Population Dynamics of Strains of *Xanthomonas campestris* Differing in Aggressiveness on Swingle Citrumelo and Grapefruit

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ABSTRACT

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The aggressiveness of strains of *Xanthomonas campestris* causing citrus canker (*X. c. citri*) and citrus bacterial spot (*X. c. citrumelo*) on Swingle citrumelo and Duncan grapefruit was assessed by comparing lesion expansion and population development for these strains in greenhouse, growth chamber, and field experiments, using different inoculation techniques and sampling methods. When leaves were pinprick inoculated and resultant lesions sampled over time, there was a positive relationship between internal populations (detected upon macerating lesions) and external populations (detected by swabbing the surface of moist lesions) and between each population and lesion diameter for the different pathovars and aggressiveness types of *X. c. citrumelo*. Correlations among internal and external populations and lesion diameter were higher in the field than under dew-forming conditions in the growth chamber. A leaf-infiltration method revealed few differences in internal populations among pathovars and strains. Strain × host interactions based on the populations and expansion of lesions were apparent for the different aggressiveness types of *X. c. citrumelo* in the field. The highly aggressive strain of *X. c. citrumelo* on Swingle citrumelo most consistently produced the highest bacterial populations and largest lesions. In the field, internal populations were indicative of external populations and therefore might be predictive of the ability of a strain of *X. campestris* to spread on a given host.

Additional keyword: epiphyte.

In Florida, two diseases of citrus are caused by xanthomonads. Asiatic citrus canker, caused by *Xanthomonas campestris* pv. *citri* (syn. *X. citri*) group A strains, has a worldwide distribution and is characterized by erumpent lesions on leaves, stems, and fruits of many citrus cultivars (3). Economic losses from citrus canker may result from fruit lesions (which decrease fresh fruit value), abscission of fruit or leaves, and regulatory measures (e.g., shipping restrictions, eradication) designed to halt the spread of the disease (3,24). Citrus bacterial spot, caused by *X. c. citrumelo* (11) (syn. *X. c. citri* strain E), has only been associated with nursery plants in Florida (21). The majority of outbreaks have occurred on Swingle citrumelo (*Poncirus trifoliata* (L.) Raf. × *Citrus paradisi* Macfady.) or grapefruit varieties (*C. paradisi*). Lesions on stems and leaves are flat, variously water-soaked, and/or necrotic (15). Although *X. c. citrumelo* has not caused severe disease loss, regulatory actions to eradicate citrus canker have been applied to citrus bacterial spot because of uncertainty surrounding the biological relationships of these two diseases and their causal bacteria (21).

The epidemiological significance of strains of *X. c. citrumelo* compared to strains of *X. c. citri* is not fully resolved. The strains causing citrus bacterial spot have been classified into aggressiveness types based on the extensiveness of water-soaking and necrosis on wound-inoculated leaves (15) and by different interactions on Swingle citrumelo, Duncan grapefruit, and other

cultivars (15,16). The most severe reactions are associated with the highly aggressive strain on Swingle citrumelo. Graham et al suggested, based on host reactions and the genetic uniformity of the highly aggressive strains, that they are the only strains that should be classified as *X. c. citrumelo* (16,17).

Less aggressive strains, however, have been found on several citrus cultivars in field nurseries, and when leaves of Swingle citrumelo and Duncan grapefruit are inoculated, lesions are readily formed and undergo limited expansion (15–17). While the highly aggressive strains appear to be spread by windblown rain in nurseries, the less aggressive strains are spread only mechanically down nursery rows on wounded plants (13,15). In a greenhouse study, internal populations, defined here as bacteria recovered upon maceration of lesions, differed according to aggressiveness type on citrus cultivars (16). However, such differences have not been demonstrated in the field. In addition, it is not clear how external populations, defined here as bacteria recovered by swabbing moist lesions, differ among aggressiveness types. The windblown-rain spread of external populations is important in the epidemiology of citrus canker (8,14,23). If internal and external populations are well correlated, it may be possible to use internal population data to estimate external populations, since the latter parameter is quite variable and thus difficult to determine.

