Rapid Identification of *Xanthomonas fragariae* in Infected Strawberry Leaves by Enzyme-Linked Immunosorbent Assay

A. ROWHANI, A. J. FELICIANO, T. LIPS, and W. D. GUBLER, Department of Plant Pathology, University of California, Davis 95616

ABSTRACT

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An indirect enzyme-linked immunosorbent assay using the F(ab′)$_2$ fragment as a trapping antibody for *Xanthomonas fragariae* was developed. The pathogen was detected in extracts of single 4-mm leaf disks containing a 4- to 5-day-old lesion obtained by artificial inoculation or lesions in field collections from plants showing symptoms of angular leaf spot disease. Our antiserum reacted positively with 34 strains of *X. fragariae* but not with 16 of 17 pathovars of *X. campestris* and strains of *Agrobacterium* spp., *Erwinia amylovora, Pseudomonas* spp., *Rhodococcus fascians,* and nonpathogenic bacteria isolated from strawberry plants.

Additional keywords: crown rot, *Fragaria* × *ananassa* Duchesne, vascular collapse

Xanthomonas fragariae Kennedy & King causes an angular leaf spot and occasionally crown rot of strawberry (*Fragaria* × *ananassa* Duchesne). This pathogen is a slow growing, gram-negative bacterium, 0.4 × 1.3 μm with a single polar flagellum (9). Angular leaf spot was first found in Minnesota in 1960 (9) and has since been reported in other parts of the United States (7,8,17) and the world (4,13–16). The pathogen appears to have spread rapidly to other strawberry-growing areas of the world through contaminated planting stocks (12).

Leaf symptoms consist of water-soaked angular lesions on the lower leaf surface that are translucent when viewed with transmitted light and dark green with reflected light. The lesions coalesce and appear on the upper leaf surface as irregular reddish brown spots (10). Symptoms at this stage are difficult to distinguish from leaf spot caused by *Mycosphaerella fragariae* (Tul.) Lindau or leaf scorch incited by *Diplocarpon earlianum* (Ellis & Everh.) F.A. Wolf (12). In addition to the foliar phase, crown rot and vascular collapse of plants was attributed to *X. fragariae* in California (7). Prior to the rapid death of plants, the only observable external symptom was water-soaking at the base of newly emerged leaves. This phase of the bacterial disease has been difficult to distinguish from a vascular collapse caused by *Phytophthora cactorum* (Lebert & Cohn) J. Schröt. (12).

Disease diagnosis is based on symptomatology confirmed by isolation of the pathogen, pathogenicity tests, and/or microscopic observation for bacterial streaming. Symptoms are a useful diagnostic tool at an early stage of lesion development. More importantly, isolation of the causal organism is difficult since *X. fragariae* grows slowly, and faster growing saprophytes often mask its presence in culture plates. Pathogenicity tests are reliable but time-consuming and require two or more weeks to complete. Bacterial streaming is not a sufficiently specific test for *X. fragariae.*

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) has been used to detect plant pathogenic bacteria. The method is simple, specific, rapid, and economical, and is especially useful for routine diagnosis of pathogens in large numbers of samples (1,3,19). DAS-ELISA uses antibacterial antibodies to recover bacteria from the samples by binding them onto an ELISA plate and to detect bound bacteria. Detection is quantitated by a colorimetric reaction catalyzed by an enzyme (e.g., peroxidase, alkaline phosphatase, or other alternate enzymes) conjugated to the detecting antibody, which is applied after the bacterium is bound by a different antibody. Indirect (I)-ELISA compared to DAS-ELISA has the advantage of higher sensitivity and lower background levels (11). This is accomplished by the use of a different animal (e.g., chicken instead of rabbit) as a source of the second antibody. However, it is possible to retain the benefit of I-ELISA while avoiding the necessity for two sources of antiserum by using the F(ab′)$_2$ fragment technique (2,11). This paper describes the application of this methodology for the rapid detection of *X. fragariae* in strawberry.

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MATERIALS AND METHODS

Source and maintenance of plants and bacteria. Strawberries (cv. Pajaro) were grown in pots and kept in the greenhouse, where day length ranged from 10 to 12 hr and the temperature was maintained at 21–25 C. Plants were fertilized once a week with 2.4 g/L of complete fertilizer (24-10-18). These plants were used in pathogenicity tests and as sources of infected leaves for ELISA analyses after inoculation. Field collections of Pajaro were made from a strawberry field plot at University of California, Davis; and collections of cv. Selva were made from a commercial nursery located in San Joaquin County in the Central Valley. Leaves of Selva were collected in December 1991 and stored at −18 C. All other leaf samples were used fresh.

