Relationship Between the Hypersensitive Reaction and Field Resistance to Tomato Race 1 of *Xanthomonas campestris* pv. *vesicatoria*

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ABSTRACT

Somodi, G. C., Jones, J. B., Scott, J. W., Wang, J. F., and Stall, R. E. 1996. Relationship between the hypersensitive reaction and field resistance to tomato race 1 of *Xanthomonas campestris* pv. *vesicatoria*. Plant Dis. 80:1151-1154.

In this study the relationship between hypersensitivity to tomato race 1 (T1) of *Xanthomonas campestris* pv. *vesicatoria* and field resistance in an F_2 population derived from crosses made between Hawaii 7998 (H7998) (resistant to T1) and Walter (susceptible to T1) was determined. Two techniques were used for quantifying the hypersensitive reaction: (i) measurement of speed of confluent necrosis following infiltration of a high concentration of the bacterium and ii) measurement of internal populations of *X. campestris* pv. *vesicatoria* after infiltration of a low concentration of the bacterium into leaflets. Bacterial populations were assayed by an indirect enzyme-linked immunosorbent assay (ELISA) procedure termed lysozyme ELISA (L-ELISA), which when used in combination with an amplification system allowed the detection of populations as low as 10^4 CFU. When the cultigens H7998 and Walter were infiltrated with 10^5 CFU/ml and population levels were determined over time by direct plating and amplified L-ELISA, relationships between the two parameters were significant ($r^2 = 0.86$ and 0.78) in two experiments. Comparisons were made between field susceptibility and the speed of confluent necrosis and internal populations of *X. campestris* pv. *vesicatoria* as measured by amplified L-ELISA in an F_2 population (a cross between Walter and H7998). There was a significant correlation (0.52) between the speed of confluent necrosis and populations as detected by amplified L-ELISA. When amplified L-ELISA and confluent necrosis were compared with field susceptibility, significant correlation coefficients were 0.26 and 0.20, respectively, for the first field rating; and 0.52 and 0.34, respectively, for the second field disease rating. In a second experiment in which confluent necrosis was compared with field susceptibility, the correlation was 0.31. Confluent necrosis appears to provide some information regarding resistant genotypes; however, much of the field reaction could not be explained by hypersensitive reaction.

Bacterial spot of tomato (*Lycopersicon esculentum* Mill.) caused by *Xanthomonas campestris* pv. *vesicatoria* is extremely difficult to control under the moist, warm conditions prevalent in tropical and subtropical tomato growing areas (4,14). Bactericides may provide adequate control when environmental conditions are marginal for the disease, but are ineffective when environmental conditions are favorable for disease development (7). The search for effective bacterial spot control has focused on the manipulation of cultural factors (11–13) and plant resistance to the bacterium (15–18). Bacterial spot resistance to tomato race 1 (T1) has been found in the tomato cultigen Hawaii 7998 (H7998) (9); incorporation of this resistance into commercially acceptable genotypes has been an important priority (17). However, the resistance to T1 in H7998 is inherited quantitatively and selection of progeny with high levels of resistance is difficult (16). Furthermore, the presence of tomato race 3 (T3) in Florida has made it difficult to screen for resistance to the T1 strain in the field (10) since T3 is pathogenic on H7998, and is more prevalent than T1 in Florida (J. B. Jones, unpublished).

While a seedling screening technique (19) was developed to identify genotypes with resistance to T1 strains, another approach that had been used for selecting resistant genotypes was based on resistant genotypes developing confluent necrosis following infiltration with high concentrations of the T1 strain (20). A major problem with the second approach is that confluent necrosis (i.e., rapid reaction indicative of hypersensitive reaction [HR]) associated with H7998 resistance is inherited quantitatively (20), and thus selection of highly resistant genotypes that express rapid confluent necrosis is not totally reliable.

With many bacterial-related HRs, strong negative relationships between rapid confluent necrosis and internal populations have been demonstrated (5,9,10). Furthermore, it was theorized that quantification of internal bacterial populations in leaf tissue infiltrated with the pathogen might be more accurate in discerning resistant genotypes than measuring the time to confluent necrosis. Enumeration of populations with dilution plating is extremely labor intensive; however, enzyme-linked immunosorbent assay (ELISA) would be a more efficient method for quantifying bacterial populations.

