Strains of the Group I Lineage of *Acidovorax citrulli*, the Causal Agent of Bacterial Fruit Blotch of Cucurbitaceous Crops, are Predominant in Brazil

Gustavo M. Silva, Ricardo M. Souza, Lichun Yan, Rui S. Júnior, Flavio H. V. Medeiros, and Ron R. Walcott

First, second, and fifth authors: Department of Plant Pathology, Federal University of Lavras, Lavras, MG, Brazil; third author: College of Plant Protection and Key Laboratory of Integrated Management of Crop Disease and Pests, Ministry of Education, Nanjing Agricultural University, Nanjing, China; fourth author: Department of Plant Sciences, Federal University of Semi-Arido, Mossoró, RN, Brazil; and sixth author: Department of Plant Pathology, University of Georgia, Athens.
Accepted for publication 6 August 2016.

ABSTRACT

Silva, G. M., Souza, R. M., Yan, L., Júnior, R. S., Medeiros, F. H. V., and Walcott, R. R. 2016. Strains of the group I lineage of *Acidovorax citrulli*, the causal agent of bacterial fruit blotch of cucurbitaceous crops, are predominant in Brazil. *Phytopathology* 106:1486-1494.

Bacterial fruit blotch (BFB), caused by the seedborne bacterium *Acidovorax citrulli*, is an economically important threat to cucurbitaceous crops worldwide. Since the first report of BFB in Brazil in 1990, outbreaks have occurred sporadically on watermelon and, more frequently, on melon, resulting in significant yield losses. At present, the genetic diversity and the population structure of *A. citrulli* strains in Brazil remain unclear. A collection of 74 *A. citrulli* strains isolated from naturally infected tissues of different cucurbit hosts in Brazil between 2000 and 2014 and 18 *A. citrulli* reference strains from other countries were compared by pulsed-field gel electrophoresis (PFGE), multilocus sequence analysis (MLSA) of housekeeping and virulence-associated genes, and pathogenicity tests on seedlings of different cucurbit species. The Brazilian population comprised predominantly group I strains (98%), regardless of the year of isolation, geographical region, or host. Whole-genome restriction digestion and PFGE analysis revealed that three unique and previously unreported *A. citrulli* haplotypes

(assigned as haplotypes B22, B23, and B24) occurred in Brazil. The greatest diversity of *A. citrulli* (four haplotypes) was found among strains collected from the northeastern region of Brazil, which accounts for more than 90% of the country's melon production. MLSA clearly distinguished *A. citrulli* strains into two well-supported clades, in agreement with observations based on PFGE analysis. Five Brazilian *A. citrulli* strains, representing different group I haplotypes, were moderately aggressive on watermelon seedlings compared with four group II strains that were highly aggressive. In contrast, no significant differences in BFB severity were observed between group I and II *A. citrulli* strains on melon and squash seedlings. Finally, we observed a differential effect of temperature on in vitro growth of representative group I and II *A. citrulli* haplotypes. Specifically, of 18 group II strains tested, all grew at 40 and 41°C, whereas only 3 of 15 group I strains (haplotypes B8 [P], B3[K], and B15) grew at 40°C. Three strains representing haplotype B8 (P) were the only group I strains that grew at 41°C. These results contribute to a better understanding of the genetic diversity of *A. citrulli* associated with BFB outbreaks in Brazil, and reinforce the efficiency of MLSA and PFGE analysis for assessing population structure. This study also provides the first evidence to suggest that temperature might be a driver in the ecological adaptation of *A. citrulli* populations.

Bacterial fruit blotch (BFB), caused by the gram-negative bacterium *Acidovorax citrulli*, is an economically important disease of cucurbitaceous crop species worldwide (Bahar and Burdman 2010; Burdman and Walcott 2012; Wall and Santos 1988; Yan et al. 2013). The pathogen was first reported in Georgia in the United States in the 1960s, causing necrotic lesions on watermelon cotyledons of different plant introductions (PI) from Turkey (Webb and Goth 1965). Initially, the disease was reported to only affect the seedling stage, with limited damage potential on fruit, and the causal agent was subsequently classified as *Pseudomonas pseudoalcaligenes* subsp. *citrulli* (Schaad et al. 1978). More recently, the pathogen was renamed *A. citrulli* (syn. *A. avenae* subsp. *citrulli*) (Schaad et al. 2008; Willems et al. 1992). The economic impact of BFB was realized in 1987, when the first outbreak occurred in the Mariana Islands, causing up to 100% watermelon yield losses (Wall and Santos 1988). To date, BFB management has been difficult to achieve because there are no commercially available sources of disease resistance and chemical control has limited efficacy in warm and wet field conditions (Burdman and Walcott 2012).

A. citrulli strains can be divided into at least two genetically distinct groups (Burdman and Walcott 2012; Eckshtain-Levi et al. 2014; Feng et al. 2009; Walcott et al. 2000, 2004). These include group I strains that have been isolated from a range of cucurbit hosts, and group II strains that are more aggressive to and have been isolated predominantly from watermelon. Group I and II *A. citrulli* strains can be clearly distinguished by restriction enzyme (*SpeI*) digestion and pulsed-field gel electrophoresis (PFGE), gas chromatography fatty acid methyl ester profiles, repetitive element palindromic polymerase chain reaction (rep-PCR), and multilocus sequence typing of housekeeping genes (HKG) (Burdman et al. 2005; Feng et al. 2009; Walcott et al. 2000). A recent comparative analysis of putative type III secreted (T3S) effector genes revealed that group I and II *A. citrulli* strains differed in their effector protein repertoires, which may contribute to their host preference association (Eckshtain-Levi et al. 2014). Moreover, this analysis led to the identification of a third, weakly virulent *A. citrulli* group comprising two *A. citrulli* strains recovered from watermelon in India (Eckshtain-Levi et al. 2014).

Since the first major BFB outbreak in 1990 in a watermelon field located in São Paulo state (Robbs et al. 1991), BFB has become a serious threat to Brazilian watermelon and melon production. The disease has occurred in the states of Rio Grande do Norte (Assis et al. 1999), Ceará (Santos and Viana 2000), Minas Gerais (Macagnan et al. 2003), Rio Grande do Sul (Ueno et al. 2003), Pernambuco (Mariano and Silveira 2004), Bahia (Mariano et al. 2004), and Roraima (Halfeld-Vieira and Nechet 2007). Despite its wide distribution, BFB outbreaks

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

*The e-Xtra logo stands for "electronic extra" and indicates that three supplementary figures and two supplementary tables are published online.

