Detection, Survival, and Sources of Inoculum for Bacterial Diseases of Leafy Crucifers in Oklahoma

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ABSTRACT

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Xanthomonas campestris pv. *campestris*, *X. campestris* pv. *armoraciae*, and *Pseudomonas syringae* pv. *maculicola* are bacterial pathogens that cause leaf spot diseases on leafy crucifers in Oklahoma. Polymerase chain reaction (PCR) amplification of the *cfl* gene from the gene cluster encoding the phytotoxin coronatine was used to identify coronatine-producing strains of *P. syringae*, and the expected 0.65-kb PCR product was detected in 19 strains of *P. syringae* pv. *maculicola* originating from diseased crucifers in Oklahoma. A simple, rapid PCR method based on primers from the *cfl* gene was developed to detect coronatine-producing strains of *P. syringae* in planta. Pathogenicity tests confirmed the *cfl*-positive strains to be *P. syringae* pv. *maculicola*. To monitor the survival of *X. campestris* pv. *armoraciae* and *P. syringae* pv. *maculicola* in the field, turnip and collards were inoculated with rifampicin-resistant strains and were buried beneath the soil or left on the soil surface. Both pathogens were recovered from turnip and collard debris up to 2 months following burial, but neither pathogen was recovered from soil after the debris had decomposed. However, both pathogens were recovered from plant debris left on the soil surface for up to 5 months. Four production fields were surveyed for sources of inoculum of the bacterial pathogens from October 1999 to May 2000. *X. campestris* pv. *campestris* was isolated from the weed shepherd's purse (*Capsella bursa-pastoris*) in all fields, and from volunteer turnip and kale in three fields. *X. campestris* pv. *campestris* and *P. syringae* pv. *maculicola* were isolated from surface debris and regrowth from crop stubble left in one field after harvest in the fall. *X. campestris* pv. *campestris* was detected in 6 of 51 lots of crucifer seed assayed. *X. campestris* pv. *armoraciae* and *P. syringae* pv. *maculicola* were not recovered from weeds, volunteer plants, or seed lots.

Since 1994, collards (*Brassica oleracea* var. *viridis* L.), kale (*B. oleracea* var. *sabellica* L.), spinach mustard (*B. rapa* L. var. *perviridis* Bailey), and turnip (*B. rapa* var. *utilis* (D.C.) Metz.) have been damaged periodically by bacterial leaf spots in eastern Oklahoma (33,34). Severely damaged fields have been rejected for harvest by processors, causing total economic loss. *Xanthomonas campestris* pv. *campestris*, *X. campestris* pv. *armoraciae*, and *Pseudomonas syringae* pv. *maculicola* were identified as causal agents of leaf spots on these crops (33,34). These bacteria often occurred together within an affected field. Genetic and phenotypic diversities were high for strains of *X. campestris* originating from different fields, but were often similar within fields. Strains of *P. syringae* pv. *maculicola* from most fields were relatively homogeneous and within the same genetic group.

Control of bacterial leaf spots on leafy crucifers in Oklahoma has proven to be

difficult. The use of bactericidal sprays containing copper compounds has not been effective. Therefore, other management strategies are necessary to reduce losses. The identification of inoculum sources for the diseases should result in cultural practices designed to prevent disease outbreaks through a reduction and/or elimination of primary inoculum.

X. campestris pv. *campestris*, the cause of black rot, also causes leaf spot on leafy crucifers (33). Plant debris (16,25), seed (18,26), and weeds (24,30) are reported most often to be important sources of inoculum for black rot. However, the survival of *X. campestris* pv. *campestris* on plant debris depends on field conditions and temperature (2,16,25).

X. campestris pv. *armoraciae*, the cause of Xanthomonas leaf spot (5), and *P. syringae* pv. *maculicola*, the cause of bacterial leaf spot (6,28), are important bacterial pathogens of leafy crucifers in Oklahoma (33,34). Although these two pathogens may be seedborne (18,27), there is little information available on their survival in plant debris, and it is unclear whether weeds also serve as inoculum sources for these pathogens.

This study was undertaken to identify important sources of inoculum for bacterial leaf spot disease of leafy crucifers in Oklahoma. Specific objectives were to deter-

mine the survival of *X. campestris* pv. *armoraciae* and *P. syringae* pv. *maculicola* in plant debris, and to assess the potential for plant debris, weeds, seed, or volunteer plants to serve as inoculum sources for these pathogens as well as *X. campestris* pv. *campestris*. Because selective media are not available for *P. syringae*, a rapid polymerase chain reaction (PCR) method was developed to detect coronatine-producing strains in order to putatively differentiate *P. syringae* pv. *maculicola* from xanthomonads and other fluorescent pseudomonads. Preliminary reports have been published (31,32).

MATERIALS AND METHODS

Bacterial strains and pathogenicity.

The strains of *P. syringae* used in this study (Table 1) were routinely cultured on King's medium B (KB; 15) or mannitol-glutamate medium (MG; 14) at 28°C and stored in 15% glycerol at -70°C. *X. campestris* pv. *armoraciae* strain BR16 and *P. syringae* pv. *maculicola* strain F18 were previously isolated from turnip in Oklahoma (Table 1). Spontaneous rifampicin-resistant mutants of both strains were selected by plating bacterial cells onto Luria-Bertani (LB; 21) or MG medium containing rifampicin at 100 µg/ml.