Immunoassays have been useful for quantifying bacterial populations in plant tissue (1,3). De Boer and McCann (3) detected *Clavibacter michiganensis* subsp. *sepedonicus* in potato stems at about 10^5 immunofluorescing units per gram of tissue and found ELISA useful for detecting the bacterium in asymptomatic tissue; furthermore, there was a strong positive correlation between populations measured by immunofluorescence and those measured by ELISA. In preliminary studies using ELISA to quantify *X. campestris* pv. *vesicatoria* populations in the plant, the minimum detectable level was approximately 10^7 CFU/ml. An indirect ELISA procedure termed lysozyme ELISA (L-ELISA) increased sensitivity to below 10^6 CFU/ml with 20 *X. campestris* pv. *vesicatoria* strains (J. B. Jones et al., unpublished).

The objective of this study was to determine the relationship between field resistance and HR with two methods for determining HR: (i) ELISA used to quantify populations of *X. campestris* pv. *vesicatoria* in leaflets of tomato genotypes with varying levels of resistance after infiltrating with low concentrations of the bacterium, and (ii) the time necessary for confluent necrosis to occur (when genetic material was infiltrated with a high bacterial concentration) as described by Wang et al. (20).

MATERIALS AND METHODS

Inoculum production. Strain 90-14 of *X. campestris* pv. *vesicatoria* was utilized in these studies and was stored at –80°C in 15% glycerin. Nutrient agar (Difco, Detroit, MI) plates were inoculated with cells from single colonies and plates were incu-

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Florida Agricultural Experiment Station Journal Series R-04637.

This research was supported in part by the USDA under CSRS Special Grant 92-34135-7283 managed by the Caribbean Basin Advisory Group.

Accepted for publication 1 July 1996.

Publication no. D-1996-0802-04R

bated for 48 h at 28°C. Bacterial cells from the plates were suspended in either 0.85% saline or 0.01 M $MgSO_4 \cdot 7H_20$ and concentrations were standardized turbidimetrically.

ELISA protocols. All assays utilized the indirect double antibody sandwich technique in which Immulon 2 (Dynatech Laboratories, Chantilly, VA) flat bottom 96-well microtiter plates were coated with a polyclonal antibody, developed in New Zealand White rabbits against *X. campestris* pv. *vesicatoria* strain 75-3, and incubated at 4°C overnight. Buffers used in the ELISAs were as described by Clark and Adams (2) except where indicated. An extraction buffer found to increase detection sensitivity of the bacterium was used in place of phosphate buffered saline (PBS) in the step following coating and prior to addition of the monoclonal antibody (J. B. Jones et al., unpublished). The extraction buffer contained the following: 2 g of KH_2PO_4; 11.5 g of Na_2HPO_4; 0.14 g of EDTA disodium; 0.02 g of thimerosal; and 0.2 g of lysozyme per liter. Two 7-mm-diameter leaf disks were ground in small glass tissue grinders (Corning, Corning, NY) in 2 ml of 0.85% NaCl (wt/vol) containing known concentrations of *X. campestris* pv. *vesicatoria* and diluted 1:1 with PBS (in two of three experiments) or in 2× extraction buffer. Samples were incubated for 16 h at room temperature. Microtiter plates were washed with a sodium chloride–Tween 20 (SCT) solution containing 0.8% NaCl and 0.1% Tween 20 in deionized water. Plates were then blocked with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in PBS and shaken to remove excess liquid. A 100-µl quantity of each bacterial suspension in extraction buffer or PBS was added to wells on a microtiter plate and incubated for 1 h at 37°C. Plates were rinsed with SCT, a monoclonal antibody (2H10) prepared against *X. campestris* pv. *vesicatoria* strain 75-3 (8) was added to all wells, and plates were incubated for 1 h at 37°C. Plates were rinsed with SCT and alkaline phosphatase conjugated goat antimouse (Sigma) was applied to each well. Plates were incubated 1 h at 37°C and then rinsed with SCT.

The procedures for amplified L-ELISA were the same as for L-ELISA until the final washing step, at which time the plates were treated according to the ELISA Amplification System protocol (Gibco BRL, Gaithersburg, MD). Plates were washed four times with Tris-buffered saline (0.05 M Tris-HCl [pH 7.5]; and 0.15% NaCl). Fifty microliters of reconstituted substrate was added to each well and the plates were incubated for 15 min at 25°C. Fifty microliters of reconstituted amplifier was then added and the plates were incubated for an additional 15 min at 25°C. The plates were then read on a microplate reader (SLT Instruments, Austria) at A_{492}.