<http://dx.doi.org/10.1094/PHYTO-05-16-0205-R>
© 2016 The American Phytopathological Society

have only occurred sporadically in watermelon fields. On the other hand, significant yield losses have been common in melon-producing areas during the rainy season in the northeastern region of Brazil. More precisely, BFB outbreaks in melon have been common in the states of Rio Grande do Norte and Ceará, which account for >80% of Brazil's melon production (Assis et al. 1999; Conceição et al. 2014; Melo et al. 2014).

Previous studies have reported variability among Brazilian *A. citrulli* strains with regards to aggressiveness on melon and watermelon seedlings, biochemical properties, and genetic relatedness based on rep-PCR analysis (Melo et al. 2014; Oliveira et al. 2007). However, none have reported the genetic relatedness of Brazilian strains in accordance with the two well-documented and genetically distinct groups of *A. citrulli* (Eckshain-Levi et al. 2014; Feng et al. 2009; Walcott et al. 2004; Walcott et al. 2000). These studies were conducted primarily with Brazilian *A. citrulli* strains; therefore, it is important to clarify the genetic diversity of *A. citrulli* strains in Brazil by comparison with well-characterized reference strains.

To further examine the population structure of *A. citrulli* in Brazil, develop a fingerprinting database, and provide a basis for the integrated management of BFB, we used PFGE, multilocus sequence analysis (MLSA) of housekeeping and virulence-associated genes, and pathogenicity tests on different cucurbit seedlings to characterize a Brazilian population of *A. citrulli* strains collected from different hosts and geographical regions between 2000 and 2014. We also investigated the effect of temperature as a potential factor driving *A. citrulli* adaptation.

MATERIALS AND METHODS

Bacterial strains. *A. citrulli* strains used in this study are listed in Table 1. Seventy-four *A. citrulli* strains were isolated from naturally infected tissues of cucurbitaceous crops during BFB outbreaks in Brazil between 2000 and 2014. Additionally, some Brazilian strains were provided by collaborators, as well as several well-characterized reference strains from international sources (Table 2). *A. citrulli* strains were routinely grown on King's B medium (King et al. 1954)

at 28°C for 48 h, and single colonies were confirmed as *A. citrulli* by PCR assay with primers WFB1/WFB2 (Walcott and Gitaitis 2000), BX-S/BX-L (Bahar et al. 2008), or BOXAACF/BOXAACR2 (Ha et al. 2009). Bacterial DNA was extracted using the UltraClean Microbial DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA). Strains were stored in 15% sterilized glycerol at -80°C at the University of Georgia Seed Pathology Laboratory, Athens.

DNA fingerprinting by *SpeI* digestion and PFGE. DNA extraction, *SpeI* restriction enzyme digestion, and PFGE were conducted as previously described (Walcott et al. 2000). After electrophoresis, agarose gels were stained with an ethidium bromide solution of 0.5 µg/ml for 30 min, destained with distilled water for 30 min and digital images were captured in tagged image file (TIF) format under UV transillumination with an Eagle Eye II Still Video System (Stratagene, La Jolla, CA). DNA fingerprint profiles for each unique haplotype were compared using Dice's (1945) coefficient of analysis with the aid of the BioNumerics software package (Applied Math, Kortrijk, Belgium), and the unweighted pairwise group method with arithmetic mean algorithm was used to generate a dendrogram indicating strain relatedness.

MLSA. Seven genes, representing a total of 5,681 bp, were sequenced for MLSA. The genes were chosen based on a previous multilocus sequence typing (MLST) scheme developed for *A. citrulli* (Feng et al. 2009). Additional genes were chosen by comparing two sequenced *A. citrulli* genomes using Geneious software version 8.1.7 (Biomatters Ltd., Auckland, New Zealand): reference group II strain AAC00-1 (National Center for Biotechnology Information [NCBI] accession number NC_008752) and the draft genome of the reference group I strain M6 (Eckshain-Levi et al. 2016) (GenBank accession number LKUW000000000). Four of these genes were HKG (*adk*, *gyrB*, *pilT*, and *gltA*) and three were virulence-associated genes (*luxR* homolog, *avrRxo1* and *Aave_1548*) (Table 3). For selection of the HKG, we considered the gene distribution across the genome so that a single recombination event would be unlikely to affect more than one locus. The selected virulence-associated genes are interspersed between the HKG. For MLSA, genes were amplified using primers designed from the genome of *A. citrulli* strain AAC00-1 with Primer3

TABLE 1. Brazilian *Acidovorax citrulli* strains used in this study including the host of origin, geographical origin, date collected (Year), source, and *SpeI*-digested pulsed-field gel electrophoresis (PFGE) haplotype and group

Strains (other designation)	Host	Origin ^a	Year	Source ^b	Group	Haplotype ^c
AC 24 (Aac 5.3)	Melon, Santa Claus	RN	2001	UFRPE	I	B1(F)
AC 50 (Aac 1.12)	Melon, Yellow	RN	2001	UFRPE	I	B6(N)
AC 59 (AC 604)	Melon	RR	2009	Embrapa RR	I	B6(N)
AC 36 (AGR 009.1-P)	Watermelon	RS	2013	Agronomica	I	B24
AC 35 (AGR 011.1-P)	Watermelon	RS	2013	Agronomica	I	B24
AC 33 (AGR 012.1-P)	Watermelon	RS	2013	Agronomica	I	B24
AC 37 (AGR 008.1-P)	Watermelon	RS	2013	Agronomica	I	B24
AC 05, 01, 02, 03, 04, 06, 07, 08, 09, 10, 11	Melon, Yellow	RN	2014	UFLA	I	B23
AC 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22	Melon, Yellow	RN	2014	UFLA	I	B23
AC 27, 28, 29, 30, 31, 32, 34, 38, 39, 40, 41	Melon, Yellow	RN	2014	UFLA	I	B23
AC 42, 43, 44, 45, 46, 47, 48, 49, 53, 54, 55	Melon, Yellow	RN	2014	UFLA	I	B23
AC 56, 57, 58, 61, 62, 63, 64, 65, 66, 67, 68	Melon, Yellow	RN	2014	UFLA	I	B23
AC 51 (Aac 180)	Watermelon	PE	2001	UFRPE	I	B23
AC 52 (Aac R2)	Cantaloupe	RN	2001	UFRPE	I	B23
AC 25(Aac 5.16)	Melon, Santa Claus	RN	2001	UFRPE	I	B22
AC 23 (Aac 1.45)	Melon, Yellow	RN	2001	UFRPE	I	B22
Emb.C587 (Aac 1.31)	Melon	RN	2002	Embrapa	I	ND
Emb.I97	Melon	RS	Unknown	Embrapa	I	ND
Emb.E117 (UFV Ac 14)	Watermelon	MG	2004	Embrapa	I	ND
Emb.H530	Melon	RS	Unknown	Embrapa	I	ND
Emb.A11-19	Melon	RN	2000	Embrapa	I	ND
Emb.D348 (CNPH Aac Maisa 2)	Melon	RN	2003	Embrapa	I	ND
AC60 (Aac 11)	Unknown	ND	Unknown	UFLA	II	A3(C)
Emb.D349 (CNPH Aac 1213)	Melon	ND	2003	Embrapa	II	ND

^a Brazilian state: RN = Rio Grande do Norte; RR = Roraima; RS = Rio Grande do Sul; PE = Pernambuco; MG = Minas Gerais. ND = not determined.

