

# Cultivar-Specific Interactions for Strains of *Xanthomonas campestris* from Florida that Cause Citrus Canker and Citrus Bacterial Spot

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## ABSTRACT

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Lesion expansion and development of bacterial populations in leaves after pin-prick inoculation were used to characterize the host interaction of eight citrus cultivars with an Asiatic citrus canker strain of *Xanthomonas campestris* pv. *citri* (group A) and with three strains of *X. campestris* pv. *citrumelo* that differ in aggressiveness and cause citrus bacterial spot in Florida nurseries. Populations of *X. c.* pv. *citri* increased or were maintained in all cultivars, and differences among cultivars in rates of lesion expansion up to 40 days were slight except for trifoliolate orange, which is a citrus relative. In contrast, populations of the aggressive strain of *X. c.* pv. *citrumelo* were maintained in trifoliolate orange and its hybrids, Swingle citrumelo and Carrizo citrange, but declined in Duncan grapefruit, sweet orange, and other citrus cultivars. In general, the size of lesions caused by the aggressive strain on trifoliolate orange, Swingle citrumelo, and Carrizo citrange was greater in the greenhouse and field than on other cultivars where lesion expansion ceased after 20–30 days. Less aggressive strains of *X. c.* pv. *citrumelo* caused smaller lesions than the aggressive strain, and populations declined to  $10^2$  or less in lesions after 40 days on all cultivars. Bacterial population in lesions and lesion size were correlated when all cultivars and strains were considered. We conclude that the aggressive strain of *X. c.* pv. *citrumelo* has a host range different from the Asiatic strain of *X. c.* pv. *citri*. The less aggressive strains, even though they are isolated from citrus, are apparently incompatible with citrus and perhaps should not be classified in *X. c.* pv. *citrumelo*.

Citrus bacterial spot has been identified on at least 20 different citrus cultivars in Florida nurseries, but over 75% of the outbreaks were associated primarily with the rootstock Swingle citrumelo (8,14). Hence, the strains of *Xanthomonas campestris* (Pammel) Dowson from nurseries have been classified as pathovar *citrumelo* Gabriel (4; syn. = *X. campestris* pv. *citri* [Hasse] Dye group E). Swingle citrumelo is a hybrid between Duncan grapefruit (*Citrus paradisi* Macf.) and *Poncirus trifoliata* (L.) Raf., both of which are susceptible to Asiatic citrus canker (1,11–13) caused by *X. c.* pv. *citri* group A (syn. = *X. citri* [Hasse] Dowson; 4). Because Swingle citrumelo has become widely used as a rootstock only in recent years, its susceptibility to citrus canker was not known previously. Observations in Argentina of 8- to 10-yr-old fruiting trees of Swingle citrumelo in comparison to other commercial rootstock cultivars from 1985 to 1987 indicated that Swingle citrumelo was susceptible to Asiatic citrus canker (Graham,

unpublished data). Inoculation of detached leaves and leaves on greenhouse-grown plants with strains of *X. c.* pv. *citrumelo* and *X. c.* pv. *citri* revealed that Swingle citrumelo was somewhat less susceptible to citrus canker than Duncan grapefruit, whereas Swingle citrumelo was more susceptible to the aggressive strain of *X. c.* pv. *citrumelo* than Duncan grapefruit (8). Furthermore, there were differences in lesion development caused by strains of *X. c.* pv. *citrumelo* on the two citrus cultivars, which in part lead to the classification of these strains into aggressiveness types (8).

The principal technique used previously (8) for evaluation of citrus cultivar × strain interactions was lesion diameter measured 30–40 days after pin-prick or Carborundum-rub inoculation of leaves. The pin-prick inoculation procedure was first used by Koizumi and Kuhara (11,12) for evaluation of resistance of citrus species, hybrids, and relatives to Asiatic citrus canker. Garran (5) compared several inoculation techniques for quantification of resistance of 13 different clones of citrumelo and seven commercial citrus cultivars to an aggressive strain of *X. c.* pv. *citrumelo*. Of the four different inoculation procedures (pin-prick, leaf-injection infiltration, leaf-rubbing with Carborundum, and leaf spraying), lesion diameters on leaves after pin-prick inoculation resulted in the most quantitative and reliable evalua-

tion of resistance. Furthermore, bacterial populations in lesions from susceptible cultivars remained high, whereas those in more resistant cultivars could not be detected after 24–30 days (5).

