Role of Blossoms in Watermelon Seed Infestation by *Acidovorax avenae* subsp. *citrulli*

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ABSTRACT

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The role of watermelon blossom inoculation in seed infestation by *Acidovorax avenae* subsp. *citrulli* was investigated. Approximately 98% (84/87) of fruit developed from blossoms inoculated with 1×10^7 or 1×10^9 CFU of *A. avenae* subsp. *citrulli* per blossom were asymptomatic. Using immunomagnetic separation and the polymerase chain reaction, *A. avenae* subsp. *citrulli* was detected in 44% of the seed lots assayed, despite the lack of fruit symptoms. Furthermore, viable colonies were recovered from 31% of the seed lots. Of these lots, 27% also yielded seedlings expressing bacterial fruit blotch symptoms when planted under conditions of 30°C and 90% relative humidity. *A. avenae* subsp. *citrulli* was detected and recovered from the pulp of 33 and 19%, respectively, of symptomless fruit whose blossoms were inoculated with *A. avenae* subsp. *citrulli*. The ability to penetrate watermelon flowers was not unique to *A. avenae* subsp. *citrulli*, because blossoms inoculated with *Pantoea ananatis* also resulted in infested seed and pulp. The data indicate that watermelon blossoms are a potential site of ingress for fruit and seed infestation by *A. avenae* subsp. *citrulli*.

Additional keywords: *Citrullus lanatus*, *Pseudomonas pseudoalcaligenes* subsp. *citrulli*, stigma.

The phytopathogenic bacterium, *Acidovorax avenae* subsp. *citrulli* (Willems et al.), (formerly *Pseudomonas pseudoalcaligenes* subsp. *citrulli* (Schaad et al.)) (29,42) causes bacterial fruit blotch (BFB), a devastating disease of watermelon (*Citrulus lanatus* (Thunb.) Matsum. & Nakai) and other cucurbits (3,12,13, 18,20,39). Since the first observation in commercial production fields in 1988 (31), BFB has caused millions of dollars in losses in many watermelon-producing regions of the United States and other parts of the world (1,2,5,8,14,24,25,41). Infested seed represent the most important source of inoculum for BFB epidemics (19,26). Once the pathogen has been introduced into transplant houses, high relative humidity, high temperatures, and overhead irrigation contribute to rapid disease development and secondary spread. To mitigate the effects of seedborne *A. avenae* subsp. *citrulli*, a combination of management tactics have been employed, including seed production in cool, dry climates; visual inspection of seed production fields; and seed health testing. Despite these activities, BFB incidence increased in the United States between 1999 and 2000, with seed being the primary inoculum source.

Currently, it is not clear how seed become infested with *A. avenae* subsp. *citrulli*. In preliminary investigations, seed from symptomless watermelons proximal to fruit displaying BFB symptoms in commercial fields in Georgia in 1996 and 1997 were determined to be infested with the bacterium (40). This prompted speculation about the mechanisms by which *A. avenae* subsp. *citrulli* gains access to watermelon seed. According to Maude (21), seed can become infested by three general mechanisms. First, seed become contaminated by incidental contact with infected ovary tissues during pre- and post-extraction stages. Second, seed also may become infested by systemic movement of the pathogen from infected mother plants. However, no evidence exists for the systemic migration of *A. avenae* subsp. *citrulli* in watermelon plants (26). Finally, seed may become infested by invasion of floral structures. Precedents for the latter include bacterial blight of soybean (16) and the well-established role of blossoms in the epidemiology of fire blight of apple and pear (9,27,35,43). The objective of this research was to determine if *A. avenae* subsp. *citrulli* invades watermelon blossoms and eventually infests developing seed.

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MATERIALS AND METHODS

Bacterial strains. *A. avenae* subsp. *citrulli* strains AAC94-21 and AAC94-48 were isolated from infected watermelon fruit in 1994. These strains were characterized by fatty acid analysis, DNA fingerprinting, and pathogenicity tests on watermelon seedlings (38). Green fluorescent protein (GFP)-tagged (34) mutants of *A. avenae* subsp. *citrulli* were generated by transposon mutagenesis (30) using a standard conjugation protocol (4). The donor strain, *Escherichia coli* S17.1 (λ *pir*), carrying the plasmid pAG408 with a mini-Tn5 transposon containing a promotorless *gfp* gene (34), was provided by T. Denny (Department of Plant Pathology, University of Georgia, Athens) and the recipient was AAC94-48. Transconjugants were selected on M9 minimal medium (6) amended with 20% sodium citrate and kanamycin (100 μg/ml) and visually screened for the green fluorescent phenotype under UV light.

