Role of Blossom Colonization in Pepper Seed Infestation by *Xanthomonas euvesicatoria*

B. Dutta, R. Gitaitis, H. Sanders, C. Booth, S. Smith, and D. B. Langston, Jr.

Department of Plant Pathology, University of Georgia, Coastal Plain Experiment Station, Tifton 31793-0748.
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ABSTRACT

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Colonization of *Xanthomonas euvesicatoria* was investigated in pepper blossoms and the relationship between inoculum concentrations and seed infestation was determined. Inoculation of blossoms resulted in asymptomatic pepper fruit. However, real-time polymerase chain reaction detected *X. euvesicatoria* in 39% of the seed lots assayed and viable colonies were recovered from 35% of them. Successful transmission occurred in 16% of the seed lots tested. In a separate experiment, *X. euvesicatoria* reached populations of up to 1×10^5 CFU/blossom on stigmas 96 h after inoculation. Bacteria colonized stilar and ovary tissues

with populations ranging from 1×10^5 to 1×10^6 CFU/blossom 96 h after inoculation. A positive correlation existed between inoculum concentration and percentage of infested seedlots. Blossoms inoculated with *Acidovorax citrulli* also resulted in infested pepper seedlots. Furthermore, *A. citrulli* colonized pepper blossoms significantly better than *X. euvesicatoria* by 96 h postinoculation. It was concluded that pepper blossoms can be a potential site of ingress for *X. euvesicatoria* into seed, and blossom colonization may be involved in pepper seed infestation. Data also indicated that seed infestation via blossoms may be nonspecific because nonhost plants can be colonized by incompatible pathogens. Thus, host-pathogen interactions may not be important for bacterial ingress through blossoms.

Bacterial spot (BS) of pepper (*Capsicum annuum* L.) and tomato (*Solanum lycopersicum* L.) is caused by the BS-causing xanthomonads (BSX) *Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri* (13), and is an economically important disease of these crops worldwide (13,26). The classification of BSX has evolved over the years (10,12,26,29,30). Using polyphasic approaches of classification, the bacterium was classified into two genetically and phenotypically distinct groups (A and B) (26) which, later, Vauterin et al. (29) placed into two species; namely, *X. axonopodis* pv. *vesicatoria* (A) and *X. vesicatoria* (B) (29). Although *X. axonopodis* pv. *vesicatoria* group A strains were unable to or weakly hydrolyzed starch (amylolytic activity), *X. vesicatoria* group B strains hydrolyzed starch and were pectinolytic (12). Jones et al. (10,12,13) further reported two other BSX strains (C and D) from tomato that differed from those in group A and B. In addition, the strains from groups A, C, and D had <70% DNA relatedness with each other, and also with the type strain of *X. axonopodis*. As a result, the authors renamed A, C, and D groups as *X. euvesicatoria*, *X. perforans*, and *X. gardneri*, respectively, whereas strains from group B were retained as *X. vesicatoria*. The *X. euvesicatoria* species designation was given to only the original strains that were weakly amylolytic and originally identified by Doidge (12). Further multi-locus sequence analysis and amplified fragment length polymorphism studies showed close-relatedness between *X. euvesicatoria* and *X. perforans*, and *X. gardneri* and *X. cynarae* (9,34). The strains belonging to *X. euvesicatoria*, *X. vesicatoria*, and *X. gardneri* are pathogenic on both tomato and pepper whereas strains of *X. perforans* are pathogenic only on tomato (12).

Bacterial spot of pepper is characterized by necrotic lesions on leaves, stems, and fruit. The symptoms on leaves start as water-

soaked spots that gradually turn into necrotic-angular lesions of 1.6 to 6.4 mm in diameter. The necrotic spots have light gray centers with dark margins surrounded by yellow haloes. In severe cases, tissues in the center of a lesion become dry and fall out, leading to "shot-hole" symptoms (24). Leaves with numerous necrotic spots usually cause premature leaf abscission. Narrow, elongated, raised cankers may occur on stems and, over time, become rough and light brown in color. Symptoms on fruit start with small, pale, water-soaked spots that gradually turn into dark-brown, raised, necrotic lesions. Symptomatic fruit are not suitable for market. Under favorable conditions, BSX can cause yield losses up to 100% (24).

X. euvesicatoria is a seedborne pathogen, and infested pepper seed serve as a main source of primary inoculum in transplant and fruit production systems (11,15). Once the pathogen is introduced into greenhouses, high temperatures, high plant density, and high humidity favor disease development. The spread of disease is further facilitated by overhead irrigation. Hence, the use of pathogen-free seed and transplants is one of the important disease management strategies for BS, apart from host resistance (20) and copper-based bactericides (11,17). Seed health testing and seed treatments are routinely employed to exclude seedborne inoculum. In seed health testing, a minimum of 2,000 (protected cultivation in temperate conditions) or 30,000 (outdoor cropping in humid and warm conditions) seeds are usually tested to detect seed infestation levels of 0.15 and 0.01%, respectively, with 95% reliability (8). Despite these efforts, sporadic outbreaks of BS due to seedborne *X. euvesicatoria* inoculum still occur in different parts of the world. Hence, better disease management is required in seed-production fields which, in turn, requires good knowledge of mechanisms of pepper seed infection by *X. euvesicatoria*.

At present, it is not clear how pepper seed become infested with *X. euvesicatoria*. Maude (18) suggested three general pathways by which seed can become infested by phytopathogenic bacteria. First, ingress of pathogen can occur through fruit surface infections, resulting in seed infestation. Bashan et al. (2) success-

