Bacterial Leaf Spot of Leafy Crucifers in Oklahoma Caused by *Pseudomonas syringae* pv. *maculicola*

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ABSTRACT

Zhao, Y. F., Damicone, J. P., Demezas, D. H., Rangaswamy, V., and Bender, C. L. 2000. Bacterial leaf spot of leafy crucifers in Oklahoma caused by *Pseudomonas syringae* pv. *maculicola*. Plant Dis. 84:1015-1020.

During 1995 and 1996, bacterial leaf spots severely damaged fields of kale, spinach mustard, and turnip in Oklahoma. Symptoms were small, brown, necrotic spots with irregular edges surrounded by chlorotic halos. Lesion margins were often water-soaked on the abaxial surface. The spots enlarged and coalesced, causing extensive leaf yellowing and necrosis. Nineteen strains of a fluorescent *Pseudomonas* spp. were isolated from symptomatic plants. LOPAT tests and carbon source oxidation using Biolog GN MicroPlates were used to classify the strains as *P. syringae*. Cluster analysis of carbon source oxidation profiles for the local strains and selected reference strains of *P. syringae* pv. *maculicola* and pv. *tomato* produced one group with 79.5% similarity. In spray inoculations, all local strains caused chlorotic or water-soaked lesions on collards, kale, cauliflower, and tomato. A few local strains caused necrotic lesions on mustard. Most local strains caused one of the three lesion types on turnip and spinach mustard. Reference strains of *P. syringae* pv. *maculicola* caused similar symptoms. All but three of the local strains produced coronatine in vitro. The local strains were thus classified as *P. syringae* pv. *maculicola*, the cause of bacterial leaf spot of crucifers. Two distinct groups of *P. syringae* pv. *maculicola* were identified by repetitive sequence-based polymerase chain reaction (rep-PCR) with both REP and BOXA1R primers. Three subgroups within each group were further identified using the BOXA1R primer. Except for two strains of *P. syringae* pv. *tomato* which were pathogenic on crucifers, the pathovars *maculicola* and *tomato* had different genetic fingerprints. The pathogen was recovered from seven of ten fields sampled during 1994 to 1996. In five of the fields with *P. syringae* pv. *maculicola*, pathovars of *Xanthomonas campestris* were also isolated from lesions forming a bacterial disease complex. This is the first report of bacterial leaf spot caused by *P. syringae* pv. *maculicola* on leafy crucifers in Oklahoma.

The leafy crucifers collard (*Brassica oleracea* var. *viridis* L.), kale (*B. oleracea* var. *sabellica* L.), mustard (*B. juncea* L. var. *crispifolia* Bailey), spinach mustard (*B. rapa* L. var. *perviridis* Bailey), and turnip (*B. rapa* var. *utilis* (D.C.) Metz.) are important minor crops in Oklahoma that are grown for processing. About 600 ha are grown each year during both spring and fall cropping seasons (2). The crops are direct seeded and harvested in bulk with non-selective cutters up to three times per cropping season. Because of the non-selective harvesting procedure, leaf blemishing from disease can reduce product grade and value. Product from severely diseased fields may be discounted or even rejected by processors, resulting in total economic loss.

During 1994 to 1996, fields of turnip, spinach mustard, mustard, and kale in the Arkansas River Valley of eastern Oklahoma were severely damaged by bacterial leaf spots. Symptoms were circular to irregular shaped necrotic lesions with yellow halos. Lesion size ranged from 2 mm to over 1 cm in size. Chlorotic haloes surrounding isolated spots were narrow (1 to 2 mm). However, chlorosis was general on areas of leaves where lesions were numerous. The margins of lesions were often water-soaked on the abaxial leaf surface. On severely diseased leaves, lesions coalesced to cause extensive leaf necrosis and death. Up to 70% of leaves were affected. Where disease incidence was lower, affected leaves were usually of a similar age. Fluorescent *Pseudomonas* spp. and *Xanthomonas campestris* were isolated from diseased leaves. Two pathovars of *X. campestris*, pv. *campestris*, the cause of black rot, and pv. *armoraciae*, the cause of *Xanthomonas* leaf spot, were identified as important components of the leaf spot diseases (31,32).