Strains of *X. fragariae* and other bacterial spp. used in these studies are listed in Table 1. Stock cultures were maintained at 1 C and subcultured at 28 C every 10–14 days on fresh medium containing 0.8% nutrient broth, 0.5% glucose, 0.1% yeast extract, 1% potato starch, 2.3% nutrient agar, and 0.005% cycloheximide (NPCA). This medium allowed for rapid multiplication of all test cultures of *X. fragariae* (T. Lips and W. D. Gubler, *unpublished*).

Antiserum production. Strain 61 of *X. fragariae,* isolated from a diseased strawberry plant in Shasta County, California, was cultured on NPCA medium. After 4 days at 28 C, bacterial colonies were suspended in sterile phosphate-buffered saline (PBS) containing 8.0 g of NaCl, 0.2 g of KH_2PO_4, 1.15 g of Na_2HPO_4 anhydrous, and 0.2 g of KCl per liter, pH 7.4, and washed twice by centrifugation at 10,000 *g* for 10 min. Washed cells were heated to 80 C for 10 min and suspended in PBS to give an absorbance value of A_{600nm} = 0.1. This value is equivalent to approximately 10^8 colony-forming units (cfu) per milliliter, as determined by previously plating 10-fold dilutions onto NPCA. The cell suspension was stored at 4 C until use.

A white female New Zealand rabbit was initially immunized with 1.0 ml of cell suspension emulsified with an equal volume of complete Freund's adjuvant followed by a booster injection of a second milliliter emulsified with incomplete adjuvant 14 days after the initial immunization. The injections were administered subcutaneously with 0.2 ml of emulsion in 10 locations along the back of the rabbit. Blood was collected

28 and 34 days after the booster injection, and antibody titers were evaluated by ELISA as outlined below and indirect immunofluorescent method (5). The specific antibody (Ab) and $F(ab')_2$ fragments for ELISA were prepared as previously described (18).

Sample preparation. Antigens from in vitro culturing of the different *Xanthomonas* spp. and other bacterial spp. were prepared by suspending 7- to 12-day-old bacterial cells in extraction buffer (PBS + 2.0% polyvinylpyrrolidone [PVP40], 0.2% bovine serum albumen + 0.05% Tween 20) and adjusting the turbidity of the suspension to $A_{600nm} = 0.1$ (approximately 10^8 cfu/ml). Tissue samples were prepared from fresh and frozen leaf collections with lesions caused by *X. fragariae*. Samples from artificially inoculated plants were first washed in running water for 30 min to remove applied inoculum that might still be on the leaf surface. Naturally infected leaves were used without washing. Disks (4 mm diameter) containing the lesions were excised from artificially inoculated leaves and individually macerated with 0.2 ml of extraction buffer using a mortar and pestle. Naturally infected leaf samples were diluted 1:10 (w/v) in extraction buffer and homogenized with a Polytron. Noninfected controls were prepared in the same manner as the infected samples. To determine if heating improved the sensitivity of the assay, cell suspensions of *X. fragariae* were treated at 80 C for 30 min or not treated and compared by ELISA.

ELISA. The $F(ab')_2$ method of Barbara and Clark (2) was used with some modifications. Polystyrene, flat-bottom, microtiter plates were coated with 200 μl of *X. fragariae*-specific $F(ab')_2$ at 1.0–2.0 μg/ml in coating buffer (1.59 g of Na_2CO_3, 2.93 g of $NaHCOO_3$, and 0.2 g of NaN_3 per liter, pH 9.6) and incubated at 37 C for 1.5 hr. The plates were washed three times in PBST (PBS containing 0.05% Tween 20), spotted with 100–200 μl of the test sample in duplicate wells, incubated overnight at 4 C, and rewashed three times. Then, 200 μl of purified Ab (at 2.0 μg/ml) premixed with protein A-alkaline phosphatase conjugate (BMB at 0.1 μg/ml) diluted in extraction buffer was added, and the mixture was incubated at 37 C for 1.5 hr. After a thorough washing, 200 μl of 0.7 mg/ml of *p*-nitrophenylphosphate in substrate buffer (97 ml of ethanolamine and 1.0 g of $MgCl_2 \cdot 6H_2O$ per liter, pH 9.8) was added to each well. The plates were incubated for 2 hr at room temperature and read in an ELISA reader at A_{405nm}. A positive reaction was determined to be at least 3.5 times higher than the values for healthy tissues and *X. campestris* pv. *campestris* UCD36-63-8 (negative controls).