Corresponding author: B. Dutta; E-mail address: bhabesh@uga.edu

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fully demonstrated that infected immature pepper fruit can be an avenue of bacterial ingress into seed. Artificial inoculation of fruit showed that the bacterium can penetrate deep into the fruit and infest pepper seed under greenhouse conditions. These researchers also showed that the bacterial multiplication occurred on the warts (small protuberance) of developing fruit. Second, systemic movement of pathogens from infected mother plants to developing seed may also cause seed infestation. However, no evidence exists for the systemic migration of *X. euvesicatoria* in pepper plants (2). Finally, seed invasion by plant pathogens can also occur through the ingress of floral structures (5,6,16,28). Bacterial invasion through the floral structures leading to seed infestation has been demonstrated in a number of different phytopathogenic bacterial pathosystems. For example, inoculation of blossom stigmas led to infestation of seed within symptomless fruit with both *Pseudomonas syringae* subsp. *glycinea* (bacterial blight of bean) (14) and *X. campestris* pv. *campestris* (black rot of cauliflower) (28). Blossom invasion leading to seed infestation has also been documented with *Acidovorax citrulli* (bacterial fruit blotch of cucurbits), where inoculation of female watermelon blossoms resulted in infested seed within symptomless watermelon fruit (5,16,31). However, in the case of the *X. euvesicatoria*–pepper pathosystem, neither the ingress of the pathogen through blossoms nor the ability of bacteria to colonize blossoms has been explored. The objectives of this research were to (i) determine the role pepper blossoms in seed infestation by *X. euvesicatoria*, (ii) determine whether *X. euvesicatoria* is able to colonize pepper floral tissues, (iii) compare the temporal population dynamics of *X. euvesicatoria* with *A. citrulli* (a known blossom-colonizing pathogen of watermelon) during pepper blossom colonization, and (iv) investigate the relationship between *X. euvesicatoria*–blossom inoculum density and percentage of infested seedlots.

MATERIALS AND METHODS

Bacterial strains and inoculum preparation. Bacterial strains of *X. euvesicatoria* and *A. citrulli* used in this study are described in Table 1. *X. euvesicatoria* and *A. citrulli* strains were routinely grown on yeast extract-dextrose–CaCO₃ (YDC) or nutrient agar (NA) media (Becton-Dickinson, Sparks, MD) for 48 h at 28°C. The identity of *Xanthomonas* strains as *X. euvesicatoria* was ascertained by species-specific polymerase chain reaction (PCR) assay, as described by Moretti et al. (21). In addition, the strains did not display amylolytic and pectinolytic activities on respective differential media but did utilize cis-aconitic acid, which were the diagnostic phenotypic characteristics of this species (12). Inoculum was prepared by transferring single colonies of each bacterial strain from 48-h-old cultures on NA or YDC media to nutrient broth. This was followed by overnight incubation at 28°C on a rotary shaker (Innova; New Brunswick Scientific Co., Edison, NJ) at 150 rpm. After overnight incubation, bacterial cultures were centrifuged at 5,000 × *g* (Allegra 25R; Beckman Coulter, Fullerton, CA) for 3 min and the supernatant was decanted, leaving a pellet of bacterial cells. The pellet was resuspended in 0.1 M phosphate (KH₂PO₄–K₂HPO₄)-buffered saline (0.75% NaCl) solution at pH 7.0 (PBS) and the concentration of bacterial cells was adjusted using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY) to an optical density of 0.3 at 600 nm (≈1 × 10⁸ CFU/ml). This was serially diluted in PBS to obtain

desired concentrations of ≈1 × 10⁶ and ≈1 × 10² CFU/ml. The strains of *A. citrulli* (AAC 00-1 and AAC 94-24) used in this study produced hypersensitive reactions on bell-pepper leaves (‘Aristotle’) upon inoculation with 1 × 10⁸ CFU/ml. This confirmed that *A. citrulli* strains used in this study were incompatible with bell-pepper cultivar used.

Effect of blossom inoculation on pepper seedlot infestation by *X. euvesicatoria*. In two independent trials, 50 bell-pepper plants (‘Aristotle’) per trial were grown in 10-liter plastic pots containing commercial potting mix (Scotts Miracle-Gro Company, Marysville, OH). Pots were spaced evenly on benches under greenhouse conditions of 25 to 30°C, a mean relative humidity (RH) of 76%, and 12 h of natural light daily. Plants were fertilized every 2 weeks with Osmocote smart-release plant food (14-14-14) (Scotts Miracle-Gro Company, Marysville, OH) and sprayed with Enstar II (Zoecon, Palo Alto, CA), Merit (Bayer, Montvale, NJ), and Avid (Syngenta, Greensboro, NC), as needed, to control whiteflies. Pepper plants were maintained under greenhouse conditions for ≈90 days. At anthesis, 10 µl of inoculum containing ≈1 × 10⁸ *X. euvesicatoria* (strain Xcv 04-100) CFU/ml (≈1 × 10⁶ CFU/blossom) were deposited onto the stigmas. At least 10 blossoms per plant were inoculated and left to mature for 40 to 45 days. Fruit were harvested at maturity and seed were extracted manually. Seedlots generated by inoculating pepper blossoms with 0.1 M PBS served as a negative control. For seed extraction, fruit were surface sterilized by wiping with 70% ethanol and excised with a sterilized knife. Seed were extracted and air dried in sterile petri dishes with partially open lids under a laminar air flow chamber for 72 h. Seed from individual fruit were maintained as a separate seedlot and stored in paper bags at 4°C.

For detection, individual pepper seedlots were divided into two samples of equal weight (2 g, ≈150 seeds/seedlot) and tested for *X. euvesicatoria* by (i) seed maceration followed by plating and PCR and (ii) seedling grow-out (SGO), as described below.

Seed maceration followed by plating and PCR. In the seed maceration procedure, seed (*n* = 150 per seedlot) were placed in an extraction bag (15 by 28.5 cm) (Bioreba, Reinach, Switzerland) and homogenized (without using buffer) using a tissue homogenizer (Homex 6; Bioreba). Seed macerates were suspended in 10 ml of PBS in the extraction bag and mixed carefully by gently shaking. The resulting suspensions (10 ml) were transferred to 50-ml polypropylene screw-cap tubes. Macerates were vortexed for 30 s and centrifuged at 8,000 × *g* for 4 min. After centrifugation, the supernatant was decanted and the pellet was resuspended in 1 ml of PBS by vortexing. Seed extracts were divided into two fractions (0.9 and 0.1 ml). Total microbial genomic DNA was extracted from the 0.9-ml fraction using an UltraClean Microbial DNA Kit (MO BIO, Carlsbad, CA) according to the manufacturer’s instructions. Extracted DNA was subjected to SYBR-green-based real-time PCR using *X. euvesicatoria*-specific primers (21). For real-time PCR, 5 µl of DNA was amplified in 25 µl of PCR master-mix containing 12.5 µl of SsoFast EvaGreen Supermix (Bio-Rad Laboratories Inc., Hercules, CA), and 1 µl each of 25 µM primers Xeu2.4 (CTG GGA AAC TCA TTC GCA GT) and Xeu2.5 (TTG TGG CGC TCT TAT TTC CT) (21) were used per sample. Real-time PCR was carried out in a Cepheid Smart Cyclyer (Sunnyvale, CA) using the following thermal profile: denaturation at 94°C for 180 s and 35 cycles each of denaturation at 94°C for 45 s, annealing at 64°C for 50 s, and extension at 72°C for 50 s. Samples with cycle