The pathogenicity of the rifampicin-resistant strains of *X. campestris* pv. *armoraciae* (BR16R) and *P. syringae* pv. *maculicola* (F18R) was confirmed to be similar to the wild-types (BR16 and F18) using spray inoculation as described previously (33,34). Briefly, strains were grown on LB or MG with or without rifampicin (50 µg/ml) for the resistant mutants or wild-types, respectively, at 28°C for 48 h. Cells were then suspended in sterile distilled water (SDW) and adjusted to 10⁷ CFU/ml as determined by dilution plating and OD readings ($A_{600} = 0.05$). Plants were grown in the greenhouse at 20 to 25°C until three to four true leaves formed. Collard (cv. Vates) and turnip (cv. Hybrid All-top) plants were incubated in polyethylene 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 the leaves were uniformly wet. After inoculation, plants were removed from the chambers and maintained in the greenhouse until symptoms were assessed 7 to 14 days after inoculation.

PCR detection of *P. syringae*. Bacterial strains (Table 1) were grown in 5 ml of LB

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broth at 28°C, 280 rpm, for 18 h. Aliquots (1 ml) were centrifuged, and pellets were washed in 0.5 M NaCl (0.5 ml) and resuspended in SDW to 10⁸ CFU/ml. PCR amplification was performed in 25- μ l reaction volumes. Each reaction contained *Taq* reaction buffer, 1.5 mM MgCl₂, 50 pmoles of each primer, 0.05 mM of each dNTP (Boehringer Mannheim, Indianapolis, IN), 0.5 U *Taq* DNA polymerase (Promega, Madison, WI), and 1 μ l of the bacterial suspension. Oligonucleotides with the sequence 5'-GGCGCTCCCTCGCACTT (primer 1) and 5'-GGTATTGGCGGG GGTGC (primer 2) were used to amplify the *cfl* gene, which is encoded within the coronatine (COR) gene cluster (4). Primers were synthesized by the Oklahoma State University Recombinant DNA/Protein Resource Facility. PCR was carried out as follows: 1 cycle at 94°C for 3 min, 30 cycles at 94°C (1 min), 67°C (1 min), and 72°C (1 min), followed by 72°C for 10 min. A 10- μ l aliquot of the reaction was analyzed by electrophoresis on a 1% agarose gel at 70 V for 2 h in Tris acetate buffer (21). Gels were stained with ethidium bromide.

A simple PCR assay for detecting COR-producing pathovars of *P. syringae* in plant tissue was developed. Briefly, a 1 cm² diameter leaf disk with a lesion was excised, surface-sterilized in 0.25% aqueous sodium hypochlorite for 30 s, and washed three times in SDW. The disk was cut into several pieces in 500 μ l SDW, allowed to

soak for 10 min, and pelleted at 325 $\times$ g for 2 min. The supernatant was centrifuged at 16,000 $\times$ g for 10 min; the pellet was washed with 0.5 M NaCl (0.5 ml), resuspended in 20 μ l SDW, and used as template in the PCR reaction.

The PCR assay was verified using plants spray-inoculated with known bacteria and with unknown samples of diseased leaves collected in the field. Turnip, collard, and tomato (cv. Summer Flavor 3000) plants were inoculated as described above with *P. syringae* pv. *maculicola* strains 84-59 and F18, and *P. syringae* pv. *tomato* DC3000. Healthy plants and plants inoculated with *X. campestris* pv. *armoraciae* or *P. viridiflava* were used as negative controls. Disks excised from three leaves for each plant type and inoculation treatment were analyzed for the presence of the *cfl* gene by PCR as described above. The assay was performed three times. Diseased turnip and spinach mustard leaves were sampled from the fields described below over a 2-year period. Disks excised from three to five leaves per field were analyzed by PCR using the *cfl* primer set. Diseased turnip and collard leaves produced by spray inoculation and healthy leaves served as positive and negative controls, respectively. Bacterial isolations were made from adjacent leaf disks on MG amended with cycloheximide. At least one colony resembling *P. syringae* from each *cfl*-positive leaf disk was characterized by PCR using the *cfl* primer set and pathogenicity tests on collards or turnip.

Survival of *X. campestris* pv. *armoraciae* and *P. syringae* pv. *maculicola* in plant debris. Survival studies were conducted at the OSU Plant Pathology Farm in Stillwater in microplots that contained Norge loam soil (pH 6.2). Microplots were 2 $\times$ 2 m, raised 25 cm high with railroad timbers, and were each surrounded by a 3-m-wide alley. Crucifers had not been grown in these plots for at least 5 years, and wheat was the only crop planted in the surrounding area. Granular fertilizer (42-0-0 N-P-K) at 84 kg/ha was incorporated into the soil prior to direct seeding. Two plots each of turnip and collard were seeded on 20 September 1998, 9 October 1998, and 14 September 1999. In 1998, the two late-seeded plots of collard were later discarded because of frost damage prior to inoculation. One plot of each crop was inoculated with *P. syringae* pv. *maculicola* F18R and the other with *X. campestris* pv. *armoraciae* BR16R on 5 and 19 November for the early- and late-planted plots in 1998, respectively, and on 14 October in 1999. The inoculations consisted of spraying all plants in a plot to runoff with bacteria (10⁷ CFU/ml) suspended in SDW. Plants were uprooted and then buried ca. 10 cm below the soil surface on 23 November and 14 December for the early- and late-planted plots in 1998, respectively, and on 30 October in 1999. Additionally, half of the uprooted plants were left on the soil surface for the late-planted plots in 1998 and for the plots planted in 1999. Beginning immediately after burial, six or more plants were unearthed from each plot at 30-day intervals. The surface debris also was sampled at 30-day intervals. When buried and surface debris were decayed and could no longer be recovered, three to five soil samples (2 g each) were collected from the treated area and assayed for rifampicin-resistant *X. campestris* and *P. syringae*.

