Role of Stomatal Opening and Frequency on Infection of *Lycopersicon* spp. by *Xanthomonas campestris* pv. *vesicatoria*

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Florida Agricultural Experiment Stations Journal Series No. 7355.
Accepted for publication 26 January 1987.

ABSTRACT

Ramos, L. J., and Volin, R. B. 1987. Role of stomatal opening and frequency on infection of *Lycopersicon* spp. by *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology 77:1311-1317.

In *Lycopersicon esculentum* (tomato) and related *Lycopersicon* spp., bacterial spot disease was significantly reduced when leaf stomatal closure was physiologically induced or the stomatal opening was chemically suppressed by abscisic acid and phenylmercuric acetate before inoculation with *Xanthomonas campestris* pv. *vesicatoria*. Both disease incidence and severity were related to stomatal opening at the time of, and immediately following, inoculations. Stomatal frequency on adaxial and abaxial surfaces was correlated with the number of spot lesions produced after the infection. Higher stomatal frequency and number of bacterial lesions were found in *L. esculentum* 'Flora-Dade' and 'Florida 1B', whereas *L. hirsutum* had lower stomatal frequency and fewer lesions. Midrange values in the disease response and stomatal frequency of hybrids (*L. esculentum* × *L. hirsutum*) suggested that a moderate level of resistance may be heritable and quantitatively based. Inoculations by misting versus infiltration of the mesophyll provided evidence that the lower disease level of the host is mainly due to an external, morphological resistance (i.e., fewer stomata on the surface of leaves) rather than to an internal, intrinsic resistance. An improved level of control may be achieved by combining low stomatal number with stomatal closure by application of antitranspirants.

Additional key words: bacterial ingress, bacteriology, genetics, stomatal agents, stomatal behavior.

The bacterial spot disease in tomato, caused by *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye, is one of the most serious diseases of tomato plants in south Florida, affecting yield and quality of fresh-market tomatoes (14). Control of the disease using bactericides and conventional plant breeding methods has proved to be ineffective. New physiological and genetic approaches must be developed for the reduction of the disease. The identification of morphological and biochemical traits associated with a polygenic resistance should be very valuable in the control of the disease. In addition, some relevant questions related to the ingress and invasion of tomato plants by the bacterium and, in particular, the role of stomata on infection need to be better understood. Natural openings like stomata, hydathodes, lenticels, and nectaries are portals of entry for bacterial pathogens (2,9–11,15) and are considered relevant in the etiology of bacterial diseases because bacteria cannot breach intact leaf surfaces (21). *X. c.* pv. *vesicatoria* enter tomato leaves through epidermal wounds produced mechanically (19). *X. c.* pv. *vesicatoria* as residents of the leaf surface probably can be splashed to other leaves, infecting them through stomata (7). However, bacterial invasion through stomata is not well documented (4). A possible relationship between stomatal opening, frequency of stomata, structure and arrangement of stomata, and the resistance to pathogen entry has been suggested, but in only a few instances has it been firmly established (15,20,21).

This paper describes the roles of stomatal opening and stomatal closure as chemically induced by the antitranspirants abscisic acid and phenylmercuric acetate or physiologically induced by dark conditions on the subsequent infection by *X. c.* pv. *vesicatoria*.

MATERIALS AND METHODS

Host plants. Test plants were grown in a growth chamber in sterilized metal flats containing a mixture of 1:1 vermiculite and peat moss with nutrients added. Fifteen to 40 seedlings were grown per flat depending on the test.

Before inoculation, plants were placed in a growth chamber for 17–24 days under a 10-hr photoperiod. Illumination consisted of a combination of cool-white and Gro-lux Sylvania and incandescent light delivering a mean intensity of 450 μE m^{-2} sec^{-1} (4,500 lx) measured with a Lambda LI, 185A quantum photometer (Lambda Instruments Corp., Lincoln, NE) at the bench surface. In the growth chamber, the mean relative humidity was 65% and temperature 25 C.

