Molecular, Physiological, and Host-Range Characterization of *Acidovorax avenae* subsp. *citrulli* Isolates from Watermelon and Melon in Israel

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ABSTRACT

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Bacterial fruit blotch (BFB), caused by *Acidovorax avenae* subsp. *citrulli*, is a serious disease of cucurbit plants. The first important occurrence of BFB in Israel was during 2000 to 2003 on watermelon and melon. Twelve bacterial isolates associated with these outbreaks were confirmed as *A. avenae* subsp. *citrulli* by pathogenicity assays, gas chromatography of fatty-acid methyl esters, and substrate-utilization profiles. The isolates were characterized in terms of their aggressiveness in different hosts by seed, seedling, and fruit inoculations, and according to their DNA fingerprinting profiles using pulse-field gel electrophoresis (PFGE) and repetitive-PCR approaches. Results from the present work agree with previous studies supporting the existence of two differentiated groups within *A. avenae* subsp. *citrulli*, one including strains that are more associated with watermelon (group II), the other consisting of strains that are usually associated with nonwatermelon cucurbits (group I). This study indicates that isolates from both groups have been introduced to Israel. PFGE analysis revealed that the 12 analyzed isolates can be divided into five different haplotypes, of which four were previously unreported. Additional differentiating features between group I and II strains are presented.

Additional keywords: *Citrullus lanatus*, *Cucumis melo*, Cucurbitaceae

The gram-negative bacterium *Acidovorax avenae* subsp. *citrulli*, formerly *Pseudomonas pseudoalcaligenes* subsp. *citrulli*, is the causal agent of bacterial fruit blotch (BFB) of cucurbits (22,30). Although the disease was first reported in 1965 in watermelon seedlings of several plant introduction lines in Georgia, in the United States (24), BFB gained importance after devastating outbreaks in watermelon fields in the Mariana Islands (28), Indiana (13), and Florida (23) in the late 1980s. Since then, BFB has emerged as a major threat to watermelon in the southeastern and midwestern United States. In recent years, significant economic losses caused by *A. avenae* subsp. *citrulli* on other cultivated cucurbits, including melon, cucumber, squash, and pumpkin, have been reported in many parts of the world, including the United States, Nicaragua, Costa Rica, Brazil, Australia, Turkey, Japan, Korea, Thailand, Taiwan, and China (7,8,11,15,18,21,25).

A. avenae subsp. *citrulli* can be highly destructive. Under favorable conditions, the bacterium spreads rapidly throughout transplant houses and in the field, leading to seedling blight and/or fruit rot. Chemical control of BFB has been ineffective (10,19,21,24), and complete/monogenic disease resistance is not currently available in commercial cultivars. *A. avenae* subsp. *citrulli* is transmitted by seeds that usually serve as the primary source of inoculum for BFB outbreaks (6,21).

Although chemical treatments can reduce *A. avenae* subsp. *citrulli* populations in infested seeds, they fail to eradicate the bacterium (5,21). Therefore, the main strategy for combating BFB is the elimination of infested seed lots prior to planting. Several seed assays for *A. avenae* subsp. *citrulli* detection have been developed; however, all have limitations (21,26). Because of the costly lawsuits filed by growers against watermelon seed companies, BFB represents a serious threat to the watermelon industry. For instance, in the mid-1990s, to avoid costly litigation, several seed companies in the United States suspended the sale of watermelon seeds in several southeastern states that were considered to be at high risk for BFB outbreaks (12,21).

Based on pathogenicity assays, analyses of DNA fingerprinting profiles, and whole-

cell fatty-acid analyses of many isolates, Walcott et al. (25,27) confirmed previous evidence (18,23) indicating that *A. avenae* subsp. *citrulli* strains can be divided into at least two well-differentiated groups: group I includes strains that were mainly isolated from nonwatermelon cucurbits and the species type strain, ATCC29625, which was isolated from watermelon PI lines (22); group II includes the typical watermelon BFB-causing strains that were responsible for the late 1980s outbreaks in the United States. It was also suggested that inconsistent results from different attempts to screen watermelon varieties for BFB resistance could be due, at least in part, to significant differences in aggressiveness between group I and II strains (27).

In Israel, a strange phenomenon of seedling collapse was observed in a few spots of a single watermelon field in the southern part of the Golan Heights region in 1979. Bacteria isolated from diseased seedlings were shown to be aggressive on watermelon, and several biochemical assays showed the highest similarity to *P. pseudoalcaligenes* subsp. *citrulli* (G. Kritzman, unpublished data). Later, *A. avenae* subsp. *citrulli* was intercepted in imported watermelon seeds in the years 1992 to 1994 (1). Interestingly, the pathogen was shown to be transmitted by solanaceous seeds since the bacterium was isolated from several shipments of tomato seeds imported to Israel, and from eggplant seedlings grown from imported seeds (2). Natural BFB outbreaks occurred in several watermelon and melon fields and greenhouses from different Israeli regions during 2000 to 2003, causing economic losses, especially in 2002. The objective of this work was to characterize *A. avenae* subsp. *citrulli* isolates associated with these outbreaks in terms of aggressiveness, DNA fingerprinting profiles, and several biochemical features.