Comparison of amplified L-ELISA with direct plating for determining populations in tomato leaf tissue. H7998 and Walter plants were grown in the greenhouse until the fourth leaf was fully expanded. Two 7-mm-diameter circular areas on a leaflet were infiltrated with *X. campestris* pv. *vesicatoria* at 10^5 CFU/ml, using a hypodermic syringe with a 26G 3/8 intradermal beveled needle (Becton Dickinson and Co., Franklin Lakes, NJ). Leaves were infiltrated 0, 1, 4, and 7 days before sampling in the first experiment, and at 0, 1, 4, 7, and 14 days before sampling in the second experiment. Five replications were used for each genotype. After infiltration, plants were placed in a 28°C growth chamber with 12 h of 110 $\mu E \cdot s^{-1} \cdot m^{-2}$ light per 24 h. Samples were assayed by the amplified L-ELISA procedure previously described and by a direct plating procedure (18).

Relationship between amplified L-ELISA, measurement of speed of confluent necrosis, and field disease severity. Plants of H7998, Walter, Walter × H7998 F_1, and Walter × H7998 F_2 were grown as previously described. There were five plants per line with the exception of the F_2, which had 50 plants. A portion of one leaflet per plant was infiltrated with 10^8 CFU/ml of *X. campestris* pv. *vesicatoria* and plants were placed in a 24°C growth chamber with 12 h of 110 $\mu E \cdot s^{-1} \cdot m^{-2}$ light per 24 h. Confluent necrosis was recorded 24, 36, 48, 60, and 72 h after infiltration: each time, an HR was considered positive if >50% of the infiltrated area exhibited confluent necrosis. In addition, 7-mm-diameter areas were infiltrated as described previously and amplified L-ELISA was done 7 days after infiltration. The only change in procedure was that disks were placed into a microcentrifuge tube containing 100 µl of phosphate buffer and ground with a small plastic pestle (suitable for microcentrifuge tubes) attached to an electric drill. Sterile pestles were used for each sample.

After populations in leaflets were sampled, the plants were set in the field on EauGallie fine sand beds and tracked so that HR determinations could be correlated with field disease ratings. The beds had

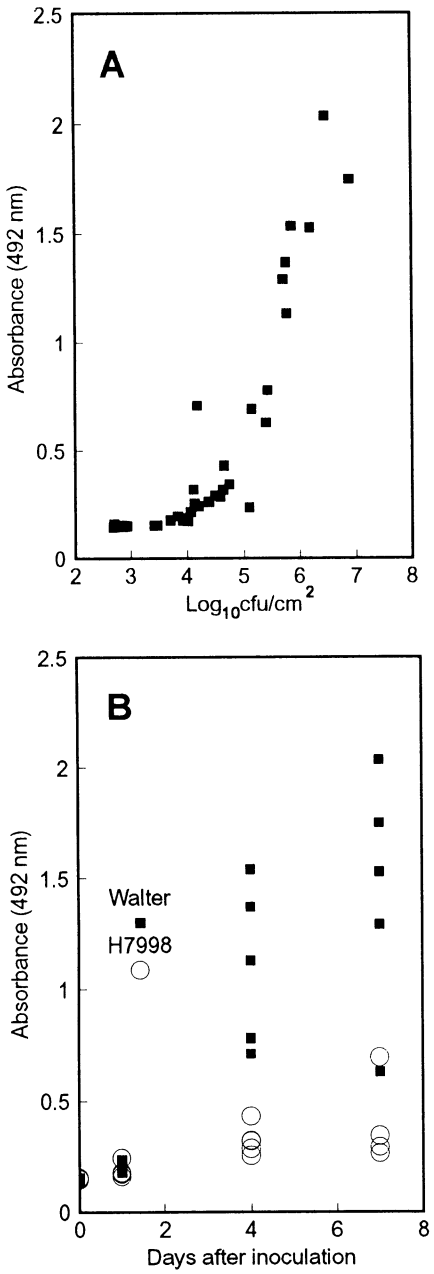

Fig. 1. (A) Relationship between amplified lysozyme enzyme-linked immunosorbent assay (amplified L-ELISA) and *Xanthomonas campestris* pv. *vesicatoria* populations (CFU/cm² leaf tissue) in infiltrated Hawaii 7998 (resistant) and Walter (susceptible) tomato leaflets sampled 0, 1, 4, and 7 days after infiltration, as determined by dilution plating. **(B)** Populations of *X. campestris* pv. *vesicatoria* in infiltrated Hawaii 7998 (resistant) and Walter (susceptible) tomato leaflets, as determined by amplified L-ELISA 0, 1, 4, and 7 days after infiltration.

