Use of Serology to Detect *Xanthomonas campestris* pv. *pelargonii* in Aqueous Extracts of Geranium Plants

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ABSTRACT

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A direct double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) proved superior to latex bead aggutination (LBA) for rapid detection of *Xanthomonas campestris* pv. *pelargonii* in aqueous extracts of geranium plants. The antisera, obtained from rabbits injected with live cells of *X. c.* pv. *pelargonii*, did not react with strains of *Erwinia, Pseudomonas, Clavibacter*, or *Agrobacterium* in either the DAS-ELISA or the LBA tests. The antiserum reacted strongly with two strains each of *X. c.* pv. *campestris* and pv. *vesicatoria*, but higher values were obtained with strains of pv. *pelargonii*. With the DAS-ELISA test, cells of *X. c.* pv. *pelargonii* were detected in aqueous extracts of plants that had been inoculated and incubated and either developed symptoms or were symptomless but infected. In contrast, none of the extracts of noninoculated plants were positive in the DAS-ELISA. The assays were completed within 24 hr, as compared with the 4–5 days needed for isolation, culture, identification, and inoculation. False-positive or false-negative results were recorded frequently in the LBA assay of the same extracts. Only 56% of the extracts of diseased geranium plants appeared positive.

Bacterial wilt, also known as bacterial stem rot and leaf spot, of cutting geraniums (*Pelargonium* × *hortorum* Bailey) is caused by *Xanthomonas campestris* pv. *pelargonii* (Brown) Dye (5). Symptoms, ranging from leaf spots to discoloration of the vascular tissue, wilt, and plant death, progress most rapidly under high relative humidity and temperature (5,9–12,14,16,22). Under conditions unfavorable for disease development, the bacterium can survive on plants as epiphytic populations or in plants as latent infections. The movement of such asymptomatic plants between greenhouses is the major means of long-distance dispersal of the pathogen in the industry, whereas the cutting and propagation of plants and the use of overhead irrigation readily disperse the pathogen within greenhouses (17).

Ease of dissemination of the bacterium and the potential severity of disease outbreaks increase the importance of effective control strategies. Chemical sprays, such as antibiotics or fixed coppers used for control of some bacterial plant diseases, are ineffective (10). The severity of the disease can be minimized by growing plants at optimum nutritional levels and temperatures (14), but these practices will not stop disease spread, cure diseased plants, or clean contaminated plants. Currently, the only practical control is to avoid the introduction of contaminated plant material into the greenhouse.

Rapid, accurate, and user-friendly techniques are not available for detection of latent infections or epiphytic populations of the pathogen. Available detection involves isolation of the pathogen on nonselective medium, presumptive identification with biochemical and physiological tests, and proof of pathogenicity (Koch's postulates). However, the 4- to 5-day period needed for completion of the necessary tests (not including Koch's postulates) is not compatible with commercial practice. In other host-pathogen systems, selective media (19), phage typing (7), and serology (18,26) are being used. These techniques require much less time than the standard isolation, identification, verification system.

One of the serological techniques, the Ouchterlony double-diffusion test, has been successfully used to identify bacteria found in plant tissue. For example, strains of *Erwinia carotovora* subsp. *atroseptica*, the cause of potato blackleg, were detected in asymptomatic potato tubers with a variation of the Ouchterlony technique. Strains of *E. c.* subsp. *carotovora* commonly found on potato tubers did not trigger a positive response. Ouchterlony tests have also been used to detect *Cornyebacterium sepedonicum* in infected potato and *Rhizobium* sp. in galls in legume roots, as well as to confirm isolation of *Agrobacterium* sp. from stone fruit galls (4,8,13,20). Such tests include sterile technique and laboratory items that are not compatible with commercial practices, however.

Two other quick and inexpensive serological tests that might be used in greenhouse identification of *X. c.* pv. *pelargonii* are the direct double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and the latex bead agglutination (LBA) tests (3). These tests were originally developed for the detection of viruses in plant tissue, but ELISA has been adapted for detection of plant-pathogenic bacteria (2,27). Alvarez and Lou (2) developed a rapid method for identification of *X. c.* pv. *campestris* based on DAS-ELISA. Slack and co-workers (20,21) have examined the use of this test for detection of *C. sepedonicum* in potato tubers. The objective of the research reported here was to examine the potential use of ELISA and LBA tests for the detection of *X. c.* pv. *pelargonii* in geranium plant tissue under greenhouse conditions.

