

Strains of *Xanthomonas campestris* pv. *campestris* with Atypical Pigmentation Isolated from Commercial Crucifer Seeds

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ABSTRACT

Poplawsky, A. R., and Chun, W. 1995. Strains of *Xanthomonas campestris* pv. *campestris* with atypical pigmentation isolated from commercial crucifer seeds. *Plant Dis.* 79:1021-1024.

Assays of commercial crucifer seed using a semiselective agar medium yielded three bacterial strains that were similar to the black rot pathogen except for atypical pigmentation. Cultural traits and carbon source utilization (BIOLOG) identified these strains, along with a previously isolated, nonpigmented strain, as *Xanthomonas campestris*. Further characterization by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membrane proteins, monoclonal antibody typing, and host symptomatology indicated that the first three strains were *X. campestris* pv. *campestris*. Wild-type *X. c.* pv. *campestris* strains show a standard DNA hybridization pattern for the pigment-encoding region (*pig*). However, the three atypical strains each showed one of two different molecular rearrangements in *pig*, indicating multiple mutational events. Over a 4-year period, atypically pigmented *X. c.* pv. *campestris* strains occurred in 1.8% of the samples that were positive for the pathogen. Thus, in screening crucifer seed for *X. c.* pv. *campestris* strains, it is necessary to test for nonpigmented and partially pigmented strains in addition to those with typical yellow pigmentation.

Additional keywords: mutants, seedborne pathogen, xanthomonadins, yellow pigments

Black rot is a persistent problem in crucifer production worldwide (19). The causal agent, *Xanthomonas campestris* pv. *campestris*, is a seedborne vascular pathogen that causes progressive vein blackening, chlorosis, and leaf wilt (14). The production in culture of yellow pigments (xanthomonadins), extracellular polysaccharide slime (EPS), and starch-degrading enzyme are diagnostic for preliminary identification of the pathogen. These three typical traits, however, should not be considered inviolate since mutations may occur at a high frequency due to mechanisms such as the insertion of transposable elements (5). Transposable elements were recently discovered in *X. c.* pv. *campestris* (11), as well as *X. c.* pv. *vesicatoria* (7).

In conducting tests of crucifer seed for the presence of *X. c.* pv. *campestris*, bacterial strains possessing all three of the typical traits are tested further for pathogenicity (14). However, in 1990 we also started pathogenicity testing of strains positive for the production of EPS and starch-degrading enzyme, but lacking typical yellow pigmentation. This work describes the observation, frequency, and characterization of these strains during the 4-year period from 1990 to 1993. We

sought to determine whether these strains were *X. c.* pv. *campestris* and, if so, whether atypical pigmentation resulted from the same or different mutational events.

MATERIALS AND METHODS

Isolation and characterization of atypical strains. Forty-gram samples of seed from commercial seedlots were washed at 4°C for 4 h, and dilutions from concentrated washes were plated on the semiselective agar medium FS(M), as described previously (14). Suspect colonies were further characterized by visual determination of starch degradation on nutrient starch agar (15), and EPS production on yeast extract-dextrose-calcium carbonate medium (YDC) (20). Yellow pigments (xanthomonadins) were also observed on YDC, and quantified by extraction in boiling methanol and measurement of spectrophotometric absorbance at 441 nm (16,18).

Pathogenicity was determined by inoculation of leaf mid-veins of 2- to 3-week-old cauliflower or cabbage plants with a syringe and 26 gauge needle (16). Plants were then incubated for 10 days in a temperature-controlled (25°C) dew chamber (Percival Mfg. Co., Boone, Iowa) on a 16-h photoperiod. Bacteria were reisolated by grinding symptomatic leaves in sterile phosphate buffer (0.01 M, pH 7.0), and dilution plating on FS(M).

Biochemical and molecular techniques. Membrane proteins were isolated using sonication and differential centrifugation (13). Protein preparations were

quantified using the Bio-Rad Protein Assay (Bio-Rad Chemical Division, Richmond, Calif.), and 15- μ g aliquots from each strain were separated on sodium dodecyl sulfate (SDS) 12% polyacrylamide gels (3,8). For identification using carbon source utilization (BIOLOG), strains were cultured on Biolog Universal Growth Medium, and all manipulations were performed with GN MicroPlates according to the manufacturer's instructions (BIOLOG Inc., Hayward, Calif.).