Inoculation and isolation procedures. Leaves were first dipped in 70% ethanol for 5 sec, then in 0.5% sodium hypochlorite for 1 min and rinsed twice in sterile distilled water. The lesions were excised and plated directly onto NPCA and incubated at 28 C for 5 days or more.

Pathogenicity of the different bacterial strains was tested on greenhouse-grown Pajaro plants inoculated by a spray infiltration procedure (6). The inoculum was applied with an airbrush sprayer at a pressure of 1.0 kg/cm^2 approximately 3 cm from the leaves. The youngest and second youngest fully expanded leaves were sprayed on the lower surfaces with 10^8 cfu/ml of a 24- to 48-hr-old culture suspended in sterile water. Leaves were sprayed for 2–4 sec or until slight water-soaking was apparent. Inoculated plants were incubated under intermittent mist in a greenhouse at 21–25 C, and symptom development was observed up to 14 days.

In a separate test, the minimum inoculum potential to induce symptoms was determined by inoculating leaves with cell suspensions ranging from 10^3 to 10^8 cfu/ml of *X. fragariae* as described above. Symptom development was observed up to 21 days after inoculation.

RESULTS

Serum specificity. In repeated tests, ELISA readings ranged from A_{405nm} = 1.38 to 2.48 with cell suspensions (10^8 cfu/ml) of all 34 strains of *X. fragariae*. At comparable dilutions, strains representing six known species (totaling 44 strains), an unidentified species of *Pseudomonas* (three strains), and nonpathogenic bacterial species isolated from strawberries (eight strains) consistently yielded readings of $A_{405nm} < 0.35$, with the exception of an unidentified isolate of *X. campestris* (A_{405nm} = 0.75) isolated from *Nerium oleander*.

Detection of *X. fragariae*. A single 4-mm leaf disk with a 4- to 5-day-old lesion obtained by artificial inoculation and homogenized in 0.2 ml of buffer provided sufficient numbers of cells to achieve an ELISA reading of 0.59 ± 0.07 nm. The A_{405nm} values almost doubled (0.96 ± 0.05) when single disks from 21-day-old lesions were used. *X. fragariae* was detected in frozen and nonfrozen extracts of naturally infected leaves. Two hundred milligrams of fresh (cv. Pajaro) or frozen (cv. Selva) tissues containing single and coalesced lesions homogenized in 2.0 ml of buffer gave A_{405nm} readings of 1.19 ± 0.45 and 1.95 ± 0.25, respectively. Healthy leaves collected in the greenhouse or field gave A_{405nm} readings of 0.17 ± 0.07. The tests were repeated at least twice for each sample,

Table 1. Reaction of different bacterial strains to an antiserum specific to *Xanthomonas fragariae* strain 61 in ELISA and a pathogenicity comparison

Bacteria	Sources[a]	Total tested	No. tested positive	
			ELISA[b]	Pathogenicity[c]
Agrobacterium rubi	A	1	0	0
A. tumefaciens	A	1	0	0
Erwinia amylovora	A	1	0	0
Pseudomonas sp.	A	3	0	0
P. syringae pv. *syringae*	A	1	0	0
Rhodococcus fascians	A	1	0	0
Xanthomonas campestris				
pv. *campestris*	A,B	9	0	0
pv. *corylina*	A	1	0	0
pv. *dieffenbachiae*	A,D	3	0	0
pv. *incannae*	A	1	0	0
pv. *juglandis*	A	1	0	0
pv. *malvacearum*	B	1	0	0
pv. *pelargonii*	A,D	4	0	0
pv. *phaseoli*	A	6	0	0
pv. *raphani*	A	1	0	0
pv. *syngonii*	A	3	0	0
pv. *translucens*	A	2	0	0
pv. *undulosa*	A	2	0	0
pv. *vesicatoria*	A	4	0	0
pv. *zinnae*	A	1	0	0
Isolated from *Nerium oleander*	A	1	1	0
X. fragariae	A,B,C,D	34	34	34
Xanthomonas spp. Yellow bacteria from strawberry leaves or petioles	A,B	8	0	0
Total strains tested		90	35	34

[a]Strains obtained from: A = California Department of Food and Agriculture, Sacramento; B = Department of Plant Pathology, University of California, Davis; C = A. R. Chase, University of Florida, Apopka; and D = American Type Culture Collection, Rockville, Maryland.

[b]A reaction is termed positive if the A_{405nm} value was 3.5 times or higher than the negative control.