MATERIALS AND METHODS

Bacterial isolates. Bacteria were isolated from leaves, fruits, and seeds of watermelon and melon from five fields and three greenhouses located at different geographical regions, as well as from rotted fruits from a local market, during 2000 to 2003 (Table 1). Following preliminary pathogenicity testing, 12 isolates, named

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W1 to W6 and M1 to M6 for those isolated from watermelon and melon (Table 1), respectively, were selected for further characterization. Gas chromatographic analysis of fatty-acid methyl esters (GC-FAME) was performed by the Israeli Plant Protection and Inspection Services (Volcani Center, Bet Dagan, Israel), according to described procedures (16), after growing bacteria on trypticase soy broth agar (TSBA; Difco Laboratories, Detroit, MI) for 24 h at 28°C. GC-FAME similarity indexes were determined by statistical comparison to the MIDI TSBA40 library through the MIDI Sherlock Microbial Identification System (Midi Inc., Newark, DE). Carbon-source metabolic fingerprinting was carried out using the Biolog system, according to manufacturer's instructions (Biolog Inc., Hayward, CA). Biolog similarity indexes were determined by analysis of the data using the Microlog software (Biolog Inc.). *A. avenae* subsp. *citrulli* isolates were stored in 20% sterile glycerol at -80°C.

Growth curves, and physiological and biochemical assays. For growth curves, individual colonies from nutrient agar (NA; Becton, Dickinson and Co., Sparks, MD) plates were used to inoculate 5-ml nutrient broth (NB; Becton, Dickinson and Co.) starter cultures that were incubated at 28°C with shaking (200 rpm) for 24 h. Then, 1 ml of each starter culture was added to 100 ml of NB in 250-ml Erlenmeyer flasks. Cultures were grown as described and cell density was measured every 2 h in a Helios Gamma spectrophotometer (Thermo Electron Corp., Rochester, NY) at 600 nm. Overall differences in growth curves between M and W isolates were assessed by logistic curve comparison using JMP software (JMP, Cary, NC). Extracellular enzyme activities were tested by growing cells for 48 h at 28°C on the following solid media: milk-agar (70% wt/vol skim milk in 2% wt/vol agar) for protease activity; 0.5% (wt/vol) starch in NA for starch hydrolysis; 0.5% (wt/vol) carboxymethylcellulose in NA for cellulase activity; and a medium containing 15

g of sodium polypectate, 3 g of trisodium citrate dehydrate, 0.8 g of NaNO₃, 0.3 g of CaCl₂, 50 mg of sodium dodecyl sulfate, 0.75 mg of crystal violet, and 0.2% agar for pectinase activity per liter. Activities were estimated by measuring the diameters of corresponding haloes around colonies. For starch hydrolysis, a few drops of IKI solution (0.1 g of iodine and 0.2 g of KI per liter) were added to stain nonhydrolyzed starch in the medium. Each experiment was carried out at least twice. Positive and negative controls consisted of different species (local isolates) from our laboratory collection, including *Bacillus megaterium* (protease⁺, starch hydrolysis⁺, pectinase⁻), *Serratia marcescens* (protease⁺, starch hydrolysis⁺, cellulase⁻, pectinase⁻), *Pectobacterium carotovorum* subsp. *carotovorum* (cellulase⁺, pectinase⁺), and *Micrococcus* sp. (protease⁻, starch hydrolysis⁻, cellulase⁻). *Escherichia coli* DH10B (Invitrogen, Carlsbad CA; starch hydrolysis⁻, cellulase⁻, pectinase⁻) was also utilized. To assess significant differences between W and M isolates, data were subjected to statistical analysis by *t* test. Catalase activity was assessed by adding a few drops of H₂O₂ on 48-h-grown NA cultures. Sensitivity of isolates was assessed for the following antibiotics according to standard concentrations (3,20): ampicillin (100 µg/ml), cephalexin (30 µg/ml), chloramphenicol (17 µg/ml), and rifampicin (40 µg/ml).

Seedling pathogenicity assays. Three-week-old seedlings of watermelon (*Citrus lanatus*) cv. Malali (Hazera Genetics), melon (*Cucumis melo* var. *reticulatus*) cv. Ideal (Galia muskmelon type; Syngenta), melon (*Cucumis melo* var. *cantalupensis*) cv. 6404 (Ananas cantaloupe type, Hazera Genetics), and cucumber (*Cucumis sativus*) cv. Olympus (Syngenta), were inoculated with each of the 12 isolates. The plants were grown in 650-ml plastic pots in a medium containing equal parts sand, peat, and vermiculite under standard greenhouse conditions (25 to 28°C). For inoculation, bacteria were grown on NA containing 100 µg/ml ampicillin for 48 h at

28°C. Following resuspension in sterile water, bacterial suspensions were adjusted to ~10⁸ CFU/ml using a spectrophotometer. Carborundum was added to the suspensions at 100 mg/ml, and inoculation was performed by spreading the resulting mixture on one cotyledon and one of the true leaves of each seedling with a brush. Negative controls were seedlings treated with a mixture of sterile water and Carborundum. Seedlings were covered with plastic bags for 48 h to increase humidity, then the bags were removed and seedlings were kept in the greenhouse. Seven days after inoculation (DAI), the seedlings were scored based on a 0 to 4 disease index: 0, no symptoms; 1, slight water-soaking on cotyledon (water-soaking observed mainly on margins) and necrotic spots on true leaf; 2, massive water-soaking on cotyledon and necrotic spots on true leaf; 3, collapse of the infected cotyledon and/or leaf; and 4, spread of the infection to other leaves and collapse of the whole plant. Each isolate/host combination included four replicates (plants) per experiment. The experiment was carried out three times, and data were subjected to statistical analysis using nonparametric two-way analysis of variance.