Bacterial leaf spot, also known as pepper spot, is a previously reported bacterial disease of crucifers caused by *P. syringae*

pv. *maculicola* (6). The disease was first reported by McCulloch on cauliflower (*B. oleracea* var. *botrytis* L.) in 1911 (22). Since then, it has been reported in at least 32 countries where cauliflower and other *Brassica* spp. are grown (6). Bacterial leaf spot is prevalent in cool coastal areas (27) where it causes severe losses on cauliflower and broccoli (*B. oleracea* L. var. *italica* Plenck. [30]). In Argentina, a severe epidemic was recently reported on Brussels sprouts (*B. oleracea* L. var. *gemmifera* Zenk. [1]). *P. syringae* pv. *maculicola* is also pathogenic on tomato (*Lycopersicon esculentum* Mill. [6,28,30]), and is closely related to *P. syringae* pv. *tomato*, the cause of bacterial speck of tomato (9,11,28,30). A new, unnamed pathovar of *P. syringae* was recently reported to cause leaf spot and blight of broccoli raab (*B. ruvo* Bailey [16]). The pathovar produced symptoms on crucifers that were similar to those caused by *P. syringae* pv. *maculicola*, but it was nonpathogenic on tomato and caused disease on some grass species (16).

Typical symptoms caused by *P. syringae* pv. *maculicola* are small, brown, necrotic spots with irregular edges surrounded by chlorotic halos (27). However, the pathogen causes a variety of symptoms that include chlorotic, water-soaked, and necrotic lesions (7,22,30). *P. syringae* pv. *maculicola* is known to produce coronatine, a chlorosis-inducing, non-host specific phytotoxin that functions as an important virulence factor (5,30). Coronatine production has been used as a diagnostic tool to identify *P. syringae* pv. *maculicola* and other coronatine-producing pathovars of *P. syringae* (3–5).

Repetitive sequence-based polymerase chain reaction fingerprinting, referred to as rep-PCR, has been successfully used to identify and classify bacteria (20,21). Three families of repetitive sequences have been successfully used, including the 35 to 40 bp repetitive extragenic palindromic (REP) sequence, the 124 to 127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp BOX element. Genomic fingerprints generated with REP-, ERIC-, and BOX-PCR have been used for the rapid identification of pathovars and bacterial strains (19,20,21) and for studies on genetic diversity (12).

In a recent national assessment of the importance of diseases of leafy green vegetables and their management, bacte-

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Accepted for publication 2 June 2000.

rial leaf spots were not recognized (2). The objective of this study was to identify and characterize the strains of fluorescent *Pseudomonas* spp. isolated from leafy crucifers in Oklahoma. A preliminary report of this work has been published (32).

MATERIALS AND METHODS

Bacterial strains. Bacterial strains were isolated from leaves of kale, spinach mustard, and turnip with symptoms of leaf spot that were collected from 10 fields in eastern Oklahoma from 1994 to 1996 (Table 1). Leaf pieces with lesions were excised from diseased leaves, surface sterilized in 0.25% aqueous sodium hypochlorite for 30 sec, and rinsed in sterile tap water. The leaf pieces were macerated in a few drops of sterile tap water and the extract was streaked onto nutrient agar (NA). Plates were incubated at 28°C and examined at 3 to 5 days for bacterial growth. Colonies resembling *Pseudomonas* spp. were purified on NA and tested for fluorescence on King's medium B (KB [15]) and gram reaction using standard methods (26). Fluorescent, gram-negative strains were retained and subjected to LOPAT tests, which include production of arginine dihydrolase, levan, and oxidase; ability to rot potato; and induction of the hypersensitive reaction in tobacco leaves (17). Known strains of *P. syringae* pv. *maculicola* and pv. *tomato* were obtained from various sources and used as reference strains (Table 1). Bacterial strains were stored in 15% glycerol at -70°C and were routinely cultured on KB, mannitol-glutamate me-

dium (MG), or MG containing 0.01% yeast extract (14) at 28°C.