^b Culture collections of phytobacteriology: UFRPE = Federal Rural University of Pernambuco; Embrapa RR = The Brazilian Agricultural Research Corporation, Roraima unit; Agronomica = Phytosanitary Diagnostic Laboratory; UFLA = Federal University of Lavras; and Embrapa = The Brazilian Agricultural Research Corporation, Plant Quarantine unit.

^c PFGE haplotype. Letters in parenthesis represent previously reported haplotypes (Walcott et al. 2000; 2004) while letter/number combinations represent current haplotype designations.

software v.0.4.0 (<http://bioinfo.ut.ee/primer3-0.4.0/>) (Rozen and Skaletsky 2000). Each reaction mixture contained DNA template at approximately 20 to 30 ng/μl, 1× buffer solution (New England Biolabs Inc., Ipswich, MA), 0.2 mM deoxynucleotide triphosphates mixture (New England Biolabs Inc.), 1 μM each primer, and 0.25 U of Taq polymerase (New England Biolabs Inc.) for a 25-μl reaction volume. The PCR thermal conditions included an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 90 s. The final extension step was at 72°C for 5 min. The sequences of the primers used in this study are listed in Supplementary Table S1. The PCR amplicons were purified with the ExoSap-IT Clean-up system (USB Co., Cleveland) according to the manufacturer's instructions, and direct sequencing of the amplicons was performed in the forward and reverse directions at Eurofins Genomics (Huntsville, AL). The seven genes selected for MLSA were sequenced for 20 *A. citrulli* strains, including 18 reference strains (Table 2) and 2 Brazilian strains representing the PFGE-determined haplotypes B6(N) and B23. Additionally, the *gyrB* gene sequences from six additional Brazilian strains (Emb.197, Emb.E117, Emb.H530, Emb.A11-19, Emb.D348, and Emb.D349) were included. Sequences of the closely related bacterium *A. avenae* strain ATCC 19860 (NCBI accession number NC_015138) served as an outgroup. All gene sequences were deposited in NCBI GenBank (accession numbers KU984110 to KU984250).

Phylogenetic analysis and single-nucleotide polymorphisms. Gene sequences were aligned using ClustalX software (Thompson et al. 1997). Phylogenetic trees were generated from the nucleotide

sequences using the maximum-parsimony method in MEGA6 software (Tamura et al. 2013). Bootstrap values were derived from 1,000 replicates to validate tree topology. Phylogenetic trees were generated for individual gene sequences, as well as for concatenated sequences. The phylogenetic information (trees and nucleotide sequences) was deposited in TreeBASE (<https://treebase.org>) under study number S19309. The DNA sequence polymorphisms were determined using DnaSP software V5 (Librado and Rozas 2009).

Seedling virulence assays. The virulence of selected *A. citrulli* strains representing the most abundant PFGE-determined haplotypes observed in Brazil and other reference strains was evaluated on watermelon (*Citrullus lanatus*), melon (*Cucumis melo*), and squash (*Cucurbita pepo*) seedlings, as previously described (Hopkins and Thompson 2002; Walcott et al. 2004). Briefly, watermelon 'Crimson Sweet', melon 'Joaquin Gold', and squash 'Early Yellow Crookneck' seedlings were grown in 1.4-liter plastic pots under greenhouse conditions (28 to 30°C, approximately 70% relative humidity [RH], and 12 h of sunlight daily). Seedlings were cultivated in a 3:1 ratio of fine-grade composted pine bark and vermiculite. Two weeks after planting, seedlings were spray inoculated with cell suspensions of group I Brazilian strains AC 05, AC 23, AC 36, AC 50, and AC 59 and group II strains AAC 00-1, AAC 213-44, AAC213-46, and AAC 213-47 at approximately 1×10^6 CFU/ml. To generate inoculum, -80°C stock cultures of each strain were cultured on King's B medium at 28°C for 48 h. Subsequently, a single colony of each culture was transferred to 5 ml of nutrient broth (NB) and incubated at 28°C for 16 h with agitation at 250 rpm in an incubator shaker (Innova, New Brunswick,

TABLE 2. Representative *Acidovorax citrulli* strains used for multilocus sequence analysis and virulence assays

Strain (other designation)	Host	Geographical origin	Date collected	Source	Group	Haplotype ^a
AAC 00-1	Watermelon	Georgia, United States	1990	R. Walcott	II	A1(A)
AAC 213-44	Unknown	Unknown	Unknown	R. Walcott	II	A3(C)
AAC 213-46	Unknown	Unknown	Unknown	R. Walcott	II	A11(W)
AAC 213-47	Unknown	Thailand	Unknown	R. Walcott	II	A2(B)
AAC 213-41	Unknown	Unknown	Unknown	R. Walcott	II	A2(B)
AAC 213-42	Unknown	Unknown	Unknown	R. Walcott	II	A4(D)
AAC 213-48	Unknown	Unknown	Unknown	R. Walcott	II	A5(E)
AAC 213-45	Unknown	Unknown	Unknown	R. Walcott	II	A28
AAC 213-49	Unknown	Unknown	Unknown	R. Walcott	II	A26
ATCC 29625 (AAC92-300)	Watermelon	Georgia, United States	1969	ATCC ^b	I	B3(K)
M6	Melon	Israel	2002	S. Burdman	I	B21
AAC 213-50	Unknown	Unknown	Unknown	R. Walcott	I	B3(K)
AAC 213-51	Pumpkin	China	Unknown	R. Walcott	I	B8(P)
AAC 213-52	Unknown	Unknown	Unknown	R. Walcott	I	B13(Y)
AAC 213-53	Melon	Brazil	2002	R. Walcott	I	B13(Y)
AAC 213-54	Unknown	Unknown	Unknown	R. Walcott	I	B12(X)
AAC 213-55	Unknown	Unknown	Unknown	R. Walcott	I	B9(R)
AAC 213-58	Unknown	Unknown	Unknown	R. Walcott	I	B5(M)
AAC 213-59	Melon	China	2009	R. Walcott	I	B5(M)
AAC 213-60	Unknown	Unknown	Unknown	R. Walcott	I	B5(M)
AAC 213-61	Melon	Mexico	2002	R. Walcott	I	B5(M)

^a Pulsed-field gel electrophoresis haplotypes. Letters in parenthesis represent previously reported haplotypes (Walcott et al. 2000; 2004) while letter/number combinations represent current haplotype designations.