TABLE 1. Identity, origin, and source of bacterial strains used in this study

Bacterium	Strain	Host	Inoculum source	Source	Reference
<i>Xanthomonas euvesicatoria</i>	XCV04-100	Pepper	Leaves	R. Gitaitis, University of Georgia	This study
<i>X. euvesicatoria</i>	XCV98-3	Pepper	Leaves	R. Gitaitis, University of Georgia	This study
<i>Acidovorax citrulli</i>	AAC00-1	Watermelon	Fruit	R. Walcott, University of Georgia	6,31
<i>A. citrulli</i>	AAC94-24	Watermelon	Seed	R. Gitaitis, University of Georgia	31

threshold (Ct) values <35 were considered positive for *X. euvesicatoria*. Samples of seed extract from negative control seedlots were also processed as described above. To test for PCR inhibition in samples that yielded negative results, *X. euvesicatoria* genomic DNA at 10 ng/μl was added to seed macerates from five negative control lots (blossom-inoculated with PBS), which were then processed separately and assayed by real-time PCR, as described above.

The 0.1-ml aliquot of seed extract was serially diluted 9:1 in PBS and spread plated in triplicate onto YDC agar. Dishes were incubated for 3 days at 28°C and *X. euvesicatoria* colonies (yellow, round, mucoid colonies with smooth margins) were enumerated and recorded. For further confirmation of identity, genomic DNA from two colonies per isolation was extracted and amplified with SYBR-green-based real-time PCR using *X. euvesicatoria*-specific primers, as described above. Seed extracts from negative control seedlots generated by inoculating blossoms with PBS were processed as described above. The mean percentages of *X. euvesicatoria*-positive seedlots detected by each detection assay were determined.

SGO assay. SGO assays were conducted using seed samples ($n = 150$ seeds per seedlot) planted in transparent plastic boxes on two layers of blotter paper saturated with sterile water for 18 days under conditions of >90% RH and 28°C under continuous fluorescent light. After 18 days, percent germination (number of seedlings germinated/total number of seeds planted $\times 100$) and percent BS-positive lots (ratio of number of lots with at least one seedling with BS symptoms [seedlings displaying water-soaked lesions that gradually turned necrotic]/total number of lots planted $\times 100$) were determined. Seed samples from negative control lots were also evaluated by SGO assay, as described above. To confirm putative BS symptoms on seedlings, isolations were conducted from 10 symptomatic seedlings/lot. Isolations were made from small ($\approx 4\text{-mm}^2$) pieces of leaf tissue along lesion margins. Tissues were excised and chopped in 100 μl of PBS followed by streaking on YDC medium. After 5 days of incubation at 28°C, yellow-colored colonies were tentatively identified as *X. euvesicatoria* colonies. Confirmation of bacterial identity was achieved by extracting genomic DNA from two colonies per isolation and amplifying them with a SYBR-green-based real-time PCR assay using *X. euvesicatoria*-specific primers, as described above.

The study was repeated two times and analysis of variance was conducted to determine the significance of the blossom treatments on pepper seedlot infestation with *X. euvesicatoria* by plating, real-time PCR, and SGO assay by General Linear Model of the SAS (version 9.2 for Windows; SAS Institute Inc., Cary, NC) package. Additionally, mean percentage of *X. euvesicatoria*-seedlots detected by detection assays were compared using Fischer's least significant difference (LSD) test ($P < 0.05$).

Temporal bacterial population dynamics on pepper blossoms. To investigate the temporal population dynamics of *X. euvesicatoria* in pepper blossoms, 25 mature pepper plants were established under greenhouse conditions and maintained as described above. At anthesis, 15 to 20 blossoms per plant were inoculated with two strains *X. euvesicatoria* (Xcv 04-100 and Xcv 98-3). A 10-μl suspension containing 1×10^4 CFU/ml (1×10^2 CFU/blossom) of each strain was deposited onto the stigma of pepper blossoms. Each inoculation was marked by tagging. At each sampling period (0, 6, 12, 24, 48, and 96 h postinoculation [hpi]), five inoculated blossoms were harvested and their petals were removed. Using a sterile scalpel, stigma and style were excised together from the pepper blossom and placed in a 1.5-ml microcentrifuge tubes which were then macerated with a disposable plastic pestle (ACT Gene AgileGrinder portable disposable plastic pestle; Thomas Scientific, Swedesboro, NJ). Tissue macerates were suspended in 1 ml of PBS and vortexed vigorously for 1 min. Pepper blossoms inoculated with *A. citrulli* (causal

agent of bacterial fruit blotch of cucurbits) strains (AAC 00-1 and AAC 94-24) were used for comparison and processed as described above. Then, 10-fold serial dilutions of tissue macerates were made in 1 ml of PBS and 100-μl aliquots were spread plated onto Tween B (25) and Nunhem's proprietary semi-selective media for *X. euvesicatoria* and *A. citrulli*, respectively. After 4 days of incubation at 28°C, *X. euvesicatoria* and *A. citrulli* colonies were enumerated and recorded from each treated blossom at each sampling point. Pepper blossoms inoculated with PBS and processed as described above served as a negative control.

The experiment was repeated twice and plots of $\log_{10}$ *X. euvesicatoria* and *A. citrulli* CFU per blossom against time were generated and used to calculate area under the growth progress curve (AUGPC) as follows: $\sum_{i=1}^n [(Y_{i+1} + Y_i)]/2[X_{i+1} - X_i]$, where Y_i = bacterial population at the i th observation, X_i = time in hours at the i th observation, and n = the total number of observations. Analysis of variance was conducted on the AUGPC values to determine significance of treatment effects on blossom colonization, and Fischer's LSD test ($P < 0.05$) was used for mean separation.

