Efficacy of a Nonpathogenic *Acidovorax citrulli* Strain as a Biocontrol Seed Treatment for Bacterial Fruit Blotch of Cucurbits

K. L. Johnson, Department of Plant Pathology, University of Georgia, Athens 30602; G. V. Minsavage, Department of Plant Pathology, University of Florida, Gainesville 32611; T. Le, Department of Plant Pathology, University of Georgia; J. B. Jones, Department of Plant Pathology, University of Florida; and R. R. Walcott, Department of Plant Pathology, University of Georgia

Abstract

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

Bacterial fruit blotch (BFB), caused by the seedborne, gram-negative bacterium *Acidovorax citrulli*, is a serious threat to cucurbit seed and fruit production worldwide. Because of the lack of effective management strategies, we investigated the efficacy of a nonpathogenic *A. citrulli* strain as a biological control seed treatment for BFB. For this study, we generated a type III secretion system mutant of *A. citrulli*, AAC00-1 Δ hrcC, that was nonpathogenic on watermelon but retained its ability to colonize germinating watermelon seed. With watermelon seed naturally infested with *A. citrulli*, AAC00-1 Δ hrcC reduced BFB seedling transmission by 81.8% relative to control seed. In comparison,

another *A. citrulli* antagonist, *A. avenae* strain AAA 99-2, reduced BFB seedling transmission by 74.6% for seed samples from the same lot. Additionally, when female watermelon blossoms were protected with AAC00-1 Δ hrcC and subsequently challenged with AAC00-1, the resulting seedlots displayed 8% BFB seedling transmission. This was not significantly different than seed from blossoms protected with AAA 99-2 (4%) but significantly less than those from blossoms protected with 0.1 M phosphate-buffered saline (36%). These results suggest that nonpathogenic *A. citrulli* has potential as a biological control seed treatment component in a comprehensive BFB management program.

Bacterial fruit blotch (BFB) of cucurbits, caused by the gram-negative bacterium *Acidovorax citrulli* (33) (formerly *A. avenae* subsp. *citrulli*; 42), poses a significant threat to cucurbit seed and fruit production worldwide (32). BFB was reported in commercial watermelon fields in Florida in 1989, and frequent outbreaks caused significant economic losses across the southeastern United States in the early 1990s (35). At present, the disease is a sporadic threat to cucurbit producers worldwide (4,26,29), because an outbreak can lead to 100% yield loss under ideal environmental conditions.

Seed are the most important source of primary inoculum for *A. citrulli* and infested seed can readily initiate BFB epidemics under transplant house conditions (18). Watermelon seed can become infested via infection of immature fruit through open stomata (up to 3 weeks after anthesis) or by bacterial penetration of the pistil of female flowers (10,40). Commercial seed producers bear the responsibility for producing *A. citrulli*-free seed to limit BFB outbreaks. To date, the most effective strategy for managing BFB has been excluding the pathogen from seed, fruit, and transplant production systems. This involves producing seed in regions of countries with cool, dry climates that limit BFB development, combined with visual seed field inspection and rigorous seed health testing (40). Seed treatments, including disinfestants and fermentation, are also routinely employed (15,16); however, because the pathogen can be localized under the seed coat (30), the efficacy of externally applied seed treatments can vary widely (16,30).

Currently, there are no commercially available BFB-resistant watermelon cultivars. Early reports of resistance in some cultivars were disputed and, although five cultivars with BFB resistance were reported, the horticultural traits of these lines were undesirable (17). Identification of effective resistant cultivars is further

complicated by the fact that *A. citrulli* can infect all growth stages of the watermelon plant, and the level of resistance may vary depending on the plant stage being screened and the bacterial strains used. For example, Bahar et al. recently reported that, whereas certain melon cultivars had BFB-tolerant foliage, the BFB seed-to-seedling transmission rates were still high (2). In the absence of resistance there is a need to develop a comprehensive integrated disease management strategy for BFB. One environmentally sound component that could be incorporated into this strategy is biological control seed treatments.

Biological control has been previously explored for BFB management (9,25,41). Fessehaie and Walcott (9) demonstrated that seed treatments with *A. avenae* (formerly *A. avenae* subsp. *avenae*; 33) strain AAA99-2, a pathogen of maize, reduced BFB seed-to-seedling transmission (9). Additionally, when applied as a protectant to female watermelon blossoms, the bacterium significantly reduced *A. citrulli* seed infestation. Because AAA 99-2 was pathogenic on maize, it was unsuitable as a commercial seed treatment for BFB. One approach for developing a more suitable BFB biocontrol candidate is the generation of a nonpathogenic *A. citrulli* strain. Nonpathogenic strains of phytopathogens have been used previously to manage plant diseases (27,37,38). These strains can occur naturally or can be generated by mutagenesis. One strategy that can efficiently abolish pathogenicity in phyto bacteria is mutagenesis of the type III secretion system (T3SS) (3,5). Type III secretion (T3S) is important for pathogenesis because it facilitates the translocation of virulence proteins from the bacterial cell directly into the cytoplasm of the host plant cell (13). Mutations in critical T3SS genes result in loss of pathogenicity and the ability to induce the hypersensitive response (HR) in nonhost plants (21). The use of T3SS mutants as biocontrol agents has been reported for *Xanthomonas campestris* pv. *vesicatoria* and *Erwinia amylovora* (27,37), the causal agents of bacterial spot of pepper and fire blight of pear and apple, respectively. However, there have been no attempts to develop a biocontrol seed treatment for BFB based on a T3SS *A. citrulli* mutant. Hence, the objective of this study was to develop and assess the efficacy of a T3SS mutant of *A. citrulli* as a biocontrol seed treatment for BFB.

Corresponding author: R. Walcott, E-mail: rwalcott@uga.edu

Accepted for publication 10 February 2011.

Materials and Methods

Bacterial cultures, media, and inoculum preparation. *A. citrulli*, *A. avenae*, and *Escherichia coli* strains used in this study are listed in Table 1. *A. citrulli* and *A. avenae* strains were routinely grown on nutrient agar (Difco, Becton Dickinson and Co., Sparks, MD) with appropriate antibiotics for 48 h at 28°C. *E. coli* strains were grown in Luria Bertani (LB) broth or agar (Difco) with appropriate antibiotics for 24 h at 37°C. To prepare *A. citrulli* inoculum, bacterial cells were pelleted from 1.5 ml of overnight LB broth culture by centrifugation at 13,000 rpm for 1 min. Cells were washed with and resuspended in 0.1 M phosphate-buffered saline (PBS; 137 mM NaCl, 5.6 mM Na₂HPO₄, 2.7 mM KCl, and 1.5 mM KH₂PO₄). Bacterial suspensions containing approximately 1 × 10⁸ CFU/ml were estimated using a spectrophotometer (optical density at 600 nm of approximately 0.3) (Spectronic 20; Bausch and Lomb, Rochester, NY) and 10-fold serially diluted with PBS to generate cell suspensions with desired concentrations. When used, antibiotics were incorporated into media at the following concentrations: rifamycin (100 µg/ml, *A. citrulli*), spectinomycin (100 µg/ml, *A. citrulli* and *E. coli*), kanamycin (50 µg/ml, *A. citrulli* and *E. coli*), and cycloheximide (200 µg/ml, *A. citrulli*) (Sigma-Aldrich, St. Louis).

