Rapid Identification of *Xanthomonas campestris* pv. *campestris* by ELISA

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ABSTRACT

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A double-antibody sandwich (DAS)-ELISA was developed to detect *Xanthomonas campestris* pv. *campestris* in leaf disks sampled from cabbage in the field. Assay time was reduced from 3–5 days with semiselective media to 5 hr with DAS-ELISA. Sheep and rabbit antisera were used as primary and secondary antibodies. Goat antirabbit peroxidase and purified 5-aminosalicylic acid were used as the enzyme indicator system, measured at 450 nm. On the basis of 510 samples with a disease incidence of 56%, 96.8% of known positives were detected by DAS-ELISA as confirmed by isolation and pathogenicity tests. DAS-ELISA permitted the processing of large numbers of samples and facilitated identification of the pathogen in seedbeds, on weeds, and on cabbage plants that showed unusual symptoms.

Additional key words: black rot

Xanthomonas campestris pv. *campestris*, cause of black rot of crucifers, is usually associated with black veins and characteristic chlorotic, dry, V-shaped lesions extending from leaf margins (18). However, atypical symptoms are often observed during extended periods of high humidity (11), and if presence of the pathogen needs to be known, it must be confirmed through other techniques. Culturing requires 3–5 days and limits the numbers of samples that can be collected to determine infection rates in epidemiological studies.

The purpose of this work was to develop a serological assay that can be used conveniently to process large numbers of field samples without culture media.

MATERIALS AND METHODS

Antisera. The primary antibody was prepared in sheep. After a preimmunization bleeding, a sheep was injected intravenously with 3 ml of a saline suspension of *X. c.* pv. *campestris* strain A249. The bacteria were grown on yeast extract-glycerol agar slants (2), washed three times with 0.85% saline, and maintained in 0.5% formalin in 0.01 M phosphate-buffered saline (PBS), pH 7.4. As required, small volumes were aseptically removed and washed with 0.85% sterile saline before use. The sheep was injected three times a week for 3 wk with about 3×10^8 cells per injection (total of 10 injections). After the animal had rested 6 days, a trial bleeding was taken and the titer and specificity of the serum was determined. After a short rest period, the sheep was primed with three injections of live bacteria and subsequently bled. Agglutination tests were performed on the three bleedings. The secondary antibody was prepared in rabbit, and its specificity was described previously (2,8). Additional heterologous strains were used to test the specificity of the DAS-ELISA.

DAS-ELISA method. The parameters of this assay were examined in detail. They included incubation times and temperatures for coating plates (1, 2, 4, and 10 hr at 4, 24, and 37 C), incubation times and temperatures for enzyme and substrate reactions (1, 2, and 4 hr at 24, 37, and 70 C), coating method (liquid incubation vs. air-dry), type of conjugate (alkaline phosphatase- or peroxidase-labeled goat antirabbit globulin) and substrate (*p*-nitrophenyl phosphate, *o*-phenylenediamine, and 5-aminosalicylic acid [5-AS]). The latter substrate was purified by the method of Ellens and Gielkens (9). The most suitable assay for large-scale field testing was performed by the protocol described under Results.

Controls included all reagents except homologous antigen, rabbit immune serum, enzyme-labeled goat antirabbit globulin (ELGARG), and substrate. Saline was substituted for the missing reagent. Additional controls included homologous antigen with normal serum and ELGARG, absorbed specific antiserum and ELGARG, and uninfected leaf disks. A standard curve was made from a twofold dilution series of a cell suspension of the homologous antigen *X. c.* pv. *campestris* strain A249. A similar dilution series was made for a heterologous antigen, *E. herbicola* strain Eh-1. Values for antigen controls were established with stock suspensions of whole cells maintained in 0.5% formalin. Before each assay, cells were washed four times and suspended in PBS to give an absorbance value of $A_{600} = 0.1$. A 10-fold dilution series of this suspension was made and 0.1-ml aliquots of dilutions 6, 7, and 8 were plated on tetrazolium chloride medium (TZC) (10) for viable counts.