[c]The lower leaf surface was inoculated with approximately 10^8 cfu/ml of a 24- to 48-hr-old culture suspended in sterile distilled water, and symptoms were observed up to 14 days after inoculation.

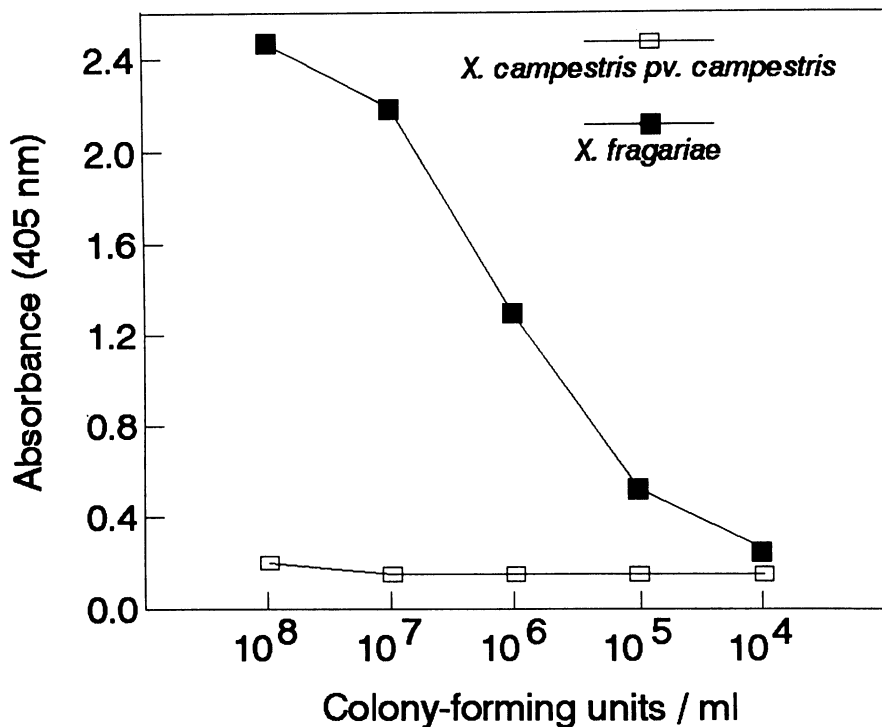

Fig. 1. Absorbance values (A_{405nm}) for enzyme-linked immunosorbent assay (ELISA) of *Xanthomonas fragariae* from American Type Culture Collection (ATCC33239) and *X. campestris* pv. *campestris* from UC Davis (UCD36-63-8) using an antibody prepared from *X. fragariae* strain 61. The number of colony-forming units/ml in the initial suspension (A_{600nm} = 0.1) was determined by standard dilution plating.

and the results were consistent.

Sensitivity of ELISA. The average A_{405nm} values for preparations of *X. fragariae* from the American Type Culture Collection (ATCC33239) were 0.26, 0.52, 1.29, 2.12, and 2.48, for cfu/ml of 10^4, 10^5, 10^6, 10^7, and 10^8, respectively (Fig. 1). The lowest detection threshold for *X. fragariae* under the conditions of the present test was 10^5 cfu/ml. In the same test, the average A_{405nm} value for preparation of *X. campestris* pv. *campestris* from a UC Davis collection (UCD36-63-8) containing 10^8 cfu/ml was 0.19. Sensitivity was not improved by heating the preparation.

Pathogenicity tests. Strawberry leaves sprayed with 10^8 cfu/ml of *X. fragariae* developed typical angular leaf spots in 4–5 days. In contrast, other bacterial species tested at 10^8 cfu/ml were nonpathogenic. However, 21 days of incubation was required for lesions to appear when leaves were inoculated with 10^4 cfu/ml.

DISCUSSION

Although symptoms are useful in field diagnosis of angular leaf spot disease, a confirmatory, objective assay was needed to satisfy certification requirements. ELISA readily detected *X. fragariae* in leaves with visible angular leaf spots. The procedure was simple and rapid compared to isolating the pathogen and performing pathogenicity tests.

In comparison, ELISA was less sensitive than the pathogenicity test. It was sensitive enough, however, to detect *X. fragariae* in extracts of single lesions. Heating the antigen preparations did not enhance activity in ELISA, which is contrary to published reports (1,3).

The simplicity, speed, and specificity of ELISA make it attractive for testing large numbers of field samples. Also, the pathogen can be detected in frozen tissues, which is an added advantage because collections made over an extended period can be safely stored and tested later.

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