MATERIALS AND METHODS

Antisera production. Antisera were prepared against glutaraldehyde-fixed and live bacterial cells of three pathogenic strains of *X. c.* pv. *pelargonii*, two isolated from geraniums grown in Michigan and one from geraniums grown in Kansas (courtesy of the Kansas State University Department of Plant Pathology). Bacteria were grown in a broth preparation of Lederberg's complete medium (LCM) (15) at 27 C for 3 days. The broth contained 10 g casamino acids, 5 g yeast extract, 3 g K_2HPO_4, and 1 g KH_2PO_4 in 1,000 ml of distilled water. Bacteria harvested from these cultures were washed three times with, and resuspended in, phosphate buffered saline (PBS; 0.01 M potassium phosphate and 0.15 M NaCl, pH 7.2) and then stored at 4 C. Half of these suspensions were used to prepare glutaraldehyde-fixed immunogen and the other half, for live immunogen.

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To fix the immunogen, washed bacteria were dialyzed against 2% glutaraldehyde for 4 hr at room temperature, then against PBS at 4 C for 20 hr. PBS was changed three times at 2-hr intervals during the first 6 hr and then for the final (fourth) time after an additional 14 hr (1). Both glutaraldehyde-fixed and live bacterial preparations were diluted with PBS to a final concentration of 10^6 cells per milliliter based on counts made with a hemacytometer.

A 1.5-ml sample of the appropriate bacterial suspension was emulsified in 1.5 ml of Freund's incomplete adjuvant, and 3-mo-old female New Zealand White rabbits received 2-ml intramuscular injections of the emulsion weekly for 9 wk. Blood was collected for normal sera before injections began, and then every week, beginning 6 days after the fourth injection (28). After collection, the blood was left undisturbed for 4 hr at room temperature, then refrigerated overnight. Serum drawn from the clotted blood was centrifuged at 1,800 *g* for 15 min and stored in sterile glass vials at −15 C.

The titer of the serum obtained from each blood sample was determined by the microagglutination test adapted from Ball (3). A grid was placed on the bottom of plastic petri dishes, and dilutions of the test serum and live bacteria were placed onto the grids. The plates were covered, sealed with Parafilm, and incubated at room temperature for 2 hr. Positive agglutination was detected by observation with a dissection microscope, a dark background, and indirect lighting. The serum with the highest titer in the successive blood samplings was retained for use.

Gamma globulin (IgG) was fractionated as described by Clark and Adams (6) and further purified with DE 23 cellulose. The IgG preparations were stored either at 4 C for less than 14 days or at −20 C for more than 14 days.

Enzyme-linked immunosorbent assay. The IgG was conjugated with alkaline phosphatase, following the methods of Clark and Adams (6). Flat-bottomed microtitration plates (Immulon 1, Dynatech Laboratories, Inc., Alexandria, VA) were coated with a 1:1,000 dilution of IgG in coating buffer (pH 9.6) and incubated for 4 hr at 37 C. Plates were washed three times with 0.01 M PBS containing Tween 20 (PBS-T) (6), after which test samples were added. Plates were incubated 20 hr at 37 C because no reaction was observed within a 4- to 6-hr incubation. After the plates had been washed with PBS-T, 1 mg/ml of 2-nitrophenyl phosphate (Sigma 04 phosphate substrate, Sigma Chemical Co., St. Louis, MO) was added to substrate buffer. Absorbance at 405 nm was measured every 20 min over a 1-hr period with a Dynatech ELISA minireader II. Visual observations were made simultaneously. Each sample was placed in a minimum of five wells in each of five plates for a total of 25 wells per sample. Samples with absorbance readings greater than three times the means of control readings were considered positive.

