J. Phytopathology **152**, 277–285 (2004)

ISSN 0931-1785

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Differences in Pathogenicity between two Genetically Distinct Groups of *Acidovorax avenae* subsp. *citrulli* on Cucurbit Hosts

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With 4 figures

Received October 22, 2003; accepted March 2, 2004

Keywords: *Cucurbita pepo*, *Cucurbita maxima*, *Cucurbita melonis*, *Citrullus lanatus*, watermelon fruit blotch, REP-polymerase chain reaction, pulse-field gel electrophoresis

Abstract

Using DNA fingerprinting by pulse-field gel electrophoresis and repetitive extragenic pallindromic (REP)-polymerase chain reaction (PCR), two distinct groups were confirmed among 64 *Acidovorax avenae* subsp. *citrulli* strains collected from a range of cucurbitaceous hosts in the USA, China, Taiwan, Thailand, Canada, Australia, Brazil and Israel. Eighty-two percent of the group I strains were recovered from non-watermelon hosts and the subspecies type strain was the only member of this group that utilized L-leucine as a sole carbon source. On the contrary, 94% of the group II strains were recovered from watermelon and 96% of them utilized L-leucine. Two-week-old watermelon cv. Crimson sweet, cantaloupe cv. Athena, pumpkin cv. Lumina and squash cv. Early yellow crookneck seedlings were susceptible to *A. avenae* subsp. *citrulli* strains representing each group with the exception of the subspecies type strain. Overall, seedlings of watermelon cv. Crimson Sweet were most susceptible to *A. avenae* subsp. *citrulli* infection followed by cantaloupe, pumpkin and squash. Group II strains were more aggressive watermelon than on other hosts. On the contrary, group I strains were moderately aggressive on all cucurbit hosts tested.

Introduction

Bacterial fruit blotch (BFB) is a devastating disease of watermelon (*Citrullus lanatus* [Thunb.] Matsum. & Nakai) caused by *Acidovorax avenae* subsp. *citrulli* (Schaad et al., 1978; Willems et al., 1992), a Gram-negative, rod-shaped bacterium that can be seed transmitted by a range of cucurbit hosts (Hopkins and Thompson, 2002a). *Acidovorax avenae* subsp. *citrulli* was first recovered from watermelon foliage at the USDA plant introduction station, Griffin, GA in 1965 (Webb and Goth, 1965; Schaad et al., 1978); however, in 1989 a highly aggressive strain was recovered from outbreaks in commercial watermelons in Florida (Somodi et al., 1991). Subsequently, numerous costly BFB outbreaks occurred throughout south-eastern and mid-western USA between 1989 and 1994 (Latin and Rane, 1990; Jacobs et al., 1992; Black et al., 1994).

While it was acknowledged that *A. avenae* subsp. *citrulli* could infect other cucurbits, the disease was a threat mainly to watermelons (Latin, 1996). However, since 1996 BFB has been responsible for significant economic losses in other cultivated cucurbits including melons (*Cucumis melo* var. *cantalupensis*), honeydew (*C. melo* var. *indorus* Jacq.), cucumber (*C. sativus* L.), squash and pumpkin (*Cucurbita pepo*, *Cucur. maxima* and *Cucur. moschata*), citronmelon (*C. lanatus* [Thunb.] Matsum. & Nakai var. *citroides*), prickly paddy melon (*C. myriocarpus* subsp. *myriocarpus*) and several types of gourds in the USA, Australia, Costa Rica, Nicaragua, Taiwan, China, Japan and Brazil (Isakeit et al., 1997, 1998; Assis et al., 1999; Langston et al., 1999; Martin and O'Brien, 1999; O'Brien and Martin, 1999; Cheng et al., 2000; Shirakawa et al., 2000; Zhao et al., 2001; Martin and Horlock, 2002; Mora-Umana and Araya, 2002; Munoz and Monterroso, 2002).

One possible explanation for the apparent change in the pattern of BFB outbreaks in cucurbits is the introduction of non-indigenous *A. avenae* subsp. *citrulli* strains. At least two groups of *A. avenae* subsp. *citrulli* strains have been identified based on gas chromatography-fatty acid methyl ester (GC-FAME) profiles, sole carbon substrate utilization patterns, pathogenicity on different seedling hosts, and DNA fingerprinting using pulse-field gel electrophoresis (PFGE) of *Spe*I-digested DNA fragments (Somodi et al., 1991; O'Brien and Martin, 1999; Walcott et al., 2000). Unfortunately, these studies only included strains from the USA and Australia, and pathogenicity studies were limited to the seedling stages of a few cucurbits. The overall objective of this study was to understand the nature of

the apparent host range expansion for *A. avenae* subsp. *citrulli*. Specifically, the objectives were to (1) further examine the population structure of *A. avenae* subsp. *citrulli* using strains from a wide range of geographical origins and (2) investigate differences in pathogenicity between the two genetically distinct groups of *A. avenae* subsp. *citrulli* on different cucurbit hosts.

Methods and Materials

A. avenae subsp. *citrulli* strains

Acidovorax avenae subsp. *citrulli* strains were collected and submitted by contributors in the USA, China, Brazil, Australia, Thailand, Taiwan, Israel and Canada (Table 1). Strains were confirmed as *A. avenae* subsp. *citrulli* by PCR with subspecies-specific primers SEQID4^{m}/SEQID5 (Schaad et al., 1999; Walcott et al., 2003) and stored in 15% sterile glycerol at −80°C at the Seed Pathology laboratory at the University of Georgia, Athens, GA. Due to import restrictions, purified genomic DNA from eight strains was submitted by contributors in Taiwan (Table 1). Hence, substrate utilization, PFGE analysis and pathogenicity studies could not be conducted on these strains.

Copper sensitivity

All strains were tested for copper sensitivity on nutrient agar plates amended with 1.25 mM cupric sulphate ($CuSO_4{\cdot}5H_2O$) (NACu). Using a sterile loop, a single colony of each strain was transferred from 48 h nutrient agar cultures onto NACu medium. Plates were incubated at 28°C for 48–72 h and observed for bacterial growth. Copper sensitivity was further tested using nutrient broth amended with the 1.25 mM or 0.63 mM $CuSO_4$. Optical density was measured at 600 nm for each tube prior to inoculation with each isolate and again after incubation at 30°C with agitation at 180 rpm.

