Xanthomonas campestris pv. *campestris* in Western Washington Crucifer Seed Fields: Occurrence and Survival

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ABSTRACT

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The crucifer black rot pathogen *Xanthomonas campestris* pv. *campestris* recently has been reported for the first time in crucifer seed lots produced in western Washington. The occurrence, survival in residues, and host colonization by this pathogen were investigated. The pathogen was not detected by surveying in western Washington during three growing seasons. *X. c.* pv. *campestris* was recovered from buried, artificially infected cabbage residues as long as the residues persisted. The data indicated that *X. c.* pv. *campestris* could survive for 507 days in cabbage stem residues. The pathogen colonized and persisted in association with leaves of inoculated cabbage, radish, and wild turnip internally and externally under field conditions, although plants generally remained symptomless.

Black rot is considered the most destructive seedborne disease of crucifers in the world (20). In 1980, the causal agent, *Xanthomonas campestris* pv. *campestris* (Pam.) Dows., was reported for the first time in seed produced in western Washington (9). Western Washington has long been considered as a source of crucifer seed free of the black rot organism (17).

Because black rot has not been observed in the field in western Washington, little is known of the biology of the pathogen in this important seed-producing area. A program was initiated to determine the occurrence and survival of *X. c.* pv. *campestris* in crucifer seed fields in western Washington. Results of field surveys of crucifer seed crops and associated weeds for *X. c.* pv. *campestris*, and experiments to determine its ability to survive in infested cabbage stem residues and to colonize leaves of crucifer hosts under conditions in western Washington are reported herein. A preliminary report of this work has been published (15).

MATERIALS AND METHODS

Field surveys. Crucifer seed-crops, volunteers, and weeds at 22 farms and roadside weed sources in the cabbage seed-growing area of western Washington were surveyed for *X. c.* pv. *campestris* from 1981 to 1983. This area included western Skagit, northern Snohomish, and northern Island counties. Fields surveyed included those in which cabbage seeds contaminated with *X. c.* pv. *campestris* had been produced. Less than 300 ha of cabbage are grown here annually for seed (1).

Plants were observed for symptoms of black rot. Typical foliar symptoms that occur in other areas include yellowed, V-shaped lesions extending inward from leaf edges with black venation and dark brown or black vascular discoloration in stems or leaf petioles. Atypical symptoms include yellow and or brown lesions that were not V-shaped and lacked black venation (7).

Tissues of crucifer plants showing any of the above symptoms were brought to the laboratory, surfaced sterilized in 0.5% sodium hypochlorite for 3 min, and rinsed in sterile distilled water. The area adjacent to the leading edge of the lesion was excised and macerated in a drop of saline (0.85% NaCl) with a sterile razor blade. The macerate was streaked onto yeast extract-dextrose-calcium carbonate agar (YDC) (21) and incubated at 28 C. Any yellow, mucoid colonies that developed were transferred to nutrient starch-cyclohexamide agar (NSCA) (11) or SX agar (12). Yellow (on NSCA) or purple (on SX) mucoid colonies that hydrolyzed starch were tested for pathogenicity by using the methods described below.

Symptomless leaves of cruciferous weeds *Sisymbrium officinale* (L.) Scop. (hedge mustard), *Capsella bursa-pastoris* (L.) Medik. (shepherd's purse), and *Brassica campestris* L. (wild turnip) were occasionally collected and washed with sterile saline plus one drop of Tween-20 in a 250-ml flask on a rotary shaker for 20 min. Washings were diluted tenfold and 0.1 ml was plated onto NSCA and SX agar. Plates were incubated at 30 C and observed each day for 7 days for possible colonies of *X. c.* pv. *campestris*. Suspected isolates of *X. c.* pv. *campestris* were further tested for pathogenicity.

