Penetration Through Leaf Stomata and Growth of Strains of *Xanthomonas campestris* in Citrus Cultivars Varying in Susceptibility to Bacterial Diseases

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ABSTRACT

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Leaf stomata and the pressures required to effect water congestion of tissue and bacterial penetration and growth in leaves were compared for selected cultivars of citrus species and relatives that vary in susceptibility to Asiatic citrus canker and citrus bacterial spot caused by *Xanthomonas campestris* pv. *citri* and *X. c.* pv. *citrumelo*, respectively. The differences among cultivars in structure and density of stomata on leaves expanded by two thirds (most susceptible stage to infection) and leaves fully expanded (least susceptible) were not related to previously reported susceptibility to citrus canker. Leaves, two-thirds expanded, of citrus cultivars were inoculated with *X. c. citri* or *X. c. citrumelo* after pretreatment at three impact pressures to yield incipient water congestion of tissue, full congestion, and congestion with damage to the epidermis. The number of lesions of citrus canker and citrus bacterial spot increased with degree of water congestion, but there was no interaction among cultivars with impact pressure. The number of bacteria that penetrated and the growth of either *X. c. citri* or *X. c. citrumelo* in leaves did not vary significantly among cultivars from 5 to 48 h. Populations continued to increase up to 168 h in citrus cultivars susceptible to citrus canker and in trifoliate orange and its hybrids susceptible to citrus bacterial spot. After 48–72 h, populations of *X. c. citri* were significantly lower in Cleopatra mandarin and in trifoliate orange, which are moderately resistant to citrus canker, and growth of *X. c. citrumelo* ceased in citrus species that are highly resistant to citrus bacterial spot. The number of bacteria recovered from within the infiltrated area at 5 h corresponded with the number of lesions of citrus canker and citrus bacterial spot at 168 h, suggesting that individual lesions developed from infections of stomata. In susceptible cultivars, lesion development was often correlated with bacterial populations at 168 h, but these factors were not correlated in cultivars resistant to citrus bacterial spot. Thus, resistance of citrus leaf tissue was expressed not as reduction in the number of bacteria that penetrated through stomata, but as a reduction in bacterial growth after 72 h.

Additional keywords: stomatal inoculation apparatus.

Variation in susceptibility of *Citrus* species and hybrids of the genus *Citrus* with citrus relatives to *Xanthomonas campestris* pv. *citri* was extensively studied after the first outbreak of Asiatic citrus canker in the United States in the early 1910s (19,23). Comparisons of cultivars were subjective; ratings were commonly based on development of secondary infections after spray or wound inoculation of susceptible immature foliage. In the 1970s, Koizumi (13–16) developed several quantitative inoculation methods for the study of the infection process in resistant and susceptible citrus cultivars. Koizumi and Kuhara (17) used pinprick inoculation and measured lesion extension and bacterial populations in lesions for evaluation of citrus cultivars for resistance to Asiatic citrus canker. This inoculation technique was further applied to determine the susceptibility of *Citrus* species and hybrids with the citrus relative, trifoliate orange (*Poncirus trifoliata* (L.) Raf.), to *X. c.* pv. *citrumelo*, the cause of citrus bacterial spot in Florida citrus nurseries (5,9). The host range of *X. c. citri* is broad, encompassing many citrus species, hybrids with trifoliate orange, and several near-citrus relatives (3,9,17,19,23). The host range of *X. c. citrumelo* is apparently confined to trifoliate orange and hybrids with *Citrus* species, including two rootstock cultivars, Swingle citrumelo and Carrizo citrange (9). Commercial fruit-bearing cultivars of citrus, including grapefruit, sweet orange, mandarins, lemons, and limes, are resistant to citrus bacterial spot (8,9,10).

Pinprick inoculation of leaves bypasses bacterial penetration through stomatal openings by creating a wound in the leaf epidermis and introducing a high concentration of bacteria into the leaf mesophyll tissue (5). Wound inoculation has been necessary to standardize the comparison among disparate leaf types (trifoliate vs. entire) and stages of leaf development because resistance of leaves to stomatal infection increases markedly as leaves mature (6,20,26).

Stall et al (26) attributed the decrease in susceptibility to citrus canker with leaf age to the development of mesophyll resistance

to bacterial multiplication in the mature leaf. On the basis of lesion development, levels of resistance among grapefruit (highly susceptible), sweet orange cultivars (susceptible), and mandarin (moderately resistant) were distinguished after infiltration of 14- to 21-day-old field leaves with 10^3 colony-forming units (cfu) per milliliter, but not when older leaves were used for inoculation.

McLean (21) hypothesized that structural characteristics of stomata, which differed between grapefruit (highly susceptible) and mandarin (moderately resistant), were responsible for the genotypic variation in resistance to citrus canker. Although the stomata of the two cultivars were similar in morphology, the stomatal opening of grapefruit was larger than that of mandarin. This was later correlated with greater pressure required to force water into leaves of mandarin than into those of grapefruit (22).

More recently, Gottwald and Graham (6) determined that the pressures required for water congestion and to obtain bacterial penetration increased dramatically as grapefruit leaves developed from two thirds of full leaf expansion to the fully expanded stage. Stomata on the abaxial surface of the leaf were the point of entry for water and suspensions of *X. c. citri* or *X. c. citrumelo*. Leaves treated on the adaxial surface without stomata or leaves treated at the less than 50% expanded stage, when stomatal pores were not fully open, were not water-congested, and bacterial lesions were not produced. They hypothesized that leaf characteristics that change with leaf expansion must affect the ease with which the leaf becomes congested and bacteria gain entry into mesophyll tissue.

In this report, we reexamine the role of stomatal structure in bacterial penetration of leaves in eight cultivars, including *Citrus* species, trifoliate orange, and citrus hybrids, that range in their susceptibility to citrus canker and citrus bacterial spot. For inoculations, we used a previously described stomatal inoculation apparatus that precisely controls the pressure with which water impacts the leaf surface (impact pressure) and the number of bacterial cells that gain entry through stomata without injury to the leaf (6). Bacterial penetration and growth were quantified by measurement of populations in the leaf and numbers of discrete lesions in the infiltrated area of the leaf up to 168 h after inoculation.