Carbon source oxidation and utilization. Strains were typed for oxidation of the 95 carbon sources available on the GN MicroPlates (Biolog Inc., Hayward, CA). Bacteria were grown on trypticase soy agar (TSA; Bacto Tryptic Soy Broth, 15 g/liter agar; Difco Laboratories, Detroit, MI) for 24 h at 28°C. Cells were transferred from the plates to 30 ml test tubes containing 20 ml of sterile saline (0.85% NaCl). The optical density (OD) of the suspension was determined at 590 nm and adjusted as recommended by the manufacturer (13). Microplates were inoculated with 150 µl of bacterial suspension per well and incubated at 28°C. The absorbance (A_{590}) of the wells was recorded at 24 h using a plate reader (Molecular Devices Corp., Sunnyvale, CA) controlled by SOFTmax (version 1.02). The data were converted to ASCII format with SMAX123. MicroLog 2 (version 3.5, Biolog Inc., Hayward, CA), and the GN database (version 3.5) was used for strain identification. Similarity coefficients were determined using NTSYS-PC (24). Cluster analysis was performed using the unweighted pair-group method, and dendrograms were generated using the SAHN subroutine of NTSYS-PC. The repeatability of the assay was confirmed by running a set of 18 arbitrarily selected strains twice. The assay then was performed on the 19 local strains and on four reference strains each of *P. syringae* pv. *maculicola* and pv. *tomato*.

Utilization of sorbitol and homoserine, useful for grouping strains of *P. syringae*

pv. *maculicola* and pv. *tomato* (9), was determined for each strain. Ayer's minimal salt medium (11) was amended with 0.1% sorbitol or homoserine, inoculated, and incubated at 28°C. Growth at 14 days was indicative of substrate utilization.

Pathogenicity tests. Pathogenicity and host range was determined by spray inoculation of cauliflower (cv. Snow Crown), collard (cv. Vates), kale (cv. Dwarf Blue Curled Scotch), mustard (cv. Southern Giant Curled), spinach mustard (cv. Tendergreen), turnip (cv. Hybrid Alltop), and tomato (cv. Summer Flavor 3000). Strains were grown on NA or KB at 28°C for 48 h and cells were suspended in sterile tap water to a concentration of approximately 1×10^7 CFU/ml as determined by dilution plating and OD readings ($A_{600} = 0.05$). Plants were produced from seed in 12.7-cm-diameter pots containing soilless media (Metro-Mix 200, Scotts-Sierra Horticultural Products Co., Marysville, OH). Plants were maintained in a greenhouse at 20 to 25°C, thinned to one per pot at the cotyledon stage, and fertilized every 2 weeks with soluble 20-20-20 (N-P-K) fertilizer. Plants were incubated in polyethylene humidity chambers within the greenhouse at 100% relative humidity for 24 to 48 h before and after inoculation. Bacterial suspensions were applied with an artist's airbrush at 172 kPa until leaves were uniformly wet. After removal from the chambers, plants were maintained in the greenhouse until symptoms were assessed 7 to 14 days after inoculation. Symptoms were classified by lesion type as previously described (30). Chlorotic lesions were brown,

Table 1. Bacterial strains used in this study^x

Strain	Field-year	Host	Location	Source (reference)
<i>Pseudomonas syringae</i> pv. <i>maculicola</i>				
F1	2-1995	Spinach mustard ^y	Oklahoma	This study
NF2, NF3, NF3a	3-1995	Turnip	Oklahoma	This study
F6, F18, F19	4-1995	Turnip	Oklahoma	This study
NF4, NF5, F7, NF12	5-1995	Spinach mustard ^z	Oklahoma	This study
F16, F17	6-1995	Spinach mustard ^z	Oklahoma	This study
F9, F10a, F15	7-1995	Kale	Oklahoma	This study
F20, F21, F22	9-1996	Spinach mustard ^y	Oklahoma	This study
16391, 16392		Cauliflower	Florida	R. Stall
17223, 17225		Brassica sp.	Florida	R. Stall
1820	1965	Radish	California	D. Cuppels (9)
84-59, 84-67	1984	Cauliflower	California	D. Cuppels (9,30)
88-10	1988	Cauliflower	California	D. Cuppels (9,30)
4981	1970	Cauliflower	Zimbabwe	D. Cuppels (9,30)
90-32	1990	Cauliflower	California	D. Cuppels (9)
4326	1965	Radish	United States	C. Bender (30)
<i>Pseudomonas syringae</i> pv. <i>tomato</i>				
6198		Tomato	Oklahoma	R. Stall
9861, 9865, 9867		Tomato	Florida	R. Stall
OH314		Nettle	Ohio	D. Cuppels (9)
DC84-1	1984	Tomato	Ontario	D. Cuppels (9)
DC3000		Tomato	United Kingdom	D. Cuppels
487	1979	Tomato	Greece	D. Cuppels (9)
3357	1972	Tomato	New Zealand	D. Cuppels (9)

^x Reference strains were from C. Bender, co-author; D. Cuppels, Agriculture and Agri-Food Canada, London, Ontario N5V 4T3; and R. Stall, Department of Plant Pathology, University of Florida, Gainesville 32611. Local strains were isolated from leaf spots on leaves from seven fields in eastern Oklahoma and were identified based on results of this study.

