Use of Monoclonal Antibodies to Monitor the Dissemination of *Xanthomonas campestris* pv. *campestris*

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ABSTRACT

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Monoclonal antibodies (MCA) were used to detect and differentiate strains of *Xanthomonas campestris* pv. *campestris* isolated from cabbage black rot lesions collected from the field. Extracts from leaf lesions were spotted onto a semiselective medium (FS). Four-day-old cultures that appeared to contain *Xanthomonas* were tested without further purification with four MCA using indirect enzyme-linked immunosorbent assay. The MCA reactivity patterns of four strains of *X. c.* pv. *campestris* were stable through four sets of serial transfers onto culture media and serial passages through cabbage. The four strains were introduced to replicate field plots on inoculated transplants, and dissemination of each strain was tracked independently with the MCA. *Xanthomonas*-like colonies were recovered on FS from 98% of samples (341 of 348) of lesions with definite black rot symptoms; 88% of the 341 presumptive *Xanthomonas* cultures on FS were identified as one of the four test strains by serotyping cultures directly from the isolation medium. The method also was useful in testing 484 lesions with symptoms that did not resemble those of black rot; the inoculated strains of *X. c.* pv. *campestris* were recovered from 21%. Pathogenic and nonpathogenic xanthomonads with MCA patterns unlike those of the four introduced strains of *X. c.* pv. *campestris* also were found in the field trials by this method. By monitoring the spread of the four inoculated strains of *X. c.* pv. *campestris* with MCA, disease progression and spatial patterns of infections were determined for each strain.

Additional key words: *Brassica oleracea*, serology.

Ecological and epidemiological studies of bacterial plant pathogens typically address population level phenomena. Techniques that allow accurate identification and monitoring of individual strains within given populations have been limited to those involving phage typing, serotyping, or mutant strains with antibiotic resistance (5,8,9,11,14,17). Only a small number of strains can be investigated using antibiotic resistance markers, whereas phage-typing and serotyping provide means to identify many strains.

In studies on black rot of crucifers caused by *Xanthomonas campestris* pv. *campestris*, phage typing and serotyping have provided varying degrees of success. Use of five bacteriophages specific to *X. c.* pv. *campestris* allowed separation of 145 strains of *X. c.* pv. *campestris* from different geographic origins into two lysotypes and seven subtypes (12). Phage typing was used to distinguish infections caused by three strains of *X. c.* pv. *campestris* in a field study by introducing strains selected from different geographic locations for their distinct phage-sensitivity patterns (11). However, differentiation of strains in Hawaii by phage typing was limited because 97% of the strains isolated from local cabbage farms were of a single lysotype (12). Monoclonal antibodies (MCA) subsequently were produced that differentiated Hawaiian strains of *X. c.* pv. *campestris* and separated them into six major groups and several subgroups (1).

This study was initiated to test the feasibility of using MCA to identify the strain causing black rot in the field, to simultaneously follow the progress of black rot caused by two serologically distinct strains originating from different inoculum sources, and to determine infection rates and spatial patterns for each strain.

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MATERIALS AND METHODS

Bacterial strains and pathogenicity tests. Four strains of *X. c.* pv. *campestris* were selected for field studies on the basis of reactivity patterns with four MCA that distinguished one strain from another (Table 1). The pathogenicity of each strain was tested on cabbage by spraying a susceptible cabbage (CG hybrid) with a suspension of log-phase, washed bacteria (10^5 to 10^6 colony forming units [cfu] per milliliter) in a solution of 0.85% NaCl and 0.5% Tween 80. Plants were kept under high humidity for 24 hr after inoculation and maintained in the greenhouse at 23–37 C. Symptoms were recorded at 4-day intervals over a 21-day period.