DNA fingerprinting by pulse-field gel electrophoresis

DNA preparation and *Spe*I restriction enzyme digestion and PFGE were conducted according to previously described methods (Walcott et al., 2000). After electrophoresis, gels were stained with a 0.5 μg/ml ethidium bromide solution for 30 min and under ultraviolet transillumination, digital images were captured in tagged image file format with an Eagle Eye II Still Video System (Stratagene, La Jolla, CA, USA). 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 (UPGMA) algorithm was used to generate a dendrogram indicating strain relatedness.

DNA fingerprinting by REP-PCR

Each strain was grown overnight in 5 ml nutrient broth at 30°C with continuous agitation at 180 rpm and DNA was extracted using standard protocols (Moore, 1999). Using 2 ng of DNA from each strain as template, REP-PCR was conducted using primer BOXA1R (5′-CTA CGG CAA GGC GAC GCT GAC G-3′). PCR amplification was conducted in 25 μl reaction volumes with PureTaq Ready-To-Go PCR Beads (Amersham, Biosciences, Piscatway, NJ, USA) according to the manufacturer's instructions. Using a Mastercycler Gradient programmable thermal cycler (Eppendorf, Hamburg, Germany), the PCR amplification protocol included an initial denaturation at 95°C for 7 min followed by 30 cycles of PCR consisting of denaturation at 95°C for 1 min, annealing at 53°C for 1 min, and extension at 65°C for 8 min. Ten microlitres of PCR product was separated by electrophoresis at 125 V for 6 h on a 1.5% agarose gel in 1X Tris-acetate ethylenediaminetetraacetic acid (EDTA) buffer. A molecular marker consisting of a 100 bp ladder (Sigma-Aldrich, St Louis, MO, USA) was included in the first, middle and last lanes of each gel to facilitate normalization and analysis. Subsequently gels were stained with ethidium bromide and the gel images were captured as previously described. Dice's (1945) coefficient of similarity was used to determine a distance matrix, and the UPGMA algorithm was used to generate a dendrogam.

Substrate utilization profiles

Each *A. avenae* subsp. *citrulli* strain was grown on universal growth medium (Biolog Inc., Hayward, CA, USA) for 24 h at 28°C and used to generate a cell suspension with 1×10^8 CFU/ml of inoculating fluid (Biolog Inc.) determined spectrophotometrically. For each strain, 150 μl of cell suspension was transferred into each well of a Biolog GN microtitre plate followed by incubation at 28°C for 12 h. Substrate utilization, indicated by a purple colour change in each well, was scored visually, and the binary data were analysed using Microlog software V.4.01B, (Biolog Inc).

Seedling pathogenicity assays

Two-week-old seedlings of four cucurbit hosts were inoculated with 10 strains representing the two genetically distinct groups (group I: ATCC29625, AAC92-301, AAC92-305, AACAU-2, AAC98-17 and group II: AAC00-1, AAC94-21, AAC92-17, AAC94-48, AAC94-87). ATCC29625 was included because it was the type strain for *A. avenae* subsp. *citrulli*; however, the other strains were arbitrarily selected to represent a range of haplotypes within each group. Inoculum of each strain was prepared by inoculating 5 ml of nutrient broth (Difco, Sparks, MD, USA) with one colony of a 48 h culture, grown on King's medium B (KMB) (King et al., 1956). After 12 h, the cells in 2 ml of broth culture were harvested by centrifugation at 10 000 × ***g*** for 2 min, rinsed once with, and resuspended in 3 ml of sterile 0.1 M phosphate-buffered saline (PBS). The optical density of the cell suspension was adjusted to 0.3 at a wavelength of 600 nm and diluted to a concentration of approximately 0.5×10^6 CFU/ml. Two-week-old seedlings of cantaloupe cv. Athena, pumpkin cv.

Table 1
Acidovorax avenae subsp. *citrulli* strains included in this study including their original host, geographical origin, date collected, contributors, sensitivity to $CuSO_4$, pulse-field gel electrophoresis (PFGE)-determined haplotype and grouping based REP-polymerase chain reaction (PCR) of genomic DNA

Strains (other designation)	Host	Origin	Date collected	Source	Cu^{2+} sensitivity[1]	PFGE haplotype	Group
AAC00-1	Watermelon	Georgia, USA	1990	R. Walcott	S	A	II
AAC94-36	Watermelon	Georgia, USA	1994	R. Walcott	S	B	II
AAC94-21	Watermelon	Georgia, USA	1994	R. Walcott	S	C	II
AAC92-17	Watermelon	Georgia, USA	1992	R. Walcott	S	D	II
AAC94-48	Watermelon	Georgia, USA	1994	R. Walcott	S	U	II
AAC94-55	Watermelon	Georgia USA	1994	R. Walcott	ND	E	II
AAC92-301	Watermelon	Georgia, USA	1992	R. Walcott	I	F	I
AAC94-87	Watermelon	Georgia, USA	1994	R. Walcott	S	G	II
AAC94-11	Watermelon	Georgia, USA	1994	R. Walcott	ND	H	II
AAC94-12	Watermelon	Georgia, USA	1994	R. Walcott	S	W	II
AAC92-305	Watermelon	Georgia, USA	1992	R. Walcott	S	I	I
AAC61*	Watermelon	Taiwan	2000	W. Yang	ND	ND	II
AAC70*	Muskmelon	Taiwan	2000	W. Yang	ND	ND	I
AAC126*	Muskmelon	Taiwan	2000	W. Yang	ND	ND	I
AAC5*	Watermelon	Taiwan	2000	W. Yang	ND	ND	II
AAC160*	Bitter gourd	Taiwan	2000	W. Yang	ND	ND	I
AAC128*	Muskmelon	Taiwan	2000	W. Yang	ND	ND	I
AAC55*	Watermelon	Taiwan	2000	W. Yang	ND	ND	II
AAC31*	Watermelon	Taiwan	2000	W. Yang	ND	ND	II
AAC94-39	Watermelon	Georgia, USA	1994	R. Gitaitis	S	J	II
ATCC29625	Watermelon	Georgia, USA	1969	ATCC	I	K	I
AACAU-2	Rockmelon	Australia	1999	R. Walcott	I	L	I
AACAU-9	Rockmelon	Australia	1999	R. Walcott	I	M	I
AAC98-17	Pumpkin cv. Lumina	Georgia USA	1999	R. Walcott	I	N	I
AAC99-5	Cantaloupe	Georgia, USA	1999	R. Walcott	I	O	I
IACANT58-1	Cantaloupe	Iowa, USA	1999	C. Block	I	P	I
Saticoy B	Cantaloupe cv. Saticoy	Florida, USA	2000	D. Hopkins	S	Q	II
AAC200-6	Cantaloupe	Georgia, USA	2000	R. Walcott	I	R	I
AAC200-23	Watermelon	Oklahoma, USA	2000	B. Bruton	I	P	I
AAC200-30	Cantaloupe	California, USA	2000	R. Walcott	I	S	I
AAC201-12	Watermelon	South Carolina, USA	2001	R. Walcott	ND	T	II
AAC201-13, -14	Honeydew	Queensland, Australia	2000	H. Martin	I	V	I
AAC201-15 (3433)	Rockmelon cv. Meteor	Queensland, Australia	2000	H. Martin	I	V	I
AAC201-16 (3552)	Watermelon cv. Babylee	Queensland, Australia	2000	H. Martin	I	V	I
AAC201-17 (3692)	Rockmelon cv Amira	Queensland, Australia	2000	H. Martin	I	L	I
AAC201-18 (3723)	Watermelon cv. Red Tiger	Queensland Australia	1997	H. Martin	I	L	I
AAC201-19 (4391)	Watermelon cv. Wall Heart	Queensland, Australia	1996	H. Martin	I	B	II
AAC201-20 (4884)	Prickly paddymelon	Queensland, Australia	1997	H. Martin	S	C	II
AAC 201-21, -22, -23	Melon	Rio Grande do Norte, Brazil	1999	R. Mariano	I	F	I
AAC201-24	Melon	Rio Grande do Norte, Brazil	1999	R. Marian	S	N	I
AAC201-26	Watermelon	Ontario, Canada	1998	D. Cuppels	S	A	II
AAC202-1, -3, -5, -6,	Muskmelon	Nanjing, China	2001	H. Baishi	I	M	I
AAC202-2, -4, -7	Muskmelon	Nanjing, China	2001	H. Baishi	S	M	I
AAC202-66	Melon	Israel	2002	Anonymous	S	X	I
AAC202-67	Watermelon	Israel	2002	Anonymous	S	E	II
AAC202-68, -69, -70, -71, -72, -73, -74, -75, -76, -78	Watermelon	Khon Kaen, Thailand	2002	V. Dittapongpitch	S	W	II