^y Cultivar Savannah, which is used by the processor as a mustard green.

^z Cultivar Tendergreen, which is used by the processor as a turnip green.

water-soaked, and surrounded by yellow zones of chlorosis. Water-soaked lesions were the same as chlorotic lesions, but lacked a chlorotic border. Necrotic lesions were tan to brown, dry, and also lacked a chlorotic border. Three replicate plants of each crop were inoculated with each strain and the entire set of inoculations was repeated. Water was used as a control for each set of inoculations.

Coronatine production. A rapid extraction method was used to determine in vitro production of coronatine as described previously (23). Briefly, bacteria were cultured in 10 ml Hoitink-Sinden medium optimized for coronatine production and incubated at 18°C for 5 days at 280 rpm. Coronafacoyl compounds were extracted from culture supernatants with ethyl acetate, evaporated, and resuspended in 0.5 ml of 10% acetonitrile in 0.05% trifluoroacetic acid. Extracts were analyzed by high-pressure liquid chromatography (HPLC) as described previously (23). Three extractions per strain were performed. *P. syringae* pv. *glycinea* strains PG4180.N9 and PG4180.G109 were included as positive and negative controls, respectively.

Rep-PCR. Rep-PCR was carried out as described by Versalovic et al. (29). The REP primers REP1R-1[5'-IIICGICGICAT CIGGC] and REP2-1[5'-ICGICTTATCIG GCCTAC] were synthesized by Genosys (The Woodlands, TX) (20,29). The BOX primer BOXA1R [5'-CTACGCAAGG CGACGCTGACG] was synthesized by the Oklahoma State University Recombinant DNA/Protein Resource Facility. Bacteria initially were grown on NA at 28°C for 24 h, and then transferred to 5 ml of Luria-Bertani broth (25) and incubated at 28°C, 280 rpm, for 18 h. Aliquots (1 ml) were centrifuged, and pellets were washed in 0.5 ml of 0.5 M NaCl and then resuspended in 50 µl sterile distilled water to a final density of 10¹⁰ CFU/ml. Repetitive DNA elements were amplified using REP and BOX primers in 25 µl reaction volumes. Each reaction consisted of 1× Gitschier buffer (16.6 mM [NH₄]₂SO₄; 67 mM HCl, pH 8.8; 6.7 mM MgCl₂; 6.7 mM EDTA; 30 mM β-mercaptoethanol), 4 µg bovine serum albumin, 10% dimethyl sulfoxide (v/v) (Fluka Chemical Corp., Ronkonkoma, NY), 50 pmoles of each primer, 1.25 mM of each dNTP (Boehringer Mannheim, Indianapolis, IN), 2 units of *Taq* polymerase (Promega, Madison, WI), and 1 µl of the bacterial suspension. Each reaction set included a negative control containing water only. Reaction mixtures were overlaid with 25 µl of sterile mineral oil. The PCR was carried out in a DNA thermal cycler 480 (Perkin-Elmer Cetus, Norwalk, CN) as follows: 1 cycle at 95°C for 6 min, 30 cycles at 94°C for 1 min, 40°C (REP) or 50°C (BOX) for 1 min, and 65°C for 8 min, followed by 65°C for 16 min. An 8 µl aliquot of the PCR product was analyzed by gel electrophoresis on 1.5% agarose gel

at 70V for 5 h in 1× TBE buffer (pH 8.3). Gels were stained with ethidium bromide and differences in DNA fingerprint patterns were assessed visually. Each strain was assayed twice.