MATERIALS AND METHODS

Stomatal characteristics of citrus cultivars. Trees, 3 yr old, located in an orchard at the Citrus Research and Education Center at Lake Alfred, Florida, were used to sample leaf flushes in the spring of 1987. In March, several branches of the following cultivars were cut back: Red Blush grapefruit (*Citrus paradisi* Macf.), Marsh grapefruit, Valencia sweet orange (*C. sinensis* (L.) Osbeck), sour orange (*C. aurantium* L.), Cleopatra mandarin (*C. reticulata* Blanco), Orlando tangelo (*C. reticulata* × *C. paradisi*), and Swingle citrumelo (*P. trifoliata* × *C. paradisi*). In early June, the basal leaves of the new flushes reached full expansion but were still immature. This leaf and one approximately two-thirds of full expansion were selected on the basis of lengths of the leaf blade from the petiole to the tip. Leaf disks 5 mm in diameter were removed from each side of the midrib at the point of maximum leaf width. Five disks from each expansion stage of leaves were collected from each cultivar and immersed in 3%

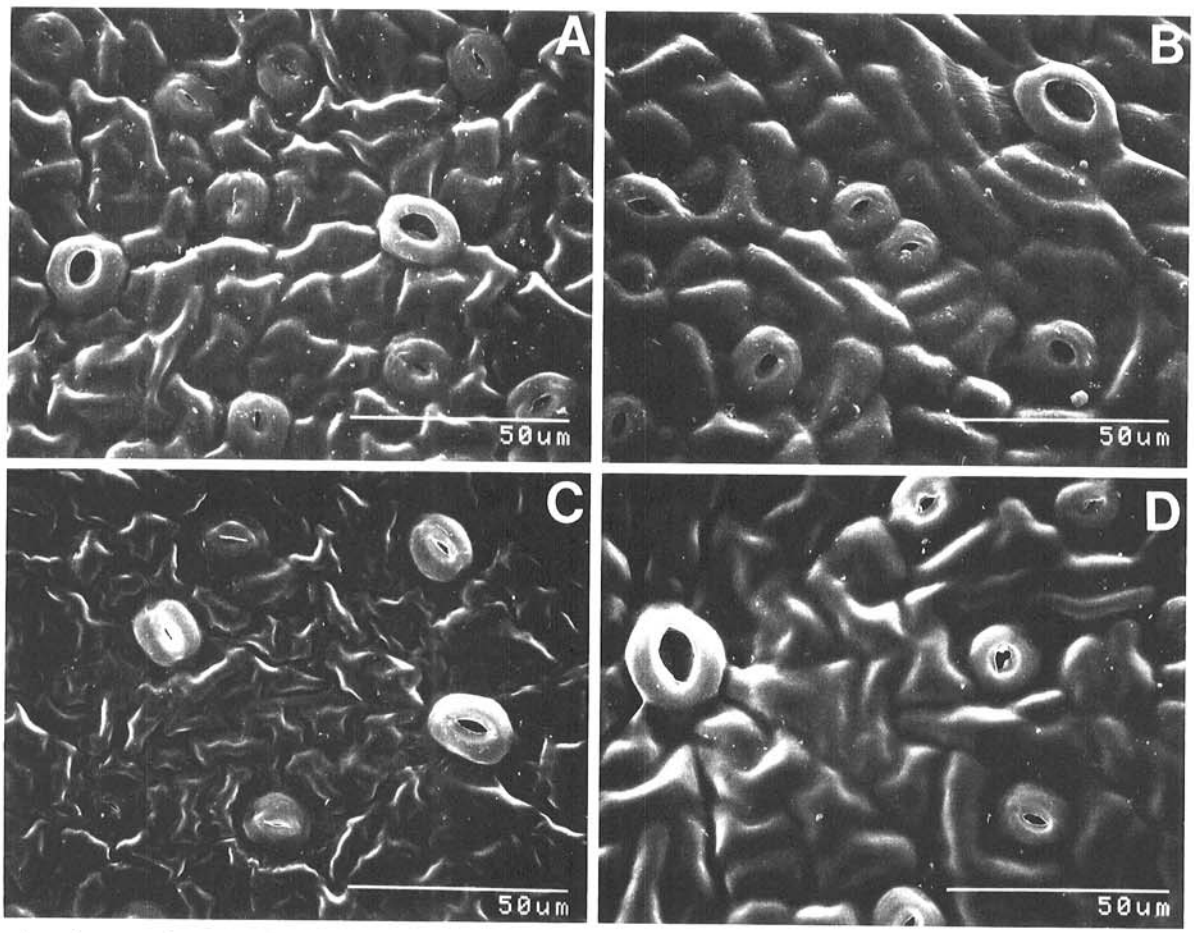

Fig. 1. Scanning electron microscopy of stomata on the leaf surface of Duncan grapefruit (susceptible to citrus canker) and Cleopatra mandarin (moderately resistant to citrus canker) grown under greenhouse conditions. Note that the guard cells in both cultivars form a cuticular hood creating an antichamber over the actual stomatal pore. **A,** Grapefruit leaf surface at the two-thirds–expansion stage shows two large stomata with antichambers open. Small stomata arranged around the large stomata have antichambers in various stages of opening. **B,** Grapefruit leaf surface at full expansion has large and small stomata with fully open antichambers. **C,** Cleopatra mandarin leaves at two-thirds expansion have large and small stomata that resemble those of grapefruit in **A**, with respect to the morphology and size of antichamber openings. **D,** Cleopatra mandarin with open antichambers of large and small stomata that resemble those of grapefruit in **B**.

glutaraldehyde in 0.10 M potassium phosphate buffer (pH 6.8). After fixation overnight at 4 C, the disks were washed with buffer, dehydrated in ethanol, critical point–dried, mounted on a metal stub, and sputter-coated with 10 nm of gold-palladium. To observe stomata, the abaxial surface of the leaf disk was scanned with a Hitachi S530 scanning electron microscope. Two 0.059-mm^2 fields of view on each leaf disk were scanned, and the following observations made: 1) number of large stomata (usually one per field); 2) number of small stomata (stomatal density); 3) number of stomata with antichambers open and with the cuticle still covering, to calculate the percentage of open antichambers (Fig. 1); and 4) the length and width of the antichamber opening to calculate the area of the opening. Thus, seven cultivars were examined at two leaf stages, and five samples were collected from each leaf stage. Parameters were subjected to the General Linear Models (GLM) procedure (SAS Institute, Cary, NC), and mean separation among cultivars was made by the Student–Newman-Keuls multiple range test.