After inoculation, plants were maintained 4 days in a dew chamber under 95–100% relative humidity and 32 C temperature to ensure a postinoculation period of high humidity. Plants were subirrigated to prevent splash dispersal of bacteria. To assess the effects of stomatal opening and stomatal closure as chemically induced by antitranspirants or physiologically induced by dark conditions on the subsequent infection by the bacterium, *L. esculentum* Mill. 'Flora-Dade' was used (Tables 1, 2, and 3). To study the relationship between stomatal frequency and disease severity, three *Lycopersicon* species (*L. esculentum* 'Flora-Dade' and 'Florida 1B', *L. hirsutum* Humb. & Bonpl. PI 127826-D1, and *L. pimpinellifolium* (L.) Mill. PI 127805-D1) were used in two different experiments (Tables 4 and 5). Interspecific F_1 hybrids between Flora-Dade and PI 127826-D1 and between Florida 1B and PI 127805-D1 were included in one experiment (Table 5). Two very susceptible breeding stocks, 720635-1 and USDA 28, were used in the experiments presented in Tables 4 and 5, whereas two moderately resistant stocks, USDA 79B156 and Florida 3075-1, were included in the experiment presented in Table 4.

Bacterial strains and inoculation procedures. For pathogenicity tests, *X. c.* pv. *vesicatoria* was isolated from naturally infected tomato plants in Dade County, FL. The bacterium was grown on nutrient agar amended with 1% sucrose and incubated 48 hr at 25–30 C. For inoculation by misting, bacteria from agar cultures were suspended in sterile tap water. Bacterial numbers were adjusted to approximately 2×10^{-6} bacterial colony-forming units (cfu) per milliliter, using a standard curve of the relationship between absorption and colony-forming units. Bacterial suspensions were sprayed on both surfaces of test plant leaves with a low-pressure hand sprayer. To inoculate by infiltration, a cell suspension containing 10^{-5} cfu/ml was injected into the intercellular spaces of the leaf by a sterile syringe with a fine

TABLE 1. Relationship of stomatal opening to disease incidence in tomato (*Lycopersicon esculentum*) after inoculation by *Xanthomonas campestris* pv. *vesicatoria* (Experiment 1)[t]

	Infected plants after inoculation[u] (%)			Mean leaf spot lesion counts/cm^2[v]	Mean spot size (mm^2)[w]	Total foliage area per plant (cm^2)[x]
	4 days	7 days	12 days			
Stomata open—light	32.5 a[y]	88.8 a	100.0 a	6.5 a	4.6 a	124.0 b
Stomata closed—dark	0.0 b	16.3 b	31.3 b	1.9 b	1.6 b	191.8 a
Stomata closed—phenylmercuric acetate[z]	2.5 b	15.0 b	21.3 c	1.4 b	1.8 b	188.1 a
Uninoculated control	0.0 b	0.0 c	0.0 d	0.0 c	0.0 c	210.7 a
F value	72.6	240.8	291.3	85.9	97.7	4.9
Probability	0.0001	0.0001	0.0001	0.001	0.0001	0.0026
Minimum significant difference	1.14	1.56	1.56	0.92	0.59	58.47

[t] The experiment included four blocks, four treatments, and 20 plants per plot.
[u] Mean number of tomato plants showing one or more bacterial leaf spot lesions per leaf in 20 plants per plot. The plants were 17 days old when inoculated with cell suspension of the bacterium.
[v] Counts per square centimeter of leaf surface for those plants infected by the bacterium.
[w] Mean spot size in those plants infected with the bacterium 12 days after inoculation.
[x] Determined in 39-day-old plants, 21 days after inoculation.
[y] Mean separation with lines by Duncan's multiple range test, 5% level.
[z] 5×10^{-5} M phenylmercuric acetate.

TABLE 2. Relationship of stomatal opening to disease incidence in tomato (*Lycopersicon esculentum*) after inoculation with *Xanthomonas campestris* pv. *vesicatoria* (Experiment 2)[w]

	Infected plants 7 days after inoculation[x] (no.)	Leaf spot lesion counts/cm^2[y]
Stomata open—light	68.33 a[z]	3.85
Stomata closed—dark	23.33 b	2.63 b
Stomata closed—phenylmercuric acetate	16.67 b	1.50 b
Uninoculated control	10.0 b	1.0 bc
F value	8.28	6.50
Probability	0.0001	0.005
Minimum significant difference	3.64	1.40

[w] The experiment included four blocks, four replicates, and 15 plants per plot.
[x] Mean number of tomato plants showing one or more bacterial leaf spot lesions per leaf in 15 plants per plot.
[y] Counts per square centimeter of leaf surface for those plants infected by the bacterium 12 days after inoculation.
[z] Mean separation within lines by Duncan's multiple range test, 5% level.

hypodermic needle. The injection was made, as previously described (5,6), near or parallel to the lateral veins of the abaxial surface where the tissues are thicker. The injected suspension spread over a relatively large area between veins. After a few hours, the dark-green areas produced disappeared. Three or four days after injection of a susceptible plant with this pathogenic bacteria, the injected area became covered by local, sometimes coalescing lesions.