*Only DNA was available.
[1]S indicates that strains did not grow on nutrient agar plates amended with 1.25 or 0.63 mM $CuSO_4$; I indicates that strains grew on nutrient plates amended with 1.25 or 0.63 mM $CuSO_4$; and ND indicates that the test was not done.

Lumina, squash cv. Early yellow crookneck and watermelon cv. Crimson Sweet were established in fine-grade composted pine bark mixed with vermiculite in a 3 : 1 ratio in 250 ml plastic pots under greenhouse conditions. Each plant was sprayed until run-off with a suspension of the appropriate strain. For a negative control, plants were inoculated with sterile PBS. Seedlings were incubated in a plastic bag for 48 h at approximately 100% relative humidity (RH). The plastic bags were then removed and seedlings were incubated under greenhouse conditions of 30–35°C and 80–90% RH with 12 h of natural sunlight daily. Ten days after inoculation the BFB severity of each seedling was evaluated according to the 1–9 scale previously described (Hopkins and Thompson, 2002b). Each strain/host combination was replicated five times and the experiment was arranged in a randomized complete block design. This experiment was repeated twice and ANOVA was conducted using the general linear model (GLM) procedure. Additionally, pairwise comparisons of mean BFB severity were conducted using the Fisher's test of least significant difference (LSD). Linear contrasts were used to compare the BFB severity induced by groups I and II strains on different cucurbit seedling hosts. Statistical analysis was conducted using Statistical Analysis Systems v. 8 (SAS Institute, Cary, NC, USA).

Fruit pathogenicity assays

Due to difficulties in consistently producing squash and pumpkin fruit under greenhouse conditions, only cantaloupe and watermelon were included in the fruit inoculation assays. Cantaloupe and watermelon plants were established in 15 l plastic pots as described above. Plants were fertilized (Peters Professional 20-10-20 Peat Lite, Special, Scotts-Sierra Horticultural Products Co., Marysville, OH, USA) once a week and allowed to develop to anthesis. Female blossoms on each plant were hand-pollinated as previously described (Walcott et al., 2003), and fruits were inoculated 3–9 days after pollination with each of the above-mentioned strains, and PBS as a negative control. Inoculum was prepared as previously described and fruits were inoculated by applying bacterial cell suspension to the fruit surface using a saturated sterile cotton tip applicator. Care was taken to avoid wounding fruits during inoculation. Each fruit was incubated in a plastic bag for 48 h, followed by removal of the plastic bag and incubation under standard greenhouse conditions for 25–30 days with natural diurnal light cycles. Fruits were harvested and evaluated for BFB severity according to the following scale: 0 = no symptoms, 1 = surface lesions/water-soaking no penetration into rind; 2 = lesions penetrating into the rind; 3 = lesions penetrating into the flesh of the fruit; 4 = extensive penetration and necrosis of fruit tissues; 5 = complete fruit rot. Each strain was applied to four cantaloupe and watermelon fruits, each on a separate plant. Plants were arranged in a randomized complete block design, and the experiment was conducted twice. Attempts were made to recover *A. avenae* subsp. *citrulli* from infected fruits and the identity of the recovered isolates was confirmed by PCR. Additionally, the strains were confirmed as belonging to group I or II by REP-PCR using the BOXA1R primer as described above. ANOVA was conducted on disease severity estimates using the GLM procedure and Fisher's protected test of LSD was used to conduct pairwise mean comparisons. Linear contrasts were also used to compare the effect of groups I and II strains on BFB severity on cantaloupe and watermelon fruit. Statistical analysis was conducted using SAS.

Results

Strain identification

All strains were confirmed as *A. avenae* subsp. *citrulli* by PCR with subspecies-specific primers and substrate utilization profiles based on Biolog.

Copper sensitivity

Approximately 46% (24 of 52) of the *A. avenae* subsp. *citrulli* strains displayed restricted growth on NACu (Table 1). Only one of the group I strains AC92-301, was sensitive to copper sulphate. In contrast, when assayed in nutrient broth amended with the same concentration of $CuSO_4$, only AAC202-6, AAC200-6 and AAC200-30 grew after 4 days at 30°C. In 0.63 mM $CuSO_4$-amended nutrient broth, 42% of the strains grew. Two group II strains, AAC94-39, and AAC94-87, were insensitive to 0.63 mM $CuSO_4$.

