three different germination techniques were assessed to determine the best method. In each method, all materials used were sterilized. In the soil germination method, 2" diameter plastic pots were filled with ProMix Flex growth media (Griffin Greenhouse Suppliers), moistened with sterile, deionized (DI) water and seeds planted at a depth of 1 inch (Figure 1). The pots were kept in a deep tray and watered from below with sterile DI water every other day, or as needed. The trays were covered with humidity hoods and kept under fluorescent lighting at 22°C.

Figure 1. Soil Germination Method. Pictures taken with humidity hoods removed. Top image taken after seeds were planted. Bottom image of seedlings after 5 days.

In the test tube germination method, cotton balls were placed in 15 ml Falcon test tubes, moistened with 1 ml sterile, DI water and a single seed deposited on its surface (Figure 2). The test tube caps were tightly closed to preserve humidity. Sterile DI water was added every other day or as needed as the cotton dried. The seedlings were kept under fluorescent lighting at 22°C.

In the paper method, blue blotter germination paper (Anchor Paper Co., Saint Paul, MN) was placed on shallow trays and saturated with 6 ml sterile, DI water (Figure 3). Six seeds were placed on each paper card and the trays were sealed for humidity using plastic wrap. Germination blotters were misted with sterile DI water every other day. The RH inside of the trays was 90% and they were kept under fluorescent lighting at 22°C.

In both the test tube and germination paper methods, fungi developed on the germinating seeds (Figures 2, 3). After this was observed, a Captan 50WP fungicide seed treatment was

incorporated into later trials and applied by shaking the seeds in a paper bag with ¼ teaspoon of wettable powder fungicide, per the label.

After each trial, seedlings and plastic trays, pots, and humidity hoods were decontaminated by soaking in a 10% bleach solution for one hour, rinsed, and disposed of in a biohazard unit.

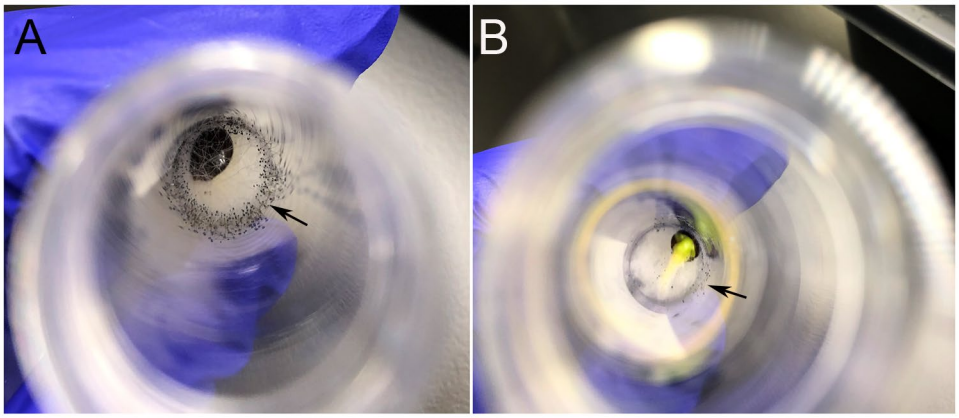


Figure 2. Top view of the test tube germination method. A. Watermelon seed that never germinated, was over-run with fungal mycelium and fruiting bodies (arrow). B. Germinating seedling with apparent fungal mycelium and fruiting bodies (arrow).

Figure 3. Examples of the germination paper method showing fungal growth. A. 'Crimson Sweet' seed (uninoculated – control) did not germinate due to fungal growth.

B. ‘Crimson Sweet’ seedling inoculated with wild type *A. citrulli* OD600:1.0 (soaking infiltration method), displaying fungal mycelium and fruiting bodies. C. ‘Crimson Sweet’ seedling inoculated with wild type *A. citrulli* OD600:1.0 (vacuum infiltration method), displaying fungal mycelium.

Seedling growth and analysis of infection

For each method, seeds were grown under regular fluorescent lighting at 22°C, 90% RH. To assess whether seeds inoculated with *A. citrulli* contained sufficient bacterial presence to display BFB infection, seedling roots and cotyledons were analyzed at 4 days post-germination for presence of GFP using an Olympus SZX12 epifluorescence stereomicroscope. Seedlings also were assessed for water-soaking lesions visually and under a stereomicroscope at either 4 days or 14 days post-germination.

In each trial, the frequency with which GFP was observed in the cotyledons and the frequency of water-soaked lesions was calculated as a percentage and then arcsine transformed to create a normally distributed population. Values for the two trials of each phenotype were compared by One-way ANOVA (GraphPad software).

Green tea treatment

Green tea was chosen as a potential anti-bacterial seed treatment because of its proven efficacy in combating *Pseudomonas* bacteria, which are phylogenetically similar to *A. citrulli.* Additionally, *in vitro* studies indicated that a green tea treatment inhibited BFB (Merve Kiremit, personal communication). Japanese ryokucha sencha green tea (Yamamoto brand) was brewed in the autoclave liquid cycle for 20 minutes at the rate of 5g loose tea leaves per 100mL DI water.