Bacteria were reisolated from plant material on a semiselective medium developed by D. Fieldhouse and M. Sasser (*personal communication*). The medium (FS) contained 10 g of potato starch, 500 mg of KNO_3, 800 mg of K_2HPO_4, 800 mg of KH_2PO_4, 100 mg of $MgSO_4$, 100 mg of yeast extract, 15 mg of methyl green, and 15 g of agar per liter. Antibiotics (30 mg of trimethoprim, 50 mg of cephalexin, and 150 mg of cycloheximide), 1 mg of pyridoxine-HCl and 3 mg of D-methionine were added aseptically after autoclaving. In preliminary experiments, this medium was

TABLE 1. Reactivity[a] to four monoclonal antibodies of strains of *Xanthomonas campestris* pv. *campestris* used in field experiments

	Antibody			
Strain	X3	X9	X11	X20
Experiment 1				
A249	+	+	+	–
EEab	–	–	+	–
Experiment 2				
SR1	+	+	+	–
XC114[b]	–	–	+	+

[a] Tested by indirect enzyme-linked immunosorbent assay.
[b] Provided by E. Echandi, North Carolina State University. All other strains were isolated in Hawaii.

equivalent in plating efficiency to starch-methionine medium (7) and yeast extract glycerol agar (YGA) (3) and was 100 times more sensitive than starch-methionine medium in recovery of *X. c.* pv. *campestris* from soil (*unpublished data*). Strains of *X. c.* pv. *campestris*, several other *X. campestris* pathovars, and some nonxanthomonads grow on FS. Colonies of *X. c.* pv. *campestris* are greenish blue, translucent, raised, and surrounded by clear zones of starch hydrolysis. Some other pathovars of *X. campestris* and a few other genera of bacteria resemble *X. c.* pv. *campestris* on FS.

Serotyping. The characteristics and specificities of three of the four antibodies used in this study (X3, X9, X11) have been reported previously (1). X11 was specific to *Xanthomonas* species. The fourth antibody, X20, reacts with some but not all strains of *X. c.* pv. *campestris* in group 5 (1). An indirect enzyme-linked immunosorbent assay (ELISA) was used. Strains were cultured on YGA for about 48 hr, harvested in 0.01 M phosphate-buffered saline (PBS) containing 0.5% formalin, and stored at 4 C. Before coating, cells were washed three times with 0.85% saline, suspended in 0.05 M carbonate-bicarbonate buffer (pH 9.6), and adjusted to A_{600} = 0.1 on a spectrophotometer (Spectronic 20, Bausch & Lomb). Microtiter plates (Immulon 2 TM, Dynatech Laboratories, Inc.) were coated with 200 μl of suspension per well and dried in a forced-air incubator at 37 C. To assay, wells were washed three times with PBS-containing 0.05% Tween (PBS-T) and then treated with 200 μl of the following reagents added separately in this order: i) mouse ascitic fluids, containing X3, X9, or X11, or hybridoma culture fluid, containing X20, diluted 1:2,000 and 1:10, respectively, with PBS-T; ii) goat antimouse immunoglobulin conjugated to horseradish peroxidase (Cooper Biomedical, Inc.) diluted 1,000-fold with PBS-T; and iii) 0.04% 5-aminosalicylic acid and 0.003% H_2O_2 in phosphate-EDTA buffer, pH 6.8 (4). Addition of each reagent was preceded by three washes with PBS-T, and each reagent was allowed to react for 1 hr. Color was measured at 450 nm on a microtiter plate reader (Multiscan, Flow Laboratories).

Reactions of test strains with MCA were compared to reactions of 10 strains of *X. c.* pv. *campestris*, serving as known positive and negative controls for each antibody, and with *Erwinia herbicola* strain Eh-1, a negative control for all antibodies. Initial assays also included reagent controls. However, this was discontinued because reactions of reagent controls were always lower than those of Eh-1.

Evaluation of antigenic stability. The stability of antigenic determinants was tested by repeated passage of strains of *X. c.* pv. *campestris* through culture media and through plants. Cultures of strains A249, XC114, SR1, and EEab were successively transferred four times onto YGA and onto FS. After 3–4 days of incubation at 28 C, cells were harvested from each culture and preserved in PBS with 0.5% formalin. For serial passage through plants, cabbage plants were inoculated with bacterial suspensions as described for pathogenicity tests. Ten disks per plant were excised from resulting black rot lesions with a 5-mm cork borer, surface sterilized in 0.3% NaOCl for 3 min, rinsed, and shaken for 1 hr in 10 ml of saline. A loopful of fluid then was streaked onto a medium containing tetrazolium chloride (TZC) (10). The samples were shaken an additional 24 hr at 28 C, and the contents of each tube were sprayed onto the surface of healthy cabbage plants. The inoculation and reisolation were repeated through four serial passages. *Xanthomonas* colonies recovered on TZC from each plant were transferred onto YGA and harvested in formalin containing PBS. Cells from each passage were serotyped separately by ELISA.