Survival of *X. c.* pv. *campestris* in cabbage residues. Inoculum was prepared by growing *X. c.* pv. *campestris* (strain B-24, N. W. Schaad, Department of Plant Science, University of Idaho, Moscow) in Difco nutrient broth for 14–16 hr at approximately 27 C on a rotary shaker. Eight-week-old Early Jersey Wakefield cabbage growing in the field was sprayed with the undiluted cell suspension. Leaves were sprayed to runoff in the early morning when guttation droplets were present. Plants were harvested 9 wk later. Stem sections, 1.5–3.5 cm wide by 7 cm long, showing black vascular tissue were buried in soil in 15- × 20-cm nylon mesh bags at depths of 5 and 15 cm at two sites on both Whidbey Island and at Puyallup in October 1982. Residues were sampled at 0, 30, and every 60 days thereafter. Sampling had used all tissues by July 1983 (275 days) for residues buried at 5 cm and by September 1983 (334 days) for residues buried at 15 cm.

On every sampling date, two tissue slices 2–3 mm thick were removed from one end of every stem section at each site. All slices were individually weighed and one section of each pair was dried to determine grams of dry tissue per slice. The other slice of each pair was assayed for bacteria. These sections were ground with a mortar and pestle and homogenized with a Waring Blendor in 40 ml of 0.85% saline. The suspension was filtered through cheesecloth, diluted by tenfold intervals to 10^{-5}, and these dilutions were plated in triplicate on SX agar. Plates were incubated at 30 C for 7 days. Colonies suspected of being *X. c.* pv. *campestris* were tested for pathogenicity and the number of colony-forming units (cfu) per gram of dry tissue was estimated.

Colonization of leaves by *X. c.* pv. *campestris*. A rifampin-resistant strain of *X. c.* pv. *campestris* was selected by growing

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wild-type strain B-24 on rifampin agar medium (RAM) (18). Mutants were subcultured again on RAM and finally onto YDC agar to provide inoculum for pathogenicity tests. A single virulent mutant (RrB-24) was used to study leaf colonization following inoculation of field and greenhouse-grown plants.

Cabbage (*B. oleracea* var. *captitata* 'Early Jersey Wakefield'), radish (*Rhaphanus sativus* 'Cherry Belle'), and wild turnip were field-grown 6–8 wk before inoculation. Inoculum was prepared by growing RrB-24 on YDC agar for 48 hr before making a cell suspension of 10^7 cfu/ml in sterile water. The suspension was sprayed onto upper and lower leaf surfaces until runoff on all expanded leaves of radish and cabbage 1 October 1982 and of all three species 25 August 1983.

Assays for Rr24-B were done by harvesting the oldest leaves of inoculated plants 24 hr after inoculation and approximately every 14 days thereafter during an 85-day period. Leaves were divided into three replications per plant type with fresh weights varying from 8 to 46 gm per replication. Samples were homogenized in 40–50 ml of sterile saline for 2 min in a Waring Blendor. Suspensions were filtered through cheesecloth and 0.1 ml from serial dilutions of $0–10^{-5}$ was plated in triplicate on RAM and incubated at 28 C. Yellow, mucoid, rifampin-resistant colonies were counted 3–6 days later. Colony-forming units reported were based on a calculated leaf area of 40 cm^2 per host. Data was analyzed by using Duncan's new multiple range test.

Two methods were used to determine if recovered RrB-24 were internal or external. First, the upper or lower surface of sections or whole leaves was gently pressed onto the surface of RAM for 90 sec. Growth of RrB-24 from leaf pressings was scored as follows: 0 = no colonies, + = 1–25% of print area with colonies, ++ = 26–50% of print area with colonies. Leaves also were bisected along the midrib and one section of each pair was surface sterilized for 4–5 min in 0.5% sodium hypochlorite. Each was ground separately with a mortar and pestle in 10 ml of sterile saline and streaked onto RAM. Plates were incubated at 28 C and colonies were counted 3–6 days later.

Four plant species including shepherd's purse, *Rorippa islandica* (Oeder) Borbas (marsh yellowcress), *Chenopodium album* L. (common lamb's-quarters) and cabbage were grown for 8 wk and used to study colonization of leaves by *X. c.* pv. *campestris* in the greenhouse. Common lamb's-quarters was included because it is neither a member of the Cruciferae or a known host of *X. c.* pv. *campestris*. As in the field experiments, plants were spray inoculated with RrB-24 at 10^8 cfu/ml. Temperatures in the greenhouse varied from 15 to 24 C. To assay, leaves were homogenized in a blender using methods similar to those used for field-grown leaves. Leaves were harvested 24 hr after inoculating and at 1- to 2-wk intervals up to 68 days. Recovered RrB-24 were expressed as colony-forming units per gram of fresh leaf tissue. All survival and colonization studies both in the field and the greenhouse were conducted well away from commercial seed production areas.