Then, the tea was diluted with distilled water to a concentration of OD390:4.0. First, watermelon seeds were inoculated via soaking infiltration method described above with the M6-GFP bacteria at concentrations of OD600:1.0 and OD600:0.001. Then, seeds were placed in a sterile 50ml flask with 22°C green tea mixture covering the seeds and gently agitated for 2 hours. The seeds were then dried and stored in a desiccator with charged sodium silicate desiccator beads for 4 days until the RH in the desiccator dropped to 40%. Green tea-treated seeds were germinated at 90% relative humidity for 4 days via the soil method and assessed visually and under the stereomicroscope for BFB lesions and presence of GFP as above. Frequencies of lesions and GFP presence in the cotyledons were analyzed as above.

Results

Comparison of germination methods

Of the three types of germination methods used, BFB-type lesioning was observed only in the soil germination method (Figure 4). In both the test tube and germination paper methods, fungi developed on the germinating seeds, despite fungicide treatment (Figures 2, 3). It is possible that the presence of the fungi may have inhibited bacterial colonization by *A. citrulli*, thus preventing the appearance of BFB-type lesioning.

Efficacy of cantaloupe versus watermelon infection

'Crimson Sweet' watermelon and 'Hales Best Jumbo' cantaloupe seeds inoculated with GFP-tagged M6 *A. citrulli* (OD600:1.0) via the vacuum infiltration inoculation method were grown by the soil germination method and harvested at 4 days old to screen for the presence of lesions and GFP (Figures 4, 5). Two trials, each including 60 cantaloupe seedlings and 60

watermelon seedlings, were performed. In both trials and in both groups, 100% of seedlings had GFP fluorescence in the roots, indicating highly efficient transfer of the bacteria (Figures 6, 7). At the time of harvest, the watermelon seedlings had cotyledons, but no primary leaves (Figure 4), whereas the cantaloupe seedlings were beginning to form their primary leaves (Figure 5). At the time of analysis, 23% of the watermelon seedlings had lesions, whereas only 5% of the cantaloupe seedlings had lesions (Figures 4, 5); this difference in lesion frequency was significant (Figure 8; $p=0.0097$ One-way ANOVA). The watermelon seedlings were etiolated, indicative of insufficient light, which could also account for cotyledon distortion (Figure 4).

Screening for the presence of GFP in the seedling cotyledons also demonstrated a difference between the cantaloupe and watermelon seedlings (Figures 6, 7). While 45% of the watermelon seedlings displayed GFP in the cotyledon, only 7.5% of the cantaloupe seedlings displayed GFP in the cotyledon. The frequencies of GFP detection in the cotyledons were significantly different (Figure 8; $p=0.0015$, One-way ANOVA).

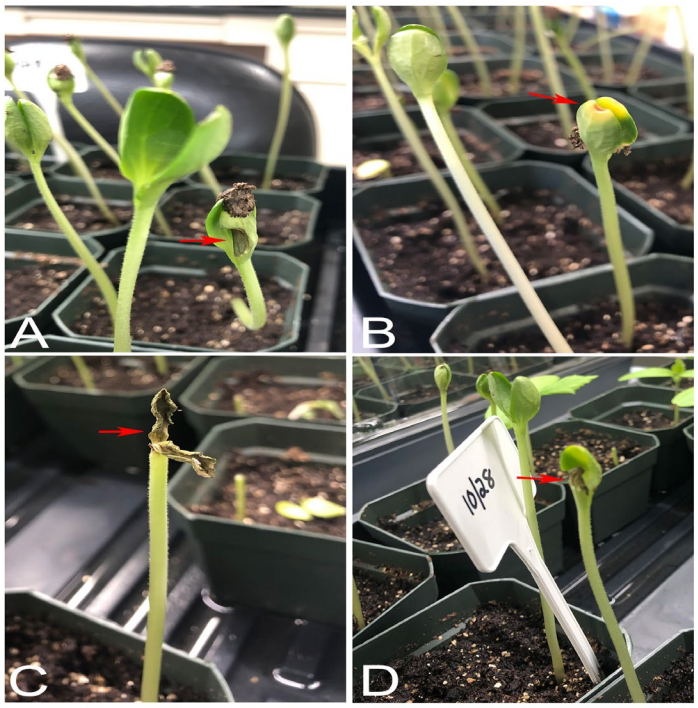
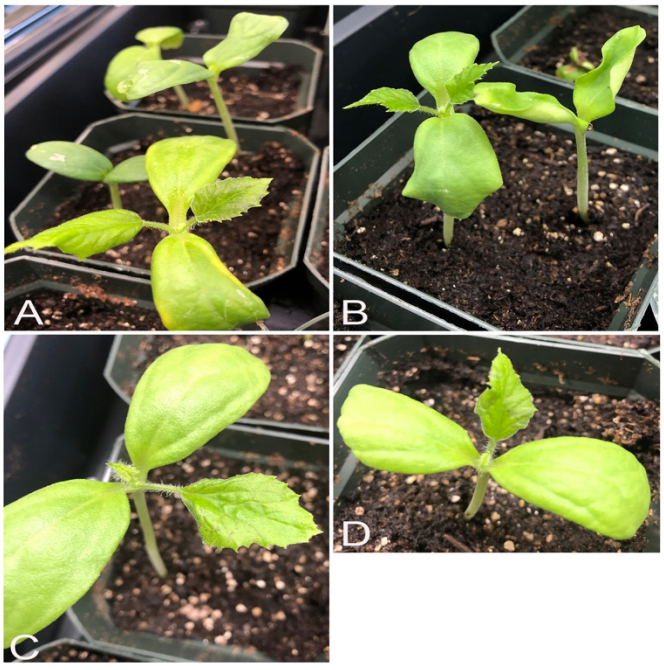


