Pathogenic and Genetic Relatedness Among *Xanthomonas axonopodis* pv. *allii* and Other Pathovars of *X. axonopodis*

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ABSTRACT

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Xanthomonas axonopodis pv. *allii* is phenotypically and genetically diverse and its relationship to other *X. axonopodis* pathovars within DNA homology group 9.2 is unknown. In growth chamber experiments, disease symptoms were produced on onion only by inoculation with *X. axonopodis* pv. *allii*. Citrus bacterial spot symptoms were induced by *X. axonopodis* pvs. *alfalfae*, *citrumelo*, and *allii* on Duncan grapefruit and key lime. *X. axonopodis* pv. *allii* multiplication and persistence in Duncan grapefruit were equal to those of an aggressive strain of *X. axonopodis* pv. *citrumelo*, but populations of *X. axonopodis* pvs. *alfalfae*, *betlicola*, *citrumelo*, *phaseoli*, and *vesicatoria* were 1.3 to 4.0 log units less than *X. axonopodis* pv. *allii* in onion. Genomic fingerprinting by repetitive sequence-based polymerase chain reaction demonstrated that *X. axonopodis* pvs. *allii*, *alfalfae*, and *citrumelo* are distinct from other *Xanthomonas* species and *X. axonopodis* pathovars, but these pathovars were indistinguishable from each other. Three genotype groups were apparent among DNA homology group 9.2 strains, and generally correspond to the aggressiveness and genotype groups previously described for *X. axonopodis* pv. *citrumelo*. *X. axonopodis* pvs. *allii*, *alfalfae*, and *citrumelo* appear to have recently diverged from a common ancestral strain.

Additional keywords: *Allium cepa*, citrus canker, *Citrus aurantifolia*, *C. paradisi*, *Xanthomonas campestris* pv. *allii*, *X. campestris* pv. *citri* E.

Xanthomonas leaf blight, caused by *Xanthomonas axonopodis* pv. *allii*, is an emerging disease of onion (*Allium cepa*) in the United States and the world (10). Disease symptoms are varied and include leaves with lenticular water-soaked lesions that elongate into chlorotic streaks most prominent on the flattened sides of leaves, necrosis, and tip dieback. Plants become stunted and bulb development is reduced or ceased. A bulb rot has never been reported and apparently does not occur, but yield losses related to the loss of photosynthetic area can be significant (31,40,43).

A leaf blight of onion caused by a *Xanthomonas* sp. was described originally by Alvarez et al. (2) in Hawaii in 1978. The disease appeared in a new onion production region in Hawaii cleared from native vegetation. Attempts to isolate the pathogen from nearby plants and crops were unsuccessful, and seed transmission was speculated. Later reports have confirmed the pathogen is seedborne (36) and seed may be an epidemiologically important inoculum source (35). The pathogen has since been introduced into many onion-producing regions of the world, and Xanthomonas leaf blight has been reported from the continental United States (23,28,38,41), the east Caribbean (31), South Africa (43), and Reunion Island, France (36,37). *X. axonopodis* pv. *allii* also causes a related disease on Welsh onion (*A. fistulosum*) (11,24,35). Interestingly, a foliar blight of onion caused by *X. striaformans* was reported from southern Colorado in 1953 (45), and the disease description appears identical to Xanthomonas leaf blight symptomology. However, the description provided for *X. striaformans* differs from the descriptions of *X. axonopodis* pv. *allii* by eight physiological characteristics (2,45). Comparative studies of *X. striaformans* and *X. axonopodis* pv. *allii* are not possible because cultures of *X. striaformans* were not preserved.

The taxonomy of *Xanthomonas* strains pathogenic to *Allium* spp. has been in flux since 1978. The original description by Alvarez et al. (2) simply recognized the leaf blight pathogen as a *Xanthomonas* spp., but ensuing reports referred to the pathogen as *Xanthomonas campestris* (23,28,41). Later, Kadota et al. (24) proposed the epithet *X. campestris* pv. *allii* for *Xanthomonas* strains recovered from diseased *A. fistulosum*. In independent studies, Roumagnac et al. (35) and Gent et al. (11) conducted polyphasic characterizations of a broad collection of *Xanthomonas* strains from *Allium* spp. Each discovered *X. campestris* pv. *allii* is pathogenic to *A. cepa*, and that these strains are indistinguishable genetically, phenotypically, and pathogenically from other *Xanthomonas* strains recovered from *A. cepa*. The researchers suggested the correct species and pathovar designation should be *X. axonopodis* pv. *allii* to represent the true phylogenetic position of the organism.

The pathogenic capabilities and host range of *X. axonopodis* pv. *allii* are not clear. The host range of *X. axonopodis* pv. *allii* is reported in some literature as limited to *Allium* sp. (2,4,35). How-

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ever, strains from Barbados are reportedly pathogenic to leguminous hosts such as snap bean (*Phaseolus vulgaris*), lima bean (*Phaseolus lunatus*), soybean (*Glycine max*), winged bean (*Psophocarpus tetragonolobus*), moth bean (*Vigna aconitifolia)*, and field pea (*Pisum sativum*) (29), but these collections of *X. axonopodis* pv. *allii* from diverse sources and researchers have not been tested on all reported hosts. Some *X. axonopodis* pv. *allii* strains occasionally cause small, water-soaked lesions on snap bean, but lesions remain small and become necrotic within 7 days (35). Gent et al. (11) found that although typical common bacterial blight symptoms were absent on dry bean (*P. vulgaris*), *X. axonopodis* pv. *allii* multiplied and attained high population levels in dry bean leaves. The bacterium may also persist epiphytically on some leguminous hosts (10).

Previous studies indicated some strains of *X. axonopodis* pvs. *alfalfae* and *citrumelo* can induce disease symptoms on alfalfa (*Medicago sativa*) and citrus (*Citrus* sp.) (9), but no studies have evaluated onion as a host of these and other *X. axonopodis* pathovars. Likewise, no studies have determined the compatibility of *X. axonopodis* pv. *allii* on host plants of closely related *X. axonopodis* pathovars. The relationship among these pathovars may be important for developing management strategies for the diseases these pathogens cause on their primary hosts.