To detect rifampicin-resistant strains, samples were weighed and macerated in 10 to 20 ml SDW using a mortar and pestle. Serial dilutions were made in SDW, and 25- μ l aliquots were spotted onto the surface of four replicate plates per dilution. Nutrient agar (NA; 19) and MG medium amended with cycloheximide (100 μ g/ml) and rifampicin (50 μ g/ml) were used to isolate *X. campestris* and *P. syringae*, respectively. The plates were dried in a laminar flow hood and incubated at 28°C for 3 to 5 days. Rifampicin-resistant colonies of *X. campestris* and *P. syringae* were counted, and the recovery of bacteria per gram fresh weight was calculated.

Field surveys. In the fall of 1999 and spring of 2000, four fields in the Arkansas River Valley, each with a history of bacterial disease(s), were surveyed for potential sources of inoculum. Fields 1 and 2 were located near Tucker, OK. Field 1 had a history of bacterial leaf spot (*P. syringae* pv. *maculicola*), and field 2 had a history of black rot (*X. campestris* pv. *campestris*).

Table 1. Detection of coronatine-producing strains of *Pseudomonas syringae* by polymerase chain reaction (PCR)

Strain	Host	<i>cfl</i> -PCR ^a	Source (reference) ^b
<i>P. syringae</i> pv. <i>maculicola</i>			
NF2, F6, F18, F19, NF3, NF3a	Turnip	+	OK (34)
NF4, NF5, NF12, F1, F7, F16, F17, F20, F21, F22	Spinach mustard	+	OK (34)
F9, F10a, F15	Kale	+	OK (34)
16391, 16392	Cauliflower	+	R. Stall (34)
17223, 17225	Brassica sp.	+	R. Stall (34)
84-67	Cauliflower	-	D. Cuppels (28,34)
84-59, 88-10, 88-11, 90-32	Cauliflower	+	D. Cuppels (28,34)
4326	Radish	+	C. Bender (28,34)
<i>P. syringae</i> pv. <i>tomato</i>			
DC3000	Tomato	+	D. Cuppels (34)
<i>P. syringae</i> pv. <i>glycinea</i>			
PG4180	Soybean	+	C. Bender
18a/90	Soybean	-	C. Bender
<i>P. viridiflava</i>	Brassica sp.	-	OK (34)
<i>P. putida</i>	Brassica sp.	-	OK (34)
<i>P. fluorescens</i>	Brassica sp.	-	OK (34)
<i>Xanthomonas campestris</i> pv. <i>campestris</i>			
BR40	Cabbage	-	OK (33)
Xcc528	Brussels sprouts	-	ATCC 33913 (1,33)
<i>X. campestris</i> pv. <i>armoraciae</i>			
BR 16	Turnip	-	OK (33)
XLS6	Cabbage	-	L. Black (1,33)

^a PCR analysis with primers from the *cfl* gene (4) was carried out using bacterial cells as template. +, the 650-bp PCR product was detected; -, no PCR product was observed.

^b Strains were from C. Bender, coauthor; D. Cuppels, Agriculture and Agri-Food Canada, London, Ontario, Canada N5V 4T3; R. Stall, Department of Plant Pathology, University of Florida, Gainesville 32611; and L. Black, Department of Plant Pathology and Crop Physiology, Louisiana State University, Baton Rouge 70803. Strains from OK were isolated from diseased leaves sampled from local fields as previously reported (33,34).

Fields 3 and 4 were located in Webbers Falls, OK. Field 3 had a history of bacterial leaf spots caused by *X. campestris* pvs. *campestris* and *armoraciae* and *P. syringae* pv. *maculicola*, while field 4 had a history of black rot. Fields were surveyed twice each in the fall and spring production seasons. The first survey was on 29 October 1999 when the fall crop of leafy crucifers was near harvest and diseases were fully developed, and the second was on 16 December 1999 after harvest. The third survey was on 16 March 2000 during field preparation for the spring production season, and the final survey was on 17 May 2000 when the spring crop of leafy crucifers was near harvest and diseases were fully developed. Fields varied in crop rotations, but each was planted to leafy crucifers at least once during the survey period (Table 2).

Fields and surrounding vegetation were sampled in a w-shaped pattern. Leaves of cruciferous crops, plant stubble remaining after harvest, crop debris, and volunteer cruciferous plants with symptoms resembling bacterial disease were collected, placed in plastic bags, chilled, and transported to the laboratory. Broadleaf weeds were collected regardless of symptom manifestation. Bacteria were isolated from suspected lesions as previously described (33,34). Asymptomatic weeds were assayed for bacterial pathogens by shaking leaf sections in SDW (10 ml) for 20 min at 4°C. Serial dilutions of the suspension were made in SDW, and 0.1-ml aliquots were plated on Fieldhouse-Sasser (FS) medium (9) and MG amended with cycloheximide (100 µg/ml). Suspected xanthomonads were selected, and pathogenicity was determined using the cabbage cotyledon assay which differentiates the pathovars *campestris* and *armoraciae* (1).