In this study, we sought to determine whether there are different host ranges for *X. c.* pv. *citri* group A and strains of *X. c.* pv. *citrumelo* with the use of the pin-prick method of inoculation for eight cultivars known to be susceptible to Asiatic citrus canker (1,11–13).

## MATERIALS AND METHODS

**Bacterial strains.** Strains F1 (DPI X84-3048), F6 (DPI X84-3401), and F100 (DPI X85-12689) were isolated by the Florida Department of Agriculture and Consumer Services, Division of Plant Industry (DPI) from plants in three different nurseries where outbreaks of citrus bacterial spot occurred. The identification, pathogenicity, and aggressiveness of these strains were reported previously (2,8). The strains were characterized as aggressive (F1), moderately aggressive (F6), or weakly aggressive (F100) on Swingle citrumelo and Duncan grapefruit. Likewise, a previously characterized strain of *X. c.* pv. *citri* (R-1) (from Manatee County, Florida, in 1988) was used for comparison with *X. c.* pv. *citrumelo* strains. Bacteria were suspended in a mixture of milk and glycerin, mixed with silica gel (15), and stored at 5 C until tests were conducted.

**Plant material.** Citrus seeds of the following cultivars from registered seed-source trees were obtained from the DPI: trifoliolate orange (*P. trifoliata*), Duncan grapefruit (*C. paradisi*), Swingle citrumelo (*C. paradisi* × *P. trifoliata*), Ridge Pineapple sweet orange (*C. sinensis* [L.] Osb.), Carrizo citrange (*C. sinensis* × *P. trifoliata*), sour orange (*C. aurantium* L.), Cleopatra mandarin (*C. reticulata* Blanco), and Volkamer lemon (*C. volkameriana* Pasq.). Seedlings were grown for 10 mo under greenhouse conditions. Two tests were conducted from January to April 1989 in the DPI quarantine facility in Gainesville, Florida, where greenhouse conditions ranged from 25 to 30 C and 60 to 100% RH. Plants were pruned 1 mo before the inoculations to produce uniformly susceptible young leaves. Similar results were obtained from each test; therefore, the results of only one of the tests are reported.

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For the field test, seedlings of most cultivars were used, except for Ridge Pineapple sweet orange and trifoliolate orange, which were propagated on Swingle citrumelo rootstock. Ridge Pineapple sweet orange was propagated to avoid problems with *Phytophthora* diseases, and trifoliolate orange was propagated to impart more vigor in the cultivar. Rough lemon (*C. jambhiri* Lush.) was used instead of Volkamer lemon. Trees were about 1 yr old when planted in the field in June 1988. They were pruned in the spring and summer of 1989 to produce uniformly susceptible young leaves among cultivars for two inoculation trials. The results of the two trials were consistent, therefore, only one of the tests is reported.

**Greenhouse inoculation.** Bacterial strains were grown in nutrient glucose broth overnight, pelleted by centrifugation, and resuspended in sterile, distilled water to obtain  $10^8$  cfu/ml (determined

turbidimetrically and confirmed by serial dilution plating on nutrient glucose agar). Four immature leaves of 75–100% expansion on each plant were punctured twice on each side of the midrib with a 26-gauge syringe needle. Leaves were inoculated with two of the four strains (F1, F6, F100, and R-1) on each side of the midrib in different combinations of strains by hanging a 10  $\mu$ l droplet of a suspension of the test strain from the adaxial side of the leaf. A second leaf at a different stage of expansion was inoculated on the same plant with each strain. On a noninoculated plant, needle wounds were treated with sterile, distilled water. There were 10–15 inoculated plants of each cultivar except for trifoliolate orange (five replications), and two observations were made per plant. At 6, 10, 20, 30, and 40 days after inoculation, two lesions per plant were measured under a dissecting microscope at  $\times 8$  magnification with an ocular micrometer. The diameter of the wound reaction on