The phylogenetic relationship among *X. axonopodis* pv. *allii* and other *Xanthomonas* species and pathovars has been studied, but generally only the type strains of representative *Xanthomonas* species and pathovars were included (11,35), and pathogenicity assays of strains apparently closely related genetically were not included. The use of a single type strain to represent an entire pathovar may not be adequate to determine the true phylogenetic relationship of *X. axonopodis* pv. *allii* to closely related pathovars of *X. axonopodis*. Gent et al. (11) reported that some strains of *X. axonopodis* pv. *allii* are indistinguishable from the type strain of *X. axonopodis* pv. *citrumelo*. However, *X. axonopodis* pv. *citrumelo* is a genetically, pathogenically, and serologically diverse pathogen (1,8,18,19,22). Consequently, the genetic relationships among *X. axonopodis* pv. *allii* and other pathovars of *X. axonopodis* remain unclear. The "*axonopodis*" group appears to be comprised of six distinct genetic clusters labeled as group 9.1 to 9.6 (32). *X. axonopodis* pv. *allii* repetitive sequence-based polymerase chain reaction (rep-PCR) fingerprints appear highly similar to other *X. axonopodis* pathovars within DNA homology group 9.2 (11,34), namely *X. axonopodis* pv. *alfalfae* and *X. axonopodis* pv. *citrumelo*, but characterization of a broader collection of strains is necessary to properly determine their genetic relatedness.

Knowledge of the genetic and pathogenic relationships among *X. axonopodis* pv. *allii* and other pathovars of *X. axonopodis* is essential to the development of effective management strategies for Xanthomonas leaf blight of onion. This study was initiated to determine the genetic and pathogenic relationships among *X. axonopodis* pv. *allii* and apparently closely related pathovars.

MATERIALS AND METHODS

Bacterial strains, culture, and DNA isolation. Fifty-nine strains of *Xanthomonas* isolated from 20 different plants species were used in this study, but only data from 39 strains are presented in detail (Table 1). The other strains were included as positive controls to assure DNA fingerprinting experiments correctly classified strains to the appropriate pathovar. Strains were routinely cultured on nutrient agar or broth during incubation at 26 to 29°C. When recovered from onion leaf homogenates, *X. axonopodis* strains were cultured on nutrient agar amended with 60 µg/ml of kasugamycin and 50 µg/ml of cycloheximide to reduce growth of secondary organisms. The addition of these antibiotics to the nutrient agar was not essential to recover *X. axonopodis* from tissue homogenates, but all strains used in this study were resistant to both antibiotics and their addition reduced or eliminated the growth of other bacteria and fungi and simplified enumeration of the pathogens. All antibiotics were purchased from Sigma Chemical Co. (St. Louis, MO). Strains were preserved in 15% nutrient glycerol broth at –80°C for long-term storage (11).

Strains were cultured in 1.5 ml of nutrient broth for 24 h for DNA isolation procedures. The culture was adjusted to an optical density of 0.1 at 600 nm in sterile 0.02 M potassium phosphate buffer pH 7.2 (PB), and DNA was isolated using the CTAB (hexadecyltrimethylammonium bromide) method (3). DNA was stored in Tris-EDTA buffer (TE [10 mM Tris, 1 mM EDTA, pH 8.0]) at –20°C.

Pathogenicity and host range. Strains of *X. axonopodis* pv. *allii* (14 strains), *X. axonopodis* pv. *citrumelo* (11 strains), *X. axonopodis* pv. *alfalfae* (5 strains), *X. axonopodis* pv. *betlicola* (2 strains), and *X. axonopodis* pv. *cyamopsidis* (1 strain) were tested for pathogenicity on onion (cvs. Blanco Duro and Vantage) in growth chamber assays. Colonies of the strain to be tested were inoculated into 3 ml of nutrient broth in 15-ml culture tubes and were incubated at 26°C with vigorous shaking (250 oscillations per min) for 24 h. The cultures were washed with sterile PB, adjusted to approximately 10^7 CFU/ml in sterile PB, and sprayed (Crown SpraTool; Aerovoe Industries, Inc., Gardnerville, NV) onto the foliage of 6- to 8-week-old plants to runoff with the bacterial suspension. Control plants were inoculated with sterile PB. The plants were placed in a growth chamber and incubated for 7 days with a 28°C/24°C day/night temperature regimen, light intensity of 350 $\mu Ms^{-1}m^{-2}$, 100% relative humidity, and daily misting with tap water to runoff. At least four plants were inoculated with each strain. If disease symptoms did not develop, a pinprick inoculation procedure was conducted. The youngest, fully extended leaves of 8-week-old onion plants (cv. Vantage) were pinpricked seven times at 1-cm intervals with a 22-gauge needle bearing bacterial cells of a given *X. axonopodis* strain removed from a 72-h-old nutrient agar culture plate. The strains were grown in pure culture for inoculations and thus did not require antibiotic amendment to the nutrient agar for selection. Each pinpricked leaf area was inoculated with bacterial cells approximately equal in size to the needle tip, which consistently delivered 10^6 CFU/pinprick. Plants were observed daily for symptom development, and the pathogen was isolated from characteristic lesions by grinding leaf sections in 1 ml of PB with a sterile mortar and pestle and streaking loopfuls of the homogenate onto nutrient agar. The experiment was repeated at least once for each strain.