Polymerase chain reaction conditions. *A. avenae* subsp. *citrulli* colonies expressing the GFP were purified and confirmed by polymerase chain reaction (PCR) with primers SEQID4^m (SEQID4 with two nucleotides removed from the 5′ end: 5′-GTC-ATTACTGAATTTCAACA-3′) and SEQID5 (5′-CCTCCACCA-ACCAATACGCT-3′) (28). These primers, designed based on the 16S-23S internally transcribed spacer region of the ribosomal DNA sequence of *A. avenae* subsp. *citrulli*, directed the amplification of a 246-bp amplicon and were screened for specificity

with a wide range of bacteria (Table 1). Cell suspensions of each strain were generated by suspending bacterial cells grown on King's medium B (17) for 48 h at 28°C, in sterile high-pressure liquid chromatography (HPLC)-grade water (J. T. Baker, Phillipsburg, NJ). The concentration of each cell suspension was adjusted to ≈0.5×10^8 CFU/ml and 2 ml was lysed by boiling for 15 min to release genomic DNA. Samples of 2 µl of lysed cells were used as template and amplified in 25 µl of PCR master-mix containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each nucleotide (dATP, dCTP, dGTP, and dTTP), 25 pM of each primer, and 1 unit of *Taq* DNA polymerase (Promega Corp., Madison, WI) per reaction. DNA amplification was carried out in a Mastercycler Gradient programmable thermal cycler (Eppendorf, Hamburg, Germany). The PCR conditions included denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing of primers at 53°C for 30 s, and elongation at 72°C for 30 s. The PCR was completed after incubation at 72°C for 5 min and results were analyzed after electrophoresis for 1 h at 90 V in 1% agarose gels stained with ethidium bromide at 0.5 µg/ml.

Characterization of the GFP mutant of *A. avenae* subsp. *citrulli*. The pathogenicity of the GFP mutants of *A. avenae* subsp. *citrulli* was determined by injecting bacterial suspensions containing ≈1×10^8 CFU/ml in 0.1 M phosphate-buffered saline (PBS) into the cotyledons of 2-week-old watermelon seedlings (cv. Jubilee), followed by incubation under greenhouse conditions (12 h of sunlight, 30°C, and 80 to 90% relative humidity [RH]) for 7 days. The in vitro growth rate of the GFP mutant of *A. avenae* subsp. *citrulli* was compared with that of AAC94-48, AAC8-1ST, and AAC9-21. Nutrient broth (100 ml) in sidearm flasks (Wheaton Science Products, Millwille, NJ) were inoculated separately with each strain to generate cell suspensions containing 10^4 CFU/ml. Changes in optical density of the broth were monitored over time with a colorimeter (Spectronic 20; Bausch and Lomb, Rochester, NY) at a wavelength of 600 nm. This was repeated three times for each strain. The ability of the mutant to be seed transmitted was compared with that of AAC94-48 and AAC94-21 by vacuum infiltrating 100 watermelon seed (cv. Crimson Sweet) with suspensions containing each strain at 10^8 CFU/ml. Seed were air dried and planted on two layers of blotter paper (Hoffman Manufacturing Inc., Albany, OR) saturated with sterile water in closed, transparent, plastic boxes (6 cm high by 24 cm wide by 33.5 cm long) (Tri-State Plastics, Dixon, KY). Seed were incubated under conditions of continuous fluorescent light at 30°C and 100% RH for 14 days and the proportion of seedlings exhibiting BFB symptoms was determined. This experiment was repeated three times.

Greenhouse blossom inoculations. To determine the role of blossoms in seed infestation, female watermelon blossoms were pollinated and inoculated with GFP-tagged (AAC8-1ST) and wild-type (AAC94-21) *A. avenae* subsp. *citrulli* under greenhouse conditions. Fifty watermelon plants (cv. Crimson Sweet) were established in fine-grade composted pine bark mixed with vermiculite in a 3:1 ratio in 15-liter plastic pots under greenhouse conditions. Plants were fertilized (Peters Professional 20-10-20 Peat Lite, Special; Scotts-Sierra Horticultural Products Co., Marysville, OH) once a week and allowed to develop to anthesis. As female blossoms opened, pollen was applied evenly to their stigmas by rubbing the anthers of male blossoms onto them. Simultaneously, female blossoms were subjected to one of the following treatments: (i) 10 µl of PBS buffer as a negative control, (ii) ≈1×10^7 CFU of *Pantoea ananatis* (PNA 97-1, originally isolated from onion (*Allium cepa* L), (iii) ≈1×10^7 CFU of AAC94-21 (wild type), (iv) ≈1×10^7 CFU of AAC8-1ST, and (v) ≈1×10^9 CFU of AAC8-1ST. For each treatment, bacterial suspensions containing ≈0.5×10^8 CFU/ml were generated by a colorimeter and adjusted to the desired inoculum level. Using a pipettor (Pipet-Lite; Rainin Instruments LLC, Oakland CA), 10 µl of each treatment was applied to the stigmas of 10 female blossoms, each on a different plant. Inoculations were conducted between 0700 and 1000 h daily for 3 weeks and blossoms were allowed to dry completely before watering, to prevent splash-dispersal of inoculum. Treated blossoms were tagged and plants were maintained until fruit developed to harvest maturity (30 days after pollination). Fruit were harvested and stored at 4°C until seed were extracted (up to 2 weeks). For seed extraction, fruit were surface-sterilized by wiping with 0.5% NaOCl solution and cut with a flame-sterilized knife. Seed from each fruit were extracted manually without rinsing and maintained as a separate lot. After air drying on paper towels, seed were stored in paper bags at 4°C until they were tested.