DNA fingerprinting by restriction enzyme digestion and PFGE

DNA fingerprinting by PFGE yielded 9–14 unique markers (Fig. 1). Amongst the 56 strains analysed 24 unique haplotypes were observed (Table 1). Of these, 10 were previously unreported and were designated O–X in the order that they were first observed (Fig. 1). Cluster analysis of DNA fingerprints revealed two major groups that were approximately 45% dissimilar. Group I included 50% of the haplotypes (I, F, K, L, M, N, O, P, R, S, V and X) and comprised 52.7% (29 of 55) of the *A. avenae* subsp. *citrulli* strains analysed. Approximately 80% (23 of 29) of these were recovered from non-watermelon hosts. Approximately 93% (25 of 27) of the group II strains, comprising 14 haplotypes were recovered from watermelon (Table 5). One exception was AAC SaticoyB, recovered from cantaloupe seedlings produced from seed harvested from plants that had been exposed to a group II strain (D. Hopkins, personal communication).

DNA fingerprinting by REP-PCR

Based on DNA fingerprints generated by BOXA1R primers, 47 unique haplotypes were observed among the 64 strains analysed (Fig. 2). However, in some cases DNA amplicons were difficult to score. As with PFGE-based DNA fingerprinting, analysis of the BOXA1R-generated DNA profiles revealed two major clusters that were approximately 35% dissimilar (Fig. 2). Cluster I included strains recovered from can-

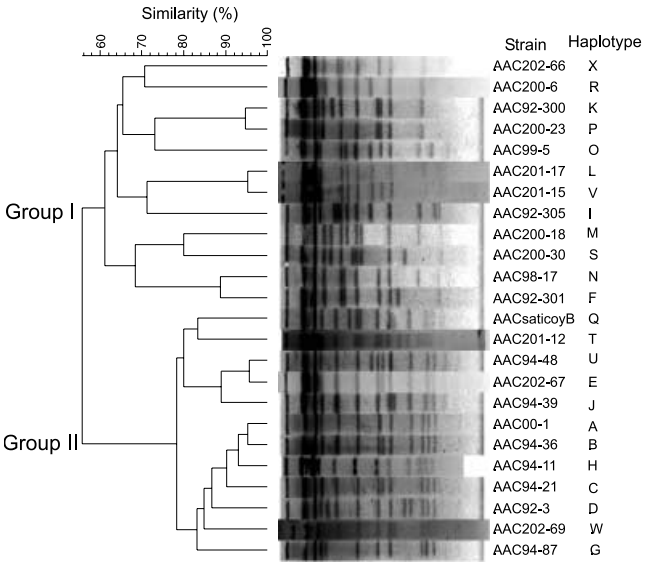

Fig. 1 Dendrogram indicating the relationship among haplotypes of *Acidovorax avenae* subsp. *citrulli* based on polymorphisms among restriction fragments obtained following *Spe*I digestion of whole-cell genomic DNA and separation of fragments by pulse-field gel electrophoresis. Distance matrix for the dendrogram was generated by Dice's (1945) coefficient of similarity and the dendrogram was generated based on the unweighted pairwise group method with arithmetic mean (UPGMA) algorithm. Letters adjacent to the strain profiles indicate the unique haplotype designations

taloupe, pumpkin, gourds and watermelon and composed of 56% of the strains analysed. Cluster II included 44% of the strains tested, of which, 100% were isolated from watermelon. This cluster corresponded to the PFGE-determined group II. REP-PCR group I was more heterogenous than group II and corresponded to the PFGE-determined group I.

Substrate utilization profiles

Approximately 64% (61 of 95) of the substrates included in the Biolog GN plates were utilized by *A. avenae* subsp. *citrulli* strains. The most commonly utilized carbon sources included, Tween 80, monomethyl succinate, acetic acid, β-hydroxybutyric acid, D-L-lactic acid, sebacic acid, succinic acid, bromo succinic acid, L-asparagine, L-aspartic acid, L-pyroglutamic acid. In general, strain clusters based on substrate utilization profiles did not correspond to DNA-based groups; however, 96.3% (26 of 27) of the group II strains utilized L-leucine, as opposed to one group I strain ATCC29625 (Table 2). Other substrates that were differentially utilized included α-hydroxybutyric acid and to a lesser extent, 2-amino ethanol (Table 2).

Seedling pathogenicity assays

All cucurbit seedlings used in this study were susceptible to attack by the 10 strains selected with a few exceptions. ATCC296265 failed to induce BFB on pumpkin or squash and AAC94-21 failed to infect squash. In general, BFB symptoms including water-soaking and coalescing reddish-brown lesions on cotyledons were visible after 10 days. Additionally, symptoms on true leaves included reddish-brown lesions that developed along the venation. BFB symptoms on pumpkin often included extensive chlorosis. As expected, none of the seedlings inoculated with PBS developed BFB, and data for these treatments were excluded from the statistical analysis. The effects of experiment on BFB severity were not significant ($P = 0.95$), hence, data from the two experiments were pooled for analysis. The effect of host on BFB severity was statistically significant ($P < 0.0001$) and overall, watermelon seedlings displayed the highest severity ratings (Fig. 3). BFB severity on cantaloupe and pumpkin seedlings was significantly lower than for watermelon ($\alpha = 0.05$) but the differences between these two hosts were not statistically significant. BFB severity was lowest on squash seedlings. Overall, the effect of *A. avenae* subsp. *citrulli* strain on BFB severity was statistically significant ($P < 0.0001$). Linear contrast analysis indicated differences in the response of groups I and II strains on seedling hosts. Differences in BFB severity caused by groups I and II strains were significant on watermelon ($P < 0.0001$), cantaloupe ($P = 0.04$) and squash ($P < 0.0001$), but not on pumpkin ($P = 0.34$) (Table 3). Additionally, the mean BFB severity for group II strains on watermelon (4.68) was significantly greater than on cantaloupe (3.16), pumpkin (3.12) and squash (1.62) (Fig. 3; Table 3). BFB severity caused by group II strains was also significantly higher on cantaloupe and pumpkin than on squash, although the differences between cantaloupe and pumpkin were not significant ($P = 0.83$). In contrast, the mean BFB severity for group I strains on watermelon (3.52) was not significantly greater than on cantaloupe (3.74) or pumpkin (3.38) (Fig. 3; Table 3). The difference in mean BFB severity caused by group I strains was higher ($P < 0.007$) on watermelon than on squash. The differences in BFB severity caused by group I strains on cantaloupe and pumpkin were not significant ($P = 0.19$). Finally, the differences between mean BFB severity on watermelon seedlings caused the group II strains was significantly higher than on cantaloupe, pumpkin or squash collectively ($P < 0.0001$). In contrast, a similar comparison was not statistically significant for group I strains ($P = 0.31$) (Table 3). REP-PCR confirmed that the strains recovered from the infected seedlings belonged to the same groups as those applied.