Influence of stomatal opening on the infection. Stomatal closure was physiologically induced by placing plants in the dark for 4 hr before inoculation. The inoculated plants were incubated for 2 days in a darkened dew chamber and returned to the growth chamber. To ensure that stomata would not reopen during routine inoculation procedures, those exposed to physiological closing under dark regime pretreatments were inoculated in a dark room under low-intensity safelight illumination made from a flashlight passing through a filter of two layers of green cellophane paper. In a second set, stomata were closed by chemical treatments. Both the adaxial and abaxial leaf surfaces of seedlings were sprayed with 5×10^{-5} M phenylmercuric acetate or with 10^{-4} M abscisic acid 4 hr before inoculation. Stomatal closure was verified by microscopic examination of the epidermis of treated leaves. Control plants were sprayed with sterile water.

Disease severity was evaluated 4, 7, and 12 days after treatment. Three methods were used: number of plants infected, number of lesions per square centimeter of leaf surface, and leaf lesion size. The number of lesions per square centimeter was determined by counting 4- or 5-cm^2 areas from the center of treated leaf blades of plants on the abaxial surface. The lesion size was determined by measuring the diameter of the lesions with a caliper and calculating area assuming a circular shape for the spots. To assess the reduction in the total foliage area per plant by the bacterial infection in the different treatments, the total foliage area per plant was determined by adding the area of every single leaflet by means of an area meter, Lambda model LI-3050A with an LI-3000 readout control unit (Lambda Instruments Corp., Lincoln, NE).

To assess the influence of stomatal openings on the severity of the infection by *X. c.* pv. *vesicatoria*, a randomized block design was used. Statistics included analysis of variance, and where *F* tests indicated significant differences in arrays, means were separated by Waller-Duncan's method at $P < 0.05$ (17).

Influence of stomatal frequency on infection by *X. c.* pv. *vesicatoria*. Several *Lycopersicon* species, hybrids, and breeding lines that had a wide range of differences in stomatal frequency were assessed for disease response.

The stomatal frequency was calculated in four or five locations per leaflet for each plant on both the adaxial and abaxial surfaces. Only the central portion of full expanded or nearly fully expanded leaves was examined. When possible, only leaves occupying similar positions on the plant were examined.

Disease severity was evaluated after 12 days by counting the number of lesions per square centimeter of leaf surface of several leaflets of each plant. Inoculated plants were arranged in the growth chamber in a randomized complete block design with individual plants as repeated measurements within a block. In one experiment, four blocks, seven treatments, and eight plants per plot were used. In another experiment (Table 5) three blocks, eight treatments, and 10 plants per plot were used. A nested model was assumed in the analysis of variance. The means were separated by Waller-Duncan's method at $P < 0.05$. However, the minimum significant difference was also calculated. Stomatal frequency was compared with disease severity by correlation coefficients and regression curve fitting. Regression models included linear ($Y = a + bX$), logarithmic ($\log Y = \log x + b$), and quadratic ($Y = a + bX + CX^2$). The curve-fitting statistic included *R*-square and the sum of squares method, using the significant test of departure from linearity (17).

Effects of inoculation by misting vs. inoculation by infiltration on the infection. To determine what percentage of less disease incidence and severity in the hosts was due to fewer stomata or

stomatal closure and what percentage was due to an intrinsic (not morphological) resistance, a 3 × 2 × 2 factorial experiment was designed. The experiment included inoculation by misting vs. inoculation by infiltration, two stomatal closing agents, phenylmercuric acetate and abscisic acid, and two *Lycopersicon*: *L. esculentum* 'Flora-Dade' and *L. hirsutum* PI 127826-D1. *Y* = Number of leaf spot on leaf; X = frequency.

