

# Bacterial Leaf Spot Diseases of Leafy Crucifers in Oklahoma Caused by Pathovars of *Xanthomonas campestris*

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## ABSTRACT

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Fields of kale, spinach mustard, and turnip were severely damaged by bacterial leaf spots during 1994 to 1996. Symptoms included circular to angular necrotic lesions with yellow halos and water-soaking on the abaxial leaf surface. Yellow, mucoid strains isolated from leaf spots were identified as *Xanthomonas campestris* using Biolog. Four strains caused black lesions on stems of cabbage seedlings in an excised cotyledon assay, leaf spots and sunken dark lesions on petioles of spray-inoculated crucifers, and leaf spots on spray-inoculated tomato. These strains were classified as *X. campestris* pv. *armoraciae*. Most other strains from leafy crucifers and all strains from a cabbage field caused black rot in the cotyledon assay and in spray-inoculations. Many of these strains also caused leaf spots on collard and kale but not stem and petiole lesions. The strains causing black rot were classified as *X. campestris* pv. *campestris*. Cluster analysis of Biolog profiles yielded a small group that contained local strains of both pathovars, and a large group comprised of reference and local strains of each pathovar, and some local, nonpathogenic strains. Five fingerprint groups were identified by rep-polymerase chain reaction using the BOXAIR primer. Local and reference strains of each pathovar occurred in two of the groups. Two pathovars of *X. campestris* are involved in the leaf spot diseases. Both pathovars were recovered within several fields, and also were recovered along with *Pseudomonas syringae* pv. *maculicola*. This is the first report of *Xanthomonas* leaf spot caused by *X. campestris* pv. *armoraciae* in Oklahoma.

The leafy crucifers collards (*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.) Metzg.) are important minor crops that are grown for processing in Oklahoma. 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 production season. Because of the non-selective harvesting procedure, leaf blemishing from disease can reduce product grade and value. Severely diseased fields may be discounted or even rejected by processors, resulting in total economic loss.

During 1994 to 1996, fields of kale, spinach mustard, and turnip in eastern Oklahoma were severely damaged by bacterial leaf spots. Symptoms included circular to angular, interveinal, necrotic lesions with yellow halos. Lesions ranged in

size 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 small lesions were numerous. Lesion margins were usually water-soaked on the abaxial surface. Spots coalesced and resulted in extensive leaf necrosis and death. Up to 70% of leaves were affected. Where disease incidence was lower, affected leaves were often of a similar age. In some fields, dark elliptical lesions, 2 to 4 mm in length, were numerous on petioles and large, abaxial leaf veins. Both *Pseudomonas syringae* and *Xanthomonas campestris* were isolated from diseased leaves. Bacterial leaf spot, caused by *P. syringae* pv. *maculicola*, has since been identified as an important component of the leaf spot diseases (38,39).

Several pathovars of *X. campestris*, including pv. *campestris*, the cause of black rot (35); pv. *armoraciae*, the cause of *Xanthomonas* leaf spot of horseradish (*Armoracia rusticana* Gaertn. et al. [16]); and pv. *raphani*, the cause of bacterial leaf spot of radish (*Raphanus sativus* var. *sativus* L.) and turnip (34); are known to cause leaf spots on crucifers (4). These pathogens have been reported to cause severe losses on leafy crucifers (1,2,28,35).

Black rot has been reported in all countries where crucifers are grown and re-

mains a persistent problem (18,35). Typical symptoms include V-shaped lesions on leaf margins and blackened veins associated with systemic movement of the pathogen (1,35). As a vascular pathogen, *X. campestris* pv. *campestris* is normally not associated with leaf spot symptoms. However, it has been reported to cause atypical symptoms that mimic those of other bacterial pathogens (7,17,19,36). These include leaf blights (1,6,36), water-soaked lesions, and soft rot (26). In Australia, a bacterial leaf spot disease of cabbage (*B. oleracea* var. *capitata* L.), cauliflower (*B. oleracea* var. *botrytis* L.), and broccoli (*B. oleracea* L. var. *italica* Plenck.) was reported to have symptoms differing from black rot (17). The pathogen was indistinguishable from *X. campestris* pv. *campestris* both morphologically and physiologically, and could not be differentiated on the basis of symptoms. *X. campestris* pv. *campestris* is also known to infect several cruciferous weeds (4,28,29,31).

*Xanthomonas* leaf spot of crucifers, caused by *X. campestris* pv. *armoraciae* or pv. *raphani* was first reported on horseradish in 1929 and on radish and turnip in 1930 (16,34). The disease has been reported in many countries including the United States, Japan (30), Brazil, China (37), Turkey, and India (4). Typical symptoms include small, water-soaked, olive green lesions with a yellow halo (3,16,28). Black, sunken, and elongated lesions on stems and petioles may rapidly kill young plants (4). The symptoms of *Xanthomonas* leaf spot closely resemble those of bacterial leaf spot of cauliflower caused by *P. syringae* pv. *maculicola* (23,28). A different pathotype of *X. campestris* pv. *armoraciae*, recently reported in Ohio, differed in host range from previously reported strains (23). Additional hosts for *X. campestris* pv. *armoraciae* include tomato (*Lycopersicon esculentum* Mill.), pepper (*Capsicum annum* L.), and tobacco (*Nicotiana tabacum* L. [3,4,15, 30,37]).

