Location of *Acidovorax citrulli* in Infested Watermelon Seeds Is Influenced by the Pathway of Bacterial Invasion

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ABSTRACT

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Watermelon seeds can become infested by *Acidovorax citrulli*, the causal agent of bacterial fruit blotch (BFB) of cucurbits via penetration of the ovary pericarp or by invasion of the pistil. This study investigated the effect of these invasion pathways on *A. citrulli* localization in seeds. Seed samples ($n = 20$ or 50 seeds/lot) from pistil- and pericarp-inoculated lots were dissected into testa, perisperm-endosperm (PE) layer, and embryo tissues and tested for *A. citrulli* by species-specific polymerase chain reaction (PCR) and by plating on semiselective media. Less than 8% of the testa samples were *A. citrulli*-positive regardless of the method of seed inoculation. Additionally, the difference in percentages of contaminated testae between the two seed lot types was not significant ($P = 0.64$).

The percentage of *A. citrulli*-positive PE layer samples as determined by real-time PCR assay was significantly greater for seeds from pistil-inoculated lots (97%) than for seeds from pericarp-inoculated lots (80.3%). The mean percentage of *A. citrulli*-positive embryo samples was significantly greater for seeds from pistil-inoculated lots (94%) than for seeds from pericarp-inoculated lots ($\approx 8.8\%$) ($P = 0.0001$). Removal of PE layers and testae resulted in a significant reduction in BFB seed-to-seedling transmission percentage for seeds from pericarp-inoculated lots (14.8%) relative to those from pistil-inoculated lots (72%). Additionally, using immunofluorescence microscopy, *A. citrulli* cells were observed in the PE layers and the cotyledons of pistil-inoculated seeds but only in the PE layers of pericarp-inoculated seeds. These results suggest that pericarp invasion results in superficial contamination of the testae and PE layers while pistil invasion results in the deposition of *A. citrulli* in seed embryos.

Bacterial fruit blotch (BFB) is an economically important disease of cucurbits, caused by the Gram-negative, beta-proteobacterium, *Acidovorax citrulli* (= *A. avenae* subsp. *citrulli* formerly *Pseudomonas pseudoalcaligenes* subsp. *citrulli*) (29,30,43). Under environmental conditions of high relative humidity (RH) and high temperatures, the bacterium causes seedling blight and fruit rot of a range of cucurbitaceous plants including watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), melon (*Cucumis melo* L. var. *cantalupensis* Naudin), and pumpkin (*Cucurbita pepo* L.) (16,20,21,38). In the United States BFB was first observed in commercial watermelon fields in Florida in 1989 (34). Since then, it has emerged as a sporadic threat to cucurbit seed, seedling, and fruit production worldwide (3,6,11,15,17,28). Infested seeds are the most important source of primary inoculum for *A. citrulli* and when introduced into transplant houses, they can initiate costly BFB outbreaks (13,26). Hence, exclusion of *A. citrulli* from cucurbit production systems is critical for effective BFB management (8,38).

While seed treatments like peroxyacetic acid, hydrochloric acid, and sodium hypochlorite can reduce seedborne *A. citrulli* inoculum, they generally fail to eradicate the bacterium (12,14). To develop more effective seed treatments for BFB, it is important to know the location of *A. citrulli* in naturally infested seeds. The inability of seed treatments to eradicate the bacterium combined with its ability to survive more than 30 years in stored seeds

suggest that *A. citrulli* may be located deep within seed tissues (5,14). Rane and Latin (26) detected the bacterium in testae and embryos of naturally and artificially infested seeds and since they did not recover the pathogen from the peduncle of the fruit, they concluded that it did not move systemically from the mother plant to the ovary. While Rane and Latin dissected watermelon seeds in their attempts to determine the location of *A. citrulli*, they did not fully explore the localization patterns of the pathogen in different seed tissues nor did they consider influence of different pathways of seed colonization.

