Embryo Localization Enhances the Survival of *Acidovorax citrulli* in Watermelon Seeds

Bhabesh Dutta, Raymond W. Schneider, Clark L. Robertson, and Ronald R. Walcott

First author: Department of Plant Pathology, Coastal Plain Experiment Station, University of Georgia, Tifton 31793; second and third authors: Department of Plant Pathology and Crop Physiology, Louisiana State University Agricultural Center, Baton Rouge 70803; and fourth author: Department of Plant Pathology, University of Georgia, Athens 30602.
Accepted for publication 6 January 2016.

ABSTRACT

Dutta, B., Schneider, R. W., Robertson, C. L., and Walcott, R. R. 2016. Embryo localization enhances the survival of *Acidovorax citrulli* in watermelon seeds. *Phytopathology* 106:330-338.

Acidovorax citrulli, the causal agent of bacterial fruit blotch (BFB) of cucurbits has been observed to survive for >34 years in stored melon and watermelon seeds. To better understand this remarkable longevity, we investigated the bacterium's tolerance to desiccation and the effect of bacterial localization in different watermelon seed tissues on its survival. We compared the ability of *A. citrulli* to tolerate desiccation on filter paper discs and on host (watermelon) and nonhost (cabbage, corn and tomato) seeds to two seedborne (*Xanthomonas campestris* pv. *campestris* and *Pantoea stewartii* subsp. *stewartii*) and one soilborne (*Ralstonia solanacearum*) plant-pathogenic bacteria. *A. citrulli* survival on dry filter paper (>12 weeks) was similar to that of *X. campestris* pv. *campestris* but longer than *P. stewartii* subsp. *stewartii*. *Ralstonia solanacearum* survived longer than all other bacteria tested. On all seeds tested, *A. citrulli* and *X. campestris* pv. *campestris* populations declined by 5 orders of magnitude after 12 weeks of incubation at 4°C and 50% relative humidity, while *R. solanacearum* populations declined by 3 orders.

P. stewartii subsp. *stewartii* was not recovered after 12 weeks of incubation. To determine the effect of tissue localization on bacterial survival, watermelon seeds infested with *A. citrulli* by flower stigma inoculation (resulting in bacterial localization in the embryo/endosperm) or by ovary pericarp inoculations (resulting in bacterial localization under the testa) were treated with peroxyacetic acid or chlorine (Cl₂) gas. Following these treatments, a significantly higher reduction in BFB seed-to-seedling transmission was observed for seeds generated by ovary pericarp inoculation (≥89.5%) than for those generated by stigma inoculation (≤76.5%) ($P < 0.05$). Additionally, higher populations of *A. citrulli* survived when the bacteria were localized to the embryo/endosperm versus the seed coat, suggesting that tissue localization is important for bacterial survival in seed. This observation was confirmed when *P. stewartii* subsp. *stewartii* survived significantly longer in stigma-inoculated (embryo/endosperm-localized) watermelon seeds than in vacuum-infiltrated (testa-localized) seeds. Based on these results we conclude that *A. citrulli* cells are not intrinsically tolerant to desiccation and that localization of the bacterium to testa tissues does not enhance *A. citrulli* survival. In contrast, it is likely that embryo/endosperm localization enhances the survival of *A. citrulli* and other bacteria in seeds.

Since the first outbreak in Florida in 1989, bacterial fruit blotch (BFB), caused by *Acidovorax citrulli* (syn. *Acidovorax avenae* subsp. *citrulli* formerly *Pseudomonas pseudoalcaligenes* subsp. *citrulli*) (Schaad et al. 1978, 2008; Willems et al. 1992) has become a sporadic but devastating threat to watermelon (*Citrullus lanatus* [Thunb.] Matsum. & Nakai) and other cucurbit crops worldwide (Burdman et al. 2005; Latin and Rane 1990; Latin and Hopkins 1995; Schaad et al. 2003; Somodi et al. 1991). *A. citrulli* is seedborne and seed-to-seedling transmitted in many cucurbitaceous crops (Hopkins and Thompson 2002; Rane and Latin 1992). Infested seeds are the most important source of primary inoculum for BFB epidemics (Hopkins and Thompson 2002; Rane and Latin 1992); however, little is known about the factors that influence *A. citrulli* survival in seeds. Block and Shepherd (2008) reported that *A. citrulli* survived for more than 34 years on watermelon and melon (*Cucumis melo* L. var. *cantalupensis* Naudin) seeds stored at 5°C at the USDA North Central Plant Introduction Station, Ames, IA. BFB seed-to-seedling transmission was reported for 2 and 8% of these melon and watermelon seedlots, respectively (Block and Shepherd 2008), and the authors suggested that the pathogen might survive as long as the seeds remained viable.

In general, the longevity of a seedborne pathogen is influenced by a range of factors including seed storage conditions; intrinsic

characteristics of the pathogen, e.g., tolerance to desiccation; and the location of bacteria in seeds (Schuster and Coyne 1974). Latin et al. (1995) reported that *A. citrulli* survived for only 63 days on the surface of untreated Speedling polystyrene trays (TSC Polyform, Minneapolis, MN) containing potting soil and seedling root debris (Latin et al. 1995). This observation suggests that the bacterium's longevity in stored cucurbit seed might not be due to its intrinsic tolerance to desiccation. One alternative explanation for *A. citrulli*'s extraordinary longevity is its location in cucurbit seeds.

Seed treatments with hydrochloric acid, sodium hypochlorite and peroxyacetic acid are commonly used for BFB management (Hopkins et al. 2003; Walcott 2008); however, they usually do not eradicate the pathogen (Hopkins et al. 2003; Rane and Latin 1992), suggesting that the pathogen can be localized in protected seed tissues under the testa. Dutta et al. (2012) reported that *A. citrulli* localization in watermelon seeds was influenced by the pathway of bacterial invasion (Dutta et al. 2012). More specifically, ingress through the ovary pericarp resulted in the localization of *A. citrulli* cells just under the testa while ingress through the pistil via stigma inoculation resulted in bacterial localization in the embryo/endosperm (Dutta et al. 2012). Despite these observations, to date there have been no efforts to determine how tissue localization affects *A. citrulli* survival in seeds. Hence, the goal of this study was to investigate the factors that influence *A. citrulli* survival in watermelon seeds. Specific objectives were to (i) compare *A. citrulli* tolerance to desiccation to other phytopathogenic bacteria; (ii) determine if seed species affects *A. citrulli* survival;

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

<http://dx.doi.org/10.1094/PHYTO-09-15-0232-R>
© 2016 The American Phytopathological Society

and (iii) determine the effect of tissue localization on bacterial survival in watermelon seed.