MG and KB amended with cycloheximide were used to isolate *P. syringae*, and coronatine-producing strains of *P. syringae* were differentiated from other fluorescent pseudomonads by PCR.

Seed assays. Preliminary assays were performed to assess recovery of *X. campestris* pv. *armoraciae* and *P. syringae* pv. *maculicola* from artificially inoculated seed. Noninfested seed was soaked in a bacterial suspension (10^8 CFU/ml) of BR16R or F18R for 5 min and air-dried. Inoculated seed was added to noninfested seed at ratios of 0, 1, 10, and 100 inoculated seeds per 20 g of clean seed. Bacteria were isolated from seed as described by Schaad (22). Briefly, seeds (20 g) were shaken in 50 ml of 0.1 M phosphate-buffered saline (PBS; pH 7.2) on a rotary shaker for 1 h at 4°C. The supernatant was filtered through two layers of cheesecloth and centrifuged at $17,600 \times g$ for 30 min. The pellet was resuspended in 2 ml SDW, diluted in SDW, and plated in 0.1-ml aliquots to FS medium and nutrient starch cycloheximide antibiotic agar (NSCAA) to detect xanthomonads (20). FS and NSCAA plates were incubated for 3 to 5 days, and colonies with a clear zone of starch hydrolysis were selected. The cabbage cotyledon assay was performed to differentiate the two pathovars of *X. campestris*. MG and KB amended with cycloheximide were used to isolate *P. syringae*. After a 48-h incubation period, Bio-PCR (23) was performed on selected plates using the *cfl* primer set. Four days after incubation, suspected colonies were used in a standard PCR reaction using the *cfl* primer set.

The seed assays were performed on 51 commercial seed lots collected from five different companies during 1995 to 1999. The seed lots consisted of nine lots of collard, eight lots of kale, seven lots of mus-

tard, twelve lots of turnip, five lots of spinach mustard, four lots of Chinese cabbage, five lots of broccoli, and one lot of cabbage.

RESULTS

PCR detection of *P. syringae*. With the exception of strains 84-67 and 18a/90, which served as negative controls, PCR analysis of strains of *P. syringae* pvs. *maculicola*, *glycinea*, and *tomato* resulted in the expected 0.65-kb PCR product (Table 1, Fig. 1A). Analysis of four strains of *P. syringae* pv. *maculicola* (NF3, NF4, 16391, and 16392) that did not produce detectable COR in vitro (34) resulted in the 0.65-kb PCR product (Table 1; Fig. 1A, lanes 5 and 6). No PCR products were detected for the COR-nonproducing bacteria that included *P. viridiflava* (4 strains), *P. fluorescens* (4 strains), *P. putida* (3 strains), and *X. campestris* pvs. *campestris* (2 strains) and *armoraciae* (2 strains) (Table 1; Fig. 1A, lane 10).

PCR amplification with the *cfl* primers was also conducted with healthy and diseased leaf extracts. The expected 0.65-kb PCR product was detected from leaf extracts of tomato, turnip, and collard inoculated with all of the COR-producing strains of *P. syringae* (Fig. 1B; lanes 2, 4, and 7). No PCR product was observed for healthy turnip, tomato, and collard; or for leaves inoculated with *X. campestris* pv. *campestris*, *X. campestris* pv. *armoraciae*, and *P. viridiflava* (Fig. 1B, lanes 1, 5, and 6). The expected 0.65-kb PCR product was detected from leaf extracts of turnip and spinach mustard showing leaf spot symptoms in the field (Fig. 1B, lane 3). When bacteria were isolated from leaves that were *cfl*-positive, the 0.65-kb product was obtained from individual colonies when further analyzed by PCR, and they were

Table 2. Recovery of bacterial leaf spot pathogens of leafy crucifers during field surveys

Field	Crop		Pathogen ^a	Site of recovery ^b			
	Fall 99	Spring 00		29 Oct 99	16 Dec 99	16 Mar 00	17 May 00
1	Spinach mustard	Spinach mustard	PSM	Crop	NR	NR	Crop
			XCA	NR	NR	NR	Crop
			XCC	Crop	Volunteers	Volunteers	Crop
2	Spinach mustard	Snap bean	PSM	NR	NR	NR	Weeds
			XCA	NR	NR	NR	Weeds
			XCC	Crop	Volunteers	Volunteers	NR
3	Collards, turnip	Corn	PSM	Weeds	Weeds	Weeds	NR
			XCA	Crop	Debris	Debris	NR
			XCC	NR	Regrowth	Regrowth	NR
4	Spinach mustard	Spinach mustard	PSM	Crop	NR	NR	Weeds
			XCA	Weeds	Debris	Debris	NR
			XCC	NR	Weeds	Weeds	Crop
					Regrowth	Regrowth	Weeds
					Volunteers	Volunteers	

^a PSM = *Pseudomonas syringae* pv. *maculicola*; XCA = *Xanthomonas campestris* pv. *armoraciae*; XCC = *X. campestris* pv. *campestris*.