^b American Type Culture Collection.

TABLE 3. Single-nucleotide polymorphisms (SNP) and amino acid substitutions of seven genes of *Acidovorax citrulli* used for multilocus sequence analysis

Gene name ^b	Biological function ^c	ORF (nt) ^d	Substitutions between groups I and II ^a	
			SNP (nt)	Amino acid
<i>Adk</i>	Cellular energy	657	1	0
<i>gyrB</i>	DNA replication	2,634	11	0
<i>pilT</i>	Twitching motility	1,044	1	0
<i>Aave_1548</i>	Putative T3S effector	1,458	33	16
<i>gluA</i>	Citrate cycle	1,311	5	0
<i>avrRx01</i>	Putative T3S effector	1,245	16	6
<i>luxR</i>	Transcriptional regulator	729	3	2

^a SNP substitutions (nucleotides [nt]) and amino acid substitutions between groups I and II.

^b Genes named according to AAC 00-1 annotation in the National Center for Biotechnology Information.

^c T3S = type III secreted.

^d Length of the open reading frame (ORF).

NJ). Cells in 5 ml of each NB culture were pelleted by centrifugation at $10,000 \times g$ for 2 min, rinsed once with 0.1 M phosphate-buffered saline (PBS), and resuspended in 3 ml of PBS. The optical density of the cell suspensions at a wavelength of 600 nm (OD_{600}) was adjusted to 0.5 (approximately 0.5×10^8 CFU/ml) using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY), and 10-fold serial dilutions were performed to generate cell suspensions of approximately 1×10^6 CFU/ml. Using plastic, hand-held spray bottles, cell suspensions of each strain were sprayed until run-off onto 10-day-old seedlings of each cucurbit species. Seedlings treated in the same manner with sterilized PBS served as negative controls. Each seedling was incubated in a transparent plastic bag (approximately 100% RH) for 48 h and then the plastic bags were removed and the seedlings were incubated under greenhouse conditions. Seven days after inoculation, seedlings were evaluated for BFB severity according to a previously described 1-to-9 rating scale (Hopkins and Thompson 2002). This experiment was repeated three times from June to August 2015 and each experiment included four replicates (individual plants) for each strain–host combination. Each plant was assessed for BFB severity by three independent evaluators. The experiment was arranged in a randomized complete block design. Statistical analysis was performed using the R environment. The R package multcomp was used for multiple comparison of groups (Herberich et al. 2010). The R package GGPlot2 was used to plot the disease severity data.

Effect of temperature on in vitro growth of *A. citrulli* strains.

A. citrulli strains representing 15 group I and 18 group II haplotypes (Supplementary Table S2) were used to determine the effect of temperature on in vitro growth of *A. citrulli* groups. Strains were grown in 5 ml of Lysogeny broth (LB) at 28°C for 16 h, with agitation at 250 rpm in an incubator shaker. Cells suspensions were adjusted spectrophotometrically to a concentration of 10^4 CFU/ml in PBS. Cells suspensions of each strain (40 μ l) were transferred into a sterilized Honeycomb microplate containing LB at 360 μ l/well. Each strain was replicated in 10 wells. Bacterial growth was measured at OD_{600} using a Bioscreen microplate reader (Bioscreen-C Automated Growth Curve Analysis System; Growth Curves USA, Piscataway, NJ). Cultures were grown at 40, 41, and 45°C with agitation at 270 rpm. OD was measured every 30 min for 39 h. This experiment was conducted twice and statistical analysis was performed using the R environment. One-way analysis of variance was used to analyze the mean values of final optical densities for each *A. citrulli* group at different temperatures.

RESULTS

Strain identification. All Brazilian strains were confirmed as *A. citrulli* by PCR with species-specific primers. However, *A. citrulli* strains representing the two common Brazilian PFGE haplotypes (B23 and B24) were negative when assayed by PCR with the BOXAACF/BOXAACR2 primer set. This was expected because this primer set does not amplify DNA from some group I *A. citrulli* strains (e.g., haplotypes B3[K], B8[P], B9[R], and B17) (Ha et al. 2009).

SpeI-PFGE analysis clustered the Brazilian strains into group I. Of 74 Brazilian *A. citrulli* strains, 67 were analyzed by PFGE. Among these 67 strains, seven distinct PFGE haplotypes were observed. Six haplotypes, representing 98% of the strains tested, clustered into group I and one strain, AC 60, was determined to be haplotype A3(C) (group II) (Table 1). Unfortunately, no data were available on the geographical origin or the host of origin for AC 60. Of the six group I haplotypes observed in the Brazilian population, haplotypes B1(F) and B6(N) were previously identified (Walcott et al. 2004) and three haplotypes (B22, B23, and B24) were previously unreported (Fig. 1). Haplotypes B24 (AC 33, AC 35, AC 36, and AC 37 from watermelon) and B6(N) (AC 59 from melon) were recovered from southern and northern regions of Brazil, respectively. The greatest *A. citrulli* diversity (haplotypes B1[F], B6[N], B23, and B22) was observed in northeastern Brazil, where haplotype B23 was the most prevalent (85.58% of the strains tested)

(Table 1). The other seven Brazilian strains, that were not fingerprinted by PFGE, were assigned to group I or II based on a PCR assay using a primer set (G1/G2) targeting the putative T3S effector gene *Aave_2166* (Zivanovic 2014) and sequence analysis of the HKG *gyrB* (Fig. 2A).