Cucurbit seeds, including muskmelon and watermelon, have a thin envelope called the perisperm-endosperm (PE) layer that encloses the embryo (25,27,41,42). This envelope is composed of a single layer of endospermous cells covered by a noncellular layer of callose-rich material and a thinner, waxy outer layer composed of lipids and suberin (25,41,42). Welbaum et al. (41) suggested that the lipid layer contributes to the semi-permeability of the endosperm envelope in muskmelon. Additionally, the PE layer in cucumber, muskmelon, and other cucurbitaceous seeds acts as a primary physical barrier to radicle emergence (25,41,42). During germination, this barrier becomes weakened by hydrolytic enzymes that facilitate radicle emergence (25). Because the PE layer completely encloses the embryo and is semi-permeable, we hypothesized that it played a role in embryo contamination by *A. citrulli*. Additionally, we hypothesized that the location of *A. citrulli* in watermelon seeds is influenced the pathway of bacterial invasion. Watermelon seeds can become infested by bacterial ingress through stomata on the ovary pericarp or by bacterial invasion through the pistil of the female blossom (7). Generally, *A. citrulli* ingress through the ovary pericarp leads to typical BFB fruit symptom development (7). This pathway is accessible for approximately 2 weeks after anthesis, at which time a wax layer develops on the ovary pericarp (7). In contrast, pistil penetration

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*The e-Xtra logo stands for "electronic extra" and indicates that Figure 3 appears in color online.

by *A. citrulli* leads to seed infestation within asymptomatic fruits (40). Based on these observations, we hypothesized that *A. citrulli* cells become deep-seated in seeds infested via the pistil invasion pathway. Hence, the objective of this work was to investigate the effect of the pathway of infestation on the localization of *A. citrulli* in watermelon seeds.

MATERIALS AND METHODS

Bacterial strains and inoculum preparation. *A. citrulli* strain AAC00-1 was used in this study and was routinely grown on King's medium B (19) or nutrient agar (Becton-Dickinson, Sparks, MD) for 48 h at 28°C. To prepare inoculum, nutrient broth was inoculated with a single colony of AAC00-1 from a 48 h agar culture and incubated overnight at 30°C on a rotary shaker (Innova; New Brunswick Scientific Co., Edison, NJ) at 250 rpm. The culture was centrifuged at 6,000 × *g* (Allegra 25R, Beckman Coulter, Fullerton, CA) for 5 min and the supernatant was decanted. The pellet was resuspended in 0.1 M phosphate buffer saline solution (PBS) and the bacterial concentration was adjusted to an optical density of 0.3 at 600 nm ($\approx 1 \times 10^8$ CFU/ml) spectrophotometrically (Spectronic 20; Bausch and Lomb, Rochester, NY) and diluted to a final concentration of $\approx 1 \times 10^6$ CFU/ml in PBS.

Generation of infested watermelon seed lots. Four *A. citrulli* infested seed lots (two seed lots each by pericarp- and pistil-inoculation) were generated for this study (Table 1). Pericarp-inoculated seed lots (1 and 2) were generated under greenhouse conditions in 2008 and 2009, respectively, as follows. Briefly, 20 watermelon plants ('Crimson Sweet') were grown in 15-liter plastic pots and at anthesis, 30 to 40 attached female blossoms were pollinated and simultaneously inoculated by gently rubbing their ovaries with a cotton swab saturated with a cell suspension with $\approx 1 \times 10^6$ AAC00-1 CFU/ml. Blossoms were enclosed in plastic bags for 24 h and fruits were allowed to develop for 30 days after pollination. Fruits were harvested and stored at 4°C until seed extraction. For seed extraction, fruits were surface sterilized with either 70% ethanol or 0.5% NaOCl and seeds were manually extracted and air-dried at 25°C on paper towels. Seeds from different fruits that were treated similarly were pooled and stored at 4°C until they were used. Pistil-inoculated seed lots (3 and 4) were generated in 2008 and 2009, respectively, by pollinating female watermelon blossoms and simultaneously inoculating their stigmas with AAC00-1 as previously described (24, 41). Briefly, 20 to 25 watermelon plants ('Crimson Sweet') were

grown in 15-liter plastic pots under greenhouse conditions and at anthesis, stigmas were pollinated. Subsequently, 10 µl of a suspension containing $\approx 1 \times 10^8$ AAC00-1 CFU/ml ($\approx 1 \times 10^6$ CFU/blossom) was transferred to each stigma using a micropipette. Five blossoms were inoculated per plant, tagged, and allowed to develop for 30 days after pollination. Thirty-five to forty fruits were harvested at maturity and seeds were extracted as described above and pooled to a produce each seed lot.