^b Volunteers were turnip and kale in fields 1 and 2, and turnip in field 3. Among the weeds sampled in each field, shepherd's purse (*Capsella bursa-pastoris*) was the only weed that harbored pathogens. Debris = surface crop debris; regrowth = new growth from crop stubble; NR = no recovery.

pathogenic to crucifers and tomato (data not shown). The estimated concentration of total bacteria at the time of detection was approximately 10^7 CFU/g fresh weight as determined by dilution plating.

Pathogen survival. Mean monthly temperatures and total rainfall recorded within 0.5 km from the test site were 11.3°C and 14 cm for November 1998, 4.3°C and 3.8 cm for December 1998, 3.1°C and 4.1 cm for January 1999, 9.4°C and 1.8 cm for February 1999, 8.8°C and 10.4 cm for March 1999, and 16.0°C and 17.5 cm for April 1999. Rifampicin-resistant *X. campestris* pv. *armoraciae* and *P. syringae* pv. *maculicola* were recovered from plant debris for 2 months after burial during 1998 and 1999 (Fig. 2). Each pathogen survived on both early- and late-planted turnip, and on early-planted collards. The plant debris remained intact for the first 2 months, but was decomposed within 3 months and could not be recovered. Rifampicin-resistant *X. campestris* pv. *armoraciae* and *P. syringae* pv. *maculicola* were not isolated from the soil after the debris had decayed. However, both pathogens were recovered from turnip debris left on the soil surface for up to 4 months (Fig. 2). No debris remained on the soil surface after 5 months, and the pathogens were not recovered from surrounding soil. While populations of *P. syringae* pv. *maculicola* were often higher than for *X. campestris* pv. *armoraciae*, population trends and the duration of survival were similar for both pathogens over the sampling periods.

Mean monthly temperatures and rainfall were 12.9°C and 0 cm for November 1999, 5.6°C and 15.1 cm for December 1999, 3.7°C and 2.0 cm for January 2000, 8.5°C and 3.0 cm for February 2000, 11.3°C and

11.2 cm for March 2000, and 15.0°C and 9.1 cm for April 2000. Plant debris remained intact for less than a month when buried in 1999. Rifampicin-resistant *X. campestris* pv. *armoraciae* and *P. syringae* pv. *maculicola* were not recovered from soil after the debris had decayed. However, debris on the soil surface remained intact, and rifampicin-resistant *X. campestris* pv. *armoraciae* and *P. syringae* pv. *maculicola* were recovered from both collard and turnip for 4 and 5 months, respectively (Fig. 3). Pathogen survival was again similar for the two hosts and pathogens during the sampling periods.

Field surveys. *X. campestris* pv. *campestris* was recovered from shepherd's purse (*Capsella bursa-pastoris*) in two fields in fall 1999 and from all four fields in spring 2000 (Table 2). Shepherd's purse was the primary cruciferous weed observed in the fields. Symptoms on this weed included marginal, V-shaped, chlorotic, and necrotic lesions on the leaves. The cotyledon assay confirmed that bacterium isolated from shepherd's purse was *X. campestris* pv. *campestris*. No pathogenic bacteria were recovered from other broad-leaf weeds, including *Rumex* sp. and Virginia pepperweed (*Lepidium virginicum*). Although fluorescent pseudomonads were isolated from most weeds, none were *cfl*-positive when analyzed by PCR.

In December 1999 and March 2000, volunteer kale and turnip were common in and around the surveyed fields. *X. campestris* pv. *campestris* was recovered from volunteer plants in three of the four fields (Table 2). In October 1999 and May 2000, bacterial leaf spot diseases were observed in all of the fields where crucifers were cropped (Table 2). All three pathogens

were recovered from field 1 in the spring of 2000. In field 3, crop stubble was left undisturbed after harvest in the fall, and regrowth from the stubble occurred during the winter months. *X. campestris* pv. *campestris* and *P. syringae* pv. *maculicola* were isolated from the regrowth and surface debris of this field in December 1999 and March 2000 (Table 2).

Seed assays. In the assay of artificially inoculated seed, both *X. campestris* pv. *armoraciae* and *P. syringae* pv. *maculicola* were recovered when only one inoculated seed was added to 20 g of pathogen-free seeds. When two seeds, one inoculated with *X. campestris* pv. *armoraciae* and a second with *P. syringae* pv. *maculicola*, were added to 20 g of healthy seeds, both pathogens were recovered.

X. campestris pv. *campestris* was recovered from 6 of the 51 commercial seed lots surveyed. Infested seed lots included turnip (two seed lots), Chinese cabbage (two seed lots), spinach mustard (one seed lot), and

Fig. 1. Polymerase chain reaction (PCR) products amplified using the *cfl* primer set (4). **A**, PCR products from bacterial cells. Lanes: 1, PG4180; 2, 18a/90; 3, F18; 4, 84-67; 5, NF4; 6, 16392; 7, 84-59; 8, F20; 9, 4326; 10, *Pseudomonas viridiflava*; M, molecular size marker (1-kb ladder). **B**, PCR products from leaf extracts. Lanes: 1, healthy turnip leaf; 2, tomato leaf inoculated with DC3000; 3, diseased turnip leaf from a field with bacterial leaf spot caused by *Pseudomonas syringae* pv. *maculicola*; 4, collard leaf inoculated with *P. syringae* pv. *maculicola* 84-59; 5, turnip leaf inoculated with *P. viridiflava*; 6, turnip leaf inoculated with *Xanthomonas campestris* pv. *campestris*; 7, turnip leaf inoculated with *P. syringae* pv. *maculicola* F18.