Bacterial genomic DNA was isolated by standard procedures, and plasmid (pIG102) DNA was isolated using alkaline lysis (4). Standard methods (9) were used for restriction endonuclease digestion, agarose gel electrophoresis, and Southern transfer to Zeta-Probe nylon membranes (Bio-Rad Laboratories, Richmond, Calif.).

The previously cloned pigment-encoding region (*pig*) from *X. c.* pv. *campestris* was used as a DNA probe (Fig. 1). The probe was obtained by digestion of pIG102 with *Eco*RI and *Hind*III, electrophoresis in SeaKem GTG agarose (FMC BioProducts, Rockland, Maine), and electro-elution of the three nonvector digestion products in dialysis membranes (4). Subsequently, the 10.3-, 8.5-, and 5.7-kb fragments were labeled with digoxigenin using the Genius kit (Boehringer Mannheim Corp., Indianapolis, Ind.). Total cellular DNA was digested with *Eco*RI and *Hind*III, separated on an agarose gel and transferred to nylon membranes. Southern hybridizations, subsequent washes, and detection of hybrids were performed as recommended by the manufacturer (no formamide and 68°C, and two 15-min washes with 0.1 \times SSC [1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% SDS at 68°C, respectively).

Atypical strains were tested with monoclonal antibodies 197, X1, and X21 in the laboratory of A. M. Alvarez (University of Hawaii at Manoa, Honolulu) using previously described enzyme-linked immunosorbent assay methods (21). MAb 197 was prepared against *Pseudomonas solanacearum* (A. M. Alvarez, personal communication), and was used to assess background levels of reaction. MAb X1 is specific to the genus *Xanthomonas* (1), and MAb X21 reacts with most strains of *X. c.* pv. *campestris* and a few strains of *X. c.* pv. *amoraciae*, but with no other pathogens, species, or genera (2). Non-*Xanthomonas* and *X. c.* pv. *campestris* controls were run in all tests.

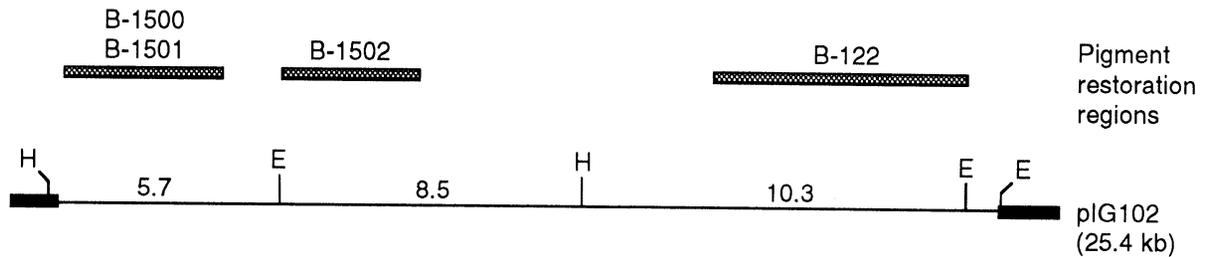


Fig. 1. The cloned *X. c. pv. campestris* pigment-encoding region (*pig*, thin line), and regions that restored pigmentation to the strains with atypical pigmentation (from 12). Abbreviations are as follows: H, *Hind*III; E, *Eco*RI. Numbers refer to sizes in kilobase pairs.

Table 1. Characteristics of standard *Xanthomonas campestris* pv. *campestris* strains and atypical strains

Strain	Origin/source	Pigment ^w	Starch ^x	EPS ^y	Pathogenicity ^z
<i>X. c. pv. campestris</i>					
B-293	ATCC33913/ATCC	+ 0.49 a	+	+	+
B-24	Oreg., U.S./L. Moore	+ 0.38 b	+	+	+
Atypical strains					
B-122	Ariz., U.S./R. Morrison, 1984	- 0.04 d	+	+	±
B-1500	Western U.S./this study	± 0.19 c	+	+	+
B-1501	Western U.S./this study	± 0.20 c	+	+	+
B-1502	Western U.S./this study	- 0.04 d	+	+	+

^w + = yellow; ± = light, creamy yellow; - = white. Numerical values are A_{441} measurements of methanol extracts, averages of two replicates. Values followed by different letters were statistically different [LSD(0.05) = 0.034].

^x + = a clear zone, indicating starch hydrolysis was observed on nutrient starch agar medium.

