Indirect Immunofluorescent Staining for Detection and Identification of *Xanthomonas campestris* pv. *phaseoli* in Naturally Infected Bean Seed

EILEEN M. MALIN, Graduate Assistant, DON A. ROTH, Assistant Professor, Department of Plant Science, and E. LEE BELDEN, Professor, Department of Microbiology and Veterinary Medicine, University of Wyoming, Laramie 82071

ABSTRACT

Malin, E. M., Roth, D. A., and Belden, E. L. 1983. Indirect immunofluorescent staining for detection and identification of *Xanthomonas campestris* pv. *phaseoli* in naturally infected bean seed. Plant Disease 67:645-647.

Indirect immunofluorescence (IF) was evaluated for detecting and identifying *Xanthomonas campestris* pv. *phaseoli* in pure and mixed culture and in Pinto and Navy bean seed. IF was specific for 24 *X. c.* pv. *phaseoli* isolates tested that were collected from diverse geographical locations. Strong positive cross-reacting fluorescence was not observed when antiserum was tested against numerous *X. campestris* pathovars as well as other pseudomonads and common bean seed bacteria. Cells of *X. c.* pv. *phaseoli* were detected on prepared slides in mixed bacterial populations containing as few as 250 colony-forming units (cfu) per milliliter of *X. c.* pv. *phaseoli* and 10^8 cfu/ml of common bean seed bacteria. Cells of *X. c.* pv. *phaseoli* were detected in naturally infected bean seed lots with infection levels of 0.01% and in bean seed leachate to which 10^2 cfu/ml of *X. c.* pv. *phaseoli* were added. IF provides a reliable method for rapid screening of bean seed lots for *X. c.* pv. *phaseoli* although it is not viewed as the sole test for final determination of seed certification.

Common bacterial blight incited by *Xanthomonas campestris* pv. *phaseoli* is a major seedborne disease of dry beans (*Phaseolus vulgaris* L.) (17). Disease management is based primarily on planting of pathogen-free bean seed. Although no data exist correlating percent seed lot infection or inoculum density within individual seeds with field losses, a zero tolerance for *X. c.* pv. *phaseoli* exists in Wyoming certified seed lots. Thus, to aid in compliance with certification tolerances, a sensitive and reliable method is required for detecting *X. c.* pv. *phaseoli* in naturally infected bean seed. Also, especially from an industry standpoint, rapid evaluation of seed is very important.

Currently used tests for *X. c.* pv. *phaseoli* infection of bean seed include pathogenicity tests (9,12,15), bacteriophage typing (3,7), and double-diffusion serological (13) procedures. These procedures are relatively insensitive (9,12,13) and/or time-consuming (3,7,9,12,15).

Various serological procedures have been used for detecting and identifying phytopathogenic bacteria (1,4,6,10,11,13). Schaad (10) used indirect immunofluorescent staining to identify *X. campestris* pv. *campestris*. Trujillo and Saettler (13) combined a semiselective enrichment medium and a double-diffusion assay to detect *X. c.* pv. *phaseoli* in Navy bean seed.

This paper presents information on application of an immunofluorescent staining procedure for detecting and identifying *X. c.* pv. *phaseoli* in pure and mixed cultures and in naturally infected bean seed. A preliminary report has been given (8).

Accepted for publication 22 November 1982.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

MATERIALS AND METHODS

Bacterial isolates. Bacterial isolates used to evaluate serological procedures are listed in Table 1. Cultures were maintained on yeast extract calcium carbonate agar (YDC) (16). Bacteria were subcultured no more than five times past the stock culture (stored at −10 C in YDC in 40% aqueous glycerol or at 4 C in pulverized diseased leaves). Isolates of *X. c.* pv. *phaseoli* (10^6 colony-forming units (cfu) per milliliter were periodically injected into or sprayed onto 10-day-old kidney bean seedlings (cultivar Manitou) and reisolated from developing lesions (9,12).