Fruit pathogenicity assays

Cantaloupe and watermelon fruit inoculation with *A. avenae* subsp. *citrulli* strains resulted in external BFB symptoms that ranged from small, restricted water-soaked spots to blackened areas. Internal fruit symptoms ranged from small lesions that barely penetrated the rind to black cavities within the flesh of the fruit. Especially in the case of cantaloupe, extensive internal fruit rot developed with only minor external symptoms. In some cases, infected watermelon fruits displayed a dry, mummified rot.

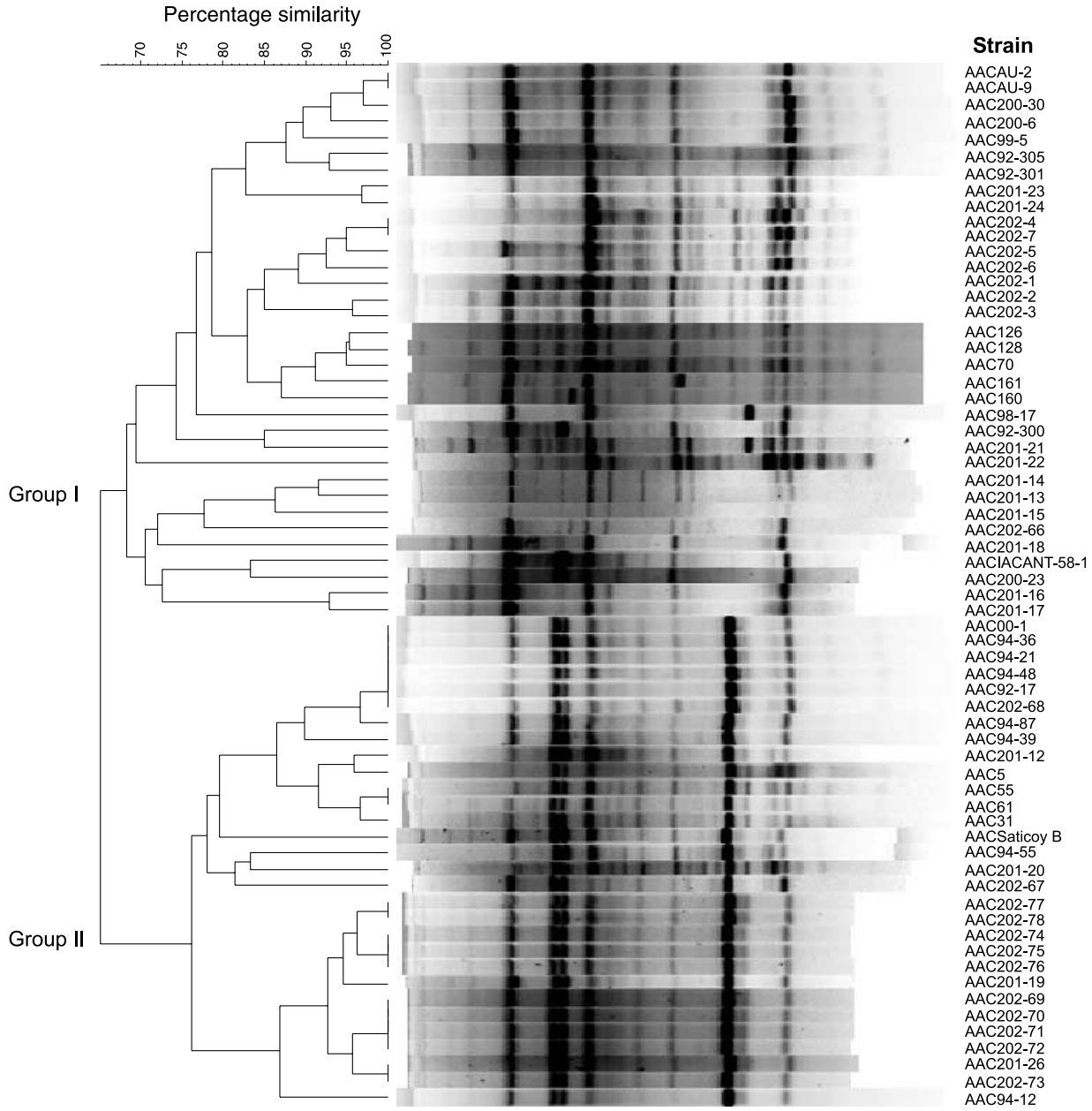

Fig. 2 Cluster analysis of *Acidovorax avenae* subsp. *citrulli* strains based on DNA fingerprint profiles generated by polymerase chain reaction (PCR) amplification of repetitive BOX elements. Distance matrix data was generated using Dice's (1945) coefficient of similarity and the dendrogram was constructed by the unweighted pairwise group method with arithmetic mean (UPGMA) algorithm

Table 2
Sole carbon substrates differentially utilized by groups I and II strains of *Acidovorax avenae* subsp. *citrulli*

	Proportion of strains utilizing substrate (%)	
Substrates	Group I	Group II
α-Hydroxybutyric acid	28.6 (8/28)	85.2 (23/27)
L-Leucine	3.57 (1/28)	96.3 (26/27)
2-Amino ethanol	82.8 (24/28)	48.1 (13/27)

As expected, none of the fruits inoculated with PBS developed BFB symptoms and data for the control fruits were excluded from statistical analysis. Overall, the differences between the two experiments were not significant ($P = 0.36$); hence, the data were pooled for analysis. The effect of fruit host on BFB severity was statistically significant ($P = 0.0013$) and overall, watermelon cv. Crimson Sweet (2.40) was more susceptible to infection than cantaloupe cv. Athena (1.33) (Fig. 4). With the exception of ATCC29625, all strains induced BFB symptoms on cantaloupe and watermelon fruits. The effect of strain on BFB severity was statistically significant ($P < 0.0001$). Linear contrast analysis revealed that while there were no significant differences in BFB severity between groups I and II strains on cantaloupe ($P = 0.10$), the differences on watermelon were significant ($P < 0.0001$) (Table 4). The mean BFB severity induced by group II strains was significantly