Seed testing. Seed were tested for *A. avenae* subsp. *citrulli* and *P. ananatis* by (i) immunomagnetic separation followed by PCR (IMS-PCR) (36), (ii) IMS followed by plating captured bacteria on semiselective agar medium, and (iii) the seedling grow-out assay. IMS was employed because compounds present in seed tis-

TABLE 1. Specificity of polymerase chain reaction (PCR) primers SEQID4[m]/SEQID5 developed for the detection of *Acidovorax avenae* subsp. *citrulli*

Bacterium	Strain number	Source	Number of strains yielding 246-bp amplicon[a]
Acidovorax avenae subsp. *citrulli*	…	R. Walcott	42/42
A. avenae subsp. *avenae*	AAA99-2	R. Stall	0/1
A. avenae subsp. *cattelyeae*	AACAT99-2	R. Walcott	0/1
A. konjaci	AK202-1	R. Walcott	0/1
A. facilis	AF94-1	R. Gitaitis	0/1
Pantoea ananatis	PNA97-1	R. Gitaitis	0/1
Pseudomonas syringae pv. *syringae*	PSS99-1	R. Gitaitis	0/1
P. syringae pv. *phaseolicola*	PPH00-1	R. Gitaitis	0/1
P. syringae pv. *lachrymans*	PSLNK344	R. Gitaitis	0/1
P. coronofaciens	PCF83-300	R. Gitaitis	0/1
P. viridiflava	PV200-1	R. Gitaitis	0/1
Comomonas acidovorans	CA201-1	R. Stall	0/1
Burkholderia cepacia	BC83-1	R. Walcott	0/1
Agrobacterium tumefaciens	ATC58	R. Walcott	0/1
A. radiobacter	AR79-1	R. Walcott	0/1
P. aeruginosa	PA84-1	R. Gitaitis	0/1
B. gladioli pv. *alliicola*	BGA00-1	R. Gitaitis	0/1
Pantoea agglomerans	PNG99-3	R. Gitaitis	0/1
Ralstonia solanacearum	AW-1	T. Denny	0/1
Xanthomonas campestris pv. *campestris*	XCC00-1	R. Walcott	0/1
X. campestris pv. *vesicatoria*	XCV00-1	R. Walcott	0/1
Pseudomonas corrugata	PCG84-1	R. Gitaitis	0/1

[a] PCR was conducted on 2 µl of boiled cell suspension containing ≈0.5×10^8 CFU/ml in sterile deionized, distilled water.

sues inhibit conventional PCR resulting in false-negative results. IMS overcomes the inhibitory effects and concentrates target bacteria, resulting in detection thresholds of 10 CFU/ml (36). Additionally, IMS followed by plating was employed to confirm the PCR results by recovering viable CFU from seed tissues, and seedling grow-out was employed to assess the potential of the infested seed to transmit BFB. IMS-PCR was conducted on 15 g of seed (≈500 seed) from each lot as previously described using genus-specific antibodies, primers SEQID4^{m}/SEQID5 for *A. avenae* subsp. *citrulli* and primers PANITS1/EC5 for *P. ananatis* (36,37). Seed lots from blossoms inoculated with PNA 97-1 were tested for *P. ananatis* as well as for *A. avenae* subsp. *citrulli.* Attempts were made to recover viable bacteria from seed lots by IMS followed by plating captured bacteria on nutrient agar (Difco, Beckton Dickinson, Sparks, MD) with kanamycin (NAK) and without kanamycin (100 µg/ml). In the case of seed lots from blossoms inoculated with AAC94-21, a proprietary semiselective medium (Nunhems Seed Company, Haelen, Holland) was used. Additionally, seedling grow-out assays were conducted using 50 to 100 seed from each seed lot, depending on availability. Seed samples were incubated in transparent plastic boxes on two layers of blotter paper saturated with sterile water for 14 days under conditions of >90% RH at 30°C under continuous fluorescent light. The germination percentage (number of seedling with established root systems and cotyledons) and BFB transmission (number of seedlings with persistent water-soaked lesions on cotyledons divided by the number of germinated seedlings × 100) were determined for each seed lot. Seedlings were allowed to air dry for 1 h before they were assessed for BFB. Seedlings with typical water-soaked lesions were considered to be infected and bacterial isolation was attempted on a subsample of infected seedlings. Bacterial isolations also were made from seedlings with atypical BFB symptoms.