Figure 4. Examples of inoculated 'Crimson Sweet' seedlings (M6-GFP OD600:1.0 vacuum infiltration method) displaying lesions. A. Large brown lesion on the side of the cotyledon (red arrow). B. Chlorosis and lesion on the cotyledon tip (red arrow). C. Withered cotyledon (red arrow). D. Brown necrotic spot on the side of the cotyledon (red arrow).



F**igure 5**. Examples of inoculated 'Hales Best Jumbo' seedlings (M6-GFP OD600:1.0 vacuum infiltration method). A. Seedlings beginning to grow their secondary leaves without lesions. Spotting on the cotyledons is due to material from the seed coat, not lesions. B. Distortion in the cotyledon, which could be indicative of many types of stress. C. Seedling not displaying any obvious lesions. D. Seedling not displaying any obvious lesions.

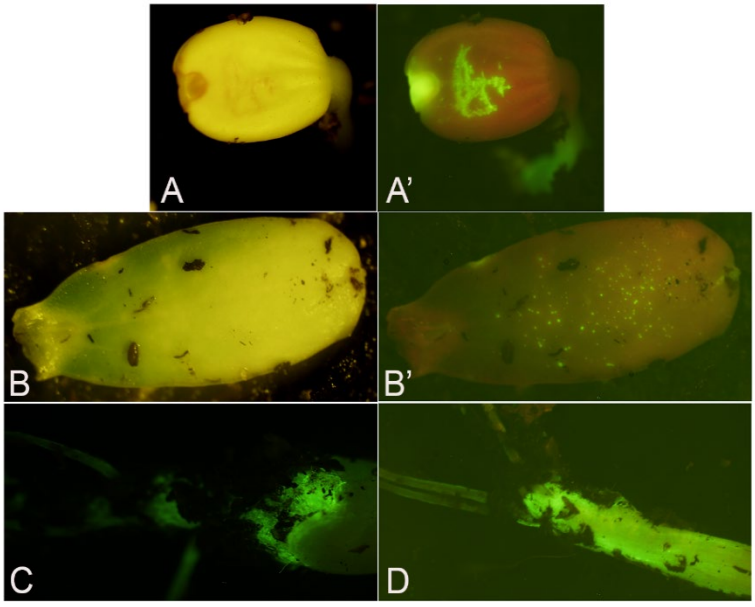


Figure 6. Detection of GFP in 'Crimson Sweet' Watermelon seedlings (M6-GFP OD600:1.0, vacuum infiltration). A. Brightfield illumination of cotyledon showing apparent lesion in cotyledon tip. A'. Same cotyledon under epifluorescence showing GFP in cotyledon tip lesion as well as cotyledon body and root. B. Brightfield illumination of cotyledon with no apparent lesions. B'. Same cotyledon under epifluorescence showing GFP speckled throughout cotyledon and one small lesion on the upper left-hand portion of the cotyledon. C, D. Significant GFP presence in two different seedling roots.