In a recent national assessment of the importance of diseases of leafy green vegetables and their management, bacterial leaf spots were not recognized (2). The objective of this study was to identify and characterize the strains of *X. campestris* isolated from leaf spots of leafy crucifers in Oklahoma. A preliminary report of this work has been published (38).

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## MATERIALS AND METHODS

**Bacterial strains.** Leaves with leaf spot symptoms were collected from 10 fields cropped to either kale, spinach mustard, or turnip in eastern Oklahoma from 1994 to 1996. Sections of leaves with lesions were excised, surface sterilized in 0.25% aqueous sodium hypochlorite for 30 sec, and rinsed in sterile tap water. Leaf sections were macerated in sterile tap water and the extract was inoculated to nutrient agar (NA) and several semi-selective media (5), including Fieldhouse-Sasser medium (FS [8]), basal starch cycloheximide antibiotic agar (BSCAA [21]) and nutrient starch cycloheximide antibiotic agar (NSCAA [21]). Plates were incubated at 28°C and examined after 3 to 5 days for bacterial growth and cleared zones indicative of starch hydrolysis. Single, yellow (NA, NSCAA and BSCAA) or bluish white (FS) bacterial colonies that utilized the starch on semi-selective media were selected and purified on NA for further identification and characterization. Twenty-nine strains of *Xanthomonas* spp. from leafy crucifers were recovered for characterization (Table 1). A cabbage field in western Oklahoma with severe black rot also was sampled in

1997. Isolations were made from V-shaped lesions on leaf margins and from leaf spots using the methods described above. Sixteen strains of *Xanthomonas* spp. were recovered from the cabbage field and also characterized (Table 1). Gram stains were performed on all local strains. Thirteen strains of *X. campestris* pv. *campestris* or pv. *armoraciae* were obtained from various sources and used as reference strains (Table 1). All strains used in this study were stored in 15% glycerol at -70°C and were routinely cultured on NA or Luria-Bertani medium (LB [24]) at 28°C.

**Carbon source oxidation.** Strains were assayed 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 TSA plates to 30 ml test tubes containing 20 ml of sterile saline (0.85% NaCl). The optical density (OD) of the suspension was adjusted as recommended by the manufacturer. Microplates were inoculated with 150 µl of suspension per well and incubated at 28°C. The absorbency of the wells

at 590 nm was determined at both 24 and 48 h using a plate reader (Molecular Devices Corp., Sunnyvale, CA). MicroLog 2 (version 3.5, Biolog Inc.) and the GN database (version 3.5) were used for strain identification. Similarity coefficients were determined using NTSYS-PC (22). 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 was then performed on all 45 local strains and nine of 13 reference strains.

**Pathogenicity.** To assess the pathogenicity and host range of bacterial strains, inoculum was prepared by growing strains on NA at 28°C for 48 h. Cells were suspended in sterile tap water and adjusted to a concentration of approximately 10<sup>7</sup> CFU/ml as determined by OD readings and bacterial counts by dilution plating.

Various crops were produced from seed in 12.7-cm-diameter pots containing soil-less media (Metro-Mix 200, Scotts-Sierra Horticultural Products Co., Marysville, OH). Plants were maintained in a green-

**Table 1.** Strains of *Xanthomonas campestris* used in this study

Strain	Field	Host	Location	Source* (reference)
<i>pv. campestris</i>				
BR1, BR4, BR5, BR6, BR7, BR9, BR13	1-1994	Turnip	Oklahoma	This study
BR15, BR23	2-1995	Spinach mustard <sup>x</sup>	Oklahoma	This study
BR24	3-1995	Turnip	Oklahoma	This study
BR17, BR27	4-1995	Turnip	Oklahoma	This study
BR18	5-1995	Spinach mustard <sup>y</sup>	Oklahoma	This study
BR19	6-1995	Spinach mustard <sup>y</sup>	Oklahoma	This study
BR20, BR26	7-1995	Kale	Oklahoma	This study
BR21, BR22, BR28	8-1995	Kale	Oklahoma	This study
BR29, BR30, BR31	9-1996	Spinach mustard <sup>x</sup>	Oklahoma	This study
BR33, BR34, BR35	10-1996	Turnip	Oklahoma	This study
BR37, BR39, BR40, BR43, BR46, BR47, BR48	1997	Cabbage (black rot)	Oklahoma	This study
BR41, BR42, BR44, BR45, BR49, BR50, BR51, BR52, BR53	1997	Cabbage (leaf spot)	Oklahoma	This study
Xcc528		Brussels sprouts	United Kingdom	ATCC 33913 (1,6)
PHW-117		Cabbage	Wisconsin	D. Cuppels (1)
Xcc1205		Cabbage	Wisconsin	P. Williams
3150		?	Florida	R. Stall
16936		Cabbage ("A")	Florida	R. Stall
16937		Cabbage ("B")	Florida	R. Stall
JT1		Cabbage	Michigan	D. Cuppels (13)
JT4		Arabidopsis	Michigan	D. Cuppels (13)
<i>pv. armoraciae</i>				
BR11	1 - 1994	Turnip	Oklahoma	This study
BR16	3 - 1995	Turnip	Oklahoma	This study
BR25	5 - 1995	Spinach mustard <sup>y</sup>	Oklahoma	This study
BR32	9 - 1996	Spinach mustard <sup>x</sup>	Oklahoma	This study
XLS6		Cabbage	N. Carolina	L. Black (1)
756		Cabbage seed	E. Asia	D. Gabriel (1)
3145 <sup>z</sup>		?	Florida	R. Stall
3152		?	Florida	R. Stall
69-2 <sup>z</sup>		Radish	Florida	D. Cuppels