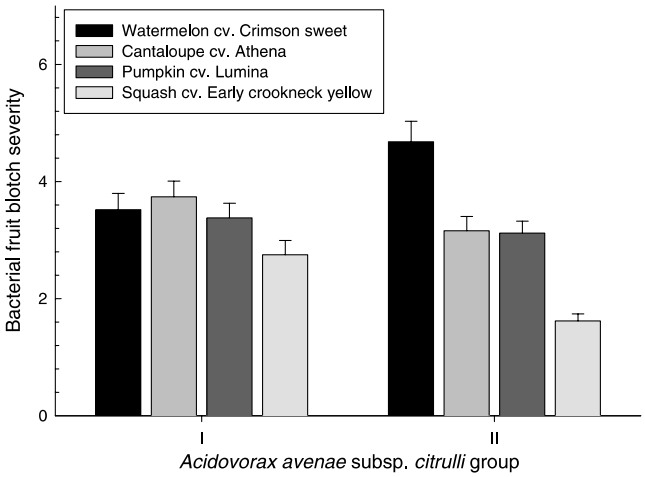

Fig. 3 Mean bacterial fruit blotch (BFB) severity estimates from 2-week-old seedlings of watermelon cv. Crimson sweet, cantaloupe cv. Athens, pumpkin cv. Lumina and squash cv. Early yellow crookneck spray-inoculated with 10 different strains of *Acidovorax avenae* subsp. *citrulli* representing groups I and II. Bars indicate mean BFB severity estimates for each group [$n = 50$ seedlings (five strains inoculated into 10 seedlings each)] and lines indicate the standard errors of the mean

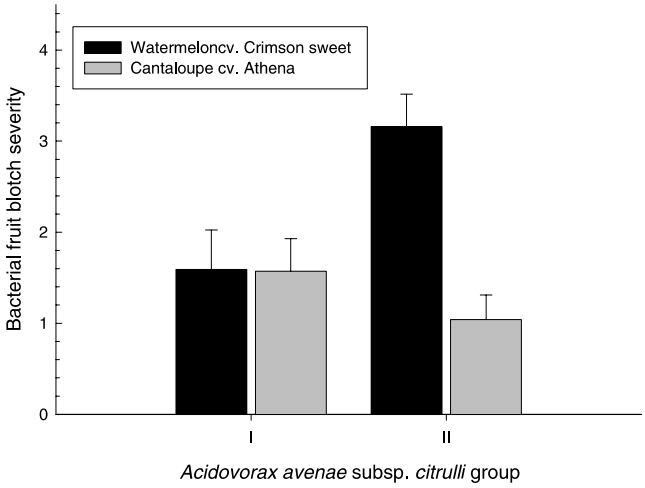

Fig. 4 Mean bacterial fruit blotch (BFB) severity of cantaloupe cv. Athena and watermelon cv. Crimson sweet fruits inoculated with *Acidovorax avenae* subsp. *citrulli* strains representing groups I and II. Bars represent mean BFB severity estimates for each group [$n = 40$ fruits (five strains inoculated onto eight fruits)] and lines represent the standard error of the mean

greater for watermelon (3.16) than for cantaloupe (1.04) ($P = 0.006$). Additionally, while the group II strains were more aggressive on watermelon than cantaloupe ($P < 0.0001$), group I strains were equally aggressive on both hosts ($P = 0.89$) (Table 4). REP-PCR confirmed that the strains recovered from the infected fruits belonged to the same groups as those applied.

Discussion

Since 1989 BFB has been a sporadic but serious threat to watermelon production. Recent trends of devastating BFB outbreaks in melons, pumpkin, cucumber, bitter and bottle gourds in Costa Rica, Brazil, China, Australia, Nicaragua and Taiwan suggest that the host range of *A. avenae* subsp. *citrulli* has expanded (Assis et al., 1999; Langston et al., 1999; Martin and O'Brien, 1999; O'Brien and Martin, 1999; Cheng et al., 2000; Zhao et al., 2001; Martin and Horlock, 2002; Munoz and Monterroso, 2002). While the recent increase in reliance on greenhouse-grown transplants for cucurbit production could be contributing to BFB outbreak frequency, it is more likely that increases in BFB on non-watermelon hosts are due to changes in the population structure of the pathogen. Diversity among *A. avenae* subsp. *citrulli* populations was suggested in 1991 when the subspecies type strain and strains from the 1989 BFB outbreak in Florida were found to differ based on fatty acid profiles and the ability to cause a hypersensitive response on tobacco (Somodi et al., 1991). O'Brien and Martin (1999), reported two distinct groups of *A. avenae* subsp. *citrulli* from North and

Table 3
Linear contrasts comparing bacterial fruit blotch (BFB) severity caused by groups I and II *Acidovorax avenae* subsp. *citrulli* strains on 2-week-old seedlings of watermelon cv. Crimson sweet, cantaloupe cv. Athena, pumpkin cv. Lumina and squash cv. Early yellow crookneck

Treatment comparison[1]	*F*-value	$P > F$
Group I (3.52) vs. group II (4.68) on watermelon	18.16	< 0.0001
Group I (3.74) vs. group II (3.16) on cantaloupe	4.18	0.042
Group I (3.38) vs. group II (3.12) on pumpkin	0.91	0.34
Group I (2.75) vs. group II (1.62) on squash	15.89	< 0.0001
Group I on watermelon (3.52) vs. cantaloupe (3.74)	0.65	0.42
Group I on watermelon (3.52) vs. pumpkin (3.38)	0.26	0.61
Group I on watermelon (3.52) vs. squash (2.75)	13.60	0.0071
Group I on cantaloupe (3.74) vs. pumpkin (3.38)	1.75	0.19
Group I on cantaloupe (3.74) vs. squash (2.75)	12.17	0.0005
Group I on pumpkin (3.38) vs. squash (2.75)	4.9	0.03
Group I on watermelon (3.52) vs. cantaloupe, pumpkin and squash (3.23)	1.04	0.31
Group II on watermelon (4.68) vs. cantaloupe (3.16)	30.02	< 0.0001
Group II on watermelon (4.68) vs. pumpkin (3.12)	32.84	< 0.0001
Group II on watermelon (4.68) vs. squash (1.62)	22.80	< 0.0001
Group II on cantaloupe (3.16) vs. pumpkin (3.12)	0.05	0.83
Group II on cantaloupe (3.16) vs. squash (1.62)	31.25	< 0.0001
Group II on pumpkin (3.12) vs. squash (1.62)	29.24	< 0.0001
Group II on watermelon (4.68) vs. cantaloupe, squash and pumpkin (2.67)	83.67	< 0.0001

[1]The numbers in parentheses represent the mean BFB severity rating on a scale of 1–9 for each group/host combination.