Watermelon pulp testing. Fruit that developed from blossoms inoculated with *A. avenae* subsp. *citrulli* and PNA 97-1 were tested for *A. avenae* subsp. *citrulli* and *P. ananatis*. Watermelon pulp (100 to 200 g) was crushed for 15 min in a stomacher blender (Seward Ltd., London, UK) and the watermelon extract was filtered through two layers of cheesecloth and one layer of Whatman No. 1 filter paper (Whatman, Springfield Mill, UK). Bacterial cells were concentrated by centrifugation at 3,200 × *g* for 15 min, resuspended in 6 ml of PBS with 0.2% bovine serum albumin, and assayed by IMS-PCR and IMS followed by plating on selective media as described previously.

Statistical analysis. This study was repeated three times and analysis of variance was conducted to determine the significance of the blossom treatments on seed lot infestation with *A. avenae* subsp. *citrulli*, seed germination, and seedling transmission of BFB. Additionally, correlation analysis was conducted to determine the relationship between pulp and seed infestation.

RESULTS

PCR primers. SEQID4^{m}/SEQID5 were found to be highly specific for *A. avenae* subsp. *citrulli* and, thus, could be used for detecting the bacterium in watermelon seed (Table 1). The expected 246-bp amplicon was produced with 100% of the *A. avenae* subsp. *citrulli* strains tested but not with the other bacterial strains.

Characterization of GFP mutants of *A. avenae* subsp. *citrulli*. When the eight GFP-expressing mutants of *A. avenae* subsp. *citrulli* AAC94-48 that were generated using plasmid pAG408 were subjected to PCR with SEQID4^{m}/SEQID5, they all produced a 246-bp fragment. One of the mutants, AAC8-1ST, caused lesions on watermelon seedlings and, when inoculated onto watermelon seed by vacuum infiltration, led to BFB seedling transmission. AAC8-1ST was re-isolated from infected watermelon tissue and stored at –80°C in 15% glycerol. The in vitro growth for AAC8-1ST was slower than that of the wild-type parent, AAC94-48, and AAC94-21 (Fig. 1). However, the BFB progress curves for seed inoculated with AAC8-1ST were comparable to those of AAC94-21 and AAC94-48 (Fig. 2).

Watermelon production. In three trials, 143 watermelons were produced. Of these, 30, 29, 29, 27, and 28 fruit were produced from blossoms treated with 0.1 M PBS, 1×10^7 CFU of AAC94-21, 1×10^7 CFU of AAC8-1ST, 1×10^9 CFU of AAC8-1ST, and 1×10^7 CFU of *P. ananatis*, respectively. Two of the fruit (one from a blossom inoculated with 1×10^7 CFU of AAC8-1ST and one from a blossom inoculated with 1×10^9 CFU of AAC8-1ST) displayed atypical BFB symptoms, including small restricted rind lesions and cracks. The infection status of these fruit was confirmed by bacterial isolation followed by visual observation of the GFP marker and PCR using primers SEQID4^{m}/SEQID5. Data collected from the symptomatic fruit were not included in this study. Analysis of variance indicated that there were no statistically significant differences between experiments; therefore, data from the three experiments were pooled and analyzed.