DNA manipulations. A nonpathogenic *A. citrulli* T3SS mutant (AAC00-1Δ*hrcC*) was generated by deletion of the *hrcC* gene that encodes the T3SS outer membrane pore (22). Briefly, a 3.6-kb fragment containing the *hrcC* gene was identified in the genome library of AAC00-1. The 3.6-kb fragment was digested from a cosmid clone using *Bam*HI (Promega Corp., Madison, WI), cloned into pBluescript, and transformed into *E. coli* strain DH5α. In all, 673 bp were deleted from the cloned fragment by restriction digestion with *Stu*I and *Bst*EII (Promega Corp.) and the fragment was religated, resulting in deletion of the *hrcC* gene. The flanking open reading frames, SAM methyl transferase and *hrpD5*, remained unaffected. The deleted *hrcC* gene construct was cloned into the suicide vector pOK1, which carries the levansucrase *SacB* gene, to generate pOK-HRCC. pOK-HRCC was transformed into *E. coli* strain S17-1 (λpir), which was mated with AAC00-1, and single-crossover transconjugants were selected based on resistance to spectinomycin. A second homologous recombination was promoted by growth on M9 agar plates containing sucrose, which retards the growth of bacteria expressing *SacB*. Generation of the deletion mutation was confirmed by polymerase chain reaction (PCR) amplification using primers HrcC_{for} (5'-TGTATTCTACCCGGGCAAGTCC-3') and HrcC_{rev} (5'-TCCTCGATGTAGAGACTCAGCTTG-3'). One *hrcC* deletion mutant, designated AAC00-1Δ*hrcC*, was suitable for our studies. The *hrcC* deletion mutant was complemented by subcloning the 3.6-kb *Bam*HI fragment containing the *hrcC* gene into the cosmid pUFR043 to pro-

duce pUFR034-*hrcC*. pUFR043-*hrcC* was subsequently transformed into *E. coli* S17-1 (λpir) for use in triparental mating. pUFR043-*hrcC* was then transformed into AAC00-1Δ*hrcC* to create AAC00-1Δ*hrcC*comp (*hrcC* mutation complement).

Pathogenicity and HR assays. The effect of *hrcC* deletion on the ability of *A. citrulli* to induce an HR was evaluated using 6-week-old tobacco plants (*Nicotiana tabacum*). Using a hypodermic syringe without a needle, AAC00-1, AAC00-1Δ*hrcC*, AAC00-1Δ*hrcC*comp at approximately 1 × 10⁸ CFU/ml, or PBS was infiltrated into the intercellular spaces between the major veins of a fully expanded tobacco leaf. Plants were incubated under conditions of approximately 75% relative humidity (RH), 28°C, and 12 h of fluorescent light daily in a growth chamber (Percival, Perry, IA) for 2 days. Plants were subsequently observed for cell death associated with a typical HR. To determine the effect of the T3SS mutation on *A. citrulli* pathogenicity, 2-week-old watermelon seedlings ('Sugar Baby') were infiltrated as described above with AAC00-1Δ*hrcC*, AAC00-1Δ*hrcC*comp AAC00-1 (wild-type) at approximately 1 × 10⁶ CFU/ml, or PBS as a negative control. The cotyledons of three seedlings were inoculated (28) and plants were incubated for 2 days at 28°C with approximately 75% RH and 12 h of fluorescent light daily in a growth chamber. Plants were then visually observed for BFB symptoms (necrosis and water soaking). This experiment was conducted three times.

Role of T3SS in *A. citrulli* colonization of watermelon seed during germination. The ability of AAC00-1Δ*hrcC* to colonize watermelon seed (Sugar Baby) during seed germination was determined by infiltrating seed with AAC00-1, AAC00-1Δ*hrcC*, or AAC00-1Δ*hrcC*comp at approximately 1 × 10³ CFU/ml as previously described (39). Watermelon seed (*n* = 35) were planted in plastic boxes (Tri-State Plastics, Dixon, KY) on moist blotter paper (Hoffman Manufacturing, Albany, OR) and incubated at 30°C with 100% RH and continuous fluorescent light. Seed samples (*n* = 5 seeds) were collected at 0, 6, 12, 24, 48, 72, and 96 h after planting and bacterial populations were estimated by macerating each seed in 900 µl of PBS in a sterile microcentrifuge tube. Appropriate 10-fold serial dilutions (100 µl) of each seed homogenate were spotted onto nutrient agar plates with appropriate antibiotics. Plates were incubated for 48 to 72 h at 28°C and, subsequently, *A. citrulli* colonies were enumerated. The experiment was conducted three times and mean bacterial populations were plotted over time to generate area under population dynamics curves (AUPDCs). Analysis of variance (ANOVA) was conducted on AUPDC data using JMP statistical analysis software (version 8 for Windows; SAS Institute Inc, Cary, NC) and the effect of the T3SS mutation on colonization of watermelon seed was determined using Tukey-Kramer's honestly significant difference (HSD).

Role of T3S in *A. citrulli* virulence. To determine the role of T3S in virulence, suspensions with AAC00-1, AAC00-1Δ*hrcC*, or

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics ^a	Source
Bacteria		
<i>Acidovorax citrulli</i> (AAC)		
00-1	Wild-type, Rf ^r	9
00-1Δ <i>hrcC</i>	<i>hrcC</i> deletion mutant	This study
00-1Δ <i>hrcC</i> comp	<i>hrcC</i> complemented mutant	This study
<i>A. avenae</i> (AAA)		
99-2	Maize pathogen	9
<i>Escherichia coli</i>		
DH5α	F – Φ80 <i>lacZ</i> Δ <i>M15</i> Δ (<i>lacZ</i> Y _A -argF) U169 <i>recA1</i> <i>endA1</i> <i>hsdR17</i> (rK–, mK+)	Invitrogen
S17-1 (λpir)	<i>phoA</i> <i>supE44</i> λ – <i>thi-1</i> <i>gyrA96</i> <i>relA1</i> Δ <i>lacU169</i> (Φ <i>lacZ</i> Δ <i>M15</i>), <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i> , λpir	Jeff Jones, UFL
Plasmids		
pBluescript	Ap ^r	Invitrogen
pOK1	Sp ^r , <i>SacB</i>	19
pOK-HRCC	truncated <i>hrcC</i> gene construct in pOK1	This study
pUFR043	Gm ^r , Km ^r	8
pUFR043- <i>hrcC</i>	Complete <i>hrcC</i> gene in pUFR043	This study

^a Rf^r, Ap^r, Sp^r, Gm^r, and Km^r = resistant to rifamycin, ampicillin, spectinomycin, gentamycin, and kanamycin, respectively.