\* Local strains from kale, spinach mustard, and turnip were isolated from leaf spots on leaves collected in 10 fields in eastern Oklahoma. Local strains from cabbage were isolated from either V-shaped lesions (black rot) or leaf spots collected from a cabbage field in western Oklahoma. All local strains were identified based on results in this paper. Reference strains were received from: L. L. Black, Department of Plant Pathology and Crop Physiology, Louisiana State University, Baton Rouge 70803; D. Cuppels, Agriculture and Agri-Food Canada, London, Ontario N5V 4T3; D. Gabriel and R. Stall, Department of Plant Pathology, University of Florida, Gainesville 32611; P. Williams, Department of Plant Pathology, University of Wisconsin, Madison 53706.

<sup>x</sup> Cultivar Savannah used by processor as mustard greens.

<sup>y</sup> Cultivar Tendergreen used by processor as turnip greens.

<sup>z</sup> Strains were received as *pv. raphani*, which was considered synonymous with *pv. armoraciae* in this study (1).

house 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 grown for 3 weeks until they had three to four true leaves at the time of inoculation except for cabbage and tomato, which were 6 weeks old. Horseradish was produced vegetatively from retail roots that were sectioned and planted in pots. Horseradish was maintained as described above for 8 weeks prior to inoculation.

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 airbrush at 172 kPa until leaves were uniformly wet. Collards (cv. Vates), kale (cv. Dwarf Blue Curled Scotch), mustard (cv. Southern Giant Curled), spinach mustard (cv. Tendergreen), turnip (cv. Hybrid Alltop), radish (cv. Scarlett Knight), and tomato (cv. Summer Flavor 3000) were inoculated with each local strain and nine of the 13 reference strains. Horseradish was inoculated with selected strains. After incubation, plants were removed from the humidity chambers and maintained in the greenhouse until symptoms were assessed, 7 to 14 days after inoculation. The presence or absence of leaf spot and black rot symptoms were recorded for each crop. Symptoms of leaf spot included interveinal necrotic lesions surrounded by chlorotic halos. Symptoms of black rot included V-shaped lesions along leaf margins bordered by chlorosis and darkening of leaf veins. 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.

An excised-cotyledon assay on cabbage seedlings was performed on all strains to differentiate *X. campestris* pv. *campestris* and pv. *armoraciae* as described by Alvarez et al. (1). Cabbage (cv. Early Jersey) was grown from seed in the greenhouse for 8 to 10 days until the first true leaf was visible and cotyledons were fully expanded. One cotyledon per seedling was excised close to the stem and the wound surface was inoculated with a mass of bacteria from a single colony grown on NA for 24 h at 28°C. Three replicate plants were inoculated with each strain, and the entire set of inoculations was repeated. After inoculation, plants were maintained in the greenhouse until symptoms were assessed at 4, 7, and 14 days after inoculation. Strains that caused black stem lesions by 4 to 7 days after inoculation were classified as *X. campestris* pv. *armoraciae* (1). Strains that did not cause stem lesions at 4 to 7 days after inoculation and induced the systemic symptoms typical of black rot, were classified as *X. campestris* pv. *campestris* (1). The severity of black rot for strains of *X. campestris* pv. *campestris* was

assessed at 14 days after inoculation, using a disease index (DI) of 0 to 9, in which 0 = healthy and 9 = dead (1). A mean DI was calculated for each strain and strains with a DI of 0 to 1 were considered avirulent, 1.1 to 3 were weakly virulent, 3.1 to 6 were moderately virulent, 6.1 to 8 were virulent, and 8.1 to 9 were highly virulent (1).