MATERIALS AND METHODS

Bacterial strains. The strains of *A. citrulli*, *Xanthomonas campestris* pv. *campestris*, *Pantoea stewartii* subsp. *stewartii*, and *Ralstonia solanacearum* used in this study were AAC00-1 (Walcott and Gitaitis 2000), XCC00-1 (from culture collection of R. Walcott, University of Georgia), DS-203 (provided by D. Coplin, Ohio State University), and AW-1 (Brumbley and Denny 1990), respectively. XCC00-1 (resistant to streptomycin) and DS-203 (resistant to nalidixic acid) were isolated from cabbage and corn, respectively, while AAC00-1 (*A. citrulli*) and AW-1 (*R. solanacearum*) were isolated from watermelon and tomato, respectively. *A. citrulli* AAC00-1 was cultured on King's medium B (King et al. 1956), *X. campestris* pv. *campestris* XCC00-1 was grown on yeast dextrose calcium carbonate agar (YDC) (Schaad et al. 2001) amended with 100 µg/ml of streptomycin, *P. stewartii* subsp. *stewartii* DS-203 was grown on Luria Bertani (LB) (Schaad et al. 2001) agar amended with nalidixic acid at 50 µg/ml, and *R. solanacearum* AW-1 was grown on LB agar. Inoculum for each bacterium was prepared by transferring a single colony from a 24 to 48 h agar plate culture into nutrient broth followed by overnight incubation at 28°C on a rotary shaker (Innova; New Brunswick Scientific Co., Edison, NJ) at 250 rpm. Subsequently, cultures were centrifuged at 6,000 × *g* (Allegra 25R centrifuge, Beckman Coulter, Fullerton, CA) for 5 min and the supernatant was decanted. The resulting pellets were resuspended in 0.01 M phosphate buffered saline (PBS) and the bacterial concentrations were adjusted to optical densities of 0.3, 0.5, 0.3, and 0.1 for *A. citrulli*, *P. stewartii* subsp. *stewartii*, *X. campestris* pv. *campestris*, and *R. solanacearum*, respectively, at 600 nm (~1 × 10⁸ CFU/ml) (Spectronic 20; Bausch and Lomb, Rochester, NY).

***A. citrulli* tolerance to desiccation.** Since tolerance to desiccation is an important factor for bacterial survival, we compared the desiccation tolerance of *A. citrulli* to other phytopathogenic bacteria. Bacterial suspensions containing ~1 × 10⁸ CFU/ml of *A. citrulli* AAC00-1, *X. campestris* pv. *campestris* XCC00-1, *P. stewartii* subsp. *stewartii* DS-203 and *R. solanacearum* AW-1 were generated as described above and pieces (~40 cm²) of sterile filter paper (Whatman Inc., Sanford, ME) were incubated in each suspension for 10 min followed by air drying at ~25°C for 12 h. After drying, each piece of filter paper was incubated in a partially open Petri dish in a desiccator cabinet (Secador 4.0, Scienceware, Pequannock, NJ) at 50% relative humidity (RH). The desired RH was achieved by placing a 1:1 mixture of terpentine oil (Pine Chemicals Pvt. Ltd., Singapore) and water in the desiccator and incubating at 4°C. Pieces of filter paper saturated with PBS served as negative controls. After 0 (filter paper processed immediately after inoculation), 1, 2, 3, 4, 8, and 12 weeks postinoculation (WPI), samples of filter paper (~4 mm²) were removed and macerated using a sterile glass rod in a sterile 1.5-ml microcentrifuge tube containing 1 ml of PBS. Macerates were vortexed for 30 s (Vortex Genie2; Fisher Scientific International, Pittsburgh, PA) and bacterial populations were estimated by 10-fold serial dilution in PBS followed by spread plating 100 µl of aliquots onto respective semiselective agar media as described above. For *A. citrulli*, the proprietary Nunhem's agar (Nunhems Seed Company, Haelen, Holland) was used as a semiselective medium (Walcott et al. 2003). Bacterial colonies were counted after 24 to 72 h of incubation. In addition, confirmation of the identity of the bacterial colonies was conducted by PCR assay using *A. citrulli*-, *P. stewartii* subsp. *stewartii*-, *X. campestris* pv. *campestris*-, and *R. solanacearum*-specific primers, as previously described (Chen et al. 2010; Fessehaie et al. 2006; Ha et al. 2009; Park et al. 2004). This experiment was conducted two times and each treatment was replicated three times per experiment. Bacterial populations (CFU/mm²) were

log₁₀-transformed prior to analysis. Data were pooled to determine mean bacterial populations after preliminary statistical analysis showed no significant (*P* > 0.05) effect of replication and experiment. Repeated measures analysis of variance was conducted on log₁₀-transformed bacterial population data, where storage time and bacterial species served as repeated measures. The statistical analysis was conducted using the General Linear Model (PROC GLM) in SAS (version 9.2 for Windows; SAS Institute Inc., Cary, NC) to determine the effect of storage time on mean bacterial population and the differences in the abilities of bacterial species to survive on filter paper at *P* < 0.05.

Survival of phytopathogenic bacteria on host and nonhost seed species. It is possible that association with seeds of host and nonhost plants influence bacterial longevity in storage. Hence, we assessed if *A. citrulli* association with host or nonhost seeds affected its survival and how this survival compared with that of other phytopathogenic bacteria. To achieve this, *A. citrulli*, *P. stewartii* subsp. *stewartii*, *X. campestris* pv. *campestris*, and *R. solanacearum* strains were inoculated separately onto their respective host and nonhost seeds by vacuum infiltration as described below. Each bacterial strain was inoculated separately onto watermelon ('Crimson Sweet'), corn (*Zea mays* L. 'Tom Tom'), cabbage (*Brassica oleracea* L. 'Saratoga'), and tomato (*Lycopersicon esculentum* L. 'Bonnie Best') seeds, which represented the natural hosts for *A. citrulli*, *P. stewartii* subsp. *stewartii*, *X. campestris* pv. *campestris*, and *R. solanacearum*, respectively. Seeds (*n* = 25 g) were placed in a sterile 250 ml side-arm flask containing 20 ml of each bacterial cell suspension (~1 × 10⁸ CFU/ml), and the flask was attached to a vacuum line. Cell suspensions were infiltrated into the seeds by applying vacuum to the flask for 1 h. Seeds were subsequently removed from the cell suspensions and air-dried at ~25°C for 24 h. Seeds were then stored in open Petri plates at 4°C and 50% RH. At 0, 1, 2, 3, 4, 8, and 12 WPI, samples (*n* = 1 g of seed) were crushed using a tissue homogenizer (Homex 6, Bioreba, Reinach, Switzerland). Seed macerates were suspended in 10 ml PBS and transferred to 50 ml polypropylene screw cap tubes (Corning Inc., Corning, NY). Macerates were agitated at 100 rpm (Fisher Vortex Genie2; Fisher Scientific International) for 30 s and centrifuged at 8,000 × *g* (Allegra 25R) for 5 min. Subsequently, the supernatant was decanted and the pellet was suspended in 1 ml PBS. Ten-fold serial dilutions of the seed macerates were made in PBS and 100 µl aliquots were spread onto respective semiselective agar media. After 24 to 72 h of incubation at 28°C, bacterial colonies were counted. For each treatment, three seed samples were tested per time point and the experiment was conducted two times. Bacterial populations (CFU/g) were log₁₀-transformed prior to analysis. Student's *t* test was used to compare treatments at *P* < 0.05. Data from the replicates of the two experiments were pooled to determine mean bacterial populations after preliminary analysis showed no significant (*P* > 0.05) effect of replication and experiment. Repeated measures analysis of variance was used to determine the effect of host and nonhost seeds on bacterial survival at *P* < 0.05, where storage time, seed type and bacterial species served as repeated measures.