Seed transmission assays. Seeds of watermelon cv. Malali, melon cv. 6404, and cucumber cv. Olympus were incubated in *A. avenae* subsp. *citrulli* suspensions (10⁸ CFU/ml) with gentle shaking (100 rpm) for 1 h at room temperature. Bacterial suspensions were prepared as indicated for seedling pathogenicity assays. Sterile water was used as a negative control. After incubation, suspensions were removed and seeds were gently washed with sterile water, air-dried for 4 h at room temperature, and sowed in 1.3-liter plastic pots containing sand for growth in the greenhouse at a temperature of 25 to 28°C. Percentages of symptomatic seedlings and of seedling death were assessed 18 DAI. Each isolate-host combination included 12 plants distributed in two pots per experiment. The experiment was carried out twice, and data were subjected to analysis of variance with

Table 1. *Acidovorax avenae* subsp. *citrulli* isolates included in this study

Isolate	Host and part of the plant	Geographical origin	Year collected ^a	GC-FAME SI ^b	Biolog SI ^b
W1	Watermelon, leaf from symptomatic plant	Dead Sea region	2002	0.756	0.530
W2	Watermelon, leaf from symptomatic plant	Northern Negev	2003	0.731	0.540
W3	Watermelon, fruit from symptomatic plant	Northern Negev	2003	0.663	0.655
W4	Watermelon, leaf from symptomatic plant	Golan Heights	2002	0.694	0.595
W5	Watermelon, rotted fruit from market	Central region	2003	0.756	0.757
W6	Watermelon, symptomatic seedling	Northern Negev	2001	0.805	0.881
M1	Melon, leaf from symptomatic seedling	Northern Negev	2000	0.530	0.560
M2	Melon, leaf from symptomatic plant	Northern Negev	2002	0.825	0.780
M3	Melon, fruit from symptomatic plant	Northern Negev	2002	0.926	0.724
M4	Melon, seeds from symptomatic plant	Northern Negev	2002	0.620	0.805
M5	Melon, rotted fruit from market	Central region	2001	0.907	0.952
M6	Melon, rotted fruit from symptomatic plant	Central region	2002	0.806	0.622

^a All isolates were collected by G. Kritzman.

^b SI (similarity index), similarity with *A. avenae* subsp. *citrulli*. GC-FAME SI was determined through statistical comparison with the TSBA40 library using the MIDI Sherlock Microbial Identification System. Biolog SI was determined through analysis with the Microlog software. For all isolates, *A. avenae* subsp. *citrulli* was the highest hit for both GC-FAME and Biolog profiles.

isolates nested in groups (W and M), using JMP software.

Fruit pathogenicity assays. Mature fruits of watermelon (*Citrullus lanatus*) cv. Crimson Sweet and melon (*Cucumis melo* var. *cantalupensis*) cv. Ananas were purchased from a local market. *A. avenae* subsp. *citrulli* inoculum was prepared as previously described, and fruits were inoculated by injecting 1 ml of bacterial suspensions at 10^6 or 10^8 CFU/ml with a 5-ml syringe through the rind, no deeper than 1 cm. As negative controls, fruits were injected with sterile water. Following inoculation, the fruits were placed individually into plastic bags and incubated at 25 to 28°C. After 48 h, the bags were removed and fruits were arranged in separate plastic boxes. Symptom severity was evaluated 7 DAI according to a previously determined scale (25): 0, no symptoms; 1, small water-soaking lesions on the rind (surface lesions); 2, large lesions penetrating into the rind; 3, lesions penetrating into the flesh of the fruit; 4, extensive penetration and collapse of fruit tissues; 5, complete fruit rot. Each isolate was applied to three watermelon and melon fruits, at four and two different sites per fruit, respectively. The experiments were carried out twice, and data were subjected to statistical analysis using nonparametric two-way analysis of variance.

DNA digestions with *SpeI* and pulsed-field gel electrophoresis (PFGE). Bacteria were grown on NA at 28°C for 48 h. Cells were suspended in a buffer containing 100 mM EDTA and 100 mM NaCl (pH 8.0), and adjusted to an OD₆₀₀ of 0.3. Cells were pelleted at $10,780 \times g$ for 2 min and resuspended in 300 µl of the same buffer, followed by the addition of 60 µl of a 10 mg/ml lysozyme solution. Preparation of agarose plugs and restriction with the rare-cutting endonuclease *SpeI* (New England Biolabs, Beverly, MA) were done as described (27). PFGE was carried out in 15 × 15 cm 1% Seakem Gold agarose gels using a CHEF DR-II apparatus (Bio-Rad, Hercules, CA). The gels were run in 0.5× TBE buffer at a temperature of 14°C. The *SpeI*-digested fragments were resolved as described (27). Concatemeric lambda DNA (New England Biolabs) was used as a marker. Following electrophoresis, gels were stained in an ethidium-bromide solution (0.5 µg/ml) and photographed with transmitted UV light at 295 nm. Two independent PFGEs (from different restriction digestion reactions) were carried out for each isolate, with identical results.

Repetitive-PCR (Rep-PCR) fingerprinting. Genomic DNA was prepared from 1.5 ml of a 24-h NB culture of *A. avenae* subsp. *citrulli* using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). Primers REP1R-I and REP2-I (for repetitive extragenic palindromic-PCR; REP-PCR), ERIC1R and ERIC2 (for enterobacterial repetitive inter-

genic consensus-PCR; ERIC-PCR), and BOXA1R (for BOX element-PCR; BOX-PCR) were from IDT, Inc. (Coralville, IA), as described by Louws et al. (14). The PCR mixtures (25 µl) contained: 1 U of Red *Taq* Polymerase (Sigma Chemical Co., Saint Louis MO), 2.5 µl 10× buffer (Sigma), 2.5 µl of 25 mM MgCl₂, 0.2 µM of each primer (0.4 µM of BOXA1R in BOX-PCR), 0.2 mM of each dNTP (Promega), 2.75 mM bovine serum albumin (Roche, Basel, Switzerland), and 20 ng of template DNA. Negative controls were PCR mixtures without template. Amplifications were performed in an Eppendorf Thermal Cycler (Eppendorf, Hamburg, Germany) with an initial denaturation cycle of 7 min at 95°C, followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 40, 42, or 53°C (for REP-, ERIC-, and BOX-PCR, respectively), and elongation at 65°C for 8 min. A final extension step was performed at 65°C for 16 min. Samples of 12 to 15 µl from each reaction were run in 1.5% agarose gels for 4 h at 60 V. The gels were stained with an ethidium bromide solution (0.5 µg/ml) and photographed with transmitted UV light at 295 nm. Three independent experiments were carried out for each Rep-PCR technique.