We investigated the relationships among internal and external bacterial populations and lesion expansion on leaves for *X. c. citri* and *X. c. citrumelo* on Swingle citrumelo and grapefruit, the cultivars most commonly affected by citrus bacterial spot in Florida nurseries. We used different inoculation and sampling methods in an attempt to relate aggressiveness of the two pathovars and strains of *X. c. citrumelo* to differences in population dynamics in leaves and the availability of external populations for spread. A preliminary report of a portion of this study has been published (9).

MATERIALS AND METHODS

Bacterial strains. All strains were isolated by the Florida Department of Agriculture and Consumer Services, Division of Plant Industry (DPI), except for *X. c. citri* strain MF23P, which we isolated. Three strains of *X. c. citrumelo*, F1 (DPI no. 84-3048), F6 (DPI no. 84-3401), and F100 (DPI no. 85-12869), were previously determined to be highly, moderately, and weakly aggressive, respectively, on Swingle citrumelo and Duncan grapefruit (15). Strains were classified as either *X. c. citri* or *X. c. citrumelo* based on morphological and physiological tests, which identified strains to the species *X. campestris* (21), and their reaction on the host, which determined the pathovar (11,15,16). The two strains of *X. c. citri*, MF23P and 9771, were equally aggressive in wound inoculations, like those reported previously (15). For inoculation, bacteria were cultured 12–15 h in Difco nutrient broth, harvested by centrifugation at 8,000 *g* for 15 min, and resuspended in sterile tap water. Bacteria were adjusted turbidimetrically to 10^8 colony-forming units (cfu)/ml (0.1 absorbance at 600 nm), and appropriate dilutions were made. Final populations were determined by plating on Difco nutrient agar or nutrient-glucose agar (Difco nutrient agar amended with 0.1 g of glucose per liter).

Population dynamics after injection-infiltration of leaves. Experiments were conducted with strain 9771 of *X. c. citri* and the three strains of *X. c. citrumelo* (F1, F6, and F100) on Swingle citrumelo and Duncan grapefruit in a quarantine greenhouse at DPI, Gainesville, FL. Seedlings were cut back to produce uniformly susceptible immature leaves (three-fourths to fully expanded) and were inoculated by injecting 10^4 cfu/ml into the mesophyll of the leaf with a 26-gauge needle, which resulted in initial populations of 10^2–10^3 cfu/cm^2 of leaf area. Populations of bacteria per square centimeter were estimated at 0, 1, 5, 10, 20, 30, and 40 days by harvesting a leaf disk (0.6 or 0.28 cm^2) from a randomly chosen site within the inoculated area and grinding the tissue in 1 ml of phosphate buffer (0.075 M, pH 7.0) in a glass tissue homogenizer. Serial dilutions of the extracts were plated on nutrient agar amended with chlorothalonil (Bravo 720, a.i. 12 mg/L). Populations were expressed as the log transformation of cfu/cm^2 of leaf area. Each treatment was replicated five times, and each replicate was represented by one leaf per strain per seedling. Each experiment involving a particular host was repeated at least once.

Population dynamics under growth chamber conditions. Growth chamber experiments were conducted with Swingle citrumelo seedlings in a dew chamber (Percival model 1-35 DL, Boone, IA) at USDA quarantine facilities in Plymouth, Florida. This experiment was repeated once. Photoperiods were 10 h of light (28 C, 92% RH) and 14 h of dark (30 C, 96% RH). Although dew formed daily, a mist was applied for 4 h before sampling, thereby augmenting moisture on the leaf surface. The experimental design was a randomized, complete block with five replicates for each treatment; seedlings were placed randomly on chamber shelves and rearranged every third day.

Six-month-old seedlings were cut back to produce uniformly susceptible immature leaves, which were inoculated by puncturing each side of the midvein with a 26-gauge syringe needle and applying a drop (10 μl) of a suspension (10^8 cfu/ml) to the adaxial side of the puncture wounds. All four strains (MF23P, F1, F6, and F100) were inoculated onto each leaf at four separate sites, and at least five leaves were treated.