Effect of watermelon seed tissue localization on *A. citrulli* survival. To determine how localization in seed tissues affects *A. citrulli* longevity watermelon seedlots were inoculated with *A. citrulli* strain AAC00-1 by flower stigma and ovary pericarp inoculation, as described previously (Dutta et al. 2012; Lessl et al. 2007; Walcott et al. 2003). Four *A. citrulli*-infested seedlots (two each by stigma and ovary pericarp inoculation) were generated (Table 1). Pericarp-inoculated seedlots (1 and 2) were generated in 2009 and 2010, respectively, as follows. Twenty-five watermelon plants (Crimson Sweet) were grown in pots under greenhouse conditions at approximately 24°C and 65% RH with 12 to 14 h of natural sunlight daily. At anthesis, 30 to 40 attached female flowers were hand-pollinated and the pericarps of the ovaries were uniformly inoculated by gently rubbing them with cotton-tipped applicators

saturated with *A. citrulli* AAC00-1 cell suspension (1×10^6 *A. citrulli* CFU/ml). Inoculated ovaries were covered with plastic bags and incubated for 24 h. After incubation, bags were removed and ovaries were allowed to develop for 30 days. Fruits ($n = 20$) were then harvested and stored at 4°C until seed extraction. Seeds were manually extracted from each fruit, air-dried overnight at 25°C on paper towels, and pooled before storing in paper bags at 4°C.

Seedlots with infested embryo/endosperm tissues were generated by pollinating female watermelon flowers at anthesis and inoculating their stigmas with AAC00-1 (Dutta et al. 2012; Walcott et al. 2003). Stigma-inoculated seedlots (3 and 4) were generated under greenhouse conditions in 2009 and 2010, respectively. Briefly, 25 watermelon plants (Crimson Sweet) were established in pots under greenhouse conditions as described above and at anthesis female blossoms were pollinated manually followed immediately by the inoculation of the stigmas with 10 µl of a bacterial suspension containing $\sim 1 \times 10^8$ AAC00-1 CFU/ml ($\sim 1 \times 10^6$ CFU/flower). Inoculum was applied to the stigmas with a micropipette. At least five flowers were inoculated and tagged per plant, which were later thinned to one fruit per pot. Fruits were allowed to develop for 30 days after pollination, at which time, they were harvested and seeds from individual fruit were extracted and air-dried. Seeds from each fruit were combined and stored at 4°C until they were used. Seeds harvested from fruits ($n = 20$ fruits/inoculation type) that developed from flowers treated with PBS by each inoculation method served as negative controls.

Seedlot characteristics. To determine the percentage of *A. citrulli*-infested seeds in each lot, four samples ($n = 100$ seeds/lot) were planted in transparent plastic boxes (6 cm [H] $\times$ 24 cm [W] $\times$ 33.5 cm [L]; Tri-State Plastics, Dixon, KY) on blotter paper (Anchor Paper Co., St. Paul, MN) saturated with sterile water and maintained at 28°C and 85% RH for 14 days. Seeds from fruits that developed after stigma- and pericarp-inoculation with PBS served as negative controls. Seed germination and BFB seed-to-seedling transmission percentages (number of seedlings with symptoms divided by the total number of seeds germinated $\times$ 100) were determined 14 days after planting (DAP). Additionally, mean *A. citrulli* CFU/seed were determined for samples ($n = 30$ seeds) from each lot by 10-fold serial dilution of individually macerated seeds followed by spread-plating onto Nunhem's semiselective agar. Bacterial colonies were counted after incubating agar plates for 72 h at 28°C and mean CFU/seed was estimated for each lot.

Confirmation of the location of *A. citrulli* in seed tissues after stigma and pericarp inoculation. The location of *A. citrulli* cells in pericarp- and stigma-inoculated watermelon seed samples was confirmed as previously described (Dutta et al. 2012). Fifty seeds from each lot were individually dissected into sections including testa, perisperm-endosperm (PE) layer, and embryo. Testa, PE layer, and embryo sections dissected from 50 seeds each from fruit whose stigmas and pericarps were treated with PBS served as negative controls. Each seed section was macerated separately with

a sterilized glass rod in a microcentrifuge tube containing 1 ml of PBS. One hundred microliter aliquots of tissue macerate were spread onto Nunhems medium in triplicate and incubated for 72 h at 28°C. Plates were visually inspected for colonies with typical *A. citrulli* morphology to determine if tissue sections were infested. This experiment was conducted two times and the mean percentage of *A. citrulli*-positive seed sections was determined.

Effect of watermelon seed tissue localization on the efficacy of seed treatments against *A. citrulli*. Because a multiyear pathogen survival study with naturally infested seeds was impractical, we used liquid and gaseous antimicrobial seed treatments to determine the effect of seed tissue localization on *A. citrulli* response to stress. We hypothesized that *A. citrulli* in stigma-inoculated seeds would survive externally applied liquid seed treatments better than in pericarp-inoculated seeds. We also hypothesized that gaseous chlorine treatments that penetrated watermelon seed tissues would have a greater negative effect on *A. citrulli* survival in stigma-inoculated seeds than externally applied antimicrobial compounds.

Watermelon seeds generated by pericarp and stigma inoculation were treated with peroxyacetic acid (Tsunami 100, Ecolab, McDonough, GA) or chlorine (Cl₂) gas. For treatment with peroxyacetic acid, seeds [$n = 20$ g/lot (~ 450 seeds)] were placed in a 250 ml side-arm flask with 100 ml of peroxyacetic acid (1,600 µg/ml). The flask was sealed with a rubber stopper and a vacuum was drawn for 20 min. The peroxyacetic acid was decanted; and seeds were rinsed with sterile water for 10 min followed by drying for 48 h at 40°C in a seed dryer (Grainman Machinery Manufacturing Corp., Miami, FL). Pericarp- and stigma-inoculated seeds ($n = 20$ g) vacuum-infiltrated with PBS served as positive controls. Seeds ($n = 20$ g) from PBS-treated flowers (stigma and pericarp inoculation) that were vacuum-infiltrated with peroxyacetic acid served as a negative controls.

For chlorine gas treatment, seeds ($n = 20$ g/lot) were placed in a glass chamber (50 cm $\times$ 25 cm $\times$ 30 cm) fitted with plastic support racks, a 400-ml mixing vat, a pH probe, wet and dry bulb thermometers, a propeller for mixing the atmosphere, and a glass top with sample septa. The propeller was attached to a stainless steel shaft that protruded through an impervious gland in the glass top. The shaft was attached to a variable speed electric motor, which was operated at ~ 200 rpm. Chlorine gas was generated by mixing 100 ml of a freshly made solution of 6.0% NaOCl and 100 ml of 0.6 M monobasic potassium phosphate buffer in the mixing vat via a funnel and tube that passed through a sealed port in the glass top as described previously (Stephens et al. 2008). Chlorine concentrations inside the chamber were monitored with a Kitagawa gas detector system (Matheson Trigas, Newark, CA). Seeds were placed in open glass Petri dishes that were fully exposed to the atmosphere inside the glass chamber for 9 h. The concentration of chlorine gas inside the airtight chamber was maintained at a range of 400 to 500 ppm. Seeds from PBS-treated flowers (pericarp and stigma) exposed to chlorine gas served as negative controls. Untreated pericarp- and stigma-inoculated seeds from corresponding lots were used as positive controls.