MLSA confirms that most Brazilian *A. citrulli* strains belong to group I. Based on a previously described MLST scheme for *A. citrulli* (Feng et al. 2009), three HKG (*gltA*, *ugpB*, and *pilT*) were initially selected for our study. The other HKG (*gmc*, *lepA*, *trpB*, and *phaC*) were monomorphic or noninformative (data not shown). In addition to the three HKG, a putative T3S effector gene (*Aave_1548*) (Eckshtain-Levi et al. 2014) was included, as well as *Aave_3810* (a *luxR* homolog) and *Aave_3062* (*avrRxoI*) (Johnson and Walcott 2013; Liu et al. 2014). These gene designations are according to the AAC00-1 annotation. Additional HKG (*gyrB* and *adk*) were included based on other MLST schemes generally reported for plant-pathogenic bacteria (Harayama and Kasai 2006). Only two Brazilian strains, representing haplotypes B23 (strain AC 05) and B6(N) (strain AC 50), and the *gyrB* sequence of additional strains (Emb.I97, Emb.E117, Emb.H530, Emb.A11-19, Emb.D348, and Emb.D349) were used in MLSA because the Brazilian population generally lacked genetic diversity based on PFGE data. DNA and amino acid sequence data were used for MLSA. Nucleotide sequence data analysis of the genes *gyrB* and *gltA* (Fig. 2A and B, respectively) clustered the Brazilian strains into group I, in agreement with PFGE analysis. The phylogenetic analysis of the putative T3S effector gene *Aave_1548* (Supplementary Fig. S1), the homolog *luxR* gene, and the concatenated sequence data (Fig. 2C and D) clustered strains AAC 213-50 (haplotype B3[K]) and AAC 213-51 (haplotype B8[P]) as a distinct group, closely related to group I strains.

Phylogenetic analysis and single nucleotide polymorphisms. The most variable HKG was *gyrB* (11 single-nucleotide polymorphisms [SNP] between group I and II), followed by *gltA* (5 SNP) and *adk* and *pilT* (1 SNP each). No amino acid substitutions were detected among the HKG. Among the virulence-associated genes, *Aave_1548* had 33 SNP and 16 amino acid substitutions and was the most variable gene (Table 3). Finally, the putative T3S effector gene *avrRxoI* had a 1-bp deletion at position 558 of the open reading frame (ORF) in all tested group I strains, resulting in a truncated protein, in agreement with Eckshtain-Levi et al. (2014).

Seedling virulence assays. The virulence of five group I Brazilian strains (AC 05, AC 23, AC 36, AC 50, and AC 59), each strain representing a different haplotype, and four group II strains (AAC00-1, AAC213-44, AAC213-46, and AAC213-47) was compared on melon, watermelon, and squash seedlings. We observed high variability in disease severity for the Brazilian strains on watermelon seedlings, with strains AC 05, AC 50, and AC 59 being more virulent (mean severity ratings of 6.5, 6, and 7, respectively) than strains AC 23 and AC 36 (mean severity ratings of 4.2 and 4.5, respectively). No significant difference in virulence was observed between group I and II strains on melon and squash ($P = 0.13$ and 0.97 , respectively). However, group I *A. citrulli* strains were significantly less virulent than the group II strains on watermelon seedlings ($P < 0.001$) (Fig. 3).

***A. citrulli* group II strains have higher growth temperature maxima.** Among the 15 group I and 18 group II strains tested (each strain representing a distinct PFGE haplotype), we observed a significant reduction in growth of the group I strains at 40°C ($P = 0.003$) and 41°C ($P < 0.001$) compared with the group II strains (Fig. 4). Interestingly, some group I strains (AAC 207-41, haplotype B15; AAC 92-300, haplotype B3[K]; and AAC 208-12, IA 58-116, and IA58-1, haplotype B8[P]) grew at 40°C. At 41°C, the only group I strains that grew were representatives of haplotype B8(P) (AAC 208-12, IA 58-116, and IA 58-1). In contrast, all group II strains tested grew at 40 and 41°C. No *A. citrulli* strain grew at 45°C (Fig. 4).

DISCUSSION

In Brazil, efforts to manage BFB outbreaks in cucurbit crop fields include the use of disease-free seed, rotations with noncucurbit crops,

and applications of copper-based compounds combined with plant resistance-inducing chemicals (Júnior et al. 2007). Despite this, BFB outbreaks are still common, particularly in melon-producing regions (Conceição et al. 2014). To characterize Brazilian *A. citrulli* strains representing different years, hosts, and geographical origin, we collected strains from natural BFB outbreaks in the field and requested older reference strains from Brazilian culture collections (Table 1). It is important to note that the strains that we collected from the field

were mostly recovered from naturally infected fruit in the northeastern region of Brazil, where melon production is heavily concentrated and BFB outbreaks are frequently reported. Because BFB epidemics in other regions of Brazil occur sporadically, we assume that our collection adequately represents the genetic spectrum of *A. citrulli* strains that has occurred in the country. Considering that (i) the Brazilian strains were mainly group I, regardless of the year of isolation and host; (ii) we found three unique and previously unreported haplotypes in

Fig. 1. Dendrogram showing the relationship between reference and Brazilian (in shaded gray boxes) *Acidovorax citrulli* strains based on *SpeI* genomic DNA digestion and pulsed-field gel electrophoresis (PFGE). PFGE haplotypes in parenthesis are old designations. Distance matrix generated by Dice's coefficient of similarity and dendrogram generated by unweighted pairwise group method with arithmetic mean.

Brazil; and (iii) BFB outbreaks occur predominantly on melon rather than watermelon, we hypothesize that group IA. *citrulli* strains are ecologically adapted to Brazil. Because contaminated seedlots represent the main source of inoculum for long-distance dissemination, it seems unlikely that only group I strains would be introduced into Brazil on cucurbit seed. In contrast, it seems more likely that group I strains are better adapted in Brazil.

In northeastern Brazil, where the weather is typically hot, melon fields are extensively planted year-round, due to the high demand for its export and domestic consumption. Watermelon and melon are cultivated concomitantly; however, the watermelon acreage is generally smaller during the rainy season of the year (from May to August), when the weather conditions are more conducive for BFB outbreaks. In contrast, most growers usually keep melon fields during this period of the year, in order to supply the domestic and international demand. As a result, a major portion of watermelon production generally escapes BFB epidemics, while melon fields are frequently attacked during this season. This recurring situation, along with the genetic variability among *A. citrulli* populations, may promote outbreaks and a better establishment of group IA. *citrulli* strains in Brazilian melon fields. Agricultural practices, including large areas of monoculture, may dramatically change the selection pressure, allowing bacterial populations to adapt to a single host species, given the dynamic nature of bacterial genomes (Baltrus et al. 2011; Nunney et al. 2012; Vinatzer et al. 2014). Additionally, although not addressed in this study, it is important to mention the potential role of

volunteer seedlings or cucurbitaceous weeds on BFB epidemiology in Brazil. These sources might contribute to *A. citrulli* inoculum which, under conducive conditions, would incite BFB outbreaks on subsequent crops (Dutta et al. 2014; Latin and Hopkins 1995).