Latex bead agglutination. The LBA test was performed in microtitration plates by the methods of Van Regenmortel (25). Latex beads (0.81 μm diameter, Difco Laboratories, Detroit, MI) were sensitized with different dilutions of gamma globulin to determine the optimum ratio of antibody to latex beads. Tenfold dilutions of bacteria and sensitized latex beads spotted in a grid design were visually observed for flocculation clumps. Then, 25 μl of sensitized beads of the optimum bead-to-gamma globulin ratio was mixed with 50 μl of antigen in the tests. This mixture of beads and antigen was oscillated at 120 rpm for 15 min and observed for flocculation clumps with a dissection microscope.

Antigen preparation. Samples included suspensions of pure bacterial cultures or aqueous extracts of diseased and pathogen-free plant material. The bacterial suspension cultures used in the serological tests were prepared from 4- to 5-day-old cultures. Each culture was flooded with 5 ml of PBS. After 5 min, sterile glass rods were used to suspend the colonies of bacteria. The suspensions were pipeted into sterile test tubes. Tenfold serial dilutions were made in LCM broth. Colony counts were made in the initial aliquot to determine colony-forming units (15).

Aqueous extracts prepared from plant tissue were ground in PBS-T; 2% polyvinylpyrrolidone (PVP) (w/v) was added as a stabilizer. Tween-HCl PVP buffer (28) was used as a stabilizer in latex bead agglutination tests. The plant samples consisting of less than 1-cm squares of leaves, stems, or petioles were soaked in the buffer approximately 1 hr to allow the bacteria to elute from the tissue.

Specificity and sensitivity of ELISA and LBA assays. Bacteria representing various plant pathogens and soil and water saprophytes were used in the ELISA and LBA tests with rabbit antiserum prepared to *X. c.* pv. *pelargonii* to eliminate the potential for cross-reaction. Preliminary tests were conducted to determine the optimum bacterial concentration needed to obtain an ELISA reaction. The bacterial strains were grown on LCM. A single colony was removed from the plate, suspended in 1.5 ml of PBS, and 10-fold serially diluted. The ELISA tests were conducted with each dilution.

The sensitivity of the serological tests for detection of *X. c.* pv. *pelargonii* in plants with leaf spots and systemic invasion was determined. Half of a group of pathogen-free, 10-wk-old plants were spray-inoculated with 10^6 cfu/ml and stems of the remaining 10 plants were stab-inoculated with 10^6 cfu/ml (29). The two groups were subdivided and one group was stored at 16 C and the other at 24 C, temperatures favorable and unfavorable, respectively, for disease development. Some of the plants were ground in sterile saline buffer and spread onto LCM. Suspect yellow bacterial colonies were subcultured until they appeared to be free from contaminants. The presumptive *X. c.* pv. *pelargonii* colonies were stab-inoculated into geranium stems. Localized browning of the vascular system within 2–3 days constituted a positive reaction (29).

Plants suspected of having bacterial wilt were collected from the Michigan State University Plant Diagnostic Clinic and from growers. These plants were assayed with ELISA, LBA, and our standard bacterial isolation procedures.

A related experiment was carried out using geraniums infected with *Botrytis cinerea*, which produces leaf spots on geraniums that are similar in appearance to the localized lesions produced by the bacterial wilt organism.

RESULTS AND DISCUSSION

Antisera production. Serum taken from blood obtained 10 wk after the first injection of the live-immunogen-injected rabbits had a titer of 1/1,024. Serum taken from blood obtained before or after 20 wk had substantially lower titers. Blood sera from the glutaraldehyde-immunogen-injected rabbits had considerably lower titers than those of sera prepared from the live-immunogen-injected rabbits (1/256 vs. 1/1,024 at 10 wk). After the reduction in titer at week 11, the amount of bacteria in the sixth injection was increased to 1×10^{11} cfu/ml for both types of immunogens. This did not lead to an increase in titer. Blood serum obtained at 10 wk from the live-immunogen-injected rabbits (titer = 1/1,024) and blood serum obtained at 11 wk from the glutaradehyde-immunogen-injected rabbits (titer = 1/512) were purified for use in the serological tests.

Enzyme-linked immunosorbent assay. The DAS-ELISA alpha optimum of the conjugated vs. the normal IgG was determined to be 1/800 and 1/200, respectively. In further tests, longer sample incubation periods led to improved absorbance readings; incubation of the bacteria in the microplate wells for 20 hr provided the highest absorbance readings. Strong color reactions could be visually observed within 10–15 min after adding the enzyme substrate.