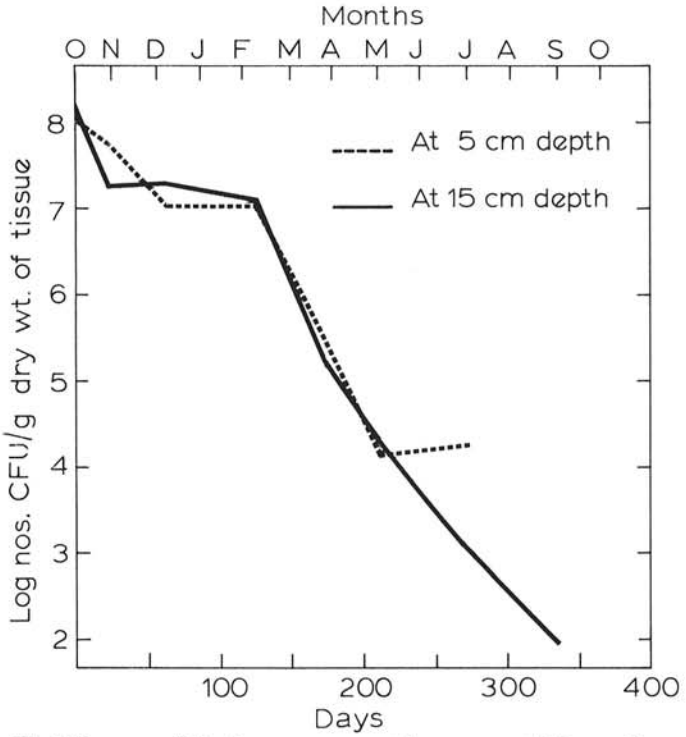

Fig. 1. Recovery of *Xanthomonas campestris* pv. *campestris* from cabbage stem residue buried in soil.

Pathogenicity tests. Two methods were used to determine pathogenicity. A suspension ($10^5–10^6$ cfu/ml) of log-phase cells of the suspected isolate was injected into leaf petioles of four young cabbage plants (8). Plants were placed in a growth chamber (Percival Manufacturing Co., Boone, IA) set for 28 C, 14 hr days, 20 C nights, and 80% relative humidity.

In the second method, four young cabbage plants were inoculated with fresh cells of the isolate grown on YDC agar for 48 hr. Leaf petioles were punctured with a 25-gauge needle covered with cells. After inoculation, plants were placed in the growth chamber described above. With both methods plants were observed 2–3 wk later for symptoms of black rot. These symptoms included systemic vein blackening, yellowing, wilting, and necrosis distal to the point of inoculation. Controls were treated similarly, except sterile water was used in the first method and a sterile needle without inoculum for the second method. No symptoms were observed on controls in either test.

RESULTS

Field surveys. Typical black rot symptoms were never observed on crucifer seed crops or weeds during the 3-yr period. Moreover,

TABLE 1. Recovery of rifampin-resistant *Xanthomonas campestris* pv. *campestris* from leaf pressings and homogenized leaves of inoculated, field-grown crucifers[a]

Days after inoculation	Cabbage		Radish		Wild turnip	
	Press[b]	Homogenized leaves[c]	Press	Homogenized leaves	Press	Homogenized leaves
1	···	1.3×10^2 c[d]	···	4.8×10^1 b	···	4.7×10^1 b
15	0	1.4×10^4 c	0	5.5×10^2 b	+	1.1×10^4 b
29	0	2.2×10^5 b,c	0	8.7×10^2 a	+	3.2×10^5 a,b
43	++	5.4×10^5 b	+	2.3×10^2 b	+	4.7×10^5 a
57	++	9.6×10^5 a	++	2.1×10^3 b		···
73	++	1.8×10^5 b,c	0	3.0×10^{-1} b	+	4.9 b
85	++	1.5×10^4 c	0	7.0×10^{-1} b	0	2.0×10^{-1} b