Seed lot characteristics. To determine the percentage of *A. citrulli*-infested seeds infestation for each lot, four samples ($n = 100$ seeds/lot) were planted on blotter paper (Anchor Paper Co., St. Paul, MN) saturated with sterile water in transparent plastic boxes (6 cm [H] × 24 cm [W] × 33.5 cm [L]; Tri-State Plastics, Dixon, KY) and maintained at 30°C and 85% RH with continuous fluorescent light for 14 days. Seeds from the fruits of blossoms whose pistils were treated with PBS were used as negative controls. Seed germination percentages and BFB seedling transmission percentages [(number of seedlings with BFB symptoms divided by the total number of seeds germinated) × 100] were recorded at 14 days after planting (DAP). Additionally, mean *A. citrulli* CFU/seed for samples ($n = 30$ seeds) from each lot were determined by 10-fold serial dilution of individually macerated seeds followed by spread-plating onto Nunhems semi-selective agar (Nunhems Seed Company, Haelen, The Netherlands). After incubation for 5 to 6 days at 28°C, *A. citrulli* colonies were enumerated and mean CFU per seed was determined for each lot.

Localization of *A. citrulli* in infested watermelon seeds. To compare the effect of invasion pathway on *A. citrulli* location, seeds from pericarp- and pistil-inoculated fruits were studied. Seed samples ($n = 50$ seeds) from each lot were dissected into testa, PE layer, and embryo tissues and assayed for *A. citrulli*. To separate seed sections, testae were first removed from dry seeds using a sterile scalpel. Each decoated seed was soaked in distilled water for 3 h at 25°C in the dark and then the PE layer was removed using sterile forceps (DR Instruments, Palos Hills, IL). Soaking in distilled water was required to facilitate the complete removal of PE layers from seeds. However, we acknowledge that this process may result in the cross-contamination of tissues which, in turn, may prevent determination of the exact location of *A. citrulli* cells in seed tissues (e.g., inner versus outer surfaces of testae or PE layers). Similar sections dissected from 10 seeds from fruits of blossoms whose pistils were treated with PBS served as negative controls. To detect *A. citrulli*, each section was placed in a separate microcentrifuge tube containing 1 ml of PBS, macerated using a sterile glass rod and vigorously agitated for 30 s (Vortex Genie2; Fisher Scientific International, Pittsburgh, PA). An UltraClean Microbial DNA Kit (MO BIO Laboratories, Inc., Carlsbad, CA) was used to extract microbial genomic DNA from seed tissues according to the manufacturer's instructions. DNA (10 to 15 ng/µl) was subjected to real-time PCR assay using *A. citrulli*-specific primers as previously described (10). Samples with cycle threshold (Ct) values less than 35 were considered to be positive for *A. citrulli*. To ensure that there was no PCR inhibition in samples that yielded negative results, 5 ng/µl of *A. citrulli* genomic DNA was added to DNA preparations from 10 control seed samples and subjected to real-time PCR as described (9). The mean percentage of *A. citrulli*-positive seed sections was determined for each seed lot. To confirm real-time PCR assay results, attempts were made to isolate *A. citrulli* from seed sections. Samples ($n = 20$ seeds/lot) were dissected into testa, PE layer and embryo tissues as described above and the tissues were macerated separately in PBS and 100-µl aliquots were spread onto Nunhems semi-selective medium. Plates were incubated for 5 days at 28°C and the percentage of *A. citrulli*-positive seed sections was compared for seeds inoculated by each method. For statistical analysis the mean percentage of *A. citrulli*-positive seed tissues for each lot (as determined by real-time PCR assay and plating on

TABLE 1. Characteristics of watermelon seed lots generated for this study

Seed lot	Mechanism of seed inoculation	Mean <i>Acidovorax citrulli</i> population per seed (CFU) ^w	Mean seed germination (%) ^x	Mean BFB seedling transmission (%) ^y
1	Pericarp	3.5×10^4 (2.3×10^4) ^z	78 (1.6)	82 (3.6)
2	Pericarp	2.8×10^4 (1.0×10^4)	74 (2.6)	77 (1.7)
3	Pistil	4.5×10^4 (1.8×10^4)	80 (3.3)	74 (2.5)
4	Pistil	1.8×10^4 (8.4×10^3)	72 (0.8)	75 (4.6)

^w 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.

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

^y Mean percentages of bacterial fruit blotch (BFB) seedling transmission for four replicates of seed sample ($n = 100$) from each lot. Seeds were planted in transparent, closed, plastic boxes on saturated blotter paper and incubated for 14 days at 28°C and 80% relative humidity. BFB seedling transmission was determined as the number of seedlings showing typical BFB symptoms divided by total number of seeds germinated × 100.

^z Numbers in parentheses denote standard error of the means.

semiselective media) were compared by factorial analysis using the Student's *t* test ($P < 0.05$) in SAS (version 8.1 for Windows; SAS Institute Inc., Cary, NC).