RESULTS

Influence of stomatal opening on the infection by the bacterium. Less disease was observed among plants that were inoculated, incubated, and maintained for 2 days in the dark or treated with phenylmercuric acetate, a stomatal closing agent. Seven days after inoculation the incidence of disease was significantly lower in plants inoculated under dark conditions (16.3%) or treated with phenylmercuric acetate (15%) than for plants inoculated and maintained under light conditions 88.8% (Table 1). Even at the end of the 12-day period, the number of infected plants was significantly lower when the plants were inoculated under dark conditions as compared with plants infected in the light treatment (Table 1). In a quantitative evaluation of disease severity 12 days after the inoculations (Table 1), the number of leaf spot lesions was significantly reduced on plants grown and inoculated under dark conditions (1.89 lesions per square centimeter) or those treated with phenylmercuric acetate (1.44 lesions per square centimeter) as compared with plants grown and inoculated under light (6.48 lesions per square centimeter). In addition, lesion size was more significantly reduced when the stomata were closed in plants grown under dark conditions or (1.55 mm^2) pretreated with phenylmercuric acetate (1.83 mm^2) (Table 1) than were those on plants inoculated with their stomata open (4.62 mm^2). The differences found in the severity of infection in each treatment were correlated with stomatal opening. Similar reductions in both percentage of disease incidence and number of lesions per leaf surface area were obtained in a second experiment (Table 2), when the stomata were closed by dark or chemical treatments with phenylmercuric acetate. Thus, it was concluded that the infection by *X. c.* pv. *vesicatoria* is significantly reduced when the stomata are closed and that stomata play a major role in the infection.

Finally, as a result of the infection of tomato plants with the bacterium, the total foliage area of the plant 18 days after the inoculation was significantly reduced when the plants were inoculated and maintained under light conditions (124 cm^2) as compared with the control, which was not inoculated (210.7 cm^2), or those held under the dark (191.8 cm^2) or treated with phenylmercuric acetate (188.1 cm^2) (Table 1).

Influence of stomatal frequency on the infection by the bacterium. The first group of experiments with several lines displaying different levels of resistance to the bacterium showed

TABLE 3. Effects of two antitranspirants and two different types of inoculation with *Xanthomonas campestris* pv. *vesicatoria* on the percentage of disease incidence and disease severity of *Lycopersicon esculentum* 'Flora-Dade' and *Lycopersicon hirsutum* PI 127826-D1[a]

	Disease incidence and disease severity							
	Misting[b]					Infiltration[c]		
	L. esculentum		*L. hirsutum*			*L. esculentum*	*L. hirsutum*	
Antitranspirant	Plants infected (%)	Number lesions/ cm^2	Plants infected (%)	Number lesions/ cm^2	Total anti-transpirant	Plants infected (%)	Plants infected (%)	Total anti-transpirant
Phenylmercuric acetate	50.0	7.65	37.4	1.93	45.83	95.8	91.6	93.75
Abscisic acid	66.7	6.19	20.8	0.92	43.75	100.0	75.0	87.50
Control	91.7	13.75	54.2	2.49	72.92	100.0	95.8	97.92
Total species	69.44		38.88			98.61	87.50	

[a] Analysis as a factorial 3×2×2 *F* test: antitranspirants $F = 33.4$, $P \leq 0.0006$; inoculations $F = 35.6$, $P \leq 0.0094$; species $F = 16.5$, $P \leq 0.0270$; antitranspirant × species $F = 15.2$, $P \leq 0.0450$; inoculation × species $F = 11.31$, $P \leq 0.0436$. Analysis as a 3 × 1 × 2 (inoculation by misting only) *F* test: antitranspirants $F = 9.0$, $P \leq 0.0156$. Analysis as a 3 × 1 × 2 (inoculation by infiltration only) *F* test: antitranspirants $F = 3.35$, $P \leq 0.1053$. No sign.
[b] Total by misting = 54.2%.
[c] Total by infiltration = 93.0%.

TABLE 4. Frequency of stomata on the adaxial and abaxial surfaces of the leaf and number of leaf spot lesions produced after inoculation with *Xanthomonas campestris* pv. *vesicatoria* for a group of very susceptible lines and a group with measurable resistance[y]

	Frequency of stomata				Leaf lesions/ cm^2 12 days after inoculation (no.)	
	Adaxial		Abaxial			
Line	Range	Mean	Range	Mean	Range	Mean
720635-1	135–202	174 b[z]	390–471	437 a	3.25–4.12	3.75 a
USDA 28	207–236	220 a	345–372	356 b	2.50–4.50	3.75 a
USDA 79B156	107–109	108 c	209–216	212 e	2.12–3.75	2.75 ab
720518 (*L. hirsutum*)	32–58	42 e	241–271	256 cd	1.50–2.88	2.09 bc
PI 127805-D1 (*L. pimpinellifolium*)	58–66	62 de	122–152	137 f	1.25–2.25	1.75 bc
PI 127826-D1 (*L. hirsutum*)	32–40	36 e	197–266	232 de	1.25–2.00	1.75 bc
Fla 3075-1 (SAD × MH-11) × (Melintka-101)	58–82	72 d	257–285	268 c	1.25–1.63	1.40 c
F value	50.44		78.47		5.49	
Probability	0.0001		0.0001		0.0060	
Minimum significant difference	27.89		31.26		1.335	
Coefficient of variation	16%		7%		20%	