In addition to the assay parameters, the sampling method was evaluated by comparing results obtained with the following variations: 1) Leaf disks were macerated or left intact to compare numbers of bacteria released into the suspending fluid; 2) water, 0.85% saline, PBS, and nutrient broth were compared as suspending fluids; 3) size (0.5–1.5 cm) and number (one to five) of leaf disks per milliliter of suspending fluid were compared; and 4) time needed for natural elution of bacteria into the suspending fluid was varied using intervals of 0.5, 1, 4, 8, 24, and 48 hr.

Numbers of viable bacteria associated with various lesion types were calculated from viable plate counts on starch-methionine (SM) medium (6). These values were compared with those obtained by ELISA and pathogenicity tests for the same samples.

To perform simultaneous assays on leaf samples, the sample volume was increased. Three leaf disks (5 mm in diameter) were excised from the lesion and suspended in 3 ml of sterile distilled water. Bacteria were eluted for 2 hr. For viable counts, a 1-ml sample was carried through serial 10-fold dilutions in 0.85% saline and 0.1 ml was spread onto SM medium. For pathogenicity tests, 0.5-ml samples were injection-infiltrated in duplicate into interveinal areas of cabbage leaves; plants were maintained on the laboratory bench at 24 C, and symptoms were observed 7–21 days after inoculation. For ELISA, duplicate 200-μl samples were assayed as described and readings were compared with a standard curve based on duplicate twofold serial dilutions of strain A249.

Sensitivity and specificity of the assays were tested by comparison with direct isolation onto culture media. Tests were evaluated by standard clinical procedures (12,16). Samples were rated independently as diseased and healthy, and tests were evaluated according to the ability of the

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assay to pick out the diseased samples (positives) from the healthy samples (negatives). Tests were run double-blind by a coding procedure. Disease-positives were determined on the basis of obvious symptoms and were confirmed by isolation of the pathogen followed by tests for pathogenicity. Healthy samples were excised from symptomless areas of leaves on the same or adjacent plants.

RESULTS

Specificity. The rabbit antiserum prepared to *X. c.* pv. *campestris* strain A249 reacted with 49 of 54 *X. c.* pv. *campestris* strains tested but not with unrelated genera or other pathovars of *X. campestris*, except two strains of *X. c.* pv. *armoraciae*, 10 strains of *X. c.* pv. *vesicatoria*, and 10 strains of *X. c.* pv. *vitians* (Table 1). Nonspecific reactions could not be removed without destroying reactivity to the crucifer strains. The sheep antiserum was identical in specificity. Of the 157 strains tested by the DAS-ELISA, only the strains of the above pathovars showed nonspecific reactions (Table 1).

Field sampling and assay. After all parameters were tested and compared, the most efficient and reproducible procedure for a rapid DAS-ELISA was achieved by the following protocol: 1) Sheep anti-A249 serum was diluted 1:500 in 0.05 M carbonate buffer (pH 9.6), and 200 μl of antiserum was placed in each well of a polystyrene microtiter plate (Immulon-2, flat-bottom; Dynatech Laboratories, Alexandria, VA). Plates were air-dried at 37 C with a circulating air incubator. 2) The plate was washed four times with 0.01 M PBS containing 0.5% Tween 20 (PBS-T); fluid removal and washing were facilitated by a Nunc-Immuno Wash 12 (Vangard International, Neptune, NJ). 3) The test sample (200 μl) was placed in each well and incubated for 1 hr at room temperature (RT). 4) Fluid was removed and wells were washed four times with PBS-T. 5) Rabbit anti-A249 serum was diluted 1:2,500 in PBS-T; 200 μl was added to each well and incubated 1 hr at RT. 6) Fluid was removed and wells were washed four times with PBS-T. 7) Peroxidase-labeled goat antirabbit globulin (GARP) (Cappel Laboratories, West Chester, PA) was diluted 1:3,000 in PBS-T; 200 μl was added to each well and incubated 1 hr at RT. 8) Fluid was removed and wells were washed four times with PBS-T. 9) Purified 5-AS was prepared as a 0.03% solution in 0.01 M phosphate-EDTA buffer, pH 6.8 (ethylenediaminetetraacetic acid disodium salt, 35 mg/L); 100 μl of 3% H_2O_2 was added to 100 ml of 5-AS to give a final concentration of 0.003% H_2O_2, and 200 μl of the substrate was added to each well of the microtiter plate. 10) Color was read at 450 nm on a microtiter plate reader (Titertek Multiskan) 30 min after addition of substrate. No enzymatic terminator was used. Preliminary testing with terminators confirmed earlier reports (5,9) that terminators were unnecessary.