Effect of seed layer removal on BFB seedling transmission. To further confirm the effect of the pathway of invasion on *A. citrulli* localization in watermelon seeds, we investigated the effect of removing seed layers on BFB seedling transmission. Samples ($n = 100$ seeds/treatment) from pistil-inoculated and pericarp-inoculated lots were dissected as described above. Treatments in this study included (i) removal of the testae; (ii) removal of the testae and PE layer tissues; and (iii) intact seeds (positive control). Each seed was planted in a capped, sterile 10-ml test tube containing cheese cloth saturated with sterile deionized distilled water and maintained at 85% RH. Seeds were incubated at 28°C and 85% RH with continuous fluorescent light, and seed germination and BFB seedling transmission percentages were determined after 14 days. Germination and BFB seedling transmission percentages were compared by factorial analysis using the Student's *t* test ($P < 0.05$) in SAS (version 8.1 for Windows; SAS Institute Inc.). To confirm that putative BFB seedling symptoms were caused by *A. citrulli*, bacterial isolation was attempted from at least five symptomatic seedlings from each treatment. Small (2 to 4 mm²) pieces of symptomatic cotyledon tissue were macerated in 200 µl of sterile PBS and a loopful (≈20 µl) of macerate was streaked onto Nunhems medium. After incubation for 5 days at 28°C, putative *A. citrulli* colonies (round, red colonies with smooth margins) were tested by real-time PCR assay using an *A. citrulli*-specific TaqMan assay (10).

Visualization of *A. citrulli* in pericarp-inoculated and pistil-inoculated seeds. To visually observe the location of *A. citrulli* cells in seed tissues, five seeds from pericarp-inoculated and pistil-inoculated lots were arbitrarily selected and decoated. Seeds were decoated to facilitate fixation and infiltration of resins into tissues. Decoated seeds were fixed for 24 h in 1.6% (wt/vol) paraformaldehyde and 0.2% (wt/vol) glutaraldehyde in 25 mM sodium phosphate buffer at pH 7.1. Seeds were washed with sodium phosphate buffer (twice for 15 min each) and water (twice for 15 min each) and dehydrated using an ascending ethanol series (35, 50, 75, 85, 95, 100, 100, and 100% [vol/vol] ethanol) for 30 min at each step. The dehydrated tissue was gradually infiltrated with LR White embedding resin (Ted Pella Inc., Redding, CA) using 33% (vol/vol) and 66% (vol/vol) resin in 100% ethanol for 24 h each, followed by 100% resin for 24 h three times. For embedding, the resin-infiltrated seeds were transferred to gelatin capsules containing 100% resin and polymerized by incubation at 45°C for 24 h followed by 55°C for an additional 24 h. Seed sections (250 nm thick) were generated using an ultramicrotome (Leica EM UC6, Leica Microsystems, Buffalo Grove, IL) and mounted on glass slides (ColorFrost Plus; Fisher Scientific). For immunolabeling of *A. citrulli*, sections were blocked with 3% (wt/vol) non-fat dry milk in 0.01 M potassium phosphate, pH 7.1, containing 0.5 M NaCl (KPBS) for 1 h and washed with 10 mM KPBS three times for 5 min each. The primary antibody, polyclonal rabbit anti-AAC (39), was diluted 1:10 in 3% BSA in 10 mM KPBS and 10 µl was applied to the seed sections and incubated for 60 to 90 min. Sections were then washed with BSA-KPBS three times for 2 min each, and 10 µl of a 1:100 dilution of anti-rabbit immunoglobulin G (IgG) conjugated to Alexa-fluor 488 (Life Technologies Corp., Grand Island, NY) in BSA-KPBS was applied and incubated for 60 to 90 min. Finally, sections were washed with BSA-KPBS and then with distilled water for 5 min each. Before placing the coverslip on the sections, 10 µl of Citifluor antifade mounting medium AF1 (Electron Microscopy Sciences, Hatfield, PA) was applied. Separate seed sections were stained with 0.05% toluidine blue to visualize the basic cellular arrangement of watermelon seed tissues. The immunolabeled seed sections were observed with an Eclipse 80i microscope (Nikon Instruments Inc., Melville, NY)

equipped with epifluorescence optics. A Nikon DS-Ri1 camera was used to capture the images configured with a NIS-Elements Basic Research software. Images were subsequently assembled using Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