Pathogenicity assays were conducted on key lime (*Citrus aurantifolia*) and Duncan grapefruit (*Citrus paradisi* var. Duncan) leaves with *X. axonopodis* pv. *allii* strains O177, JV 595, Xcu 200-2, *X. axonopodis* pv. *citrumelo* strain 3048, *X. axonopodis* pv. *alfalfae* strain KX-1, and *X. axonopodis* pv. *citri* strain 3213 as described previously (6,12). Briefly, *Xanthomonas* strains were grown in peptone/yeast extract/glycerol/MOPS (PYGM) medium overnight at 28°C, pelleted, and rinsed with sterile tap water before dilution to approximately 10^5 CFU/ml in sterile tap water. Newly expanded leaves were inoculated by pressure infiltrating the abaxial surface to water soaking with approximately 10^5 CFU/ml of each strain with the blunt end of a tuberculin syringe. Strains were classified as aggressive, moderately aggressive, or weakly aggressive on the basis of the extent of water soaking and necrosis (18) 7 days after inoculation. Pathogen isolations from lesions were not performed, but this assay is robust and has been used extensively to differentiate the aggressiveness groups of citrus bacterial spot pathogens (9,12). Inoculations were repeated at least once.

In planta multiplication in citrus. In planta population dynamics of *X. axonopodis* pv. *allii* strain Xcu 200-2, isolated from onion in Georgia (39), and the holopathotype strain *X. axonopodis*

pv. *citrumelo* 3048 were monitored in attached leaves of Duncan grapefruit. Strain Xcu 200-2 was selected for this study because it is phenotypically representative of most *X. axonopodis* pv. *allii* strains and it was among several *X. axonopodis* pv. *allii* strains that induced symptoms on attached leaves of Duncan grapefruit and key lime that resembled citrus bacterial spot. For inoculations, cultures of *Xanthomonas* strains were grown in PYGM medium overnight at 28°C (6), pelleted, washed with sterile tap water, and diluted to approximately 5×10^5 CFU/ml in sterile tap water saturated with $CaCO_3$. This method for enumerating multiplication of *Xanthomonas* strains in citrus was selected because it is well established (8,9,14,18,19). Each strain was pressure infiltrated into the abaxial leaf surface of attached grapefruit leaves using the blunt end of a tuberculin syringe so that most of the leaf was soaked. Three attached grapefruit leaves were infiltrated for each strain in a given experiment. A sterile cork borer (no. 3, approximately 0.28 cm^2) was used to remove three leaf disks from each leaf at 0, 1, 5, 12, and 18 days after inoculation. These disks from each inoculated leaf were macerated together in 1 ml of sterile tap water using a sterile mortar and pestle. The homogenate was serially diluted in sterile tap water before plating 10 µl of each dilution (10^{-1} to 10^{-9}) onto PYGM medium. *Xanthomonas* colonies were counted after incubation at 28°C for 48 h. The experiment was conducted three times.

In planta multiplication in onion. Populations of strains O177, 11677, 81-23, 3048, 82.1, and 49199 of *X. axonopodis* pvs. *allii*, *betlicola*, *vesicatoria*, *citrumelo*, *alfalfae*, and *phaseoli*, respectively, were monitored in onion as described by O'Garro and

TABLE 1. *Xanthomonas* strains used in this study

Strain	Other strain designations[a]	Pathovar	Origin		
			Host	Location	Source[b]
82.1	…	*alfalfae*	*Medicago sativa*	Florida	D. Gabriel
334	…	*alfalfae*	*M. sativa*	Florida	D. Gabriel
676	…	*alfalfae*	*M. sativa*	Florida	D. Gabriel
KX-1	…	*alfalfae*	*M. sativa*	Florida	D. Gabriel
A551-3		*allii*	*Allium cepa*	Hawaii	A. Alvarez
JV 594	…	*allii*	*A. cepa*	Brazil	O. Pruvost
JV 595	CFBP 6362	*allii*	*A. cepa*	Brazil	O. Pruvost
O130	CFBP 6363	*allii*	*A. cepa*	Colorado	H. Schwartz
O177	ATCC 504	*allii*	*A. cepa*	Colorado	H. Schwartz
F2:22	ATCC 508	*allii*	*A. cepa*	Barbados	L. O'Garro
TX-3	…	*allii*	*A. cepa*	Texas	T. Isakeit
TX-10	…	*allii*	*A. cepa*	Texas	T. Isakeit
MAFF 311173	…	*allii*	*A. fistulosum*	Japan	MAFF
MAFF 311174	…	*allii*	*A. fistulosum*	Japan	MAFF
ATCC BAA 575	…	*allii*	*A. cepa*	South Africa	ARC-PPRI
Xcu 200-2	BD 142	*allii*	*A. cepa*	Georgia	R. Gitaitis
Calon-1	…	*allii*	*A. cepa*	California	R. Gilbertson
Calon-5	…	*allii*	*A. cepa*	California	R. Gilbertson
3213	…	*citri*	*Citrus* sp.	Florida	D. Gabriel
3048	ATCC 49120	*citrumelo*	*Citrus* sp.	Florida	D. Gabriel
3162	…	*citrumelo*	*Citrus* sp.	Florida	D. Gabriel
3294	…	*citrumelo*	*Citrus* sp.	Florida	D. Gabriel
3328	…	*citrumelo*	*Citrus* sp.	Florida	D. Gabriel
3401	…	*citrumelo*	*Citrus* sp.	Florida	D. Gabriel
4600	…	*citrumelo*	*Citrus* sp.	Florida	D. Gabriel
4754	…	*citrumelo*	*Citrus* sp.	Florida	D. Gabriel
5436	…	*citrumelo*	*Citrus* sp.	Florida	D. Gabriel
6260	…	*citrumelo*	*Citrus* sp.	Florida	D. Gabriel
6572	…	*citrumelo*	*Citrus* sp.	Florida	D. Gabriel
6774	…	*citrumelo*	*Citrus* sp.	Florida	D. Gabriel
7364	…	*citrumelo*	*Citrus* sp.	Florida	D. Gabriel
13D5	…	*cyamopsidis*	*Cyamopsis tetragonoloba*	…	D. Gabriel
ATCC 19312	ICMP 312 LMG 555 NCPPB 2972 ITCCF B45	*axonopodis*	*Axonopu scoparius*	Colombia	ATCC
ATCC 11677	NCPPB 2968	*betlicola*	*Piper betle*	India	ATCC
ATCC 49119	LMG 682 NCPPB 409 PDDCC 24	*phaseoli*	*Phaseolus vulgaris*	USA	ATCC
ATCC 35938	LGM 892 NCPPB 2821 PDDCC 5755	*vasculorum*	*Saccharum officinarum*	Mauritius	ATCC
81-23	…	*vesicatoria*	*Capsicum annuum*	Florida	J. Jones
910	…	*vesicatoria*	*C. annuum*	USA	F. Louws
ATCC 11633	XV 23	*vesicatoria*	*C. annuum*	USA	ATCC

[a] … Indicates information was unknown.

