Evaluation of Indirect Immunofluorescence and Ice Nucleation Activity as Rapid Tests for Identifying Foliar Diseases of Tomato Transplants Incited by Fluorescent Pseudomonads

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ABSTRACT

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Indirect immunofluorescence (IIF) and ice nucleation activity (INA) were evaluated for detection and identification of two bacterial foliar pathogens of tomato transplants. When 127 lesions from 11 transplant fields and two research plots were triturated in water and tested by IIF with two antisera prepared against *Pseudomonas syringae* pv. *tomato* (PST), 74% reacted positively with one antiserum. Similar results were obtained when the IIF procedure was used on 117 pure cultures isolated from the lesions. Only 24% of the 117 cultures, however, were PST, whereas most of the remainder were *P. syringae* pv. *syringae* (PSS). The antisera (one was cross-absorbed with *Xanthomonas campestris* pv. *vesicatoria* [XCV]) reacted negatively with pure cultures of four strains of XCV and with lesions from artificially inoculated plants. All lesions that yielded PST tested negatively for INA, whereas 76% of those with PSS tested positively. All pure cultures of PST from field plants tested negatively, whereas 95% of PSS cultures were positive. These results suggest that IIF and INA, when used in combination, are useful diagnostic tools for distinguishing PSS from PST on tomato transplants and will assist in certification efforts.

Southern Georgia is a major area for production of field-grown transplants of tomato (*Lycopersicon esculentum* Mill.) that are shipped to the northern United States and Canada. Bacterial foliar diseases cause losses to transplants and tomato crops where transplants are shipped. Historically, bacterial spot caused by *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye (XCV) has been of greatest concern (14). Bacterial speck caused by *Pseudomonas syringae* pv. *tomato* (Okabe) Young, Dye, & Wilkie (PST) was first recognized as an important disease of tomato transplants in southern Georgia in 1978, which coincided with increased incidence of the disease worldwide (3). More recently, a less damaging leaf spot disease caused by *Pseudomonas syringae* pv. *syringae* van Hall (PSS) has appeared in transplant fields (8).

Because Georgia-grown plants may introduce inoculum into northern production fields, stringent certification procedures are needed to minimize the shipment of diseased plants. Rapid and accurate diagnosis is essential in the certification process. Although the three major bacterial foliar diseases of tomato transplants can be identified by isolation followed by a few key determinative tests, the procedures require considerable time (7 days or more). Identification is complicated by the potential presence of two taxonomically similar pseudomonads (PST and PSS) that are considered indistinguishable except by specialized laboratory (4,18) or pathogenicity tests (8).

Earlier studies (1,16,17) have suggested using serodiagnostic assays for rapid detection and identification of certain phytopathogenic bacteria. Hirano et al (5) suggested that ice nucleation activity (INA) may be useful for characterization of certain strains of *P. syringae*. Determination of INA appeared especially useful for separating PSS and PST that may occur on tomato transplants because PSS is generally INA positive and PST is nearly always INA negative (5,8). The purpose of this study was to evaluate indirect immunofluorescence (IIF) and INA as rapid methods for detection and identification of the bacterial foliar pathogens of tomato transplants.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in the studies and described later were grown on either nutrient-yeast dextrose agar (NYDA, nutrient agar 23 g, yeast extract 5 g, dextrose 10 g, and water 1 L, pH 6.8) or medium B of King et al (KMB) (9). They were maintained between studies on slants of NYDA at 6 C.

Production of antisera and serological testing. In earlier studies (7), PST was found to be composed of two major serotypes (serotypes I and II). Antisera were prepared against bacterial strains representative of the two serotypes to facilitate field detection. A strain provided by D. L. Coplin (OARDC, Wooster, OH) was used for serotype I and a strain from tomato transplants in Georgia for serotype II. The strains were grown on KMB for 24 hr at 25 C. Bacterial cells were suspended in 0.85% saline (40 ml/plate) and the resulting suspension was autoclaved for 30 min at 121 C. The autoclaved suspension was centrifuged for 10 min at 12,000 *g* at 4 C and the pellet was washed twice in saline. The cells were resuspended in saline and adjusted to 10^{10} cells per milliliter. One milliliter of each antigen preparation was mixed 1:1 with Freund's incomplete adjuvant and injected intramuscularly

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into New Zealand rabbits at weekly intervals for 3 wk. One week after the final injection, the rabbits were bled at the marginal ear vein. Bleeding was continued at weekly intervals for 4–6 wk.