RESULTS

Seed lot characteristics. Pericarp-inoculated seed lots 1 and 2 displayed mean seed germination of 78 and 74%, respectively, and mean BFB seedling transmission of 82 and 76.5%, respectively (Table 1). The mean *A. citrulli* populations per seed for these lots were 3.5×10^4 and 2.8×10^4 CFU/seed, respectively (Table 1). The pistil-inoculated seed lots 3 and 4 displayed mean seed germination of 80 and 72%, respectively, and BFB seedling transmission of 74 and 75%, respectively (Table 1). *A. citrulli* populations were 4.5×10^4 and 1.8×10^4 CFU/seed for seeds from lots 3 and 4, respectively (Table 1). *A. citrulli* colonies were not recovered from seeds from the negative control lots whose stigmas were treated with PBS, and samples from these lots displayed 0% BFB seedling transmission (data not shown).

Localization of *A. citrulli* in watermelon seed. The expected amplicon was produced with 100% of the extracts from pericarp- and pistil inoculated seed samples that were spiked with *A. citrulli* DNA (data not shown) indicating no PCR inhibition. Overall, the tissue colonization results obtained by the real-time PCR assay were not significantly different to those obtained by plating on semiselective agar media ($P > 0.05$) (Table 2). For pericarp-inoculated seed lots, the real-time PCR assay indicated that 5.3% of the testae samples were contaminated with *A. citrulli* while 6.3% of testae samples from pistil-inoculated lots were positive (Table 2). This difference was not statistically significant ($P = 0.64$). With regards to PE layer tissues, 97% of the samples from pistil-inoculated seeds were contaminated with *A. citrulli* as compared with 80.3% for seeds from pericarp-inoculated seeds, as determined by the real time PCR assay (Table 2). For both seed infestation types, the percentage of *A. citrulli*-positive PE layer samples was significantly greater than the percentage of contaminated testa samples ($P = 0.002$). Additionally, the percentage of *A. citrulli*-positive PE layer samples was significantly greater for seeds from pistil-inoculated lots (97%) than for those from

TABLE 2. Comparison of *Acidovorax citrulli*-positive testae, perisperm-endosperm (PE) layer, and embryo samples from pericarp-infested and pistil-infested seed lots by real-time polymerase chain reaction (PCR) assay and plating on semiselective media

Seed sections	Pistil-infested (%)		Pericarp-infested (%)	
	Plating ^x	Real-time PCR ^y	Plating	Real-time PCR ^z
Testae	7.5 c	6.25 C	2.5 c	5.25 C
PE layers	77.5 b	97 A	95.0 a	80.25 B
Embryos	90 ab	94 AB	6.5 c	8.75 C

^x Mean percentage of *A. citrulli*-positive seed sections by plating on semiselective media. Fifty seeds per seed lot were dissected individually into testa, PE layer, and embryo tissues and assayed for *A. citrulli* by crushing individual seed sections in 0.1 M phosphate-buffered saline followed by spread plating on semiselective medium. Fifty seeds per lot of each infection type were used per experiment and the experiment was repeated twice. Means with similar lowercase letters are not significantly different according to factorial analysis by *t* test ($P < 0.05$).

^y Mean percentage of seed section tested positive for the bacterium by real-time PCR. Fifty seeds were dissected individually into testa, PE layer, and embryo tissues and assayed for the presence of *A. citrulli* by total microbial DNA extraction from individual sections followed by real-time PCR with *A. citrulli*-specific primers. Fifty seeds per lot of each infection type were used per experiment and the experiment was repeated twice.

^z Thirty fluorescence units were used as a threshold and the cycle at which fluorescence exceeded the threshold was used as the cycle threshold (CT) value. Samples with CT values below 35 were considered to be positive for *A. citrulli*. Means with similar uppercase letters are not significantly different according to factorial analysis by *t* test ($P < 0.05$).

pericarp-inoculated lots (80.3%) ($P = 0.0001$). The percentage of *A. citrulli*-positive embryo samples for seeds from pistil-inoculated lots (94%) was significantly greater than for seeds from pericarp-inoculated lots (8.8%) ($P = 0.0001$). While there was no significant difference ($P = 0.43$) between the percentage of *A. citrulli*-positive embryos (94%) and *A. citrulli*-positive PE layer samples (97%) for pistil-inoculated, for pericarp-infested seeds

Fig. 1. The effect of watermelon seed layer removal on seed germination percentage. Bars represent mean percentage of seed germination for pericarp-infested (symptomatic fruit) (lots 1 and 2) and pistil-infested (asymptomatic fruit) (lots 3 and 4) seeds in two independent experiments. Error bars represent the standard deviation of the mean germination percentages for two seed lots of each infection type in two experiments. **A**, Mean germination percentages for seeds with intact testae and perisperm-endosperm (PE) layers (control). **B**, Mean germination percentages for seeds with testae removed. **C**, Mean germination percentages for seeds with testae and PE layers removed. Means with similar uppercase (column-wise comparisons) and lowercase (row-wise comparisons) letters are not significantly different according to factorial analysis by *t* test ($P < 0.05$).

the percentage of infested embryos (8.8%) was significantly lower ($P = 0.0001$) than the percentage of infested PE layers (80.3%).