AAC00-1 Δ hrcComp at approximately 1×10^3 CFU/ml were infiltrated separately into the cotyledons of three 2-week-old watermelon seedlings (Sugar Baby). Infiltrated leaves were allowed to air dry for 1 h; then, six leaf discs, 3 mm each (one from each cotyledon) were collected at 1, 12, 24, 48, 72, and 96 h after inoculation. Each leaf disc was macerated in a separate microcentrifuge tube containing 900 μ l of PBS and bacterial populations were enumerated by serial dilution plating as described above. The experiment was conducted three times and bacterial populations were plotted over time to generate AUPDC data. AUPDC data were used to compare the effect of the T3SS mutation on *A. citrulli* virulence using Tukey-Kramer's HSD.

Efficacy of AAC00-1 Δ hrcC as a biocontrol seed treatment for BFB under growth chamber conditions. To evaluate the efficacy of AAC00-1 Δ hrcC as a biocontrol seed treatment, naturally infested seed extracted from symptomatic watermelon fruit from a BFB outbreak in Reidsville, GA in 2005 were used. Samples (four replicates of 100 seeds) of this seedlot displayed 75% mean BFB seedling transmission and 76% mean germination using a modified seedling grow-out assay (40). Infested seed ($n = 100$) were incubated in 20 ml of cell suspension containing AAC00-1 Δ hrcC, AAA 99-2 at approximately 1×10^8 CFU/ml, or PBS as a negative control. Watermelon seed were then subjected to continuous vacuum infiltration for 20 min, which allowed the biocontrol agents to be deposited under the testae. After inoculation, seed were air dried at approximately 25°C for 24 h and then assayed for BFB seed-to-seedling transmission using a modified seedling grow-out assay. Briefly, seed ($n = 100$) were planted in transparent plastic boxes on two layers of blotter paper (Hoffman Manufacturing) saturated with deionized water. Germination boxes were incubated in a growth chamber (Percival) under conditions of 100% RH, 30°C, and continuous fluorescent light for 12 days. Seed were monitored daily and germination percentage (number of seed that produced radicles and plumules divided by the total number of seed planted $\times 100$) and BFB seedling transmission (number of seedlings displaying typical BFB symptoms divided by the number of seedlings that germinated $\times 100$) percentages were recorded daily. The experiment was conducted twice and mean BFB seedling transmission percentage data were plotted over time to generate AUDPC data. AUDPC data were used to conduct ANOVA and to determine the significance of the effect of AAC00-1 Δ hrcC on BFB seedling transmission using Tukey-Kramer's HSD. Seed germination data were used to determine the effect of AAC00-1 Δ hrcC on seed physiology. The ability of AAC00-1 Δ hrcC and AAA 99-2 to limit BFB seedling transmission was determined using the formula described by Moss et al. (27).

Efficacy of AAC00-1 Δ hrcC as a biocontrol seed treatment for BFB under greenhouse conditions. Infested watermelon seed ('Crimson Sweet') were generated by hand pollinating (rubbing pollen from male blossoms onto the stigmatic surfaces), then inoculating the stigmas of female blossoms of greenhouse-grown plants with AAC00-1 (approximately 1×10^8 CFU/ml) (40). Samples (four replicates of 100 seeds) of this seedlot displayed 75% mean BFB seedling transmission and 72% mean germination using a modified seedling grow-out assay (40). Fruit from inoculated blossoms were allowed to develop to maturity (35 days after anthesis) and seed were extracted by hand. After extraction, seed were air dried at 25°C for 24 h and samples ($n = 100$ seed) were vacuum-infiltrated with AAC00-1 Δ hrcC at approximately 1×10^8 CFU/ml or PBS as a negative control as described above. Treated seed were air dried at approximately 25°C for 24 h and then assayed for BFB seed-to-seedling transmission under greenhouse conditions as follows. Seed ($n = 100$) were planted in 128-cell polystyrene flats (Speedling Inc., Sun City, FL) filled with pasteurized potting mix (90% composted pine bark and 10% vermiculite). Flats were incubated in plastic chambers (on a greenhouse bench) used to generate conditions of 30°C and approximately 100% RH for 21 days. Seedlings were monitored daily and germination and BFB seedling transmission percentages were recorded. The experiment was conducted three times and

mean BFB seedling transmission percentages were plotted over time to generate AUDPC data. AUDPC data were used to conduct ANOVA and to determine the significance of the effect of AAC00-1 Δ hrcC on BFB seedling transmission using the Student's *t* test.

Efficacy of AAC00-1 Δ hrcC as a blossom protectant to prevent seed infestation by *A. citrulli*. The ability of AAC00-1 Δ hrcC to protect female blossoms from invasion by *A. citrulli* was evaluated. Watermelon plants (Crimson Sweet) were maintained under standard greenhouse conditions in 15-liter pots containing 90% composted pine bark and 10% vermiculite until anthesis. At anthesis, female blossoms were manually pollinated as described above, then immediately inoculated by depositing 10 μ l of a suspension containing AAC00-1 Δ hrcC, AAA99-2 at approximately 1×10^8 CFU/ml, or PBS as a negative control onto the stigmas. Five hours after inoculation, blossoms were challenged with 10 μ l of a cell suspension containing AAC00-1 at 1×10^9 CFU/ml. Each treatment was applied to 10 blossoms, each on a separate plant, and fruit were allowed to develop for 35 days after pollination. At harvest maturity, fruit were surface sterilized and seed were manually extracted (seed from each fruit were maintained as a separate lot), air dried at 25°C for 24 h, and stored at 4°C. To determine the level of seed infestation by *A. citrulli*, samples ($n = 100$ seed) from each lot were subjected to the modified seedling grow-out assay as described above. Seedlings were monitored daily for 14 days and germination and BFB seedling transmission percentages were recorded daily. This experiment was conducted twice and mean BFB seedling transmission percentage data were plotted over time and used to generate AUDPC data. Germination data were also collected and used to determine the effect of biocontrol blossom treatments on seed physiology. AUDPC data were analyzed using the Tukey-Kramer's HSD to determine the significance of blossom protection on seed infestation by *A. citrulli*.