Internal populations of leaves inoculated as above were determined by removing lesions with a cork-borer. The lesion diameter was measured to the nearest 0.5 mm with a micrometer. The tissue was ground in 2 ml of phosphate buffer, and the suspension was plated on KCB semiselective medium (nutrient agar plus kasugamycin 16.0 mg/L, cephalexin 16.0 mg/L, and chlorothalonil [Bravo 720] 12.0 mg/L) (15). Lesions were sampled at 10, 20, 32, and 41 days after inoculation. Populations were expressed as log cfu per lesion.

External populations on leaves were evaluated by absorbing the moisture from the adaxial surface of individual lesions with a sterile cotton swab after an overnight dew cycle. The swabs were placed in 5 ml of phosphate buffer, sonicated for 3 min, followed by 30 min on a rotary shaker. Sonication did not adversely affect the viability of bacteria. The suspension was plated onto KCB medium. External bacterial populations were expressed as log cfu per lesion. Sampling times were as above.

Population dynamics under simulated nursery conditions. Field experiments included *X. c. citrumelo* strains F1, F6, and F100 inoculated onto Swingle citrumelo and Duncan grapefruit at a quarantine facility in Hastings, Florida. The use of strains of *X. c. citri* in field experiments was prohibited by federal and DPI quarantine regulations.

Simulated nurseries consisted of four rows of 25 seedlings (20–30 cm tall) of each cultivar, spaced 10 cm apart within rows and 30 cm between rows, with 10 m between plots. Each plot was separated by nylon screening to act as windbreaks to prevent the spread of bacteria among plots. Plants were inoculated by mechanically rubbing a suspension (10^8 cfu/ml) of each strain with Carborundum onto the upper and lower leaf surface. The experiment was a 3×2 factorial in which each treatment consisted of a strain-cultivar combination. Minimum temperatures during the experiment (spring 1989) ranged from 8 to 23 C, and maximum temperatures ranged from 25 to 35 C. Total rainfall for the experimental period was 117 mm. Seedlings were inoculated on 1 September 1988 and 10 May 1989. Although conducted during different times of the year, the results of the two experiments reinforced each other; data from the second experiment are reported.

After symptoms appeared (about 14 days), seven seedlings were chosen as replicates in each treatment, and five lesions were chosen on each seedling. These lesions were measured to the nearest 0.5 mm with a micrometer at approximately 7-day intervals until 56 days postinoculation. To estimate external populations, the adaxial surfaces of lesions were swabbed at about 8–9:00 a.m., when dew was present. The moisture present on all five lesions of a single plant was absorbed onto a single sterile cotton swab. On some dates the dew formation was too low to supply a sample,

in which case it was augmented by overhead irrigation for 30 min prior to sampling (on days 15 and 36). Each swab absorbed, on average, 25 μl of moisture per seedling. Swabs were placed in 5 ml of phosphate buffer and held at 4 C (for not more than 24 h) until samples could be processed. Vials were vortexed for 10–20 sec, and 0.5 ml was removed and plated at the appropriate dilutions on KCB medium.

Internal populations in leaf lesions were estimated from seven randomly chosen lesions from each treatment at 7-day intervals until 70 days postinoculation. Lesions were removed with a cork-borer and held at 4 C until processed. Lesions were macerated in 2 ml of phosphate buffer in a tissue homogenizer, and the extract plated onto KCB medium.

Statistical analysis. Population and lesion diameter data for each date were compared by ANOVA; if the F test was significant at the $\alpha = 0.05$ level, means were compared by Tukey's HSD procedure ($\alpha = 0.05$) for each date. GLM and Tukey procedures were run using SAS (Statistical Analysis Systems, Cary, NC). In the simulated nursery experiment, significant interactions were present at the $\alpha = 0.05$ level for the cultivar × strain on several dates; therefore, strains were compared separately on each cultivar. The significance of correlation coefficients was determined from tabular information (25).

RESULTS

Population dynamics after injection-infiltration. Strains F1 and F6 of *X. c. citrumelo* caused indistinguishable flat lesions, with water-soaked, necrotic centers, and chlorotic halos 5–7 days after inoculation. Lesions elicited by *X. c. citrumelo* strain F100 developed slowly (8–10 days after inoculation) as small, reddish, raised spots and expanded into slightly raised necrotic areas with little water-soaking or chlorosis. In contrast, lesions caused by *X. c. citri* strain 9771 appeared as raised green spots (5–7 days after inoculation), expanded quickly, and eventually became erumpent, necrotic lesions with marginal water-soaking and chlorosis.