Effect of seed layer removal on germination and on BFB seedling transmission. Confirmation of the location of *A. citrulli* in seeds was sought by removing seed layers and assaying the seeds for BFB seedling transmission. We assumed that *A. citrulli* cells associated with each tissue would be eliminated as the seed layers were removed, thereby reducing BFB seedling transmission. The mean germination percentages for seeds with only the testae removed (78.5 and 75.7% for pericarp- and pistil-inoculated lots, respectively) were not significantly different to intact seeds (76.5 and 81.0% for pericarp and pistil-inoculated seeds, respectively) (Fig. 1A and B). However, the germination percentages for seeds from which the testae and PE layers were removed (45.5 and 51.0% for pericarp- and pistil-inoculated lots, respectively) were significantly lower than for intact seeds and seeds from which only the testae were removed (Fig. 1C). With regards to BFB seedling transmission, intact seeds from pericarp- and pistil-inoculated lots yielded seedlings with 76.3 and 75.0% BFB incidence, respectively (Fig. 2A). Testa removal did not have a significant effect on BFB seedling transmission for either seed inoculation method ($P = 0.74$). In contrast, the removal of the testae and PE layers from pericarp-inoculated seeds resulted in a statistically significant reduction in BFB seedling transmission (14.8%) compared with intact pericarp-inoculated seeds (76.5%) ($P < 0.0001$) and seeds with the testae removed (85%) ($P = 0.003$). This was not the case for pistil-inoculated lots, for which testa and PE layer removal did not have a significant effect ($P = 0.89$) on BFB seedling transmission (72%) relative to intact seeds or seeds with testae removed (Fig. 2). Finally, testa and PE layer removal from pericarp-inoculated seeds resulted in a statistically significant ($P = 0.003$) reduction in BFB seedling transmission relative to pistil-inoculated seeds treated in the same manner (Fig. 2). All seedlings that displayed putative BFB symptoms were confirmed as infected with *A. citrulli* by bacterial recovery on semiselective media and subsequent species-specific real-time PCR assays (data not shown).

Visualization of *A. citrulli* in pericarp- and pistil-inoculated seeds. Sections from pistil- and pericarp-inoculated watermelon seeds that were labeled with anti-rabbit IgG conjugated with Alexa-fluor 488 alone did not yield fluorescent signals. This indicated nonspecific binding of the secondary antibody to watermelon seed tissues did not occur (data not shown). Additionally, fluorescence was not observed in sections of negative control seeds from PBS-treated blossoms when labeled with the anti-*A. citrulli* primary antibody. Toluidine blue-stained seed sections (Fig. 3A and B) showed the PE envelope enclosing the cotyledons. In sections of pistil-inoculated seeds, *A. citrulli* cells were observed not only in the inner cells of the PE envelope, but also in the intercellular spaces of parenchyma tissue of the embryo (Fig. 3C). In pericarp-inoculated seeds, *A. citrulli* was observed only in the outer layer of PE envelope (Fig. 3D). *A. citrulli* cells were not observed in the embryo of pericarp-inoculated seeds.

DISCUSSION

Seed contamination by phytopathogens can occur systemically through the plant vasculature, via the pistils of flowers or via direct penetration of ovary walls (1). For phytopathogens, the most common pathway of seed infestation is through the pericarp of the ovary. For example, bacterial ingress through stomata on the pods of common bean (*Phaseolus vulgaris* L.) and pea (*Pisum sativum* L.) led to seed infestation by *Xanthomonas axonopodis* pv. *phaseoli* and *Pseudomonas syringae* pv. *pisi*, respectively (33,44). Similar observations were reported for *Clavibacter michiganensis* subsp. *michiganensis* in tomato (*Lycopersicon esculentum* L.) seeds and *Curtobacterium flaccumfaciens* subsp. *flaccumfaciens* in common bean (*Phaseolus vulgaris* L.) seeds (22,31).