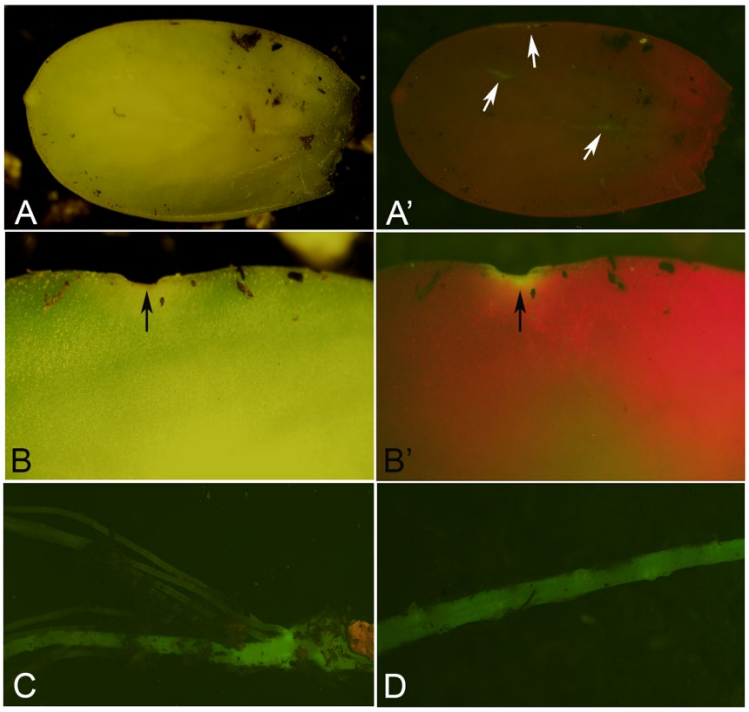


Figure 7. Detection of GFP in 'Hales Best Jumbo' cantaloupe seedlings (M6-GFP OD600:1.0 vacuum infiltration). A. Brightfield illumination of cotyledon with no apparent lesions. A'. Same cotyledon under epifluorescence showing barely detectible GFP in cotyledon (white arrows). B. Brightfield illumination of cotyledon showing small lesion on edge (black arrow). B'. Same cotyledon under epifluorescence showing minimal GFP at site of lesion (black arrow). C, D. Presence of GFP in two different in seedling roots.

Figure 8

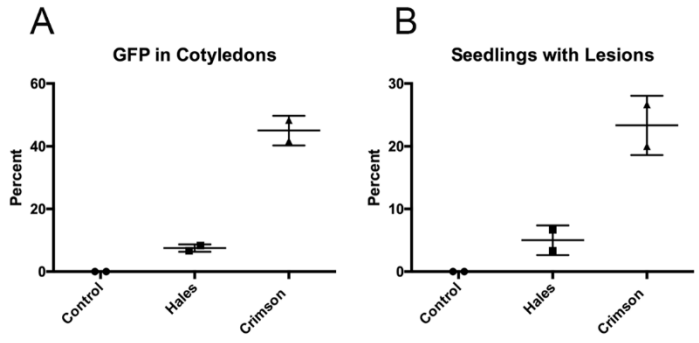


Figure. 8. Comparison of the proportion of infection phenotypes observed across two trials in infected "Hales Best Jumbo" cantaloupe (Hales) and "Crimson Sweet" watermelon (Crimson) seedlings. A. GFP detected in cotyledons. B. Lesions detected in cotyledons or leaves. The percentage of infection phenotypes were subjected to arcsine transformation and the values for the two trials of each phenotype compared by One-way ANOVA. GFP frequency in the cotyledons was significantly different at p=0.0015 and lesion frequency was significantly different at p=0.0097. Bars indicate standard deviation between the two trials.

Comparison of BFB lesion frequency between vacuum and soaking infiltration methods

Based on the greater frequencies of GFP presence in cotyledons and lesions in watermelon seedlings (Figure 8), the efficacies of vacuum versus soaking infiltration methods of inoculation were evaluated only with watermelon seeds. For each of two trials, twenty seeds inoculated via soaking infiltration (M6-GFP OD600:1.0), 20 seeds inoculated via vacuum infiltration (M6-GFP OD600:1.0) and 20 uninoculated control seeds from the same seed lot were grown by the soil germination method and assessed for the frequency of BFB symptoms at 14 days post-germination. The longer growth period was used because the lesion-like symptoms

observed after 4 days (Figure 4) might be explained by high humidity or damage caused by the seed coat which stayed attached to the cotyledon. No significant lesions were observed in any of the three groups in either trial (total n=40/group). This experiment was repeated with seeds inoculated with a wild type (wt) M6 *A. citrulli* (OD600:1.0), i.e., not carrying the *gfp* transgene, to control for the possibility that the GFP construct was decreasing the pathogenicity and therefore decreasing the disease/water soaking symptoms. Uninoculated 'Crimson Sweet' control (n=60 X 2 trials), 'Crimson Sweet' inoculated with M6-wt OD600:1.0 soaking infiltration method (n=60 X 2 trials), and 'Crimson Sweet' inoculated with M6-wt OD600:1.0 vacuum infiltration method (n=60 X 2 trials) were grown. Fungal infections occurred on these seedlings, even with applications of fungicide, which interfered with plant growth (Figures 2, 3). Additionally, no lesions were observed on the seedlings that survived. These experiments could not discern a difference between vacuum and soaking infiltration methods.