^y Extracellular polysaccharide slime: + = mucoid appearance on yeast extract-dextrose-calcium carbonate agar medium.

^z + = typical symptoms 5 to 7 days after inoculation of cauliflower included chlorosis and small black lesions spreading throughout the entire vascular system of the leaf; ± = chlorosis and large necrotic lesions limited to major leaf veins.

RESULTS

Recovery of atypical strains. From 1990 to 1993, 161 of 548 crucifer seed samples tested yielded bacterial strains possessing the three typical cultural traits as well as pathogenicity in crucifer. However, three samples yielded strains that appeared bluish-white instead of green on FS(M) medium, produced copious amounts of EPS, hydrolyzed starch, but did not develop typical yellow pigmentation on YDC. These three strains resembled a previously isolated strain with atypical pigmentation (B-122, Table 1). Strains B-1502 and B-122 were white, and strains B-1500 and B-1501 were white initially, but became light, creamy yellow after several days in culture. The A_{441} measurements of methanol extracts confirmed the two different levels of pigmentation (Table 1). Each strain originated from a different seedlot in which no pathogenic strains with normal pigmentation were detected. All strains except for B-122 caused chlorosis, vein blackening throughout the entire vascular system of the leaf, and an eventual dry wilt of these leaves. Plants inoculated with strain B-122 developed large, necrotic lesions along leaf mid-veins near the inoculation points, but never developed vein blackening. In all cases, the atypical strains were recovered from symptomatic leaves of plants.

Characterization of membrane proteins. SDS-polyacrylamide gel electrophoresis (PAGE) of membrane protein preparations from the two *X. c. pv. campestris* standard strains B-24 and B-293 detected over

25 proteins in the 14.2 to 50 kDa size range (Fig. 2). These strains were identical except for protein bands at approximately 16 and 27 kDa. Each of the atypical strains, except for B-122, showed from zero to two protein band differences from the standard strains. Strain B-122 and each of the other *X. campestris* pathovars showed approximately ten or more protein band differences from the *X. c. pv. campestris* standard strains.

Carbon source utilization studies. The two standard strains and the four atypical strains were identified as *X. campestris* with a similarity coefficient of 0.70 or higher. Several different pathovar designations were indicated for these strains, none of which was *pv. campestris*.

pig hybridization patterns. The standard *X. c. pv. campestris* strains had hybridizing fragments of 10.3, 8.5, and 6.4 kb, and each of the atypical strains exhibited an altered hybridization pattern (Fig. 3). Strain B-122 had the greatest alterations, retaining only the 6.4-kb fragment, with the addition of two new strongly hybridizing bands (6.9 and 12.5 kb). Strain B-1502 had a pattern similar to the standard strains retaining the 10.3- and 6.4-kb fragments, but with loss of the 8.5-kb fragment and addition of 11.5- and 1.9-kb fragments. Strains B-1500 and B-1501 showed identical patterns with the retention of the 10.3- and 8.5-kb fragments, loss of the 6.4-kb fragment, and the addition of 9.3- and 2.0-kb fragments.

Monoclonal antibody reactions. Average absorbance values for reaction of the *P. solanacearum* MAb 197 were less than

0.100 with each of the four atypical strains. With the *Xanthomonas*-specific antibody MAb X1, non-xanthomonads had an average absorbance value of 0.103, known *X. campestris* strains had an average absorbance value of 0.976, and each of the four atypical strains had an average absorbance value of 0.571 or higher. Average absorbance values for reaction of MAb X21 (specific for most strains of *X. c. pv. campestris* and a few strains of *X. c. pv. amarae*) were 0.124 with non-xanthomonads, 0.480 with known *X. c. pv. campestris* strains, and 0.544 or higher with each of the atypical strains except B-122, which gave a value of 0.118.