Tracing dissemination of black rot in the field. Two experiments were conducted at the University of Hawaii Agricultural Experiment Station, Waimanalo. The first experiment was initiated on February 1, 1985, and the second on March 21, 1985. Plots were in fields with no history of cropping with crucifers and were at least 200 m from existing crucifer plantings. No cruciferous weeds were found when the fields were inspected before plowing. The CG hybrid cabbage was planted in a greenhouse, and seedlings were transplanted after 4–6 wk into subplots, each containing 64 plants arranged in an 8 × 8 square with 0.45-m spacing between each row and column of plants. In experiment 1, 3.6 m of fallow ground separated adjacent subplots. The intersubplot spacing was increased to 5.4 m in experiment 2 because dissemination of black rot inoculum between subplots was detected in experiment 1. In both experiments, 12 subplots were arranged in a 3 × 4 array. For each treatment there were three replicate subplots placed in a randomized complete block design. For each treatment, strains of *X. c.* pv. *campestris* were sprayed onto cabbage seedlings 1 wk before transplanting. Inoculations were performed in the laboratory, and inoculated seedlings were isolated in a separate greenhouse to prevent infestation of uninoculated seedlings. For an individual inoculum treatment, four seedlings that had been inoculated with a single strain were transplanted into the center positions of each subplot. For a mixed inoculum treatment, two seedlings inoculated with one strain and two seedlings inoculated with another strain were placed in the four center positions, with homologous strains in the diagonal position. Two strains of *X. c.* pv. *campestris* were used as inocula in each experiment: A249 and EEab in experiment 1, XC114 and SR1 in experiment 2. Control subplots contained only healthy transplants. Plants were irrigated twice weekly by overhead sprinkling for 30 min/day during periods of low rainfall. The frequency was increased to five times per week after the sixth week in experiment 2 to facilitate the spread of disease.

Plants were inspected individually for symptoms at weekly intervals. The location of each plant displaying symptoms was recorded. Plants with symptoms were sampled each week for 9–10 wk after initial observation of disease in order to detect possible infection by a second strain of *X. c.* pv. *campestris*. Portions of lesions were removed with a plastic bag used as a glove to prevent contamination among samples and were transported to the laboratory for processing. When several lesions occurred on one plant, two to three lesions were sampled and pooled. Samples were processed on the same or following day after storage at 5 C. Three to four disks were excised from each sample with a sterilized 5-mm cork borer and eluted overnight in 1.0 ml of sterile distilled water. Ten-microliter volumes of the fluid were spotted onto FS medium, and the cultures were incubated for 4 days at 28 C.

Bacteria from colonies that resembled *Xanthomonas* were tested by ELISA with slight modifications. Cells were taken directly from FS, suspended in carbonate-bicarbonate buffer without washing, and adjusted visually to a density corresponding to A_{600} = 0.05–0.10. In preliminary experiments within this range, unwashed cells from FS did not have appreciably different reactions with MCA, compared to washed cells. Reactions of test strains to antibodies were interpreted as positive when ELISA readings were greater than 0.05 absorbancy units above that of the negative control, Eh-1. If the reactivity pattern of the sample corresponded to that of one of the two inoculated strains, the sample was not tested further. If a variant of either pattern was found, the sample was retested. If no recognizable pattern was detected during retesting, the bacteria from that sample were purified, reserotyped, and tested for pathogenicity on cabbage.

The spatial pattern of infection for each strain was determined. Ordinary runs analysis (13) was used to evaluate randomness of the pattern. In this analysis, rows within each plot were combined to form a single abstract row, and the orientation of the rows were chosen to give the least number of runs. Disease incidence data were transformed using the monomolecular, logistic (16), and Gompertz (6) functions. Regression analysis was performed using Statistical Analysis System programs (SAS Institute, Inc., Cary, NC). Coefficients of determination (R^2) and plots of residuals were used to assess the fit of each model to the data.