Comparison of bacterial colonization of stigma, style, and ovary tissues of pepper blossoms. A second greenhouse study was conducted to determine the ability of *X. euvesicatoria* and *A. citrulli* to colonize different pepper-floral tissues (stigma, style, and ovary). Fifty mature pepper plants were established under greenhouse conditions and, at anthesis, stigmas of 15 to 20 female blossoms per plant were inoculated with bacterial suspensions. Treatments included (i) 10 μl of 1×10^4 *X. euvesicatoria* (Xcv 04-100) CFU/ml ($\approx 1 \times 10^2$ CFU/blossom), (ii) 10 μl of 1×10^4 *A. citrulli* (AAC 00-1) CFU/ml ($\approx 1 \times 10^2$ CFU/blossom), and (iii) 10 μl of PBS as a negative control. At each sampling period (0, 6, 12, 24, 48, and 96 hpi), five inoculated blossoms per treatment were harvested and processed as follows. The petals from individual blossoms were removed and, using a sterile scalpel and forceps, stigma, style, and ovary were excised. Excised floral tissues were placed individually in separate 1-ml microcentrifuge tubes and macerated using disposable plastic pestles. Tissue macerates were suspended in 1 ml of PBS and agitated vigorously (Vortex Genie 2; Fisher Scientific International, Fairlawn, NJ) for 1 min. To determine bacterial populations recovered from floral tissues, 10-fold serial dilutions were made in 1 ml of PBS followed by spread plating 100-μl aliquots onto Tween B (25) and Nunhem's proprietary media for *X. euvesicatoria* and *A. citrulli*, respectively. After 4 days of incubation at 28°C, *X. euvesicatoria* and *A. citrulli* colonies were enumerated and recorded from each treated blossom from each sampling period. The experiment was repeated twice and plots of $\log_{10}$ of the mean number of bacterial CFU per blossom against time were generated and used to calculate AUGPC values. Analysis of variance was conducted on the AUGPC values to determine significance of treatment effects on bacterial growth in stigma, style, and ovary tissues, and Fischer's LSD test ($P < 0.05$) was used for mean separation.

Relationship between *X. euvesicatoria*-blossom inoculum dose and seed infestation. One hundred pepper plants ('Aristotle') were established in the greenhouse as described previously. At anthesis, stigmas of 15 to 20 blossoms per plant were inoculated separately with different levels of *X. euvesicatoria* inoculum (0, 1×10^1 , 1×10^3 , 1×10^5 , and 1×10^7 CFU/blossom) as described above. Each inoculum level was applied to the blossoms of 20 individual plants. Inoculated blossoms were allowed to develop into fruit for 40 to 45 days. At harvest maturity, fruit were harvested and seed from individual fruit were extracted as described above. Seed from individual pepper fruit was maintained as separate seedlots and stored at 4°C.

Seed from each lot per inoculum concentration were divided into two samples of 2 g each ($n = 150$ seeds per seedlot) and subjected to the detection assays (PCR and SGO) for *X. euvesicatoria*, as described above. Percentages of *X. euvesicatoria*-

infested seedlots detected by PCR and plating for each blossom inoculum dosage were determined.

For SGO, seedlings were evaluated for BS symptoms at 18 days after planting, as described above. A seedlot was considered to have transmitted the disease when the inoculated seedling in a tray developed typical BS symptoms. *X. euvesicatoria* isolations from suspected seedlings were performed and bacterial colonies were assayed for *X. euvesicatoria* by real-time PCR, as described above. Percentages of infested seedlots (ratio of number of lots with at least one seedling with BS symptoms/total number of lots planted $\times$ 100) from each blossom inoculum concentration-treatment by SGO were determined. Additionally, within an individual lot, mean seed germination (ratio of seedlings germinated/total number of seeds planted $\times$ 100) and mean BS transmission (ratio of number of seedlings with BS symptoms/total number of seedlings germinated $\times$ 100) were also determined.

The relationship between *X. euvesicatoria* blossom inoculum load and percentage of *X. euvesicatoria*-infested seedlots detected by PCR, plating, and SGO was determined by regression analysis using SigmaPlot (version 10.0; Systat, San Jose, CA). The regression analysis was based on means from the two experiments after a preliminary analysis of variance (PROC GLM in SAS, version 9.2; SAS Institute) showed no significant effect of the experiment factor at $P = 0.05$. Regression analysis was also used to determine the relationship between blossom inoculum concentrations and the mean percent BS seedling transmission.

RESULTS

Effect of blossom inoculation on pepper seedlot infestation by *X. euvesicatoria*. In two separate greenhouse trials, 95 and 98 fruit were produced from the blossoms that were treated with *X. euvesicatoria* (Xcv 04-100) and PBS, respectively. BS symptoms were not observed on fruit that were developed from either the *X. euvesicatoria*- or the PBS-inoculated blossoms. However, *X.*

Fig. 1. Percentage of *Xanthomonas euvesicatoria*-infested pepper seed lots that developed in fruit from blossoms-inoculated with 1×10^6 CFU of Xcv 04-100 determined by real-time polymerase chain reaction (PCR), plating, and seedling grow-out (SGO) assays. Samples of seed ($n = 150$ seeds/seed lot) were macerated followed by **A**, plating on yeast extract-dextrose- CaCO_3 (YDC) agar medium or **B**, real-time PCR assay using species-specific primers. For SGO assay, seed samples ($n = 150$ /seedlot/treatment) were planted on two layers of blotter paper saturated with sterile water for 18 days under conditions of $>90\%$ relative humidity at 28°C under continuous fluorescent light. Seedlings were evaluated for bacterial spot symptoms at 18 days after planting. A seedlot was considered to have transmitted the disease when the inoculated seedling in a tray developed typical bacterial spot symptoms. Each bar represents the mean incidence of infested seed lots for two independent experiments and the lines indicate the standard errors of the means.

euvesicatoria was detected by PCR from 39% (37/95) of the seedlots from fruit that developed from *X. euvesicatoria*-inoculated blossoms (Fig. 1). The bacterium was not detected from any lots (0/98) inoculated with PBS. *X. euvesicatoria* was detected in 100% of the seed extracts that were spiked with *X. euvesicatoria* DNA (data not shown), indicating that PCR inhibition did not occur. The viable *X. euvesicatoria* colonies were recovered from 35% (33/95) of the seedlots tested (Fig. 1). In all, 100% of the colonies assayed were identified as *X. euvesicatoria* by PCR. In total, 16% (15/95) of the seedlots yielded seedlings expressing BS symptoms when planted under conditions of 28°C and $>90\%$ RH (Fig. 1). Additionally, 100% of the seedlings that displayed putative BS symptoms were confirmed as infected with *X. euvesicatoria* by bacterial recovery on YDC medium and subsequent PCR assays. As expected, regardless of the detection assay, *X. euvesicatoria* was not detected from seedlots that were developed from PBS-inoculated blossoms. Overall, blossom inoculations significantly affected pepper seed infestation by *X. euvesicatoria* ($P = 0.001$) as determined by PCR, plating, and SGO assays. Additionally, significantly higher percentages of blossom-inoculated seedlots were detected by PCR and plating than by SGO.