**Genetic fingerprinting.** To assess the genetic diversity of strains of *X. campestris*, repetitive sequence-based polymerase chain reaction fingerprinting (rep-PCR) was performed on all strains. The BOX primer BOXA1R [5'-CTACGGCAAGGC GACGCTGACG] was synthesized by the Oklahoma State University Recombinant DNA/Protein Resource Facility. Rep-PCR was carried out as previously described (12). Bacteria were initially grown on NA at 28°C for 24 h, and then transferred to 5 ml of LB broth (24) and incubated at 28°C, 280 rpm, for 18 h. Cultures (1 ml) were harvested by centrifugation, and pellets were washed in 0.5 ml of 0.5 M NaCl and resuspended in 50 µl sterile distilled water to a final concentration of 10<sup>10</sup> CFU/ml. Repetitive DNA elements were amplified in a 25 µl reaction volume. Each reaction consisted of 1× Gitschier buffer (16.6 mM [NH<sub>4</sub>]<sub>2</sub>SO<sub>4</sub>; 67 mM HCl, pH 8.8; 6.7 mM MgCl<sub>2</sub>; 6.7 mM EDTA; 30 mM β-mercaptoethanol), 4 µg bovine serum albumin, 10% dimethyl sulfoxide (vol/vol) (Fluka Chemical Corp., Ronkonkoma, NY), 50 pmoles of the 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 performed in a thermal cycler (Model 480, Perkin-Elmer Cetus, Norwalk, CT) under the following conditions: 1 cycle at 95°C for 6 min, 30 cycles at 94°C for 1 min, 50°C for 1 min, and 65°C for 8 min, followed by 65°C for 16 min. An 8-µl aliquot of the PCR reaction was analyzed by gel electrophoresis on a 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. The assay was performed twice for each strain.

## RESULTS

**Bacterial strains.** Of the 45 local strains of *Xanthomonas* spp., including 29 strains from leafy crucifers and 16 strains from cabbage, all were gram-negative and yellow-pigmented on NA, and utilized starch on semi-selective media. On FS medium, the colonies were bluish-white, translucent, raised, and mucoid. On NSCAA and BSCAA, the colonies were greenish-yellow, translucent, and mucoid. Based on these characteristics all of the local strains were identified as *X. campestris* (25). *X. campestris* was recovered from each of the

10 fields of leafy crucifers with leaf spot symptoms.

**Carbon source oxidation.** For *Xanthomonas* spp., an incubation time of 48 h was necessary for full color development on the Biolog GN MicroPlates, an observation also noted elsewhere (32). All local strains and reference strains were identified as *X. campestris*. Seventeen strains were further classified as *X. campestris* pv. *campestris*, with similarity coefficients ranging from 43.2 to 88.2%. The Biolog GN database placed another nine strains close to *X. campestris* pv. *campestris*, but with lower similarity coefficients (<40%). Other strains were assigned to other pathovars of *X. campestris* by the database.

Cluster analysis of the carbon source utilization profiles for local strains and for reference strains of *X. campestris* pv. *armoraciae* and pv. *campestris* produced two groups, A and B (Fig. 1). The similarity within each group was 80% or greater. Local strains, identified by pathogenicity as *X. campestris* pv. *campestris* and pv. *armoraciae* (see below), were found in both groups, but all reference strains of *X. campestris* pv. *campestris* and pv. *armoraciae* were in group B.

**Pathogenicity.** Among the 45 local strains, only four caused black lesions on stems of cabbage seedlings within 7 days after inoculation using the cotyledon assay (Table 2). There was no further symptom development for these strains. Except for strain 3152, reference strains of *X. campestris* pv. *armoraciae* produced the same symptoms. The four local strains that caused stem lesions, each recovered from a different field, were designated as *X. campestris* pv. *armoraciae*. In spray inoculations of various crucifers and tomato, the four strains caused localized leaf spots with chlorotic halos, but no symptoms of systemic infection. The four strains also caused elongated, black, sunken lesions on petioles and stems of collard, kale, turnip, and spinach-mustard when inoculated by spraying. Except for strain 3152, reference strains of *X. campestris* pv. *armoraciae* produced similar symptoms. All strains of *X. campestris* pv. *armoraciae* were very aggressive and caused severe leaf spotting on tomato and all crucifers except horseradish (Table 2). Strains were less aggressive on horseradish, and two local strains were non-pathogenic on this host. The reference strain 16936, received as *X. campestris* "A", was classified here as *X. campestris* pv. *armoraciae* based on pathogenicity tests.

Among the remaining 25 local strains of *X. campestris* from leafy crucifers, all but eight caused systemic symptoms of black rot, but not stem lesions, in the excised cotyledon assay (Table 2). However, these local strains varied in virulence in the cotyledon assay. Thirteen of the local strains induced severe blight symptoms within 10 days and caused seedling death

within 14 days. These were ranked as highly virulent to virulent based on the disease index. Four local strains caused black rot symptoms in at least one true leaf within 14 days and were ranked as moderately to weakly virulent. Most of the reference strains of *X. campestris* pv. *campestris* caused similar symptoms and also varied in virulence (Table 2). The seventeen local strains that produced black rot symptoms, recovered from eight of the ten fields of leafy crucifers sampled, were designated as *X. campestris* pv. *campestris*. In addition, all sixteen strains from the cabbage field caused black rot and were highly virulent to virulent in the cotyledon assay. Reference strain 3152, received as *X. campestris* pv. *armoraciae*, was reclassified as *X. campestris* pv. *campestris* based on the cotyledon assay. The other eight local strains and reference strain 16937 were avirulent in the cotyledon assay.