Efficiency of seed treatment via female watermelon blossom inoculation. To determine whether blossom inoculation was an efficient method for inoculating seed with biocontrol agents, female blossoms were inoculated with AAC00-1 Δ hrcC and the resulting seed were assayed for the bacterium by species-specific quantitative real-time PCR. Watermelon plants (Crimson Sweet) were cultivated under greenhouse conditions in 15-liter pots as described above. At anthesis, female blossoms were hand pollinated and stigmas were immediately inoculated with 10 μ l of a suspension of AAC00-1 Δ hrcC or AAC00-1 at approximately 1×10^8 CFU/ml as described above. Inoculated blossoms were allowed to develop to harvest maturity (approximately 35 days after anthesis). For each treatment, five mature fruit were harvested and seed were extracted and stored at 4°C. The level of seed infestation was determined by extracting total microbial DNA from seed and conducting quantitative real-time PCR as follows. Seed ($n = 50$) were macerated for 5 min using a homogenizer (Bioreba Homex 6; Bioreba, Gilroy, CA) and suspended in 10 ml of PBS. The suspension (1 ml) was pelleted by centrifugation at 13,000 rpm for 3 min. The PBS was decanted and total microbial DNA was extracted from the pellet using a Mobio Ultraclean Microbial DNA isolation kit (Mobio Laboratories Inc., Carlsbad, CA), according to the manufacturer's instructions. Extracted DNA was dissolved in 20 μ l of water and 5 μ l was used for real-time PCR. For real-time PCR, the master-mix comprised 12.5 μ l of Bio-Rad real-time PCR mastermix (Bio-Rad Laboratories, Hercules, CA), 7.5 pM BOXAACF and BOXAACR2 primers, and 5 pM TaqMan probe (11). Thermal cycling conditions included denaturation at 95°C for 2 min., 35 cycles of denaturation at 95°C for 15 s, and annealing and extension at 55°C for 45 s. The experiment was conducted twice and mean cycle threshold (Ct) values were recorded. A standard curve was generated using 10-fold serial dilutions of *A. citrulli* cell suspensions (1×10^8 to 1×10^0 CFU/ml). An amplification threshold value of 30 fluorescence units was routinely used, and the standard curve was based on four independent replicates. The standard curve was constructed by plotting mean Ct values against log₁₀ A.

citrulli cell dilutions and it was used to estimate *A. citrulli* populations in seed. The effect of the T3SS on colonization of watermelon seed tissue via blossoms was determined using the Student's *t* test.

Fig. 1. Ethidium bromide-stained agarose gel of polymerase chain reaction amplicons confirming the deletion of the *hrcC* gene in *Acidovorax citrulli* strain 00-1. Lane 1, 1-kb DNA ladder (Fermentas, Glen Burnie, MD); lane 2, AAC00-1 Δ *hrcC* (nonpathogenic *hrcC* mutant of AAC00-1); lane 3, AAC00-1 Δ *hrcC*comp (complemented *hrcC* mutant of AAC00-1); lane 4, AAC00-1 (wild-type *A. citrulli*); lane 5, water (negative control).

Results

Generation and characterization of a T3SS mutant of *A. citrulli*. Deletion of the AAC00-1 *hrcC* gene was confirmed by PCR amplification (Fig. 1). Using primers for the *hrcC* gene, a 1.08-kb DNA fragment was amplified from wild-type AAC00-1 and the complemented strain, AAC00-1 Δ *hrcC*comp. However, the 1.08-kb fragment was absent from AAC00-1 Δ *hrcC* and a smaller 0.4-kb fragment was amplified, indicating deletion of the *hrcC* gene. As expected, both amplicons were produced with DNA from AAC00-1 Δ *hrcC*comp (Fig. 1). *hrcC* deletion resulted in the loss of T3S in AAC00-1, which was confirmed by lack of an HR when AAC00-1 Δ *hrcC* was infiltrated into tobacco leaves (Fig. 2A). In contrast, AAC00-1 (wild-type) and AAC00-1 Δ *hrcC*comp induced a typical HR on tobacco leaves (Fig. 2A). As expected, leaf infiltrations with PBS did not yield an HR on tobacco. When infiltrated into watermelon seedlings, AAC00-1 and AAC00-1 Δ *hrcC*comp produced typical water-soaking and necrosis by 2 days post inoculation (dpi) (Fig. 2B). In contrast, seedlings infiltrated with AAC00-1 Δ *hrcC* did not develop BFB symptoms and yielded results that were comparable with seedlings inoculated with PBS (Fig. 2B).

Fig. 2A, Induction of the hypersensitive response on tobacco (*Nicotiana tabacum*) by different *Acidovorax citrulli* strains. Leaves were infiltrated with 1, AAC00-1 Δ *hrcC* (nonpathogenic *hrcC* mutant of AAC00-1); 2, AAC00-1 Δ *hrcC*comp (complemented *hrcC* mutant of AAC00-1); 3, AAC00-1 (wild-type *A. citrulli*); and 4, 0.1 M phosphate-buffered saline (PBS, negative control) at 1×10^6 CFU/ml. **B,** Response of 2-week-old watermelon ('Crimson Sweet') cotyledons infiltrated with different *A. citrulli* strains, including: 1, PBS (negative control); 2, AAC00-1 Δ *hrcC*; 3, AAC00-1 Δ *hrcC*comp; and 4, AAC00-1.

Role of T3S in *A. citrulli* colonization of watermelon seed during germination. Both AAC00-1 Δ *hrcC* and AAC00-1 Δ *hrcC*comp colonized watermelon seed at wild-type levels during the first 96 h of germination. For all strains, populations increased on watermelon seed by 6 h after planting (Fig. 3A). AAC00-1 Δ *hrcC* and AAC00-1 Δ *hrcC*comp increased continuously over the course of the experiment (96 h), similar to AAC00-1. By 96 h after planting, the mean populations of AAC00-1, AAC00-1 Δ *hrcC*, and AAC00-1 Δ *hrcC*comp were 1.7×10^9 , 3.9×10^8 , and 1.6×10^9 CFU/g of seed, respectively (Fig. 3A). For seed inoculated with AAC00-1 and AAC00-1 Δ *hrcC*comp, seedlings emerged and developed typical BFB symptoms on cotyledons by 14 days after planting (dap). In contrast, seedlings from seed inoculated with AAC00-1 Δ *hrcC* emerged and remained asymptomatic by 14 dap. ANOVA conducted on AUPDC data indicated that the effect of experiment was not statistically significant ($P = 0.94$); therefore, data from three experiments were pooled and analyzed. There was no significant difference in the colonization of watermelon seed by AAC00-1 Δ *hrcC*, AAC00-1, and AAC00-1 Δ *hrcC*comp according to Tukey-Kramer's HSD ($P = 0.19$; Fig. 3B).