Temporal bacterial population dynamics on pepper blossoms. *X. euvesicatoria* and *A. citrulli* CFU were not recovered from blossoms that were inoculated with PBS. *A. citrulli* was used for comparison because it is a seedborne bacterium of watermelon that infests seed via female watermelon blossoms, which is one of the modes of seed infestation. Additionally, *A. citrulli* is a successful blossom colonizer and known to colonize blossoms within 96 hpi (16). The populations of *X. euvesicatoria* strains (Xcv 04-100 and Xcv 98-3) increased exponentially and reached a population of 1.8×10^5 CFU/blossom at 96 hpi (Fig. 2). In contrast, during the same period, populations of *A. citrulli* strains (AAC 00-1 and AAC 94-24) increased to populations that were 100-fold higher (1.5×10^7 CFU/blossom) (Fig. 2). Based on AUGPC values, significant differences were not observed within *X. euvesicatoria* and *A. citrulli* strains ($P \geq 0.153$) for their ability to colonize pepper blossoms. However, between bacterial genera, AUGPC values for *A. citrulli* strains were significantly higher than AUGPC values for *X. euvesicatoria* strains ($P < 0.05$). Hence, strains Xcv 04-100 and AAC 00-1 were used in further studies.

Fig. 2. Temporal dynamics of *Xanthomonas euvesicatoria* (Xcv 04-100 and Xcv 98-3) and *Acidovorax citrulli* (AAC 00-1 and AAC 94-24) populations on pepper blossoms during 96 h postinoculation (hpi). Five blossoms per treatment were sampled at 0, 6, 12, 24, 48, and 96 hpi and populations of *X. euvesicatoria* and *A. citrulli* were enumerated on Tween B and Nunhem's media, respectively. Data points represent the mean bacterial populations at each sampling period in two independent experiments. Bars indicate the standard error of the mean.

Comparison of bacterial colonization of stigma, style, and ovary tissues of pepper blossoms. As expected, *X. euvesicatoria* and *A. citrulli* CFU were not recovered from the stigma, style, and ovary tissues of PBS-inoculated blossoms at all sampling periods. *X. euvesicatoria* and *A. citrulli* populations were recovered immediately after inoculation from the stigma tissues whereas both bacterial species were detected from the style and ovary tissues only after 6 and 12 h, respectively. In stigmas, populations of *X. euvesicatoria* and *A. citrulli* increased to 3.9×10^5 and 7.42×10^7 CFU/stigma, respectively, after 96 hpi (Fig. 3). Based on AUGPC values, *A. citrulli* populations were significantly higher than *X. euvesicatoria* populations ($P = 0.032$). Despite the lag in bacterial growth (for both bacteria) during the first 0 to 12 h in the style and ovary tissues, *X. euvesicatoria* and *A. citrulli* populations reached levels of 2.6×10^5 and 1.8×10^5 CFU, respectively, in both tissues at 96 hpi (Fig. 3). The trend in bacterial

Fig. 3. Temporal dynamics of *Xanthomonas euvesicatoria* (Xcv 04-100) and *Acidovorax citrulli* (AAC 00-1) populations on stigma, style, and ovary tissues of pepper blossoms during 96 h postinoculation (hpi). **A**, Stigma; **B**, style; and **C**, ovary tissues of five inoculated blossoms were sampled at 0, 6, 12, 24, 48, and 96 hpi and populations of *X. euvesicatoria* and *A. citrulli* were enumerated on Tween B and Nunhem's media, respectively. Data points represent the mean bacterial populations in each floral tissues enumerated in two independent experiments. Bars indicate the standard error of the mean values.

growth for both bacteria was not different in these tissues because AUGPC values for *X. euvesicatoria* and *A. citrulli* were not significantly different ($P = 0.016$).

Relationship between *X. euvesicatoria*-blossom inoculum dose and seed infestation. In two experiments, 350 seedlots were produced from symptomless pepper fruit. All concentrations of *X. euvesicatoria* inoculated in pepper blossoms led to seed infestation and BS transmission. On average, seedlots from blossoms inoculated with 0, 1×10^1 , 1×10^3 , 1×10^5 , and 1×10^7 CFU/blossom of *X. euvesicatoria* led to seed infestation of 0, 14.3, 38.1, 52.4, and 66.7% of the seedlots, respectively, as determined by real-time PCR (Fig. 4). When samples from the same lots were plated, viable colonies of *X. euvesicatoria* were detected from 0, 4.8, 33.3, 47.6, and 57.1% of the seedlots for the blossoms treatment with 0, 1×10^1 , 1×10^3 , 1×10^5 , and 1×10^7 CFU/blossom, respectively (Fig. 4). Additionally, blossom inoculations of 0, 1×10^1 , 1×10^3 , 1×10^5 , and 1×10^7 CFU/blossom produced 0, 2, 12, 23, and 32% of the infested seedlots, respectively, as determined by SGO assay (Fig. 4). A strong linear relationship between *X. euvesicatoria* blossom inoculum concentrations and percentage of infested seedlots was observed regardless of the detection assays used. The regression statistics generated by the PCR, plating, and SGO assays were $R^2 \geq 0.98$, $P \leq 0.002$; $R^2 \geq 0.96$, $P \leq 0.001$; and $R^2 \geq 0.94$, $P \leq 0.003$, respectively (Fig. 4).