To compare stomata of different species under a single environmental condition, leaves were sampled from greenhouse-grown seedlings of the following cultivars: Duncan grapefruit, Ridge Pineapple sweet orange, sour orange, Cleopatra mandarin, trifoliate orange, Swingle citrumelo, and Carrizo citrange (*P. trifoliata* × *C. sinensis*). Seedlings, 8 mo old, were cut back in June of 1988 to produce shoot flushes so that leaves of full and two-thirds expansion developed at the same time for all cultivars. Leaves from greenhouse-grown seedlings were sampled and analyzed as described for the field study.

Effect of impact pressure on bacterial infection of citrus cultivars. A stomatal inoculation apparatus and strains of *X. c. citri* and *X. c. citrumelo* were used to inoculate leaves of two-thirds expansion at different impact pressures as previously described (6). The citrus cultivars used were the same as those in the study of stomata in the greenhouse, with the addition of Volkamer lemon (*C. volkameriana* Pasq. and Tan.) and were cut back to produce leaves of two-thirds expansion, as described above. Individual leaves were mounted in the apparatus, and an area 3–4 mm in diameter on the abaxial side of the leaf was pretreated with five 200-μl aliquots of sterile water at each of three impact pressures (6.28, 9.81, and 11.57 kPa). The area was then treated at an impact pressure of 6.28 kPa with a 200-μl suspension of strain MF23P of *X. c. citri* or strain F1 of *X. c. citrumelo* containing 1×10^6 cfu/ml of buffer. The volume of water entering the leaf mesophyll over this range of impact pressures varied from 0.1 to 1 $\mu l/cm^2$ leaf (6). For each bacterial strain, seven leaves (plants) of two-thirds expansion of each of the eight cultivars were treated at the three impact pressures. Plants inoculated with one of the bacterial strains were placed in a completely randomized design in a dew chamber (Percival model I-35 DL, Boone, IA) located at the U.S. Department of Agriculture quarantine facility in Plymouth, Florida. The photoperiod was 10 h of light (28 C, 92% RH) and 14 h of dark (30 C, 96% RH). The number of citrus canker or citrus bacterial spot lesions within the treated area on each leaf was counted 7–10 days after inoculation. Because the results of two separate tests with each bacterial strain were very similar, they were combined. The data for each bacterial strain were subjected to the GLM procedure to evaluate effects of cultivar, impact pressure, and their interaction.

Bacterial populations in leaves of citrus cultivars after stomatal inoculation. Leaves of two-thirds expansion for the cultivars used in the previous experiment were pretreated with five 200-μl aliquots of sterile water and inoculated with 200 μl of 10^6 cfu/ml of strain MF23P or F1 at an impact pressure of 9.81 kPa at several locations on each leaf, as described above. Immediately before assaying the internal populations of *X. c. citri* and *X. c. citrumelo* in the leaf, the inoculation site was swabbed with 70% ethanol to surface-disinfest the leaf. Previously, this treatment was very effective for surface disinfestation of detached citrus leaves (7). A leaf disk 4 mm in diameter was removed with a cork borer from 10 different inoculation sites (leaves) for each of the eight cultivars at 5, 24, 48, 72, and 168 h after inoculation (i.e., 10 leaves [plants] for each of the cultivars were repeatedly sampled through time). The tissue was ground in sterile 0.075 M sodium-potassium phosphate buffer (pH 7.0), and appropriate dilutions of the suspension were plated on kasugamycin-cephalexin-Bravo medium as previously described (5,9,10). Bacterial populations were expressed as the log cfu per inoculation site. At 168 h after inoculation, the number of lesions at the inoculation site on 10 leaves was counted. Population data for citrus cultivars were subjected to the GLM procedure for repeated measures analysis of variance with time after inoculation as a repeated measure of populations. Linear contrasts of cultivars were made in the univariate mode after adjustment for correlation among repeated measures. Bacterial populations at 168 h and number of lesions were compared among cultivars by the Student–Newman-Keuls test, and the relationship between the two parameters was examined for each cultivar and all cultivars combined by correlation analysis.

RESULTS

Stomatal characteristics of citrus cultivars. Cultivars in Tables 1 and 2 are listed in descending order from susceptible to moderately resistant to citrus canker on the basis of previous studies (3,9,17,19,23).

In the initial evaluation of the susceptibility of cultivars, we examined the characteristics of large and small stomata for two-thirds and fully expanded leaves. The guard cells of Duncan grapefruit and Cleopatra mandarin (Fig. 1), as well as those of the others tested (not shown), form a cuticular hood, creating an antichamber over the actual stomatal pore. For two-thirds–expanded leaves from field trees, the range in the area of the antichamber opening of the large stomata appeared to coincide

TABLE 1. Characteristics of stomata on leaves of two-thirds and full expansion of field-grown citrus cultivars varying in susceptibility to citrus canker