An uninfected leaf sample served as a negative control, and cell suspensions ($A_{600} = 0.1$) of *X. c.* pv. *campestris* strain A249 and *Erwinia herbicola* strain Eh-1 served as additional positive and negative controls, respectively. On the basis of viable counts on TZC and formalin-killed cells counted in a Petroff-Hauser chamber, this suspension contained 1–3 $\times 10^8$ colony-forming units (cfu) per milliliter. After assay, 200 μl of this suspension gave ELISA absorbance values between 1.1 and 1.2 at 450 nm for strain A249 and <0.05 for Eh-1. Net readings were obtained after subtracting the highest background readings (average of duplicates) from the sample value. Higher background readings always were observed in the *E. herbicola* controls than in uninfected tissue controls. In preliminary tests, none of the other negative controls reached values as high as those for Eh-1, thus they were not included in large-scale field assays. All tests were performed in duplicate.

Values for the unknowns were compared with a standard curve made from a twofold dilution series of strain A249 cells ranging from 10^8 to 10^4 cells per milliliter prepared for each ELISA (Fig. 1). The initial suspension gave readings varying from 1.1 to 1.2 ELISA absorbance units. The lowest limit of detection, arbitrarily established at 0.05 ELISA absorbance units above background, was 2×10^5 cells per milliliter (4×10^4 cells per well). On the basis of an

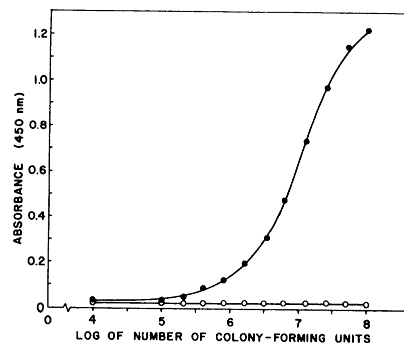

Fig. 1. Absorbance values (A_{450nm}) in double-antibody sandwich ELISA using antisera prepared against intact cells of *Xanthomonas campestris* pv. *campestris* strain A249. The number of viable cells per milliliter in the initial suspension ($A_{600} = 0.1$) was determined by plating appropriate 10-fold dilutions on tetrazolium chloride medium. ●——● = *X. c.* pv. *campestris*; ○——○ = *Erwinia herbicola*.

Table 1. Bacterial strains reacting with antisera prepared to *Xanthomonas campestris* pv. *campestris* strain A249 in the double-antibody sandwich ELISA

Bacteria tested	Strains showing ELISA reaction (no.)		Strains tested (no.)
	Negative	Positive	
Xanthomonas campestris			
pv. *armoraciae*	0	2	2
pv. *begoniae*	3	0	3
pv. *campestris*	5	49	54
pv. *dieffenbachiae*	3	0	3
pv. *euphorbiae*	2	0	2
pv. *hederae*	1	0	1
pv. *manihotis*	2	0	2
pv. *oryzae*	2	0	2
pv. *pelargonii*	1	0	1
pv. *phaseoli*	2	0	2
pv. *poinsettiicola*	2	0	2
pv. *vesicatoria*	4	10	14
pv. *vitians*	3	10	13
Undescribed pathovars			
From *Allium cepa*	9	0	9
From *Cordyline terminalis*	2	0	2
From *Pellionia* sp.	2	0	2
X. albilineans	1	0	1
X. fragariae	1	0	1
Agrobacterium tumefaciens	1	0	1
Erwinia carotovora			
subsp. *carotovora*	1	0	1
subsp. *atroseptica*	1	0	1
E. chrysanthemi	1	0	1
E. herbicola	2	0	2
Pseudomonas cichorii	1	0	1
P. syringae pv. *phaseolicola*	2	0	2
Corynebacterium sepedonicum	1	0	1
C. michiganense	1	0	1
Other genera of human, animal, and soil origin	10	0	10
Yellow, gram-negative, oxidative leaf epiphytes	20	0	20
Total strains tested			157

average of 10 replicates, the coefficient of variation was 4.4%.

Using the parameters determined to produce standard curves, we used the numbers of cells recovered from leaf disks as a quantitative measure for comparing sampling methods and selecting a standard sampling procedure.