The IIF staining procedure described by Schaad (19) was used. Prepared slides were examined at ×250 with a Wild Dual Illumination Microscope (Wild Herrburgg Instrument, Inc., Herrburgg, Switzerland) equipped with an HBO-200 mercury vapor lamp (burner) and an oil immersion condensor. The BG12 filter and a barrier filter in the OG1 position were used to provide a suitable wavelength (transmission range above 530 nm).

The antiserum prepared against the serotype I isolate of PST also reacted weakly with certain strains of XCV. This reaction was eliminated by cross-absorption of the antiserum with cells of four strains of XCV. Cultures of four strains of XCV were grown in nutrient broth on a rotary shaker (200 rpm) for 24 hr at 25 C. The cells were harvested by centrifuging for 10 min at 12,000 *g*. The resulting pellet was suspended in 10 ml of antiserum diluted 1:10 with saline. The suspension was incubated 24 hr at 25 C and centrifuged 15 min at 12,000 *g*. The supernatant was recovered and the procedure repeated until no observable microagglutination of the absorbed antiserum and homologous antigen occurred.

Preliminary evaluation of the IIF procedure. Before field evaluation, the two antisera were tested for their reaction against nine strains of PST obtained from widely separated areas of the United States. Four were isolated from tomato transplants in Georgia. One was from tomato seed used for transplant production and four were provided by workers outside Georgia (three from D. L. Coplin, OARDC, and one from M. Sasser, University of Delaware). Efficiency of the IIF procedure for detecting PST in lesions was also tested using plants inoculated in the greenhouse. Chico III plants were inoculated with six strains. Lesions on Chico III tomato plants produced by inoculating with XCV (four strains described earlier) were also tested with the two antisera. Individual lesions were triturated in a drop of sterile distilled water and incubated 20 min at room temperature. Loopfuls of each suspension were placed on IIF slides, treated separately with each antiserum, and examined as described previously.

Ice nucleation testing. Suspensions of bacteria prepared either from foliar lesions collected in tomato fields and/or from pure culture isolated from the lesions were tested for INA by the procedure described by Lindow (12) with certain modifications (8). Suspensions from lesions prepared as described later were pipetted into drops of 0.1 M phosphate buffer (pH 7.0) placed in 6-cm aluminum weighing pans floated on an ethanol-ice water mixture held at −5 to −10 C. Cells from pure cultures grown for 24 hr at 25 C on KMB were suspended (10^8 cells/ml) in phosphate buffer and tested for INA by placing 10 10-μl droplets on the surface of the aluminum pans. A sample was considered to be positive for INA if more than one droplet froze within 30 sec.

Field evaluation of the IIF and INA procedures. The methods were evaluated with lesions suspected of being incited by bacteria for 11 tomato transplant fields in southern Georgia in 1981. Lesion samples were also collected from two tomato field plots near Athens, GA. Five samples of diseased foliage were collected at random from widely separated areas in each field or plot area. Four lesions were removed from different leaves of each sample with a sterile razor blade and two lesions were triturated per drop (0.1 ml) of SDW, giving 10 samples per field. The drops containing triturated tissue were incubated 20 min at room temperature and subsamples of each drop were used as follows: 1) streaked with a loop on a plate of KMB, 2) placed in four wells of IIF slides and stained as described before, and 3) tested for INA as described before. The streaked KMB plates were incubated for 48 hr at 25 C, and individual representative colonies (eight to 11) were transferred to plates of KMB for later testing.