	Two-thirds expansion				Full expansion			
	Large stomata	Small stomata			Large Stomata	Small stomata		
Cultivar[w]	Area[x] (μm^2)	Area (μm^2)	Density (no./0.059 mm^2)	Open[y] (%)	Area (μm^2)	Area (μm^2)	Density (no./0.059 mm^2)	Open (%)
Red Blush grapefruit	46.5 a[z]	13.4 a	48.0 a	65.0 a	61.8 b	14.3 a	28.2 bc	76.2 bc
Marsh grapefruit	37.8 ab	4.5 c	30.3 b	34.2 bc	60.7 b	13.7 a	25.9 c	88.1 a
Swingle citrumelo	35.1 ab	10.1 b	33.9 b	24.8 c	90.9 a	16.4 a	29.4 bc	60.0 d
Valencia sweet orange	45.2 a	9.9 b	45.0 a	54.4 a	59.9 b	15.3 a	38.6 a	75.2 bc
Orlando tangelo	28.5 b	6.1 c	49.2 a	21.9 cd	50.9 b	14.8 a	32.0 b	69.3 c
Sour orange	22.6 b	3.7 c	32.4 b	10.2 d	41.7 b	13.3 a	21.2 d	91.7 a
Cleopatra mandarin	27.2 b	4.0 c	34.8 b	38.0 b	62.6 b	14.0 a	31.3 b	81.1 ab

[w] Cultivars are listed in descending order of susceptibility to citrus canker based on artificial inoculations and field observations.
[x] Area of the opening of antichambers (see Fig. 1).
[y] Percentage of stomata with open antichambers (see Fig. 1).
[z] Means (of 10 fields, each measuring 0.059 mm^2) in columns followed by the same letter do not differ significantly according to the Student–Newman-Keuls multiple range test ($P \leq 0.05$).

TABLE 2. Characteristics of stomata on leaves of two-thirds and full expansion of greenhouse-grown citrus cultivars varying in susceptibility to citrus canker

Cultivar[w]	Two-thirds expansion				Full expansion			
	Large stomata	Small stomata			Large Stomata	Small stomata		
	Area[x] (μm^2)	Area (μm^2)	Density (no./0.059 mm^2)	Open[y] (%)	Area (μm^2)	Area (μm^2)	Density (no./0.059 mm^2)	Open (%)
Duncan grapefruit	137 b[z]	34.8 b	50.0 b	39.1 b	214 d	60.6 c	31.3 b	99.3 a
Ridge Pineapple sweet orange	161 b	27.1 bc	52.6 ab	34.3 bc	404 abc	112.4 b	27.0 b	98.7 ab
Swingle citrumelo	122 b	20.9 c	58.2 a	28.0 cd	479 a	192.2 a	26.4 bc	98.8 ab
Carrizo citrange	164 b	30.1 bc	56.6 ab	36.3 bc	453 ab	97.3 b	29.4 b	97.0 ab
Sour orange	125 b	27.1 bc	50.6 b	24.4 d	318 b	49.6 c	21.7 c	98.7 ab
Cleopatra mandarin	143 b	34.0 b	50.1 b	34.7 bc	305 bcd	64.7 c	28.5 b	95.6 b
Trifoliate orange	243 a	60.4 a	31.0 c	97.1 a	277 cd	115.7 b	40.7 a	98.1 ab

[w] Cultivars are listed in descending order of susceptibility to citrus canker based on artificial inoculations and field observations.
[x] Area of the opening of antichambers (see Fig. 1).
[y] Percentage of stomata with open antichambers (see Fig. 1).
[z] Means (of 10 fields, each measuring 0.059 mm^2) in columns followed by the same letter do not differ significantly according to the Student–Newman-Keuls multiple range test ($P \leq 0.05$).

with cultivar susceptibility to citrus canker (Table 1). However, there was no relationship between area of the antichamber opening of large stomata and canker susceptibility. Small stomata were 30–50 times more numerous than large stomata and also showed a range in the area of the antichamber opening. A relationship of area of the antichamber opening to cultivar susceptibility was not apparent. Neither the density of small stomata or the percentage of antichambers that were open at the two-thirds or fully expanded stages were related to cultivar susceptibility. The percentage of open antichambers on two-thirds–expanded leaves varied widely among cultivars (10–65%), whereas on fully expanded leaves, a much higher percentage of the antichambers of small stomata were open (60–92%).

Because leaf flushes of the cultivars in the field develop at different times and therefore under varying environmental conditions, measurements of stomatal characteristics of greenhouse-grown plants were performed on leaves that developed at the same time. Cultivars examined were somewhat different than in the field but still represented a range of susceptibility to citrus canker (Table 2). For leaves of two-thirds expansion, the antichamber openings of large and small stomates were four to five times larger for greenhouse than for field leaves (Tables 1 and 2). This would be expected for seedlings grown under a controlled environmental condition (i.e., lower light intensity and reduced heat and water stress). Under greenhouse conditions, only trifoliate orange had stomatal characteristics that deviated consistently from the other cultivars. This cultivar had the largest area of antichamber openings for large and small stomata, the lowest density of small stomata, and the highest percentage of open antichambers. Almost all of the antichambers of trifoliate orange were open at the two-thirds expansion stage, compared to 25–40% open antichambers for other cultivars. For fully expanded leaves, the area of antichamber openings was larger than on leaves two-thirds expanded, and >95% of the antichambers of small stomata were open. There were differences among cultivars in the area of opening for large and small stomata and density of small stomata, but they were not related to cultivar susceptibility to citrus canker. The comparison of stomata for Duncan grapefruit (highly susceptible) and Cleopatra mandarin (moderately resistant) by scanning electron microscopy showed little difference between the two cultivars in the morphology of the antichamber hood and area of the opening (Fig. 1A–D).

Effect of impact pressure on bacterial infection of citrus cultivars. The degree of congestion of leaf tissue resulting from stomatal inoculation apparatus treatments was similar for all cultivars. An impact pressure of 6.28 kPa gave incipient water congestion of tissues, a pressure of 9.81 kPa consistently produced congestion within the 3- to 4-mm-diameter treated area, and a pressure of 11.57 kPa yielded tissue congestion that extended outside of the treated area. No leaf damage was observed at the low and intermediate impact pressures, but collapse of leaf epidermal cells occurred at the highest impact pressure, as revealed by transmission electron microscopy (Graham and Achor, *unpublished*). Regardless of cultivar, the higher the impact pressure, the greater the number of lesions that developed after inoculation with strain MF23P of *X. c. citri* ($P \leq 0.001$) (Fig. 2). Cultivar had no significant effect on lesion development at any of the