When inoculated by spraying, local and reference strains of *X. campestris* pv. *campestris* that were weakly to highly virulent in the cotyledon assay, generally caused black rot on all crucifers except horseradish and were nonpathogenic on tomato (Table 2). Several strains were nonpathogenic on mustard, and a few were nonpathogenic on radish and collards. Generally, symptoms were most severe on collards, kale, turnip, and spinach-mustard. Most local strains of *X. campestris* pv. *campestris*, including all strains from the cabbage field, also caused leaf spots on collard and kale. The symptoms were very similar to those caused by strains of *X. campestris* pv. *armoraciae*. However, none of the strains of *X. campestris* pv. *campestris* caused leaf spots on the other crucifers or caused lesions on stems and petioles of any of the crucifers. Except for strain BR1, which caused mild black rot symptoms on turnip and radish, strains that were avirulent in the cotyledon assay also did not cause symptoms on any of the crops inoculated by spraying.

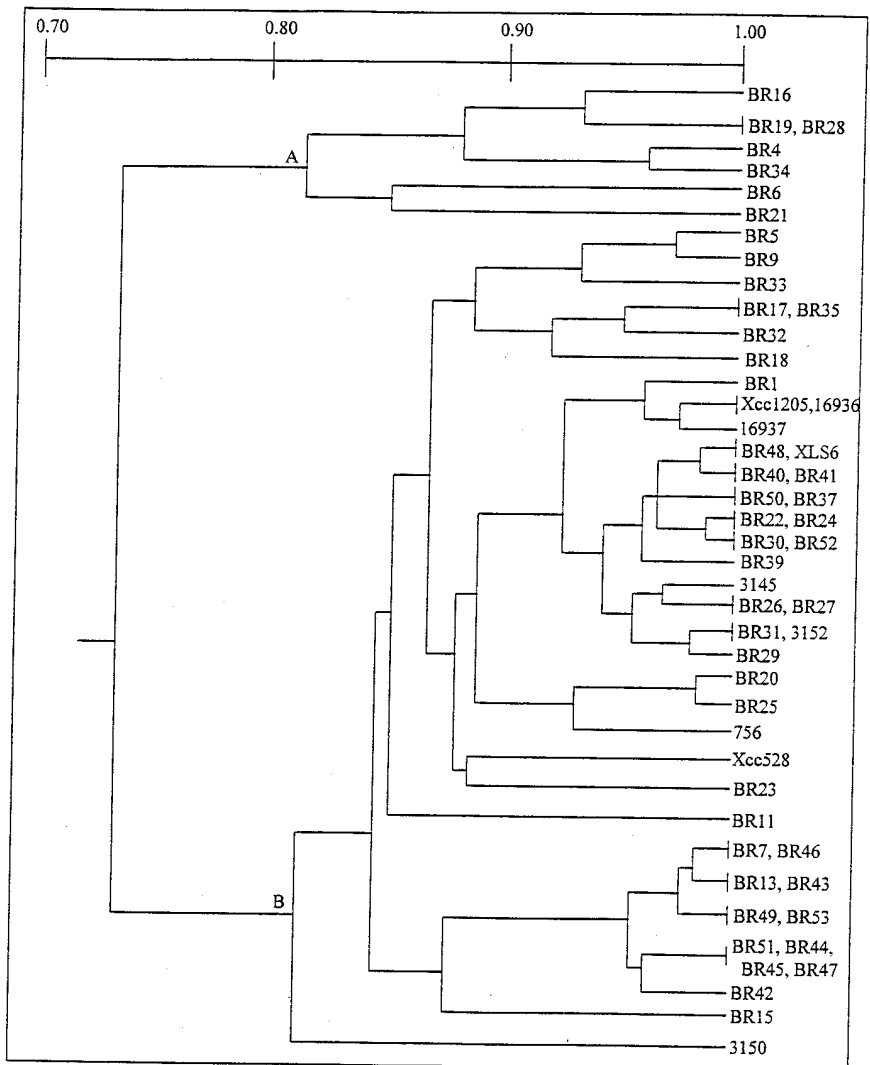
**Genetic fingerprinting.** In the fingerprints generated for strains of *X. campestris*, more than 20 DNA fragments, ranging in size from approximately 0.3 to 4.0 kb, were amplified with the BOXA1R primer. Five distinct groups of strains identified by these fingerprint patterns were arbitrarily designated as A–E. Most strains (35 of 58) were assigned to group A (Table 2), which included weakly to highly virulent local strains of *X. campestris* pv. *campestris* (Fig. 2, lanes 4 to 6), local strains of nonpathogenic *X. campestris* (lane 7), local strains of *X. campestris* pv. *armoraciae* (lane 8), and reference strains of both pathovars (lanes 1 to 3, 9). All 16 local strains of *X. campestris* pv. *campestris* isolated from the cabbage field belonged to this group (Table 2). Three nonpathogenic strains of *X. campestris* had fingerprints identical to those of highly virulent strains of *X. campestris* pv. *campestris* in group A.

Group B (Fig. 2, lanes 10 to 11) included ten local and reference strains of *X. campestris* pv. *campestris* that were moderately to highly virulent (Table 2). Group C (Fig. 2, lanes 12 to 14) consisted of five local or reference strains of *X. campestris* pv. *armoraciae* and four nonpathogenic strains of *X. campestris* (Table 2). Group D included only one reference strain of *X. campestris* pv. *armoraciae* (Fig. 2, lane 15). Three local strains of nonpathogenic *X. campestris* and a highly virulent reference strain of *X. campestris* pv. *campestris* formed group E (Fig. 2, lanes 16 to 18). Group E was variable, but differed from other groups by the presence of a 0.3-kb band (Fig. 2, shown with an arrow).