RESULTS

Bacterial strains. A total of 29 strains of fluorescent pseudomonads was recovered from the fields of leafy crucifers sampled. Colonies were white, circular, entire, and convex. All strains were gram negative and had strong bluish green fluorescence on both KB and MG plates. In LOPAT tests, 19 strains were positive for levan production and the hypersensitive response on tobacco; but negative for oxidase, arginine dihydrolase, and potato rot. The 19 strains belonged to LOPAT group Ia and were presumably *P. syringae*. These 19 strains of *P. syringae* were recovered from seven of the ten sampled fields and were isolated from kale, spinach mustard, and turnip (Table 1). The remaining 10 strains belonged to LOPAT groups II, IVa, and Va, and likely were *P. viridiflava* (3 strains), *P. fluorescens* (3 strains), and *P. putida* (4 strains), respectively.

Carbon source oxidation and utilization. The Biolog GN database classified

the 19 putative strains of *P. syringae* (described above) and the four reference strains of each *P. syringae* pv. *maculicola* and pv. *tomato* that were assayed as *P. syringae*. The database classified 13 of the 19 local strains and three of the eight reference strains as *P. syringae* pv. *pisi*, with similarity coefficients ranging from 52 to 87%. Other strains were classified as *P. syringae* pv. *tabaci*, pv. *tomato*, pv. *hibisci*, and pv. *porri* with similarity ranging from 60 to 89%. Only reference strain 9867 was correctly classified as *P. syringae* pv. *tomato*. Cluster analysis of the carbon source oxidation profiles for the 19 local strains of *P. syringae*, including the four reference strains of each pathovar produced one group at 79.5% similarity (Fig. 1). Subgroups at >90% similarity contained local and reference strains of both pathovars.

All 19 local strains of *P. syringae*, three of the reference strains of *P. syringae* pv. *maculicola*, and all reference strains of *P. syringae* pv. *tomato* utilized sorbitol, but not homoserine (Table 2). However, five reference strains of *P. syringae* pv. *maculicola* utilized neither carbon source.

Pathogenicity. The 19 local strains of *P. syringae* caused chlorotic or water-soaked lesions on cauliflower, collards, and kale

Fig. 1. Dendrogram showing the similarity among strains of *Pseudomonas syringae* for carbon source oxidation as determined with GN MicroPlates (Biolog, Inc., Hayward, CA).

(Table 2). Pathogenicity of the local strains of *P. syringae* was more variable on mustard, spinach mustard, and turnip (Table 2). Except for strains F18, F20, F21, and F22, which caused necrotic lesions, most strains were nonpathogenic on mustard. Thirteen of the strains were pathogenic on turnip and caused necrotic, water-soaked, or chlorotic lesions. All but four strains were pathogenic on turnip, causing either chlorotic or water-soaked lesions. Chlorotic and water-soaked lesions were interveinal, circular to irregular in shape, brown, and water-soaked on the abaxial surface. On tomato, all 19 strains caused either chlorotic or water-soaked lesions on leaves. Spots were small (1 to 2 mm diameter) and brown. *P. syringae* was isolated from lesions on representative plants and was confirmed to be the same bacteria after characterization.

Reference strains of *P. syringae* pv. *maculicola* caused similar symptoms when inoculated on these crops (Table 2). However, reference strains induced a more uniform symptom expression across crops. Except for strains from Florida (16391,

16392, 17223, and 17225) which caused chlorotic and water-soaked lesions, reference strains caused chlorotic lesions on all crops tested. All reference strains of *P. syringae* pv. *tomato* except DC3000 and OH314 caused chlorotic or water-soaked lesions on tomato, but were nonpathogenic on crucifers (Table 2). Strains DC3000 and OH314 caused chlorotic lesions on crucifers and tomato and were similar in pathogenicity to reference strains of *P. syringae* pv. *maculicola*.

Based on pathogenicity, the 19 local strains of *P. syringae* were designated as pv. *maculicola*. The 10 local strains of *Pseudomonas* spp. which were classified as *P. viridiflava*, *P. fluorescens*, or *P. putida*, were nonpathogenic on all crops inoculated (*data not shown*). Control plants inoculated with water remained symptomless.

Coronatine production. Among the 19 local strains of *P. syringae* pv. *maculicola*, 16 produced coronatine in vitro (Table 2). Most reference strains also produced coronatine in vitro, except for strains 16391 and 16392 of *P. syringae* pv. *maculicola* and strain 6198 of *P. syringae* pv. *tomato*.