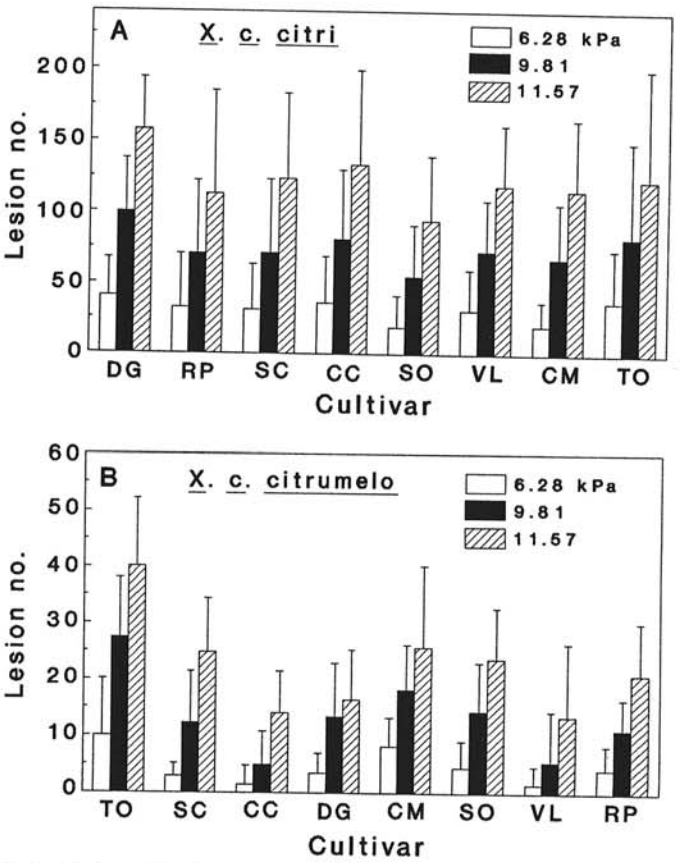

Fig. 2. Relationships between impact pressure for inoculation with **A,** *Xanthomonas campestris* pv. *citri*, and **B,** *X. c.* pv. *citrumelo*, on two-thirds–expanded leaves of citrus cultivars, and the development of lesions of citrus canker and citrus bacterial spot, respectively, 7–10 days later. Impact pressures of 6.28, 9.21, and 11.52 kPa were used to produce incipient tissue congestion, full congestion, and congestion with damage to the epidermis, respectively, before inoculation with 2×10^5 cfu/ml at an impact pressure of 6.28 kPa. Citrus cultivars are listed from left to right in decreasing order of observed susceptibility to citrus canker, **A,** and citrus bacterial spot, **B,** in previous reports (see text). DG = Duncan grapefruit, RP = Ridge Pineapple sweet orange, SW = Swingle citrumelo, CC = Carrizo citrange, SO = sour orange, VL = Volkamer lemon, CM = Cleopatra mandarin, and TO = trifoliate orange. Bars represent 95% confidence interval.

pressures. Thus, the interaction between impact pressure and cultivar was not significant.

Inoculation with *X. c. citrumelo* strain F1 produced significantly ($P \leq 0.01$) greater lesion numbers on trifoliate orange, the most susceptible cultivar (9), than on other less susceptible hybrids and nonsusceptible citrus species at all three impact pressures (Fig. 2B). Higher impact pressures significantly ($P \leq 0.001$) increased lesion numbers, but there was no interaction between impact pressure and cultivar.

Bacterial populations in leaves of citrus cultivars after stomatal inoculation. Recovery of *X. c. citri* strain MF23P at 5 h after inoculation ranged from 2.2 to 22.2 cfu per inoculation site and did not differ significantly ($P \leq 0.05$) among cultivars. Population development up to 168 h was similar among cultivars, except for Cleopatra mandarin and trifoliate orange (Fig. 3A). Bacterial populations in these cultivars were comparable to the other cultivars up to 48 h but by 168 h were significantly lower. At 168 h, there was a significant correlation between bacterial populations and number of lesions that developed within the inoculation sites for six of the eight cultivars (Table 3). Overall, when all cultivars were considered, the correlation between population and lesion development was not as strong ($r = 0.57$) but was significant ($P \leq 0.01$).

Recovery of *X. c. citrumelo* strain F1 at 5 h after inoculation ranged from 5.0 to 28.5 cfu per site. Populations of strain F1 were comparable among cultivars up to 48 h after inoculation, except for Volkamer lemon (Fig. 2B). By 168 h, population growth had slowed in Swingle citrumelo, trifoliate orange, Carrizo citrange, and Cleopatra mandarin, but populations in these cultivars were significantly greater than in the other cultivars where bacterial growth had stopped. For several cultivars, populations of strain F1 at 168 h were lower, but greater numbers of lesions