BS seedling transmission in individual blossom-inoculated seedlots varied with different *X. euvesicatoria* inoculum concentrations. The mean BS transmission from seedlots generated by blossom inoculation with 1×10^1 , 1×10^3 , 1×10^5 , and 1×10^7 CFU/blossom was 2.8, 11.2, 27.3, and 42.5%, respectively. As expected, disease transmission did not occur in any of the seedlots developed from PBS-inoculated blossoms. The relationship be-

Fig. 4. Linear regression analysis for the relationship between *Xanthomonas euvesicatoria* (Xcv 04-100) populations applied to pepper blossoms and mean percent seedlot infestation as determined by real-time polymerase chain reaction (PCR), plating, and seedling grow-out (SGO) assay. Seed samples ($n = 150$ /seedlot/treatment) developed from blossoms inoculated with *X. euvesicatoria* populations (0, 1×10^1 , 1×10^3 , 1×10^5 , and 1×10^7 CFU/blossom) were assayed for *X. euvesicatoria* by real-time PCR, plating, and SGO assay. For real-time PCR and plating, seed samples were macerated followed by plating on Tween B agar medium or real-time PCR assay using species-specific primers. For SGO assay, seed samples ($n = 150$ /seedlot/treatment) were planted on two layers of blotter paper saturated with sterile water for 18 days under conditions of >90% relative humidity at 28°C under continuous fluorescent light. Seedlings were evaluated for bacterial spot symptoms at 18 days after planting. A seedlot was considered to have transmitted the disease when the inoculated seedling in a tray developed typical bacterial spot symptoms. Data point represents mean percentage of *X. euvesicatoria*-infested seedlots determined in two independent experiments. Bars indicate the standard error of the mean.

tween blossom inoculum dosage and mean percentage of BS transmission was linear ($R^2 = 0.95$; $P < 0.001$) (Fig. 5).

DISCUSSION

Several researchers have demonstrated in different seedborne-bacterial pathosystems that inoculation of blossoms led to seed infestation within symptomless fruit (5,6,14,16,31). Similarly, in our study, pepper blossom inoculations with *X. euvesicatoria* did not lead to BS symptoms on fruit but produced infested seed. In total, 100% of the fruit that developed from blossoms inoculated with *X. euvesicatoria* were symptomless. Despite lack of fruit symptoms, $\leq 35\%$ of the seedlots were detected positive for *X. euvesicatoria* by both real-time PCR and plating assay (Fig. 1). In contrast, the bacterium was detected from only 16% of the seedlots by the SGO assay. However, the seed-to-seedling transmission percentages in individual lots varied widely (5 to 64%). This phenomenon was expected because seedling transmission depends on a number of factors, including inoculum density per seed, distribution of inoculum within a seedlot, host susceptibility, and environmental factors (8). Among these, pathogen population per seed is of paramount importance. The results also suggest that blossom inoculations led to non-uniform seed infestation; as such, some seed were infested with low bacterial populations. Seed with low bacterial populations could be well below the transmission threshold (minimum pathogen population required for disease transmission). Decline in bacterial populations can also occur during seed extraction and seed processing steps, leading to non-uniform seed infestation. Additionally, it is possible that incubation periods used in this study for the SGO assay were not sufficient for those seed with low bacterial populations to develop symptoms; hence, a longer incubation period may be required for more consistent disease transmission results. Nevertheless, the results showed that BS seedling transmission can occur from infested pepper seed developed by blossom inoculation. In zero-tolerance environments such as transplant houses, such seedlots stemming from blossom infestations may be of significant epidemiological importance.

Although we confirmed that pepper blossoms can be a pathway for seed infestation, it is still unclear how bacteria gain access to the seed. It is possible that pollen germ tubes can act as conduits for bacterial delivery to developing ovules. This process may occur during directed growth of pollen germ tubes toward the ovules. This mechanism of bacterial invasion and its association with pollen germ tubes has been reported in *A. citrulli*-watermelon pathosystem (5). A recent study (5) showed that *A. citrulli* cells were able to co-localize with pollen germ tubes in the stigma, style, and ovary tissues of the watermelon. Such a phenomenon may also be occurring in this system. Future research should be conducted to determine the role of pollen germ tubes in blossom invasion by *X. euvesicatoria*.

In this study, it was demonstrated that inoculations of pepper blossoms with 1×10^6 CFU/blossom resulted in infestations of pepper seed. However, it is unlikely that such high levels of bacteria occur under natural seed-production conditions. Hence, for effective seed infestation via blossoms to occur under normal seed-production conditions, it is likely that *X. euvesicatoria* must colonize blossom tissues, a facet which has not been investigated. This study showed that *X. euvesicatoria* could colonize pepper blossoms reaching populations of 1×10^5 CFU/blossom by 96 hpi. Moreover, variability within *X. euvesicatoria* strains was not observed because both strains tested could colonize pepper blossoms equally well and, as a result, strain Xcv 04-100 was used for further studies. However, further blossom colonization studies with more *X. euvesicatoria* strains along with the strains of other *Xanthomonas* spp. that infect pepper are required to strengthen this claim. Bacterial growth on the stigma can be supported by copious amount of stigma secretions (nutrients like carbohy-

drates, lipids, and proteins) released during pollination. This has been well established with *A. citrulli* and *Erwinia amylovora*, where bacteria that colonized flowers reached populations of 1×10^7 to 1×10^8 CFU/blossom without inciting disease symptoms (16,19,27,32). Researchers also showed that although *E. amylovora* population growth is epiphytic in apple and pear blossoms, infection of the hypanthium can occur when bacterial populations reached 1×10^7 CFU/blossom (19,27). In the case of *X. euvesicatoria*, pepper blossoms were colonized without any symptoms resulting on developing fruit, which was similar to the results reported for *A. citrulli* and watermelon (16). Despite *X. euvesicatoria* and *A. citrulli* populations reached a level of $\geq 1 \times 10^5$ CFU/blossom within 96 hpi, symptoms were never observed on developing pepper and watermelon fruit, respectively.

It was also observed that *A. citrulli* could colonize pepper blossoms significantly better than *X. euvesicatoria*. *X. euvesicatoria* populations in pepper blossoms reached a level of 1×10^5 CFU/blossom as compared with 1×10^7 CFU/blossom for *A. citrulli* by 96 hpi. It is possible that *A. citrulli* is a better blossom colonizer compared with *X. euvesicatoria*, and even blossoms of incompatible hosts like pepper ('Aristotle') can be colonized, leading to infestation of nonhost seed. Although previous studies showed compatible interactions of *A. citrulli* with pepper and other solanaceous hosts such as eggplant and tomato (1,22), strains used in this study produced hypersensitive reactions upon inoculation in pepper leaves ('Aristotle'). This indicated that *A. citrulli* strains used in this study were incompatible with the pepper cultivar used. Darrasse et al. (4) determined the efficiency of pathogen transmission to seed of a nonhost plant and evaluated bacterial and plant behaviors at emergence. Transmission of *X. campestris* pv. *campestris* (causal agent of black rot of cabbage) from flowers to seed and from seed to resulting seedlings was evaluated in an incompatible situation. Seedling transmission were also evaluated for *X. citri* pv. *phaseoli* var. *fuscans* (as a compatible interaction) and *Escherichia coli* (a human pathogen, as null interaction) with bean. Results showed that inoculation of bean flowers with *X. campestris* pv. *campestris*