[y] The experiment included four blocks, seven treatments, and eight plants per plot.
[z] Duncan-Waller multiple range test. Those lines followed by the same letter are not significantly different.

that there was an association between the frequency of stomata and the number of leaf lesions per square centimeter developed after inoculation with *X. c.* pv. *vesicatoria* (Table 4). The stomatal frequency on both the adaxial and abaxial surfaces of the leaf was positively correlated with bacterial leaf spot lesions per square centimeter, with correlation coefficients of $r = 0.73$ and $r = 0.62$, respectively (Fig. 1C and D). Significant differences between numbers of stomata bacterial leaf lesions were found by using the Waller-Duncan multiple range test. In a second group of experiments, susceptible and moderately resistant *Lycopersicon* species and their hybrids were included (Table 5). Evidence was also found of a positive correlation between stomatal frequency on both adaxial and abaxial leaf surfaces and leaf lesion counts developed after the infection. Values of $r = 0.77$ and $r = 0.73$ were obtained for the association between stomatal frequency on the adaxial and abaxial surfaces and lesion count, respectively. The strength of the relationship of the two variables was studied by regression analysis using linear, logarithmic, and quadratic models. As shown in the curve-fitting statistic analysis (Table 6), the highest *r*-square ($r^2 = 0.61, r^2 = 0.60$) was obtained by using the quadratic and linear models. In some instances, the larger the r^2 the better the model fit. But the significant test of departure from linearity showed no significance. Thus, the hypothesis of curvilinear regression was rejected (Table 6). The linear model was adapted and is represented in Figure 1. Thus, it was found that as much as 60% of variance of Y (lesion counts) is associated with variations in X (stomatal frequency) in the adaxial and abaxial surface, respectively. Significant differences were also found among the stomatal frequency means, as well as among bacterial spot lesion counts of different *Lycopersicon* spp. and hybrids. Plants with highest stomatal frequency (*L. esculentum* 'Flora-Dade' and 'Florida 1B') had the highest incidence of disease. Those with lowest stomatal frequency (*L. hirsutum* PI 127826-D1 and *L. pimpinellifolium* PI 127805-D1) had the lowest incidence of disease. The midrange values in disease response and stomatal frequency of hybrids (*L. esculentum* × *L. hirsutum* and *L. pimpinellifolium*) suggest that a reduced level of susceptibility may be heritable and quantitatively based. Finally, the variation per plant sample within each treatment (each *Lycopersicon* sp. or tomato cultivar) was analyzed in a randomized block nested design and was not significant.

In both experiments, the relationship between the adaxial stomatal frequency and bacterial lesion counts was stronger than for the abaxial relationship with lesion counts.

Effects of inoculation by misting vs. inoculation by infiltration. Additional evidence for the reduction in bacterial leaf spot infection by application of antitranspirants was obtained in a factorial 3 × 2 × 2 experiment using susceptible vs. moderately resistant plants, two antitranspirants, and inoculations by misting vs. infiltration of the mesophyll (Table 3). In those plants inoculated by misting, abscisic acid and phenylmercuric acetate reduced the disease incidence 33.4 and 16.8% in *L. hirsutum* PI 127826-D1 and 25.0 and 41.7% in *L. esculentum* 'Flora-Dade', respectively. The antitranspirants significantly reduced the disease incidence in those plants inoculated by misting but not in plants inoculated by infiltration. This was demonstrated when a separate analysis was made for each type of inoculation (Table 3, footnote). The interaction between antitranspirants and species was found to be significant. Both the percentage of disease incidence and the disease severity (number of leaf lesions per square centimeter) were reduced when antitranspirants were applied 4 hr before the inoculation (Table 3). *L. hirsutum* PI 127826-D1 control plants were 37.5% more resistant than *L. esculentum* 'Flora-Dade' when inoculated by misting but only 4.2% more when inoculated by infiltration. These inoculations by misting vs. infiltration of the intercellular spaces provided evidence that the lower disease level of the host *L. hirsutum* is mainly due to an external, morphological resistance (i.e., fewer stomata on the surface of leaves) rather than on an internal and intrinsic resistance. This result also indicated that antitranspirants or stomatal closing agents can be used for the reduction of the bacterial leaf spot disease in tomato and that an acceptable level of control of the disease can be achieved by combining low stomatal number with stomatal closure by application of antitranspirants.