Coronatine was detected for the positive control (PG4180.N9), but not for the negative control (PG4180.G109).

Symptom expression for several strains varied on different host plants despite coronatine production in vitro (Table 2). The coronatine-producing strains F20, 17223, 17225 of *P. syringae* pv. *maculicola* caused water-soaked lesions without chlorosis on one or more crucifer crops. Strains NF3, NF3a, NF4, 16391 and 16392 which did not produce coronatine, caused chlorotic lesions on one or more crops.

Rep-PCR. Genomic fingerprints were generated for all strains of *P. syringae* (Table 1). More than 20 DNA fragments ranging in size from 0.2 to 4.0-kb were amplified with the REP or BOXA1R primers. Based on the genomic fingerprints using both the REP (*data not shown*) and BOXA1R primers, two distinct groups were identified for strains of *P. syringae* pv. *maculicola*, which were arbitrarily designated as group A and B (Fig. 2). Strains DC3000 and OH314 of *P. syringae* pv. *tomato* showed identical fingerprints to strains of *P. syringae* pv. *maculicola* in

Table 2. Pathogenicity, host range, carbon source utilization, coronatine production, and genetic fingerprint groups using repetitive sequence-based polymerase chain reaction fingerprinting (rep-PCR) for strains of *Pseudomonas syringae* pv. *maculicola* from crucifers and *P. syringae* pv. *tomato*

Strain	Pathogenicity ^v							S/H ^w	COR ^x	rep-PCR group ^y
	Collards	Kale	Mustard	Spinach mustard	Turnip	Cauliflower	Tomato			
pv. <i>maculicola</i>										
F1	CL	CL	–	CL	CL	CL	CL	+/–	+	A1
F7	CL	CL	–	–	CL	CL	CL	+/–	+	A1
F6	CL	CL	–	NL	–	CL	CL	+/–	+	A2
F9, F10a	CL	CL	–	CL	CL	CL	CL	+/–	+	A2
F16	CL	CL	–	–	–	CL	CL	+/–	+	A2
F17	CL	CL	–	–	–	CL	CL	+/–	+	A2
F18	CL	CL	NL	CL	CL	CL	CL	+/–	+	A2
F19	CL	CL	–	–	CL	CL	CL	+/–	+	A2
F15	CL	CL	–	–	–	CL	CL	+/–	+	A3
NF2, NF5	CL	CL	–	NL	CL	CL	CL	+/–	+	B1
NF12	WS	WS	–	NL	WS	WS	WS	+/–	+	B1
NF3	WS	CL	–	NL	CL	WS	CL	+/–	–	B1
NF3a	CL	CL	–	NL	CL	CL	WS	+/–	–	B1
NF4	CL	WS	–	WS	WS	CL	WS	+/–	–	B1
F20	CL	CL	NL	WS	CL	CL	CL	+/–	+	B3
F21	CL	CL	NL	–	CL	CL	CL	+/–	+	B3
F22	WS	WS	NL	WS	WS	WS	WS	+/–	+	B3
84–67	CL	CL	...	CL	CL	CL	CL	–/– ^z	+	A1
84–59, 88–10, 90–32	CL	CL	...	CL	CL	CL	CL	–/– ^z	+	A2
4981	...	...	...	...	...	...	...	+/– ^z	+ ^z	A3
16391	WS	WS	NL	CL	WS	WS	WS	+/–	–	B1
16392	WS	CL	WS	CL	WS	WS	CL	+/–	–	B1
17223	WS	WS	CL	CL	CL	WS	CL	–/–	+	B2
17225	CL	WS	CL	CL	CL	CL	CL	–/–	+	B2
4326	CL	CL	...	CL	CL	CL	CL	–/–	+	B2
1820	...	...	...	...	...	...	...	–/– ^z	+ ^z	B2
pv. <i>tomato</i>										
DC3000, OH314	CL	CL	...	CL	CL	CL	CL	–/– ^z	+	A2
6198	–	–	–	–	–	–	WS	+/–	–	C
9861, 9865	–	–	–	–	–	–	CL	+/–	+	C
9867	–	–	–	–	–	–	WS	+/–	+	C
DC84–1, 487, 3357	–	–	–	–	–	–	CL	+/– ^z	+ ^z	C

^v Symptom type 7 to 14 days after spray inoculation and incubation in humidity chambers: (CL) = chlorotic lesion, (NL) = necrotic lesion, (WS) = water-soaked lesion, (—) = no symptoms, (...) = not determined. Three replicate plants of each crop were inoculated per strain and the experiment was repeated.