Bacteria flowed from leaf disks into the suspending fluid in sufficient numbers to eliminate the need for crushing tissues, provided disks were floated in the suspending fluid for at least 2 hr after excision from the leaf. Longer soaking periods (up to 48 hr after sampling) did not significantly alter the ELISA readings. Highest ELISA readings were achieved when bacteria were eluted in water as opposed to suspending in fluids with higher osmotic potential, such as normal saline, PBS, or nutrient broth. The antigen-antibody binding reaction was not significantly altered when water rather than PBS was used as the diluent for live bacterial cells. A single 0.5-cm leaf disk per milliliter of water provided sufficient numbers of bacteria to achieve ELISA readings of 0.12–1.2 at 450 nm. This corresponded to about 1×10^6 to $>1 \times 10^8$ cfu/ml on the basis of viable counts on TZC and SM plates. Higher readings (>1.1) were achieved with two and three disks per milliliter, respectively. One disk per milliliter was selected as the sample size, because sufficient numbers of bacteria were eluted to achieve a reading that could be interpreted as a definite positive while still permitting semi-quantification of results. Since increased sensitivity of ELISA was reportedly achieved by heating culture or tissue antigens of *X. c.* pv. *citri* (7), we heated (100 C, 30 min) cell suspensions and leaf-disk elution fluids containing live cells. We were unable to increase the sensitivity by heating the elution fluids, but we observed some increased activity when heating washed, formalin-killed cell suspensions (Fig. 2).

The numbers of bacteria detected in the suspending fluid after 2-hr elution in water from various symptom types are shown in Table 2. Higher numbers were detected by ELISA since antisera bind both to live and dead cells. However, most cells were viable and virulent as indicated by growth on SM medium and production of black rot symptoms on cabbage 7 days after injection of suspending fluids into interveinal areas of cabbage leaves. Results of pathogenicity tests did not change when plants were observed after 10–21 days. A closer estimate of the numbers of bacteria eluted from representative disks under these conditions was made from the twofold serial dilutions of the suspending fluids (Table 3). Dilution end points were 1:800 and 1:1,600 for ELISA and pathogenicity methods, respectively. Isolation onto the selective medium was more sensitive for detecting low numbers of bacteria.

The sensitivity and specificity of the ELISA method was based on evaluation of 510 samples with an actual disease incidence of 55.5% based on typical black rot symptom expression followed by isolation of the pathogen in culture (Table 4). The term "prevalence" is equivalent to "disease incidence" in the described methods used for analysis (12,16). Predictive values of positive and

Table 2. Populations of *Xanthomonas campestris* pv. *campestris* detected in cabbage leaf tissue exhibiting a range of symptom types

Symptom type	Viable count with SM medium[a] (cfu/ml)	Total count with DAS-ELISA[b] (cells/ml)	Pathogenicity[c] (symptoms on cabbage leaves)
Necrotic lesion, center	2.0×10^8	$>10^8$	+
Water-soaked lesion, center	2.1×10^6	$>10^8$	+
Water-soaked lesion, margin	1.6×10^9	$>10^8$	+
Chlorotic tissue	1.4×10^9	$>10^8$	+
Black veins	3.9×10^9	$>10^8$	+
Necrotic flecks	5.9×10^6	5×10^7	+
No symptoms	0	0	0

[a] Three leaf disks (5 mm in diameter) suspended in 3 ml of sterile, distilled water for 2 hr. Samples were serially diluted 10-fold and 0.1-ml aliquots of 10^{-4}, 10^{-5}, 10^{-6} dilutions were spread in duplicate on SM medium.

[b] Two hundred microliters of elution fluid was assayed in duplicate by double-antibody sandwich ELISA. Values are based on standard curve.

[c] Aliquots (0.5 ml) of elution fluid were injection-infiltrated in duplicate into interveinal areas of cabbage leaves. Symptoms were recorded 7 days after inoculation. No additional infections were observed on plants observed after 10–21 days.