RESULTS

Stability of antigenic determinants. Serotypes of the four strains of *X. c.* pv. *campestris* were unchanged after four serial transfers through culture media. There were no significant quantitative differences in reactivity to the MCA between cells cultured on YGA and those on FS. Similarly, no changes in serotype were detected when the strains were passed four times successively through cabbage plants.

Identification of *X. c.* pv. *campestris* from field samples. Strains of *X. c.* pv. *campestris* in leaf samples collected from the two field experiments were readily identified by culturing on FS and serotyping. Colonies appearing to be *Xanthomonas* were recovered by spot-plating on FS medium from 341 of 348 samples (98%) showing typical black rot lesions. Serotyping of cultures not recognized as *Xanthomonas* on FS yielded only three additional strains of *X. c.* pv. *campestris*; thus, detection of *X. c.* pv. *campestris* by FS alone resulted in false negative results in less than 1% of the samples. No *Xanthomonas* colonies were recovered from 16 samples from healthy plants.

The reliability of serotyping for identification of *X. c.* pv. *campestris* directly from mixed cultures on FS was high. Serotyping of the 341 cultures identified as *Xanthomonas* on FS showed that 299 (88%) were identical to one of the four strains of *X. c.* pv. *campestris* introduced into the field. Of the remaining 42 cultures, 14 were identified as one of the four inoculated strains after purification and reserotyping. Eight cultures were xanthomonads that were not experimentally introduced into the field plots and yet were pathogenic in subsequent plant inoculations. From the remaining 20 mixed cultures, no xanthomonads were recovered; none of the purified cultures resembled *Xanthomonas* on FS, and none were pathogenic.

We assayed an additional 484 plant samples that had symptoms not recognized as black rot. Most of these were angular, tan, necrotic lesions that lacked vein-blackening and chlorosis. They were tested because previous studies (4,18) showed that such symptoms can result from infection by *X. c.* pv. *campestris*. Serotyping showed that 101 of these samples carried strains of *X. c.* pv. *campestris* identical to the experimentally inoculated strains. Thirty-two strains had serotypes unlike those of introduced strains, and they were not pathogenic to cabbage. The remaining 351 strains cultured from unusual lesions were not xanthomonads as determined by colony morphology, failure to hydrolyze starch on FS medium, and failure to react with X11.

Spatial patterns. In experiment 1, black rot caused by strains A249 and EEab showed markedly different spatial patterns on day 68 (Fig. 1A). Strain EEab was recovered only from inoculated transplants. In contrast, strain A249 was detected in high numbers of infections and in every subplot. It spread from the inoculum source toward the northeast, corresponding to the direction of surface water flow. Heavy rainfall (23 cm) occurred during a storm on days 13 and 14 after transplanting and caused flooding in the northeastern half of the field. Spatial patterns of black rot caused by strain A249 were determined by ordinary runs analysis (Table 2). Disease was aggregated in most of the subplots inoculated with strain A249, whereas infections by A249 were random in the other subplots.

Differences between spatial patterns of black rot infections

TABLE 2. Ordinary runs analysis of the distributions of cabbage plants infected with strain A249 in experiment 1, 68 days after planting

Inoculated strain(s)[a] in subplot	Subplot no.[b]	Distribution[c]	Z_u
A249	3	Nonrandom	−1.88
	5	Nonrandom	−2.05
	10	Nonrandom	−2.48
A249 and EEab	2	Random	−0.93
	7	Nonrandom	−3.16
	9	Random	−1.52
EEab	1	Random[d]	−0.21
	8	Random[d]	0.73
	12	ND[e]	ND
Uninoculated	4	Random[d]	−0.17
	6	Random[d]	1.03
	11	ND	ND

[a] Seedlings were sprayed 1 wk before transplanting with suspensions of *Xanthomonas campestris* pv. *campestris*, one strain per seedling. Four inoculated seedlings were planted among 60 uninoculated transplants in each subplot.

[b] Subplot numbers are designated in Figure 1A.

[c] Rows were combined into one abstract row, and the orientation of rows in each plot was chosen to give the smallest number of runs. The distribution was considered nonrandom when $Z_u < -1.64$ ($P = 0.05$).