All the pure culture isolates were tested for IIF reaction and INA as described earlier. Also, a series of tests was completed to determine pathogenicity and identify pathogenic strains. Because XCV was not detected or isolated from any of the fields, emphasis was placed on determinative tests (4,15,18) used to separate PST from PSS. To identify the isolates, each one was tested for oxidase reaction (11) using Taxo differentiation disks (BBL, Div. of Becton, Dickinson & Co., Cockeysville, MD 21030), arginine dihydrolase activity by Thornley's medium 2A (20), tobacco hypersensitivity (10) by infiltrating suspensions (10^8 cfu/ml) into leaf sections of *Nicotiana tabacum* 'Hicks,' and finally by inoculating Chico III tomato plants as described previously (8). Other key determinative tests used to separate pathovars of *P. syringae* included use of the organic substrates DL-lactate, D(−)tartrate, and erythritol as described by Misaghi and Grogan (15); pectate degradation at pH 5.1 and 8.3 with Hildebrand and Schroth's medium (4); acid production from sucrose using Hugh and Leifson's procedure (6); and syringomycin production as described by DeVay et al (2) with slight modification (8).

RESULTS

Preliminary evaluation of the IIF procedure. One or the other of the two antisera reacted strongly with all strains of PST tested before field evaluation (Table 1). Antiserum specific to serotype I reacted more strongly with more strains than did serotype II antiserum. Serotype I antiserum gave a moderate to strong reaction with eight of the nine strains of PST, whereas serotype II antiserum reacted similarly with only four strains. Serotype I antiserum was highly useful for detecting PST in lesions of artificially inoculated leaves. PST was detected in 59 of 60 lesions produced by inoculating tomato plants with six strains of PST (Table 1). The only lesion giving negative results was initiated by a serotype II strain. Serotype II antiserum was highly effective (100% of lesions positive) for detecting the strain not detected by serotype I. Both antisera were considered useful for inclusion in the field tests. In tests on lesions induced by four XCV strains, results were negative when either serotype II or cross-absorbed antiserum of serotype I was used (Table 1).

Field evaluation of the IIF and INA procedures. About 74% of the 127 lesions from 13 tomato fields tested gave positive IIF reactions when serotype I antiserum was used, whereas 29% gave positive reactions with serotype II antiserum (Table 2). Samples from 12 fields gave some positive IIF reactions although the percent positive among fields ranged from 50 to 100% for serotype I antiserum

Table 1. Indirect immunofluorescent reactions of lesions infected with strains of *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria* when tested with two antisera

Organism	Strain	Source	Antiserum		
			Serotype I	I[a]	Serotype II
P. syringae pv. *tomato*	BL 13	D. L. Coplin	10/10	8/8	7/10
	BL 36	D. L. Coplin	10/10	ND[b]	7/10
	BL 72	D. L. Coplin	10/10	ND	9/10
	Field 3	S. M. McCarter	9/10	8/8	10/10
	Field 4	S. M. McCarter	10/10	ND	10/10
	Field 7	S. M. McCarter	10/10	ND	8/10
X. campestris pv. *vesicatoria*	GS3	R. E. Stall	8/8	0/8	0/8
	JJ7	J. B. Jones	7/8	0/8	0/8
	FS1	F. M. Shokes	8/8	0/8	0/8
	FS3	F. M. Shokes	7/8	0/8	0/8

[a]Serotype I antiserum absorbed with whole cells of *X. campestris* pv. *vesicatoria* strain FS3.
[b]ND = Not determined.

and from 10 to 100% for serotype II. Neither of the antisera gave a positive reaction with lesions collected from one field and no bacteria were isolated. About 67% of the PST isolates reacted positively with serotype I antiserum and 20% reacted positively with serotype II antiserum.

About 48% of lesions collected from 11 fields (two fields were not tested) gave a positive test for INA (Table 2). Seven of the 11 fields had some lesions that were positive. Sixty-seven percent of pure culture isolates from the lesions in nine fields (designated 1–9) were positive for INA. Lesions from fields 10, 11, 12, and 13 all tested negatively for INA, and all pure cultures from fields 10, 12, and 13 were also negative.