TABLE 1. Characteristics of watermelon seedlots generated for this study

Seedlot	Inoculation method	Mean <i>Acidovorax citrulli</i> population per seed (CFU) ^a	Mean seed germination (%) ^b	Mean BFB seedling transmission (%) ^c
1	Pericarp	3.8×10^4 (1.2×10^2) ^d	77.5 (2.67)	61.3 (1.42)
2	Pericarp	2.1×10^4 (3.4×10^2)	68.0 (1.34)	64.7 (2.54)
3	Stigma	4.7×10^4 (3.7×10^2)	74.0 (3.26)	56.8 (3.87)
4	Stigma	6.5×10^4 (3.4×10^3)	85.0 (1.84)	63.5 (2.17)

^a Mean *A. citrulli* population per seed recovered by crushing individual seeds ($n = 30$ seeds/lot) in 0.1 M phosphate-buffered saline followed by dilution plating on Nunhem's semiselective medium.

^b Mean seed germination percentages for four replicates of seed samples ($n = 100$ /replicate) from each lot. Seeds were planted in transparent, closed, plastic boxes with saturated blotter paper and incubated for 14 days at 28°C and 80% relative humidity.

^c Mean bacterial fruit blotch (BFB) seedling transmission percentages for four replicates of seed samples ($n = 100$ /replicate) from each lot. Seeds were planted in transparent, closed, plastic boxes with saturated blotter paper and incubated for 14 days at 28°C and 80% relative humidity. BFB seedling transmission percentages were recorded as number of seedlings showing typical BFB symptoms divided by total number of seeds germinated $\times$ 100.

^d Numbers in parentheses denote standard error of the mean.

For the two treatments (Cl_2 versus peroxyacetic acid), three replicates of seeds from each seedlot of each inoculation type (pericarp or stigma) were used in a single experiment and the experiment was conducted twice. To determine the effect of the seed treatments on *A. citrulli* survival, the percent reduction in BFB seed-to-seedling transmission ([BFB transmission percentage for the positive control lot – BFB transmission percentage for the treated lot]/BFB transmission percentage for the positive control lot $\times$ 100) and percent reduction in infested seeds ([percentage of infested seeds for the positive control lot – percentage of infested seeds after seed treatment]/percentage of seeds for the positive control lot $\times$ 100) was determined for each seed sample. Mean reductions of BFB seed-to-seedling transmission percentage and percentage of infested seeds were calculated for seed samples from individual lots.

To determine the effect of peroxyacetic acid and Cl_2 gas seed treatments on BFB seed-to-seedling transmission, samples ($n = 100$ seeds/replicate/lot) were planted in transparent plastic boxes on blotter paper and maintained at 28°C and $\sim 85\%$ RH as described above. Seed germination and BFB seed-to-seedling transmission percentages were recorded at 14 DAP. Seed samples from positive and negative control lots were also tested and percent reduction in BFB seed-to-seedling transmission was determined. To estimate the percentage of *A. citrulli* infested seeds, samples ($n = 20$ seeds/replicate/lot) from each treatment were crushed individually in 1 ml of PBS and 100 μl aliquots were spread onto Nunhem's agar plates. After 72 h of incubation, plates were visually examined for the presence of *A. citrulli* colonies and the percentage of *A. citrulli* infested seeds was recorded. A seed was considered positive when at least one *A. citrulli* colony was detected on the agar plate. The percent reduction in infested seeds was calculated as described above. The significance of the effect of seed treatment on reduction of BFB seed-to-seedling transmission and *A. citrulli*-infested seeds was determined by analysis of variance (ANOVA) using PROC GLM of SAS. Means separation was conducted using least significant difference (LSD) ($P < 0.05$).

Effect of watermelon seed localization on *P. stewartii* subsp. *stewartii* survival. The effect of seed tissue localization on bacterial survival was further explored using watermelon seeds internally infested with *P. stewartii* subsp. *stewartii* by stigma inoculation. *P. stewartii* subsp. *stewartii* is a seedborne pathogen of maize, but it does not survive for long periods on corn seed (Michener et al. 2002). We hypothesized that when localized in the embryo/endosperm of watermelon seeds, *P. stewartii* subsp. *stewartii* would survive longer than on the surface or just under the testa. Using our knowledge of the pathways of seed infection by *A. citrulli*, we localized *P. stewartii* subsp. *stewartii* cells in the embryo/endosperm of watermelon seed. We then compared the ability of *P. stewartii* subsp. *stewartii* to survive in the watermelon seed endosperm/embryo to its ability to survive just under the testa. Two watermelon seedlots infested with *P. stewartii* subsp. *stewartii* DS203 were generated in May and September of 2010, respectively by stigma inoculation as described above. Twenty-five and thirty fruits were harvested from the greenhouse experiments in May and September (2010), respectively. Seeds from fruits ($n = 20$) whose stigmas were treated with PBS served as negative controls.

To confirm that stigma inoculation deposited *P. stewartii* subsp. *stewartii* cells in the embryos of watermelon seeds, seed samples ($n = 50$ seeds/lot) were dissected into seed coat (testa), perisperm-endosperm (PE) layer, and embryo tissues as described previously (Dutta et al. 2012). Similar tissues were dissected from 10 seeds from fruit whose stigmas were treated with PBS as negative controls. Each seed tissue type was crushed in a separate microcentrifuge tube and total microbial genomic DNA was extracted using an UltraClean Microbial DNA Kit (MO BIO, Carlsbad, CA) according to the manufacturer's instructions. DNA was used to test for *P. stewartii* subsp. *stewartii* by species-specific, real-time PCR assay and plating on LB agar amended with nalidixic acid at 50 $\mu\text{g}/\text{ml}$. For real-time PCR, 25 μM

each of primer (ES-F (TGCGAAGCGAGGACACACGTA) and ES-R (GACACCTTTTCACCTGTCACCAA) [Fessehaie et al. 2006]) was used with approximately 5 ng/ μl of DNA per sample. DNA amplification was conducted using a SYBR green mastermix (SoFast EvaGreen Supermix, Bio-Rad Laboratories Inc., Hercules, CA) and a Smart Cycler PCR machine (Cepheid, Sunnyvale, CA). The following thermal profile was used: denaturation at 95°C for 180 s, followed by 35 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 55°C for 40 s. Samples with cycle threshold (Ct) values below 35 were considered to be positive for *P. stewartii* subsp. *stewartii*. Additionally, seed sections from 50 seeds per stigma-inoculated lot were crushed individually in 1 ml of PBS, and 100 μl of tissue extract was spread onto LB agar plates amended with nalidixic acid at 50 $\mu\text{g}/\text{ml}$. Plates were incubated for 48 h at 28°C and visually examined for *P. stewartii* subsp. *stewartii* colonies. This experiment was conducted two times and the mean percentages of *P. stewartii*-positive seed sections were determined by PCR and agar plating assays. For statistical analysis the mean percentages of *P. stewartii* subsp. *stewartii*-positive seed sections (for both PCR and plating results) were compared by ANOVA using PROC GLM ($P < 0.05$) and mean separation was conducted by Fisher's LSD test.

