Populations of *Xanthomonas campestris* pv. *vesicatoria* in Lesions of Susceptible and Resistant Tomato Genotypes

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ABSTRACT

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Tomato genotypes resistant (R), susceptible (S), partially resistant (PR), and F_1 progeny of R × S and R × PR crosses developed bacterial spot lesions when inoculated with *Xanthomonas campestris* pv. *vesicatoria*. Bacterial populations in foliar lesions determined by direct plating (DP) and indirect immunofluorescence (IIF) from Hawaii 7998 (R) lesions were significantly less than populations in Walter (S). Lesion development in Hawaii 7998 appears to be a manifestation of the hypersensitive response, as exemplified by significantly lower populations in lesions of Hawaii 7998. Bacterial lesion populations of F_1 plants were intermediate between the two parents but not always significantly different from one or both parents. Partially resistant Campbell 28 had population levels similar to the susceptible cultivars, indicating these methods were not useful in identifying resistance from Campbell 28. A significant correlation existed between IIF and DP, and both methods were highly correlated to field infection. Thus, both procedures could be useful as corroborative tests for characterizing resistance levels of breeding lines derived from Hawaii 7998.

Bacterial spot of tomato (*Lycopersicon esculentum* Mill.), incited by *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye is of major concern in tropical and subtropical climates (12). The pathogen, which is reportedly seedborne, is difficult to manage by manipulating cultural practices. Control of this disease with chemical protectants often is negligible because of antibiotic resistance (24,25), copper resistance (16), and the ineffectiveness of bactericides (3) when conditions optimal for disease development are present.

Resistance in tomato was not previously considered a practical option (5) because only horizontal resistance to bacterial spot was available and the levels of resistance were not considered useful in a plant breeding program. However, a new source of resistance, Hawaii 7998, was reported in 1986 (20). This genotype exhibited a hypersensitive response when infiltrated with strains of *X. c.* pv. *vesicatoria* (13). Thus, a genotype with an adequate level of resistance that offered promise in breeding for bacterial spot resistance was identified. Progress toward incorporating bacterial spot resistance from Hawaii 7998 into horticulturally desirable genotypes has been difficult because of the complex genetic control of this resistance (21) and inconsistent selection pressure in the field. In Florida, only the summer rainy season consistently is favorable for enough disease development to avoid escapes and to detect high levels of resistance.

Before the discovery of bacterial spot resistance in Hawaii 7998, a procedure for seedling screening for resistance was described (15). Currently, seedling screening procedures have some value but do not give results consistent enough to be used alone (22). To more accurately determine resistance levels, the use of field screening in conjunction with seedling screening and/or hypersensitivity tests, such as confluent necrosis and electrolyte leakage (4,13,14), may be the best approach.

In field and greenhouse screening studies, typical bacterial spot lesions often developed on Hawaii 7998. Although disease severity was low, it was suspected that these lesions resulted from selection of virulent strains. However, the bacterium was isolated from typical lesions, and these isolates were retested on Hawaii 7998, revealing that these strains were avirulent (J. B. Jones, *unpublished data*). Thus, it was hypothesized that these lesions may be a manifestation of the hypersensitive reaction. The objective of this study was to determine if quantification of populations of *X. c.* pv. *vesicatoria* in individual lesions would allow identification of susceptible, resistant, and partially resistant tomato genotypes in seedling screening tests. Direct plating (DP) and indirect immunofluorescence (IIF) were compared to each other and to a field test to determine their accuracy and effectiveness in differentiating tomato genotypes for resistance to bacterial spot.

MATERIALS AND METHODS

Growth chamber/greenhouse studies. Seeds of Hawaii 7998 (resistant [R] to *X. c.* pv. *vesicatoria*), Walter (susceptible [S]), PI 270248 Sugar (S), Lyconorma (S), Campbell 28 (partially resistant [PR]), Ohio 4013-3 (PR), Florida 317 and Florida 325 (resistance derived from Hawaii 7998), and F_1 genotypes derived from crossing Hawaii 7998 with susceptible or partially resistant genotypes were sown in Black Beauty spent coal (Reed Minerals Div., Highland, IN) in wooden flats and covered with a layer of vermiculite. Seedlings were transplanted individually, 10 days after sowing, to 10-cm-diameter pots containing a soil/peat/sand mixture (2:1:1, v/v/v) and grown in the greenhouse. All experiments included Walter as a susceptible check and Hawaii 7998 as a resistant check.