Table 4
Linear contrasts comparing bacterial fruit blotch (BFB) severity caused by groups I and II strains on the fruit stages of watermelon cv. Crimson sweet and cantaloupe cv. Athena

Treatment comparison[1]	*F*-value	P > *F*
Group I (1.59) vs. group II (3.16) on watermelon	7.87	0.006
Group I (1.57) vs. group II (1.04) on cantaloupe	2.71	0.10
Group I on watermelon (1.59) vs. cantaloupe (1.57)	0.02	0.89
Group II on watermelon (3.16) vs. cantaloupe (1.04)	22.86	< 0.0001

[1]The numbers in parentheses represent the mean BFB severity rating on a scale of 1–5 for each group/host combination.

South Queensland, Australia based on the utilization of L-leucine and 2-amino ethanol. The authors also reported differences in pathogenicity between these groups and suggested that the North Queensland strains may have been introduced into Australia on seed.

In this study, at least two genetically and biochemically distinct groups were confirmed among strains recovered from Brazil, China, Taiwan, Australia, China, Canada, Israel, Thailand and the USA. DNA fingerprint data indicated that *A. avenae* subsp. *citrulli* groups corresponded to host of origin, with 93% of group II strains being recovered from watermelon as opposed to 18% of the group I strains (Table 5). Additionally, 96% of the group II strains utilized L-leucine when compared with 4% of the group I strains (Table 5). Similar observations were reported by O'Brien and Martin (1999) and our analysis of two strains included in their study, AAC201-19 (4391) and AAC201-20 (4884), indicates that the North and South Queensland strains corresponded to our groups I and II, respectively.

Group II strains were more aggressive than group I strains on watermelon seedlings but the opposite was true for cantaloupe and squash (Table 5). Similar findings were reported by O'Brien and Martin (1999) and this evidence, combined with the fact that 85% of the strains collected in Georgia between 1990 and 1999 were group II strains (Walcott et al., 2000), may be the reason why BFB was considered a threat to watermelon alone. While we were unable to include the original Florida strains in this study, it is likely that these were also members of group II based on the presence of 12:0, 3:OH and 17:0 *cyclo*-fatty acids (Somodi et al., 1991). These fatty acids are absent from the profiles of group I strains (Walcott et al., 2000).

The increase in BFB outbreaks on cantaloupe and other cucurbits around 1996 represents a possible introduction of group I strains into commercial cucurbit production regions. Group I strains were more aggressive on cantaloupe and squash seedlings than group II. However, no obvious differences in BFB severity were observed for group I strains on watermelon and cantaloupe fruit under greenhouse conditions. It is possible that the differences in aggressiveness on fruit may be more pronounced under natural field conditions. At present there is no compelling data to indicate the geographical origin of the group I strains; however, it is possible that contaminated seed may have been the vector as suggested by O'Brien and Martin (1999).

While widespread BFB on melon was first observed in 1996, there is evidence that group I strains were present in the USA prior to this period. Group I strains, AAC92-301 and AAC92-305, were deposited in the University of Georgia, Coastal Plain Experiment Station (UGA-CPES) bacterial collection in 1992; however, the geographical origin of these strains are unknown. Interestingly, strains with PFGE DNA fingerprints identical to AAC92-301 (Fig. 1) were recovered from melons in Brazil (AAC201-21, AAC201-22 and AAC201-23).

ATCC29625, was reported in the USA in 1965 (Webb and Goth, 1965); however, unlike other group I strains, it is able to utilize L-leucine (Schaad et al., 1978). AAC IACANT58-1 and AAC200-23, recovered from plant introductions in Iowa and Oklahoma, USA, respectively, differed from the ATCC29625 by one PFGE polymorphism, yet they did not utilize L-leucine. It is possible that the subspecies type strain represents a third group; however, more strains sharing these characteristics are required to test this hypothesis.

One interesting finding from this study was the general agreement between PFGE and REP-PCR data. While PFGE is robust and widely used for prokaryote DNA fingerprinting, it is time-consuming, and expensive equipment is required (Olive and Bean, 1999). REP-PCR is more accessible, and our results indicate that REP-PCR profiles using BOXA1R primers could distinguish between strains of groups I and II.

Table 5
Summary of the genotypic and phenotypic characteristics that distinguish groups I and II strains of *Acidovorax avenae* subsp. *citrulli*

	Characteristics					
	Proportion of strains (%)					
Group	Recovered from watermelon	Cu^{2+} sensitive	Utilize L-leucine	Aggressiveness on watermelon	Aggressiveness on non-watermelon cucurbits	Genetic uniformity
I	18 (6/33)	17 (5/29)	4 (1/28)	Moderate	Moderate	Heterogenous
II	93 (28/30)	96 (12/23)	96 (6/27)	High	Low	Homogenous

Another interesting observation was that 83% of the group I strains were insensitive to copper in *in vitro* assays. This is of practical significance since copper-based bactericides are the only effective options for in-field BFB management (Hopkins, 1991, 1995). With the increasing prevalence of group I strains, it is possible that BFB may be more difficult to manage in the future. The possible horizontal transfer copper of resistance genes to group II strains is also reason for concern.

One unresolved but troubling issue is the significance of the threat of BFB to other commercially important cucurbits. To date, there have been no reports of BFB on commercial squash in the USA, although it has been reported in Thailand (P. Siriwong, personal communication). While squash was the most resistant host in our study, it was susceptible to infection by AAC98-17. An *A. avenae* subsp. *citrulli* strain of the same haplotype (AAC201-24) was recovered from melons in Brazil and introduction of this and other group I strains may increase the likelihood of BFB occurring on squash in the future. To prevent this extra efforts must be made to exclude non-indigenous *A. avenae* subsp. *citrulli* strains.

Acknowledgements

Authors thank the following for providing *A. avenae* subsp. *citrulli* strains: V. Dittapongpich and P. Siriwong, Khon Kaen University; H. Baishi, Nanjing University; R. Mariano, UFPRE, Brasil; H. Martin, Australian Department of Plant Industries; D. Cuppels, AgriCanada; J. Jones, University of Florida; R. Gitaitis, University of Georgia; B. Bruton USDA-ARS; and C. Block USDA-ARS NCRPIS, Ames, IA.