Role of T3S in *A. citrulli* virulence. Populations of AAC00-1 and AAC00-1 Δ *hrcC*comp increased continuously over the first 96 h after inoculation. Populations of both strains increased from ap-

Fig. 3A, Colonization of watermelon seed (‘Sugar Baby’) by wild-type *Acidovorax citrulli* (AAC00-1), AAC00-1 Δ *hrcC* (nonpathogenic *hrcC* mutant), or AAC00-1 Δ *hrcC*comp (complemented *hrcC* mutant) during the first 96 h of seed germination. The experiment was conducted three times and each data point represents the mean of the three experiments ($n = 5$ seed). Lines represent the standard error of the mean. **B,** Bar chart of area under population dynamics curve (AUPDC) calculated for seed infiltrated with 10^3 CFU of AAC00-1, AAC00-1 Δ *hrcC*, or AAC00-1 Δ *hrcC*comp and germinated for 96 h. Bars represent mean AUPDC and lines indicate standard error of the means. Treatments with similar letters are not significantly different according to Tukey-Kramer's honestly significant difference.

proximately 1×10^1 CFU/disc at 1 h post inoculation (hpi) to 1×10^3 CFU/disc by 24 hpi. On the other hand, populations of AAC00-1 Δ *hrcC* remained at 1×10^1 CFU/disc for the first 72 hpi. AAC00-1 and AAC00-1 Δ *hrcC*comp colonized watermelon cotyledons at similar rates and reached mean populations of 2.2×10^7 and 4.3×10^6 CFU/disc, respectively, by 96 hpi (Fig. 4A). In contrast, AAC00-1 Δ *hrcC* reached a population of 2.0×10^2 CFU/disc by 96 hpi (Fig. 4A). The effect of the T3SS on *A. citrulli* colonization of watermelon cotyledons was statistically significant ($P < 0.01$) (Fig. 4B).

Efficacy of AAC00-1 Δ *hrcC* as a seed treatment under growth-chamber conditions. Watermelon seed naturally infested with *A. citrulli* displayed a mean BFB seedling transmission percentage of 55% ($\pm 8\%$ [standard error]) (Fig. 5A). In comparison, seed treated with AAC00-1 Δ *hrcC* displayed a BFB seedling transmission percentage of 10 ($\pm 3\%$) by 12 dap, which was lower than that observed for AAA 99-2 (14 [$\pm 4\%$]; Fig. 5A). Tukey-Kramer's HSD analysis indicated that BFB seedling transmission percentages for seed treated with AAA 99-2 and AAC00-1 Δ *hrcC* were significantly lower than for seed treated with PBS ($P < 0.01$). However, BFB seedling transmission for seed treated with AAC00-1 Δ *hrcC* and AAA 99-2 were not significantly different ($P = 0.8$; Fig. 5B). Seed treatment with AAC00-1 Δ *hrcC* delayed BFB symp-

Fig. 4A, Colonization of watermelon seedlings (‘Crimson Sweet’) by *Acidovorax citrulli* (AAC00-1) (wild-type), AAC00-1 Δ *hrcC* (*hrcC* deletion mutant), and AAC00-1 Δ *hrcC*comp (complemented *hrcC* mutant). The experiment was conducted three times and each data point represents the mean of three experiments ($n = 6$ leaf discs/treatment). Lines represent the standard error of the means. **B,** Bar chart of area under population dynamics curve calculated for watermelon seedlings infiltrated with AAC00-1, AAC00-1 Δ *hrcC*, or AAC00-1 Δ *hrcC*comp and incubated for 96 h. Bars represent the mean area under population dynamics curve and lines indicate standard error of the means. Treatments with different letters are significantly different according to Tukey-Kramer's honestly significant difference.

tom expression by 2 days. When seed were treated with AAC00-1 Δ hrcC and AAA 99-2 BFB, seedling transmission was reduced by 81.8 and 74.6%, respectively, compared with PBS.

Efficacy of AAC00-1 Δ hrcC as a seed treatment under greenhouse conditions. To determine the efficacy of AAC00-1 Δ hrcC as a seed treatment under transplant house conditions, seed naturally infested with AAC00-1 were treated and planted under greenhouse conditions highly conducive to BFB development. Seed treatment with AAC00-1 Δ hrcC reduced BFB seedling transmission and epidemic development relative to the negative control. For naturally infested seed treated with PBS, mean BFB seedling transmission percentage was 37 ($\pm$ 3)% (Fig. 6A). In contrast, seed treated with AAC00-1 Δ hrcC displayed 23 ($\pm$ 7)% BFB incidence by 21 dap (Fig. 6A), which represents a 37.8% reduction relative to PBS-treated seed. BFB seedling transmission for seed treated with AAC00-1 Δ hrcC was significantly lower than for PBS-treated seed ($P = 0.02$) according to the Student's t test. BFB transmission for AAC00-1 Δ hrcC-treated seed was delayed by 5 days in the greenhouse compared with 2 days in the growth chamber.

Efficacy of AAC00-1 Δ hrcC as a blossom protectant against seed infestation by *A. citrulli*. Watermelon fruit produced from female blossoms protected with AAC00-1 Δ hrcC or AAA 99-2 and

subsequently challenged with AAC00-1 did not develop BFB symptoms at harvest maturity. Based on ANOVA, the effect of experiment on seed infestation was not significant ($P = 0.64$); hence, the data for the two experiments were pooled for analysis. Blossom protection with biocontrol agents reduced seed infestation by *A. citrulli* and this effect was statistically significant ($P < 0.01$). Approximately 36% of seed produced from blossoms protected with PBS developed BFB symptoms in the modified seedling grow-out assay. In comparison, seedlots produced from blossoms protected with AAA 99-2 and AAC00-1 Δ hrcC had mean BFB seedling transmission percentages of 4 and 8%, respectively (Fig. 7). Tukey-Kramer's HSD analysis indicated that *A. citrulli* infestation for seed from blossoms protected with AAC00-1 Δ hrcC or AAA 99-2 was significantly less than for those treated with PBS ($P < 0.01$; Fig. 7). However, there was no significant difference between the abilities of AAC00-1 Δ hrcC and AAA 99-2 to limit seed infestation by *A. citrulli* ($P = 0.8$). ANOVA of seed germination percentage data indicated that the effect of experiment was statistically significant ($P = 0.002$). Hence, the data for the two experiments were analyzed separately. In experiment 1, mean germination percentages were 91, 86, and 81% for seed from blossoms protected with PBS, AAA 99-2, and AAC00-1 Δ hrcC, respectively.

Fig. 5A, Comparison of the effect of seed treatments with *Acidovorax citrulli* AAC00-1 Δ hrcC (type III secretion system mutant), *A. avenae* (AAA 99-2), or 0.1 M phosphate-buffered saline (PBS, negative control) on bacterial fruit blight (BFB) seedling transmission for watermelon seed naturally infested with *A. citrulli*. Data points represent the mean BFB seedling transmission percentage of two experiments ($n = 100$ seeds/treatment) and the lines represent the standard error of the means. **B,** Bar chart of area under disease progress curves (AUDPCs) calculated for naturally infested seed treated with AAC00-1 Δ hrcC, AAA99-2, or PBS. Bars represent mean AUDPC values and lines indicate standard error of the means. Treatments with different letters are significantly different according to Tukey-Kramer's honestly significant difference.