noninoculated leaves was subtracted from the lesion measurements for each respective cultivar. The average lesion diameters on all cultivars (treatment  $n = 10$ –15) at all sampling times were subjected to the General Linear Model (GLM) procedure (SAS Institute, Cary, NC) for repeated measures analysis of variance with time as a repeated measure of lesions. Linear contrasts of the cultivars were made in the univariate mode after adjustment for correlation among repeated measures. Student's *t*-test was used to compare individual strains among cultivars.

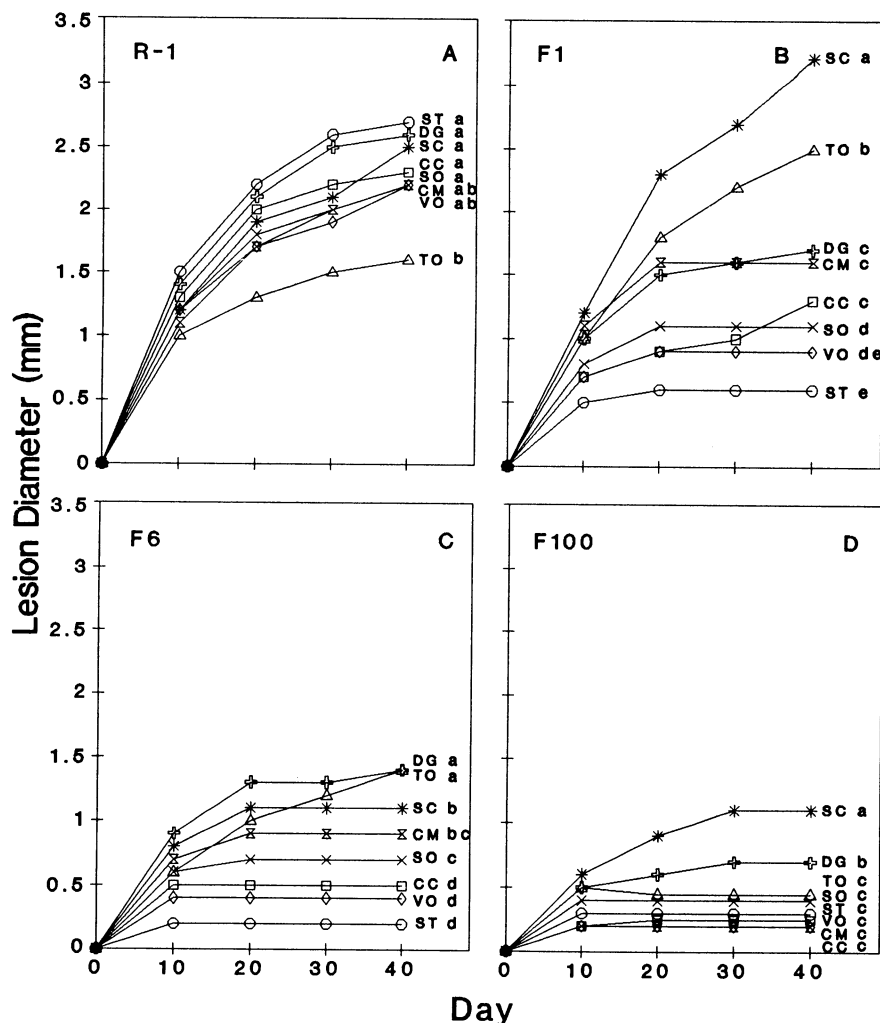
Populations of the four strains in each citrus cultivar were estimated for day 0 from the inoculum added and measured at days 20 and 40 when lesions were expanding and fully developed, respectively (Fig. 1). One leaf disk (13 mm diam.) including the lesion was removed from each of five replicate plants. The tissue was ground in sterile 0.075 M phosphate buffer (pH 7.0) and the suspension was spread on nutrient agar amended with chlorothalonil (Daconil 720 a.i., 12 mg/L). Bacterial populations were expressed as cfu per lesion and subjected to repeated measures analysis as before to contrast strain effects on each cultivar. Selected colonies of each strain recovered from lesions were tested on wound-inoculated detached leaves to confirm that the reaction was true to their aggressiveness type (8).