For inoculation by vacuum-infiltration, two watermelon (Crimson Sweet) seed samples (30 g of seeds each) were placed in a suspension containing $\sim 1 \times 10^6$ *P. stewartii* subsp. *stewartii* CFU/ml and processed as described above. Seeds were subsequently air-dried for 24 h in a laminar air-flow chamber. Seedlots vacuum-infiltrated with PBS served as negative controls. After air drying, seedlots were stored in paper for 48 h at 4°C .

Watermelon seedlots infested/infected with *P. stewartii* subsp. *stewartii* were stored at 4°C and 50% RH for 5 weeks. Seed samples ($n = 1$ g of seed/method of inoculation/sampling time) were removed at 0, 1, 2, and 3 WPI, crushed in PBS and 10-fold serially diluted. One-hundred microliter aliquots of seed extract were spread onto LB agar amended with 50 $\mu\text{g}/\text{ml}$ at nalidixic acid and bacterial colonies were counted after 48 h incubation at 28°C . This experiment was conducted twice and included three replicates per lot/inoculation method/sampling time. Bacterial populations (CFU/g) were $\log_{10}$ -transformed and pooled data from the two experiments were used for statistical analysis after ascertaining lack of significance of the effects of replication and experiment at $P < 0.05$. ANOVA using PROC GLM was used to determine the effect of bacterial location in seed tissue on *P. stewartii* subsp. *stewartii* survival at $P < 0.05$, where bacterial location and storage time served as repeated measures.

RESULTS

***A. citrulli* tolerance to desiccation.** The survival of bacteria on filter paper (at 4°C and 50% RH) was significantly affected by storage time and bacterial species ($P < 0.001$). There was a significant difference in desiccation tolerance of different bacterial species on filter paper at ($P = 0.01$). In addition, the interaction between storage time and type of bacterial species was significant ($P = 0.022$). At 0 WPI mean populations of *A. citrulli*, *R. solanacearum*, *X. campestris* pv. *campestris*, and *P. stewartii* subsp. *stewartii* on filter paper ranged from $\sim 10^7$ to 10^8 CFU/ mm^2 (Fig. 1). By 2 WPI the mean *P. stewartii* subsp. *stewartii* population declined significantly to 1.02×10^4 CFU/ mm^2 ($P \leq 0.01$), while populations of the other bacteria ranged from 2.13×10^6 to 3.63×10^6 CFU/ mm^2 (Fig. 1), which were not significantly different to each other ($P \geq 0.79$). At 4 WPI the mean *P. stewartii* subsp. *stewartii* population declined to 1.54×10^3 CFU/ mm^2 , and by 8 WPI it was 1.34×10^2 CFU/ mm^2 (Fig. 1). The population levels of *P. stewartii* subsp. *stewartii* at 4 and 8 WPI were significantly lower than that for other bacteria ($P \leq 0.002$). *P. stewartii* subsp. *stewartii* colonies were not recovered from the filter paper by 12 WPI. In contrast, by 4 WPI mean populations of *X. campestris* pv. *campestris*, *A. citrulli*, and *R. solanacearum* declined to a range of 9.33×10^4 to 9.54×10^5 CFU/ mm^2 , and by

8 WPI mean populations of *X. campestris* pv. *campestris* and *A. citrulli* declined to 2.95×10^4 to 3.23×10^4 CFU/mm², while the mean *R. solanacearum* population was 2.15×10^5 CFU/mm² (Fig. 1). The populations of *X. campestris* pv. *campestris*, *A. citrulli*, and *R. solanacearum* at 4 and 8 WPI were not significantly different ($P \geq 0.96$). At 12 WPI mean *X. campestris* pv. *campestris* and *A. citrulli* populations declined significantly to 2.29×10^2 to 3.09×10^2 CFU/mm², while *R. solanacearum* populations remained at 1.12×10^5 CFU/mm² (Fig. 1) ($P < 0.01$). As expected, bacteria were not recovered from filter paper strips that were inoculated with sterilized PBS.

Survival of phytopathogenic bacteria on host and nonhost seeds. The ability of phytopathogenic bacteria to tolerate desiccation on host and nonhost seed species after 12 weeks varied significantly ($P < 0.01$) (Fig. 2). While the interaction between storage time and bacterial species was significant ($P < 0.01$), significant interaction between storage time and seed types on bacterial survival was not observed ($P \geq 0.64$). On watermelon seed, by 12 WPI the mean population of *R. solanacearum* (3.63×10^4 CFU/g of seed) was significantly higher than other bacteria ($P < 0.01$) (Fig. 2A). During the same period, no significant differences were observed in the populations of *A. citrulli* (1.65×10^3 CFU/g of seed) and *X. campestris* pv. *campestris* (3.63×10^2 CFU/g of seed) ($P = 0.26$) (Fig. 2A). On tomato and corn seed, mean *R. solanacearum* populations at 12 WPI were 1.90×10^3 and 3.32×10^4 CFU/g, respectively, which were significantly higher than other bacterial species ($P = 0.008$) (Fig. 2B and D). On these seeds, there was no significant difference in the *A. citrulli* and *X. campestris* pv. *campestris* populations ($P = 0.99$). The mean populations of *A. citrulli* and *X. campestris* pv. *campestris* on tomato and corn seeds were 1.69×10^2 , 2.81×10^2 CFU/g of seed and 2.95×10^2 , 2.88×10^2 CFU/g of seed, respectively (Fig. 2B and D). At 12 WPI the mean *R. solanacearum* population was significantly higher on cabbage seeds ($\sim 1.31 \times 10^5$ CFU/g) than on other seed types (Fig. 2C). During the same period, *A. citrulli* and *X. campestris* pv. *campestris* populations were significantly higher on cabbage seeds than on other seed species, with mean

populations of 3.09×10^3 and 1.90×10^3 CFU/g of seed, respectively (Fig. 2C).

Overall, the difference in survival between bacterial species was statistically significant ($P < 0.01$). By 12 WPI significantly higher populations of *R. solanacearum* ($\sim 10^4$ to 10^5 CFU/g) were observed on all seed types (Fig. 2) than for all other bacteria tested. During this same period, there was no significant difference between *A. citrulli* and *X. campestris* pv. *campestris* populations ($\sim 10^2$ to 10^3 CFU/g) ($P \leq 0.27$) (Fig. 2) and viable colonies of *P. stewartii* subsp. *stewartii* were not recovered from any of the seeds tested (Fig. 2A to D).

Effect of watermelon seed tissue localization on *A. citrulli* survival. The mean germination percentages of the two pericarp-inoculated seedlots 1 and 2, were 77.5 and 68.0%, respectively, and the mean BFB seed-to-seedling transmission percentages were 61.3 and 64.7% (Table 1). The mean *A. citrulli* population per seed for lots 1 and 2, were 3.8×10^4 and 2.1×10^4 CFU, respectively. The mean seed germination percentages for the two stigma-inoculated seedlots, 3 and 4, were 74.0 and 85.0%, respectively, and they displayed BFB seed-to-seedling transmission percentages of 56.8 and 63.5%, respectively (Table 1). The mean *A. citrulli* population per seed for lots 3 and 4 were 4.7×10^4 and 6.5×10^4 CFU, respectively.