Plants were inoculated approximately 1 mo after transplanting by gently misting until runoff with a suspension of *X. c.* pv. *vesicatoria*. After inoculation, plants were incubated in a growth chamber at 27 C and nearly 100% relative humidity for 48 hr and returned to the greenhouse. Inoculum was prepared by incubating a Florida pepper strain of *X. c.* pv. *vesicatoria* for 48 hr on nutrient-yeast-dextrose agar plates at 28 C (11). Bacterial cells were suspended in 0.01 M $MgSO_4 \cdot 7H_2O$ and adjusted to approximately 10^8 cfu/ml turbidimetrically.

Leaflets with lesions were collected approximately 2 wk after inoculation. Ten randomly selected typical lesions per genotype were chosen (two lesions from each of five plants per genotype), measured with calipers in some experiments, and macerated individually in tissue grinders containing 2 ml of sterile pH 7.0 peptone buffer (5.3 g of KH_2PO_4, 8.61 g of Na_2HPO_4, and 1 g of Bacto peptone per liter of deionized water) for DP experiments or 2 ml of 0.85% sterile saline solution for the experiments where DP and IIF were compared. In four experiments, a modification of an IIF technique developed by Schaad (18) was compared with the DP method. The remaining greenhouse/growth chamber experiments were sampled only by DP.

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For DP, serial 10-fold dilutions were made in sterile phosphate buffer (pH 6.8, 0.015 M) with 1 g of NaCl and 0.005 g of $CaCO_3$ added per liter of deionized water, with 0.1 ml of the appropriate dilution plated on Tween A medium (17). Plates were incubated for 5 days at 28 C before colonies were enumerated.

For IIF samples, a 10-μl undiluted sample of each lesion ground in saline solution (zero dilution) was placed into one of eight wells in a HTC autoclavable supercured 5-mm frosted slide (Cel-Line Associates, Newfield, NJ) and allowed to air-dry. Polyclonal antiserum prepared against whole *X. c.* pv. *vesicatoria* cells in female New Zealand white rabbits (20 μl per well) was applied to the slides, and the slides were treated as described by Schaad (18). Slides were then stained and processed with antirabbit goat globulin fluorescein isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, MO), which was diluted 1:16 with deionized water (18).

Slides were mounted in glycerol adjusted with 0.05 M carbonate buffer to pH 9.0 (18) and viewed with a microscope (Olympus BH2) equipped for fluorescence work, with a ×100 oil objective, providing total magnification of ×1,000. The number of fluorescent *X. c.* pv. *vesicatoria* cells was determined in each of 10 fields for each lesion.

Field study. In the summer of 1988, seeds of Walter, Campbell 28, Hawaii 7998, Lyconorma, Ohio 4013-3, Florida 317, and Florida 325 were sown in spent coal and transplanted 10 days later in a pH 6.5 peat/vermiculite mix (1:1 v/v, amended with dolomite, superphosphate, and hydrated lime) to Todd planter flats (Speedling, Inc., Sun City, FL) with 3.8-cm^2 cells. Transplants were set in the field 1 mo later on EauGallie fine sand beds. The beds had been fumigated 2 wk earlier with methyl bromide/chloropicrin (67/33%) at 392 kg/ha and covered with white polyethylene mulch. Beds were 15 cm high, 75 cm wide, and 137 cm from center to center. Plants were spaced 61 cm apart within the rows. The experiment was in a completely randomized design with three replicates and six plants per plot. Recommended fertilizer and insecticide practices were used, and chlorothalonil was used to control fungal pathogens. Plants were staked, tied, and watered by seepage irrigation. A suspension of *X. c.* pv. *vesicatoria* was sprayed on the plants in the early morning several weeks after transplanting. Inoculum was prepared as previously described. Plants were assessed for percentage of defoliation 6 wk after transplanting. These ratings were then compared with results from greenhouse IIF and DP procedures to determine the relationship between field reactions and the screening procedures.

Statistical analyses. Analyses of variance were performed on all data and means were separated by Duncan's multiple range test ($P = 0.05$). Six experiments were sampled by DP only, and when analyzed, each experiment was considered a replication. Log_{10} transformations were used for all data, with the exception of the field study in which percentages were transformed to square root of the arcsine. Correlation analyses were done for the four experiments in which the two methods (DP and IIF) were compared and to compare field disease ratings to the two methods. Lesions where no *X. c.* pv. *vesicatoria* cells were recovered were not used in the analyses and were treated as missing data.