Fig. 2. Survival of rifampicin-resistant bacteria on plant debris during 1998 and 1999. PSM: *Pseudomonas syringae* pv. *maculicola*; XCA: *Xanthomonas campestris* pv. *armoraciae*. **A**, Pathogen recovery from early-planted turnip and collard plants buried 10 cm deep under soil. *X. campestris* pv. *armoraciae* on turnip (●) and collards (▼); *P. syringae* pv. *maculicola* on turnip (○) and collards (▽). Recovery of *X. campestris* pv. *armoraciae* (●) and *P. syringae* pv. *maculicola* (○) from late-planted turnip plants. **B**, buried 10 cm deep under soil, and **C**, left on soil surface. Vertical bars represent one standard error of the mean.

mustard (one seed lot). The cabbage cotyledon assay confirmed that the pathogen was *X. campestris* pv. *campestris*, and isolates varied in virulence (1) ranging from nonpathogenic for isolates from one seed lot of each mustard and Chinese cabbage to highly virulent for all other isolates. With the exception of mustard, the contamination level of seed lots was relatively low. The population of *X. campestris* in contaminated seed lots ranged from 1.5 to 370 CFU/g seed.

Fluorescent pseudomonads were recovered from 21 of the 51 seed lots. However, both conventional PCR and Bio-PCR indicated that the pseudomonads were not COR-producing strains of *P. syringae* pv. *maculicola*. The estimated population of fluorescent pseudomonads in the infested seed lots ranged from 4.64×10^4 to 1.31×10^6 CFU/g seed. Seed lots with the most recent harvest dates had the highest populations of fluorescent pseudomonads. *X. campestris* pv. *armoraciae* was not isolated from any of the seed lots.

DISCUSSION

P. syringae pv. *maculicola* produces COR, a chlorosis-inducing phytotoxin that is also produced by *P. syringae* pathovars *atropurpurea*, *glycinea*, *morsprunorum*, and *tomato* (3). PCR primers and nucleotide probes have been used to identify phytotoxin-producing pathovars of *P. syringae* (3), including pathovars that synthesize COR (3,7,8). In the present study, a simple method for preparing leaf extracts without isolating bacteria was developed that utilized small leaf sections (ca. 1 cm²) to de-

tect COR-producing *P. syringae* by PCR. This method can be used for the rapid detection of COR-producing pathovars of *P. syringae* since the results can be available within 24 h. However, since other COR-producing pathovars may exist as epiphytes on diseased crucifers and survive the surface sterilization, pathogenicity tests are still necessary to unequivocally establish the presence of *P. syringae* pv. *maculicola*.

X. campestris pv. *armoraciae* and *P. syringae* pv. *maculicola* were less likely to survive in buried plant debris and more likely to persist in debris left on the soil surface. These results are consistent with previous studies; for example, survival of *P. syringae* pvs. *phaseolicola* and *pisi* was longer when plant debris remained on the soil surface (11,29). *X. campestris* pv. *glycines* survived in plant debris located on the soil surface or buried at a depth of 10 cm; however, fewer bacteria were recovered from buried samples (10). In our study, survival of bacteria in plant debris was associated with the longevity of the debris, which apparently relates to its treatment after harvest and environmental conditions. Mean monthly temperatures were above normal from November to February each year, which apparently favored rapid decomposition of buried plant debris in soil. The pathogens did not survive in buried debris in either trial until March or April when these crops are planted for spring production. In the 1999-2000 trial, plants were buried earlier and temperatures were warmer than in the 1998-1999 trial. In the 1998-1999 study, buried debris remained intact for 2 months; however, buried debris decomposed in less than 1 month during the 1999-2000 study. Other studies have demonstrated that higher temperatures stimulate the rapid decomposition of plant debris; consequently, bacteria are less insulated and populations decline more quickly (13,16,17). However, bacterial survival in surface debris was less affected by environmental conditions as both pathogens persisted until spring planting time in each of the two trials reported here.

In Oklahoma, leafy crucifers are grown during spring and fall cropping seasons. Planting begins in March and September, with harvest following in May and November for the spring and fall production seasons, respectively. In general, growers moldboard plow fields at most every other year in the spring. Following harvest, fields are disk-harrowed once or twice as weather and soil conditions permit. Field preparation before planting involves one or two passes with a disk harrow. Although sampling was not performed in the summer, it is unlikely that infected plant debris would persist in the soil over the summer because high soil temperatures and irrigation during this period in Oklahoma would promote rapid residue decay. However,

because the crop is harvested in bulk with nonselective cutters, crop residue and stubble remain on the soil after harvest. In field 3 where the stubble was not managed after harvest, regrowth of the stubble occurred during the winter of 1999-2000, when weather conditions favored the regrowth of plants and survival of the pathogens. Jardine et al. (12) reported that in fall-plowed treatments, tomato debris apparently decomposed before planting. For spring-plowed treatments, a large amount of intact tomato debris was present below or on the soil surface at planting time (12). This may explain why bacterial leaf spot diseases of leafy crucifers in Oklahoma have been observed to be more severe in the spring production season than in the fall (34). In Oklahoma, volunteer kale and turnip plants may be colonized by *X. campestris* pv. *campestris*, and plant debris on the soil surface and regrowth from stubble has a high potential to serve as inoculum for the next crop. However, infected crop residue incorporated into the soil may not be an important inoculum source.