Green tea treatment

To assess whether green tea treatment could dampen *A. citrulli* infection phenotypes in watermelon seedlings, seeds were inoculated by the soaking infiltration method with M6-GFP *A. citrulli* (M6-GFP OD600:1.0), treated with green tea, and grown by the soil germination method for 4 days. As reported above, 100% of seedlings in each group (except untreated controls) displayed GFP in the roots, indicating efficacy of the inoculation method. In the M6-GFP OD600:1.0 group not treated with green tea, 45% of seedlings displayed GFP in their cotyledons, whereas only 11.2% of M6-GFP OD600:1.0 seedlings treated with green tea displayed GFP in their cotyledons. In the cotyledons of green tea treated seedlings that displayed GFP, the intensity of the signal and the size of the fluorescent patches appeared qualitatively less than in untreated seedlings (compare images in Figures 4 and 9). The frequencies of GFP detection in

the cotyledons were significantly different (Figure 10A; p=0.0054, One-way ANOVA), as were the frequencies of lesions (Figure 10B; p=0.0058, One-way ANOVA).

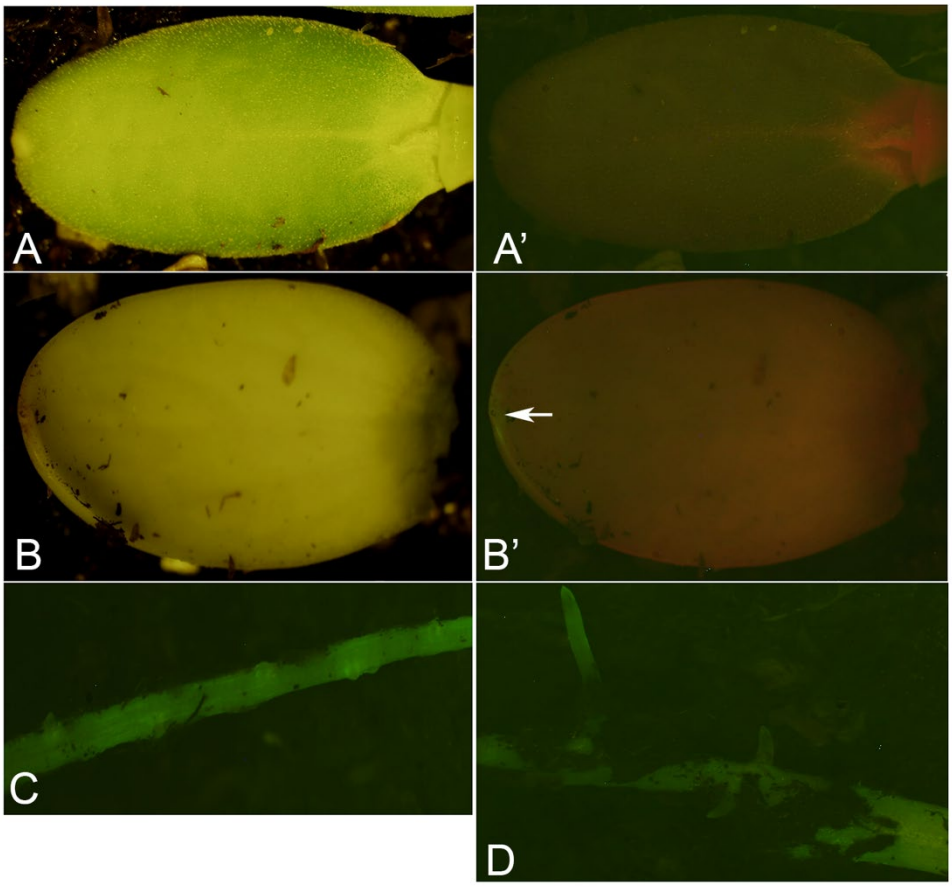


Figure 9. Detection of GFP in infected (M6-GFP OD600:1.0) 'Crimson Sweet' seedlings treated with green tea (Yamamoto brand green tea, OD390:4.0). A. Brightfield illumination of cotyledon with no apparent lesions. A'. Same cotyledon under epifluorescence showing no apparent presence of GFP (two small specks at the top of the cotyledon are part of the shiny seed coat, not GFP). B. Brightfield illumination of cotyledon with no apparent lesions. B'. Same cotyledon under epifluorescence showing very slight GFP on the cotyledon tip (arrow). C, D. GFP presence in two different seedling roots.