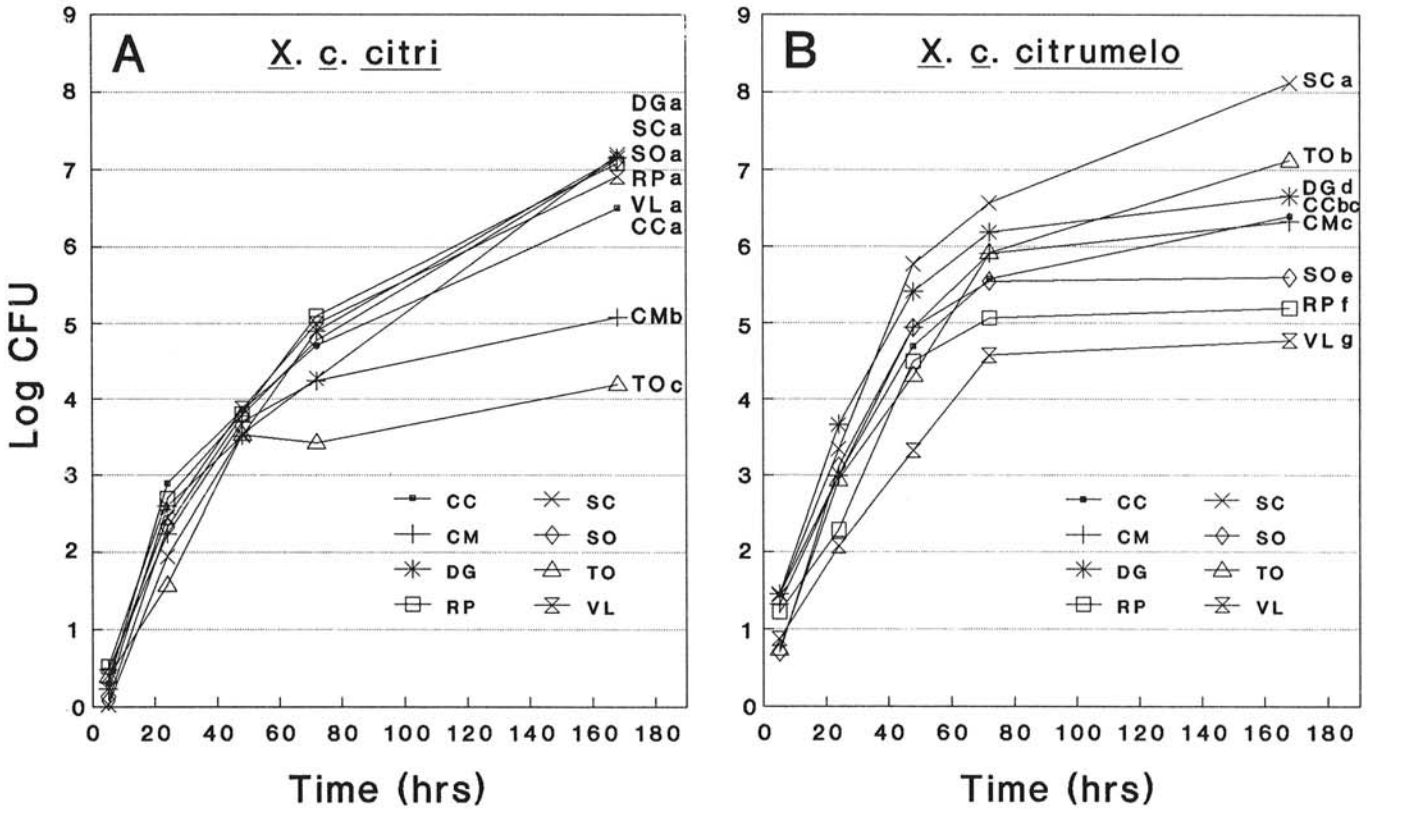

Fig. 3. Growth of **A,** *Xanthomonas campestris* pv. *citri*, and **B,** *X. c.* pv. *citrumelo*, in two-thirds–expanded leaves of citrus cultivars after pretreatment with water to produce water congestion, and inoculation with 2×10^5 cfu/ml at an impact pressure of 9.81 kPa. DG = Duncan grapefruit, SC = Swingle citrumelo, SO = sour orange, RP = Ridge Pineapple, VL = Volkamer lemon, CC = Carrizo citrange, CM = Cleopatra mandarin, and TO = trifoliate orange. Cultivars followed by unlike letters have significantly different ($P \leq 0.05$) population development with time according to linear contrast analysis.

TABLE 3. Relationship between population size and lesion number at 168 h after inoculation with *Xanthomonas campestris* pv. *citri* or *X. c.* pv. *citrumelo* in citrus cultivars

	X. c. citri			*X. c. citrumelo*		
Cultivar	Log cfu[y]	Lesions[y]	Correlation[z] (*r*)	Log cfu	Lesions	Correlation (*r*)
Swingle citrumelo	7.20 a	23.7 a	0.59*	8.12 a	51.5 a	0.90**
Duncan grapefruit	7.16 a	20.1 a	0.60*	6.65 c	50.3 a	0.19
Sour orange	7.13 a	21.6 a	0.54	5.60 d	22.9 b	0.26
Ridge Pineapple sweet orange	7.09 a	13.4 abc	0.93**	5.20 de	24.6 b	0.13
Volkamer lemon	6.90 a	22.1 ab	0.71*	4.77 e	24.5 b	0.21
Carrizo citrange	6.49 a	9.6 c	0.91**	6.39 c	14.4 b	0.96**
Cleopatra mandarin	5.35 b	11.9 bc	0.76*	6.32 c	28.1 b	0.17
Trifoliate orange	4.57 c	11.0 c	0.36	7.12 b	24.6 b	0.64*
All cultivars	6.55	17.2	0.57**	6.27	30.1	0.51**

[y] Bacterial population or lesions per inoculation site. Means ($n = 10$) in columns followed by the same letter do not differ significantly according to the Student–Newman-Keuls multiple range test at $P \leq 0.05$.

[z] Correlations between populations and lesion number significant at $P \leq 0.01$ (**) or $P \leq 0.05$ (*).

occurred, than in plants inoculated with MF23P (Table 3). Bacterial populations and lesion numbers were significantly correlated for only Swingle citrumelo, Carrizo citrange, and trifoliate orange, the three cultivars that exhibited the highest population levels over the 168-h period. The correlation coefficient for all cultivars, although significant ($P \leq 0.01$), was low ($r = 0.51$).

DISCUSSION

Leaf age has been shown to greatly influence water congestion and penetration by *X. c. citri* and *X. c. citrumelo* for Duncan grapefruit (6). In this study, we examined stomata of several citrus cultivars and citrus hybrids with trifoliate orange that range in their susceptibility to citrus canker and citrus bacterial spot. Examination of stomata on two-thirds and fully expanded leaves did not reveal major differences in stomatal structure between highly susceptible and moderately resistant cultivars, as previously suggested (21). In general, stomata were similar (Fig. 1) on the basis of morphology of the antichamber, area of the antichamber opening, density of stomata, and percentage of antichambers that were open. Paradoxically, leaves of two-thirds expansion that had smaller openings and a lower percentage of open antichambers were much more prone to water congestion and bacterial penetration than fully expanded leaves, as previously demonstrated (6).