DISCUSSION

This research clarifies the role of stomata as natural openings in the ingress infection and colonization of tomato leaves by *X. c.* pv. *vesicatoria*. The amount of infection on tomato leaves—disease incidence and disease severity—observed after their inoculation

TABLE 5. Frequency of stomata on the adaxial and abaxial surfaces of the leaf and number of leaf spot lesions produced after inoculation with *Xanthomonas campestris* pv. *vesicatoria* for two parental susceptible cultivars (P_2), two moderate resistant introductions (P_1) and hybrids (F_1), and two very susceptible lines[y]

	Frequency of stomata/mm²						Leaf lesions/cm² 12 days after inoculation (no.)		
	Adaxial (upper)			Abaxial (lower)					
Breedling line	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD
Flora-Dade (P_2) (Walter × 2153-D2)	140–185	163 a[z]	16.2	237–296	261 ab	20.8	9–12	10.00 b	1.00
Fla 1B (P_2) (Walter × 2153-D5)	135–183	159 a	19.4	200–290	249 b	31.4	8–15	10.56 ab	2.35
720635-1 (very susceptible)	142–211	170 a	25.6	250–322	278 a	22.5	9–13	11.44 a	1.33
USDA 28 (very susceptible)	137–189	157 a	19.5	227–291	266 ab	23.0	9–17	11.78 a	2.86
PI 127805-D1 (P_1) (*L. pimpinellifolium*)	95–138	118 b	13.5	109–223	185 d	22.0	6–9	7.33 c	0.86
PI 127826-D1 (P_1) (*L. hirsutum*)	34–98	63 c	18.9	185–230	201 cd	15.1	4–5	4.33 d	0.50
Flora-Dade × PI 127826-D1 (F_1)	94–141	116 b	15.5	187–225	210 c	23.5	3–6	4.89 d	1.27
Fla 1B × PI 127805-D1 (F_1)	74–138	121 b	13.5	161–218	195 cd	20.9	4–9	6.89 c	1.62
Treatments									
F value		38.82			23.70			31.92	
Probability		≤0.0001			≤0.0001			≤0.0001	
Minimum significant difference		14.64			19.22			1.32	
Coefficient of variation		13			9			18	
Variation among plants									
F value		0.34			0.49				
Probability		≤0.7120			≤0.613				

[y] Three blocks, eight treatments, ten plants per plot.

[z] Duncan-Waller multiple range test. Those lines followed by the same letter are not significantly different. The minimum significant difference is also included.