Table 3. Comparative sensitivity of three methods for detecting *Xanthomonas campestris* pv. *campestris* in cabbage leaves

Dilution of original Suspension (1/dil. factor)	Viable count with SM medium[a] (cfu/ml)	Total count with DAS-ELISA (cells/ml)	Pathogenicity (symptoms on cabbage leaves)
0	6.5×10^8	$>10^8$	+
100	1.5×10^6	6.0×10^7	+
200	1.8×10^5	9.4×10^6	+
400	1.2×10^5	4.7×10^6	+
800	5.3×10^5	2.3×10^6	+
1,600	2.8×10^5	nd[b]	+
3,200	9.0×10^4	nd	0[c]
6,400	5.6×10^4	nd	0

[a] Three leaf disks (5 mm in diameter) were exicsed from centers of a V-shaped lesion, suspended in water, and tested for bacterial populations as described in text (av. of six replicates per sample).

[b] nd = Not detected; ELISA reading was only 0.04 units above background, or about 1×10^5 cells per milliliter.

[c] 0 = No symptoms expressed within 10–21 days.

Table 4. Detection of *Xanthomonas campestris* pv. *campestris* in cabbage leaves by double-antibody sandwich ELISA[a]

Test results	Number of cabbage leaves: Diseased	Healthy	Total
Positive	274	22	296
Negative	9	205	214
Total	283	227	510

[a] Test evaluation is described in Materials and Methods. Disease incidence (283/510 = 55.5%), apparent disease incidence (296/510 = 58.0%), sensitivity (274/283 = 96.8%), specificity (205/277 = 90.3%), predictive value of a positive result (274/296 = 92.6%), and predictive value of a negative result (205/214 = 91.1%).

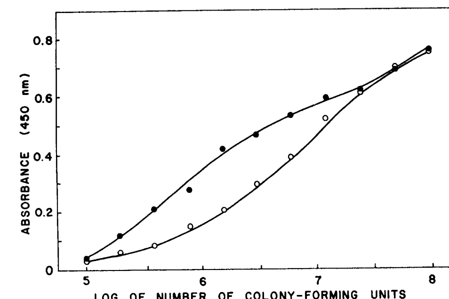

Fig. 2. Effect of heating culture antigens on ELISA absorbance values; ●——● = intact cell suspensions of *Xanthomonas campestris* pv. *campestris* maintained in 0.5% formalin; cells were washed four times, suspended in phosphate-buffered saline plus Tween 20 (PBS-T), adjusted to $A_{600} = 0.1$, diluted in a twofold series in PBS-T, and heated (100 C, 30 min) before assay; o——o = unheated cell suspensions.

negative results were calculated to be 92.6 and 91.1%, respectively.

Using this procedure to evaluate test results, we compared four assay methods by running concurrent tests on leaf-disk suspension fluids from 150 samples. In this test, disease incidence (50%) was based on expression of typical black rot symptoms alone. Tests were run double-blind and decoded after results were recorded separately. Sensitivity, specificity, estimation of apparent disease incidence, and predictive values of positive and negative results were similar for the four assay methods (Table 5).

Table 5. Evaluation of assay methods for detection of *Xanthomonas campestris* in cabbage leaves[a]

	Viable plate count		Pathogenicity test	DAS-ELISA
	TZC	SM		
Sensitivity	84.0	92.0	90.7	94.7
Specificity	97.5	91.4	95.1	97.5
Apparent disease incidence	41.7	48.7	46.2	46.8
Predictive value				
Positive result	90.8	96.8	94.4	97.3
Negative result	87.7	92.5	91.7	95.2

[a] Expressed as percentages; 150 leaves sampled with 50% disease incidence based on symptom expression.

After the reliability of the sampling procedure and ELISA for detecting the pathogen was established, two field trials were undertaken to determine the presence of *X. c.* pv. *campestris* in tissues showing unusual symptoms. In the first trial, 110 samples were collected from eight farms; 46 samples represented the range of characteristic symptoms commonly observed in previous field trials, 46 samples were excised from symptomless tissue, and 18 samples were excised from centers of large water-soaked or tan patches of interveinal tissue not usually associated with black rot. Assays were run concurrently using ELISA and viable plate counts on TZC and SM media. All of the samples showing characteristic black rot symptoms produced positive results in the three assays, and all of the symptomless samples were negative. Samples from the water-soaked, tan, and decayed tissues that produced positive results by ELISA also showed *Xanthomonas*-like colonies with starch hydrolysis on SM; TZC plates were often overgrown with saprophytes. In the second trial, 110 samples were collected from eight fields in two farms. Fifty samples showed typical black rot symptoms, and 63 samples showed unusual or mixed symptoms.