Table 1. Relationship between amplified lysozyme enzyme-linked immunosorbent assay (L-ELISA) results, speed of confluent necrosis, and bacterial spot disease ratings in a tomato F_2 population (Hawaii 7998 × Walter)

Technique[a]	Amplified L-ELISA	Field rating 1	Field rating 2
Speed of confluent necrosis	0.52***[b]	0.26**	0.52**
Amplified L-ELISA results	...	0.20*	0.34***

[a] Confluent necrosis was measured and amplified L-ELISA was performed on F_2 plants by infiltrating leaflets with high and low populations, respectively, of *Xanthomonas campestris* pv. *vesicatoria*. Data was collected prior to placing plants in the field to determine disease ratings.

[b] Correlation coefficients are as follows: * = significant at $P = 0.05$;** = significant at $P=0.01$;*** = significant at $P=0.001$.

been fumigated 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 rated 3 and 5 weeks after transplanting, according to the Horsfall-Barratt scale (6).

In a second experiment, Fla. 7060 (susceptible), H7998 (resistant), and 7060 × H7998 F_2 plants were grown in the greenhouse for approximately 3 weeks and then topped. Plants were infiltrated as described for the previous experiment and confluent necrosis was rated on a scale of 0 to 5 periodically for 90 h with 0 = no necrosis and 5 = brittle necrosis in the entire infiltrated area. Plants were transplanted to the field and rated for disease severity. Time to confluent necrosis was correlated with disease severity.

Statistical analyses. Data were analyzed by correlation or regression procedures in PC versions of SAS (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Comparison of amplified L-ELISA with direct plating for determining populations in tomato leaf tissue. A significant relationship ($r^2 = 0.85$) was shown when leaflets of H7998 and Walter plants were infiltrated with 10^5 CFU/ml and populations were sampled by dilution plating and amplified L-ELISA 0, 1, 4, and 7 days after infiltration (Fig. 1A). In a repeat experiment, with samples taken 0, 1, 4, 7, and 14 days after infiltration, populations estimated by dilution plating were significantly related ($r^2 = 0.78$) to numbers of bacteria estimated by amplified L-ELISA. Populations of *Xanthomonas campestris* pv. *vesicatoria* in H7998 and Walter were similar at day 0 and had increased slightly but were still similar at day 1. At day 4, sample populations were not overlapping in the two cultigens, as measured by both ELISA and direct plating. At day 7, populations in H7998 had increased slightly, while there was more of an increase in Walter (Fig. 1B). Amplified L-ELISA was far more efficient than direct plating for identifying genotypes with high levels of resistance to *X. campestris* pv. *vesicatoria* race T1 based on population dynamics, with minimal loss in accuracy in quantification of internal populations. With amplified L-ELISA there was no need for dilution plating since the differences between resistant and susceptible genotypes could be determined easily by measuring absorbance of undiluted samples. Also, the time required for incubation and preparation of materials needed for direct plating was eliminated.

Relationship between amplified L-ELISA, measurement of speed of confluent necrosis, and field disease severity. There were significant correlation coefficients between measurement of speed of confluent necrosis and amplified L-ELISA and the second disease severity rating, and to a lesser extent between confluent necrosis and the first disease rating (Table 1). The relationships are represented in Figure 2A and B. With H7998, three plants exhibited confluent necrosis by 24 h. These had field disease ratings of 3, 3.5, and 4, and ELISA readings of 0.37, 0.40, and 0.50. Two plants had confluent necrosis by 36 h. These had field ratings of 3.5 and 4, and ELISA readings of 0.30 and 0.62. For Walter, all plants exhibited confluent necrosis by 72 h, field disease ratings ranged from 5 to 6, and ELISA readings ranged from 1.13 to 2.15. There were four plants for the F_1. One had confluent necrosis at 36 h with a field disease rating of 4.5 and ELISA reading of 0.32; the other three had confluent necrosis at 48 h with disease ratings ranging from 4 to 5 and ELISA readings ranging from 0.32 to 0.76. Quantification of *X. campestris* pv. *vesicatoria* populations in tomato leaf tissue is possible by several methods (e.g. direct plating, ELISA, and indirect immunofluorescence). Amplified L-ELISA successfully increased sensitivity compared with conventional ELISA, and enabled detection in a resistant genotype.