Fig. 6A, Effect of seed treatment with *Acidovorax citrulli* AAC00-1 Δ hrcC (type III secretion system mutant) or 0.1 M phosphate-buffered saline (PBS, negative control) on Bacterial fruit blight (BFB) seedling transmission for watermelon seed naturally infested with *A. citrulli* under greenhouse conditions. Each data point represents the mean BFB seedling transmission for three experiments ($n = 100$ seeds/treatment) and the lines represent the standard error of the means. **B,** Bar chart of area under disease progress curves (AUDPCs) calculated for *A. citrulli*-infested watermelon seed treated with AAC00-1 Δ hrcC or PBS grown under greenhouse conditions for 21 days. Bars represent mean AUDPC values and lines indicate standard error of the means. Treatments with different letters are significantly different according to the Student's t test.

In experiment 2, seed from blossoms treated with PBS, AAA 99-2, or AAC00-1 Δ *hrcC* displayed 98, 97, and 89% germination, respectively. For both experiments, the differences in germination percentages for seed from blossoms protected with PBS, AAA 99-2, or AAC00-1 Δ *hrcC* were not statistically significant (experiment 1, $P = 0.095$; experiment 2, $P = 0.08$).

Efficiency of seed treatment via female watermelon blossom inoculation. AAC00-1 Δ *hrcC* retained its ability to colonize blossoms and infest watermelon seed compared with wild-type AAC00-1. In total, 100% of the seedlots produced in fruit from AAC00-1- or AAC00-1 Δ *hrcC*-inoculated blossoms were infested. AAC00-1 Δ *hrcC*-infested seedlots had populations that ranged from 10^2 to 10^4 CFU/g of seed. In comparison, bacterial populations on AAC00-1-infested seedlots ranged from 10^3 to 10^4 CFU/g. The mean bacterial populations for seedlots infested with AAC00-1 (2.1×10^4 CFU/g) were not significantly different from those for AAC00-1 Δ *hrcC*-infested seedlots (1.2×10^4 CFU/g) ($P = 0.33$).

Discussion

In this study, we confirmed that *A. citrulli* has a functional T3SS encoded by the *hrp* gene cluster. *hrcC* encodes a multimeric protein that forms the outer membrane pore of the T3SS (6). Deletion of *hrcC* in AAC00-1 abolished pathogenicity on watermelon seedling tissues and the ability to induce an HR on tobacco plants. These observations suggest that the T3SS is required for foliar pathogenicity. As expected, complementation of the deleted *hrcC* gene restored functionality of the T3SS. Although this is the first report of a functional T3SS in *A. citrulli*, it was expected because T3S has been reported for other phytopathogenic bacteria (1,24,34).

Biological control of plant diseases usually involves competition for nutrients or colonization sites, or antibiosis (20,31). The ability of a nonpathogenic T3SS *A. citrulli* mutant, AAC00-1 Δ *hrcC*, to colonize seed was assessed to determine whether it could compete with wild-type bacteria during seed germination and, thereby, limit BFB seedling transmission. AAC00-1 Δ *hrcC* retained its ability to colonize germinating watermelon seed and, by 96 h after planting, its populations were not significantly different to those of AAC00-1 or AAC00-1 Δ *hrcC*comp. The ability of a T3SS mutant of a phyto-bacteria to colonize seed at wild-type rates has been previously reported for *Pseudomonas syringae* pv. *syringae* B728a on bean (14). Similar to this study, Hirano et al. (14) reported that disruption of the T3SS did not affect colonization of the germinating bean seed. Based on these observations, we conclude that type III effector proteins are not essential for seed colonization by *A. citrulli* during seed germination. It is logical to conclude that, during the initial phases of germination, seedborne phyto-bacteria grow as epiphytes rather than as plant pathogens.

When applied as a seed treatment to naturally infested watermelon seed, AAC00-1 Δ *hrcC* suppressed BFB seedling transmission similarly to AAA 99-2, a strain previously reported to be antagonistic to *A. citrulli*. Under greenhouse conditions, AAC00-1 Δ *hrcC* seed treatments delayed the onset of BFB symptoms on watermelon seedlings. Interestingly, we observed better suppression of BFB seedling transmission in the growth chamber (5.5-fold reduction) than in the greenhouse assay (1.6-fold reduction). This was unexpected because conditions in the growth-chamber assay were consistently highly conducive for BFB seedling transmission, whereas conditions in the greenhouse (namely, relative humidity) were less conducive. Because plants were allowed to grow for 21 versus 12 days in the greenhouse experiment, we concluded that the biocontrol efficacy of AAC00-1 Δ *hrcC* declined as the seedlings matured. This was likely due to the inability of the mutant to colonize older watermelon foliage and compete with AAC00-1 once it entered the pathogenic phase.

Pollination of blossoms leads to the production of stigmatic exudates, including sugars and proteins, that make the blossom an ideal environment for *A. citrulli* colonization (12). *A. citrulli* can colonize and penetrate female watermelon blossoms, which can lead to seed infestation without BFB fruit symptoms (18,36). We

observed that *hrcC* was not critical for blossom colonization and penetration because AAC00-1 Δ *hrcC* was not affected in its ability to infest seed via this pathway. In two independent experiments, 100% of seedlots generated from blossoms treated with AAC00-1 Δ *hrcC* were inoculated. Seed infestation levels for blossoms treated with AAC00-1 Δ *hrcC* and AAC00-1 were not significantly different. In contrast, Darsonval et al. (7) reported that T3SS mutants of *X. fuscans* subsp. *fuscans* were impaired in their ability to be transmitted to bean seed via blossoms. This discrepancy might be explained by differences in the morphology of watermelon and bean flowers and the unique nature of the host–pathogen interactions. In the case of watermelon and *A. citrulli*, it is likely that the interaction is nonspecific and colonization and invasion of blossoms occurs passively. This explanation is supported by the observation that *Pantoea ananatis*, a seedborne onion phyto-bacterium that is nonpathogenic on watermelon, colonized blossoms and infested watermelon seed (40). Lessl et al. (23) also reported that *Pseudomonas syringae* cit7, an epiphyte recovered from tomato foliage, also colonized watermelon stigmas. It would be interesting to determine whether bean flowers could be colonized and invaded by phyto-bacteria that were not bean pathogens.

The ability of T3SS mutants of phyto-bacteria to limit plant diseases caused by their pathogenic progenitors has been reported for *X. campestris* pv. *vesicatoria* and *P. syringae* pv. *tomato* (27,43). Moss et al. (27) found that *hrp* regulatory mutants were more effective than structural mutants due to the induction of host defense responses that are greater in the presence of regulatory mutants (27). Because watermelon blossom and seed colonization does not require the T3SS, it is unlikely that *hrp* regulatory mutants of *A. citrulli* would improve biocontrol activity as seed or blossom treatments. However, regulatory mutants of *A. citrulli* might improve biocontrol activity on seedling or mature foliar tissues.