## DISCUSSION

*X. campestris* pv. *armoraciae* and pv. *campestris* were identified as causal agents of leaf spot diseases of leafy crucifers in Oklahoma. Pathogenic strains of *X. campestris* were recovered from eight of the ten fields sampled. *X. campestris* pv. *ar-*

*moraciae*, the cause of *Xanthomonas* leaf spot, was recovered from four fields. *X. campestris* pv. *armoraciae* was an aggressive pathogen that attacked leaves, stems, and petioles of collards, kale, mustard, spinach mustard, and turnip. However, it caused only a few small leaf spots on horseradish, the crop from which the pathovar was named and first described. This is the first report of *Xanthomonas* leaf spot of crucifers in Oklahoma. *X. campestris* pv. *campestris*, the cause of black rot, was recovered from leaf spots in eight fields. Although black rot was previously reported in Oklahoma on cabbage and broccoli (20), the pathogen was not associated with leaf spot symptoms. In this study, *X. campestris* pv. *campestris* caused leaf spot, but not stem and petiole lesions, on collards and kale. However, local and reference strains of *X. campestris* pv. *campestris* were not shown to cause leaf spot on spinach mustard or turnip, despite the recovery of several strains from leaf spots on these crops.



**Fig. 1.** Dendrogram showing the relationships among strains of *Xanthomonas campestris* for carbon source oxidation as determined with GN MicroPlates (Biolog Inc., Hayward, CA). (A) and (B) represent groups with >80% similarity.

*X. campestris* pv. *campestris* is typically a vascular pathogen that moves systemically in the plant and normally invades the host through wounds and hydathodes (26,28). Infections caused by *X. campestris* pv. *armoraciae* usually originate at stomatal openings (28). Since *X. campestris* pv. *campestris* has a wide host range and host susceptibility may differ (4,28), infection directly through stomata could occur in highly susceptible crops including collard, cabbage, and kale (28). Moffet et al. (17) reported a similar bacterial leaf spot disease caused by *X. campestris* pv. *campestris* on cabbage, cauliflower, and broccoli in Australia. It also was reported that virulent black rot strains caused pinpoint sized, water-soaking symptoms on seedlings prior to the development of black rot (1). Conversely, no variation in symptom expression has been reported for strains of *X. campestris* pv. *armoraciae* (1). None of the strains of *X. campestris* pv. *campestris* from the cabbage field in western Oklahoma, including the eight that were iso-

lated from leaf spots, caused leaf spot on any crop. This suggests that environmental conditions may play a role in non-vascular infection and/or expression of leaf spot symptoms by *X. campestris* pv. *campestris*. Abrasion from wind-blown sand, a common occurrence in Oklahoma, may also be involved in non-systemic infection.

The two pathovars of *X. campestris* were both isolated from leaf spots in four fields. Along with bacterial leaf spot caused by *P. syringae* pv. *maculicola*, which also was recovered from leaf spots in diseased fields (38,39), the pathovars of *X. campestris* formed a complex of leaf spot diseases. Gitaitis et al. (9) reported that mixed infections of *X. campestris* pv. *vesicatoria*, *P. syringae* pv. *syringae*, *P. syringae* pv. *tomato*, and unknown pectolytic xanthomonads occurred on both tomato and pepper transplants. Furthermore, Shen and Chen (27) isolated *X. campestris* pv. *campestris* together with pathogenic *Pseudomonas* spp. from diseased cabbage and cauliflower leaves in China.

The genus *Xanthomonas* was recently reclassified into 20 new species based on genomic relationships revealed by DNA-DNA homology (32,33). The type species, *X. campestris*, was amended to include only seven pathovars from crucifers including pvs. *campestris*, *armoraciae*, and *raphani* (32). Pathovars within the species could not be differentiated by fatty acid profiling, protein profiling, DNA-DNA hybridization, serology, or restriction fragment length polymorphisms (RFLP [1,15,32,33]).

In this study, profiles for carbon source oxidation with the Biolog GN MicroPlates were not definitive for identifying the two pathovars of *X. campestris*. The Biolog GN database correctly classified 15 strains as *X. campestris* pv. *campestris*, but based on pathogenicity, incorrectly identified two strains of *X. campestris* pv. *armoraciae* (BR32 and XLS6) as *X. campestris* pv. *campestris*. Other strains were also assigned to incorrect pathovars. Likewise, grouping the strains for similarity in car-

**Table 2.** Pathogenicity<sup>u</sup>, host range, and genetic fingerprint groups using repetitive sequence-based polymerase chain reaction fingerprinting (rep-PCR) for strains of *Xanthomonas campestris* from crucifers