RESULTS

Identification of *A. avenae* subsp. *citrulli* isolates. Bacteria isolated from symptomatic watermelon and melon plants and fruits were tested in preliminary pathogenicity assays in the greenhouse (not shown), and 12 isolates were confirmed to be *A. avenae* subsp. *citrulli* by GC-FAME analysis and carbon substrate utilization profile analysis (Table 1). Isolates were designated W1 to W6 (W isolates) and M1 to M6 (M isolates), for those isolated from watermelon and melon, respectively.

Carbon-source metabolic fingerprinting of isolates. Forty-four percent (42 of 95) of the substrates included in the Biolog plate were utilized by at least one isolate. All isolates utilized glycogen, D-galactose, methyl pyruvate, mono-methyl succinate, D-gluconic acid, β-hydroxybutyric acid, lactic acid, sebacic acid, bromo-succinic acid, L-asparagine, L-aspartic acid, L-glutamic acid, and L-pyrogutamic acid as sole carbon sources. All isolates except M1 grew on Tween 80, D-fructose, and acetic acid. Other substrates that could be utilized by most isolates were Tween 40, L-arabinose, L-proline, and 2-amino-ethanol. All W isolates, but not M isolates, grew on L-leucine, propionic acid, and α-hydroxybutyric acid as sole carbon sources. A similar pattern was observed for α-keto valeric acid and alaninamide, but in these cases, W isolates grew poorly. All W isolates, with the exception of W5, grew on D-glucose, whereas none of the M isolates grew on this carbon source. While all W isolates utilized α-keto glutaric acid, only M3 utilized this carbon substrate. Further, all W isolates grew on L-serine, whereas only M2 and M3 used this carbon source among the M isolates. Interestingly, only W2 utilized L-alanyl glycine, glycyl-L-aspartic acid, and glycyl-L-glutamic acid. Based on the PFGE results, this isolate was later found to belong to a unique, unreported haplotype.

Growth curves, and physiological and biochemical assays. Figure 1 shows the growth curves of three W and three M isolates in NB at 28°C. The other isolates showed similar growth patterns to those observed for their corresponding group (not shown). In general, all isolates showed similar growth curve dynamics, arriving at a density plateau after 18 to 20 h of growth, and no significant differences ($P = 0.05$) were found between W and M

Fig. 1. Growth curves of *Acidovorax avenae* subsp. *citrulli* isolates W2, W4, W6, M2, M4, and M6 in nutrient broth (NB) at 28°C.

isolate growth rates during the exponential phase (Fig. 1). However, W isolates showed a slightly extended exponential growth phase and reached significantly higher ($P = 0.0002$; all isolates included in the statistical analysis) levels of absorbance than M isolates. Similar differences in population growth among W and M isolates were also observed when isolates were grown at 37°C (not shown).

As expected from previous reports (22,30), all isolates were positive for catalase test, negative for starch hydrolysis, and showed weak extracellular protease activity. Weak extracellular pectinase activity was also detected, with no significant differences ($P > 0.05$) among isolates (not shown). All isolates were positive for extracellular cellulase activity. Interestingly, W isolates showed significantly higher ($P = 0.0002$) cellulase activity than M isolates: whereas W isolates showed an average halo diameter of 5.5 ± 0.7 cm, the average halo diameter for M isolates was 3.0 ± 0.3 cm (average $\pm$ standard deviation, as measured from the haloes produced in cellulase activity medium after 48 h of growth). All isolates were equally tolerant to ampicillin (100 μ g/ml) and

cephalexin (30 μ g/ml), and sensitive to chloramphenicol (17 μ g/ml). However, whereas all M isolates were sensitive to rifampicin (40 μ g/ml), all W isolates were tolerant to this antibiotic.

Seedling pathogenicity assays. All tested cucurbits were susceptible to all isolates. However, differences in aggressiveness were observed. BFB symptoms appeared 3 DAI, and severity increased with time. They included water-soaking and necrotic brown lesions on cotyledons, and reddish-brown lesions on true leaves of melon and cucumber. Similar symptoms were observed in watermelon leaves, but the lesions were gray to black (Fig. 2). None of the seedlings inoculated with sterile water developed symptoms. The experiment effect on symptom severity was not significant ($P = 0.66, 0.35, 0.42$, and 0.41 for watermelon, melon cv. 6404, melon cv. Ideal, and cucumber, respectively); hence, data from the three experiments were pooled for analysis. Results are shown in Table 2. M isolates were significantly more aggressive than W isolates on both melon cultivars and on cucumber ($P < 0.0001$ for all cases). On these hosts, M5 and M6 were found to be

particularly aggressive. Among W isolates, W1 and W6 were found to be the least aggressive on melon (Table 2).

In contrast to melon and cucumber, a differential trend of aggressiveness among M and W isolates was not observed in watermelon, since the two groups did not differ significantly in this host ($P = 0.43$). Most isolates caused the collapse of inoculated cotyledons and leaves of watermelon seedlings 7 DAI. The exceptions were isolate W5, which was slightly less aggressive than the others, and W2, which was highly aggressive, leading to the rapid spread of infection and complete collapse of watermelon seedlings (Table 2).