^w Utilization of sorbitol (S) / utilization of homoserine (H): (+) = positive, (—) = negative.

^x Coronatine production: (+) = positive, (—) = negative.

^y Groupings by banding pattern of rep-PCR products using the BOXA1R primer (see Fig. 2).

^z Data from Cuppels and Ainsworth (9).

group A (Fig. 2). All other strains of *P. syringae* pv. *tomato* formed group C, which was quite different from either group A or B (Fig. 2). The groups defined by the REP and BOXA1R primers were consistent for both pathovars and identical groupings were obtained with both primer sets. The difference between groups A and B was greater than that between groups A and C. Group A contained 10 local and 5 reference strains of *P. syringae* pv. *maculicola*, and strains DC3000 and OH314 of *P. syringae* pv. *tomato* (Table 2). Group B contained 9 local and 6 reference strains of *P. syringae* pv. *maculicola* (Table 2). Within each group, the fingerprints were similar; however, the BOXA1R primer clearly divided groups A and B into three subgroups that were arbitrarily designated as A1 to A3 and B1 to B3 (Fig. 2 and Table 2). Within group A, polymorphisms were identified in the 0.35 to 1-kb region (Fig. 2). Within group B, the polymorphisms between subgroups were located in the 1 to 2-kb region (Fig. 2).

DISCUSSION

P. syringae pv. *maculicola* was identified as a cause of bacterial leaf spot of leafy crucifers in Oklahoma. The pathogen was isolated from diseased leaves of turnip, spinach mustard, and kale in 1995 and 1996. It was recovered from seven of the ten fields sampled during 1994 to 1996. *X.*

campestris pvs. *armoraciae* and/or *campestris* were recovered from diseased leaves in the other three fields (31,32). One or both pathovars of *X. campestris* and *P. syringae* pv. *maculicola* were isolated from lesions in five fields, forming a disease complex. The symptoms caused by *P. syringae* pv. *maculicola* are similar to those incited by *X. campestris* pathovars. It is not known whether the bacteria caused mixed infections such as those reported for these genera on tomato (10). This is the first report of bacterial leaf spot of crucifers caused by *P. syringae* pv. *maculicola* in Oklahoma.

In spray inoculations, local strains of *P. syringae* pv. *maculicola* varied in their pathogenicity on different leafy crucifer crops. All strains were pathogenic on collards and kale. However, most local strains were nonpathogenic on mustard, and a few were nonpathogenic on turnip and spinach mustard. Using rub inoculation, Wiebe and Campbell (30) reported variability in the pathogenicity of three strains of *P. syringae* pv. *maculicola* on several crucifer hosts. Weak symptom development was reported for all three strains on mustard, whereas symptoms were severe on turnip for one strain, but weak for the others. In that study, temperature also influenced pathogenicity and virulence. Therefore, environmental conditions may be important for infection and disease development. In this study, spray inoculations were done in the

greenhouse where temperature ranged from 20 to 25°C. However, temperatures were within the range reported to favor moderate to severe disease development for the pathogen (30). Other potentially important environmental factors known to favor severe development of bacterial diseases in the field include wind-driven rain and aerosols, and abrasion from wind-blown sand. Preliminary inoculations with leaf infiltration provided more uniform results than spraying, but this method was rejected because symptoms were not typical of those observed in the field. Differences in cultivar reaction to the disease may also be important in pathogenicity and symptom expression.

In spray inoculations, some strains of *P. syringae* pv. *maculicola* produced variable symptoms on the different crucifer hosts. Local strains produced the three different lesion types described elsewhere for this and other pathovars of *P. syringae* (16,30). Differences in symptom expression could not be explained by the ability of a strain to produce coronatine in vitro. The three local strains that did not produce coronatine in vitro produced chlorotic lesions in planta in one or more crops. Several coronatine-producing strains produced water-soaked lesions without chlorosis on one or more crops. These results suggest that coronatine production in planta may vary with the host plant and may be differentially regulated in some strains.