DISCUSSION

In screening crucifer seed for *X. c. pv. campestris*, we considered strains with all levels of pigmentation. Three strains with atypical pigmentation were identified and divided into two classes. Strain B-1502 was nonpigmented, whereas pigment production in strains B-1500 and B-1501 was reduced to about 45% of levels typical of the pathogen. Reaction with MAb X1 indicated that these strains belong to the genus *Xanthomonas*, carbon source utilization studies identified them as *X. campestris*, and there are five lines of evidence supporting their designation as *pv. campestris*. First, all strains produced copious amounts of EPS and hydrolyzed starch, which are typical traits of the black rot pathogen (14). Second, the SDS-PAGE membrane protein profiles of these strains were identical to, or almost identical to, the two standard *X. c. pv. campestris* strains, and very different from those of ten other *X. campestris* pathovars. Previously (10), the greatest homogeneity in membrane protein profiles of fully pathogenic *X. c. pv. campestris* strains was found in the size range below 50.0 kDa; thus, we analyzed the proteins in this size range. The prior investigation showed that 26 fully pathogenic strains of *X. c. pv. campestris* had nearly identical membrane protein profiles, and these were easily distinguishable from those of other gram-negative, rod-shaped bacteria including seven other pathovars of *X. campestris*. Third, these strains showed *pig* restriction fragment length polymorphism (RFLP) patterns similar to those previously observed with fifteen known strains of *X. c.*

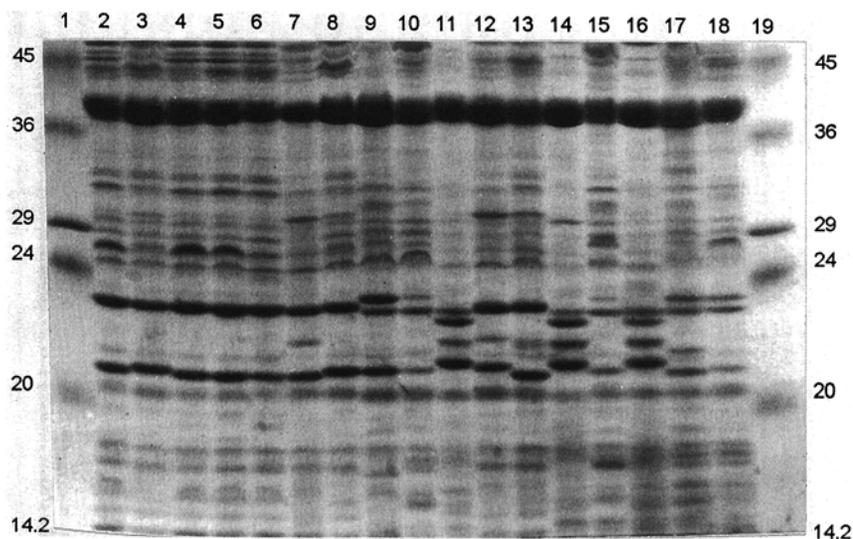


Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membrane proteins. Lanes: 1) protein standards; 2) *X. c. pv. campestris*, B-24; 3) *X. c. pv. campestris*, B-293; 4) B-1500; 5) B-1501; 6) B-1502; 7) B-122; 8) *X. c. pv. campestris*, B-293; 9) *X. c. pv. vesicatoria*, B-272; 10) *X. c. pv. phaseoli*, B-703 = ATCC 43276; 11) *X. c. pv. carotae*, B-804; 12) *X. c. pv. malvacearum*, B-412; 13) *X. c. pv. translucens*, B-451 = ATCC 19319; 14) *X. c. pv. pruni*, B-977; 15) *X. c. pv. citri*, B-769; 16) *X. c. pv. oryzae*, B-719; 17) *X. c. pv. manihotis*, B-901; 18) *X. c. pv. mangiferaeindicae*, JF26.1 = NCPPB 2438; 19) protein standards. Sizes of protein standards are in kDa.

pv. campestris (12; A. R. Poplawsky, unpublished data). Each strain showed two of the three *pig* fragments characteristic of the pathogen (Fig. 3; 10.3-, 8.5-, and 6.4-kb *EcoRI-HindIII* fragments). Fourth, all three strains were positive for reaction with the *X. c. pv. campestris/X. c. pv. amoraciae*-specific MAb X21. Fifth and most important, these strains produced typical black rot symptoms when inoculated into cauliflower or cabbage seedlings, and were subsequently reisolated from the diseased plants.

Since we were aware of only one previously isolated, naturally occurring, pigment-negative strain thought to be *X. c. pv. campestris*, it was of interest to include it in this study also. Although reaction with MAb X1 was positive, carbon source utilization studies identified it as *X. campestris*, and mucoid growth and starch hydrolysis were observed, in other tests strain B-122 was significantly different from the standard *X. c. pv. campestris* strains. The membrane protein profile showed approximately ten differences from the standard strains, the *pig* RFLP pattern had only one of the three fragments typical of the pathogen, reaction with MAb X21 was negative, and host plant symptomatology was atypical. A previous investigator concluded that B-122 was incapable of systemic infection of the host plant (6). Although this strain may not be *X. c. pv. campestris*, there is an alternative explanation for these differences. A 1.3-kb transposable element was previously isolated from this strain, and hybridization studies revealed the presence of at least seven related elements in the B-122 genome (11). Gene inactivation and homologous recombination activities of mul-

tiples, homologous transposable elements may be responsible for the major differences in this strain.