Confirmation of the location of *A. citrulli* in watermelon seed tissues after stigma and ovary pericarp inoculation. For seedlots from pericarp-inoculated fruits the mean percentages of *A. citrulli*-infested testa, PE layer, and embryo/endosperm tissues were 5.5, 80.5, and 10%, respectively. In contrast, for stigma-inoculated seedlots *A. citrulli* was detected in 16.5, 79, and 87% of the testa, PE layer, and embryo tissues, respectively. These data confirm that stigma inoculation deposited *A. citrulli* cells into the embryo/endosperm of seeds while pericarp inoculation deposited *A. citrulli* cells into the testa and PE layer. *A. citrulli* cells were not detected in tissues of seeds from negative control lots.

Effect of watermelon seed tissue localization on the efficacy of seed treatments against *A. citrulli*. Peroxyacetic acid did not affect the germination of the control seeds ($P = 0.34$) and these seeds did not transmit BFB to resulting seedlings (data not shown). Seed treatment with peroxyacetic acid resulted in a statistically significant reduction in BFB seed-to-seedling transmission percentage ($P = 0.01$); however, no significant difference was observed between lots of the same inoculation type ($P = 0.43$). The mean reduction in BFB seed-to-seedling transmission for seeds treated with peroxyacetic acid was significantly higher for pericarp-inoculated seeds (89.5%) than for stigma-inoculated seeds (61.1%) (Fig. 3A). Chlorine gas treatment did not affect watermelon seed germination ($P = 0.67$) but significantly reduced BFB seed-to-seedling transmission for both pericarp- and stigma-inoculated seedlots ($P = 0.01$). With chlorine gas, the reduction in BFB seed-to-seedling transmission for pericarp-inoculated seeds was 99.1% (Fig. 3A), which was significantly higher than for the stigma-inoculated seeds (76.5%) ($P = 0.05$) (Fig. 3A).

Overall, peroxyacetic acid significantly reduced the mean percentage of *A. citrulli*-infested seeds ($P = 0.01$). This reduction was significantly greater for seeds from pericarp-inoculated lots (74.8%) than for seeds from stigma-inoculated lots (9.63%) ($P = 0.01$) (Fig. 3B). Additionally, the reduction in the percentage of *A. citrulli*-infested seeds by chlorine gas treatment was significantly greater for seeds from pericarp-inoculated lots (80.8%) than for seeds from stigma-inoculated lots (54.02%) ($P = 0.05$) (Fig. 3B). *A. citrulli* colonies were not recovered from negative control seeds treated with peroxyacetic acid or chlorine gas.

Survival of *P. stewartii* subsp. *stewartii* in watermelon seeds. To further demonstrate that seed tissue localization affected bacterial survival we investigated if *P. stewartii* subsp. *stewartii* (a xpoor survivor on corn seed) survival could be enhanced by localization in watermelon seed embryo/endosperm. Previous experiments showed that *P. stewartii* subsp. *stewartii* was sensitive to desiccation. Since *A. citrulli* survived stress better in stigma-inoculated than in

Fig. 1. Survival of *Acidovorax citrulli*, *Pantoea stewartii* subsp. *stewartii*, *Xanthomonas campestris* pv. *campestris*, and *Ralstonia solanacearum* on filter paper at 4°C and 50% relative humidity (RH). Filter paper strips were inoculated with $\sim 10^8$ CFU/ml of each bacterium and incubated at 4°C and 50% RH for 12 weeks. Each data point represents mean colony forming units per square millimeter as estimated by dilution plating on respective semiselective agar plates. Bars represent standard errors of the means ($n = 6$).

pericarp-inoculated seeds, we hypothesized that the survival of *P. stewartii* subsp. *stewartii* would be enhanced by localization in seed embryo/endosperm tissue.

Tissue sections from negative control seedlots were negative for *P. stewartii* subsp. *stewartii* by species-specific PCR assay and plating on agar plates. In contrast, for stigma-inoculated seedlots significantly higher mean percentages of embryo/endosperm (63% by PCR assay and 58% by agar plating) and PE layer (48% by PCR and 34% by plating) tissue samples were positive for *P. stewartii* subsp. *stewartii* relative to testa (8% by PCR and 2% by plating) samples ($P < 0.01$). These data confirmed the localization of *P. stewartii* subsp. *stewartii* in embryo/endosperm tissues with stigma inoculation.

Overall, the difference between *P. stewartii* subsp. *stewartii* populations in vacuum-infiltrated and stigma-inoculated watermelon seeds was significant ($P < 0.01$). *Pantoea stewartii* subsp. *stewartii* colonies were not recovered from seedlots that were treated with PBS by stigma inoculation or vacuum infiltration at any sampling period. In contrast, at 0 WPI, the mean *P. stewartii* subsp. *stewartii* populations recovered from vacuum-infiltrated and stigma-inoculated watermelon seeds were 3.46×10^6 and 1.32×10^3 CFU/g of seed, respectively (Fig. 4). By 1 and 2 WPI, the mean bacterial populations recovered from stigma-inoculated seeds were 8.4×10^4 and 2.3×10^4 CFU/g of seed, respectively. In contrast, in the same period, mean *P. stewartii* subsp. *stewartii* populations declined to 9.4×10^2 (1 WPI) and 4.3×10^2 CFU/g of seed (2 WPI) for

Fig. 2. Survival of *Acidovorax citrulli*, *Pantoea stewartii* subsp. *stewartii*, *Xanthomonas campestris* pv. *campestris*, and *Ralstonia solanacearum* on **A**, watermelon, **B**, tomato, **C**, cabbage, and **D**, corn seeds. Each seed species was inoculated with $\sim 10^8$ CFU/g of each bacterium dried to their original weight and incubated at 4°C and 50% relative humidity for 12 weeks. Each data point represents the mean colony forming units per gram of seed estimated by dilution plating of 1 g of seed at each sample time on respective semiselective agar plates. Bars represent standard errors of the means for three replicates per treatment per experiment and the experiment was conducted twice ($n = 6$).

vacuum-infiltrated seedlots. By 3 WPI, *P. stewartii* subsp. *stewartii* colony forming units were not recovered from vacuum-infiltrated seeds, while the mean population recovered from stigma-inoculated seeds was 4.07×10^3 CFU/g of seed (Fig. 4).

DISCUSSION

A range of factors including storage conditions, e.g., temperature, relative humidity, sensitivity to desiccation, and location in seed tissues contribute to phyto-bacterial survival in seeds (Agarwal and Sinclair 1997; Leben 1981; Schuster and Coyne 1974). Low temperature (5 to 10°C) and low moisture (40 to 60% RH) storage conditions extend the viability of seedborne bacteria (Agarwal and Sinclair 1997; Schuster and Coyne 1974). For example, *Pseudomonas syringae* pv. *phaseolicola* (causal agent of halo blight of bean) and *X. campestris* pv. *manihotis* (causal agent of bacterial blight of cassava) survived longer in bean and cassava seeds, respectively, when stored at 5 to 7°C and 50 to 60% RH than at 10 to 27°C and <50% RH (Persley 1979; Taylor et al. 1979). *A. citrulli* was reported to survive for 34 to 40 years in watermelon and melon seeds, respectively, stored at 5°C and 50% RH (Block and Shepherd 2008). More recently, *A. citrulli* was recovered from citron melon (*Citrullus lanatus* var. *citroides*, a cucurbitaceous weed prevalent in southeastern United States) seeds that were stored for 7 years at 4°C and 50% RH (Dutta et al. 2014). Despite these reports of extraordinarily prolonged survival in cucurbitaceous seeds, the factors that influence longevity of *A. citrulli* have not been previously studied. The current study revealed that *A. citrulli* cells are not intrinsically tolerant to desiccation as bacterial CFUs declined rapidly on filter paper stored at 4°C and 50% RH. Even superficial inoculation of *A. citrulli* onto watermelon seeds did not enhance its survival relative to other phyto-bacterial species.