**Field inoculation.** These tests were conducted at a field quarantine facility in Hastings where introduction of *X. c. pv. citri* was prohibited. Thus, to confirm that the cultivar reactions were the same in the field as in the greenhouse, a direct comparison was made for only the aggressive strain of *X. c. pv. citrumelo* (F1). The bacterium was grown for inoculation as described previously. One fully expanded immature leaf on each plant was inoculated with a pin-prick technique similar to that employed by Koizumi and Kuhara (12). Several pins mounted in a rubber stopper attached to tongs were dipped into a bacterial suspension ( $10^8$  cfu/ml) and immediately clamped over a leaf to produce at least five pin pricks. There were 10 replicate plants of each cultivar except for Ridge Pineapple sweet orange, which had five replications. At 30 and 60 days after inoculation, the size of five lesions on each plant was measured, and the average diameter of the five lesions on each plant was subjected to the GLM procedure to examine differences among cultivars (treatment  $n = 10$ ).

Lesion size was ranked by cultivar and the rankings from the field and greenhouse tests were compared with Spearman's rank correlation coefficient test.

## RESULTS

### Lesion development on greenhouse-grown plants. Lesions on all citrus culti-



**Fig. 1.** Lesion expansion up to 40 days after inoculation of eight citrus cultivars with (A) an Asiatic strain (R-1) of *Xanthomonas campestris* pv. *citri* and (B) an aggressive (F1), (C) moderately aggressive (F6), and (D) weakly aggressive strain (F100) of *X. c. pv. citrumelo*. ST = sweet orange, DG = Duncan grapefruit, SC = Swingle citrumelo, CC = Carrizo citrange, SO = sour orange, CM = Cleopatra mandarin, VO = Volkamer lemon, and TO = trifoliolate orange. (Refer to B for identification of symbols for each cultivar.) Cultivars followed by unlike letters have significantly different ( $P \leq 0.05$ ) lesion expansion with time according to linear contrast analysis.

vars inoculated with strain R-1 of *X. c. pv. citri* continued to expand up to 40 days but the rate of expansion decreased to near zero by that time (Fig. 1A). Lesion development did not differ among cultivars, but the rate was less for trifoliolate orange than all other cultivars except Volkamer lemon and Cleopatra mandarin ( $P \leq 0.05$ ).

The aggressive strain of *X. c. pv. citrumelo*, F1, elicited a much more variable rate of lesion expansion on the eight cultivars than did strain R-1 (Fig. 1B). Lesion development ceased after 20 days on Cleopatra mandarin, Duncan grapefruit, sour orange, Volkamer lemon, and sweet orange, whereas lesions continued to expand on Swingle citrumelo, trifoliolate orange, and Carrizo citrange up to 40 days. Lesion expansion with time was ranked by linear contrast analysis approximately as follows: Swingle citrumelo > trifoliolate orange >

Duncan grapefruit = Cleopatra mandarin = Carrizo citrange > sour orange = Volkamer lemon > sweet orange ( $P \leq 0.05$ ). After 40 days, lesion size on Swingle citrumelo and trifoliolate orange exceeded that caused by *X. c. pv. citri* ( $P \leq 0.01$ ) whereas lesions caused by strain F1 on all other cultivars were significantly smaller ( $P \leq 0.01$ ).