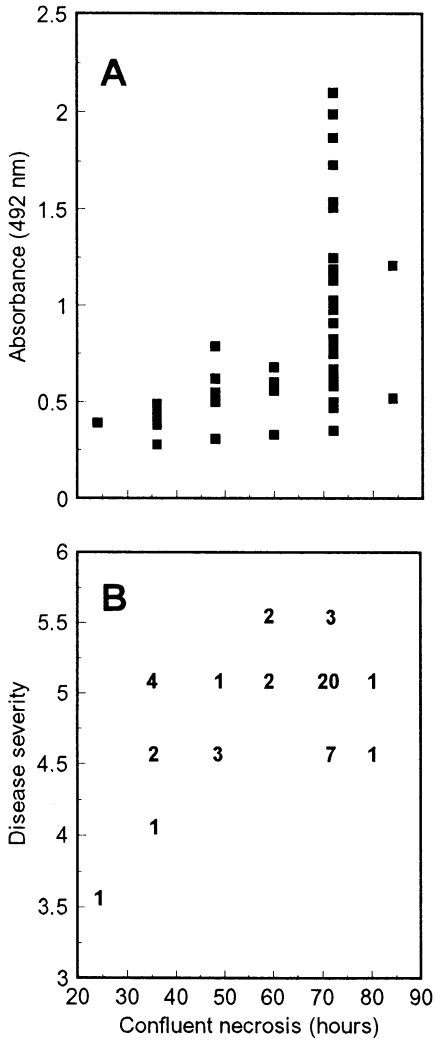

Fig. 2. Relationship between speed of confluent necrosis and **(A)** amplified lysozyme enzyme-linked immunosorbent assay (amplified L-ELISA) absorbance reading and **(B)** bacterial spot disease severity in an F_2 population (48 plants) derived from a cross between tomato cultigens Walter (susceptible) and Hawaii 7998 (resistant). Numbers on data points represent number of plants with that disease severity and hours to confluent necrosis.

In a second experiment, similar correlations were found between confluent necrosis and field ratings in an F_2 population (0.31 compared with 0.26 and 0.52 in the previous experiment). The average ratings for H7998 and 7060 were 2 and 4.45, respectively. The relationship between confluent necrosis and field disease severity is complex, with considerable variability (Fig. 3). We recommend caution in the sole use of confluent necrosis as a measure of HR as a method for resistance screening.

Identifying plant material with resistance to the T1 strain is quite difficult due to the quantitative nature of the field resistance and confluent necrosis (15,20). Wang et al. (20) determined that T1 strains will induce strong confluent necrosis in leaflets of H7998, whereas the reaction in F_1s was intermediate between H7998 and the susceptible genotype. Since the correlation between the time to confluent necrosis and field susceptibility in an F_2 (susceptible × H7998) population was low, the measurement of internal populations was expected to provide a more accurate estimate of the level of resistance in individual plants. However, the relationship between internal populations and field resistance to T1 of the bacterial spot pathogen was lower than that between the rate of confluent necrosis and field resistance. Thus, selection of genotypes with high levels of field resistance to T1 based upon rapid HR may be a challenge. Although selection of

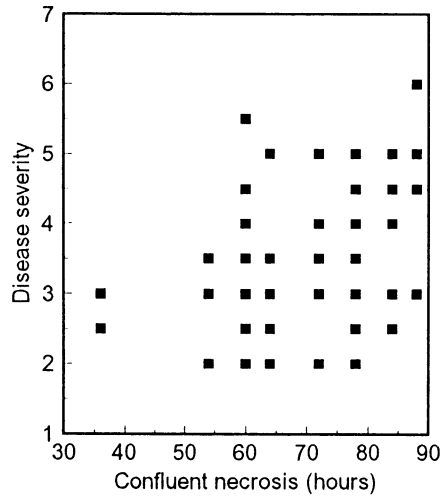

Fig. 3. Relationship between speed of confluent necrosis and disease severity in an F_2 population (98 plants) derived from a cross between tomato cultigens Fla. 7060 (susceptible) and Hawaii 7998 (resistant).

resistant genotypes based on HR (confluent necrosis rate or ELISA measurement) will eliminate much of the susceptible genetic material, secondary screening by other methods (19) will be necessary.

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