[b] Source: H. Schwartz, Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins; R. Gilbertson, Department of Plant Pathology, University of California, Davis; T. Isakeit, Department of Plant Pathology and Microbiology, Texas A&M University, College Station; L. O'Garro, Department of Biology, University of the West Indies, Bridgetown, Barbados; A. Alvarez, Department of Plant Pathology, University of Hawaii, Honolulu; R. Gitaitis, Department of Plant Pathology, University of Georgia, Tifton; D. Garbriel, Department of Plant Pathology, University of Florida, Gainesville; F. Louws, Department of Plant Pathology, North Carolina State University, Raleigh; O. Pruvost, Centre de Coopération Internationale en Recherche Agronomique pour le Dévelopement, Reunion Island, France; ARC-PPRI = Agricultural Research Council-Plant Protection Research Institute, Pretoria, South Africa; ATCC = American Type Culture Collection, Manassas, VA; CFBP = Collection Française de Bactéries Phytopathogènes, Beaucouzé Cedex, France; ICMP = International Collection of Micro-organisms from Plants, Auckland, New Zealand; LMG = Laboratorium vorr Microbiologie, Ghent, Belgium; MAFF = Ministry of Agriculture, Forestry, and Fisheries of Japan, Okinawa; NCPPB = National Collection of Plant Pathogenic Bacteria, York, United Kingdom.

Paulraj (29). Briefly, the youngest, fully extended leaves of 8-week-old onion plants (cv. Vantage) were pinpricked seven times at 1-cm intervals with a 22-gauge needle bearing a bacterial matrix of a given *X. axonopodis* strain removed from a 72-h-old nutrient agar culture plate. Each pinpricked leaf area was inoculated with a bacterial matrix approximately equal in size to the needle tip. Leaf sections (5 × 1 cm long), each having an inoculated area, were removed every 2 days, surface disinfested in 95% ethanol, rinsed in sterile PB, and ground aseptically in 1 ml of sterile PB with a mortar and pestle. Recoveries were done up to 14 days after inoculation to generate population growth curves. The homogenate was serially diluted and plated onto nutrient agar amended with 60 µg/ml of kasugamycin and 50 µg/ml of cycloheximide. *Xanthomonas* colonies were enumerated after 96 h of incubation at 29° C. The experiment was repeated once.

rep-PCR genomic fingerprinting. Genomic fingerprints were determined for 10, 12, 3, and 1 strains of *X. axonopodis* pvs. *allii*, *citrumelo*, *alfalfae*, and *cyamopsidis*, respectively, as described by Louws et al. (25–27) using primers corresponding to prokaryotic enterobacterial repetitive intergenic consensus (ERIC) sequences, repetitive extragenic palindromic (REP) sequences, and the BOXA subunit of the BOX element. *X. axonopodis* pv. *allii* strains included in this study consisted of two strains arbitrarily selected from each of the five rep-PCR genotype groups described by Gent et al. (11). Additionally, 25 other strains of phytopathogenic xanthomonads representative of 12 of 20 *Xanthomonas* species described by Vauterin et al. (47) were included. Only rep-PCR fingerprint data from DNA homology group 9.2 and 9.3 strains are reported here.

Images were imported into GelCompar software (version 4.1; Applied Maths, Kortrijk, Belgium), linearly combined, and similarity was calculated using Pearson's correlation coefficient applied to the entire densitometric curves of the gel tracks as previously described (33). Gels were standardized with an Invitrogen Corp. (Carlsbad, CA) 1-kb DNA molecular weight ladder. Cluster analysis was performed using the unweighted pair group method with arithmetic averages clustering. All PCR reactions were repeated at least once.

RESULTS

Pathogenicity and host range. Characteristic Xanthomonas leaf blight symptoms consisting of lenticular, water-soaked lesions developed on onion within 3 to 7 days of inoculation with all *X. axonopodis* pv. *allii* strains tested. In one experiment, *X. axonopodis* pv. *citrumelo* strain 3048 induced large, water-soaked lesions on onion cv. Vantage 10 to 12 days after inoculation, and the pathogen was recovered from lesions. Attempts to isolate contaminating *X. axonopodis* pv. *allii* from the lesions were unsuccessful, suggesting *X. axonopodis* pv. *citrumelo* was the only pathogen present in these lesions. Multiple attempts to reproduce this reaction on cvs. Vantage and Blanco Duro with different inoculation techniques were not successful. No other *X. axonopodis* pathovar induced disease symptoms on onion cvs. Blanco Duro or Vantage by spray or pinprick inoculation.

Duncan grapefruit inoculated with *X. axonopodis* pv. *citrumelo* strain 3048 or *X. axonopodis* pv. *alfalfae* strain KX-1 developed characteristic citrus bacterial spot symptoms, including flattened, water-soaked lesions and necrosis (Fig. 1). Distinct but limited necrosis developed at the inoculation sites with both pathovars, and the reactions were considered to be representative of moderately aggressive citrus bacterial spot pathogens (18). *X. axonopodis* pv. *citri* strain 3213 induced typical bacterial canker symptoms of faintly chlorotic, water-soaked lesions with slightly raised edges. Three strains of *X. axonopodis* pv. *allii* induced varying disease symptoms on Duncan grapefruit. *X. axonopodis* pv. *allii* strain O177 did not induce water soaking or chlorosis in most inoculations (Fig. 1), but chlorosis was observed on Duncan grapefruit following inoculation in some experiments. This reaction was classified as weakly aggressive. Strains JV 595 and Xcu 200-2 induced chlorosis and extensive water soaking, but necrosis was limited within the water-soaked area. These reactions were classified as moderately aggressive.