Lesion development after inoculation with the moderately aggressive strain of *X. c. pv. citrumelo*, F6 (Fig. 1C), was significantly less ( $P \leq 0.05$ ) on all cultivars than with the aggressive strain (Fig. 1B). Lesions stopped expanding after 20 days on Duncan grapefruit, Swingle citrumelo, and Cleopatra mandarin and after 10 days on the other cultivars. Lesions were still expanding after 40 days on trifoliolate orange. Lesion expansion varied on the eight cultivars as follows: Duncan grapefruit = trifoliolate orange > Swingle citrumelo = Cleopatra

mandarin > sour orange > Carrizo citrange = Volkamer lemon = sweet orange ( $P \leq 0.05$ ).

Lesions caused by the weakly aggressive strain of *X. c. pv. citrumelo*, F100, were even smaller ( $P \leq 0.05$ ) than those elicited by the moderately aggressive strain except on Swingle citrumelo and sweet orange (Fig. 1D). Lesion expansion ceased by 10 days on most cultivars and by 30 days on Swingle citrumelo and Duncan grapefruit. Cultivars were ranked as follows: Swingle citrumelo > Duncan grapefruit > trifoliolate orange = sour orange = sweet orange = Volkamer lemon = Cleopatra mandarin = Carrizo citrange ( $P \leq 0.05$ ).

**Bacterial populations in greenhouse-grown plants.** Different population development in leaf lesions was evident among cultivars and strains of *X. c. pv. citri* and *X. c. pv. citrumelo* (Fig. 2A-H). Populations of strain R-1 increased in all cultivars except trifoliolate orange where they remained at the inoculated levels (Fig. 2A). Swingle citrumelo was the only host where the population of strain F1 was higher after 40 days than the original inoculum level (Fig. 2B). Populations of F1 were maintained in trifoliolate orange and Carrizo citrange (Fig. 2A and C). On other cultivars, populations of F1, F6, and F100 declined to less than  $10^2$  and, in some cases, to nondetectable levels by 40 days (Fig. 2D-H).

When all cultivars and strains were considered, there was a significant correlation between populations and lesion diameter at 40 days ( $r = 0.86$ ). The correlation was the highest for Volkamer lemon ( $r = 0.98$ ) and lowest for trifoliolate orange ( $r = 0.81$ ).

**Relationship between lesion expansion and greenhouse and field plants.** Lesion development in the field after inoculation with the aggressive strain F1