We observed that *P. stewartii* subsp. *stewartii* survived for the shortest time on filter paper, and host and nonhost seeds, suggesting high desiccation sensitivity. This may in part explain the bacterium's poor ability to survive in/on corn seeds (Block et al. 1998; Michener et al. 2002). It may also explain why the corn flea beetle rather than seedborne inoculum is more important in the epidemiology of Stewart's wilt (Block et al. 1998; Block et al. 1999; Michener et al. 2002). In comparison, *R. solanacearum* survived longer than other tested bacteria. This was expected as *R. solanacearum* is a persistent,

soil borne bacterium and is likely to be tolerant to environmental stress (Alvarez et al. 2008; Milling et al. 2009). The decline in population over 12 weeks of storage for *A. citrulli* and *X. campestris* pv. *campestris* were not significantly different ($P > 0.05$) on filter paper or seed tissue. Hence, it is unlikely that the ability of *A. citrulli* to survive for more than 34 years in watermelon and melon seeds is due to its inherent tolerance to desiccation or its superficial association with watermelon seeds.

Fig. 4. Survival of *Pantoea stewartii* subsp. *stewartii* in vacuum and stigma-inoculated watermelon seeds over time. Vacuum-infiltrated seeds were inoculated with 10^6 CFU/ml of *Pantoea stewartii* subsp. *stewartii* and air-dried. Internally infested seeds were extracted from fruits whose stigmas were inoculated with bacterial suspension of $\sim 10^6$ CFU/stigma. Seed samples (30 g) from vacuum- and stigma-inoculated lots were incubated at 4°C and 50% relative humidity. Each data point represents mean $\log_{10}$ CFU/g of seed estimated by 10-fold serial dilution plating of 1 g of seed at each time point on semiselective agar plates. Bars represent standard errors of the means for three replicates and the experiment was repeated twice ($n = 6$).

Fig. 3. Reduction in **A**, bacterial fruit blotch seed-to-seedling transmission and **B**, percentage of *Acidovorax citrulli*-infested seeds after surface and subsurface antimicrobial seed treatments of watermelon seeds pericarp and stigma inoculated with *A. citrulli*. One gram of seed from two seedlots generated by each inoculation method was subjected to treatment with peroxyacetic acid (1 h followed by overnight drying) and chlorine gas for 9 h. Error bars represent the standard errors of the means with three replicates per treatment in two independent experiments. Means with the same upper or lowercase letters are not significantly different according to Student's *t* test ($P = 0.05$) ($n = 12$).

Since *A. citrulli* can become localized to different watermelon seed tissues depending on the pathway of invasion (Dutta et al. 2012), we hypothesized that seed tissue localization influences bacterial survival. Because of the practical limitations of conducting a multiyear survival study with naturally infested seeds, we used surface and subsurface antimicrobial treatments to determine the effect of tissue localization on bacterial survival. As seed treatments, we employed peroxyacetic acid, an industry standard that is effective on surface and subsurface *A. citrulli* populations (Hopkins et al. 2003) and chlorine gas, which can theoretically penetrate internal seed tissues (Stephens et al. 2008). However, the depth of seed tissue penetration by gaseous chlorine is unknown. As expected, pericarp and stigma inoculation of watermelon flowers resulted in *A. citrulli* localization mainly on the surface of the PE layer and in the embryo tissue, respectively (Dutta et al. 2012). Additionally, the levels of *A. citrulli* infestation in pericarp- and stigma-inoculated seedlots were similar based on BFB seedling transmission percentages and mean bacterial populations. Seed treatment with peroxyacetic acid and gaseous chlorine resulted in a significantly greater reduction in the percentage of *A. citrulli*-infested seeds and BFB seed-to-seedling transmission for pericarp-inoculated seedlots than for stigma-inoculated seedlots. Based on this observation, we conclude that *A. citrulli* cells in the embryo of watermelon seeds (stigma-inoculated seeds) are better protected from stress than cells localized under the testa (pericarp-inoculated seeds). Similar effects of bacterial localization on *A. citrulli* survival were observed for citron melon seed (Dutta et al. 2014). More specifically, *A. citrulli* survived longer in citron melon seeds generated by stigma inoculation than in seeds generated by pericarp inoculation when stored at 4°C and 50% RH for 7 years (Dutta et al. 2014).

To further confirm that seed tissue localization is an important factor in phytobacterial survival, *P. stewartii* subsp. *stewartii* was localized to watermelon seed embryo/endosperm tissue by stigma inoculation and its ability to survive was compared with that of watermelon seeds inoculated by vacuum infiltration. Our results showed that watermelon stigma inoculation with *P. stewartii* subsp. *stewartii* resulted in localization of the bacterium in the seed embryo. Additionally, while *P. stewartii* subsp. *stewartii* was not recovered from vacuum-infiltrated watermelon seeds after 3 weeks of storage, 4.07×10^3 CFU/g of seed of the pathogen was recovered from stigma-inoculated seeds. These data suggest that survival of *P. stewartii* subsp. *stewartii* in watermelon seeds was enhanced by localization in embryo/endosperm tissues.

Overall, our data suggest that tolerance to desiccation does not explain *A. citrulli*'s remarkable longevity in stored watermelon and melon seeds. Alternatively, it is likely that localization of the bacterium in embryo/endosperm tissues is important for long-term survival.

ACKNOWLEDGMENTS

We thank R. Christiano for assistance with statistical analysis and L. Genzlinger, I. Ontaneda, and A. Castro-Sparks for technical assistance.