Seed transmission assays. Two experiments were carried out. The effects of experiment on tested parameters were not significant ($P > 0.90$ for all parameter and host combinations); therefore, data from the experiments were pooled for further analysis. The most remarkable finding was the M isolates' clearly higher aggressiveness relative to W isolates in all tested plants (Fig. 2, for isolates M6 and W1). Differences between W and M groups were statistically significant ($P < 0.0001$) for percentage of both symptomatic and

Fig. 2. Cucurbit plants inoculated with *Acidovorax avenae* subsp. *citrulli* by different methods. **A to F**, Foliar inoculation of 3-week-old seedlings. Bacterial suspensions ($\sim 10^8$ CFU/ml) were applied to cotyledons and leaves with a brush following addition of Carborundum. Melon cv. Ideal (**A and D**), cucumber cv. Olympus (**B and E**), and watermelon cv. Malali (**C and F**) inoculated with isolates M6 (**A to C**) and W1 (**D to F**). Arrows indicate inoculated leaves. Pictures were taken 7 days after inoculation (DAI). **G**, Seed inoculation of melon cv. 6404 with bacterial suspensions of M6 (right), W1 (middle), or water (left). Seeds were inoculated with bacterial suspensions ($\sim 10^8$ CFU/ml) with gentle shaking for 1 h; then they were gently washed with sterile water and air-dried before sowing. Picture was taken 10 days after sowing. Inoculation of detached fruits (**H to J**). One milliliter of a 10^6 CFU/ml suspension was injected through the rind of the fruits with a 5-ml syringe. Watermelon cv. Malali inoculated with W1 (**H and I**); melon (Ananas type) inoculated with M6 (**J**). Pictures were taken 5 DAI.

dead seedlings in all tested hosts (Table 3). In watermelon, the percentage of mortality in seedlings inoculated with M isolates ranged from 72 to 90% 18 DAI. Inoculation of seeds with these isolates induced water-soaking and coalescing lesions on cotyledons of emerging seedlings, which were usually followed by seedling death in a few days. In contrast, BFB symptoms on watermelon seedlings from seeds inoculated with W isolates developed more slowly and to a lesser extent than with M isolates. Moreover, among W isolates, only W1 and W2 caused seedling death in watermelon, and this occurred for only 10 and 12% of the inoculated seedlings, respectively (Table 3).

Overall, melon did not significantly differ from watermelon in its response to the M isolates ($P = 0.49$ for symptomatic seedlings; $P = 0.71$ for seedling death): most melon and watermelon seeds inocu-

lated with M isolates developed symptomatic seedlings, and in most cases, inoculation led to seedling death (Table 3). In contrast, most melon seeds inoculated with W isolates developed asymptomatic seedlings. Additionally, disease severity of melon seedlings inoculated with W isolates was significantly ($P < 0.0001$) lower than in watermelons inoculated with these isolates. In fact, W1, W4, and W5 did not induce symptoms at all, and the only W isolates to cause seedling death were W2 and W6, at very low rates (5 and 10%, respectively). Similar findings were observed with two other cultivars of melon, Eden (Ananas type) and Galia (both from Hazera Genetics) (data not shown).

Cucumber seeds inoculated with M and W isolates developed a significantly ($P < 0.0001$) higher percentage of symptomatic seedlings in comparison with watermelon and melon. However, seedling death for

this host was significantly lower ($P < 0.0001$) than those observed for watermelon and melon (Table 3).

Fruit pathogenicity assays. Inoculation of mature, detached watermelon and melon fruits with different isolates resulted in a wide range of typical BFB symptoms (Fig. 3). External symptoms varied from small, restricted water-soaked areas to large, blackened water-soaked blotches. Internal fruit symptoms ranged from small lesions that barely penetrated the rind to complete rot of the fruit. In some cases, minor external symptoms were accompanied by extensive internal fruit rot, especially in melon. None of the fruits inoculated with sterile water developed BFB symptoms.

Two experiments were carried out, both including inoculation with two bacterial concentrations, 10^6 and 10^8 CFU/ml. Although a similar trend of differential aggressiveness among isolates was observed at both inoculum concentrations, the differences were more evident at the lower concentration (Fig. 3), since as expected, at 10^8 CFU/ml, all isolates caused more severe symptoms (data not shown). The effects of experiment (inoculation at 10^6 CFU/ml) on BFB severity was not significant ($P = 0.37$ and 0.44 for melon and watermelon, respectively); hence, data from the two experiments was pooled. In general, W isolates showed higher aggressiveness in watermelon than in melon fruits, whereas the opposite was observed for M isolates (Fig. 3). Differences in aggressiveness on different hosts were more pronounced within W isolates, because these were found to be consistently less aggressive on melon than M isolates. In contrast, several M isolates (M5 and M6) were found to be highly aggressive on both melon and watermelon fruits, and disease severity in watermelon fruits inoculated with these isolates was as high as in fruits inoculated with W isolates (Fig. 3). These results agree with findings from seedling

Table 2. Summary of the results of pathogenicity assays of *Acidovorax avenae* subsp. *citrulli* isolates carried out with 3-week-old seedlings^a