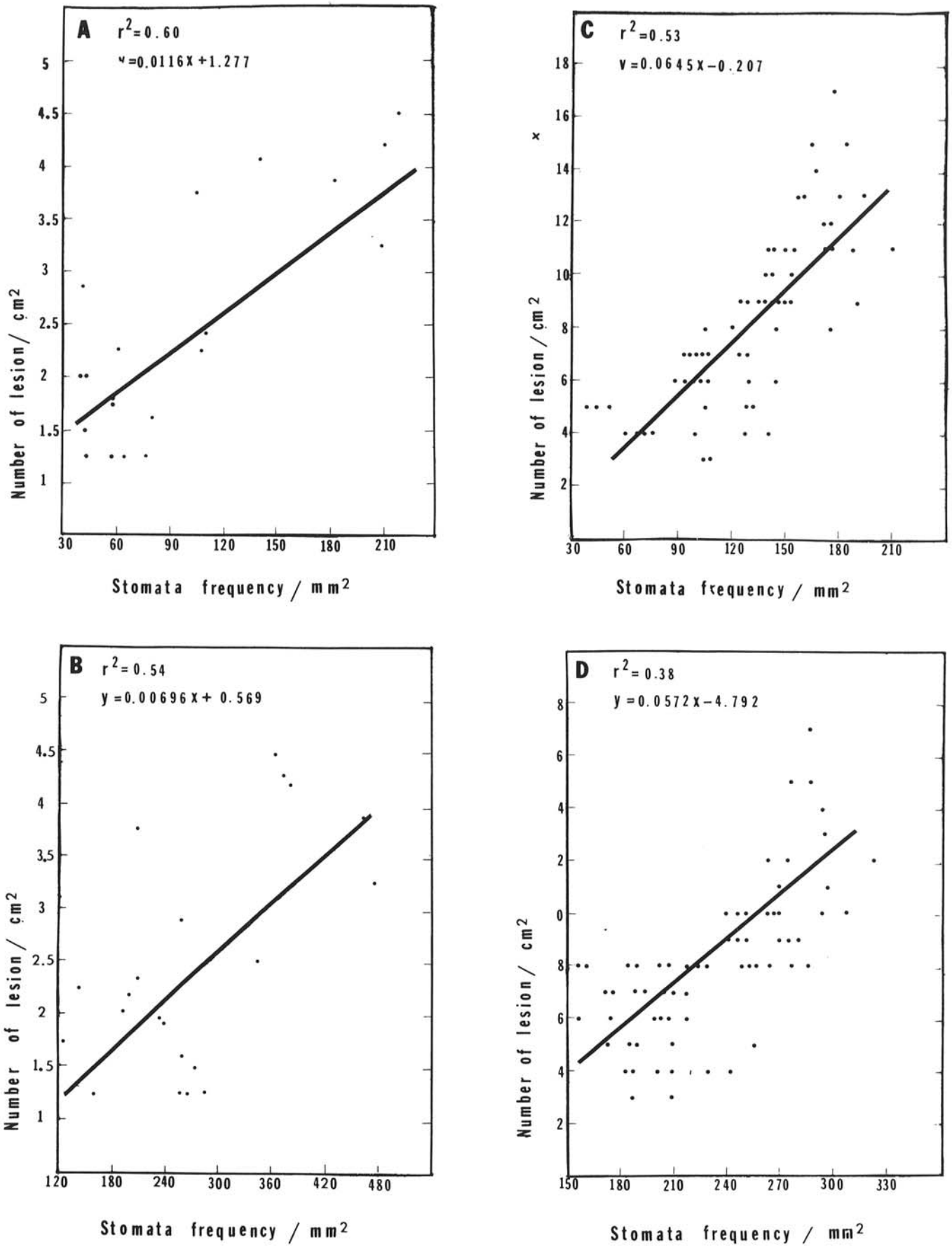

Fig. 1. Relationship between the frequency of stomata in the leaf surface and leaf lesions produced after the infection with *Xanthomonas campestris* pv. *vesicatoria*. Each diagram in the composite figure shows the distribution points and regression line, as well as the square of correlation and regression equation. **A** and **B**, The relationship of the frequency of stomata in the adaxial (upper) and abaxial (lower) surface, respectively, and the number of leaf spot lesions for the experiment presented in Table 3. **C** and **D**, The relationship of the frequency of stomata in the adaxial and abaxial surface, respectively, and the number of leaf spot lesions for the experiment presented in Table 4.

with *X. c.* pv. *vesicatoria* was found to be related to stomatal opening at the time of and immediately following inoculation. The infection was significantly higher when plants were inoculated and maintained under light conditions when stomata were mostly open. In contrast, infection was greatly reduced in tomato plants physiologically preconditioned to close most stomata and maintained in dark or when the stomatal opening was chemically suppressed by phenylmercuric acetate or abscisic acid. Closure of stomata may be an important method for practical disease control in the field. However, the application of chemicals to induce stomatal closure appears to have some adverse effects on photosynthesis and stomatal conductance, which should be investigated. Phenylmercuric acetate, for example, can cause stomatal closure at very low concentrations and extends its effects over 7 days (15), but this chemical can inhibit in some degree the photosynthetic activity of the mesophyll and can produce a substantial physiological toxicity of guard cells and subsidiary cells in some plants (13). In addition, all mercuric compounds are prohibited from agricultural use in food crops.

Inducing open stomata in plants grown under light to close by transferring them to a dark regime is probably insufficient because stomata are also under the influence of endogenous control (8). To overcome this problem, the plants were physiologically induced to close their stomata 4 hr before inoculation in late afternoon near the time when the natural stomatal closure occurs. Antitranspirants do not completely close all stomata, but observation under microscope confirmed that most of the stomata were closed at the time of inoculation.

The *r*-square values indicate that as much as 60% of the variation in the leaf spot lesion counts can be accounted for by the variation in the stomatal frequency, whereas 40% of the time it cannot predict lesion number from stomatal frequency.