Although Walcott et al. (2004) previously reported group IA. *citrulli* haplotypes B1(F) and B6(N) from Brazil, this study included only two strains that were recovered from melon. Of the three new haplotypes reported in the current study, haplotype B23 was the most prevalent, and it occurred from 2001 (AC 51 and AC 52) to 2014 in northeastern Brazil (Table 1). Using rep-PCR, Melo et al. (2014) reported that Brazilian *A. citrulli* strains were closely related to the type strain CFBP 4459, which was designated as group I, although not in accordance with the PFGE-based classification. Similar to the group I strains tested in our study, Melo et al. (2014) reported a significant reduction in virulence of the *A. citrulli* strains on watermelon seedlings relative to melon. The same strains evaluated by Melo et al. (2014) were included in our study and were placed into group I (Emb.I97, Emb.E117, Emb.H530, Emb.A11-19, Emb.D348, and Emb.C587) and group II (Emb.D349) based on a PCR assay for the T3S effector gene *Aave_2166* (Supplementary Figure S2) and sequence analysis of *gyrB* gene (Fig. 2A). Similar observations of reduced virulence of group I strains on watermelon seedlings were reported by Walcott et al. (2004). In contrast to our observations on melon seedlings, group I *A. citrulli* strains analyzed by Eckshtain- Levi et al. (2014) were generally more virulent than group II strains (including AAC00-1, used in both studies). One possible reason for the dissimilarity of the results could

Fig. 2. Phylogenetic trees generated from the nucleotide sequences of representative genes of *Acidovorax citrulli* by the maximum-parsimony method with 1,000 replicates bootstrap test. GI = group I and GII = group II. **A.** Tree based on *gyrB* sequence data. **B.** Tree based on *gltA* sequence data. **C.** Tree based on *luxR* sequence data. **D.** Tree based on sequences of all seven genes combined.

be the use of different melon cultivars: Joaquin Gold in this study versus 'Athena' in Eckshtain- Levi et al. (2014).

Using a database of PFGE fingerprints, we compared a population of *A. citrulli* strains collected in China, a major producer of melon and watermelon seed ($n = 114$ strains from 2002 to 2013) and Georgia ($n = 65$ strains from 1992 to 2012). We observed an uneven distribution of group I (80.7% from China and 6.16% from Georgia) and group II (19.3% in China and 93.84% in Georgia) strains. Specifically, haplotypes B5(M) (73.68%) and A3(C) (58.14%) were the most prevalent strains recovered from China and Georgia, respectively. In Georgia, most BFB outbreaks have occurred in watermelon crops (R. Walcott, unpublished data). Our observation of a prevalence of group I *A. citrulli* strains in China is in agreement with other studies (Feng et al. 2009; Yan et al. 2013). Although *A. citrulli*

strains are disseminated globally with cucurbit seed, our data suggest that certain haplotypes or groups are predominant in certain geographical regions. More specifically, group I strains appear to occur prevalently in China and Brazil, while group II strains are predominant in Georgia.

In the current study, MLSA clustered *A. citrulli* strains into two distinct groups that agreed with PFGE-based analysis and previous MLST analysis of HKG (Feng et al. 2009). Comparative studies of bacterial genomes have revealed that bacterial evolution is driven by composite forces acting on the "core" and "flexible" genomes (Gil et al. 2004; Sarkar and Guttman 2004). The core genome consists of essential genes such as HKG, which are less likely to undergo horizontal gene transfer. These genes evolve neutrally and are suitable for measuring the evolutionary history of clonal lineages.

Fig. 3. Bacterial fruit blotch severity induced by selected *Acidovorax citrulli* strains on different cucurbit (watermelon Crimson Sweet, melon Joaquin Gold, and squash Early Yellow Crookneck) seedlings. Ten-day-old seedlings of melon, watermelon, and squash were spray inoculated with bacterial suspensions of 10^6 CFU/ml or 0.1 M phosphate-buffered saline as a negative control. Seedlings were evaluated 10 days after inoculation using a 1-to-9 scale (Hopkins and Thompson 2002). The experiment was repeated three times. Different letters indicate significant differences ($P < 0.05$) among groups (I, II, and control) on each host plant.

Fig. 4. Effect of temperature on growth of group I and II *Acidovorax citrulli* strains. Representative strains from 15 group I and 18 group II haplotypes were grown in Lysogeny broth for 39 h. Bars represent the mean value of the final optical density of each group. Each strain had 10 technical replicates and the experiment was repeated twice. Asterisks represent significant difference between groups I and II at 40 and 41°C ($P = 0.003$ and $P < 0.001$, respectively). Phosphate-buffered saline was inoculated as a negative control.

Unlike the core genome, the flexible genome consists of genes that encode proteins involved in adaptation to specific niches, environments, or hosts, such as virulence-associated genes. These genes reflect the short-term evolution of a clonal lineage. Analysis of *A. citrulli* HKG in our study supported the existence of two groups that are distinct from the closely related species *A. avenae*, as previously reported (Eckshtain-Levi et al. 2014; Feng et al. 2009; Yan et al. 2013). This suggests that these two *A. citrulli* groups diverged long ago. Further evidence for evolutionary forces that have driven the groups apart is the presence of eight DNA fragments (ranging in size from approximately 35 to 120 kb) in the AAC00-1 (group II strain) genome, which are absent in the M6 (group I strain) genome, resulting in a difference of approximately 500 kb (Eckshtain-Levi et al. 2016). Further analyses by PCR assays targeting the eight DNA fragments, coverage analysis, G + C content, effective number of codon values, and cluster of orthologs analyses with other *A. citrulli* strains supported the conclusion that some of these fragments or a significant portion of them discriminate between groups and may have been gradually acquired by *A. citrulli* group II strains by horizontal gene transfer events (Eckshtain-Levi et al. 2016). Nevertheless, a more detailed analysis of the evolutionary history of *A. citrulli* is needed to determine how these two groups evolved.

Based on data suggesting that group I and II *A. citrulli* strains may be adapted to different ecological niches, we hypothesized that these groups may differ in their maximal growth temperatures. Growth at 41°C is a common characteristic of the genera *Comomonas*, *Hydrogenophaga*, and *Variovorax* that belong to the *Comomonadaceae* family. Accordingly, growth at 41°C differentiates phytopathogenic *Acidovorax* spp. from other plant pathogens, such as species of *Pseudomonas*, *Ralstonia*, and *Burkholderia* (Jones et al. 2001). The influence of temperature, pH, sodium chloride concentration, and carbon sources on *A. citrulli* growth was previously investigated by Cavalcanti et al. (2005). However, a limited number of strains was used in their study. More specifically, only four strains from melon were analyzed, which does not represent the genetic variability of *A. citrulli*. Although growth at 41°C was previously reported to be a general feature of *A. citrulli*, in the current study, we observed a difference in the ability of group I and II *A. citrulli* strains to grow at this temperature. More specifically, all group II strains tested grew at 41°C whereas most group I strains did not. Interestingly, a representative strain from the group I haplotype B8(P) (strain IA58-1) that grew at 41°C was recovered from plants at the United States Department of Agriculture North Central Plant Introduction Station (Ames, IA) and is closely related to the type strain ATCC 29625 (one PFGE polymorphism) (Walcott et al. 2004). However, the strain ATCC 29625 used in this study did not grow at 41°C.