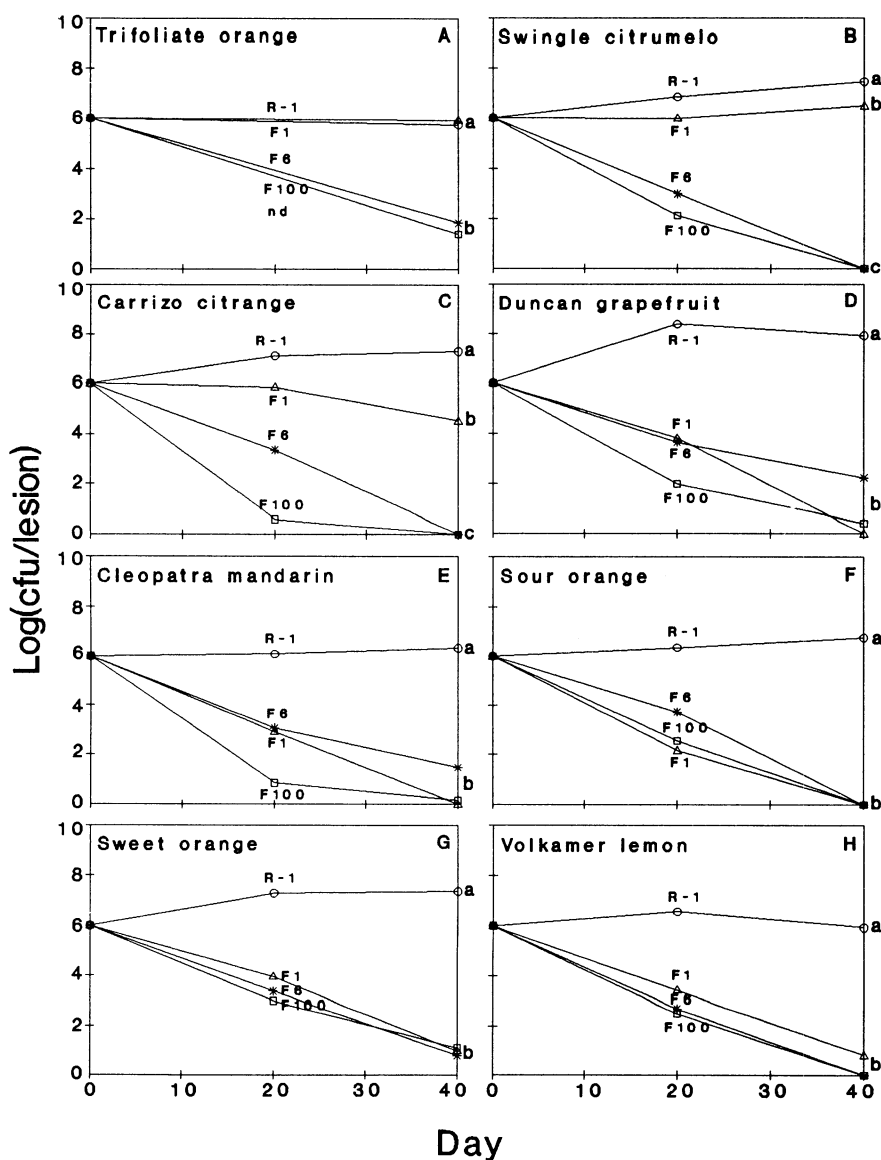


Fig. 2. Bacterial populations in lesions up to 40 days after inoculation of eight citrus cultivars with an Asiatic strain of *Xanthomonas campestris* pv. *citri* (R-1) or an aggressive (F1), moderately aggressive (F6), or weakly aggressive (F100) strain of *X. c. pv. citrumelo*. Points followed by unlike letters have significantly different ( $P \leq 0.05$ ) populations development with time according to linear contrast analysis.

Table 1. Comparison of lesion diameter at 30 days after inoculation of eight citrus cultivars with aggressive strain F1 of *Xanthomonas campestris* pv. *citrumelo* in the field and the greenhouse

Cultivar	Lesion diameter (mm) <sup>2</sup>	
	Field	Greenhouse
Trifoliolate orange	3.87 a	2.28 b
Swingle citrumelo	2.73 ab	2.79 a
Carrizo citrange	2.16 bc	1.08 d
Duncan grapefruit	1.62 c	1.68 c
Cleopatra mandarin	1.56 c	1.65 c
Sour orange	1.17 c	1.14 d
Lemon	1.35 c	0.90 de
Sweet orange	1.32 c	0.60 e

<sup>2</sup> After pin-prick inoculation with  $10^8$  cfu/ml. Values are the mean lesion diameters that differ according to Duncan's multiple range test at  $P \leq 0.05$ . The field and greenhouse rankings of the cultivars were significantly correlated ( $r = 0.71$ ) according to Spearman's rank correlation coefficient test at  $P \leq 0.05$ .

differed significantly among cultivars. Lesion diameter was greatest on trifoliolate orange, followed by Swingle citrumelo and Carrizo citrange (Table 1). Lesion development on other cultivars was substantially less and did not differ. There was a significant ( $P \leq 0.05$ ) correlation ( $r = 0.71$ ) between the rankings of lesion size on the cultivars in the field and in the greenhouse. The major exception was Carrizo citrange, which was ranked higher in the field than in the greenhouse. Greater lesion development in the field corresponded well with the greenhouse observations that lesions were still expanding and that bacterial populations were maintained in Carrizo citrange after 40 days.