Attempts have been made to establish a relationship between stomatal frequency, arrangement of stomata, and resistance to pathogen entry, but conflicting reports have appeared in the literature (15,20,21). Royle and Thomas (16) found a correlation between the number of stomata openings per unit of area at the time of inoculation of hop leaves with *Peronospora humili* Sacc. & Speg. and the severity of subsequent infection of downy mildew expressed as leaf area infected but found no evidence among the cultivar chosen that the number of stomata per unit of area was correlated with resistance to this pathogen. As indicated by research in progress, the stomatal frequency seems to be characteristic of different cultivars of tomato and related *Lycopersicon* species. However, it is necessary to take into account the variation produced by different light intensity, humidity, or position of the leaf insertion in the stem and leaf expansion as reported elsewhere (3,13). In tomato, the stomatal frequency has been reported as higher in young expanding leaves, then decreasing and remaining reasonably constant in full or near fully expanded leaves (6). During these experiments, special care was taken to sample only fully expanded leaves of approximately the same age and similar position on the plant. Fully expanded leaves of tomato plants grown in the same environment have been reported to have similar stomatal densities irrespective of their position on the plant (3). On the other hand, stomatal counts on a single leaflet were taken with caution. The unpublished data of our research supports the findings of Clements and Long (1) that the number of stomata may vary with the portion of the leaf sampled, being lower in the distal portions and higher in the proximal portions of the lamina. In this research, only the middle portions of the leaves were sampled. Also some areas near the midrib were avoided. Pazourek (13) reported that in *Iris hollandica* hor., var. Wedwood, the number of stomata per leaves is not constant, and gradients exist depending on the insertion of the leaf on stem and on plants growing at different light intensities.

Stomatal frequencies were lower in the adaxial surface of the leaf and higher in the abaxial surface. Many lesion counts were made in the abaxial surface of leaves where they were generally developed earlier, but later the necrotic lesions were observed in the abaxial surface as well. Generally, symptoms characterized by water-soaked lesions were first noted 4 or 5 days after inoculation. Necrotic spots were more conspicuous after 6 or 12 days. Delayed observations may show confluent necrotic lesions caused by enlargement of lesions.

Corrections for leaf expansion during the period between inoculation and lesion counting were unnecessary because of the small leaf size and because these samples were taken from leaves fully or near fully expanded. When studying the effects of *Pseudomonas syringae* pv. *phaseolicola* (Burkholder) Dowson on bean leaves. Panopoulos and Schroth (12) made corrections for leaf expansion. Inoculum concentration of 2×10^6 cells m^{-1} was used in the inoculations by misting, whereas concentrations of only 10^5 cells m^{-1} in the inoculation by infiltration were used. This concentration is higher than the one used in the inoculation by infiltration of pepper by *X. c.* pv. *vesicatoria* (2.5×10^3) to determine the components of horizontal resistance (18).

Inoculations by misting vs. infiltration of the mesophyll provided evidence that the lower disease level of the *L. hirsutum* is mainly due to an external, morphological resistance rather than an internal, intrinsic resistance. A moderate level of resistance found in *L. hirsutum* PI 127826-D1 and midrange values of hybrids suggest that this resistance may be heritable and quantitatively based. This mode of inheritance is being studied in this laboratory. These results also indicated that perhaps an acceptable level of disease control can be achieved by combining a low stomatal number with stomatal closure by applications of antitranspirants of stomatal closing agents.

TABLE 6. Curve fitting

Source of variation	r^2	df	Sum of squares	Error MS	*F* ratio	*F* tables 0.05	*F* tables 0.01
Disease rating vs. adaxial frequency of stomata[a]							
Logarithmic	0.5197	1	1.17	0.015	75.75	3.98	7.01
Linear	0.5996	1	428.88	4.09	104.81	3.98	7.01
Quadratic	0.6121	2	437.83	4.02	54.43	3.13	4.92
Departure from linearity		1	8.95	4.02	2.22	3.98	7.01
			$F = 8.95/4.02 = 2.22$				
Disease rating vs. abaxial frequency of stomata[b]							
Logarithmic	0.4491	1	1.0145	0.0178	57.06		
Linear	0.5399	1	386.17	4.70	82.13	3.98	7.01
Quadratic	0.5609	2	401.19	4.55	44.06	3.98	7.01
Departure from linearity		1	15.02	4.55	3.30	3.98	7.01
			$F = 15.02/4.55 = 3.30$				

[a] Linear function: $Y = -0.20 + 0.064\ X$.
Quadratic: $Y = 2.39 + 0.0183\ X + 0.000185\ X^2$.
[b] Linear function: $Y = -4.792 + 0.057\ X$.
Quadratic: $Y = 10.233 - 0.0756\ X + 0.000285\ XY^2$.

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