Strain	Cotyledon assay <sup>v</sup>		Spray inoculation <sup>w</sup>								rep-PCR group <sup>x</sup>
	Symptom	Severity	Collard	Kale	Mustard	Spinach mustard	Turnip	Radish	Horse-radish	Tomato	
<i>pv. campestris</i>											
BR4	BR	HV	BR, LS	BR	–	BR	BR	–	–	–	A
BR7, BR24, BR29	BR	HV	BR, LS	BR, LS	BR	BR	BR	BR	–	–	B
BR21, BR22	BR	HV	BR, LS	BR, LS	–	BR	BR	BR	–	–	A
BR28	BR	HV	BR	BR	–	BR	BR	BR	–	–	B
BR33	BR	HV	BR	BR	–	BR	BR	BR	–	–	A
BR34	BR	HV	BR, LS	BR	–	BR	BR	BR	–	–	A
BR39 - BR53	BR	HV	BR, LS	BR, LS	–	BR	BR	BR	...	–	A
Xcc1205	BR	HV	BR	BR, LS	BR	BR	BR	BR	...	–	A
JT1, PHW-117	BR	HV	...	...	...	...	...	...	...	...	A
JT4	BR	HV	...	...	...	...	...	...	...	...	E
Xcc528	BR	HV	BR	BR, LS	BR	BR	BR	BR	...	–	B
BR6	BR	V	BR, LS	BR, LS	–	BR	BR	BR	–	–	A
BR17	BR	V	BR, LS	BR, LS	–	BR	BR	–	–	–	A
BR30, BR31	BR	V	BR, LS	BR, LS	BR	BR	BR	–	–	–	B
BR37	BR	V	BR, LS	BR, LS	–	BR	BR	BR	...	–	A
3150	BR	V	BR	BR, LS	BR	BR	BR	BR	...	–	B
BR18	BR	MV	–	BR	BR	BR	BR	BR	–	–	B
BR19	BR	MV	–	BR	BR	BR	BR	–	–	–	B
BR5, BR9	BR	WV	BR, LS	BR, LS	–	BR	BR	BR	–	–	A
3152 <sup>y</sup>	BR	WV	BR	BR	BR	BR	BR	BR	...	–	A
BR1	–	AV	–	–	–	–	BR	BR	–	–	A
BR13, BR35	–	AV	–	–	–	–	–	–	–	–	A
BR15, BR23	–	AV	–	–	–	–	–	–	–	–	E
BR20, BR26, BR27, 16937	–	AV	–	–	–	–	–	–	–	–	C
<i>pv. armoraciae</i>											
BR11	SL	...	LS	LS	LS	LS	LS	LS	LS	LS	A
BR16, XLS6	SL	...	LS	LS	LS	LS	LS	LS	LS	LS	C
BR25, BR32	SL	...	LS	LS	LS	LS	LS	–	LS	LS	A
756, 3145, 16936 <sup>z</sup>	SL	...	LS	LS	LS	LS	LS	LS	...	LS	C
69-2	SL	...	...	...	...	...	...	...	...	...	D

<sup>u</sup> For each assay, three plants were inoculated per strain and assays were repeated; (...) = not determined.

<sup>v</sup> Excised cotyledon assay on cabbage seedlings (see text) where SL = stem lesions 4 to 7 days after inoculation, BR = symptoms of black rot 14 days after inoculation. Severity of black rot was assessed using a disease index (DI) of 0 to 9 where 0 = no symptoms and 9 = dead plant. Strains were classified as avirulent (AV) for DI = 0 to 1, weakly virulent (WV) for DI = 1.1 to 3, moderately virulent (MV) for DI = 3.1 to 6, virulent (V) for DI = 6.1 to 8, and highly virulent (HV) for DI = 8.1 to 9.

<sup>w</sup> Symptoms in spray inoculations (see text) were classified as: (–) = no symptoms; black rot (BR) = marginal, V-shaped lesions with darkened veins; and leaf spot (LS) = interveinal lesions bordered by chlorotic halos.

<sup>x</sup> Groupings by banding pattern of rep-PCR products using the BOXA1R primer (see Fig. 2).

<sup>y</sup> Strain was received as *pv. armoraciae*, however, it is classified here as *pv. campestris* based on the cotyledon assay.

<sup>z</sup> Strain was received as *X. campestris* "A", however, it is classified here as *pv. armoraciae* based on the cotyledon assay.

bon source utilization produced two groups that contained strains of each pathovar. However, Biolog typing was useful for the identification of strains to the species level. Our results were similar to those of Jones et al. (10) who examined over 600 strains of *X. campestris* and found that Biolog correctly identified 97% to species, but only 20% to pathovar.

Although rep-PCR can be a rapid and reliable method to differentiate plant pathogenic bacteria (12,13), this method did not differentiate strains of *X. campestris* pv. *campestris* and pv. *armoraciae* in the present study. Fingerprints for strains of the two pathovars were diverse, and both pathovars were present within two of the five fingerprint groups (A and C). Similar results were obtained for strains of *X. campestris* pv. *campestris* from another study that used the ERIC primer (14). Louws et al. identified at least seven dis-

tinct groups for strains from cabbage collected from North and South Carolina (14). Alvarez used RFLP analysis to compare strains of *X. campestris* pv. *campestris* and pv. *armoraciae* and found that both pathovars were genetically heterogeneous (1).