Chemical seed treatments have had limited efficacy in BFB management, most likely because *A. citrulli* may be present under the seed coat (18,30). One advantage of AAC00-1 Δ *hrcC* is its ability to infest seed in a manner similar to wild-type *A. citrulli* and, thereby, become localized in similar seed tissues as the pathogen. Although the exact mechanism of biological control is unknown, we suspect that AAC00-1 Δ *hrcC* competes with wild-type *A. citrulli* for nutrients and colonization sites on the germinating seed-

Fig. 7. Effect of female watermelon blossom protection with *Acidovorax citrulli* AAC00-1 Δ *hrcC* (type III secretion system mutant), *A. avenae* (AAA 99-2), or 0.1 M phosphate-buffered saline (PBS, negative control) on bacterial fruit blotch (BFB) seedling transmission and seed germination percentages. The experiment was conducted three times and bars represent the mean BFB incidence or germination for two experiments ($n = 100$ seeds/treatment). Lowercase letters represent the comparison of effects of blossom treatments on BFB seedling transmission, while uppercase letters indicate the results for comparing the effects of seed treatments on germination. Treatments with different letters are significantly different according to Tukey-Kramer's honestly significant difference.

ling. AAC00-1*ΔhrcC* can also be used as a blossom protectant to limit seed infestation in seed production fields. In hybrid seed production, lyophilized AAC00-1*ΔhrcC* cells could be introduced into female blossoms during hand pollination without the need for added operational steps. Unfortunately, it is unlikely that blossom protection with AAC00-1*ΔhrcC* could be a stand-alone treatment for BFB because it did not completely eliminate BFB seedling transmission. Nevertheless, blossom protection followed by seed treatment with AAC00-1*ΔhrcC* might represent an important component in a comprehensive integrated BFB management strategy that seeks to deemphasize the use of xenobiotic pest management chemicals (i.e., organic cucurbit seed production). Benefits may also be gained by developing and exploring more effective strategies and techniques for applying biocontrol agents to seed or blossoms.

Acknowledgments

We thank T. Denny, M. Schell, and A. Castro-Sparks for their technical input; and P. Chebrulo and B. Dutta for technical assistance.