Figure 10

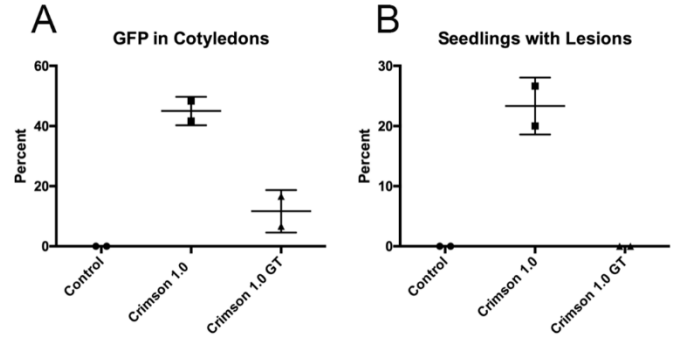


Figure 10. Comparison of the proportion of infection phenotypes (GFP detected in cotyledons; lesions detected in cotyledons or leaves) observed in untreated 'Crimson Sweet' inoculated with M6-GFP OD600:1.0 (Crimson 1.0) and 'Crimson Sweet' inoculated with M6-GFP OD600:1.0 and treated with green tea at OD390:4.0 seedlings (Crimson 1.0 GT). A. GFP detected in cotyledons. B. Lesions detected in cotyledons. The frequencies of infection phenotypes were subjected to arcsine transformation and the values for the two trials of each phenotype compared by One-way ANOVA. GFP frequency in the cotyledons was significantly different at p=0.0054 and lesion frequency was significantly different at p=0.0058. Bars indicate standard deviation between the two trials.

These data show that the M6-GFP OD600:1.0 concentration of *A. citrulli* appear to show less infectivity in the presence of green tea. To determine whether a lower inoculum of bacteria would be completely inhibited by green tea, watermelon seeds were inoculated with a low bacterial concentration (M6-GFP OD600:0.001), treated with green tea and grown for 4 days. All infected seedlings displayed GFP in their roots, indicating that the low bacterial concentration was effective in inoculating the seed. None of the high inoculum and none of the

low inoculum seedlings showed lesions. The low inoculum seedlings also did not display any GFP in their cotyledons, compared to 11.2% of the high inoculum seedlings. However, this

Figure 11

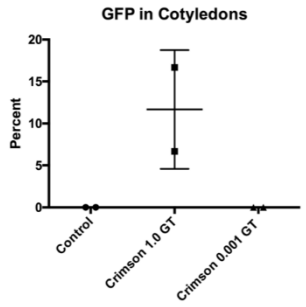


Figure 11. Comparison of the proportion of seedlings in which GFP was detected in cotyledons in 'Crimson Sweet' inoculated with M6-GFP at OD600:1.0 treated with green tea at OD390:4.0 (Crimson 1.0 GT) and 'Crimson Sweet' inoculated with M6-GFP at OD600:0.001 treated with green tea at OD390.4.0 (Crimson 0.001 GT). The frequencies were subjected to arcsine transformation and the values for the two trials compared by One-way ANOVA; the frequencies were not significantly different (p=0.0997). Bars indicate standard deviation between the two trials.

difference did not reach statistical significance (Figure 11; p=0.0997, One-way ANOVA).

Is GFP presence simply due to bacteria clinging to the seed coat?

It is not surprising that all of the inoculated seedlings showed GFP presence in their roots due to the proximity of the roots to the seed coat, which is the source of the M6-GFP bacterial inoculation, particularly with the soaking infiltration method. Potentially, GFP observed in the cotyledons might be due to proximity of the bacteria-carrying seed coat to the tip of the cotyledon before it was shed from the plant, rather than true infection of the plant. However,

while infected seedlings did display GFP in the cotyledon tip, they also displayed GFP in the body of the cotyledon (Figure 12). Furthermore, seedlings treated with green tea showed very little or no GFP in the tip of the cotyledon as compared to the untreated seedlings (Figure 9). This indicates that GFP presence in the cotyledon is not simply due to proximity to the bacteria-carrying seed coat, but also to bacterial infection in the plant. It also indicates that green tea decreased the bacterial attachment to the cotyledons or cotyledon tips.

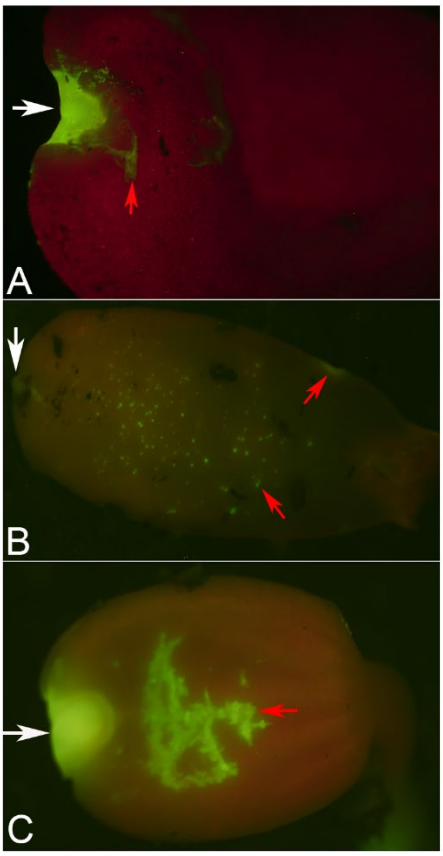


Figure 12. Patterns of bacterial colonization in cotyledons. A- C. The white arrows indicate GFP in the tip of the cotyledon that may be present due to proximity to the bacteria-carrying seed coat. B, C. In these examples, however, the red arrows indicate GFP locations that appear to represent bacterial infection of the plant.