Effects of blossom inoculation on seed lot infestation. Seed lots recovered from symptomless fruit developed from blossoms inoculated with *A. avenae* subsp. *citrulli* and *P. ananatis* were in-

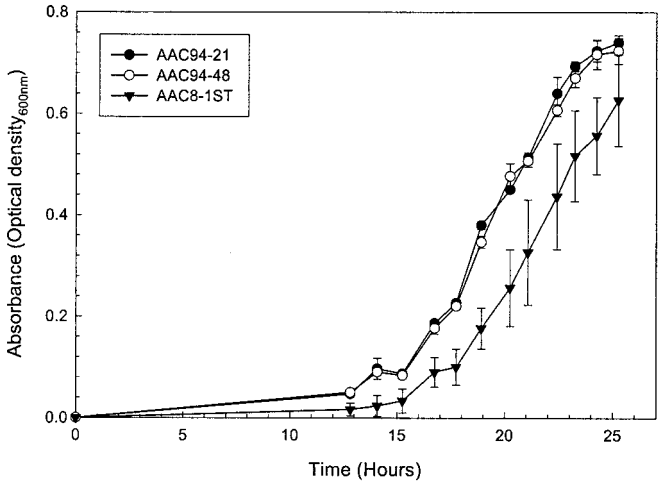

Fig. 1. Growth in nutrient broth of *Acidovorax avenae* subsp. *citrulli,* AAC94-21, AAC94-48, and the *gfp*-tagged AAC94-48 mutant, AAC8-1ST. Each data point is the mean and standard error for the three experiments.

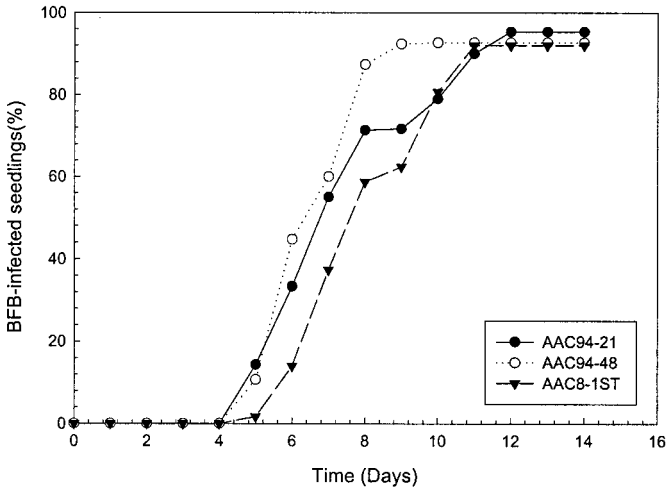

Fig. 2. Bacterial fruit blotch (BFB) disease progression on seedlings arising form seed infested with *Acidovorax avenae* subsp. *citrulli*, AAC94-21, AAC94-48, and the *gfp*-tagged AAC94-48 mutant, AAC8-1ST. The experiment was repeated three times. For each experiment, 100 seedlings per strain were examined for disease symptoms. Each data point represents the mean of the three experiments.

fested with the respective bacteria. Of the blossoms inoculated with *A. avenae* subsp. *citrulli*, 43% (37/85) yielded fruit with infested seed lots as determined by IMS-PCR (Fig. 3). *A. avenae* subsp. *citrulli* colonies were recovered from 70% (23/37) of the IMS-PCR-positive seed lots, and 62% of them gave rise to seedlings with BFB symptoms. Of the seed lots from blossoms inoculated with AAC94-21, 31% (9/29) were positive for *A. avenae* subsp. *citrulli* by IMS-PCR (Fig. 2). Although the bacterium was recovered from 17% of the seed lots, 7% gave rise to symptomatic seedlings. For blossoms inoculated with 1×10^7 and 1×10^9 CFU of AAC8-1ST, 59 and 44% of the seed lots were IMS-PCR-positive, respectively (Fig. 3). Green fluorescent colonies were recovered from seed lots from blossoms inoculated with both levels of AAC8-1ST (35 and 41% for 1×10^7 and 1×10^9 CFU per blossom, respectively). In the seedling grow-out assay, 38 and 41% of the seed lots from blossoms inoculated with 1×10^7 and 1×10^9 CFU, respectively, of AAC8-1ST yielded at least one seedling exhibiting BFB symptoms (Fig. 3). The differences among the levels of infested seed lots from blossoms inoculated with *A. avenae* subsp. *citrulli* were not statistically significant regardless of the detection technique employed. In contrast, 97% (29/30) of the seed lots from blossoms inoculated with PBS were negative for *A. avenae* subsp. *citrulli* by IMS-PCR. *A. avenae* subsp. *citrulli* was not recovered from these seed lots and none of them yielded seedlings infected with BFB. Similar results were obtained when seed from blossoms inoculated with *P. ananatis* were tested for *A. avenae subsp. citrulli*. In this case, none of the seed lots were positive by IMS-PCR, IMS-plating, or seedling grow-out. However, 43% of these lots were positive for *P. ananatis* by IMS-PCR and colonies were recovered from 43% of the seed lots.