On key lime, which is more susceptible to citrus bacterial spot than is Duncan grapefruit (14), *X. axonopodis* pv. *citrumelo* strain 3048 induced large, flattened, water-soaked lesions with indistinct necrosis (Fig. 2). Lesions were typical of aggressive strains of the pathogen. To a lesser degree than *X. axonopodis* pv. *citrumelo* strain 3048, *X. axonopodis* pv. *alfalfae* strain KX-1 induced flattened, water-soaked lesions. The necrosis associated with *X. axonopodis* pv. *alfalfae* lesions was distinct from the water soaking, but was not as extensive as compared with *X. axonopodis* pv. *citrumelo*. Necrosis induced by *X. axonopodis* pv. *alfalfae* was limited to the inoculation site. The disease reaction was characteristic of moderately aggressive strains. *X. axonopodis* pv. *citri* strain 3213 induced typical bacterial canker symptoms of faintly chlorotic, water-soaked lesions with slightly raised edges on key lime. *X. axonopodis* pv. *allii* strains provoked disease reactions characteristic of aggressive or moderately aggressive citrus bacterial spot pathogens. Strain O177 induced water soaking and indis-

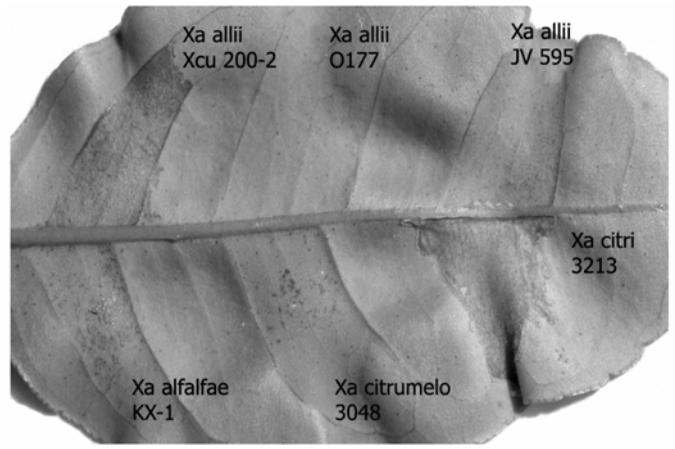

Fig. 1. Disease symptoms on Duncan grapefruit (*Citrus paradisi* var. Duncan) induced by different pathovars of *Xanthomonas axonopodis* 7 days after inoculation. Newly expanded leaves were inoculated by pressure infiltrating the abaxial surface to water soaking with approximately 10^5 CFU/ml of each strain with the blunt end of a tuberculin syringe. Strain numbers are indicated for each pathovar of *X. axonopodis*. Xa = *Xanthomonas axonopodis*.

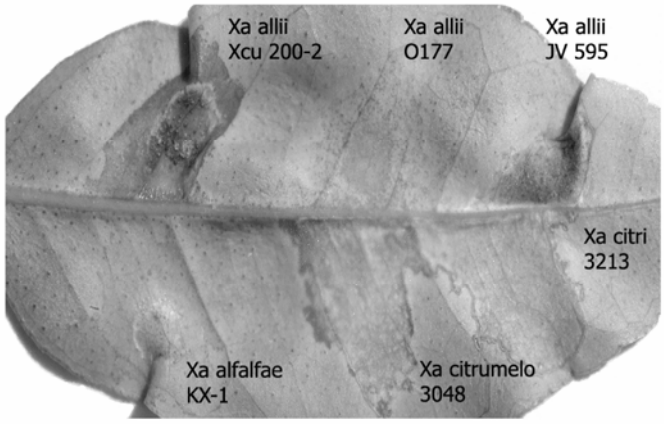

Fig. 2. Disease symptoms on key lime (*Citrus aurantifolia*) induced by different pathovars of *Xanthomonas axonopodis* 7 days after inoculation. Newly expanded leaves were inoculated by pressure infiltrating the abaxial surface to water soaking with approximately 10^5 CFU/ml of each strain with the blunt end of a tuberculin syringe. Strain numbers are indicated for each pathovar of *X. axonopodis*. Xa = *Xanthomonas axonopodis*.

tinct necrosis within the water-soaked lesions. The extent of water soaking was less than that induced by *X. axonopodis* pv. *citrumelo*, and the strain was classified as moderately aggressive. Strains JV 595 and Xcu 200-2 induced extensive water soaking that became necrotic within 7 days of inoculation, and the strains were classified as aggressive. Strain Xcu 200-2 induced more widespread water soaking as compared with JV 595, but the necrosis associated with the lesions of either strain was more extensive than that associated with the aggressive *X. axonopodis* pv. *citrumelo* strain. This reaction is typical of aggressive *X. axonopodis* pv. *citrumelo* strains on highly susceptible citrus hosts such as key lime, and therefore, investigations of whether this reaction was a hypersensitive response were not conducted.

In planta multiplication in citrus. *X. axonopodis* pv. *allii* strain Xcu 200-2 was selected for in planta population dynamic studies in Duncan grapefruit because it induced clear citrus bacterial spot symptoms in pathogenicity experiments. Multiplication and survival of *X. axonopodis* pv. *allii* strain Xcu 200-2 was equal to that of *X. axonopodis* pv. *citrumelo* strain 3048 (Fig. 3). Populations of both pathogens increased nearly 100-fold over the 14-day time course of the experiment.