Latex bead agglutination. The working ratio of gamma globulin to beads was determined to be between 1:50 and 1:100. Both ratios gave equal responses. No reactions were observed with a 1:25 ratio, whereas numerous false-positive

reactions occurred when the 1:200 ratio was used. All beads used in further tests were sensitized at a 1:100 ratio.

Specificity of serology tests to bacterial strains. High absorbance values in both the DAS-ELISA and LBA tests were recorded when pure cultivars of the xanthomonads were tested. In contrast, negative results were observed when the nonxanthomonads were used at concentrations similar to those of xanthomonads. High populations of bacteria (10^5 to $>10^{10}$ cfu/ml) were used to determine if false-positives might occur (Table 1). However, *X. c.* pv. *pelargonii* was easily detected at populations down to 5×10^3 cfu/ml (Fig. 1). Strong visible color reactions were noted within 5 min after initiation of the DAS-ELISA test with high populations of all *X. c.* pv. *pelargonii* strains, although absorbance readings were not taken until 20 min had elapsed. Thus, an earlier absorbance reading coupled with refinements of the test could presumably allow differentiation of *X. c.* pv. *pelargonii* strains from other xanthomonads. Furthermore, as xanthomonads are host-specific, it is unlikely that pathovars other than *pelargonii* would be found on geraniums in very high populations.

Strains of *X. c.* pv. *pelargonii* from pure cultures also reacted positively in the LBA test (Table 1). Some agglutination occurred with the other xanthomonads but not enough to qualify as a positive test. Determination of positive agglutination proved difficult, however, as the extracellular polysaccharide produced by these cultures appeared to cause the latex beads to string together.

Sensitivity of serological tests to plant tissue. ELISA was more accurate than LBA for detection of *X. c.* pv. *pelargonii* eluted from symptomatic and asymptomatic geraniums (Table 2). Only one of 40 inoculated plants did not appear to harbor populations of *X. c.* pv. *pelargonii* when tested by DAS-ELISA. This pattern was confirmed by direct isolation. In contrast, false-positives were noted among the controls in the LBA tests, and the pathogen was detected in only 56% of the diseased geraniums. In addition, some samples from the pathogen-free plants appeared to react positively.

In the DAS-ELISA, the absorbance was not measured until a color difference was visually observed, as probably would occur if the technique were used commercially. Low bacterial populations in samples may delay the visual reaction in some cases, however. If observations are delayed excessively, the substrate buffer in the control can begin to change color in the absence of alkaline phosphatase.

Both DAS-ELISA and LBA could be completely and relatively quickly used without excessive training or equipment. In our tests, however, DAS-ELISA was more accurate and was not confounded with false-positives. In contrast, false-negatives were never observed. Thus, the DAS-ELISA test for detection of *X. c.* pv. *pelargonii* appeared more compatible with commercial greenhouse operations. Plant growers could test any geranium stock received before introduction of the new stock into "clean" greenhouses. Shipments could be stored in an isolated greenhouse at higher temperatures (24 C) for a week and then a portion of the plants could be tested. A nondestructive sampling of petioles would make the test more attractive to growers. The DAS-ELISA test could also be used by growers who propagate geraniums for resale to ensure that customers receive pathogen-free plants. The test did not appear to be more complicated than the tests currently used in the industry, and many growers probably could run the test

Table 1. Reactions of bacterial strains to antisera prepared to *Xanthomonas campestris* pv. *pelargonii* strains in double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and latex bead agglutination (LBA) tests