Fig. 5. Linear regression analysis for the relationship between *Xanthomonas euvesicatoria* (Xcv 04-100) populations applied to pepper blossoms and mean seed-to-seedling transmission percentages of *X. euvesicatoria* as determined by seedling grow-out assay. Seed samples ($n = 150$ /seedlot/treatment) developed from blossoms inoculated with *X. euvesicatoria* populations (0 , 1×10^1 , 1×10^3 , 1×10^5 , and 1×10^7 CFU/blossom) were planted on two layers of blotter paper saturated with sterile water for 18 days under conditions of $>90\%$ relative humidity at 28°C under continuous fluorescent light. Seedlings in individual lots were evaluated for bacterial spot symptoms at 18 days after planting. Data point represents mean seed-to-seedling transmission percentage of *X. euvesicatoria*-infested lots determined in two independent experiments. Bars indicate the standard error of the mean.

resulted in a high level of infestation in bean seed. Interestingly, *X. campestris* pv. *campestris* was also transmitted efficiently to bean seedlings. Regardless of interactions tested, bacterial population dynamics were similar on bean seedlings. These results suggest a probable role of seed of nonhost plants as an inoculum source. In addition, the authors clearly demonstrated the role of nonhost blossoms in seed infestation of an incompatible host (4). Similarly, in our case, *A. citrulli* colonized pepper blossoms, leading to seed infestation. However, disease transmission by *A. citrulli* was not observed in pepper seedlings. Researchers also indicated that plant-pathogenic bacteria can efficiently transmit from seed to nonhost seedlings as a consequence of saprophytic multiplication, resulting in the establishment of a primary inoculum focus (4).

X. euvesicatoria and *A. citrulli* were able to colonize stigma, style, and ovary tissues of pepper blossoms under greenhouse conditions, which is important for the process of blossom colonization. Stigma tissues were significantly better colonized by *A. citrulli* (1×10^8 CFU/stigma) compared with *X. euvesicatoria* (1×10^5 CFU/stigma) by 96 hpi. The exact reason for this phenomenon is unclear; however, it seems that *A. citrulli* can colonize stigmas of nonhost blossoms and host-pathogen interactions may not be important in blossom colonization. Style and ovary populations of *A. citrulli* and *X. euvesicatoria* lagged behind stigma populations by 6 and 12 h, respectively, but eventually attained population levels of 1×10^6 CFU in both tissues by 96 hpi. Interestingly, style and ovary tissues were colonized by both bacteria to a similar level by 96 hpi. Lessl et al. (16) also observed a lag of 6 h between stigma and stylar population in the *A. citrulli*-watermelon pathosystem, as observed in our study for both *X. euvesicatoria* and *A. citrulli* in pepper.

A strong linear relationship was observed between *X. euvesicatoria* blossom inoculum concentrations and percentage of infested seedlots as determined by real-time PCR, plating, and SGO assays. These results suggest that, as the concentration of *X. euvesicatoria* populations increased in blossoms, so did the percentage of infested pepper seedlots. Results from this study also indicated a considerable risk of seed infestation when high concentrations of *X. euvesicatoria* inoculum were applied on pepper blossoms. Such levels of initial inoculum normally do not occur under natural conditions. However, we observed that even blossoms inoculated with low *X. euvesicatoria* populations (10 CFU/blossom) led to infestations of $\leq 14.3\%$ of the seedlots. This suggests that even low levels of initial inoculum in blossoms can pose a substantial risk to infestation of pepper seed. Similar observations were also made by Lessl et al. (16) in *A. citrulli* with blossom inoculum dosage and percentage of infested watermelon seedlots. In that study, the authors demonstrated that, with an increase in blossom-*A. citrulli* inoculum dosage, there was a linear increase in the percentage of infested seedlots. Although the lowest inoculum dosage used was 1×10^3 CFU, percentage of seedlots infested were 36 and 14.2%, as determined by Immunomagnetic-separation-PCR and SGO assays, respectively. Also, in the case of *X. campestris* pv. *campestris* in cauliflower, researchers observed a positive relationship between inoculum densities on flies, the number of cells deposited on blossoms, and the risk of seed infection (28).

Overall, to our knowledge, this is the first evidence of the role of pepper blossoms as a site of *X. euvesicatoria* ingress and subsequent seed infestation within symptomless fruit. In addition, this research provided clear evidence of the ability of *X. euvesicatoria* to colonize pepper floral tissues. Currently, the relative significance of this mechanism of seed infestation in nature is not known, nor is the epidemiological significance of blossom-inoculated seed in commercial pepper lots. Hence, future studies are required to investigate the role of pepper blossoms in seed infestation in commercial seed-production fields. Also, it is important to evaluate the potential risks of seed infestation by this mechanism.

Perhaps, blossom protection with antagonistic organisms can be effective in limiting seed infestation, as it has been successfully demonstrated in pear and watermelon blossoms against infection with *Erwinia amylovora* (23,33) and *A. citrulli* (7), respectively. Future studies with antagonists will be evaluated to protect pepper blossom against *X. euvesicatoria* seed infestation.