Most (97%) of the 117 pure-culture isolates from lesions produced a green fluorescent water-soluble pigment on KMB, suggesting they were pseudomonads (Table 3). They appeared to be pathogenic types because they were mostly oxidase negative (97%), arginine dihydrolase negative (97%), and tobacco hypersensitive positive (93%). In greenhouse pathogenicity tests, however, only 24% produced typical bacterial speck symptoms on Chico III tomato. All of the 28 isolates that produced typical speck symptoms came from three fields (10,12, and 13). Field 10 was located at Athens and was known before sampling to have plants infected with PST. Plants in field 13, also at Athens, apparently were also infected with PST. Field 12 was the only transplant field in southern Georgia that yielded isolates that caused bacterial speck symptoms on tomato. Further characterization showed all isolates from fields 10, 12, and 13 were like PST because they did not utilize erythritol or DL-lactate nor produce syringomycin but caused strong pitting on pectate medium at pH 5.1. Many of the isolates from the fields 1–9 produced small brown lesions but these were atypical of bacterial speck. Additional determinative tests showed that these isolates were PSS rather than PST because they utilized erythritol and DL-lactate and produced syringomycin but did not cause pitting on pectate medium at pH 5.1.

Table 2. Indirect immunofluorescence (IIF) reaction and ice nucleation activity (INA) in tissue extracts and pure cultures of bacteria from lesions on tomato plants with suspected bacterial diseases

Field designation[a]	No. tissue samples tested	No. pure cultures tested	IIF				INA	
			Tissue		Pure culture			
			Serotype I	Serotype II	Serotype I	Serotype II	Tissue	Pure culture
1	10[a]	11	6	2	6	1	ND[b]	10
2	10	9	8	1	6	5	ND	5
3	10	10		2	6	6	7	9
4	10	10	10	3	7	0	1	8
5	10	10	5	3	0	0	10	9
6	10	10	10	7	10	0	10	10
7	10	10	9	2	7	0	4	9
8	9	9	9	5	8	1	9	8
9	10	10	7	1	10	0	10	10
10	10	10	5	10	0	10	0	0
11	10	0	0	0	ND	ND	0	ND
12	8	8	8	1	8	0	0	0
13	10	10	10	0	10	0	0	0
Total	127	117	94	37	78	23	51	78
Percent positive			74	29	67	20	48	66

[a] All fields except 10 and 13 were commercial tomato transplant fields in southern Georgia. Numbers 10 and 13 were tomato research areas at Athens, number 10 known to have plants infected with *Pseudomonas syringae* pv. *tomato*, and number 13 had a bacterial disease of unknown cause.

[b] Numbers shown denote the number of the total tested that gave positive reactions; ND = not determined.

DISCUSSION

Fluorescent pseudomonads from tomato transplants that were oxidase and arginine dihydrolase negative and tobacco hypersensitive positive were assumed to be PST immediately after the initial outbreak of bacterial speck in 1978. The appearance of PSS as a foliar pathogen on tomato transplants in 1980 (8) complicated identification based on symptoms and required more determinative tests (4,15,18) for separating PSS from PST. Utilization of erythritol or DL-lactate, syringomycin production, degradation of sodium polypectate at pH 5.1, INA, and production of typical bacterial speck symptoms on tomato have been used (4,8) for separating PSS from PST. The present work confirmed these as reliable tests for separating the two pathovars. All these tests except INA required an additional 4–7 days of reaction time for results. INA can be done rapidly from isolation plates without an incubation period (5,12).