Preparation of antiserum. Isolate Xp6, obtained from naturally infected Pinto bean seed, was grown in nutrient broth at 25 C for 22 hr on a rotary shaker. Cells were killed by adding an equal volume of 1.25% formal-saline and incubating for 48 hr at room temperature. Killed cell suspensions (culture checked for sterility) were then washed three times in phosphate-buffered saline (PBS, 0.1M potassium phosphate buffer, pH 7.2) centrifuged at 12,000 *g* for 20 min and resuspended in PBS (concentration equivalent to 3×10^8 cfu/ml). Cells (0.5 ml of a 3×10^8 cells/ml suspension) were injected into marginal ear veins of New Zealand white rabbits (2.2 kg). Repeat injections using antigen (0.5 ml of a 3×10^8 cells/ml suspension) as prepared before were made at weekly intervals. After 6 wk, the rabbits were test-bled and the agglutination titer determined by tube agglutination (2). Antisera with end-point titers of 5,120 or greater were used for immunofluorescence reactions.

Antigen preparation. Antigen consisted of cells of *X. c.* pv. *phaseoli* from axenic cultures (as described for immunogen preparation) from naturally infected bean seed collected from symptomatic Pinto bean pods in commercial fields and from artificially infested bean seed. Bean seed samples (1.6 kg) were washed vigorously on a rotary shaker for 5 min in 0.05% Tween 80 suspension and surface-disinfested with 5.25% NaOCl for 5 min. Seed was then rinsed three times in sterile PBS and soaked for 24 hr in sterile PBS. Leachate (about 150 ml) was concentrated to about 10 ml by centrifugation at 12,000 *g* for 15 min. This cell suspension was then used as antigen in immunological procedures.

Uninfected seed lots were collected from Wyoming Foundation seedstocks grown under furrow irrigation and were extensively tested with pathogenicity tests (9,12,15) and dilution plating on a semiselective medium (13) to ensure freedom from *X. c.* pv. *phaseoli*. Artificially infested seed lots were prepared using uninfected seed that was surface-disinfested and rinsed as before. *X. c.* pv. *phaseoli* cells at varying concentrations (10^2–10^8 cfu/ml) were added to these seed lots during the bean seed PBS-soaking phase and centrifuged as described earlier.

Infected seed lots were prepared by adding naturally infected seed (as determined by dilution plating of half-seeds onto semiselective media) at varying levels to uninfected seed lots.

Fluorescent antibody staining procedures. Antigen smears were heat-fixed on slides (Fluoro-Slides, Esco American Scientific Products, McGaw Park, IL 60085) and the indirect immunofluorescent staining method was applied (5). Smears were incubated (30 min) with rabbit antiserum in paper-lined moisture-saturated chambers, rinsed three times

for 30 min in PBS followed by secondary incubation with goat antirabbit globulin conjugated with fluorescein isothiocyanate (FITC), and rinsed three times as before in PBS and once in sterile distilled water. Rhodamine-Bovine serum albumin was added to the goat antirabbit globulin-FITC conjugate as a counterstain. Immunofluorescence (IF) reaction was evaluated on a 0–4 scale where 0 = no fluorescence and 4 = maximum fluorescence. In all tests, normal rabbit serum was used as a negative control. Highest dilutions of rabbit antiserum and goat antirabbit globulin giving maximum fluorescence (1:512 and 1:15, respectively) were determined by block titrations. These standardized reagents were then used to detect antigens. Ten slides were prepared per assay.

Optical equipment. Slides were examined using a Nikon Labophot microscope with a high-pressure mercury ultraviolet (UV) lamp (HBO-50W/AC). A B excitation filter cassette, a B eyepiece-side absorption filter slide, and a UV auxilliary excitation filter were used under epiillumination at a magnification of ×1,000.

Additional detection procedures. Ouchterlony double-diffusion examination of cell suspensions of *X. c.* pv. *phaseoli* and other bacteria both in pure culture and added to bean seed leachate were performed as described by Carpenter (2). Before testing, each bacterial suspension was sonicated for 5 min to facilitate movement of antigen through the agar.