Distinct ecological characteristics between closely related bacteria often involve changes in biochemical activities that result from gene gain, gene loss, or mutations that change the biochemical activities of proteins, resulting in ecological differentiation without genetic isolation (Retchless and Lawrence 2012). It is possible that group I and II *A. citrulli* strains gained or lost genes during their evolutionary history, which might ultimately reflect the competitive fitness and adaptation to a specific ecological niche or host. Comparative genome analysis revealed that the M6 (group I strain) genome sequence possesses 123 ORF that are absent in the AAC00-1 (group II strain) genome. In comparison, the AAC00-1 genome possesses 532 ORF that are absent in M6 (Eckshtain-Levi et al. 2016), including a putative T3S effector gene (*Aave_2708*; homolog to outer protein J of *Xanthomonas campestris* pv. *vesicatoria*) (Eckshtain-Levi et al. 2014) and a *vapB-vapC*-like operon, which encodes a stable toxin (VapC) and a labile antitoxin (VapB) (toxin-antitoxin system) (Shavit et al. 2016), both present in all group II *A. citrulli* strains tested but absent in group I strains. These differences might reflect the competitive fitness and adaptation to a specific ecological niche or host.

This study showed that BFB outbreaks in Brazil are predominantly caused by group I *A. citrulli* strains, regardless of the host or region. We also identified three unique haplotypes of *A. citrulli* from

northeastern Brazil. These findings advance our understanding and should provide a basis for more effective BFB management in Brazil, more specifically in the northeastern region, where the disease seems to be endemic on melon. By analyzing a historical PFGE database, we observed that, even though *A. citrulli* is globally disseminated with cucurbit crop seed, there was a prevalence of group I strains in China, whereas BFB outbreaks in Georgia were associated predominantly with group II strains. Finally, we showed a remarkable physiological variation in growth temperature maxima between group I and II *A. citrulli* strains that must be further investigated. The genome sequences of the group II *A. citrulli* strain AAC00-1 and other group I strains (M6, pslb65, and TW6; GenBank accession numbers LKUW00000000, JYHM01000000, and JXDJ01000000, respectively) will allow a more detailed investigation of population genomics and molecular host-pathogen interactions, which should ultimately improve BFB management by identifying new targets for resistance breeding efforts.

ACKNOWLEDGMENTS

G. M. Silva thanks the National Council for Technological and Scientific Development and the Brazilian Federal Agency for Support and Evaluation of Graduate Education for his fellowship. We thank V. Duarte, R. Mariano, D. Schurt, and A. Marques for kindly providing *A. citrulli* strains for this study; and S. Burdman for critically reviewing the manuscript.