Isolate	Host			
	Watermelon cv. Malali	Melon cv. 6404	Melon cv. Ideal	Cucumber cv. Olympus
W1	3.0 ± 0	1.8 ± 0.4	1.7 ± 0.5	2.1 ± 0.3
W2	4.2 ± 0.4	2.0 ± 0	2.1 ± 0.3	3.1 ± 0.3
W3	3.0 ± 0	2.2 ± 0.4	2.1 ± 0.3	2.1 ± 0.3
W4	2.3 ± 0.5	2.3 ± 0.5	2.0 ± 0	2.1 ± 0.3
W5	2.0 ± 0	2.1 ± 0.3	1.9 ± 0.3	2.1 ± 0.3
W6	3.1 ± 0.3	1.7 ± 0.5	1.1 ± 0.1	2.0 ± 0
W _{Av}	2.9 ± 0.6	2.0 ± 0.2	1.8 ± 0.2	2.3 ± 0.2
M1	3.0 ± 0	2.2 ± 0.4	2.4 ± 0.5	2.7 ± 0.5
M2	3.0 ± 0	2.8 ± 0.4	2.1 ± 0.3	2.1 ± 0.3
M3	2.6 ± 0.5	3.1 ± 0.3	3.1 ± 0.3	3.2 ± 0.4
M4	3.1 ± 0.3	2.7 ± 0.5	3.1 ± 0.3	2.2 ± 0.4
M5	3.1 ± 0.3	3.7 ± 0.5	3.6 ± 0.5	4.0 ± 0
M6	2.4 ± 0.5	2.8 ± 0.4	3.8 ± 0.4	3.1 ± 0.3
M _{Av}	2.9 ± 0.3	2.9 ± 0.4	3.0 ± 0.5	2.9 ± 0.5

^a Symptom severity was scored 7 days after inoculation according to the following disease index scale: 0, no symptoms; 1, slight water-soaking on cotyledon (mainly on margins) and necrotic spots on true leaf; 2, massive water-soaking on cotyledon and necrotic spots on true leaf; 3, collapse of the infected cotyledon and/or leaf; and 4, spread of the infection to other leaves and collapse of the whole plant. Values are averages and standard deviations of data pooled from three independent experiments with similar results.

Table 3. Summary of the results of seed pathogenicity assays of *Acidovorax avenae* subsp. *citrulli* isolates^a

Isolate	Watermelon cv. Malali		Melon cv. 6404		Cucumber cv. Olympus	
	Symptomatic seedlings (%)	Seedling death (%)	Symptomatic seedlings (%)	Seedling death (%)	Symptomatic seedlings (%)	Seedling death (%)
W1	10	10	0	0	75	0
W2	61	12	35	5	75	0
W3	5	0	7	0	63	25
W4	25	0	0	0	30	0
W5	10	0	0	0	40	0
W6	15	0	20	10	40	0
W _{Av}	21.0	3.7	10.3	2.5	53.8	4.2
M1	85	85	90	90	100	30
M2	90	80	90	90	90	25
M3	90	90	95	80	100	20
M4	80	77	83	50	100	13
M5	92	72	95	89	100	14
M6	85	90	93	79	100	0
M _{Av}	87.0	82.3	91.0	79.7	98.3	17.0

^a Percentages of symptomatic seedlings and of seedling death occurrence as assessed 18 days after inoculation. Values are averages of data pooled from two independent experiments (24 plants per treatment per experiment). The two groups (W_{Av} and M_{Av}) differed significantly ($P < 0.0001$) for all parameters in all tested plants.

pathogenicity assays, in which M5 and M6 were among the most aggressive isolates.

DNA fingerprinting by *Spe*I digestion and PFGE. Using PFGE, Walcott et al. (25,27) reported 24 differentiated *A. avenae* subsp. *citrulli* haplotypes, 12 within

each group (I and II), which were designated A through X, following the order in which they were first observed. Because of the high reproducibility of PFGE, DNA fingerprinting profiles from these studies could be compared with ours.

PFGE analyses revealed 23 unique DNA fragments and five different profiles, each one containing 9 to 13 bands (Fig. 4). Of these profiles, four were previously unreported. The corresponding new haplotypes were designated Y, Z, E2, and E3. Isolates M1 to M6 could be clearly classified as group I isolates, while W1 to W6 clearly belonged to group II.

Isolates M1 to M6 had identical DNA fingerprinting profiles, and thus belong to a single haplotype. This haplotype was previously unreported and, following the alphabetical order, was designated Y. Although this haplotype shares similarities with the previously reported group I haplotypes R, V, and X (25), it can be clearly distinguished from them based on several differential bands (Fig. 4).

The W isolates included four different group II profiles (Fig. 4). W1 and W3 belonged to the previously reported haplotype A (27), whereas the other isolates belong to three previously unreported haplotypes. W6 had a unique profile, with some similarities with the reported haplotypes D and J (27) (Fig. 4). This new haplotype was designated Z. Isolates W4 and W5 were shown to possess identical profiles, and shared high similarities with the reported haplotype E (27) (Fig. 4). The new haplotype was named E2. Finally, W2 also showed an unreported profile. Because the W2 profile showed similarities with those of haplotypes E and E2 (Fig. 4), this new haplotype was designated E3.

Fig. 3. Bacterial fruit blotch (BFB) severity of detached watermelon (cv. Crimson Sweet) and melon (Ananas type) fruits inoculated with *Acidovorax avenae* subsp. *citrulli* isolates at 10^6 CFU/ml. Bars and lines represent average severity ratings and standard deviations, respectively, from two independent experiments that yielded similar results. In each experiment, each isolate was injected into three fruits (replicates) at four and two different sites for watermelon and melon, respectively. Symptom severity was evaluated 7 days after inoculation according to the following scale: 0, no symptoms; 1, small water-soaking lesions on the rind (surface lesions); 2, large lesions penetrating into the rind; 3, lesions penetrating into the flesh of the fruit; 4, extensive penetration and collapse of fruit tissues; 5, complete fruit rot.