Several cruciferous weeds have been reported to be hosts of *X. campestris*, including shepherd's purse, mouse-ear cress (*Arabidopsis thaliana*), and wild radish (*Raphanus raphanistrum*) (24,30). In Oklahoma, shepherd's purse occurred frequently in and around fields of leafy crucifers. Our results indicated that shepherd's purse is an alternative host for *X. campestris* pv. *campestris* in Oklahoma and may play an important role in the secondary disease cycle of black rot and leaf spot of crucifers caused by *X. campestris* pv. *campestris*, especially as a source for dispersal of the pathogen in the field. However, *X. campestris* pv. *armoraciae* and *P. syringae* pv. *maculicola* were not recovered from any of the weeds collected, suggesting that weed species may not be important in the epidemiology of these two pathogens.

The importance of seedborne inoculum of *X. campestris* pv. *campestris* has been well documented (18,26). A very low level of seed infestation by *X. campestris* pv. *campestris* can result in a high incidence of black rot in the field (20). Our results showed that 6 out of 51 seed lots were contaminated with *X. campestris* pv. *campestris*, indicating that contaminated seed is a significant inoculum source in the epidemiology of black rot. Although *X. campestris* pv. *armoraciae* and *P. syringae* pv. *maculicola* are known to be seedborne (18,27), we failed to recover these pathogens from seed. Since the detection procedure described in this study did not utilize large quantities of seed, it remains possible that *X. campestris* pv. *armoraciae* and *P. syringae* pv. *maculicola* may be seed-transmitted at a low level, and contaminated seed may play a role in the epidemiology of bacterial and *Xanthomonas* leaf spots.

Fig. 3. Survival of rifampicin-resistant bacteria on plant debris left on the soil surface during 1999 and 2000. **A**, Recovery of *Xanthomonas campestris* pv. *armoraciae* (▼) and *Pseudomonas syringae* pv. *maculicola* (▽) from collards, and **B**, *X. campestris* pv. *armoraciae* (●) and *P. syringae* pv. *maculicola* (○) from turnip. Vertical bars represent one standard error of the mean.

On the basis of our results, it is reasonable to suggest that crop residue management, elimination of volunteer plants and weeds, and the use of pathogen-free seed are important strategies in controlling leaf spot diseases of leafy crucifers in Oklahoma. A crop rotation program should also be followed to allow adequate time for the decomposition of infested debris. Thorough incorporation of crop residue would also be beneficial to hasten the decomposition of infested debris during the winter months. However, more research may be needed to identify additional sources of inoculum for *Xanthomonas* and bacterial leaf spots. In the field surveys, these diseases were present on the crops in fields 1 and 4; yet the pathogens were not detected on any of the potential inoculum sources that were sampled. Levels of these pathogens may have been low and escaped detection at the time of sampling. Assessment of additional sources of inoculum may be necessary to identify other cultural practices for control of bacterial leaf spots.