In planta multiplication in onion. *X. axonopodis* pv. *allii* populations increased linearly after inoculation into onion and increased approximately 2 log units per leaf section (Fig. 4). Populations of *X. axonopodis* pvs. *citrumelo*, *betlicola*, and *alfalfae* decreased in onion 3 to 5 days after inoculation, but after this initial decline, in planta populations increased throughout the duration of the experiment. On the final sampling date, populations of *X. axonopodis* pvs. *citrumelo*, *betlicola*, and *alfalfae* were 1.3, 1.6, and 4.0 log units per leaf section less as compared with *X. axonopodis* pv. *allii*, respectively. *X. axonopodis* pvs. *vesicatoria* and *phaseoli* decreased on most sampling dates after inoculation. Less than 20 CFU/leaf section were recovered on the last sampling date, which was greater than 7 log units less than *X. axonopodis* pv. *allii* populations on the same date.

rep-PCR genomic fingerprinting. Complex DNA fingerprints were generated from genomic DNA extracted from all *Xanthomonas* species and pathovars included in this study (Fig. 5). DNA fragments of approximately 200 bp to greater than 4 kb were amplified and revealed a high level of genetic diversity among *X. axonopodis* group 9.2 strains (34). DNA homology group 9.2 pathovars were clearly delineated from other DNA homology group 9 pathovars and species of *Xanthomonas*, but there was no clear distinction among *X. axonopodis* pvs. *allii*, *alfalfae*, and *citrumelo* strains that comprise group 9.2. The similarity of fingerprint profiles among these strains ranged from approximately 50 to 95% as determined by the product-moment correlation coefficient.

Among the 26 group 9.2 strains included in this study, some were more closely related to strains identified as belonging to another pathovar than were strains within the same pathovar. For instance, *X. axonopodis* pv. *alfalfae* strains 334 and 82.1 were more closely related to *X. axonopodis* pv. *allii* strain JV 594 and *X. axonopodis* pv. *citrumelo* strain 6774, respectively, than they were to one another. Of particular interest, *X. axonopodis* pv. *allii* strain O130 and *X. axonopodis* pv. *citrumelo* strain 7364 are virtually identical. *X. axonopodis* pv. *allii* strains included in this study generally grouped, with the exception of strain A551-3, with other *X. axonopodis* pv. *allii* strains belonging to the same genotype group as described by Gent et al. (11). When *X. axonopodis* pvs. *citrumelo*, *allii*, and *alfalfae* are considered together, three rep-PCR groups are apparent (noted in brackets in Fig. 5) and correspond approximately to the unclassified E2 and E1 restriction fragment length polymorphism (RFLP) groups differentiated by Gabriel et al. (9), respectively. Group 1 consists of four, two and one strain(s) of *X. axonopodis* pvs. *citrumelo*, *allii*, and *alfalfae*, respectively. Group 2 represents five *X. axonopodis* pv. *citrumelo* and two *X. axonopodis* pv. *allii* strains. Among *X. axonopodis* pv. *citrumelo* strains within group 2 is strain 3048, the holopathotype of the pathovar. The remaining strains were included in a heterogeneous group 3 and included three, six, and two strains of *X. axonopodis* pvs. *citrumelo*, *allii*, and *alfalfae*, respectively. The *X. axonopodis* pv. *vesicatoria* strains appeared to have distinctive rep-PCR fingerprints, clearly separating this pathovar from other group 9.2 strains included in this study.

DISCUSSION

In this study, we have established for the first time, the genetic and pathogenic relatedness of *X. axonopodis* pv. *allii* to other *X. axonopodis* pathovars in DNA homology group 9.2. Group 9.2 strains are distinct from other pathovars of *X. axonopodis* and *Xanthomonas* species but represent a heterogeneous group of genetically and pathogenically diverse plant pathogens (32). The rep-PCR did not separate the *allii*, *alfalfae*, and *citrumelo* pathovars, and in some cases demonstrated that the strains were genetically indistinguishable and of particular interest, and the pathovars evaluated in this study share the ability to induce bacterial spot symptoms on citrus. However, these strains can be distinguished based on their differential reaction on onion, and there-

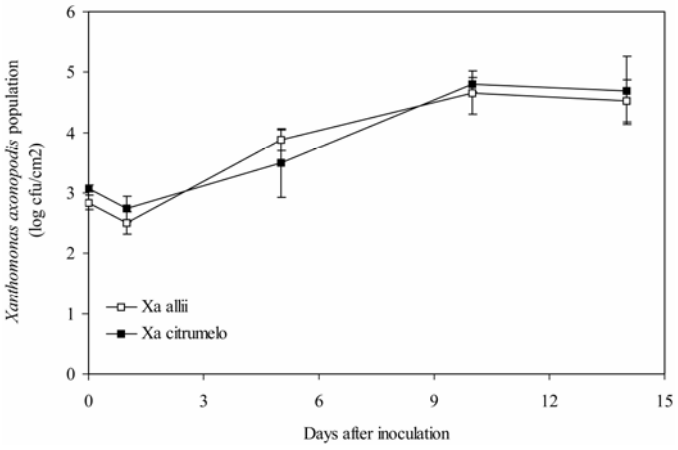

Fig. 3. Population dynamics of *Xanthomonas axonopodis* pv. *alllii* strain Xcu 200-2 and *X. axonopodis* pv. *citrumelo* strain 3048 in Duncan grapefruit (*Citrus paradisi* var. Duncan). Data are means of three replications repeated three times (n = 9) ± standard error of the mean. Xa = *Xanthomonas axonopodis*.