Discussion

This multi-faceted study lays the groundwork for more screening for antimicrobial natural compounds that can be used as seed treatments against BFB. Germination of seeds in moist potting soil at 90% RH is most likely to yield BFB symptoms in a lab. The M6 strain of *A. citrulli* has a higher pathogenicity against watermelon than cantaloupe. Watermelon seedlings are the best host in which to study the M6 strain. Creating a transgenic M6-GFP strain via BAC transformation was an effective way to transfect the bacteria with an easily observable fluorescent marker to identify infected seedlings. Vacuum infiltration and soaking infiltration were shown to be effective methods of introducing the bacteria as shown by GFP presence in the roots of seedlings inoculated via both methods. GFP staining was found in the seed micropyle where radicle emergence occurs and where the endosperm/perisperm layer is least well formed. It is possible that if bacteria concentrate here it could then be transferred to the radicle and then to the root after germination. GFP made the *A. citrulli* pathogen easily detectible in not only seedling roots, but also seedling cotyledons that indicate successful bacterial colonization. Green tea was an effective seed treatment and reduced the pathogen at all tested inoculum concentrations. A novel and effective method for treating seeds with green tea was discovered.

There are many next steps for this line of experimentation. Although minor lesions were observed on ‘Hales Best Jumbo’ cantaloupe and ‘Crimson Sweet’ watermelon, the inability to produce lesions on older seedlings may mean that initial lesions were not caused by BFB. The lesioning in the first experiment in the presence of GFP is not sufficient evidence to conclude that *A. citrulli* was the cause. It could be that the bacteria were present in the seedlings but not at a high enough concentration to produce lesions. Environmental conditions may not have been

conducive for development of disease symptoms. It is important that further studies are conducted to observe BFB symptoms on host seedlings. This, in conjunction with PCR testing of infected symptomatic seedlings, is necessary to demonstrate that both lesions and GFP signal were caused by *A. citrulli*. Fellow BFB researcher, Merve Kiremit, found that seedlings with BFB lesions did not contain *A. citrulli* bacteria when tested by PCR (unpublished data). To truly test the efficacy of a seed treatment, water-soaking lesions must be observed in conjunction with PCR detection of the presence of *A. citrulli* to conclude that the lesions are caused by this pathogen.

As was addressed in the Literature Review, it is important to note that BFB outbreaks in the field are sporadic. High infection levels are attributed to seedling production greenhouses in which environmental conditions are conducive to bacterial infection and spread. Because of this, it is important that further studies are conducted in a greenhouse environment. Initially, we believed that such trials could be conducted at the George Washington University Harlan Greenhouse, which is a plant and insect research facility. However, greenhouse experiments were not possible due to BFB's devastating damage potential and the fact that the pathogen can exist for years until finding the appropriate host to infect. These concerns, coupled with the fact that the greenhouse facility does not meet the requirements for BSL1 certification or the APHIS USDA permit to handle *A. citrulli*, prevented greenhouse experimentation. Therefore, all experiments were conducted in a laboratory. By using soil and high RH it was hoped that the laboratory would mimic comparable environmental conditions to a greenhouse. Yet, reliable BFB symptoms were difficult to produce. It is difficult to provide the warm temperatures, high light, high RH, and the high moisture in a laboratory setting that a greenhouse inherently

provides. In lieu of a greenhouse setting, a misting incubation chamber with grow lights may be an effective alternate in future studies.

The M6 strain of *A. citrulli* was chosen because as a Group I strain it is antagonistic to a larger variety of cucurbit hosts. However, because of the inability to produce BFB symptoms in a laboratory, more experimentation should be done with Group II strains which are highly virulent on watermelon hosts. Using a Group II strain such as AAC00 may be more informative for watermelons in a laboratory due to its enhanced pathogenicity.

It was not possible in this study to quantify bacterial load in the specimens by measuring highly variable GFP fluorescence in 3-dimensional structures. Instead the simple presence or absence of GFP signals in roots and cotyledons was recorded rather than the intensity of the signal. Because of this, the data are in the format of frequencies of "GFP signal" or "no GFP signal". This is valuable qualitative information. GFP-positive seedlings showed highly variable signals. Some were bright and abundant while others were faint and very localized. It would be informative to determine if the level of GFP signal correlates with BFB symptoms. Therefore, quantifying the levels of GFP as an indicator of levels of bacterial presence is a very important future goal. This could be accomplished by using quantitative PCR to measure levels of bacteria DNA and levels of GFP mRNA. Alternately, cotyledons could be homogenized and a fluorometer used to compare levels of GFP versus chlorophyll fluorescence.