Presence of *A. avenae* subsp. *citrulli* in pulp of symptomless fruit. Despite the lack of external BFB symptoms, internal tissues (pulp) of fruit produced from inoculated blossoms were infected with *A. avenae* subsp. *citrulli* and *P. ananatis*. For blossoms inoculated with 1×10^7 CFU of AAC94-21, IMS-PCR detected the bacterium in 35% (10/29) of the fruit (Fig. 4). The bacterium was recovered by IMS followed by plating on selective agar media from 7% (2/29) of these fruit (Fig. 3). IMS-PCR detected *A. avenae* subsp. *citrulli* in 24% (7/29) and 41% (11/27) of the fruits produced from blossoms inoculated with 1×10^7 and 1×10^9 CFU, respectively, of AAC8-1ST. In contrast, green fluorescent *A. avenae* subsp. *citrulli* colonies were recovered from 21% (6/29) and 33% (9/27) of the symptomless fruit inoculated with 1×10^7 and 1×10^9 CFU, respectively, of AAC8-1ST. None of the fruit from blossoms inoculated with 1×10^7 CFU of PNA97-1 were positive for *A. avenae* subsp. *citrulli*. However, 39% (7/18) of these fruit were positive for *P. ananatis* by IMS-PCR, and viable CFU were recovered from 28% (5/18) of them. As expected, *A. avenae* subsp. *citrulli* was not detected or recovered from pulp of fruit developed from blossoms inoculated with PBS. The differences between the percentage of infested fruit generated by different blossom treatments were significant as determined by IMS-PCR ($P = 0.15$).

Effect of blossom inoculation on germination and BFB transmission. The effect of blossom inoculation on germination percentage was not statistically significant ($P = 0.67$). Blossoms inoculated with PBS yielded seed lots with an average germination percentage of 87.47 (standard deviation = 10.95), while blossoms inoculated with 1×10^7 CFU of AAC94-21 and AAC8-1ST displayed mean germination of 88 and 87%, respectively (Fig. 5). Seed lots from blossoms inoculated with AAC8-1ST at $\approx 1 \times 10^9$ CFU per blossom had a lower mean germination percentage (86%). Blossom inoculation with *P. ananatis* also had no impact on seedling germination (89%). Of 85 blossoms inoculated with *A. avenae* subsp. *citrulli*, 27% (23/85) produced seed lots that resulted in at least one seedling exhibiting BFB symptoms (Fig. 5). BFB symptoms appeared on seedlings 10 to 14 days after planting as water-soaked lesions on the cotyledons. In some cases, a dry white exudate was observed on the surfaces of the cotyledons and severely infected seedlings collapsed. For blossoms inoculated with AAC8-1ST, dried exudate appeared fluorescent green with UV illumination. Blossoms inoculated with 1×10^7 CFU of AAC94-21 resulted in seed lots with a mean BFB transmission of 4%. This was significantly different from BFB transmission observed for seed lots from blossoms inoculated with PBS (0%) and 1×10^7 CFU of *P. ananatis* (0%) (Fig. 4). BFB transmission was higher for seed lots from blossoms inoculated

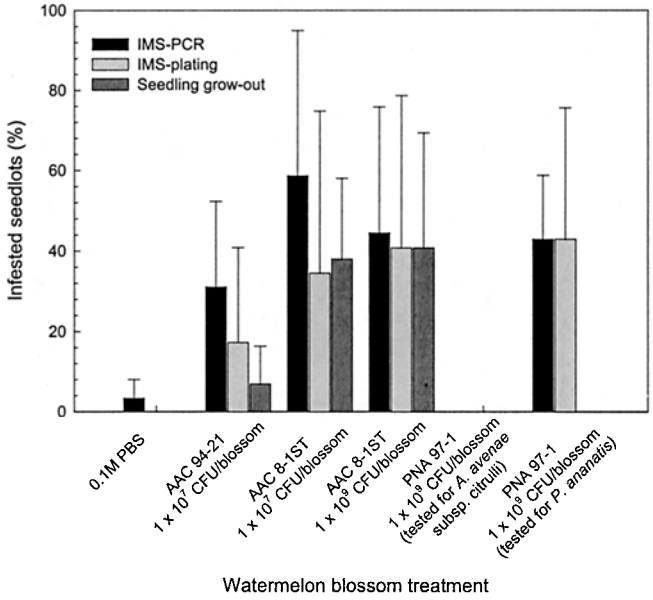

Fig. 3. Incidence of infested watermelon seed lots that developed in fruit from blossoms inoculated with 0.1 M phosphate-buffered saline (PBS), 1×10^7 CFU of AAC94-21, 1×10^7 CFU of AAC8-1ST, 1×10^9 CFU of AAC8-1ST, and 1×10^7 CFU of *Pantoea ananatis* (PNA 97-1). Each bar represents the mean incidence of infested seed lots for three replications of the experiment and the lines indicate the standard deviation of the means.