Phytophthora that enter seeds by this pathway generally become deposited in the testa; but they may also become localized in endosperm and cotyledon tissues (8,35,36). Pistil invasion is the other pathway of seed infestation reported for phytophthora. Kauffman and Leben (18) reported that inoculation of soybean blossoms with *P. syringae* subsp. *glycinea* resulted in infested seeds within symptomless pods. Ark (2) reported that when walnut pollen, artificially contaminated with *X. juglandis* (causal agent of walnut blight), was transferred to female walnut blossoms the resulting walnuts were internally contaminated with the pathogen. Similarly, van der Wolf and vander Zouwen (37) reported that cauliflower blossom inoculation with *X. campestris* pv. *campestris*-contaminated flies led to internal seed infestation. However, to date the effect of these invasion pathways on the location of phytophthora in seeds has not been studied.

Both pistil and pericarp invasion of watermelon seed have been reported for *A. citrulli* (7,40). Frankle et al. (7) reported that watermelon fruit became infested with *A. citrulli* when bacteria penetrated open stomata on the ovary pericarp. This pathway of ingress was limited to immature fruits (2 to 3 weeks postanthesis) as stomata on older fruits became covered with wax that prevented bacterial penetration. Walcott et al. (40) reported that watermelon seed infestation by *A. citrulli* occurred by pistil invasion and resulted in contaminated seeds within asymptomatic fruits. Lessl et al. (24) subsequently reported a positive linear relationship between *A. citrulli* blossom inoculum dose and percentage of infested seed lots. Additionally, using a constitutive green fluorescent protein mutant, they observed that *A. citrulli* rapidly colonized the stigmas of female blossoms, penetrated through the transmitting tract tissues of the style and entered the ovaries by 7 days postinoculation (23). Based on these observations, we hypothesized that the pathway of seed infestation may influence the location of *A. citrulli* in seeds.

Unlike other seedborne phytophthora, the testa was not a major site of *A. citrulli* accumulation in infested watermelon seeds. *A. citrulli* was detected in relatively low percentages of testae compared with that in embryo and PE layer samples. Additionally, when the testae were removed from pericarp- and pistil-inoculated seeds, BFB seedling transmission percentage was not significantly affected. This indicated that regardless of the invasion pathway, *A. citrulli* cells that accumulate in the testae may not be epidemiologically significant, especially for seeds that have been stored for long periods of time. This was unexpected based on reports on other pathosystems where phytophthora predominantly survived in the testae (9,31,35). One possible explanation for this discrepancy is that in watermelon seed testae, *A. citrulli* cells are exposed to unfavorable conditions that cause populations to decline after harvest. These conditions are influenced by factors like variation in testae anatomy and phytophthora sensitivity to desiccation that may vary based on pathogen and plant species (32). For example, testae surface features can provide protective sites that help bacteria evade stress during storage. *Xanthomonas campestris* pv. *malvacearum* was reported to survive in cotton seed fibers that remained attached to the testae after delinting (36). Interestingly, we observed that even with seeds from pistil-inoculated lots, a low percentage of testae was contaminated with *A. citrulli*. This was unexpected because we hypothesized that pistil inoculation would deposit the bacterium in the seed embryo. However, Walcott et al. (40) previously observed that fruit pulp tissues also became contaminated with *A. citrulli* by pistil inoculation. Hence, *A. citrulli* cells present in watermelon pulp could subsequently become associated with testae, similar to seeds in pericarp-inoculated fruits.

Based on our observations the watermelon seed PE acts as a barrier to *A. citrulli* invasion and colonization of the embryo via pericarp-inoculation. Irrespective of the pathway of bacterial invasion, >78% of the PE layer samples was contaminated with *A. citrulli*. However, a significantly higher percentage of embryo

samples was contaminated with *A. citrulli* in pistil-inoculated (>90%) than in pericarp-inoculated seeds (<9%). Removal of testae and PE layers significantly reduced BFB seedling transmission in pericarp-inoculated seeds but not in pistil-inoculated seeds. Clearly, removal of the PE layer eliminated *A. citrulli* inoculum from pericarp-inoculated seeds but not from pistil-inoculated seeds, for which 90% of the embryos were contami-

Fig. 2. The effect of watermelon seed layer removal on bacterial fruit blotch (BFB) seedling transmission percentage. Bars represent mean BFB seedling transmission percentages for seeds infested by pericarp invasion (symptomatic fruit) (lots 1 and 2) and pistil invasion (asymptomatic fruit) (lots 3 and 4) in two independent experiments. Error bars represent the standard deviations of the mean BFB seedling transmission percentages for two seed lots of each infection type in two experiments. **A**, BFB seedling transmission percentages for seeds with intact testae and perisperm-endosperm (PE) layers (control). **B** and **C**, BFB seedling transmission percentages for seeds with testae removed and testae and PE layers removed, respectively. Means with similar uppercase (column-wise comparisons) and lowercase (row-wise comparisons) letters are not significantly different according to factorial analysis by *t* test ($P < 0.05$).

nated. As expected, germination was significantly reduced (<51%) for seeds from which the testae and PE layers were removed.