McLean and Lee (22) reported that higher pressures were required to force water through stomata of resistant mandarin than susceptible grapefruit. We used varying impact pressures to produce a range of water congestion and inoculated two-thirds–expanded leaves. The relationship between impact pressure and number of lesions that developed, as a bioassay of bacterial penetration, did not vary among cultivars. This is consistent with the observed lack of major differences in stomata for these citrus cultivars. By contrast, Ramos and Volin (24) reported that number of lesions formed on *Lycopersicon* genotypes after mist inoculation with *X. c.* pv. *vesicatoria* was correlated with the density of stomata on the upper and lower surface of leaves.

In an earlier study, we showed that if the volume of water forced into the leaf mesophyll was at noninjurious impact pressures, as few as 2.4 bacteria were required to produce a single lesion (6). Stall et al (26) found a direct relationship between number of bacteria placed in the mesophyll by injection-infiltration and number of lesions that developed on susceptible grapefruit leaves. However, the injection-infiltration method relies on wounding the tissue to introduce bacteria into leaf mesophyll, which may explain why this method has not been useful for study of host–strain interactions of *X. c. citrumelo* strains on citrus (5). By the stomatal inoculation method, the range of bacterial populations recovered at 5 h corresponded with the number of lesions that developed 168 h later. Each lesion apparently formed at an individual stomatal infection site. Given the stomatal density and number of open antichambers on greenhouse-grown leaves (Table 2), the number of openings was more than 200 times greater than the number of bacteria that entered the leaf and produced a lesion. Given an inoculum density of 200,000 cfu of bacteria in 200 μl applied per site and an average volume of water of 0.5 $\mu l/cm^2$ of leaf entering the mesophyll, no more than 60 cfu of bacteria actually penetrated the leaf surface at the inoculation site (6). Thus, other conditions at the leaf surface must have limited bacterial penetration. We conclude that leaf characteristics that influence infiltration of the leaf surface by water and congestion of tissue are more important determinants of bacterial penetration than genotypic differences in density and structure of stomata. A rapid development of the cuticle and build-up of waxes around the antichamber opening as leaves expand from two-thirds to full expansion is coincident with a large increase in resistance of Duncan grapefruit leaves to water congestion and bacterial penetration (6; Graham and Achor, *unpublished*).

Because leaves are highly susceptible to stomatal infections only from the two-thirds to full expansion stage, cultivars with a greater frequency, size, and duration of leaf flushes, such as grapefruit, are more "field susceptible" to Asiatic citrus canker than less vigorous citrus cultivars. Hence, epidemics of citrus canker occur when leaf flushes coincide with weather conditions that are ideal for infection and spread of *X. c. citri* (18,25). Citrus rootstocks that impart vigor and increase the frequency and duration of shoot flushes by the scion increase the spread and severity of citrus canker compared to nonvigorous rootstocks (1,4). Protection of leaf flushes with bactericides for the period of weeks that leaves are susceptible is the most effective approach to reduce inoculum and subsequent infections (28).

As expected (9), differences in population growth and lesion development were small among citrus species considered highly to moderately susceptible to citrus canker. In most cases, number of lesions on these cultivars was significantly correlated with populations of *X. c. citri* at 168 h. In moderately resistant trifoliate orange, a citrus relative, bacterial populations were lower and unrelated to lesion development. Similarly, in cultivars susceptible to citrus bacterial spot, trifoliate orange and its hybrids, Swingle citrumelo and Carrizo citrange, populations of *X. c. citrumelo* and number of lesions were highly correlated; in resistant citrus species, however, they were consistently poorly correlated. For instance, in moderately resistant Duncan grapefruit, bacterial populations were intermediate among cultivars, but lesion numbers were among the highest. For susceptible trifoliate orange, lesion numbers were no greater than in several resistant citrus cultivars, yet bacterial populations were among the highest.

Up to 48 h, *X. c. citri* and *X. c. citrumelo* multiplied equally well in susceptible and resistant cultivars. Previously, Koizumi (16) observed no differences in cellular reactions between canker-susceptible *C. natsudaidai* and resistant *C. junos* until 72 h after infiltration of leaf tissue with *X. c. citri*. The resistant reaction consisted of bacterial envelopment by fibrils that resulted in cessation of bacterial growth in the intercellular spaces (14,16); in the susceptible cultivar, bacterial growth continued beyond 72 h (14,15). Similarly, in our study, growth of *X. c. citri* slowed in moderately resistant trifoliate orange and Cleopatra mandarin after 48–72 h, but populations continued to increase at a nearly logarithmic rate in the susceptible cultivars. In contrast, growth of *X. c. citrumelo* stopped after 72 h in all cultivars except trifoliate orange, Swingle citrumelo, and Carrizo citrange, which are susceptible to citrus bacterial spot (9). At 168 h after inoculation with *X. c. citrumelo*, we observed extensive envelopment of bacteria in Duncan grapefruit but not in Swingle citrumelo (Graham and Achor, *unpublished*), which may be analogous to the cellular reactions of canker-resistant and susceptible cultivars observed by Koizumi (16).

Egel et al (5) was unable to distinguish growth of *X. c. citri* and *X. c. citrumelo* in susceptible and resistant citrus hosts with an injection-infiltration method of inoculation. Even though leaves were injected with only 10–100 bacteria per cm^2 of leaf area, they judged that the injection-infiltration technique delivered bacteria to many susceptible sites in the leaf mesophyll over a large area. Here, the stomatal inoculation apparatus introduced a small number of bacteria (two to 29) through stomata into a small area of tissue without injury. Consequently, we were able to confirm previously demonstrated host–cultivar–strain interactions (9) and, furthermore, observe the expression of mesophyll resistance as previously postulated (26).