Table 1. Reaction of bacterial isolates to immunofluorescence staining

Species	Isolate	Reaction[a]	Origin	Source[b]
Pseudomonas syringae	HB-16	+1	NE-beans	5
pv. *phaseolicola*	P-33	0	Ontario-beans	8
P. putida		0	WY-unknown	1
P. syringae		0	WY-unknown	1
P. fluorescens		0	WY-unknown	1
P. glycinea		0	WY-unknown	1
P. spp.		0	NC-soil	4
Corynebacterium	CFA-1	0	NE-bean	5
flaccumfaciens	CFA-2	0	NE-bean	5
C. flaccumfaciens	CFV-1	0	NE-bean	5
subsp. *violaceum*	CFV-2	0	NE-bean	5
Xanthomonas	B-4	0	GA-unknown	2
pv. *campestris*	B-24	0	OR-broccoli	2
	B-2	0	GA-kohlrabi	2
	X-53	0	GA-unknown	2
X. campestris	X-46	0	MT-barley	3
pv. *translucens*	X-47	0	MT-barley	3
	X-52	0	MT-barley	3
	X-58	0	MT-barley	3
	X-603	0	MT-barley	3
X. campestris	B-202	0	OR-pepper	2
pv. *vesicatoria*	B-210	0	GA-unknown	2
	B-218	0	DE-tomato	2
Unidentified	1	0	WY-bean seed	6
bean seed	2	+1	WY-bean seed	6
bacterium	3	+1	WY-bean seed	6
	4	+1	WY-bean seed	6
		+1	WY-bean seed	6
	6	+1	WY-bean seed	6
	7	+1	WY-bean seed	6
	8	+1	WY-bean seed	6
	9	+1	WY-bean seed	6
	10	0	WY-bean seed	6
X. campestris	XP 6	+4	WY-bean seed	6
pv. *phaseoli*	XP 2	+4	WY-bean seed	6
	XP 8	+4	WY-bean seed	6
	XP 7	+4	WY-bean seed	6
	XP 9	+4	WY-bean seed	6
	N-6	+4	WY-bean seed	6
	N-14	+4	WY-bean seed	6
	N-13	+4	WY-bean seed	6
	10198	+4	ATCC-bean	7
	BBL-44	+4	Ontario-bean seed	8
	BBL-62	+3	Ontario-bean seed	8
	BBL-68	+4	Ontario-bean seed	8
	BBL-69	+4	Ontario-bean seed	8
	BBL-71	+4	Ontario-bean seed	8
	BBL-80	+4	Ontario-bean seed	8
	BBL-81	+4	Ontario-bean seed	8
	BBL-21	+4	Ontario-bean seed	8
	BBL-24	+4	Ontario-bean seed	8
	BBL-37	+4	Ontario-bean seed	8
	BBL-70	+4	Ontario-bean seed	8
	P-60	+4		
	103 NCPPB 1011	+3	Rumania-bean	8
	376 NCPPB E1388	+3	Hungary-bean	8
	XP 10	+4	South Dakota-bean seed	6

[a] Reaction scale: 0 = no fluorescence; +1 = slight fluorescence; +4 = maximum fluorescence.
[b] 1 = Dept. of Microbiology and Veterinary Medicine, University of Wyoming, Laramie; 2 = N.W. Schaad, Dept. Plant & Soil Sciences, University of Idaho, Moscow; 3 = D. Sands, Dept. Plant Pathology, Montana State University, Bozeman; 4 = S. Williams, Dept. Plant Sciences, U.W., Laramie; 5 = M. L. Schuster, Dept. Plant Pathology, University of Nebraska, Lincoln; 6 = Authors; 7 = ATCC; 8 = J. W. Sheppard, Ottawa, Canada, K1A 0C5.

RESULTS

Specificity of antiserum. All 24 *X. c.* pv. *phaseoli* isolates reacted positively (+3,+4) with antiserum in IF tests (Table 1). Similarly, eight *X. c.* pv. *phaseoli* isolates tested in Ouchterlony double-diffusion tests showed strong positive reactions (three or more bands). No strong (+3,+4) cross-reactive fluorescence was observed with 33 non-*X. c.* pv. *phaseoli* bacterial isolates including isolates of numerous *X. campestris* pathovars and common bean seed bacteria (Table 1), although 27% of the isolates showed weak (+1) IF reactions. Also, no band formation was observed with four non-*X. c.* pv. *phaseoli* isolates in Ouchterlony double-diffusion tests.