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LITERATURE CITED

- Alvarez, A. M., Benedict, A. A., Mizumoto, C. Y., Hunter, J. E., and Gabriel, D. W. 1994. Serological, pathological, and genetic diversity among strains of *Xanthomonas campestris* infecting crucifers. *Phytopathology* 84:1449-1457.
- Alvarez, A. M., and Cho, J. J. 1978. Black rot of cabbage in Hawaii: Inoculum sources and disease incidence. *Phytopathology* 68:1456-1459.
- Bender, C. L., Alarcón-Chaidez, F., and Gross, D. C. 1999. *Pseudomonas syringae* phytotoxins: Mode of action, regulation and biosynthesis by peptide and polyketide synthetases. *Microbiol. Mol. Biol. Rev.* 63:266-292.
- Bereswill, S., Bugert, P., Voelksch, B., Ullrich, M., Bender, C. L., and Geider, K. 1994. Identification and relatedness of coronatine-producing *Pseudomonas syringae* pathovars by PCR analysis and sequence determination of the amplification products. *Appl. Environ. Microbiol.* 60:2924-2930.
- Black, L. L., and Machmud, M. 1983. *Xanthomonas* leaf spot of crucifers. Page 126 in: *Int. Congr. Plant Pathol.*, 4th, Melbourne, Australia.
- Campbell, R. N., Greathead, A. S., and Sim, S. T. 1987. Pepper spot of crucifers in California. Pages 668-671 in: *Proc. Int. Conf. Plant Pathog. Bacteria*, 6th. C. L. Civerolo, A. Collmer, R. E. Davis, and A. G. Gillaspie, eds. Martinus Nijhoff, Dordrecht, Netherlands.
- Cuppels, D. A., and Elmhirst, J. 1999. Disease development and changes in the natural *Pseudomonas syringae* pv. *tomato* populations on field tomato plants. *Plant Dis.* 83:759-764.
- Cuppels, D. A., Moore, R. A., and Morris, V. L. 1990. Construction and use of a nonradioactive DNA hybridization probe for detection of *Pseudomonas syringae* pv. *tomato* on tomato plants. *Appl. Environ. Microbiol.* 56:1743-1749.
- Fukui, R. A., Arias, R., and Alvarez, R. 1994. Efficacy of four semiselective media for the recovery of *Xanthomonas campestris* pv. *campestris* from tropical soils. *J. Appl. Bacteriol.* 77:534-540.
- Groth, D. E., and Braun, E. J. 1989. Survival, seed transmission, and epiphytic development of *Xanthomonas campestris* pv. *glycines* in the north-central United States. *Plant Dis.* 73:328-330.
- Hollaway, G. J., and Bretag, T. W. 1997. Survival of *Pseudomonas syringae* pv. *pisi* in soil and on pea trash and their importance as a source of inoculum for a following field pea crop. *Aust. J. Exp. Agric.* 37:369-375.
- Jardine, D. J., Stephens, C. T., and Fulbright, D. W. 1988. Potential sources of initial inoculum for bacterial speck in early planted tomato crops in Michigan: Debris and volunteers from previous crops. *Plant Dis.* 72:246-249.
- Jones, J. B., Pohronezny, K. L., and Jones, J. P. 1986. Survival of *Xanthomonas campestris* pv. *vesicatoria* in Florida on tomato crop residue, weeds, seeds, and volunteer tomato plants. *Phytopathology* 76:430-434.
- Keane, P. J., Kerr, A., and New, P. B. 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. *Aust. J. Biol. Sci.* 23:585-595.
- King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the identification of pyocyanin and fluorescein. *J. Lab. Clin. Med.* 44:301-307.
- Kocks, C. G., Ruissen, M. A., Zadocks, J. C., and Duijkers, M. G. 1998. Survival and extinction of *Xanthomonas campestris* pv. *campestris* in soil. *Eur. J. Plant Pathol.* 104:911-923.
- McCarter, S. M., Jones, J. B. G., Gitaitis, R. D., and Smitley, D. R. 1983. Survival of *Pseudomonas syringae* pv. *tomato* in association with tomato seed, soil, host tissue, and epiphytic weed hosts in Georgia. *Phytopathology* 73:1393-1398.
- Mguni, C. M., Mortensen, C. N., Keswani, C. L., and Hockenhull, J. 1999. Detection of the black rot pathogen (*Xanthomonas campestris* pv. *campestris*) and other xanthomonads in Zimbabwean and imported *Brassica* seed. *Seed Sci. Technol.* 27:447-454.
- Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Randhawa, P. S., and Schaad, N. W. 1984. Selective isolation of *Xanthomonas campestris* pv. *campestris*. *Phytopathology* 74:268-272.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Schaad, N. W. 1989. Detection of *Xanthomonas campestris* pv. *campestris* in crucifers. Pages 68-75 in: *Detection of Bacteria in Seed and Other Planting Materials*. A. W. Saettler, N. W. Schaad, and D. A. Roth, eds. American Phytopathological Society, St. Paul, MN.
- Schaad, N. W., Cheong, S. S., Tamaki, S., Hatziloukas, E., and Panopoulos, N. J. 1995. A combined biological and enzymatic amplification (BIO-PCR) technique to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed extracts. *Phytopathology* 85:243-248.
- Schaad, N. W., and Dianese, J. C. 1981. Cruciferous weeds as sources of inoculum of *Xanthomonas campestris* in black rot of crucifers. *Phytopathology* 71:1215-1220.
- Schaad, N. W., and White, W. C. 1974. Survival of *Xanthomonas campestris* in soil. *Phytopathology* 64:1518-1520.
- Schultz, T., and Gabrielson, R. L. 1986. *Xanthomonas campestris* pv. *campestris* in western Washington crucifer seed fields: Occurrence and survival. *Phytopathology* 76:1306-1309.
- Sherf, A. F., and MacNab, A. A. 1986. *Vegetable Diseases and Their Control*. 2nd ed. John Wiley & Sons, New York.
- Wiebe, W. L., and Campbell, R. N. 1993. Characterization of *Pseudomonas syringae* pv. *maculicola* and comparison with *P. s. tomato*. *Plant Dis.* 77:414-419.
- Wimalajeewa, D. L. S., and Nancarrow, R. J. 1980. Survival in soil of bacteria causing common and halo blights of French bean in Victoria. *Aust. J. Exp. Agric. Animal Husb.* 20:102-104.
- Young, J. M. 1969. An alternative weed host for *Xanthomonas campestris*. *Plant Dis. Rep.* 53:820-821.
- Zhao, Y., Damicone, J. P., Jones, W., Mitchell, R., and Bender, C. L. 2000. Detection of coronatine-producing strains of *Pseudomonas syringae* by PCR and ELISA. (Abstr.) *Phytopathology* 90:S88.
- Zhao, Y. F., Damicone, J. P., and Bender, C. L. 2001. Sources of inoculum for bacterial leaf spot diseases of leafy crucifers in Oklahoma. (Abstr.) *Phytopathology* 91:S204.
- Zhao, Y. F., Damicone, J. P., Demezas, D. H., and Bender, C. L. 2000. Bacterial leaf spot diseases of leafy crucifers in Oklahoma caused by pathovars of *Xanthomonas campestris*. *Plant Dis.* 84:1008-1014.
- 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.