On both hosts, internal leaf populations of strains 9771, F1, and F6 increased rapidly up to 5 days, peaked by 20 days, and slowly declined thereafter under greenhouse conditions (Fig. 1). Except on day 5 for Swingle citrumelo, the populations of these three strains were not different on either cultivar, nor were cultivar-strain interactions detected. Populations of the weakly aggressive strain F100 were approximately 2 log units lower than any other strain; this difference was generally significant for all dates and both cultivars (Fig. 1). On Duncan grapefruit, strain F100 was not detected at 30 and 40 days after inoculation (Fig. 1B).

Population dynamics under growth chamber conditions. A larger number of differences among strains in population size in and on lesions were detected using the pinprick inoculation method than the injection-infiltration method (Figs. 1 and 2). Internal leaf populations of *X. c. citri* strain MF23P and *X. c. citrumelo* strain F1 in Swingle citrumelo were generally not different (Fig. 2A), but were significantly higher than populations of strain F6 by day 30. As previously indicated by injection-infiltration, strain F100 generally produced lower populations than any other strain (Fig. 2A).

In contrast, under dew-forming conditions in the growth chamber, fewer significant differences were observed for external populations than for internal populations. At 10 and 20 days, populations of *X. c. citri* strain MF23P were higher than strains of *X. c. citrumelo*, but populations of strains F1 and F6 were similar (Fig. 2B). Moreover, external populations of strain F100 significantly differed from strains F1 and F6 only on days 20 and 32 (Fig. 2B).

Expansion of the erumpent lesions elicited by *X. c. citri* strain MF23P was not comparable to the flat lesions produced by the strains of *X. c. citrumelo* (Fig. 2C). Lesion diameters produced by strains F1 and F6 were indistinguishable and larger than lesion diameters produced by strains F100 and MF23P, which were not significantly different from each other.

Considering both *X. c. citri* and *X. c. citrumelo* strains, external populations were well correlated with internal populations ($r = 0.64$) at 41 days but were not significantly correlated with lesion diameter ($r = 0.14$). If *X. c. citri*, which did not form a comparable lesion type, was excluded from the analysis, then internal populations and lesion diameters caused by strains of *X. c. citrumelo* were significantly correlated with each other at 41 days (Table 1). Those correlations that included external populations were lower.

Population dynamics under simulated nursery conditions. There were significant host-strain interactions among the three strains of *X. c. citrumelo* on the two cultivars when internal populations were compared (Fig. 3A and B). Internal populations usually exceeded 10^6 cfu/lesion for strain F1 on Swingle citrumelo and were higher than populations of all other host-strain combinations. Populations in lesions produced by F1 and F6 on Duncan grapefruit generally did not significantly differ. Strain F100 populations were consistently below 10^4 cfu/lesion and were not detectable after days 49 and 56 on Duncan grapefruit and Swingle citrumelo, respectively (Fig. 3A and B). Overall, populations in lesions were stable for strain F1 on both hosts, but fluctuated for strain F6 and were constantly dropping for strain F100.