Further evidence for the role of the PE layer as a barrier to embryo contamination in pericarp-inoculated seeds was provided by immunofluorescence microscopy. Immunolabeled *A. citrulli* cells were observed in both the inner layers of the PE envelope and the intercellular spaces of parenchyma tissue of the cotyledons/embryos in pistil-inoculated seeds (Fig. 3C). In contrast, *A. citrulli* cells were not observed in the embryos of pericarp-inoculated seeds but on the surfaces of PE layers (Fig. 3D).

We also found that pistil-inoculation circumvented the PE layer and deposited *A. citrulli* cells in the embryos of watermelon

seeds. This conclusion was supported by the observation that significantly higher percentages of embryos were infested with *A. citrulli* in pistil-inoculated seeds compared to pericarp-inoculated seeds. Additionally, removal of PE layers and testae did not affect BFB transmission for pistil-inoculated seeds, while BFB transmission was significantly reduced for pericarp-inoculated seeds. At present, details on the specific pathways of *A. citrulli* ingress through watermelon blossom tissues are not available; however, bacterial deposition might be influenced by the timing of development of the different seed layers. To date, PE layer development has not been described in detail for watermelon seed, but in cucumber seeds the PE layer begins to form by 15 days post-

Fig. 3. Immunolocalization of *Acidovorax citrulli* cells in pistil-infested and pericarp-infested watermelon seed sections. **A and B**, Toluidine blue-stained cross-sections of a decoated pistil- and pericarp-infested watermelon seeds, respectively, highlighting the perisperm-endosperm (PE) envelope (P) and cotyledons (c). **C and D**, Immunolocalization of *A. citrulli* cells in pistil-infested and pericarp-infested seeds, respectively. The lower right corner image in **C** shows a magnified image of the highlighted field, which shows immunolabeled *A. citrulli* cells in the PE envelope (P) and in the intercellular spaces of parenchyma tissue of the cotyledons (c). White arrows in **D** show the localization of *A. citrulli* cells in the PE layer. Bar (100 μ m) in **A** is also applicable to **C**. Bar (100 μ m) in **B** is applicable to **D**.

anthesis (DPA) and matures by 35 to 45 DPA (25,41,42). At 15 DPA, a lipid layer differentiates in the epidermis of the four- to five-layered nucellus. An immature callose layer appears on the surface of the PE layer by 25 DPA, and by 35 DPA the lipid layer matures and a callose layer is deposited on the endosperm surface (41). At 45 DPA, the nucellar cells degrade to form a mature PE layer. While similar details on watermelon PE layer development are not available, preliminary experiments showed that inoculation of female watermelon blossoms with *A. citrulli* resulted in ovule contamination by *A. citrulli* within 24 h (data not shown). Since the PE layer is not fully developed by this time, it is possible that bacteria deposited in the ovary pericarp can gain access to the seed embryos. In contrast, Frankle et al. (7) showed that the window for *A. citrulli* to migrate through the pericarp of immature watermelon fruits is 14 to 21 DPA. If the bacterium invades the ovule at this stage, it is likely that the PE layer will serve as a barrier to embryo contamination.

Overall, our results suggest that the location of *A. citrulli* in watermelon seeds is influenced by the pathway of bacterial ingress. In cool, dry conditions that generally exist in commercial seed production fields, BFB fruit symptoms are not generally observed but infested seed lots are still produced (4,38). This suggests that pistil invasion might be involved in natural seed infestation by *A. citrulli*. While the PE layer might block bacterial penetration into the embryo in pericarp-inoculated fruits, the same is not true for seed from pistil-inoculated fruits. Using the two seed inoculation methods reported in this study, we can begin to investigate how bacterial localization influences *A. citrulli* survival in seeds. Insight gained from these studies may improve the management of BFB and other seedborne phyto-bacterial diseases. In particular, this information may lead to improved techniques for *A. citrulli* extraction from seeds for improved seed health testing and for developing improved methods for effectively decontaminating seeds.

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