RESULTS AND DISCUSSION

Typical bacterial spot lesions developed on all genotypes, although under certain conditions, atypical lesions, characterized by a light tan, papery appearance, developed on resistant genotypes. These atypical lesions, possibly a hypersensitive type response, were not chosen for sampling. In several of the first experiments, diameters of individual lesions were measured before maceration, and populations were calculated per centimeter squared of lesion. However, statistical differentiation of genotypes was equally effective when lesions were not measured and, to save time, lesions were not measured in later experiments. Populations of *X. c.* pv. *vesicatoria* in lesions in Hawaii 7998 were from six to 316 times (average of 20 times) lower than in susceptible genotypes as determined by DP (Table 1). Although there was a variation in the magnitude of separation between experiments, populations of *X. c.* pv. *vesicatoria* in Hawaii 7998 were significantly lower than susceptible genotypes in each experiment. Therefore, this technique was reliable for separating resistant and susceptible genotypes. Another Florida isolate of *X. c.* pv. *vesicatoria* was tested by DP (*data not shown*) with similar results. The differential response of Hawaii 7998 and susceptible genotypes is substantiated by previous work on population dynamics in compatible and incompatible genotypes of several crops (1,2,9,19), including pepper and tomato (13,23). Stall and Cook (23) injected *X. c.* pv. *vesicatoria* at 10^5 and 10^8 cfu/ml into leaves of pepper plants resistant and susceptible to bacterial spot. *X. c.* pv. *vesicatoria* populations were consistently higher in susceptible plants than resistant plants from the fifth day after infiltration until the end of the experiment (2 wk). In tomato, *X. c.* pv. *vesicatoria* populations were considerably lower in Hawaii 7998 than in other genotypes of varying bacterial spot susceptibility when infiltrated with 10^5 cfu/ml (13). Lower populations in lesions of the resistant genotype, Hawaii 7998, compared with those in lesions of the compatible genotypes support the contention that lesion development in Hawaii 7998 resulted from an incompatible reaction. The response appears to be a manifestation of a hypersensitive response previously shown to occur in Hawaii 7998 (13).

Bacterial populations in lesions on hybrids from Hawaii 7998 crosses tended to be intermediate between the parents, but differences were not always significant (Table 1). Bacterial spot incidence on R × S hybrids has been intermediate in the field (21,22). The populations in partially resistant Campbell 28 usually were similar to populations in susceptible genotypes and could not be separated as a distinct group. Moreover, populations of hybrids with Campbell 28 as a parent generally were not less than populations from hybrids with susceptible lines. Thus, it does not appear that techniques related to determining bacterial populations in lesions are useful for detecting resistance levels provided by Campbell 28. In other work, bacterial populations on leaf surfaces of Campbell 28 were similar to susceptible genotypes and greater than that of Hawaii 7998 (R. G. McGuire and J. B. Jones, *unpublished data*). The partial resistance in Campbell 28 appears to be controlled by a different mechanism than that in Hawaii 7998 (13). Combining both of the resistance mechanisms might be useful for obtaining higher resistance levels, but the lesion population methods would not assist in detecting these higher resistance levels.

Table 1. Populations of *Xanthomonas campestris* pv. *vesicatoria* in lesions as estimated by direct plating from tomato genotypes in greenhouse experiments in Bradenton, FL

Genotype	Reaction[x]	*X. c.* pv. *vesicatoria* cells per lesion (log_{10})[y]
Walter	S	6.2 a[z]
Sugar	S	6.1 a
Campbell 28	PR	6.1 a
Hawaii 7998 × Sugar	R × S	5.8 a
Hawaii 7998 × Campbell 28	R × PR	5.4 b
Hawaii 7998 × Walter	R × S	5.3 b
Hawaii 7998	R	4.9 b

[x] Based on field observations where S = susceptible, PR = partially resistant, and R = resistant.
[y] Numbers represent average of six experiments (10 lesions per genotype).
[z] Values in a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 2. Comparison of direct plating (DP) and indirect immunofluorescence (IIF) techniques for determining *Xanthomonas campestris* pv. *vesicatoria* populations in lesions on greenhouse-grown tomato genotypes in Bradenton, FL

		Experiment							
		1		2		3		4	
Genotype	Reaction[v]	DP[w]	IIF[x]	DP	IIF	DP	IIF	DP	IIF
Walter	S	6.8 a[y]	1.2 a	6.7 a	1.5 a	6.4 a	1.3 a	6.6 a	1.7 a
Sugar	S	6.7 ab	1.3 a	5.9 b	0.7 b	···	···	···	···
Campbell 28	PR	···	0.9 b	6.3 ab	0.7 b	···	···	···	···
Hawaii 7998 × Walter	R × S	6.2 c	0.6 c	5.6 bc	0.3 c	6.2 ab	1.0 b	6.0 b	1.2 b
Hawaii 7998 × Sugar	R × S	6.4 bc	···	5.1 c	0.4 c	···	···	···	···
Hawaii 7998 × Campbell 28	R × PR	6.3 c	0.8 b	···	0.2 c	···	···	···	···
Hawaii 7998	R	5.1 d	0.1 c	4.0 d	0.2 c	5.6 c	0.4 c	5.5 c	0.5 c
Correlation coefficient (r)[z]		0.85***		0.74***		0.70***		0.90***	