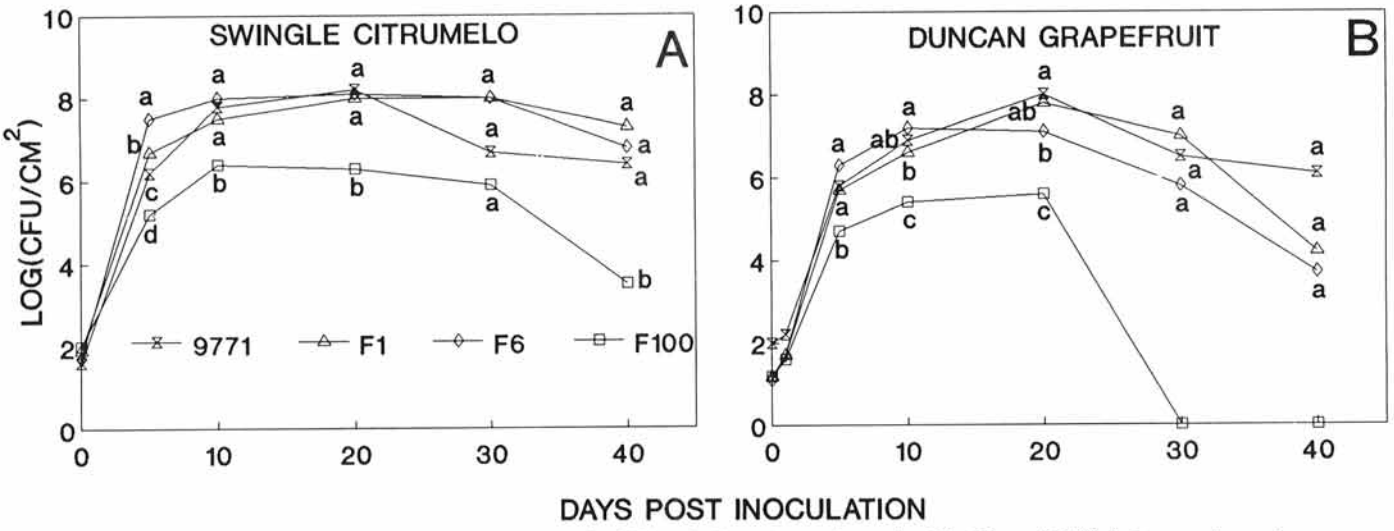

Fig. 1. Populations of *Xanthomonas campestris* pv. *citri* strain 9771 and *X. c. citrumelo* strains F1, F6, and F100 in leaves of greenhouse-grown seedlings of Swingle citrumelo (**A**) and Duncan grapefruit (**B**) inoculated by injection-infiltration. Each data point is the mean of five replications. Mean separation within sampling time by Tukey's HSD, $\alpha = 0.05$.

Bacterial populations sampled from the dew on the surface of leaf lesions followed the same general trends as internal populations (Fig. 3C and D). For Swingle citrumelo, external populations of strain F1 were usually significantly higher than populations of F6 and generally higher than any other host-strain combination (Fig. 3C and D). On Duncan grapefruit, populations of strains F1 and F6 were not significantly different throughout the sampling period (Fig. 3D). External populations on lesions produced by strain F100 were less than 10 cfu/lesion on Duncan grapefruit, but were higher on Swingle citrumelo and similar to populations of F6 (Fig. 3C and D).

The ranking of aggressiveness types by lesion expansion after 56 days (Fig. 3E and F) corresponded with rankings by bacterial population levels in and on lesions. Lesions produced by strain F1 on Swingle citrumelo continued to expand up to 49 days, and the final lesion diameter far exceeded that on other host-strain combinations (Fig. 3E and F). These water-soaked lesions sometimes coalesced, producing leaf abscission and stem dieback. In contrast to strain F1, lesions elicited by strains F6 and F100 appeared dry and stopped expanding after 20 days on both hosts. On Duncan grapefruit, lesions produced by strain F100 did not appear until after 28 days and did not expand thereafter.

When all three *X. c. citrumelo* strains were considered, lesion diameter at 41 days on Swingle citrumelo and Duncan grapefruit was significantly correlated with both internal and external populations on lesions (Table 1). Internal and external populations were also significantly correlated with each other. On Swingle citrumelo, the correlations derived from the field were higher than for the same comparisons from the growth chamber experiment.