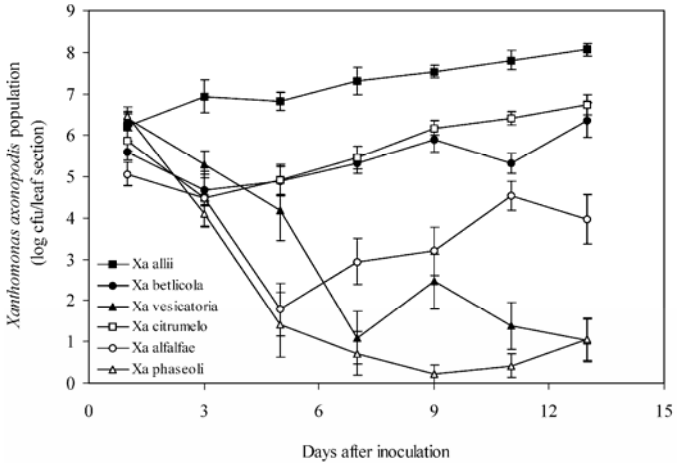

Fig. 4. Population dynamics of *Xanthomonas axonopodis* pathovars in onion cv. Vantage. Data are means of five replications repeated once (n = 10) ± standard error of the mean. Strains O177, ATCC 11677, 81-23, 3048, 82.1, and ATCC 49119 were selected for *X. axonopodis* pathovars *allii*, *betlicola*, *vesicatoria*, *citrumelo*, *alfalfae*, and *phaseoli*, respectively. Xa = *Xanthomonas axonopodis*.

fore, constitute discrete pathovars (7). Similarity coefficients among *X. axonopodis* pvs. *allii*, *alfalfae*, and *citrumelo* were at least 50% as determined by rep-PCR mediated fingerprints. Although heterogeneous, this level of similarity corresponds to at least 70% DNA:DNA homology (32), and suggests that these strains constitute pathovars within the common species *X. axonopodis*. This study supports previous findings recommending inclusion of these pathovars as the same species (11,35,47).

A limited number of strains were included in this study because most plant diseases caused by *X. axonopodis* DNA homology group 9.2 strains generally are not of economic concern, and few strains have been preserved in culture collections. Nonetheless, genomic fingerprinting of these *X. axonopodis* pathovars and strains revealed three diverse but distinct groups essentially representing the RFLP groups identified previously (9,12). Group 1 contains *X. axonopodis* pv. *allii* strains representing *X. axonopodis* pv. *allii* rep-PCR genotype 3 (11) and *X. axonopodis* pv. *citrumelo* strains that were unclassified by RFLP analysis (9). *X. axonopodis* pv. *citrumelo* strains within this group are weakly aggressive to *Citrus* sp. and are not known to carry extrachromosomal plasmids (9). Strains that cluster within group 2 of this study included *X. axonopodis* pv. *allii* rep-PCR genotype 1 (11) and *X. axonopodis* pv. *citrumelo* RFLP group E2 strains, including the holopathotype (9). The *X. axonopodis* pv. *citrumelo* strains in this group are highly aggressive to citrus hosts and share a 41-kb plasmid (9,18,19) as well as common antigens (1). Many,