It is generally accepted that *X. c. pv. campestris* is yellow pigmented (14,17,18), and we are unaware of any prior, published accounts of naturally occurring, partially pigmented or nonpigmented strains. In this study, only three of at least 164 strains were atypically pigmented. Thus, these three strains are unusual variants. Since the phenotypic variations were heritable (no changes in pigmentation were observed during repeated subculturing), and these strains could be restored to normal pigmentation by specific DNA clones from a typical strain, they are mutants of *X. c. pv. campestris*. Since the previously isolated strain, B-122, could not be positively identified as *X. c. pv. campestris*, we cannot draw the same conclusion about it.

We observed three differences between strain B-1502 and strains B-1500 and B-1501. Strain B-1502 had a different level of pigmentation, required a different *pig* subclone for pigment restoration, and had a different molecular rearrangement in *pig* than the other two strains. These differences indicate that at least two separate mutational events led to the atypical pigmentation of these three strains. In all cases, the specific region of the molecular rearrangement corresponded to the *pig* subclone capable of pigment restoration. In each of these strains, the *pig* rearrangement could be explained by the insertion of a 4.9-kb element encoding a single *EcoRI* or *HindIII* site. This would explain the loss of one of the three characteristic *EcoRI-HindIII* *pig* fragments, and the addition of two new *pig* homologous fragments with a combined size 4.9 kb

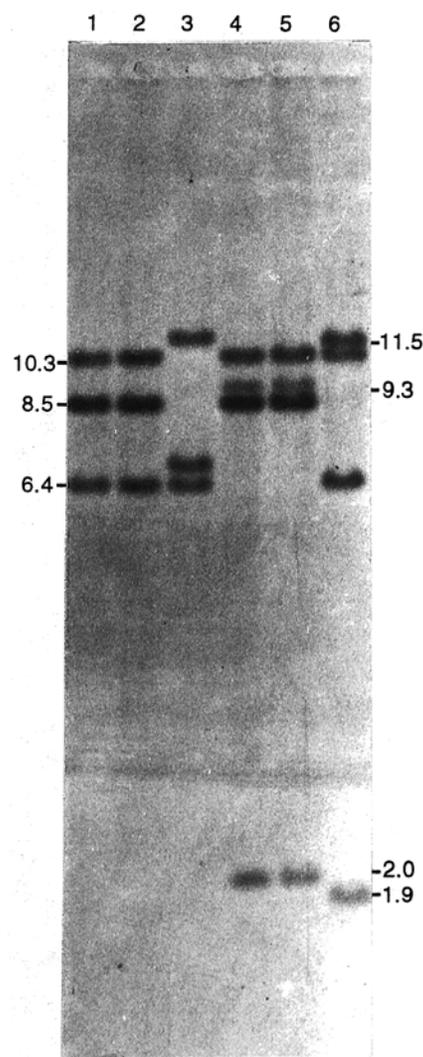


Fig. 3. Southern hybridization of *EcoRI-HindIII* digested total genomic DNA with pigment-encoding region (*pig*) probe DNA. Lanes: 1) *X. c. pv. campestris* B-293; 2) *X. c. pv. campestris* B-24; 3) B-122; 4) B-1500; 5) B-1501; 6) B-1502. Numbers on the left represent sizes of *pig* fragments from the two standard *X. c. pv. campestris* strains. Numbers on the right represent sizes of new *pig* homologous fragments found in strains B-1500, B-1501, and B-1502. All sizes are in kilobase pairs.

greater than the missing fragment. Thus, although atypical pigmentation in *X. c. pv. campestris* was not due to a single isolated genetic event, it may have been caused by the same element.