[v] Based on field observations where S = susceptible, PR = partially resistant, and R = resistant.
[w] For DP, numbers represent average *X. c.* pv. *vesicatoria* cells ($\log_{10}$) per lesion for 10 lesions per genotype.
[x] For IIF, numbers represent average *X. c.* pv. *vesicatoria* cells ($\log_{10}$) per microscope field, counting 10 fields from each of 10 lesions.
[y] Values in a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).
[z] Correlations between DP and IIF are based upon 10 individual samples per genotype; *** = significant at $P = 0.001$.

Table 3. Bacterial spot field disease severity compared to direct planting (DP) and indirect immunofluorescence (IIF) population estimates for several tomato genotypes from summer 1988 experiments in Bradenton, FL

		Field disease severity	Laboratory assays	
Genotype	Reaction[u]	(%)[v]	DP[w]	IIF[x]
Lyconorma	S	75 a[y]	6.9 a	1.4 a
Walter	S	47 b	6.2 b	1.0 b
Ohio 4013-3	PR	36 c	5.9 c	···
Florida 317	PR	33 c	6.1 bc	0.7 bc
Campbell 28	PR	25 d	6.0 bc	0.8 bc
Florida 325	R	23 d	5.6 cd	0.5 cd
Hawaii 7998	R	15 e	5.5 d	0.2 d
Correlation coefficient with field disease severity (r)			0.96***[z]	0.95**

[u] Based on field observations where S = susceptible, PR = partially resistant, and R = resistant.
[v] Disease severity defoliation percentage was transformed to square root of the arcsine for statistical analysis, nontransformed percentages are shown. Percentages represent average of three replications per genotype.
[w] For DP, numbers represent average *X. c.* pv. *vesicatoria* cells ($\log_{10}$) per lesion for 10 lesions per genotype.
[x] For IIF, numbers represent average *X. c.* pv. *vesicatoria* cells ($\log_{10}$) per microscope field, counting 10 fields from each of 10 lesions.
[y] Values in a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).
[z] ** = Significant at $P = 0.01$; *** = significant at $P = 0.001$.

In experiments in which IIF and DP techniques were compared, similar population trends occurred, as in the previous experiment in which only DP was used. Susceptible, resistant, and intermediate genotypes were equally differentiated with IIF and DP (Table 2). Susceptible genotypes had significantly higher populations of *X. c.* pv. *vesicatoria* than did Hawaii 7998. Hybrids had intermediate populations that were not always different from susceptible or resistant genotypes. Correlations between the two methods were highly significant, indicating either method would be comparable in differentiating resistant and susceptible genotypes (Table 2). Either or both methods are recommended to be used in conjunction with field screening.

It should be noted that because of variation within genotypes, these methods are probably more useful in rating lines than individual plants in a segregating population where escapes could hinder progress. IIF sampling required more time on the sampling day than did the DP technique, but DP required more advance preparation. The numbers of bacteria could be quantified immediately with IIF, whereas the number of colonies on Tween A medium could not be counted for 5 days. All cells, living and dead, could be counted with IIF, whereas DP enables one to quantify only living cells. Counting living and dead cells would be an advantage where lesion age is unknown. Effective uses of serological techniques were also found by other researchers; for example, techniques such as antibody staining and phase contrast microscopy were used successfully to enumerate the ratoon stunting disease bacterium in sugarcane extracts (6,7,10) and immunofluorescence and ELISA were used for determining population densities of *Corynebacterium sepedonicum* (Spieck. and Kotth.) Skapt. and Burkh. in potato stems with varying degrees of susceptibility, grown from inoculated seed tubers (8).

Bacterial numbers in lesions determined by DP and IIF in the growth chamber/greenhouse were highly correlated with field bacterial spot disease severity ratings (Table 3). These results show the usefulness of DP and IIF in substantiating field observations. In previous experiments (*data not shown*), lesions from field-grown plants were assayed by DP and IIF. There were sometimes problems with chosen lesions not containing *X. c.* pv. *vesicatoria* cells, preventing use of these procedures for field sampling. Hawaii 7998, Florida 325, and Florida 317 were similarly ranked by laboratory and field procedures in order from most to least resistant, respectively, even if differences were not always statistically significant. In cases like this where both field and laboratory ratings are consistent, conclusions about the relative resistance of breeding lines can be made with greater confidence. If the tests were not consistent with each other, further evaluation might be required in order to distinguish possible differences.

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