LITERATURE CITED

- Assis, S. M. P., Mariano, R. L. R., Silva-Hanlin, D. M. W., and Duarte, V. 1999. Bacterial fruit blotch caused by *Acidovorax avenae* subsp. *citrulli* in melon, in the state of Rio Grande Do Norte, Brazil. *Fitopatol. Bras.* 24:191.
- Bahar, O., and Burdman, S. 2010. Bacterial fruit blotch: A threat to the cucurbit industry. *Isr. J. Plant Sci.* 58:19-31.
- Bahar, O., Efrat, M., Hadar, E., Dutta, B., Walcott, R. R., and Burdman, S. 2008. New subspecies-specific polymerase chain reaction-based assay for the detection of *Acidovorax avenae* subsp. *citrulli*. *Plant Pathol.* 57:754-763.
- Baltrus, D. A., Nishimura, M. T., Romanchuk, A., Chang, J. H., Mukhtar, M. S., Cherkis, K., Roach, J., Grant, S. R., Corbin, D. J., and Dangl, J. L. 2011. Dynamic evolution of pathogenicity revealed by sequencing and comparative genomics of 19 *Pseudomonas syringae* isolates. *PLoS Pathog.* 7:e1002132.
- 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.
- Burdman, S., and Walcott, R. 2012. *Acidovorax citrulli*: Generating basic and applied knowledge to tackle a global threat to the cucurbit industry. *Mol. Plant Pathol.* 13:805-815.
- Cavalcanti, M. T., Silveira, E. B., Mariano, R. L. R., and Viana, I. O. 2005. Growth of *Acidovorax avenae* subsp. *citrulli* under different variable temperature, pH, sodium chloride concentrations and carbon sources. *Cienc. Rural* 35:1313-1318.
- Conceição, C. S., Felix, K. C. S., Mariano, R. L. R., Medeiros, E. V., and Souza, E. B. 2014. Combined effect of yeast and silicon on the control of bacterial fruit blotch in melon. *Sci. Hortic. (Amsterdam)* 174:164-170.
- Dice, L. R. 1945. Measures of the amount of ecologic association between species. *Ecology* 26:297-302.
- 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. *citroides*) seeds. *Plant Pathol.* 63:1130-1137.
- Eckshtain-Levi, N., Munitz, T., Živanović, M., Traore, S. M., Spröer, C., Zhao, B., Welbaum, G., Walcott, R., Sikorski, J., and Burdman, S. 2014. Comparative analysis of type III secreted effector genes reflects divergence of *Acidovorax citrulli* strains into three distinct lineages. *Phytopathology* 104:1152-1162.
- Eckshtain-Levi, N., Shkedy, D., Gershovits, M., Silva, G. M., Tamir-Ariel, D., Walcott, R., Pupko, T., and Burdman, S. 2016. Insights from the genome sequence of *Acidovorax citrulli* M6, a group I strain of the causal agent of bacterial fruit blotch of cucurbits. *Front. Microbiol.* 7:430.
- Feng, J., Schuenzel, E. L., Li, J. Q., and Schaad, N. W. 2009. Multilocus sequence typing reveals two evolutionary lineages of *Acidovorax avenae* subsp. *citrulli*. *Phytopathology* 99:913-920.
- Gil, R., Silva, F. J., Pereto, J., and Moya, A. 2004. Determination of the core of a minimal bacterial gene set. *Microbiol. Mol. Biol. Rev.* 68:518-537.
- 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.
- Halfeld-Vieira, B. A., and Nechet, K. L. 2007. Mancha-aquosa da melancia em Roraima. *Fitopatol. Bras.* 32:268.
- Harayama, S., and Kasai, H. 2006. Bacterial phylogeny reconstruction from molecular sequences. Pages 106-138 in: *Molecular Identification, Systematics, and Population Structure of Prokaryotes*. E. Stackebrandt, ed. Springer-Verlag, Berlin, Heidelberg.
- Herberich, E., Sikorski, J., and Hothorn, T. 2010. A robust procedure for comparing multiple means under heteroscedasticity in unbalanced designs. *PLoS One* 5:e9788.
- Hopkins, D. L., and Thompson, C. M. 2002. Evaluation of *Citrus* sp. germplasm for resistance to *Acidovorax avenae* subsp. *citrulli*. *Plant Dis.* 86:61-64.
- Johnson, K. L., and Walcott, R. R. 2013. Quorum sensing contributes to seed-to-seedling transmission of *Acidovorax citrulli* on watermelon. *J. Phytopathol.* 161:562-573.
- Jones, J. B., Gitaitis, R. D., and Schaad, N. W. 2001. *Acidovorax* and *Xylophilus*. Pages 121-138 in: *Laboratory Guide for Identification of Plant Pathogenic Bacteria*. N. W. Shaad, J. B. Jones, and W. Chun, eds. American Phytopathological Society Press, St. Paul, MN.
- Júnior, R. S., Pontes Filho, F. S. T., Nunes, G. H. S., and Torres, G. R. C. 2007. Eficiência de acibenzolar-S-methyl e oxicleto de cobre no controle de *Acidovorax avenae* subsp. *citrulli*, agente causal da “mancha-aquosa” no meloeiro. *Rev. Biol. Ciên. Terra* 7:66-71.
- King, E. O., Ward, M. K., and Raney, D. E. 1954. 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.
- Librado, P., and Rozas, J. 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25:1451-1452.
- Liu, H., Chang, Q., Feng, W., Zhang, B., Wu, T., Li, N., Yao, F., Ding, X., and Chu, Z. 2014. Domain dissection of *AvrRxo1* for suppressor, avirulence and cytotoxicity functions. *PLoS One* 9:e113875.
- Macagnan, D., Romeiro, R. S., Mendonça, H. L., and Barreto, R. W. 2003. Mancha bacteriana da melancia: Uma nova bacteriose no estado de Minas Gerais. *Summa Phytopathol.* 29:286-287.
- Mariano, R. L. R., Silva, V. A. V., Silva, A. M. F., Medeiros, F. H. V., and Viana, I. O. 2004. Ocorrência da mancha-aquosa do melão na Bahia. *Fitopatol. Bras.* 29:147-148.
- Mariano, R. L. R., and Silveira, E. B. 2004. Mancha-aquosa: Importante bacteriose do meloeiro no Brasil. *An. Acad. Pernambucana Cienc. Agron.* 1:79-88.
- Melo, L. A., Tebaldi, N. D., Mehta, A., and Marques, A. S. A. 2014. Comparing *Acidovorax citrulli* strains from melon and watermelon: Phenotypic characteristics, pathogenicity and genetic diversity. *Trop. Plant Pathol.* 39: 154-162.
- Nunney, L., Yuan, X., Bromley, R. E., and Stouthamer, R. 2012. Detecting genetic introgression: High levels of intersubspecific recombination found in *Xylella fastidiosa* in Brazil. *Appl. Environ. Microbiol.* 78:4702-4714.
- Oliveira, J. C., Silveira, E. B., Mariano, R. L. R., Cardoso, E., and Viana, I. O. 2007. Caracterização de isolados de *Acidovorax avenae* subsp. *citrulli*. *Fitopatol. Bras.* 32:480-487.
- Retchless, A. C., and Lawrence, J. G. 2012. Ecological adaptation in bacteria: Speciation driven by codon selection. *Mol. Biol. Evol.* 29:3669-3683.
- Robbs, C. F., Rodrigues Neto, J., Ramos, R. S., and Sinigaglia, C. 1991. Mancha bacteriana da melancia no estado de São Paulo, causada por *Pseudomonas pseudoalcaligenes* subsp. *citrulli*. (Abstr.) *Fitopatol. Bras.* 16:XLVIII.
- Rozen, S., and Skaletsky, H. J. 2000. Primer3 on the WWW for general users and for biologist programmers. Pages 365-386 in: *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. S. Krawetz and S. Misener, eds. Humana Press, Totowa, NJ.
- Santos, A. A., and Viana, F. M. 2000. Mancha-aquosa do melão. EMBRAPA-SPI, Fortaleza, Ceará, Brazil.
- Sarkar, S. F., and Guttman, D. S. 2004. Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. *Appl. Environ. Microbiol.* 70:1999-2012.
- 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.
- Shavit, R., Lebendiker, M., Pasternak, Z., Burdman, S., and Helman, Y. 2016. The vapB-vapC operon of *Acidovorax citrulli* functions as a bona-fide toxin-antitoxin module. *Front. Microbiol.* 6:1499.
- Tamura, K., Stecher, G., Peterson, D., Filipinski, A., and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30:2725-2729.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The Clustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876-4882.
- Ueno, B., Couto, M. E. O., and Uesugui, C. H. 2003. Ocorrência de Mancha-aquosa em melão no estado do Rio Grande do Sul. *Fitopatol. Bras.* 28: S246.
- Vinatz, B. A., Monteil, C. L., and Clarke, C. R. 2014. Harnessing population genomics to understand how bacterial pathogens emerge, adapt to crop hosts and disseminate. *Annu. Rev. Phytopathol.* 52:19-43.
- Walcott, R. R., Fessehaie, A., and Castro, A. C. 2004. Differences in pathogenicity between two genetically distinct groups of *Acidovorax avenae* subsp. *citrulli* on cucurbit hosts. *J. Phytopathol.* 152:277-285.
- 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., Langston, D. B., Sanders, F. H., and Gitaitis, R. D. 2000. Investigating intraspecific variation of *Acidovorax avenae* subsp. *citrulli* using DNA fingerprinting and whole cell fatty acid analysis. *Phytopathology* 90:191-196.
- Wall, G. C., and Santos, V. M. 1988. A new bacterial disease on watermelon in the Mariana Islands. (Abstr.) *Phytopathology* 78:1605.
- Webb, R. E., and Goth, R. W. 1965. A seedborne bacterium isolated from watermelon. *Plant Dis. Rep.* 49:818-821.
- 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.
- Yan, S., Yang, Y., Wang, T., Zhao, T., and Schaad, N. W. 2013. Genetic diversity analysis of *Acidovorax citrulli* in China. *Eur. J. Plant Pathol.* 136: 171-181.
- Zivanovic, M. 2014. Characterization of *Acidovorax citrulli* virulence on watermelon and melon. MSc. Thesis, The University of Georgia, Athens.