## DISCUSSION

All species and hybrids of *Citrus* and *Poncirus* were susceptible to the group A strain of *X. c. pv. citri* from the west coast of Florida based on rates of lesion expansion and development of bacterial populations after pin-prick inoculation. Thus, the host range for *X. c. pv. citri* group A strains among citrus species and hybrids is broad, and *P. trifoliata*, a citrus relative, is susceptible (13). However, lesion expansion and population development on trifoliolate orange was somewhat less than for the other cultivars after inoculation in the greenhouse.

The host range of the aggressive strain of *X. c. pv. citrumelo* was different from that of *X. c. pv. citri*. Populations of the aggressive strain of *X. c. pv. citrumelo* were maintained in leaves of trifoliolate orange and its hybrids in the same way as *X. c. pv. citri*, whereas populations uniformly declined in other citrus cultivars. Growth or maintenance of the bacterium in the leaf was correlated with sustained lesion expansion as previously found for citrus canker (11,12). These reactions define trifoliolate orange as the susceptible parent of Swingle citrumelo and Carrizo citrange and not of grapefruit and sweet orange. Thus, the aggressive type of *X. c. pv. citrumelo* is restricted in host range to trifoliolate orange and its hybrids. This is analogous to the B and C groups of *X. c. pv. citri* (syn. *X. c. pv. aurantifolia*; 4), which primarily attack lemon and lime cultivars (1,6,16).

In Florida nurseries, the aggressive and weakly aggressive strains of *X. c. pv. citrumelo* occur mostly on Swingle citrumelo, whereas moderately aggressive strains have been found as frequently on grapefruit varieties (8). The contrasting rankings of lesion development on these cultivars for the three types of strains in the greenhouse support the field observations and our previous study

(8). In the greenhouse, the moderately and weakly aggressive strains elicited reactions on all cultivars that resembled a resistance response in that lesion expansion ceased by 20 days and bacterial populations declined to  $10^2$  per lesion or less after 40 days. Koizumi and Kuhara (11,12) found that lesion development on resistant cultivars was slower and multiplication of *X. c. pv. citri* group A was less than on the susceptible varieties. Our study and another (5) corroborate the use of the pin-prick technique to determine the relationship between lesion development and population growth for the evaluation of susceptibility of citrus and citrus relatives.

The association of the aggressive strain and Swingle citrumelo with the susceptibility of trifoliolate orange and its hybrids and the other strain  $\times$  host interactions with nonsusceptibility of citrus is remarkably consistent with the epidemiology of these strains in nurseries. Natural spread and stomatal infection of leaves were associated only with the aggressive strains on Swingle citrumelo, whereas less aggressive strains were spread mechanically and wounding appeared to be the principal mode of entry of the bacterium (7,8).

We stated previously (7,8) that the moderately and weakly aggressive strains pose no threat to the citrus industry. The lack of susceptibility of citrus cultivars to less aggressive types supports the contention that these strains will not persist in the nursery in the absence of wounding and mechanical spread. We now find that trifoliolate orange, Swingle citrumelo, and possibly Carrizo citrange are hosts for the aggressive strain in Florida citrus nurseries. Significantly, scion cultivars grapefruit and sweet orange are resistant to the aggressive strains of *X. c. pv. citrumelo* but exhibit lesions in the field. Even so, when heavily infected by means of artificial inoculation, leaves with lesions drop and the infection disappears on these scion cultivars in the field (Graham and Gottwald, unpublished data).

Graham et al (9) have suggested that the aggressive strains are the only type that cause citrus bacterial spot and the less aggressive strains are other *X. campestris* pathogens attacking citrus opportunistically. The less aggressive strains are genetically very diverse in contrast with several other *X. campestris* pathovars (3,4,9,10). If only the aggressive strains are included in *X. c. pv. citrumelo*, the pathovar would be composed of a single, genetically homogeneous group (e.g. Group E2 and E; 3,4,10) with a distinctive host range that

includes *P. trifoliata*, hybrid cultivars with citrus, and perhaps other citrus species and relatives.

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