All samples showing black rot were positive by ELISA and isolation onto SM; *X. c.* pv. *campestris* was detected in 81% of the samples that showed large interveinal patches of dried tan tissue, some of which was congested with water during early morning hours.

In a survey for black rot in seedbeds of two farms, *X. c.* pv. *campestris* was detected on nine of 6,000 seedlings; eight seedlings showed mild chlorosis that resembled nutritional stress, and only one seedling showed typical black rot symptoms. In a survey of weeds in and around cabbage fields, *X. c.* pv. *campestris* was identified on leaves of *Coronopus didymus* and *Capsella bursa-pastoris* but not on noncruciferous weed species. In all cases, *X. c.* pv. *campestris* was isolated from samples showing positive ELISA readings, and pathogenicity of the strains was confirmed by inoculation.

DISCUSSION

The advantage of using a solid-phase serological assay rather than cultural methods or pathogenicity tests to detect bacterial plant pathogens in host tissue is primarily the reduced assay time required to process large numbers of samples. The DAS-ELISA method requires only 4–5 hr to obtain results, whereas culturing requires 3–5 days. Pathogenicity tests require 6–7 days for initial symptom production and an additional 10–21 days to confirm results. In addition, the ease with which several hundred samples can be handled far surpasses culturing onto media. Microtiter plates can be sensitized with the primary antibody 1 day to several months before field sampling and stored in the refrigerator until use. We observed no loss of reactivity when plates were stored 6 mo. Loss of activity may occur thereafter, and plates were unreliable after 1 yr of refrigeration.

The current protocol is more desirable than the ELISA inhibition test described earlier (3), because the test is simpler and gives more consistent results. Furthermore, 5-AS has advantages over *o*-phenylenediamine because the latter is photosensitive, requires special handling, and is reported to have mutagenic properties (4,9,17). The peroxidase-5-AS system also has advantages over the alkaline phosphatase-*p*-nitrophenyl phosphate system because the light yellow color produced by the latter reaction is more difficult to evaluate without the aid of a microtiter plate reader. The purple-brown color developed by the chromophore of 5-AS facilitates visual evaluations when test samples are compared with a twofold dilution series of a suspension of the reference strain. A terminator is not required to stop the reaction and is considered undesirable because the spectrum of the chromophore changes (5,9).

Predictive values of positive (93–97%) and negative (91–95%) results are within the range achieved by direct isolation and pathogenicity tests and are within the limits acceptable for clinical tests (12,16). Although serological methods detect both live and dead cells, the number of false positives produced by ELISA was not significantly greater than that produced by pathogenicity tests of direct-isolation methods. False positives may reflect low populations of the pathogen present on or in apparently healthy leaves.

The DAS-ELISA method is not as sensitive as immunofluorescence for detecting bacteria in seed and soil (8,13,14). However, it is sufficiently sensitive to detect levels of bacteria commonly associated with a variety of lesion types, as well as heavily decayed tissues, and the test may be performed without a fluorescence microscope.

The assay is limited by the degree of specificity of the antisera. The sheep and rabbit antisera produced by conventional methods for this assay reacted with some strains of *X. c.* pv. *vesicatoria*, pv. *vitians*, and pv. *armoraciae*. Nonspecific reactions have been reported previously (8,15), and they could not be removed by preabsorption with heterologous strains because preabsorption destroyed the reactivity of the antiserums with crucifer strains. Schaad (15) found that *X. c.* pv. *vesicatoria* was related to *X. c.* pv. *campestris* on the basis of immunological comparisons of intact ribosomes. Since pathovars are host-specific, nonspecific reactions with pv. *vesicatoria* or pv. *vitians* should not confound results of field studies unless tomato and lettuce are grown near the cabbage fields under study. On the other hand, nonspecific reactions with *X. c.* pv. *armoraciae* are a serious limitation of this assay since pv. *armoraciae* also affects cabbage and other crucifers. Monoclonal antibodies were produced in the attempt to resolve this problem, and specific antibodies were produced that clearly separated 244 strains of *X. c.* pv. *campestris* from all the other pathovars tested except two strains of pv. *armoraciae* (1). This indicates that *X. c.* pv. *campestris* and pv. *armoraciae* are closely related. Further work is under way to clarify their serological relationships.

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