[a] All data from nonsurface sterilized leaves.
[b] Whole leaves or sections gently pressed onto rifampin agar for 90 sec. 0 = no colonies, + = 0–25%, ++ = 26–50% leaf area pressed showing colonies.
[c] Colony-forming units recovered per 40 cm^2 of the leaf surface from leaves homogenized in 40 ml of 0.85% saline and washings plated onto rifampin agar.
[d] Figures within columns with the same letters are not significantly different at $\alpha = 0.05$ level according to Duncan's new multiple range test.

TABLE 2. Recovery of rifampin-resistant *Xanthomonas campestris* pv. *campestris* from homogenized leaves of inoculated, greenhouse-grown plants

Days after inoculation	Fresh leaf tissue[a] (cfu/gm)			
	Cabbage	Yellowcress	Shepherd's purse	Lamb's-quarter
1	1.37×10^7	9.07×10^6	1.41×10^7	1.80×10^3
14	3.12×10^7	3.65×10^8	7.43×10^7	0
28	1.07×10^5	···	1.14×10^7	0
35	6.95×10^2	2.57×10^4	···	···
42	2.33×10^2	0	···	···
49	6.56×10^1	0	···	···
65	0	0	···	···

[a] All data from non-surface sterilized leaves homogenized in 40 ml of 0.85% saline and washings plated onto rifampin agar.

X. c. pv. *campestris* was not recovered in over 70 attempted isolations from any chlorotic, necrotic, or symptomless leaves. One isolate acquired by washing symptomless leaves of wild turnip was morphologically indistinguishable from known strains of *X. c.* pv. *campestris* on all three media used (NSCA, SX, and YDC) but was avirulent on cabbage.

Survival in residues. Cells of *X. c.* pv. *campestris* in stem residues decreased slowly, with some still being viable after 334 days. Survival was similar in residues buried at both 5 and 15 cm. Mean number of cells decreased from 1.2×10^8 in October 1982 to 2.5×10^4 per gram of tissue in July 1983 for residues at 5 cm depth and from 2.1×10^8 per gram in October 1982 to 98 in September 1983 for residues buried at 15 cm (Fig. 1).

Softer parts of the stem cortex decayed first and harder vascular tissue was more resistant. All stem tissue at both depths at one site on Whidbey Island that had been flooded had extensive soft rot within 60 days and was not used as a sample. Soil at other sites did not flood and soft rot decay was negligible. Data used to determine survival of *X. c.* pv. *campestris* was taken from both locations for 90 days and thereafter only from the Puyallup sites.

Colonization of leaves. In 1982, *X. c.* pv. *campestris* was recovered from inoculated leaves of field-grown cabbage and radish for a maximum of 28 and 42 days, respectively. Although numbers of *X. c.* pv. *campestris* recovered from field-grown inoculated leaves differed significantly at various dates in 1983, there was no significant reduction in colonization on any of the hosts over an 85-day period (Table 1).

No symptoms of infection were observed on field-grown cabbage or radish plants in 1982. In 1983, three of 32 inoculated cabbage plants had symptoms of black rot at 57 days and two cabbage plants had symptoms at 73 days, but no symptoms were evident on any plants at 85 days after inoculation in field experiments. Radish and wild turnip remained symptomless.

In 1983, *X. c.* pv. *campestris* was recovered from leaf pressings of radish 57 days, from wild turnip 73 days, and from cabbage 85 days after inoculation (Table 1). Cells of *X. c.* pv. *campestris* were recovered from both surface sterilized and unsterilized comminuted leaves of all three species at 14 and 28 days after inoculation in 1983.

In the greenhouse, the pathogen was recovered from inoculated cabbage 49 days, from marsh yellowcress 35 days, from shepherd's purse 28 days, and from common lamb's-quarters 1 day after inoculation, respectively (Table 2). No data are available for shepherd's purse after 28 days because all available leaf material had been used. Fewer cells of *X. c.* pv. *campestris* were recovered from common lamb's-quarters 24 hr after inoculation, than from the other species. Four of eight inoculated cabbage plants had typical black rot symptoms at 28 days. None of the other species developed symptoms.