Fig. 2. Repetitive sequence-based polymerase chain reaction (rep-PCR) fingerprints obtained from representative strains of *P. syringae* pv. *maculicola* and pv. *tomato* with the BOXA1R primer. A portion of the PCR products (8 µl) was loaded to a 1.5% agarose gel, and electrophoresis was performed at 70 V for 5 h in 1× TBE buffer. M, DNA molecular size marker (λ DNA *Pst*I digestion); C, water control; sizes are indicated in kilobase pairs. Lanes 1 to 14 are Group A of *P. syringae* pv. *maculicola*. Lane 1, 4981; 2, 84-67; 3, 84-59; 4, 88-10; 5, 90-32; 6, DC3000; 7, OH314; 8, F10a; 9, F17; 10, F18; 11, F19; 12, F6; 13, F9; and 14, F1. Polymorphisms within group A are indicated within the region marked by arrowheads in lanes 13 (lanes 2 and 14 are A1, lanes 3 to 13 are A2, and lane 1 is A3). Lanes 15 to 28 are group B of *P. syringae* pv. *maculicola*. Lanes: 15, NF2; 16, NF3; 17, NF4; 18, NF5; 19, NF12; 20, 16392; 21, 16391; 22, F20; 23, F21; 24, F22; 25, 4326; 26, 1820; 27, 17223; 28, 17225. Polymorphisms in group B are indicated by arrowheads marking specific bands in lanes 21 and 24 (lanes 15 to 21 are B1, lanes 22 to 24 are B2, and lanes 25 to 28 are B3). Lanes 29 to 31 are *P. syringae* pv. *tomato* (group C). Lanes 29, 3357; 30, DC84-1; 31, 487.

Genomic fingerprints generated by rep-PCR have been used to differentiate strains of *X. campestris* pv. *vesicatoria* (21) and *P. syringae* pvs. *pisi* and *syringae* (12). It has been proposed that *P. syringae* pvs. *maculicola* and *tomato* be combined based on similarities in nutrient utilization, DNA homology, and coronatine production (5,8,28). In this study, the GN database for carbon source oxidation did not differentiate these two pathovars. Except for two strains of *P. syringae* pv. *tomato* (DC3000 and OH314), which were phenotypically similar to *P. syringae* pv. *maculicola*, our results showed that rep-PCR was useful for differentiating the two pathovars. In addition, polymorphisms within *P. syringae* pv. *maculicola* were identified. Several studies have shown that strains of *P. syringae* pv. *maculicola* are genetically more heterogeneous than strains of *P. syringae* pv. *tomato* (8,18,30). Our results support the hypothesis that genetic variation is greater in pathovars with wide host ranges (20).

There were no obvious relationships between genomic fingerprint groups and phenotypic characteristics such as Biolog group, utilization of homoserine and sorbitol, lesion type, and host range. However, except for field 5 which contained rep-PCR groups A1 and B1, and field 7 which contained groups A2 and A3, most strains recovered within a field were in the same rep-PCR group, suggesting that strains within most fields originated from a single inoculum source.

The relative importance of *P. syringae* pv. *maculicola* in relation to pathovars of *X. campestris* involved in the leaf spot disease complex is not certain. Except for the stem and petiole lesions caused by *X. c. pv. armoraciae* (31), which the other pathogens do not cause, symptoms of the different bacteria can be difficult to differentiate in the field. In addition, selective media for the tentative identification of *P. syringae* are not available. Genetic fingerprints using rep-PCR should be beneficial for rapid identification of purified isolates.

More recently, *P. syringae* pv. *maculicola* has continued to be a major pathogen in fields of leafy crucifers in Oklahoma. Severe epidemics have been observed in the spring and fall production seasons of 1998 and in the spring of 1999. In general disease problems are most severe in the spring production season when rainfall is more abundant. Although most fields are watered with overhead sprinklers, irrigation alone does not trigger epidemics because little disease develops in dry production seasons. Attempts by growers to control the disease with copper sprays have not been successful.

ACKNOWLEDGMENTS

We thank D. Cuppels, and R. Stall for providing strains of bacteria, and Jacqueline Fletcher, Bob Hunger, and Lisa Keith for reviewing the manuscript. Approved for publication by the Director, Oklahoma Agricultural Experiment Station.

This research was funded in part by the Oklahoma Agricultural Experiment Station and National Science Foundation grant MCB-9603618.

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