Fig. 4. Diagram of DNA fingerprinting haplotypes of *Acidovorax avenae* subsp. *citrulli* isolates by pulsed-field gel electrophoresis (PFGE) following DNA digestion with *Spe*I. M, concatemeric lambda DNA (New England Biolabs). Haplotypes identified in this study (isolates between brackets): E2 (W4 and W5); E3 (W2); Z (W6); and Y (M1 to M6). Haplotype A was previously reported (27) and includes isolates W1 and W3. Haplotypes D, E, and J (27), as well as R, X, and V (25), are included for comparison.

Rep-PCR fingerprinting. It has previously been shown that BOX-PCR can be used to distinguish between group I and II strains (25). A similar observation was made in our study, in which M and W isolates showed clearly differentiated BOX-PCR profiles (Fig. 5). Despite the typical reproducibility limitations of PCR-based fingerprinting techniques, BOX-PCR profiles from both studies were comparable, and this comparison supported the distribution of M and W isolates into groups I and II, respectively. In addition, similarly to BOX-PCR, ERIC- and REP-PCR clearly distinguished between M and W isolates (Fig. 5). In general, differences among M and W isolate profiles were more pronounced in BOX- and ERIC-PCR than in REP-PCR.

In all approaches, differences between M and W isolates included amplification of unique, group-specific DNA fragments such as the 2.0-, 1.2-, 0.8-, and 0.45-kb fragments in BOX-PCR, the 1.7- and 0.25-kb fragments in ERIC-PCR, and the 0.7- and 0.35-kb fragments in REP-PCR. In addition, M and W profiles shared several fragments with similar moieties but that were amplified at different intensities, such as the 1.0-kb fragment in BOX-PCR, the 1.1-kb fragment in ERIC-PCR, and the 1.0-kb fragment in REP-PCR (Fig. 5).

As expected from the PFGE results, all M isolates showed identical Rep-PCR profiles. However, no Rep-PCR technique was able to distinguish among the W isolates that were previously shown to belong to four different haplotypes based on PFGE (Fig. 5).

DISCUSSION

Until 1996, BFB was considered a serious threat mainly for the watermelon industry. It is likely that recent increases in BFB's occurrence on nonwatermelon cucurbits worldwide are due to changes in the population structure and spread of the pathogen (25). Diversity among populations of *A. avenae* subsp. *citrulli* was revealed by Somodi et al. (23), who reported that strains isolated from the late 1980s BFB outbreaks in the United States differed in several features from the ATCC type strain isolated in 1978 (22). Eight years later, O'Brien and Martin (18) distinguished between two distinct groups of *A. avenae* subsp. *citrulli* strains from Australia based on differences in pathogenicity and in L-leucine and 2-amino ethanol utilization. Recently, based on an extensive characterization of strains, Walcott et al. (25,27) confirmed the existence of at least two genetically distinct groups within this bacterium: group I included strains that were mainly isolated from nonwatermelon cucurbits such as melon and pumpkin, and group II included the typical watermelon BFB-causing strains. Our findings support this division and revealed that isolates

from both groups have been introduced into Israeli fields.

DNA fingerprinting by PFGE following *SpeI* digestion revealed that the 12 isolates belong to five different haplotypes, which could be clustered into the two defined *A. avenae* subsp. *citrulli* groups. Isolates from watermelon showed a higher degree of heterogeneity than melon isolates, consisting of four different haplotypes. Whereas W1 and W3 belong to the previously reported haplotype A (27), the other isolates belong to three different, previously unreported haplotypes. In contrast, isolates from melon were found to be monogenic, consisting of a single haplotype that had not been previously reported. Despite iden-

tical PFGE profiles, isolates M1 to M6 could be differentiated by substrate-utilization profiles and aggressiveness. Similar findings were observed for isolates W1 and W3 (haplotype A) and W4 and W5 (haplotype E2). This indicates that differences in pathogenicity and in biochemical properties between isolates go beyond their genomic haplotype classification. In other words, haplotype does not necessarily reflect strain aggressiveness.

The present study shows that ERIC- and REP-PCR can efficiently distinguish between group I and II strains of *A. avenae* subsp. *citrulli*, as reported for BOX-PCR (25). However, we found that all repetitive-PCR (Rep-PCR) techniques are less sensi-

Fig. 5. DNA fingerprinting of *Acidovorax avenae* subsp. *citrulli* isolates by Repetitive-PCR (Rep-PCR) techniques. **A**, BOX-PCR. **B**, ERIC-PCR. **C**, REP-PCR. Lanes 1 (L1) and 8 (L2) were loaded with 1-kb and 100-bp ladders (New England Biolabs), respectively. Solid arrows indicate major bands with similar moiety within W and M isolates; dashed arrows indicate unique, group-specific DNA fragments.

tive than PFGE in distinguishing among different isolates belonging to the same group. This conclusion is based on the fact that W isolates belonging to four different group II haplotypes on the basis of PFGE analysis could not be distinguished by their BOX-, ERIC-, and REP-PCR profiles.

PFGE and Rep-PCR are among the most powerful DNA fingerprinting approaches for identification and characterization of bacteria. The most significant advantage of PFGE resides in its reproducibility. Rep-PCR approaches have the advantage of being more accessible, less expensive, and faster than PFGE. However, Rep-PCR is subject to experimental limitations and may lead to reduced levels of reproducibility. Furthermore, although Walcott et al. (25) did distinguish between group I and II isolates by BOX-PCR, they reported that several DNA amplicons were difficult to score. Therefore, it can be concluded that Rep-PCR can be used to distinguish between group I and group II isolates, but PFGE is preferable if the objective is to assess the genetic diversity occurring between strains. On the other hand, the relatively high degree of conservation observed for the Rep-PCR profiles of different isolates (including several fragments that appear to be amplified from both group I and II isolates) can be exploited for the development of more specific PCR-based methods for the detection of *A. avenae* subsp. *citrulli*, for which a high demand exists (21,26).