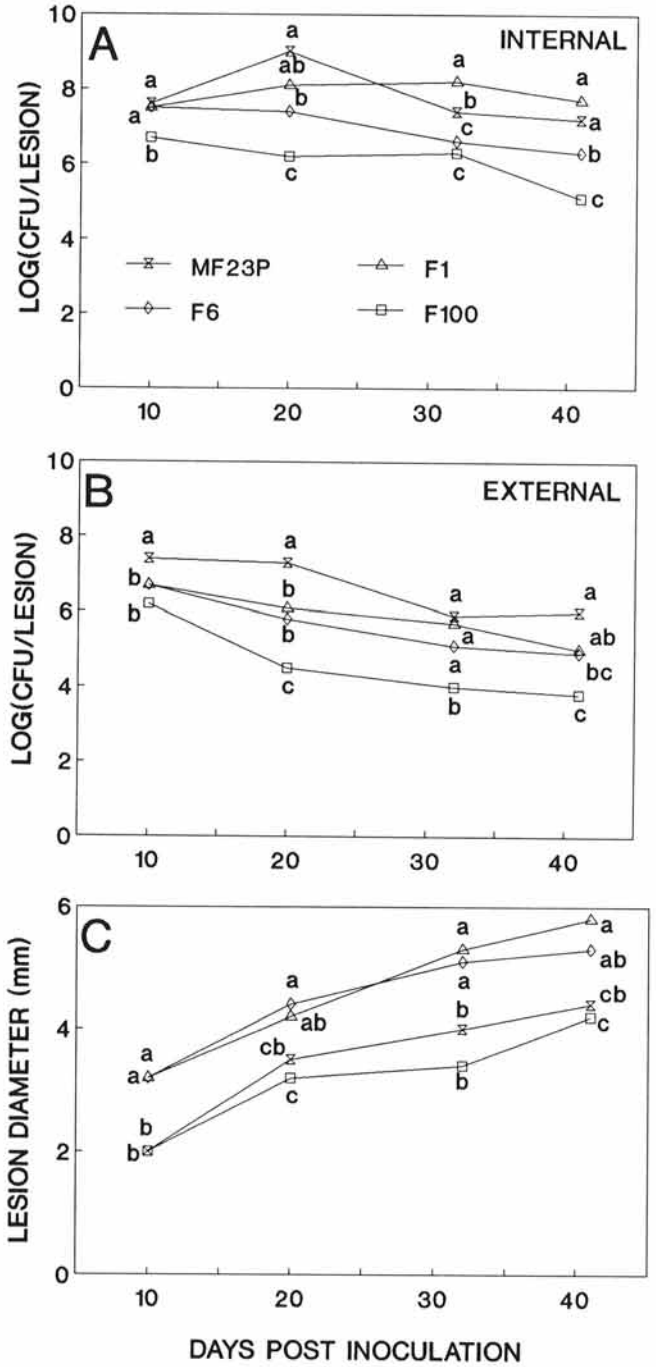

Fig. 2. Internal (**A**) and external (**B**) leaf populations of *Xanthomonas campestris* pv. *citri* strain MF23P and *X. c. citrumelo* strains F1, F6, and F100, and (**C**) expansion of lesions on Swingle citrumelo seedlings inoculated by a pinprick method under growth chamber conditions. Each data point is the mean of five replications. Mean separation within sampling time by Tukey's HSD, $\alpha = 0.05$.

DISCUSSION

Strains of *X. c. citrumelo* varied in their external and internal populations and lesion diameters produced upon wound inoculation of Swingle citrumelo and Duncan grapefruit in simulated nurseries. The highly aggressive strain F1 on Swingle citrumelo produced higher external and internal populations and larger lesion diameters than all other host-strain combinations in the field. These results confirm and extend similar greenhouse experiments, in which the highly aggressive F1 strain had higher populations in lesions on Swingle citrumelo and its parent trifoliate orange than on other citrus cultivars (16). In addition, external and internal populations and lesion diameters were strongly correlated for the different aggressiveness types of *X. c. citrumelo* in the field, although less so in the growth chamber. These findings substantiate why the highly aggressive strains of *X. c. citrumelo* appeared to be spread by wind-driven rain in field situations, but the less aggressive strains were spread only by mechanical means (13; T. R. Gottwald and J. H. Graham, *unpublished data*). Apparently, external populations represent bacteria exuded onto the moist leaf surface through the ruptured epidermis of the lesion and can then be spread by rain events. These data strengthen the contention that, among the aggressiveness types, the highly aggressive strains are the only pathogens of Swingle citrumelo and related cultivars that should be classified as *X. c. citrumelo* (16,17).