References

Assis, S. M. P., R. L. R. Mariano, D. M. W. Silva-Hanlin, V. Duart (1999): Bacterial fruit blotch caused by *Acidovorax avenae* subsp. *citrulli* in melon, in the state of Rio Grande do Norte, Brazil. Fitopathol. Brasilia **24,** 191.

Black, M. C., T. Isakeit, L. W. Barnes, T. A. Kucharek, R. J. Hoover, N. C. Hodge (1994): First report of bacterial fruit blotch of watermelon in Texas. Plant Dis. **78,** 831.

Cheng, A. H., Y. L. Hsu, T. C. Huang, H. L. Wang (2000): Susceptibility of cucurbits to *Acidovorax avenae* subsp. *citrulli* and control of fruit blotch on melon. Plant Pathol. Bull. **9,** 151–156.

Dice, L. R. (1945): Measures of the amount of ecologic association between species. Ecology **26,** 297–302.

Hopkins, D. L. (1991): Control of bacterial fruit blotch of watermelon with cupric hydroxide. Phytopathology **81,** 1228.

Hopkins, D. L. (1995): Copper-containing fungicides reduce the spread of bacterial fruit blotch of watermelon in the greenhouse. Phytopathology **85,** 510.

Hopkins, D. L., C. M. Thompson (2002a): Evaluation of *Citrullus* sp. germplasm for resistance to *Acidovorax avenae* subsp. *citrulli*. Plant Dis. **86,** 61–64.

Hopkins, D. L., C. M. Thompson (2002b): Seed transmission of *Acidovorax avenae* subsp. *citrulli* in cucurbits. Hortscience **37,** 924–926.

Isakeit, T., M. C. Black, L. W. Barnes, J. B. Jones (1997): First report of infection of honeydew with *Acidovorax avenae* subsp. *citrulli*. Plant Dis. **81,** 694.

Isakeit, T., M. C. Black, J. B. Jones (1998): Natural infection of citron-melon with *Acidovorax avenae* subsp. *citrulli*. Plant Dis. **82,** 351.

Jacobs, J. L., J. P. Damicone, B. D. McCraw (1992): First report of bacterial fruit blotch of watermelon in Oklahoma. Plant Dis. **76,** 1185.

King, E. O., M. K. Ward, D. E. Raney (1956): Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44,** 301–307.

Langston, D. B. Jr, R. R. Walcott, R. D. Gitaitis, F. H. Sanders (1999): First report of a fruit rot of pumpkin caused by *Acidovorax avenae* subsp. *citrulli* in Georgia. Plant Dis. **83,** 100.

Latin, R. X. (1996): Bacterial fruit blotch. In: Zitter, T. A., D. L. Hopkins and C. E., Thomas (eds), Compendium of Cucurbit Diseases, pp. 34–35. APS Press, St Paul, USA.

Latin, R. X., K. K. Rane (1990): Bacterial fruit blotch of watermelon in Indiana. Plant Dis. **74,** 331.

Martin, H. L., C. M. Horlock (2002): First report of *Acidovorax avenae* subsp. *citrulli* as a pathogen of Gramma in Australia. Plant Dis. **86,** 1406.

Martin, H. L., R. G. O'Brien (1999): First report of *Acidovorax avenae* subsp. *citrulli* as a pathogen of cucumber. Plant Dis. **83,** 965.

Moore, D. D. (1999): Preparation and analysis of DNA. In: Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl (eds), Short Protocols in Molecular Biology, pp. 2 : 1–2 : 52. John Wiley and Sons, Inc., NY, USA.

Mora-Umana, F., C. M. Araya (2002): Bacterial spot of fruits of melon and watermelon: integrated management of an emergency. Manajo Integrado de Plagas y Agroecologia **66,** 105–110.

Munoz, M., D. Monterroso (2002): Identification of *Acidovorax avenae* subsp. *citrulli* in watermelon seeds in Nicaragua. Manejo Integrado de Plagas y Agroecologia **66,** 101–104.

O'Brien, R. G., H. L. Martin (1999): Bacterial blotch of melons caused by strains of *Acidovorax avenae* subsp. *citrulli*. Aust. J. Exp. Agric. **39,** 479–485.

Olive, D. M., P. Bean (1999): Principles and applications of methods for DNA-based typing of microbial organisms. J. Clin. Microbiol. **37,** 1661–1669.

Schaad, N. W., G. Sowell, R. W. Goth, R. R. Colwell, R. E. Webb (1978): *Pseudomonas pseudoalcaligenes* subsp. *citrulli* subsp. nov. Int. J. Syst. Bacteriol. **28,** 117–125.

Schaad, W., Y. Song, E. Hatziloukas (1999): PCR primers for detection of plant pathogenic species and subspecies of *Acidovorax*. US Patent **6,** 146–834.

Shirakawa, T., S. Kikuchi, T. Kato, K. Abiko, A. Kaiwa (2000): Occurrence of watermelon bacterial fruit blotch in Japan. Jpn J. Plant Pathol. **66,** 223–231.

Somodi, G. C., J. B. Jones, D. L. Hopkins, R. E. Stall, T. A. Kucharek, N. C. Hodge, J. C. Watterson (1991): Occurrence of a bacterial watermelon fruit blotch in Florida. Plant Dis. **75,** 1053–1056.

Walcott, R. R., D. B. Langston, F. H. Sanders, R. D. Gitaitis (2000): Investigating intraspecific variation of *Acidovorax avenae* subsp. *citrulli* using DNA fingerprinting and whole cell fatty acid analysis. Phytopathology **90,** 191–196.

Walcott, R. R., R. D. Gitaitis, A. C. Castro (2003): Role of blossoms in watermelon seed infestation by *Acidovorax avenae* subsp. *citrulli*. Phytopathology **93,** 528–534.

Webb, R. E., R. W. Goth (1965): A seedborne bacterium isolated from watermelon. Plant Dis. Rep. **49,** 818–821.

Willems, A., M. Goor, S. Thielemans, M. Gillis, K. Kersters, J. D. Ley, J. De Ley (1992): Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov., comb. nov., *Acidovorax avenae* subsp. *citrulli*, *Acidovorax avenae* subsp. *cattleyae*, and *Acidovorax konjaci*. Int. J. Syst. Bacteriol. **42,** 107–119.

Zhao, T., F. Sun, B. Wang, W. Hui (2001): Pathogen identification of Hami melon bacterial fruit blotch. Acta Phytopathol. Sin. **31,** 357–364.