In the soil germination method, all of the seeds planted eventually germinated and grew. However, in the test tube and germination paper methods contamination with fungi was a problem. In one of the germination paper trials, even fungicide treated seeds developed fungal growth. In the control group, 21 out of 120 fungicide-treated seedlings were contaminated. Seventy out of 120 seeds inoculated with the bacteria via the soaking infiltration method and

treated with fungicide developed fungal growth. Fifty-four out of 120 seeds inoculated with the bacteria via the vacuum infiltration method and treated with fungicide developed fungal growth. This unwanted fungal growth demonstrated that the seeds must be properly disinfected before *A. citrulli* inoculation to eradicate fungal spores. Although a compound in the media may have inhibited fungal growth in soil germination, it is likely that the fungal growth can be attributed to the higher levels of moisture surrounding the seed in the test tube and germination paper tests. Fungal growth is likely an unwanted side effect the high RH necessary to induce bacterial lesioning. For these reasons, soil media germination was the most effective germination method for rearing "healthy" seedlings. Additionally, bacterial colonization may have been inhibited by fungal growth, affecting the results of some experiments. In this study, Captan 50WP fungicide was used, but different fungicides may prove to be more effective. Captan 50WP was originally formulated as a foliar treatment, trying fungicides formulated as seed treatments may be more effective. Additionally, it may be that the Captan treatment itself inhibited the BFB. Testing fungicides using a paper disc diffusion assay to determine whether they display inhibitory action against *A. citrulli* would aide in identifying an ideal fungicide for this study. Although the fungal contaminant was not identified, it was likely Rhizopus, a common saprophytic fungus that may interfere with bacterial growth.

This study found convincing evidence that a green tea seed treatment may effectively reduce *A. citrulli* depending on the inoculum load. Future research should focus on identifying which compound in green tea is antibacterial towards BFB. Knowing the mode of action will be invaluable for developing a green tea seed treatment that is stable under a wide range of environmental conditions, can be stable in long-term storage, and remain effective during seed handling and planting. Identifying these key compounds would allow the creation of highly

effective seed treatments. Based on previous *in vitro* studies, it is likely that the EGCG catechin compound in green tea is the bactericidal agent in our treatment. Using concentrated EGCG in a similar study may yield more effective results.

Green tea was chosen for this study because of its antibacterial properties (Cowan et al., 1999). Other natural compounds can control growth of *Pseudomonas* bacteria, suggesting such treatments may also work against BFB. For example, Smyth et al. (2010) showed that garlic was antagonistic to *P. aeruginosa*, and Kavanaugh and Ribbeck (2012) showed that oils derived from cassia, clove, balsam, thyme, and tea tree were effective in killing *P. aeruginosa in vitro*. Mengulluoglu and Soylu (2012), and Choi et al. (2016), found that oils derived from thyme and cinnamon were effective in combating *A. citrulli*. None of these studies incorporated the organic compounds as seed treatments for *in vivo* studies. Creating seed treatments from these organic oils and conducting similar studies would likely reveal additional effective organic control options for BFB.

In future studies it will be important to determine a range of inoculum levels wherein green tea is effective. This study examined bacterial concentrations of OD600:1.0 and OD600:0.001. Experimenting with inoculum concentrations in a full range between these two extremes would enable researchers to pinpoint treatment efficacy for a range of inoculum loads.

Lastly, although it was our intention to determine which inoculation method, soaking infiltration or vacuum infiltration, was most effective in inoculating host seeds with the M6 *A. citrulli*, BFB symptoms were not observed with either inoculation method. Additional trials will be required to determine which method is most effective. It is also important to study methodology for the green tea treatment to increase efficacy. Japanese (sencha) green tea was chosen for this study because of its high concentration of catechins. Identifying a green tea with

a higher catechin content would be beneficial for further study. Koch et al. (2018) found that South Korean (jeoncha) green tea contained higher EGCG concentrations than sencha tea, 213 mg/100ml and 124 mg/100ml respectively. Therefore, South Korean green tea may yield better results than Japanese green tea. Additionally, a green tea treatment using the vacuum infiltration method instead of the soaking infiltration method should be attempted. The vacuum infiltration method has the potential to move the green tea deeper into the seed coat and could prove more effective. Furthermore, in this study seeds were treated with a room temperature (22°C) green tea solution. It is possible that treating the seeds with warm (~37°C) green tea solution would be more effective. Heating the green tea may allow for deeper penetration of the treatment into the seed coat – however excessive temperatures may damage the seed embryo, so experimenting with a range of tea temperatures below boiling would be helpful to pinpoint an ideal tea temperature. It is also possible that adding a polymer-based binding agent, as is common with seed treatments, would improve the longevity and efficacy of the treatment.

These studies demonstrate that green tea shows promise as an effective option for BFB pathogen reduction in seeds infected with *A. citrulli.* It is especially effective at lower inoculum levels where it has the potential to significantly reduce the pathogen. However, additional experimentation is necessary to fully test its efficacy.

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