In this study, there was no consistent relationship between rep-PCR group, Biolog group, original host, virulence, or host range. These results suggest that *X. campestris* may undergo considerable genetic reassortment. Several studies have shown that genetic variation was greater among strains of pathogens with wide host ranges, such as *X. campestris* pv. *campestris*, *X. campestris* pv. *armoraciae*, and *P. syringae* pv. *syringae*, than among pathogens with a more restricted host range, such as *P. syringae* pv. *tomato* (1,11,12). Our results showed that *X. campestris* pv. *armoraciae* had a larger host range than previously

reported (4,23,30,34), including tomato, radish, and horseradish.

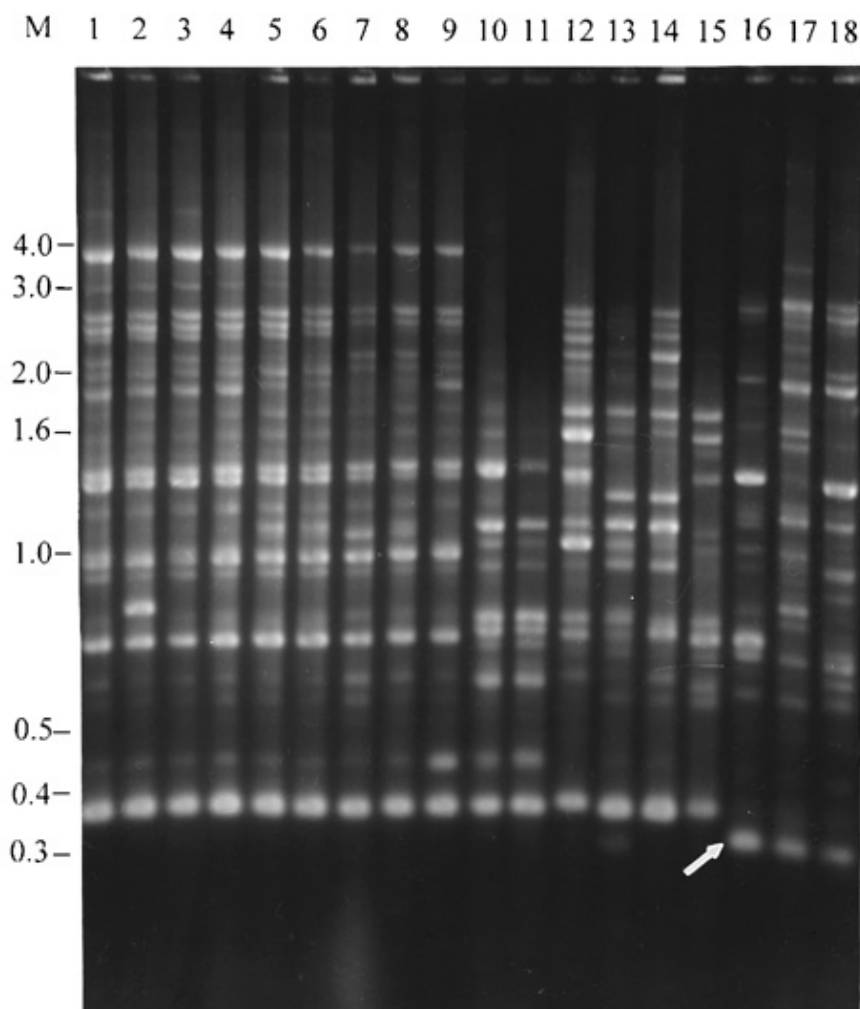
More research is needed to determine the relative importance of, and sources of inoculum for, the two pathovars of *X. campestris* in the bacterial disease complex in Oklahoma. In spray inoculations, *X. campestris* pv. *armoraciae* was a more aggressive leaf spot pathogen than *X. campestris* pv. *campestris*, and caused leaf spot on a wider range of leafy crucifers. While genetic and phenotypic diversity of strains across fields was high, strains within fields were often similar. For example, strains of *X. campestris* pv. *campestris* from the cabbage field and from three other fields in eastern Oklahoma had very similar genetic fingerprints and pathogenicity profiles. It is possible that these originated from a single source of inoculum.

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**Fig. 2.** Repetitive sequence-based polymerase chain reaction (rep-PCR) fingerprint patterns obtained from representative strains of *Xanthomonas campestris* pv. *campestris*, *X. campestris* pv. *armoraciae*, and nonpathogenic *X. campestris* with the BOXA1R primer. A portion of the PCR product (8  $\mu$ l) was loaded to a 1.5% agarose gel, and electrophoresis was performed at 70 V for 5 h in 1X TBE buffer. Group A—lane 1, Xcc1205; 2, JT-1; 3, PHW117; 4, BR42; 5, BR21; 6, BR9; 7, BR13; 8, BR32; and 9, 3152. Group B—lane 10, Xcc528; 11, 3150. Group C—lane 12, 16936; 13, 756; and 14, BR16. Group D—lane 15, 69-2; and Group E—lane 16, JT-4; 17, BR15; and 18, BR23. M, DNA molecular size marker (1 kb ladder; Gibco BRL, Gaithersburg, MD); sizes are indicated in kilobase pairs. Arrow indicates common band among strains in Group E.

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