Bacterial populations that exist on the leaf surface, whether exuded from lesions (as in this study) or as epiphytes in the absence of lesions, serve as an important source of inoculum for pathogen spread (4–7, 10). Populations of bacteria on the leaf surface have been related to internal populations of *X. c. phaseoli* (2) and *Pseudomonas phaseolicola* (22) on beans. Monitoring internal populations in leaf lesions may be used to determine the potential of a pathogen to spread and may serve as an alternative to estimating leaf surface populations, which are highly variable and thus more difficult to sample. External and internal populations measured here were well correlated at 41 days postinoculation.

TABLE 1. Correlation coefficients for strains of *Xanthomonas campestris* pv. *citrumelo* of different aggressiveness types at 41 days on Swingle citrumelo and Duncan grapefruit

	Growth chamber ($n=15$)	Field ($n=21$)	
Correlation	Swingle citrumelo	Swingle citrumelo	Duncan grapefruit
Internal populations vs external populations	0.56	0.79*[a]	0.84*
Internal populations vs lesion diameter	0.83*	0.87*	0.94*
External populations vs lesion diameter	0.55	0.85*	0.88*

[a]Asterisk indicates coefficients that are statistically significant at the $\alpha = 0.05$ level.

This time period allowed internal populations of strains of *X. c. citrumelo* to increase sufficiently to influence external populations.

Using more artificial inoculation methods under greenhouse or growth chamber conditions, we were less able to clearly distinguish the population dynamics of *X. c. citri* from *X. c. citrumelo* or among aggressiveness types. Internal populations of injection-infiltrated leaves were probably not an accurate indication of the capability of these strains to develop in lesions and, ultimately, to become available for spread in the field. For example, lesions produced by the highly and moderately aggressive strains were indistinguishable on Swingle citrumelo after injection-infiltration. Injection into the leaf mesophyll may deliver bacteria to many susceptible sites over a large area. In contrast, by inoculating with a wounding technique, population growth depended on lesion expansion from a relatively small point of introduction. The pinprick technique was previously demonstrated to be more effective than either leaf spray or injection of *X. c. citrumelo* to determine the susceptiblity of citrus cultivars (12) and the host-strain interaction of *X. c. citrumelo* strains (15,16).

Likewise, the highly conducive dew-forming conditions in the growth chamber were inappropriate for distinguishing external populations of the highly aggressive strain from the less aggressive