Overall, the results from pathogenicity assays corresponded to the genetic differentiation between W and M isolates, and were in general agreement with previous reports on differences in pathogenicity between group I and group II strains (18,23,25,27). In general, watermelon was more susceptible to *A. avenae* subsp. *citrulli* than melon in seedling and fruit pathogenicity assays. As previously reported for group II strains, W1 through W6 were more aggressive on watermelon than on nonwatermelon hosts. Also consistent with previous reports was the fact that no obvious differences were observed between different host plants in disease severity as induced by M isolates, although a trend of increased severity was observed for melon fruits inoculated with these isolates compared with watermelon fruits.

Seedling inoculation by rubbing leaves and/or cotyledons with (or without) Carborundum and inoculation of detached fruit by bacterial injection are well-accepted methods for the investigation of foliar diseases caused by plant pathogenic bacteria (9). These techniques were also used in previous studies involving inoculation of seedlings (4,29) and detached fruits (23,24,28) with *A. avenae* subsp. *citrulli*. However, it is important to state that, as with every artificial inoculation technique, these methods have potential limitations. For instance, the utilized methods may

obscure differences among isolates if motility plays an important role for bacterial penetration through natural openings such as stomata. Also, atypical symptoms may be produced with these methods (9). Hence, aggressiveness of isolates as determined in the present study may vary under natural conditions.

One of the most interesting findings from this study was the clear difference in aggressiveness among M and W isolates in seed transmission assays, where M isolates showed a significantly higher aggressiveness, on watermelon as well. Microscopic observations of the different isolates, performed during growth curve experiments and inoculum preparation, revealed that M isolates possess a consistently higher motility than W isolates (not shown). It is well-established that in addition to motility, bacterial flagella play an important role in bacteria's surface attachment and adhesion (17). A possibility exists that M isolates were able to adhere and anchor to seed surfaces in a more efficient way and remained attached to the seeds at higher levels than W isolates after seed washings and/or during the first days after sowing. Further studies, however, are required to verify if motility and flagella have a role in aggressiveness of *A. avenae* subsp. *citrulli* in seed transmission.

Walcott et al. (25) reported L-leucine and α -hydroxybutyric acid as sole carbon substrates differentially utilized by group I and group II strains: according to that study, 26 of 27 (96.3%) group II strains utilized L-leucine, while only one (the ATCC type strain) out of 28 (3.6%) group I strains utilized this substrate. Percentages of α -hydroxybutyric acid utilization were 85.2% (23/27) and 28.6% (8/28) for group II and group I strains, respectively (25). Our results agree with that finding since all W isolates, but not M isolates, were shown to utilize these substrates. It seems that several corrections are needed in the Biolog database, which currently indicates that no *A. avenae* subsp. *citrulli* strain is expected to grow in L-leucine. Similarly, five of the W isolates utilized D-glucose, whereas utilization of this substrate by *A. avenae* subsp. *citrulli* is not predicted by the Biolog database. In addition to L-leucine and α -hydroxybutyric acid, all group II isolates from our study utilized propionic acid, α -keto valeric acid, and alaninamide, while no group I isolate utilized these substrates.

This study contributes additional features that can be used to distinguish between group I and II strains. W and M isolates showed slight differences in their growth curves in NB, and clear differences between the two classes of isolates were observed in tolerance to rifampicin and extracellular cellulase activity. In contrast, no significant differences were found among isolates in terms of extracellular protease and pectinase activities, and no

isolate was able to hydrolyze starch. Whereas negative starch hydrolysis, positive protease activity, and the ability to utilize cellulose have been previously shown for *A. avenae* subsp. *citrulli* (22,27), to the best of our knowledge, this is the first study to demonstrate pectinase extracellular activities for this bacterium. Whether these features play a significant role in *A. avenae* subsp. *citrulli* virulence still needs to be assessed. Also, due to the relatively low number of isolates available for our study, we cannot state with high confidence that all differences found between M and W isolates in this work reflect features differentiating the two groups on a broader scale. Since *A. avenae* subsp. *citrulli* is a quarantined bacterium in Israel, rigid regulations prohibit its introduction from abroad, thereby limiting the number of strains available for investigation.

The interesting question remains as to whether the recent BFB occurrence in Israel was due to single events of pathogen introduction, or whether *A. avenae* subsp. *citrulli* was already present in the country and suitable environmental conditions led to disease development during the early 2000s. During the 1990s, there were several events of *A. avenae* subsp. *citrulli* detection by the Israeli Quarantine authorities, including some from noncucurbit hosts (1,2). Since there is a lack of efficient tools for detection of the pathogen, it is plausible that the pathogen could have escaped detection in seed lots with low levels of infestation. This, together with the fact that environmental conditions during the early 2000s (and especially in 2002) particularly encouraged bacterial foliar diseases, and that BFB occurred in both watermelon and melon, suggest that BFB's occurrence was not due to a single introduction event. It is more likely that the *A. avenae* subsp. *citrulli* strains were introduced into the country more often than assumed, and/or that the pathogen was already present, "waiting" for simultaneous occurrence of the three known plant-disease prerequisites: pathogen, host, and favorable environment. The relative haplotype diversity observed among the characterized haplotypes also supports this possibility.

It is still too early to predict the impact of BFB on watermelon and melon production in Israel. Although economic losses caused by BFB were limited to a few fields, growers are concerned about the disease because of its destructive potential. Moreover, BFB is considered a serious threat for local seedling nurseries and seed companies, particularly those producing watermelon and melon seeds for export. The lack of efficient pathogen detection assays, and the fact that *A. avenae* subsp. *citrulli*-infested plants can be asymptomatic under suboptimal conditions for disease development, add to this serious concern.

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