LITERATURE CITED

- Agarwal, V. K., and Sinclair, J. B. 1997. Longevity of seedborne pathogens. Pages 217-226 in: Principles of Seed Pathology. N. Levine and S. Kaplan, eds. CRC Press, New York.
- Alvarez, B., López, M. M., and Biosca, E. G. 2008. Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms. Microbiology 154:3590-3598.
- Block, C. C., Hill, J. H., and McGee, D. C. 1998. Seed transmission of *Pantoea stewartii* in field and sweet corn. Plant Dis. 82:775-780.
- Block, C. C., Hill, J. H., and McGee, D. C. 1999. Relationship between late season Stewart's bacterial wilt and seed infection in maize. Plant Dis. 83: 527-530.
- Block, C. C., and Shepherd, L. M. 2008. Long-term survival and seed transmission of *Acidovorax avenae* subsp. *citrulli* in melon and watermelon seed. Online. Plant Health Progress. doi:10.1094/PHP-2008-1219-01-BR
- Brumbley, S. M., and Denny, T. P. 1990. Cloning of *phcA* from wild-type *Pseudomonas solanacearum*, a gene that when mutated alters expression of multiple traits that contribute to virulence. J. Bacteriol. 172:5677-5685.
- Burdman, S., Kots, N., Kritzman, G., and Kopelowitz, J. 2005. Molecular, physiological, and host-range characterization of *Acidovorax avenae* subsp. *citrulli* isolates from watermelon and melon in Israel. Plant Dis. 89:1339-1347.
- Chen, Y., Zhang, W. Z., Liu, X., Ma, Z. H., Allen, C., and Guo, J. H. 2010. A real-time PCR assay for the quantitative detection of *Ralstonia solanacearum* in the horticultural soil and plant tissues. J. Microbiol. Biotechnol. 20:193-201.
- Dutta, B., Avci, U., Hahn, M. G., and Walcott, R. R. 2012. Location of *Acidovorax citrulli* in infested watermelon seeds is influenced by the pathway of bacterial ingress. Phytopathology 102:461-468.
- Dutta, B., Sanders, H., Langston, D. B., Booth, C., Smith, S., and Gitaitis, R. D. 2014. Long-term survival of *Acidovorax citrulli* in citron melon (*Citrullus lanatus* var. *citrinoides*) seeds. Plant Pathol. 63:1130-1137.
- Fessehaie, A., Shepherd, L. M., Block, C. C., and Misra, K. M. 2006. A diagnostic real-time TaqMan PCR assay for the detection of *Pantoea stewartii* subsp. *stewartii*. (Abstr.) Phytopathology 96(suppl.):S35.
- Ha, Y., Fessehaie, A., Ling, K. S., Wechter, W. P., Keinath, A. P., and Walcott, R. R. 2009. Simultaneous detection of *Acidovorax avenae* subsp. *citrulli* and *Didymella bryoniae* in cucurbit seedlots using magnetic capture hybridization and real-time polymerase chain reaction. Phytopathology 99:666-678.
- 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. 2002. Seed transmission of *Acidovorax avenae* subsp. *citrulli* in cucurbits. HortScience 37:924-926.
- King, E. O., Ward, M. K., and Raney, D. E. 1956. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44: 301-307.
- Latin, R. X., and Hopkins, D. L. 1995. Bacterial fruit blotch of watermelon: The hypothetical exam question becomes reality. Plant Dis. 79:761-765.
- Latin, R. X., and Rane, K. K. 1990. Bacterial fruit blotch of watermelon in Indiana. Plant Dis. 74:331.
- Latin, R. X., Tikhonova, I., and Rane, K. 1995. Factors affecting the survival and spread of *Acidovorax avenae* subsp. *citrulli* in watermelon transplant production facilities. Phytopathology 85:1413-1417.
- Leben, C. 1981. How plant-pathogenic bacteria survive. Plant Dis. 65:633-637.
- Lessl, J. T., Fessehaie, A., and Walcott, R. R. 2007. Colonization of female watermelon blossoms by *Acidovorax avenae* subsp. *citrulli* and the relationship between blossom inoculum dosage and seed infestation. J. Phytopathol. 155:114-121.
- Michener, P. M., Pataky, J. K., and White, D. G. 2002. Rates of transmitting *Erwinia stewartii* from seed to seedlings of a sweet corn hybrid susceptible to Stewart's wilt. Plant Dis. 86:1031-1035.
- Milling, A., Meng, F., Denny, T. P., and Allen, C. 2009. Interactions with hosts at cool temperatures, not cold tolerance, explain the unique epidemiology of *Ralstonia solanacearum* race 3 biovar 2. Phytopathology 99:1127-1134.
- Park, Y. J., Lee, B. M., Hahn, J. H., Lee, G. B., and Park, S. K. 2004. Sensitive and specific detection of *Xanthomonas campestris* pv. *campestris* by PCR using species-specific primers based on *hrpF* gene sequences. Microbiol. Res. 159:419-423.
- Persley, G. J. 1979. Studies on the survival and transmission of *Xanthomonas manihotis* on cassava seed. Ann. Appl. Biol. 93:159-166.
- Rane, K. K., and Latin, R. X. 1992. Bacterial fruit blotch of watermelon: Association of the pathogen with seed. Plant Dis. 76:509-512.
- Schaad, N. W., Jones, J. B., and Chun, W. 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria, 3rd ed. The American Phytopathological Society, St. Paul, MN.
- Schaad, N. W., Postnikova, E., and Randhawa, P. 2003. Emergence of *Acidovorax avenae* subsp. *citrulli* as a crop-threatening disease of watermelon and melon. Pages 573-581 in: *Pseudomonas syringae* and Related Pathogens: Biology and Genetics. N. A. Iacobellis, A. Collmer, S. W. Hutcheson, J. W. Mansfield, C. E. Morris, J. Murrillo, N. W. Schaad, D. E. Stead, G. E. Surico, and M. S. Ullrich, eds. Kluwer, Dordrecht, The Netherlands.
- 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. citrulli* (Schaad et al., 1978) comb.nov., and proposal of *A. oryzae* sp. nov. Syst. Appl. Microbiol. 31: 434-446.
- Schaad, N. W., Sowell, G., Goth, R. W., Colwell, R. R., and Webb, R. E. 1978. *Pseudomonas pseudoalcaligenes* subsp. *citrulli* subsp. nov. Int. J. Syst. Bacteriol. 28:117-125.
- Schuster, M. L., and Coyne, D. P. 1974. Survival mechanisms of phytopathogenic bacteria. Annu. Rev. Phytopathol. 12:199-221.

- 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.
- Stephens, D. J., Schneider, R. W., Walcott, R. R., and Johnson, C. E. 2008. A procedure, based on exposure to chlorine gas, for disinfesting watermelon seeds. (Abstr.) *Phytopathology* 98(suppl.):S150.
- Taylor, J. D., Dudley, C. L., and Presley, L. 1979. Studies of halo-blight seed infection and disease transmission. *Ann. Appl. Biol.* 93:267-277.
- Walcott, R. R. 2008. Integrated pest management of bacterial fruit blotch in cucurbits. Pages 191-209 in: *Integrated Management of Diseases Caused by Fungi, Phytoplasma and Bacteria*. A. Ciancio and K. G. Mukherji, eds. Springer Science, New York.
- Walcott, R. R., and Gitaitis, R. D. 2000. Detection of *Acidovorax avenae* subsp. *citrulli* in watermelon seed using immunomagnetic separation and the polymerase chain reaction. *Plant Dis.* 84:470-474.
- Walcott, R. R., Gitaitis, R. D., and Castro, A. C. 2003. Role of blossoms in watermelon seed infestation by *Acidovorax avenae* subsp. *citrulli*. *Phytopathology* 93:528-534.
- Willems, A., Goor, M., Thielemans, S., Gillis, M., Kersters, K., and Deley, J. 1992. Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov., comb., *Acidovorax avenae* subsp. *citrulli*, *Acidovorax avenae* subsp. *cattleyae*, and *Acidovorax avenae* subsp. *konjaci*. *Int. J. Syst. Bacteriol.* 42: 107-119.