During the 4 years of this study, three of 164 crucifer seed samples judged positive for black rot harbored *X. c. pv. campestris* strains with atypical pigmentation, and none of these three samples harbored *X. c. pv. campestris* strains with normal pigmentation. Thus, if we had not considered the strains with atypical pigmentation as suspect *X. c. pv. campestris*, these samples would have been incorrectly judged negative for black rot. A different laboratory, which independently tested two of these three seedlots for the pathogen, did judge them to be negative. Thus, in addition to

strains with typical yellow pigmentation, partially pigmented and nonpigmented strains must be considered when testing crucifer seed for the presence of *X. c. pv. campestris*.

ACKNOWLEDGMENT

We thank A. M. Alvarez for performing the monoclonal antibody tests in her laboratory.

LITERATURE CITED

1. Alvarez, A. M., Benedict, A. A., and Mizumoto, C. Y. 1985. Identification of xanthomonads and grouping of strains of *Xanthomonas campestris* pv. *campestris* with monoclonal antibodies. *Phytopathology* 75:722-728.
2. 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.
3. Ames, G. F. L. 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. *J. Biol. Chem.* 249:634-644.
4. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K., eds. 1987. Pages 2.0.1-2.9.10 in: *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.
5. Calos, M. P., and Miller, J. H. 1980. Transposable elements. *Cell* 20:579-595.
6. Kawalek, M. D. 1988. Determining biocontrol of black rot caused by *Xanthomonas campestris* pv. *campestris* through the use of genetically manipulated bacteria. M.S. thesis. University of Idaho, Moscow.
7. Kearney, B., and Staskawicz, B. J. 1990. Characterization of IS476 and its role in bacterial spot disease of tomato and pepper. *J. Bacteriol.* 172:143-148.
8. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
9. Maniatis, T. A., Fritsch, E. F., and Sambrook, J. 1982. Pages 149-186 and 247-268 in: *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. Minsavage, G. V., and Schaad, N. W. 1983. Characterization of membrane proteins of *Xanthomonas campestris* pv. *campestris*. *Phytopathology* 73:747-755.
11. Poplawsky, A. R. 1991. Isolation of a naturally occurring transposable element from a non-pigmented strain of *Xanthomonas campestris* pv. *campestris*. (Abstr.) *Phytopathology* 81:1244-1245.
12. Poplawsky, A. R., Kawalek, M. K., and Schaad, N. W. 1993. A xanthomonadin-encoding gene cluster for the identification of pathovars of *Xanthomonas campestris*. *Mol. Plant-Microbe Interact.* 6:545-552.
13. Qhobela, M., and Clafflin, L. E. 1988. Characterization of *Xanthomonas campestris* pv. *pennamericanum* pv. nov., causal agent of bacterial leaf streak of pearl millet. *Int. J. Syst. Bacteriol.* 38:362-366.
14. Schaad, N. W. 1989. Detection of *Xanthomonas campestris* pv. *campestris* in crucifers. Pages 68-75 in: *Detection of Bacteria in Seed and Other Planting Material*. A. W. Saettler, N. W. Schaad, and D. A. Roth, eds. American Phytopathological Society, St. Paul, Minn.
15. Schaad, N. W., and Kendrick, R. 1975. A qualitative method for detecting *Xanthomonas campestris* in crucifer seed. *Phytopathology* 65:1034-1036.
16. Schaad, N. W., and Stall, R. E. 1988. *Xanthomonas*. Pages 81-94 in: *Laboratory Guide for Identification of Plant Pathogenic Bacteria*. 2nd ed. N. W. Schaad, ed. American Phytopathological Society, St. Paul, Minn.
17. Starr, M. P., Jenkins, C. L., Bussey, L. B., and Andrewes, A. G. 1977. Chemotaxonomic significance of the xanthomonadins, novel brominated aryl-polyene pigments produced by bacteria of the genus *Xanthomonas*. *Arch. Microbiol.* 113:1-9.
18. Starr, M. P., and Stephens, W. L. 1963. Pigmentation and taxonomy of the genus *Xanthomonas*. *J. Bacteriol.* 87:293-302.
19. Williams, P. H. 1980. Black rot: A continuing threat to world crucifers. *Plant Dis.* 64:736-742.
20. Wilson, E. E., Zeitoun, F. M., and Fredrickson, D. L. 1967. Bacterial phloem canker, a new disease of Persian walnut trees. *Phytopathology* 57:618-621.
21. Yuen, G. Y., Alvarez, A. M., Benedict, A. A., and Trotter, K. J. 1987. Use of monoclonal antibodies to monitor the dissemination of *Xanthomonas campestris* pv. *campestris*. *Phytopathology* 77:366-370.