Rapid multiplication of the *Xanthomonas* pathovars in citrus leaves up to 48–72 h, then reduction or cessation of bacterial growth by 168 h, is consistent with quantitative expression of resistance for other diseases caused by *X. campestris* (2,11, 12,16,27). Moderate resistance of leaves of the citrus relative, trifoliate orange, to citrus canker was previously demonstrated by pinprick inoculation with *X. c. citri* as a slower rate of expansion of lesions over 40 days compared to susceptible citrus species (9). When citrus species resistant to citrus bacterial spot were inoculated with *X. c. citrumelo*, lesion expansion ceased by 20 days after inoculation (9). In this study, the reduction or cessation of growth of *X. c. citrumelo* in citrus cultivars by 168 h corresponded with an eventual decline of the population in the leaf mesophyll during the 40 days after pinprick inoculation (9). Thus, the reaction of citrus species was a resistant one based on the inability of *X. c. citrumelo* to maintain growth in the

leaf mesophyll and produce continued lesion expansion.

LITERATURE CITED

1. Agostini, J. P., Graham, J. H., and Timmer, L. W. 1985. Relationship between development of citrus canker and rootstock cultivar for young 'Valencia' orange trees in Misiones, Argentina. Proc. Fla. State Hortic. Soc. 98:19-22.
2. Civerolo, E. L. 1975. Quantitative aspects of pathogenesis of *Xanthomonas pruni* in peach leaves. Phytopathology 65:258-264.
3. Civerolo, E. L. 1984. Bacterial canker disease of citrus. J. Rio Grande Val. Hortic. Soc. 37:127-146.
4. Danos, E., Bonazzola, R., Berger, R. D., Stall, R. E., and Miller, J. W. 1981. Progress of citrus canker on some species and combinations in Argentina. Proc. Fla. State Hortic. Soc. 94:15-18.
5. Egel, D. S., Graham, J. H., and Riley, T. D. 1991. Population dynamics of strains of *Xanthomonas campestris* differing in aggressiveness on Swingle citrumelo and grapefruit. Phytopathology 81:666-671.
6. Gottwald, T. R., and Graham, J. H. 1992. A device for precise and nondisruptive stomatal inoculation of leaf tissue with bacterial pathogens. Phytopathology 82:930-935.
7. Graham, J. H., and Gottwald, T. R. 1990. Variation in aggressiveness of *Xanthomonas campestris* pv. *citrumelo* associated with citrus bacterial spot in Florida citrus nurseries. Phytopathology 80:190-196.
8. Graham, J. H., and Gottwald, T. R. 1991. Research perspectives on eradication of citrus bacterial diseases in Florida. Plant Dis. 75:1193-1200.
9. Graham, J. H., Gottwald, T. R., and Fardelmann, D. 1990. Cultivar-specific interactions for strains of *Xanthomonas campestris* from Florida that cause citrus canker and citrus bacterial spot. Plant Dis. 74:753-756.
10. Graham, J. H., Gottwald, T. R., Riley, T. D., and Bruce, M. A. 1992. Susceptibility of citrus fruit to citrus bacterial spot and citrus canker. Phytopathology 82:452-457.
11. Hibberd, A. M., Stall, R. E., and Bassett, M. J. 1988. Quantitatively assessed resistance to bacterial leaf spot in pepper that is simply inherited. Phytopathology 78:607-612.
12. Klement, Z. 1982. Hypersensitivity. Pages 149-177 in: Phytopathogenic Prokaryotes. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.
13. Koizumi, M. 1971. A quantitative determination method for *Xanthomonas citri* by inoculation in detached leaves. Bull. Hortic. Res. Stn. Ser. B. 11:167-183.
14. Koizumi, M. 1976. Behavior of *Xanthomonas citri* (Hasse) Dowson in the infection process. I. Multiplication of the bacteria and histological changes following needleprick inoculation. Ann. Phytopathol. Soc. Jpn. 42:407-416.
15. Koizumi, M. 1976. Behavior of *Xanthomonas citri* (Hasse) Dowson in the infection process. II. Multiplication of the bacteria and histological changes of the host plant following rubber-block press or infiltration inoculation. Ann. Phytopathol. Soc. Jpn. 42:517-525.
16. Koizumi, M. 1979. Ultrastructural changes in susceptible and resistant plants of citrus following artificial inoculation with *Xanthomonas citri* (Hasse) Dowson. Ann. Phytopathol. Soc. Jpn. 45:635-644.
17. Koizumi, M., and Kuhara, S. 1982. Evaluation of citrus plants for resistance to bacterial canker disease in relation to lesion extension. Bull. Fruit Tree Res. Stn. Jpn. D-4:73-92.
18. Kuhara, S. 1978. Present epidemic status and control of the citrus canker disease (*Xanthomonas citri* (Hasse) Dowson) in Japan. Rev. Plant Prot. Res. 11:132-142.
19. Lee, H. A. 1918. Further data on the susceptibility of rutaceous plants to citrus canker. J. Agric. Res. 15:661-665.
20. Lee, H. A. 1922. Relation to the age of citrus tissues to the susceptibility to citrus canker. Philipp. J. Sci. 20:311-339.
21. McLean, F. T. 1921. A study of the structure of stomata of two species of citrus in relation to citrus canker. Bull. Torrey Bot. Club 48:101-106.
22. McLean, F. T., and Lee, H. A. 1922. Pressures required to cause stomatal infections with the citrus canker organism. Philipp. J. Sci. 20:309-320.
23. Peltier, G. L., and Frederich, W. J. 1924. Further studies on the relative susceptibility to citrus canker of different species and hybrids of the genus *Citrus*, including the wild relatives. J. Agric. Res. 28:227-239.
24. 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.
25. Serizawa, S., and Inoue, K. 1974. Studies on citrus canker. III. The influence of wind on infection. Bull. Shizuoka Pref. Citrus Exp. Stn. Jpn. 47:54-67.
26. Stall, R. E., Canteros de E., B. I., and Marco, G. 1982. Importance of mesophyll in mature-leaf resistance to cancrosis of citrus. Phytopathology 72:1097-1100.
27. Stall, R. E., and Cook, A. A. 1966. Multiplication of *Xanthomonas vesicatoria* and lesion development in resistant and susceptible pepper. Phytopathology 56:1152-1154.
28. Stall, R. E., Miller, J. W., Marco, G. M., and Canteros de Echenique, B. I. 1981. Timing of sprays to control cancrosis of grapefruit in Argentina. Proc. Int. Soc. Citric. 1:414-417.