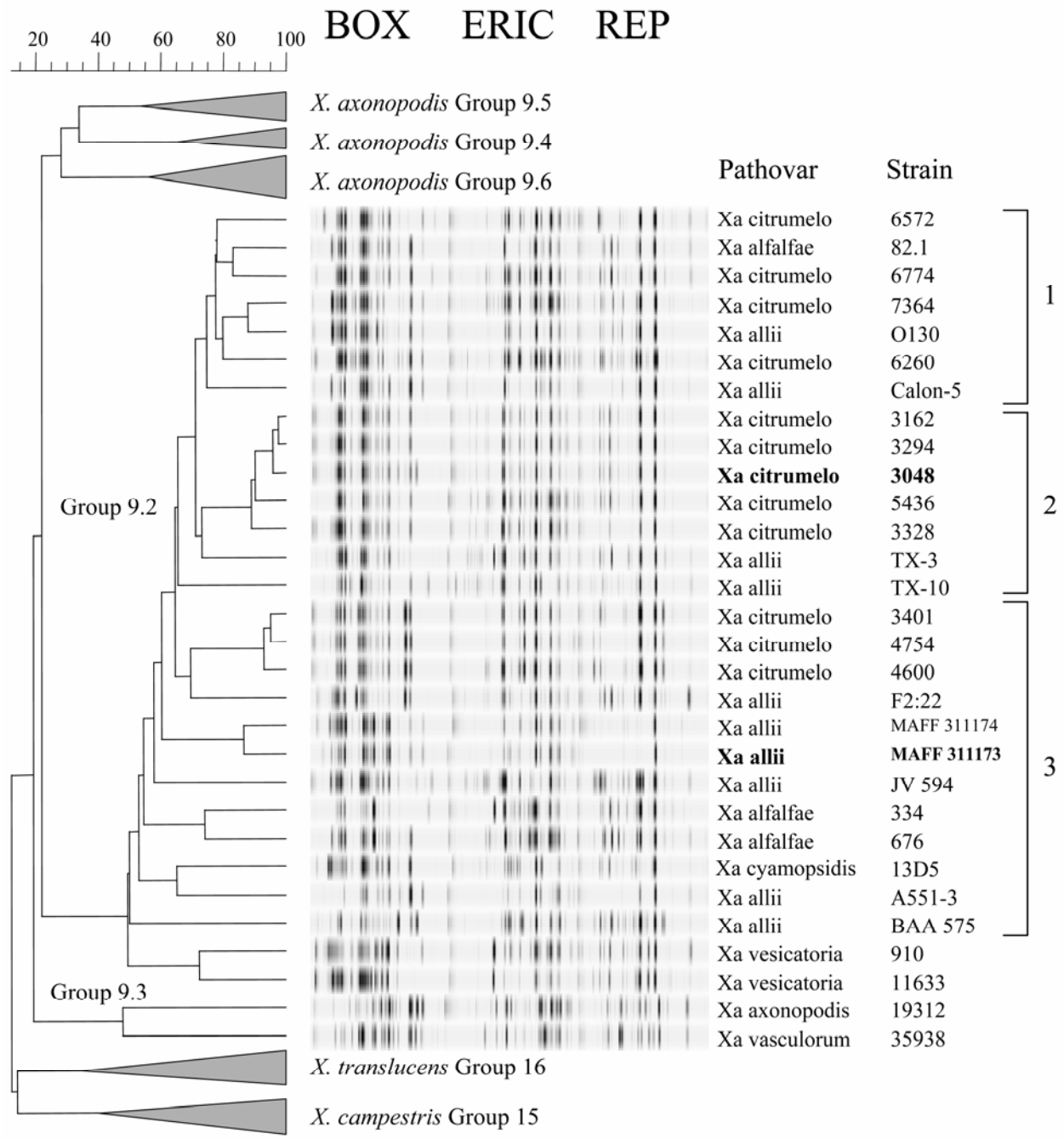

Fig. 5. Similarity among repetitive sequence-based polymerase chain reaction-mediated DNA fingerprints of *Xanthomonas axonopodis* strains within DNA homology 9.2 and other *Xanthomonas* species and pathovars. DNA fingerprints were generated using primers corresponding to prokaryotic enterobacterial repetitive intergenic consensus (ERIC) sequences, repetitive extragenic palindromic (REP) sequences, and the BOXA subunit of the BOX element (BOX) and analyzed using the product-moment correlation coefficient (*r*). DNA homology groups correspond to those reported by Rademaker et al. (32). The type strain of *Xanthomonas axonopodis* pv. *allii* MAFF 311173 and *X. axonopodis* pv. *citrumelo* 3048 are noted in bold. Genotype groups 1 to 3 within *X. axonopodis allii*, *alfalfae*, and *citrumelo* are noted with brackets. Xa = *Xanthomonas axonopodis*.

but not all, *X. axonopodis* pv. *allii* strains carry a plasmid of identical size (D. Gent, C. Ishimaru, and H. Schwartz, *unpublished*). Group 3 contains an amalgam of diverse *X. axonopodis* strains, including *X. axonopodis* pv. *allii* strains belonging to rep-PCR genotype groups 2, 4, and 5, *X. axonopodis* pv. *citrumelo*, and *X. axonopodis* pv. *alfalfae*. Strains included in this group were originally isolated from host plants in Asia, Africa, North and South America, Hawaii, and the East Caribbean. Strains of *X. axonopodis* pv. *citrumelo* in this group are weakly to moderately aggressive to their citrus hosts and belong to RFLP group E1 (9). *X. axonopodis* pvs. *allii* and *alfalfae* strains within this group also appear moderately to weakly aggressive to citrus.

X. axonopodis pv. *allii* appears to be well adapted to in planta multiplication and persistence in onion and is the only known *X. axonopodis* pathovar capable of inducing water soaking in this plant species. Culturable populations of *X. axonopodis* pv. *allii* were 10- to 1,000-fold greater in onion as compared with the other DNA homology group 9.2 strains (*X. axonopodis* pvs. *alfalfae*, *betlicola*, and *citrumelo*) and nearly 7 log units greater than the strains that are most divergent with DNA homology group 9.2 (*X. axonopodis* pv. *vesicatoria*) or outside of DNA homology group 9.2 (*X. axonopodis* pv. *phaseoli*). Under field conditions, *X. axonopodis* pv. *allii* persists epiphytically and endophytically, sometimes at populations as great as 10^7 CFU/g on onion (10,30), and can be damaging to seedling and mature onion plants (2,10,28,29,40–43). This is in complete contrast to citrus bacterial spot, in which an aggressive strain of the pathogen, favorable environmental conditions, a highly susceptible host, and wounding appear to be prerequisites for disease development in citrus nurseries (13–21). The pathogen tends to only persist in and on Swingle citrumelo (*Poncirus trifoliata* × *Citrus paradisi*) (8,19,20), produces little inoculum (46), and rarely infects fruit under field conditions (21).

The citrus bacterial spot pathogen remained widespread in Florida citrus nurseries despite repeated eradication efforts (12,13,17,18,39). Gabriel et al. (9) suggested that the primary host and source of *X. axonopodis* pv. *citrumelo* is not a rutaceous host. The close genetic, pathogenic, phenotypic, and serological relatedness among *X. axonopodis* pvs. *allii*, *alfalfae*, and *citrumelo* (1,9,11,12,18,19,35) suggests these pathovars may have originated from a common ancestral strain. Interestingly, *X. axonopodis* pv. *allii* was recovered from onion in the east Caribbean (29–31) and apparently garlic in Cuba (35) in the mid-1980s. Anecdotal evidence suggests that the pathogen may have been present in the region as early as 1971 (31,44). It is tempting to speculate the source of the citrus bacterial spot pathogens that initiated epidemics in Florida in 1984 and later were *X. axonopodis* pv. *allii* strains introduced from a neighboring country or region where they were established on a primary host such as onion or garlic. However, Xanthomonas leaf blight has not been reported on onion in Florida, and we were unable to elicit water soaking on onion by inoculation with any *X. axonopodis* pv. *citrumelo* or *alfalfae* strain in this study. The *X. axonopodis* pv. *citrumelo* strains used in this study were collected and preserved nearly 20 years ago and may have lost their ability to induce water soaking on onion because of repeated selection on artificial growth media. In the absence of selection for virulence, these strains may have lost their ability to infect onion. This raises questions about the genetics of host specificity, where one or a few genes may govern host range and thus pathovar status. Host specificity or symptomology induced by closely related strains can be controlled by single genes in the "*translucens*" or "*campestris*" groups, respectively (5,48).

The original inoculum source of *X. axonopodis* pv. *citrumelo* remains speculative. Nonetheless, we have established the close genetic and pathogenic relatedness among *X. axonopodis* DNA homology group 9.2 pathovars. Knowledge of this relationship may direct new hypotheses regarding the origin and appearance of new pathovars, and provides the foundation for future epidemiological studies of these closely related plant pathogens.

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