Literature Cited

- Alfano, J. R., and Collmer, A. 1997. The type three secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. *J. Bacteriol.* 179:5655-5662.
- Bahar, O., Kritzman, G., and Burdman, S. 2009. Bacterial fruit blotch of melon: screens for disease tolerance and role of seed transmission in pathogenicity. *Eur. J. Plant Pathol.* 123:71-83.
- Bonas, U. 1994. *hrp* Genes of phytopathogenic bacteria. *Curr. Top. Microb. Immunol.* 192:79-98.
- Burdman, S., Kots, N., Kritzman, G., and Kopelowitz, J. 2005. Molecular, physiological, and host-range characterization of *Acidovorax avenae* subsp. *citruilli* isolates from watermelon and melon in Israel. *Plant Dis.* 89:1339-1347.
- Collmer, A., and Bauer, D. W. 1994. *Erwinia chrysanthemi* and *Pseudomonas syringae*: plant pathogens trafficking in extracellular virulence proteins. *Curr. Top. Microb. Immunol.* 192:43-78.
- Cornelis, G. R., and Van Gijsegem, F. 2000. Assembly and function of type III secretory systems. *Annu. Rev. Microbiol.* 54:735-774.
- Darsonval, A., Darrasse, A., Meyer, D., Demarty, M., Durand, K., Bureau, C., Manceau, C., and Jacques, M. A. 2008. The type III secretion system of *Xanthomonas fuscans* subsp. *fuscans* is involved in the phyllosphere colonization process and in transmission to seeds of susceptible beans. *Appl. Environ. Microbiol.* 74:2669-2678.
- De Feyter, R., and Gabriel, D. W. 1991. Use of cloned DNA methylase genes to increase the frequency of transfer of foreign genes into *Xanthomonas campestris* pv. *malvacearum*. *J. Bacteriol.* 173:6421-6427.
- Fessehaie, A., and Walcott, R. R. 2005. Biological control to protect watermelon blossoms and seed from infection by *Acidovorax avenae* subsp. *citruilli*. *Phytopathology* 95:413-417.
- Frankle, W. G., Hopkins, D. L., and Stall, R. E. 1993. Ingress of the watermelon fruit blotch bacterium into fruit. *Plant Dis.* 77:1090-1092.
- Ha, Y., Fessehaie, A., Ling, K. S., Wechter, W. P., Keinath, A. P., and Walcott, R. R. 2009. Simultaneous detection of *Acidovorax avenae* subsp. *citruilli* and *Didymella bryoniae* in cucurbit seedlots using magnetic capture hybridization and real-time polymerase chain reaction. *Phytopathology* 99:666-678.
- Hawker, J. S., Sedgley, M., and Loveys, B. R. 1983. Composition of stigmatic exudate, nectar and pistil. *Aust. J. Plant Physiol.* 10:257-264.
- He, S. Y., Nomura, K., and Whittam, T. S. 2004. Type III protein secretion mechanism in mammalian and plant pathogens. *Biochim. Biophys. Acta* 1694:181-206.
- Hirano, S. S., Charkowski, A. O., Collmer, A., Willis, D. K., and Upper, C. D. 1999. Role of the Hrp type III protein secretion system in growth of *Pseudomonas syringae* pv. *syringae* B728a on host plants in the field. *Proc. Natl. Acad. Sci. USA* 96:9851-9856.
- Hopkins, D. L., Cucuzza, J. D., and Watterson, J. C. 1996. Wet seed treatments for the control of bacterial fruit blotch of watermelon. *Plant Dis.* 80:529-532.
- Hopkins, D. L., Lovic, B., Hilgren, J., and Thompson, C. M. 2003. Wet seed treatment with peroxyacetic acid for the control of bacterial fruit blotch and other seedborne diseases of watermelon. *Plant Dis.* 87:1495-1499.
- Hopkins, D. L., and Thompson, C. M. 2001. Evaluation of *Citrullus* sp. germplasm for resistance to *Acidovorax avenae* subsp. *citruilli*. *Plant Dis.* 86:61-64.
- Hopkins, D. L., and Thompson, C. M. 2002. Seed transmission of *Acidovorax avenae* subsp. *citruilli* in cucurbits. *HortScience* 37:924-926.
- Huguet, E., Hahn, K., Wengelnik, K., and Bonas, U. 1998. *hpaA* mutants of *Xanthomonas campestris* pv. *vesicatoria* are affected in pathogenicity but retain the ability to induce host-specific hypersensitive reaction. *Mol. Microbiol.* 29:1379-1390.
- Knudsen, I. M. B., Hockenhull, J., Funck Jensen, D., Gerhardson, B., Hökeberg, M., Tahvonen, R., Teperi, E., Sundheim, L., and Henriksen, B. 1997. Selection of biological control agents for controlling soil and seed-borne diseases in the field. *Eur. J. Plant Pathol.* 103:775-784.
- Kobayashi, D. Y., Tamaki, S. J., and Keen, N. T. 1989. Cloned avirulence genes from the tomato pathogen *Pseudomonas syringae* pv. *tomato* confer cultivar specificity on soybean. *Proc. Natl. Acad. Sci. USA* 86:157-161.
- Koster, M., Bitter, W., deCock, H., Allaoui, A., Cornelis, G. R., and Tommassen, J. 1997. The outer membrane component, YscC, of the Yop secretion machinery of *Yersinia enterocolitica* forms a ring-shaped multimeric complex. *Mol. Microbiol.* 26:789-797.
- Lessl, J. T., Fessehaie, A., and Walcott, R. R. 2007. Colonization of female watermelon blossoms by *Acidovorax avenae* ssp. *citruilli* and the relationship between blossom inoculum dosage and seed infestation. *J. Phytopathol.* 155:114-121.
- Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. H. 1986. Gene cluster of *Pseudomonas syringae* pv. *phaseolicola* controls pathogenicity on bean plants and hypersensitive response on nonhost plants. *J. Bacteriol.* 168:512-522.
- Medeiros, F. H. V., Moraes, I. S. F., da Silva Neto, E. B., Silveira, E. B., and de Lima R. Mariano, R. 2009. Management of melon bacterial blotch by plant beneficial bacteria. *Phytoparasitica* 37:453-460.
- Mirik, M., Aysan, Y., and Sabin, F. 2006. Occurrence of bacterial fruit blotch of watermelon caused by *Acidovorax avenae* subsp. *citruilli* in the Eastern Mediterranean region of Turkey. *Plant Dis.* 90:829-829.
- Moss, W. P., Byrne, J. M., Campbell, H. L., Ji, P., Bonas, U., Jones, J. B., and Wilson, M. 2007. Biological control of bacterial spot of tomato using *hrp* mutants of *Xanthomonas campestris* pv. *vesicatoria*. *Biol. Control* 41:199-206.
- Neipold, F., Anderson, D., and Mills, D. 1985. Cloning determinants of pathogenesis from *Pseudomonas syringae* pathovar *syringae*. *Proc. Natl. Acad. Sci. USA* 82:406-410.
- Palkovics, L., Petroczy, M., Kertesz, B., Nemeth, J., Barsony, C., Mike, Z., and Hevesi, M. 2008. First report of bacterial fruit blotch of watermelon caused by *Acidovorax avenae* subsp. *citruilli* in Hungary. *Plant Dis.* 92:834-835.
- Rane, K. K., and Latin, R. X. 1992. Bacterial fruit blotch of watermelon: association of the pathogen with seed. *Plant Dis.* 76:509-512.
- Roberts, D. P., Stromberg, E. L., Lacy, G. H., and Buyer, J. S. 1999. Biological disease control: considerations for seed treatment and stand establishment. *Acta Hort.* 504:69-74.
- Schaad, N. W., Postnikova, E., and Randhawa, P. 2003. Emergence of *Acidovorax avenae* subsp. *citruilli* as a crop-threatening disease of watermelon and melon. Pages 573-581 in: *Presentations 6th Int. Conf. Pseudomonas syringae Pathovars and Related Pathogens*. N.W. Schaad, ed. Kluwer Press, Maratea, Italy.
- Schaad, N. W., Postnikova, E., Sechler, A., Claflin, L. E., Vidaver, A. K., Jones, J. B., Agakova, I., Ignatov, A., Dickstein, E., and Ramundo, B. A. 2008. Reclassification of subspecies of *Acidovorax avenae* as *A. avenae* (Manns 1905) emend., *A. cattleyae* (Pavarino, 1911) comb. nov., *A. citruilli* (Schaad et al., 1978) comb. nov., and proposal of *A. oryzae* sp. nov. *Syst. Appl. Microbiol.* 31:434-446.
- Schulte, R., and Bonas, U. 1992. Expression of the *Xanthomonas campestris* pv. *vesicatoria* *hrp* gene cluster, which determines pathogenicity and hypersensitivity on pepper and tomato, is plant inducible. *J. Bacteriol.* 174:815-823.
- Somodi, G. C., Jones, J. B., Hopkins, D. L., Stall, R. E., Kucharek, T. A., Hodge, N. C., and Watterson, J. C. 1991. Occurrence of a bacterial watermelon fruit blotch in Florida. *Plant Dis.* 75:1053-1056.
- Sowell, G., and Schaad, N. W. 1979. *Pseudomonas pseudoalcaligenes* subsp. *citruilli* on watermelon: seed transmission and resistance of plant introductions. *Plant Dis. Rep.* 63:437-441.
- Tharaud, M., Laurent, J., Faize, M., and Paulin, J. 1997. Fire blight protection with avirulent mutants of *Erwinia amylovora*. *Microbiology* 143:625-632.
- Trigalet, A., Trigalet-Demetry, D., and Feuillade, R. 1998. Aggressiveness of French isolates of *Ralstonia solanacearum* and their potential use in biocontrol. *Bull. OEPP/EPPO Bull.* 28:101-107.
- Walcott, R. R., Castro, A. C., Fessehaie, A., and Ling, K. 2006. Progress towards commercial-scale detection of *A. avenae* subsp. *citruilli* in cucurbit seed using immunomagnetic separation and the polymerase chain reaction. *Seed Sci. Technol.* 34:101-116.
- Walcott, R. R., Gitaitis, R. D., and Castro, A. C. 2003. Role of blossoms in watermelon seed infestation by *Acidovorax avenae* subsp. *citruilli*. *Phytopathology* 93:528-534.
- Wang, X., Li, G., Jiang, D., and Huang, H. 2009. Screening of plant epiphytic yeasts for biocontrol of bacterial fruit blotch (*Acidovorax avenae* subsp. *citruilli*) of hami melon. *Biol. Control* 50:164-171.
- Willems, A., Goor, M., Thielemans, S., Gillis, M., Kersters, K., and De Ley, J. 1992. Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov., comb. nov., *Acidovorax avenae* subsp. *citruilli*, *Acidovorax avenae* subsp. *cattleyae*, and *Acidovorax konjaci*. *Int. J. Syst. Bacteriol.* 42:107-119.
- Wilson, M., Campbell, H. L., Ji, P., Jones, J. B., and Cuppels, D. A. 2002. Biological control of bacterial speck of tomato under field conditions at several locations in North America. *Phytopathology* 92:1284-1292.