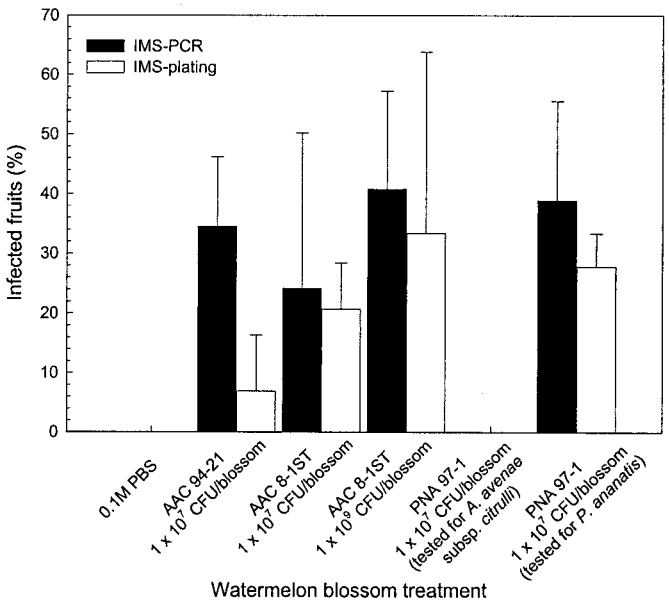

Fig. 4. Incidence of fruit with infested internal pulp that developed from blossoms inoculated with 0.1 M phosphate-buffered saline (PBS), 1×10^7 CFU of AAC94-21, 1×10^7 CFU of AAC8-1ST, 1×10^9 CFU of AAC8-1ST, and 1×10^7 CFU of *Pantoea ananatis* (PNA 97-1). Each bar represents the mean incidence of infested fruit for three replications of the experiment and the lines indicate the standard deviation of the means.

with ≈1 × 10^7 CFU of AAC8-1ST (7%) and even more so for seed lots from blossoms inoculated with 1 × 10^9 CFU (9%). However, the differences between the levels of BFB transmission were not significantly significant. Blossom inoculation resulted in highly variable BFB transmission as indicated by standard deviation values of 9.43, 20.07, and 28.6 for seed lots from blossoms inoculated with ≈1 × 10^7 CFU of AAC94-21, ≈1 × 10^7 CFU of AAC8-1ST, and ≈1 × 10^9 CFU of AAC8-1ST, respectively (Fig. 5). BFB transmission ranged from 0 to 69% for lots from *A. avenae* subsp. *citrulli*-inoculated blossoms.

A. avenae subsp. *citrulli* colonies were recovered from 100% of the symptomatic seedlings using NAK or semiselective media for the wild-type strain. In only two instances was *A. avenae* subsp. *citrulli* recovered from asymptomatic seedlings.

Relationships between pulp and seed lot infestation. In general, as the number of fruit with infested pulp increased, so too did the number of infested seed lots. The correlation coefficient indicating the strength of the relationship varied based on the technique that was employed (Table 2). A correlation coefficient of 0.71 was observed when pulp infestation determined by IMS followed by plating was compared with seed lot infestation determined by IMS-PCR. A greater correlation coefficient (R = 0.80) was observed when pulp and seed infestation was determined by IMS followed by plating on agar medium.

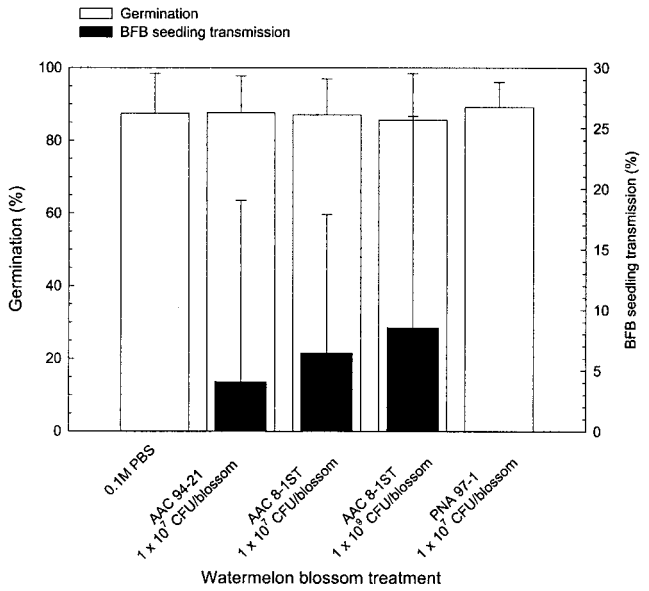

Fig. 5. Mean percent germination of seed and incidence of bacterial fruit blotch (BFB) for seed lots recovered from watermelons produced after inoculation of blossoms with 0.1 M phosphate buffered saline (PBS), 1 × 10^7 CFU of AAC94-21, 1 × 10^7 CFU of AAC8-1ST, 1 × 10^9 CFU of AAC8-1ST, and 1 × 10^7 CFU of *Pantoea ananatis* (PNA 97-1). Each bar represents the mean germination percentage per treatment for three repetitions of the experiment and lines represent the standard deviation of the means.