[d] Infections by strain A249 were due to intersubplot spread.

[e] ND = Not determined because only two or fewer plants were infected with A249 in these subplots.

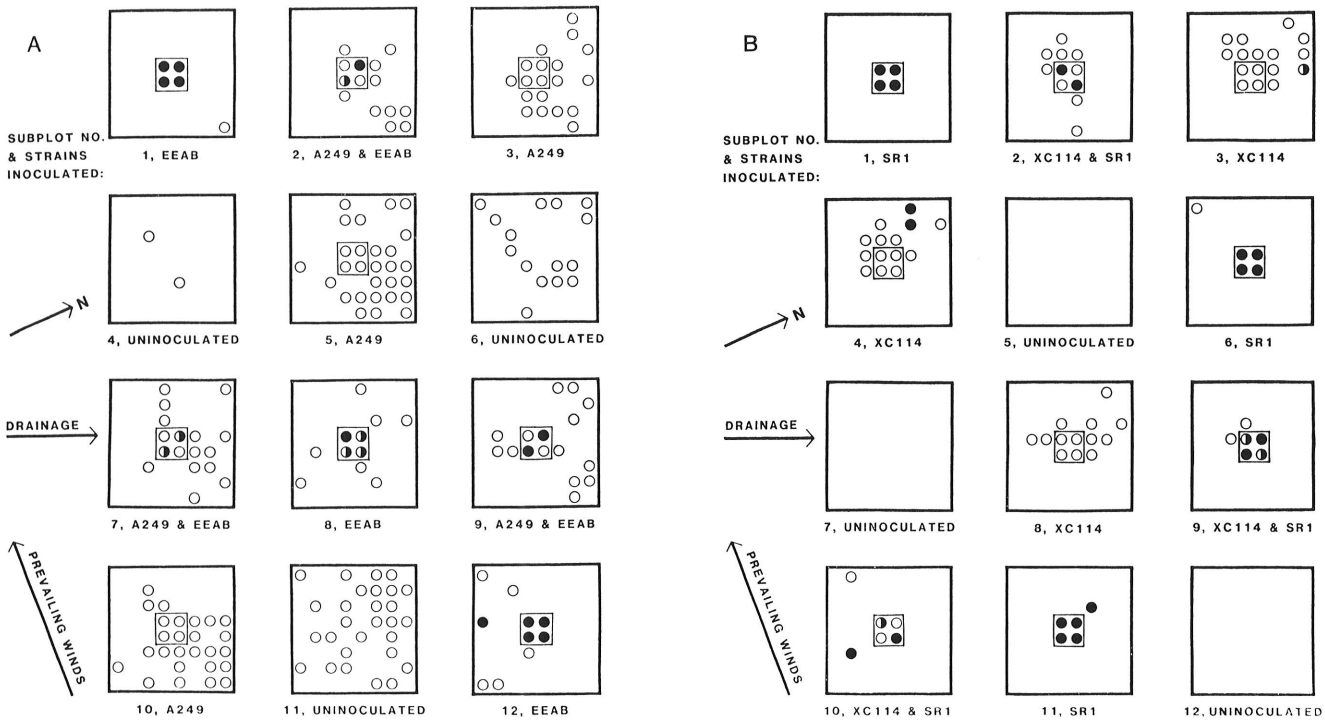

Fig. 1. Distribution of cabbage plants infected by two strains of *Xanthomonas campestris* pv. *campestris*, which were introduced simultaneously into a field and differentiated by reactions to four monoclonal antibodies. Inoculated seedlings were transplanted into the center four positions, outlined by small squares, within each subplot of 64 plants. Each subplot is identified by the strains that were inoculated. **A,** Plants infected by A249 (O), EEab (●), or both strains (◑) on day 68 in experiment 1. **B,** Plants infected by XC114 (O), SR1 (●), or both strains (◑) on day 62 in experiment 2.

caused by strains SR1 and XC114 in experiment 2 also were observed on day 62 (Fig. 1B). The distribution of infections by strain SR1 was very limited compared to those caused by strain XC114. Infections caused by XC114 generally were heavier in the western half of each subplot, in the direction of prevailing winds. Rainfall did not exceed 1 cm on any day during experiment 2. Only four instances of intersubplot spread were detected in this experiment. The distribution of plants infected by XC114 was aggregated in all subplots except one.