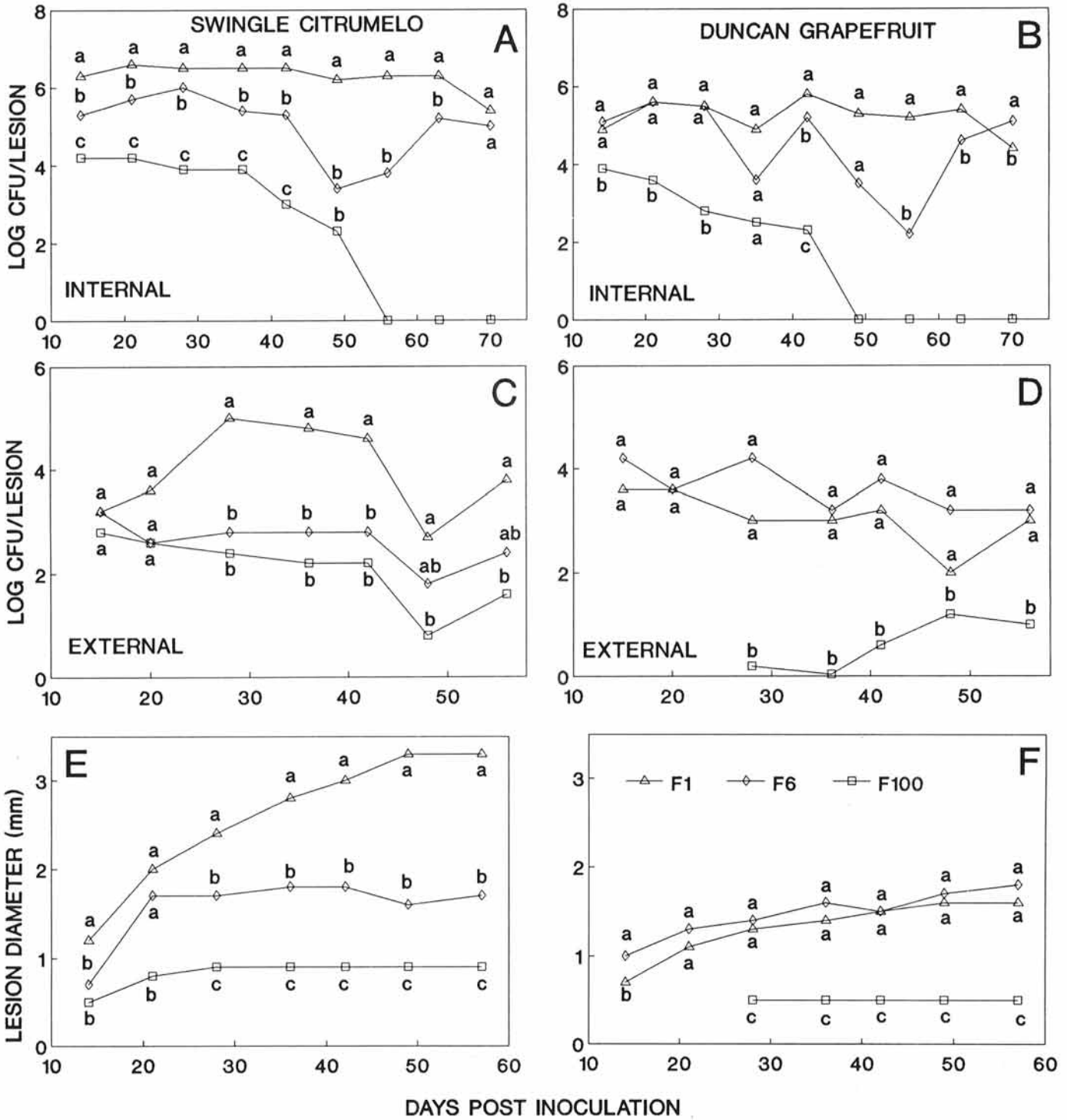

Fig. 3. Internal and external populations of *Xanthomonas campestris* pv. *citrumelo* strains F1, F6 and F100 and lesion diameter on Swingle citrumelo (**A,C,E**) and Duncan grapefruit (**B,D,F**) leaves inoculated by a Carborundum-rub method in field nurseries. Each data point is the mean of seven replications. Mean separation within sampling time by Tukey's HSD, $\alpha = 0.05$.

strains on Swingle citrumelo. O'Brien and Lindow (20) found that differences in epiphytic populations are more likely to be demonstrated with wetting and drying cycles than under constant humid conditions. In our growth chamber experiments, humidity was maintained at 92% or higher, which probably accounted for the lack of differences in leaf surface populations of the highly and moderately aggressive strains on Swingle citrumelo. Field conditions of wetting and drying cycles more truly demonstrated the greater potential for the highly aggressive strains to multiply and survive on lesion surfaces of Swingle citrumelo than in any other host-strain combination.

Furthermore, in the field we were perhaps able to ascertain whether strains of *X. c. citrumelo* behave as true epiphytes in the absence of internal populations. Internal populations of the weakly aggressive strain F100 were not detectable by days 56 and 49 on Swingle citrumelo and Duncan grapefruit respectively, whereas external populations of F100 were detected beyond this time. Sampling by absorbing moisture off of lesions was designed to detect bacteria under conditions conducive for exudation and survival (i.e., mornings, when dew formed an excellent microclimate for bacterial activity on leaves). Sampling of lesions for internal populations was conducted at midafternoon, when leaves were dry and conditions inhospitable for bacterial survival, at least on the lesion surface (J. H. Graham, *unpublished data*). Thus, bacterial populations that existed on the lesion surface were not detected during the sampling for internal populations. The continued presence of F100 on leaf surfaces in the absence of internal populations may indicate that F100 is capable of an epiphytic existence independent of lesion populations (18). The capability for epiphytic survival by *X. campestris* pathovars has been demonstrated on host and nonhost plants in the absence of disease (1,19,26,27). Strains of *X. c. citrumelo* that survived on leaf surfaces may have played a role as an inoculum source for the original outbreaks of citrus bacterial spot in citrus nurseries in which no obvious source of inoculum was observed.

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