DISCUSSION

Kauffman and Leben (16) established a precedent for seed infestation through blossoms when they demonstrated that soybean flowers inoculated with *P. syringae* subsp. *glycinea* gave rise to infested seed within symptomless pods. Likewise, in our study, watermelon blossom pollination and simultaneous inoculation with *A. avenae* subsp. *citrulli* did not lead to BFB symptoms but yielded infested seed. Of 85 blossoms inoculated with *A. avenae* subsp. *citrulli*, only two fruit displayed atypical BFB symptoms, including small restricted rind lesions. It is possible that the RH in the greenhouse prevented the development of typical watersoaking symptoms associated with BFB. Despite the lack of symptoms, fruit from blossoms inoculated with *A. avenae* subsp. *citrulli* were infected with the bacterium. The pulp from 33% of the symptomless fruit produced from blossoms inoculated with *A. avenae* subsp. *citrulli* was positive for the bacterium. For confirmation, colonies were recovered from the pulp of 57% of these IMS-PCR-positive fruit. The detection and recovery of *A. avenae* subsp. *citrulli* from symptomless watermelons was unexpected because severe fruit rot symptoms usually are associated with BFB (19,31). It is tempting to speculate that, although *A. avenae* subsp. *citrulli* causes watersoaking and rind lesions on fruit, the bacterium may not be responsible for the associated fruit rot. The rot may be induced through secondary invasion by opportunistic saprophytes. It is also possible that conditions in the immature ovary tissues (e.g., extremes in pH and the lack of usable nutrients at the time of inoculation) may inhibit the growth of *A. avenae* subsp. *citrulli* and, thus, prevent fruit rot. Such variations in the type and concentration of sugars in watermelons have been documented (7).

Blossom inoculation resulted in the production of *A. avenae* subsp. *citrulli*-infested seed within symptomless fruit. When assayed by seedling grow-out, 27% of the seed lots deemed positive by IMS-PCR resulted in BFB transmission. IMS-PCR appeared to be the most sensitive assay employed; however, it detected *A. avenae* subsp. *citrulli* in seed from one fruit whose blossom was inoculated with PBS. The bacterium was not recovered, and the pulp and seedling grow-out assays were negative; therefore, it is suspected that this particular PCR result was a false-positive, possibly due to cross-contamination.

One interesting observation in this study was that blossom inoculation with AAC8-1ST resulted in a higher level of fruit and seed infestation than the wild-type strain. This was an unexpected result, especially because the in vitro growth rate of the mutant was slower than the wild-type strain. While this could be explained in part by the kanamycin resistance of the mutant which made recovery more efficient, it still remains unclear why blossom inoculation with the GFP mutant led to higher levels of infestation and transmission.

For seed lots infested with *A. avenae* subsp. *citrulli* as a result of blossom inoculation, mean BFB transmission ranged from 5 to 9%; however, there was a considerable degree of variability. This variability was not unexpected because disease transmission requires a minimum population of bacteria per seed. It is likely that

TABLE 2. Correlation coefficients for the detection of *Acidovorax avenae* subsp. *citrulli* in watermelon seeds and pulp from inoculated blossoms[a]

	Correlation coefficient (R)				
Detection technique	IMS-PCR pulp	IMS-plating pulp	IMS-PCR seed	IMS-plating seed	Seedling grow-out assay[b]
IMS-PCR pulp	1	0.48	0.50	0.20	0.48
IMS-plating pulp	…	1	0.71	0.80	0.77
IMS-PCR seed	…	…	1	0.85	0.55
IMS-plating seeds	…	…	…	1	0.54
Seedling grow-out assay	…	…	…	…	1

[a] The detection assays employed included immunomagnetic separation and the polymerase chain reaction (IMS-PCR) conducted on watermelon pulp, IMS and plating on agar medium conducted on watermelon pulp, IMS-PCR conducted on seed, IMS-plating conducted on seed, and seedling grow-out.
[b] Seedling grow-out was conducted as described in the text.

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