Disease progress. The incidence of black rot caused by the two strains of *X. c.* pv. *campestris* in each experiment increased at markedly different rates. In experiment 1, spread of disease from transplants inoculated with strain A249 was first observed on day 32. By day 68, the mean incidence of infections caused by A249 was 39% in subplots in which A249 was introduced alone and 23% in all other subplots (Fig. 2A). In contrast, strain EEab did not spread to healthy plants. In experiment 2, spread of strain XC114 from inoculated transplants was first noted on day 34. By the end of the experiment (day 62) mean incidence of black rot caused by XC114 was 22% in subplots inoculated with XC114 and 10% in subplots inoculated with a combination of XC114 and SR1 (Fig. 2B). Strain SR1 showed little spread.

Of the three disease progression models tested, the monomolecular model gave the closest fit to the temporal increase of black rot caused by strain A249 in experiment 1 (Table 3). The coefficient of determination for the monomolecular model was 0.94 in all subplots. However, distinct patterns were evident in plots of residuals, indicating that none of the models accurately described the disease progress. In experiment 2, coefficients of determination for strain XC114 were similar for all models in all subplots (R^2 = 0.92 to 0.93). Transformations and analysis were not performed on the infection data for strains EEab or SR1 because these strains failed to spread.

DISCUSSION

The spread of different strains of *X. c.* pv. *campestris* could be monitored simultaneously in the field by serotyping cultures with MCA after enrichment on a semiselective medium. The rates at which disease increased varied markedly among the introduced strains. Differences in virulence or in capacity to disseminate may account for such variability. However, this variability was not revealed in the development of symptoms resulting from plant inoculations in the laboratory. Inherent differences among strains under field conditions may explain the prevalence of certain serotypes of *X. c.* pv. *campestris* over others in particular locations, as previously observed (1).

The spatial spread of black rot caused by individual strains also could be monitored. Strandberg (15) found the distribution of infections caused by *X. c.* pv. *campestris* to be highly aggregated. In our study, distribution of black rot was aggregated in most subplots. We also observed random patterns caused by strain A249

TABLE 3. Regression analysis of incidence of black rot caused by strains A249 and XC114 versus time[a]

Experiment Strain	Inoculum strain(s) in subplots (numbered)[b]	Monomolecular model		Logistic model		Gompertz model	
		R^{2c}	Slop ± standard error	R^2	Slope ± standard error	R^2	Slope ± standard error
Experiment 1 A249	A249 (3,5,10)	0.94	0.010 ± 0.001	0.87	0.054 ± 0.009	0.91	0.026 ± 0.004
	A249 and EEab (2,7,9)	0.94	0.006 ± 0.001	0.86	0.061 ± 0.011	0.91	0.023 ± 0.003
	EEab or uninoculated (1,4,6,8)[d]	0.94	0.006 ± 0.001	0.90	0.042 ± 0.007	0.91	0.018 ± 0.003
Experiment 2 XC114	XC114 (3,4,8)	0.93	0.006 ± 0.001	0.92	0.044 ± 0.007	0.92	0.019 ± 0.003
	XC114 and SR1 (2,9,10)	0.92	0.002 ± 0.001	0.93	0.040 ± 0.006	0.93	0.013 ± 0.002

[a] Data were transformed to monomolecular, logistic, and Gompertz models by $\ln(1/1-y)$, $\ln(y/1-y)$, and $-\ln[-\ln(y)]$, respectively, where y = proportion of plants with black rot.

[b] Seedlings were separately inoculated with strains of *Xanthomonas campestris* pv. *campestris*. Four inoculated seedlings were transplanted into the center position of each subplot and surrounded by 60 healthy transplants. Data from three subplots were averaged for each treatment. Subplot numbers are designated in Figure 1A for experiment 1 and in Figure 1B for experiment 2.

[c] R^2 = Coefficient of determination. Values for R^2 and the slopes of the transformed data were calculated using sampling dates indicated on Figure 2A and B.