Bacteria	log (cfu/ml)	DAS-ELISA[a] (A_{405nm})	LBA reaction[b]
Pathogens			
X. c. pv. *pelargonii*			
strain OLD	4.9	>2.00	−
strain UL	6.3	>2.00	+/−
strain AP10	7.9	>2.00	+
strain OLD	8.9	>2.00	++
strain PG	10.3	>2.00	++
X. c. pv. *campestris*			
strain 1	7.6	1.22	−
strain 2	8.7	1.51	+/−
X. c. pv. *vesicatoria*			
strain 1	5.0	1.82	+/−
strain 2	10.6	1.15	−
Agrobacterium radiobacter			
pv. *radiobacter*	9.5	0.05	−
pv. *tumefaciens* strain k27	9.8	0.02	−
pv. *tumefaciens* strain B6	9.5	0.10	−
pv. *rhizogenes*	ND[c]	0.07	−
Clavibacter michiganense	9.5	0.05	−
Erwinia amylovora	9.0	0.65	−
Pseudomonas syringae			
pv. *phaseolicola* strain 15	8.3	0.07	−
pv. *phaseolicola* strain 35	8.9	0.08	−
Saprophytes			
Acinetobacter lwoffii	8.0	0.12	−
Alcaligenes faecalis	6.3	0.05	−
Pseudomonas fluorescens	8.0	0.63	−

[a] Average absorbance value after a 20-min incubation of plates, average of four trials. A_{405nm} value must be at least three times greater than the control to be considered positive. Control value for all cells = 0.28.
[b] ++ = Strong agglutinations, + = mild agglutinations, +/− = variable reaction, − = no reaction.
[c] Not done.

Table 2. Detection of *Xanthomonas campestris* pv. *pelargonii* in geranium plant tissue using enzyme-linked immunosorbent assay (ELISA) and latex bead agglutination (LBA) tests

Status of plants	No. tested	No. with bacteria isolated onto media[a]	No. positive in ELISA[b]	Av. OD for ELISA	No. positive in LBA
Pathogen-free	10	0	0	0.53	1
Inoculated with *X. c.* pv. *pelargonii*[c]					
Symptomatic	30	30	30	1.73	17
Asymptomatic	10	9	9	1.80	5
With symptoms of *X. c.* pv. *pelargonii* infection[d]	17	17	17	1.85	10
Infected with *Botrytis cinerea*	7	0	0	0.55	0

[a] Standard isolations and tests performed to identify *X. c.* pv. *pelargonii*.
[b] A_{405nm} value three times that of the highest healthy control after 20 min of plate incubation. Control value for healthy sap = 0.40.
[c] Plants were spray-inoculated to induce leaf spotting and stem-inoculated to produce systemic stem rotting.
[d] Symptomatic seedlings and cutting plants collected from the Michigan State University Plant Diagnostic Clinic.

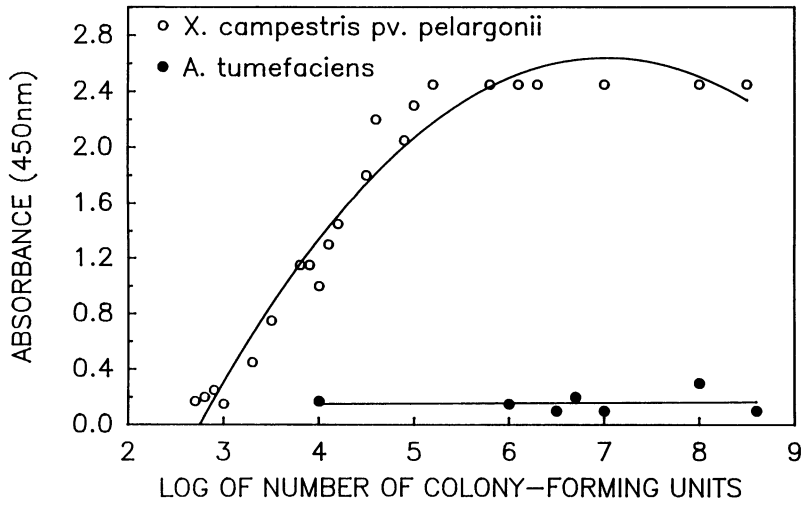

Fig. 1. Absorbance values (A_{450nm}) in double-antibody sandwich ELISA with antisera prepared against intact cells of *Xanthomonas campestris* pv. *pelargonii* strains UL3, OLD 5, and AP10 combined. The number of viable cells per milliliter in the initial suspension was determined by dilution plate analysis on Lederberg's complete medium.

themselves. Any group of plants that test positive could be held in isolation until the diagnosis is confirmed. Although useful to growers as a presumptive test for presence of *X. c.* pv. *pelargonii* in plant stock, the DAS-ELISA test must be refined before it can be used by itself in the industry or in the laboratory.

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