Comparisons of results from tests on tissue samples and from work with pure

Table 3. Reactions of bacteria isolated from foliar lesions on tomato in selected biochemical and pathogenicity tests

Field[a] (no. isolates tested)	Number of isolates with positive reaction											
	Tests to determine probable pathogenicity				Determinative tests to differentiate *Pseudomonas* strains							
	Fluorescent pigment	Oxidase	Arginine dihydrolase	Tobacco hypersensitivity	Produced speck symptoms on tomato	Utilization of substrates			Pectate degradation		Acid from sucrose	Syringomycin production
						Erythritol	D(−)tartrate	DL-lactate	pH 5.1	pH 8.3		
1 (11)	10	0	0	10	0	9	10	10	0	0	10[b]	10
2 (9)	8	3	3	5	0	6	0	9	0	0	5	5
3 (10)	10	0	0	9	0	9	8	8	1	0	10	9
4 (10)	9	1	1	9	0	10	10	10	0[b]	0[b]	2[b]	8
5 (10)	10	0	0	10	0	10	10	9	ND[c]	ND	ND	10
6 (10)	10	0	0	10	0	10	10	10	0[d]	0[d]	9[d]	10
7 (10)	10	0	0	10	0	10	10	10	0	0	10	10
8 (9)	9	0	0	9	0	9	9	9	0	1	9	9
9 (10)	10	0	0	10	0	10	10	9	0	0	10	10
10 (10)	10	0	0	10	10	0	10	0	10	0	10	0
12 (8)	8	0	0	8	8	0	8	0	8	0	8	0
13 (10)	10	0	0	10	10	0	10	0	10	0	10	0
Total (117)	114	4	4	110	28	83	105	84	29	1	94	82
Percent positive	97	3	3	93	24	71	90	72	25	<1	94	70

[a] All fields except 10 and 13 were commercial tomato transplant fields in southern Georgia. Numbers 10 and 13 were research areas at Athens, number 10 known to have plants infected with *Pseudomonas syringae* pv. *tomato*, and number 13 had a bacterial disease of unknown cause.

[b] Only three isolates tested.

[c] ND = Not determined.

[d] Only nine isolates tested.

cultures derived from the lesions support the reliability of the IIF and INA procedures for detecting PSS and PST in leaf tissue and for their identification in pure culture. The high percentages of positive IIF reactions with both leaf samples and pure cultures using the two antisera developed for PST suggested PST was the major cause of the foliar leaf spot in the fields assayed. INA of tissue samples coupled with isolation data, however, indicated PSS was the primary organism present in lesions from nine of 13 fields assayed, whereas PST was present in lesions from only three fields. Apparently, the IIF procedure is not specific enough for separating PST and PSS strains that occur on tomato transplants. Earlier work (13,17) that suggests close serological relatedness among pathogenic fluorescent pseudomonads may explain the difficulty involved. Tests with pure cultures and on lesions from artificially inoculated plants, however, gave negative IIF reactions. If similar results occur with lesions from the field, positive IIF tests would eliminate XCV and suggest either PST or PSS as the probable cause.

Attempts to use INA for detecting PST and PSS in naturally occurring lesions were reasonably successful. Lesion extracts from the three fields with plants infected with PST and one field where no bacteria were isolated all tested negative for INA, whereas 76% of lesion extracts that yielded PSS were positive for INA. INA tests were highly effective in separating pure cultures of PST and PSS. About 95% of PSS isolates were positive for INA, whereas all PST cultures were negative. These results support the suggestion by Hirano et al (5) that INA tests may be useful for separating strains of pseudomonads.

The IIF procedure with antisera developed for PST seems useful for detecting PSS and PST and eliminating XCV as the cause of foliar disease. INA tests may provide some determinative information when lesions are used but seem most useful for separating PST from PSS in pure culture, saving the reaction time required for more complicated physiological tests. Like other diagnostic tests, neither IIF nor the INA procedures can be considered completely reliable for detecting and identifying the bacterial foliar pathogens on tomato transplants. The ultimate usefulness of these two tests, however, will be in reducing the number of tests to be run when the colonies are observed to be INA positive (PSS and not PST) or the lesions are IIF negative. As observed in this study, this can be a significant benefit to the transplant industry.

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