DISCUSSION

Seed contaminated with *X. c.* pv. *campestris* is an important source of primary inoculum for black rot disease (5,6). Stock seed is imported annually from all over the world and the pathogen is undoubtedly introduced repeatedly in this manner. However, we were unable to find black rot or *X. c.* pv. *campestris* in western Washington crucifer seed fields during 3 yr of surveys. Furthermore, Washington State Department of Agriculture seed field inspectors annually inspect these crops and have never found black rot (Max Long, Washington State Department of Agriculture, Yakima, *personal communication*). The pathogen must be present in these seed fields however, because it has been reported in increasing numbers of Brassica seed lots (H. Humaydan, Joseph Harris Seed Co., Rochester, NY, *unpublished*). The greater numbers of seed lots reported recently to be infected with *X. c.* pv. *campestris* may be due in part to the use of more sensitive methods for detecting the pathogen (11). Nevertheless, the organism must be surviving, spreading, and infecting seed in some manner.

The pathogen was recovered from residues as long as they were undecayed. Regression analysis of the data in Figure 1 indicated a maximum survival for *X. c.* pv. *campestris* in buried cabbage residues to be 506 days in western Washington. Schaad and White (13) estimated that under conditions in Georgia, *X. c.* pv. *campestris* could survive in buried cabbage residues for 615 days. Because the organism only survives a short period of time in the absence of host residues (13,16), an effective rotation will be influenced by the time required for decomposition of host residues because the rate of decomposition varies with tissue and environmental conditions (20).

Crucifers were better hosts of *X. c.* pv. *campestris* than *Chenopodium* in a greenhouse test, and except for cabbage, other crucifers remained symptomless even though temperature was favorable for disease expression. Temperatures below 20 C can mask symptoms of black rot (9).

All three crucifer species were colonized by *X. c.* pv. *campestris* in the field. The pathogen could be recovered from inoculated cabbage and radish leaves longer in 1983 than 1982, which could be due to warmer temperatures when the 1983 experiment was started (mean monthly temperature 18.7 C) in contrast to when the 1982 experiment started (mean monthly temperature 11.4 C).

Growth of the pathogen without production of disease symptoms on crucifers does not exclude the possibility of internal infection, as the pathogen also was recovered from surface-sterilized, symptomless tissues. Expression of blight symptoms on bean leaves was reported to require an inoculum density of 5×10^6 cells of *X. phaseoli* per 20 cm^2 of leaf tissue (19). The concentration of *X. c.* pv. *campestris* did not reach this level on cabbage leaves in western Washington. However, inoculated cabbage leaves that had populations of 10^5–10^6 cfu/40 cm^2 did show symptoms. Other phytopathogenic bacteria are reported to inhabit the leaf surfaces of symptomless hosts when conditions are unfavorable for disease expression (4,14).

Surface-borne *X. c.* pv. *campestris* were recovered up to 73 days after inoculation on both cabbage and wild turnip (Table 1). This could be an important factor in dispersal of the pathogen in the field. The pathogen has been reported to spread up to 12 m from a single infected radish or wild turnip plant in Georgia (10) and has been isolated as an aerosol near a stand of wild turnips in California (3). Weeds such as wild turnip and radish commonly occur in the cabbage seed-growing area of Washington, and many cruciferous weeds grow here year-round. The pathogen possibly can persist as a symptomless colonizer of seed plants and seed may be contaminated at harvest by dust as reported for bean halo blight (2).

The avirulent bacterium isolated from wild turnip that morphologically resembled *X. c.* pv. *campestris* is of interest. Virulence of *X. c.* pv. *campestris* on cabbage may be attenuated in northwestern Washington. R. Morrison (Northrup-King Seed Co. Woodland, CA, *unpublished*) has reported a range of virulence for *X. c.* pv. *campestris*. Further work on relative virulence for strains of *X. c.* pv. *campestris* is needed.

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