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LITERATURE CITED

- Assouline, I., Milshtein, H., Mizrahi, M., Levy, E., and Ben-Ze'ev, I. N. 1997. *Acidovorax avenae* subsp. *citrulli* transmitted by solanaceous seeds. *Phytoparasitica* 25:117-118.
- Bashan, Y., Okon, Y., and Henis, Y. 1982. Long-term survival of *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria* in tomato and pepper seeds. *Ecol. Epidemiol.* 72:1143-1144.
- Bouzar, H., Jones, J. B., Stall, R. E., Hodge, N. C., Minsavage, G. V., Benedict, A. A., and Alvarez, A. M. 1994. Physiological, chemical, serological, and pathogenic analyses of a worldwide collection of *Xanthomonas campestris* pv. *vesicatoria* strains. *Phytopathology* 84:663-671.
- Darasse, A., Darsonval, A., Boureau, T., Brisset, M. N., Durand, K., and Jacques, M. A. 2010. Transmission of plant-pathogenic bacteria by non-host seeds without induction of an associated defense reaction at emergence. *Appl. Environ. Microbiol.* 76:6787-6796.
- Dutta, B. 2011. Localization of *Acidovorax citrulli* in watermelon seed and its influence on survival and seedling transmission of bacterial fruit blotch of cucurbits. Ph.D. dissertation, The University of Georgia, Athens.
- 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 invasion. *Phytopathology* 102:461-468.
- Fessehaie, A., and Walcott, R. R. 2005. Biological control to protect watermelon blossoms and seed from infection by *Acidovorax avenae* subsp. *citrulli*. *Phytopathology* 95:413-419.
- Gitaïtis, R., and Walcott, R. R. 2007. The epidemiology and management of seedborne bacterial diseases. *Annu. Rev. Phytopathol.* 45:371-397.
- Hamza, A. A., Robene-Soustrade, I., Jouen, E., Lefeuvre, P., Chiroleu, F., Fisher-Le-Saux, M., Gagnevin, L., and Pruvost, O. 2012. Multilocus sequence analysis- and amplified fragment length polymorphism-based characterization of xanthomonads associated with bacterial spot of tomato and pepper and their relatedness to *Xanthomonas* species. *Syst. Appl. Microbiol.* 35:183-90.
- Jones, J. B., Bouzar, H., Stall, R. E., Almira, E. C., Roberts, P. D., Bowen, B. W., Sudberry, J., Strickler, P. M., and Chun, J. 2000. Systematic analysis of xanthomonads (*Xanthomonas* spp.) associated with pepper and tomato lesions. *Int. J. Syst. Evol. Microbiol.* 50:1211-1219.
- Jones, J. B., Jones, J. P., and Woltz, S. S. 1993. The effect of selected copper bactericides on population dynamics of *Xanthomonas campestris* pv. *vesicatoria* on tomato leaflets. *Proc. Fla. State Hortic. Soc.* 106:160-163.
- Jones, J. B., Lacy, G. H., Bouzar, H., Stall, R. E., and Schaad, N. W. 2004. Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. *Syst. Appl. Microbiol.* 27:755-762.
- Jones, J. B., Stall, R. E., and Bouzar, H. 1998. Diversity among xanthomonads pathogenic on pepper and tomato. *Annu. Rev. Phytopathol.* 36:41-58.
- Kauffman, P. H., and Leben, C. 1974. Soybean bacterial blight: Flower inoculation studies. *Phytopathology* 64:329-331.
- Leite, R. P., Jones, J. B., Somodi, G. C., Minsavage, G. V., and Stall, R. E. 1995. Detection of *Xanthomonas campestris* pv. *vesicatoria* associated with pepper and tomato seed by DNA amplification. *Plant Dis.* 79:917-922.
- 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.
- Marco, G. M., and Stall, R. E. 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. *Plant Dis.* 67:779-781.
- Maude, R. B. 1996. Seedborne Disease and Their Control: Principles and Practice. CAB International, Wallingford, UK.
- Miller, T. D., and Schroth, M. N. 1972. Monitoring epiphytic populations of *Erwinia amylovora* on pear with a selective medium. *Phytopathology* 62:1175-1182.

20. Minsavage, G. V., Dahlbeck, D., Whalen, M. C., Kearney, B., Bonas, U., Staskawicz, B. J., and Stall, R. E. 1990. Gene-for-gene relationships specifying disease resistance in *Xanthomonas campestris* pv. *vesicatoria*–pepper interactions. *Mol. Plant-Microbe Interact.* 3:41-47.
21. Moretti, C., Amatulli, M. T., and Buonauro, R. 2009. PCR-based assay for the detection of *Xanthomonas euvesicatoria* causing pepper and tomato bacterial spot. *Lett. Appl. Microbiol.* 49:466-471.
22. Nascimento, A. R. P., Mariano, R. L. R., and Silva, E. I. 2004. Hospedeiros alternativos de *Acidovorax avenae* subsp. *citrulli*. *Hortic. Bras.* 22:345-349.
23. Nucló, R. L., Johnson, K. B., Stockwell, V. O., and Sugar, D. 1998. Secondary colonization of pear blossoms by two bacterial antagonists of the fire blight pathogen. *Plant Dis.* 82:661-668.
24. Ritchie, D. F. 2000. Bacterial spot of pepper and tomato. The Plant Health Instructor. Online publication. doi:10.1094/PHI-I-2000-1027-01
25. Sijam, K., Chang, C. J., and Gitaitis, R. D. 1991. An agar medium for the isolation and identification of *Xanthomonas campestris* pv. *vesicatoria* from seed. *Phytopathology* 81:831-834.
26. Stall, R. E., Beaulieu, C., Egel, D., Hodge, N. C., Leite, R. P., Minsavage, G. V., Bouzar, H., Jones, J. B., Alvarez, A. M., and Benedict, A. A. 1994. Two genetically diverse groups of strains are included in *Xanthomonas campestris* pv. *vesicatoria*. *Int. J. Syst. Bacteriol.* 44:47-53.
27. Thomson, S. V. 1986. The role of the stigma in fire blight infections. *Phytopathology* 76:476-482.
28. van der Wolf, J. M., and vander Zouwen, P. S. 2010. Colonization of cauliflower blossom (*Brassica oleracea*) by *Xanthomonas campestris* pv. *campestris*, via flies (*Calliphora vomitoria*) can result in seed infestation. *J. Phytopathol.* 158:726-732.
29. Vauterin, L., Hoste, B., Kersters, K., and Swings, J. 1995. Reclassification of *Xanthomonas*. *Int. J. Syst. Bacteriol.* 45:472-489.
30. Vauterin, L., Swings, J., Kersters, K., Gillis, M., Mew, T. W., Schroth, M. N., Palleroni, N. J., Hildebrand, D. C., Stead, D. E., Civerolo, E. L., Hayward, A. C., Marañón, H., Stall, R. E., Vidaver, A. M., and Bradbury, J. F. 1990. Towards an improved taxonomy of *Xanthomonas*. *Int. J. Syst. Bacteriol.* 40:312-316.
31. 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.
32. Wilson, M., Epton, H. A. S., and Sigeo, D. C. 1989. *Erwinia amylovora* infection of hawthorn blossom: 2. The stigma. *J. Phytopathol.* 127:15-28.
33. Wilson, M., Epton, H. A. S., and Sigeo, D. C. 1992. Interactions between *Erwinia herbicola* and *E. amylovora* on the stigma of hawthorn blossoms. *Phytopathology* 82:914-918.
34. Young, J. M., Park, D. C., Shearman, H. M., and Fargier, E. 2008. A multilocus sequence analysis of the genus *Xanthomonas*. *Syst. Appl. Microbiol.* 31:366-377.