Sensitivity of immunological tests. Sensitivity was evaluated using mixed bacterial populations and naturally infected and artificially infested bean seed. As few as 250 cfu/ml of *X. c.* pv. *phaseoli* cells were detected using IF in mixed populations containing 10^8 cfu/ml of common bean seed bacteria. Also, *X. c.* pv. *phaseoli* cells were detected by IF from Navy and Pinto bean seed lots with natural *X. c.* pv. *phaseoli* seed infection levels of 0.01% (one infected seed in 9,999 uninfected seeds) and in seed leachate artificially infested with *X. c.* pv. *phaseoli* at 10^2 cfu/ml (the lowest density tested). A strong positive fluorescence (+3,+4) from one *X. c.* pv. *phaseoli* cell was sufficient for confirming detection by IF, based on the zero tolerance certification standard that currently exists for this pathogen in certified bean seed lots.

Ouchterlony double diffusion combined with a semiselective medium assay (13) failed to detect *X. c.* pv. *phaseoli* in five infested bean seed lots where fewer than 10^9 cfu/ml of *X. c.* pv. *phaseoli* were added to 1.6 kg of uninfected seed during PBS soaking. In one test (two replicates) using seed lots with natural seed infection levels ranging from 10 to 0.01%, this assay (13) did not detect *X. c.* pv. *phaseoli* in lots with levels lower than 1.0% seed infection.

DISCUSSION

Immunofluorescence is a sensitive procedure for detecting and identifying

phytopathogenic bacteria. Schaad (10) recommended indirect IF over direct IF procedures, based on reliability and simplicity. Allan and Kelman (1) found the indirect IF assay reliable for identifying *Erwinia carotovora* var. *atroseptica* (*Eca*) on prepared slides in mixed cultures containing 500 cells of *Eca* per milliliter mixed with 10^8 cells per milliliter of *E. carotovora* var. *carotovora* and in soil (10 cells of *Eca* per gram of soil). Similarly, indirect IF is a rapid and sensitive procedure for detecting and identifying *X. c.* pv. *phaseoli* in pure and mixed cultures and in naturally infected bean seed lots.

Postharvest detection and identification procedures using double diffusion, phage typing, or pathogenicity testing are deficient in either sensitivity (9,12,13) or rapidity (3,7,9,12,15). Trujillo and Saettler (13) compared their assay combining a semiselective medium and a double-diffusion test with the standard pathogenicity assay used by the Michigan Department of Agriculture (9). They found 61 positive reactions from 175 samples using the latter assay, whereas 90 positive reactions in 175 samples were found using the double-diffusion semiselective soaking medium assay. In addition, Trujillo and Saettler (14) observed strong positive band (three or more bands) formation using their assay against *X. c.* pv. *phaseoli* cell concentrations in pure cultures only as low as 6 $\times 10^6$ cfu/ml (as steamed antigen) and 1 $\times 10^7$ cfu/ml (as live antigen). With the Trujillo and Saettler method (13), we observed positive reactions (three bands) when *X. c.* pv. *phaseoli* concentrations of 10^9 cfu/ml or greater were added to bean seed samples in the semiselective soaking phase. In contrast, the indirect IF assay detected *X. c.* pv. *phaseoli* concentrations as low as 10^2 cfu/ml in bean seed leachate.

Although bacteriophage assays (3,7) are highly sensitive (to 0.01% seed infection; J. Sheppard, *personal communication*), they are time-consuming, taking 10–14 days to complete. Similarly, pathogenicity (9,12,15) assays generally take 14–21 days to complete. Also, these procedures require extensive laboratory or greenhouse space and one (15) may not adequately account for ungerminated but infected seed in samples. The fact that IF is rapid (28 hr) and can be used by personnel without advanced training is a definite procedural asset. Compared with the IF assay, even double-diffusion assays are time-consuming (+40 hr), especially when numerous samples are tested simultaneously.

Complementary IF procedures to detect *Pseudomonas syringae* pv. *phaseolicola, P. syringae* pv. *syringae*, and *Corynebacterium flaccumfaciens* are necessary in areas where these bean seed pathogens are of concern to seed certification agencies.

Schaad (11) has correctly observed that use of specific antigens for antibody production is desirable. This consideration, however, must be moderated by the circumstances for which the procedure is developed. Relative to bean seed testing for *X. c.* pv. *phaseoli* infection, numerous undesired or potentially cross-reacting bacteria are eliminated by washing and rinsing procedures. No strong positive cross-reacting fluorescence was observed in testing of many non-*X. c.* pv. *phaseoli* isolates including common bean seed-colonizing bacteria and in over 40 bean seed tests. If positive cross-reacting fluorescence develops, however, absorption with cross-reacting antigens should eliminate undesired fluorescence. Our antiserum has been tested against numerous *X. c.* pv. *phaseoli* isolates and was strongly positive in all cases. Similarly, Trujillo and Saettler (14) used whole *X. c.* pv. *phaseoli* cells as the injected antigen and found the resultant antiserum to be highly specific for nonhomologous *X. c.* pv. *phaseoli* isolates (number unspecified) in double-diffusion tests.