[d] Infections were due to intersubplot spread of strain A249. Infections in these four subplots were averaged; the remaining two subplots (11 and 12) showed little infection. Intersubplot spread by XC114 in experiment 2 was low and therefore not included in this analysis.

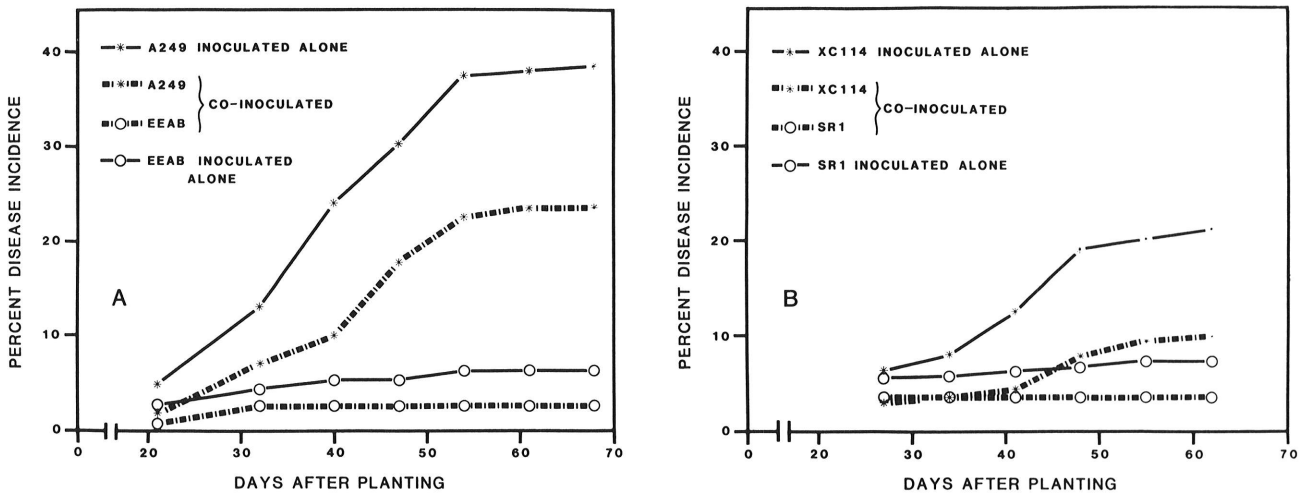

Fig. 2. Progress of black rot of cabbage caused by two strains of *Xanthomonas campestris* pv. *campestris*. Strains were identified by monoclonal antibody reaction patterns: **A,** Strains A249 and EEab in experiment 1; **B,** strains XC114 and SR1 in experiment 2. Each data point represents a mean of three measurements taken from replicate subplots.

in many subplots in experiment 1. High winds, rain, and flooding during the early weeks of the experiment may have accounted for this type of spread.

Xanthomonas strains that had not been intentionally introduced were detected in the field experiments and were distinguished from the test strains by their different MCA reactivity patterns. Because the antibody reactivity patterns of *X. c.* pv. *campestris* strains were stable in serial passage through plants and media, it was unlikely the nonintroduced strains resulted from mutations of the introduced strains. Presumably, the former were seedborne or had been established on surrounding weeds. No *Xanthomonas* strains, however, were recovered from a subsample (2,000 seeds) of the seed stock used in this study. Populations in resident weeds were not assayed, as no cruciferous weeds were found. Nonetheless, strains with similar reactivity patterns have been isolated previously from crucifer seeds and weeds in other areas (2).

Serotyping with MCA has several advantages over other methods used in epidemiological studies. MCA may be used to track any wild type strain. In contrast, the use of antibiotic-resistance markers is applicable only in the detection of mutated strains. Mutant strains may be attenuated, giving distorted results with respect to their ability to infect and disseminate under field conditions. The advantage of serotyping over phage-typing methods is that MCA can identify strains in mixed cultures, whereas phage typing must be performed on pure cultures growing in log phase. The serotyping procedure in our two-step method served as a means of screening large numbers of plant samples for *Xanthomonas* and reduced the number of strains to be purified. MCAs may be used as described for investigating other aspects in the ecology of black rot, such as epiphytic colonization, survival in soil and alternate hosts, and differential virulence among strains to crucifer cultivars.

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