The indirect IF procedure was developed for use by the Wyoming Crop Improvement Association and other certification agencies to help eliminate *X. c.* pv. *phaseoli*-infected bean seed lots from certification. Currently, one or more field inspections are carried out before certification. These inspections remain the foundation for certification efforts; however, postharvest testing of representative seed lots with the indirect IF technique offers a rapid method for initial screening of seed. Samples passing field inspections and giving a negative reaction with IF would be eligible for certification. Samples passing field inspections but giving positive IF reactions, however, would require further testing by pathogenicity (9,12,15) or, particularly because of sensitivity levels, bacteriophage (3,7) procedures before certification judgment. Thus, a positive IF assay of dead or cross-reacting cells would be an error on the safe side and would be corrected by results of subsequent assays. With this IF assay, large numbers of bean samples can be screened rapidly and seed lots with moderate to severe infection levels can be eliminated from certification.

Numerous questions relative to the influence of various *X. c.* pv. *phaseoli* inoculum levels in seed and seed lots under field conditions exist that may moderate future testing confidence levels and certification tolerance levels.

LITERATURE CITED

1. Allan, E., and Kelman, A. 1977. Immunofluorescent stain procedures for detection and identification of *Erwinia carotovora* var. *atroseptica*. Phytopathology 67:1305-1312.
2. Carpenter, P. L. 1975. Immunology and Serology. 2nd ed. Saunders, Philadelphia. 346 pp.
3. Ednie, A. B., and Needham, S. M. 1973. Laboratory test for internally-borne *Xanthomonas phaseoli* var. *fuscans* in field bean (*Phaseolus vulgaris*) seed. Proc. Assoc. Off. Seed Anal. 63:76-82.
4. French, W. J., Stassi, D. L., and Schaad, N. W. 1978. The use of immunofluorescence for the identification of phony peach bacterium. Phytopathology 68:1106-1108.
5. Goldman, M. 1968. Fluorescent Antibody Methods. Academic Press, New York. 303 pp.
6. Gutherie, J. W., Huber, D. M., and Fenwick, H. S. 1965. Serological detection of halo blight. Plant Dis. Rep. 49:297-299.
7. Katznelson, H., and Sutton, M. D. 1951. A rapid phage plaque count method for the detection of bacteria as applied to the demonstration of internally-borne bacterial infections of seed. J. Bacterial. 61:689-701.
8. Malin, E. M., Roth, D. A., and Belden, E. L. 1982. Immunological procedures for the detection of *Xanthomonas phaseoli*. (Abstr.) Phytopathology 72:944.
9. Saettler, A. W. 1971. Seedling injection as an aid in identifying bean blight bacteria. Plant Dis. Rep. 55:703-706.
10. Schaad, N. W. 1978. Use of direct and indirect immunofluorescence tests for identification of *Xanthomonas campestris*. Phytopathology 68:249-252.
11. Schaad, N. W. 1979. Serological identification of plant pathogenic bacteria. Annu. Rev. Phytopathol. 17:123-147.
12. Schuster, M. L. 1955. A method for testing resistance of beans to bacterial blight. Phytopathology 45:519-520.
13. Trujillo, G., and Saettler, A. W. 1979. A combined semi-selective medium and serology test for the detection of Xanthomonas blight bacteria in bean seed. J. Seed Technol. 4:35-41.
14. Trujillo, G., and Saettler, A. W. 1981. Production and characterization of antisera to *Xanthomonas phaseoli* and *X. phaseoli* var. *fuscans*. Pages 2-7 in: Farm Sci. Res. Rep. 414. Michigan State Experiment Station, East Lansing.
15. Venette, J. 1978. Disease and bean seed certification. N.D. Seed J. December.
16. 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.
17. Zaumeyer, W. T., and Thomas, H. R. 1957. A monographic study of bean diseases and methods for their control. U.S. Dep. Agric. Bull. 868. 255 pp.