Bacterial Exudation from Lesions of Asiatic Citrus Canker and Citrus Bacterial Spot

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ABSTRACT

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When water was added to wells surrounding young lesions of Asiatic citrus canker (caused by *Xanthomonas campestris* pv. *citri*) on detached, field-collected leaves of grapefruit, about 10^4–10^5 bacteria per milliliter were exuded immediately. Bacterial exudation into the water continued at high levels for 24 hr, and cumulative release ranged from 10^5 to 10^6 per lesion. Fewer bacteria were exuded and bacteria were exuded more slowly from old lesions than from young lesions. Bacterial exudation from lesions of citrus bacterial spot (CBS) produced by *X. c.* pv. *citrumelo* on grapefruit and Swingle citrumelo was substantially less than that from Asiatic citrus canker lesions. CBS lesions of the aggressive strain (F1) released more bacteria than those of the moderately aggressive (F6) and weakly aggressive (F100) strains, and exudation declined with all three strains as lesions aged. Lesions of Asiatic citrus canker and CBS lesions produced by the three strains in a dew chamber at 30 C exuded more than 10^6 bacteria per milliliter into water in wells surrounding new lesions. Under these conducive conditions, exudation continued at high levels for 48 hr for most strains of the CBS pathogen. The inability of the CBS pathogen to spread under field conditions unless susceptible tissue is abundant, environmental conditions are favorable, and plants have been injured may in part be the result of low inoculum production and rapid decline in bacterial exudation as lesions age.

Outbreaks of citrus bacterial spot (CBS) and Asiatic citrus canker have occurred in recent years in Florida, and regulatory programs are currently in effect (6,13,16). CBS is caused by *Xanthomonas campestris* (Pammel) Dowson, and the associated strains have been designated as a new pathovar, *X. c.* pv. *citrumelo* (2). However, it has been suggested that the less aggressive strains may be pathovars from other hosts which can infect wounded citrus tissues secondarily (7,9,10).

Asiatic citrus canker, caused by *X. c.* pv. *citri* (*X. citri* ex Hasse [2]), attacks twigs, leaves, and fruit of grapefruit, oranges, and other citrus cultivars and relatives. Infection usually occurs when immature tissues become water-soaked with bacteria-ladened moisture that enters through the stomates; mature tissues are infected only if wounded. Individual canker lesions ranging from 3 to 9 mm in diameter contain 10^6–10^7 bacteria per lesion regardless of lesion size (14). Bacterial populations in lesions formed at spring flush remain at 10^6–10^7 per lesion through the spring, summer, and fall and diminish to 10^4 only after the lesions have overwintered (14). Rainwater collected beneath canker-affected trees contained 10^5–10^6 bacteria per milliliter.

CBS occurs almost exclusively in nurseries, where young, susceptible tissue is abundant and irrigation is frequent, which may favor disease development (3,4). In epidemiological studies (3,4), spread of the pathogen, especially of the less aggressive strains, was primarily mechanical and may have depended on injury to the plants. Bacterial populations within CBS lesions in most hosts are generally lower than those in Asiatic citrus canker lesions in artificially inoculated greenhouse plants (9). Populations of most CBS strains within lesions on most hosts decline rapidly with time, but the aggressive strain maintains populations in lesions on Swingle citrumelo, indicating that citrumelo may be a primary host of this strain (8).

The dynamics of inoculum production in Asiatic citrus canker and CBS have not been compared. Spread of these pathogens is probably related to how much and how fast inoculum is released as well as to the duration of inoculum production. Thus, this study was initiated to compare the effect of time of inoculum release and lesion age on inoculum production by the pathogens of Asiatic citrus canker and CBS in order to compare their potential for rapid dissemination. A preliminary report of a portion of this research has been published (15).

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MATERIALS AND METHODS

Research sites and materials. Most of the research involving Asiatic citrus canker was conducted at the experiment station of the Instituto Nacional de Técnologia Agropecúaria in Concordia, Entre Rios, Argentina. Symptomatic leaves for the inoculum production experiments were collected from naturally infected mature Marsh grapefruit (*Citrus paradisi* Macf.) trees. Lesions referred to as "young" were from fully expanded, recently matured leaves and were usually 4–6 wk old. Those referred to as "old" were from spring flush growth and were 4–6 mo old when tested.

Leaves for experiments with CBS were collected from the quarantine site at the University of Florida experiment station near Hastings, FL. Inoculum production was measured for three strains isolated from CBS lesions in Florida citrus nurseries—F1, F6, and F100, which represent aggressive, moderately aggressive, and weakly aggressive strains, respectively (7). The leaves had been sprayed with a suspension of 10^8 cfu/ml of each strain plus Carborundum and then rubbed with inoculum-soaked gloves. Symptomatic leaves were collected from nursery plots of Duncan grapefruit and Swingle citrumelo (*Poncirus trifoliata* (L.) Raf. × *C. paradisi*) 14, 21, and 49 days after inoculation.

Bacterial exudation from CBS lesions produced by the strains F1, F6, and F100 and from Asiatic canker lesions could not be compared directly under field conditions because of regulatory measures; therefore, comparisons were done at a laboratory in Plymouth, FL, approved for laboratory research on Asiatic canker. To induce lesions for exudation studies, scratches 1 mm long were made on the lower leaf surface of immature leaves of Duncan grapefruit and Swingle citrumelo, and the wounds were then touched with a cotton swab that had been dipped in a suspension of 10^8 cfu/ml. Plants were placed in a dew chamber and held at 30 C with a 12-hr photoperiod and a 10-hr dew period. Bacterial exudation from lesions was evaluated 10 days after inoculation.

All experiments were repeated at least once. Data from representative experiments are presented.

Inoculum production from single lesions. Leaves with lesions were collected from diseased plants, and leaf pieces about 4 cm^2 in area with single lesions were prepared in the laboratory. Wells were affixed to citrus leaves over single lesions. The wells were made from 1.0-cm sections of Tygon tubing 1.0 cm in inside diameter. One edge of the well was dipped in melted paraffin and pressed to the leaf surface until the wax had hardened. This formed a watertight seal surrounding a single lesion. Sterile distilled water (0.5 ml) was placed in the well.

Samples were withdrawn periodically to measure the number of bacteria that had been exuded from the lesion. The zero-time sample was taken immediately after water was added to each of the replicate wells. Between samplings, wells were maintained in a humid chamber at or above 95% relative humidity in the dark at 27 C. At each sample time, the wells were emptied and another 0.5 ml of sterile distilled water was added. Ten replicate lesions were used in each experiment.

Samples from the wells were diluted in 0.075 M phosphate buffer, pH 6.95, containing 4.5 g of KH_2PO_4 and 5.95 g of Na_2HPO_4 per liter. Samples (0.1 ml) were spread on plates of the KCB semi-selective medium described previously (8). The medium contained nutrient agar (23 g/L), kasugamycin (16.0 mg/L), cephalexin (16.0 mg/L), and chlorothalonil (Daconil 720) (16 mg a.i./L). Plates were incubated for 3 days at 28 C. Colonies were then counted, and populations were expressed in terms of colony-forming units per milliliter.

Multiplication of bacteria in wells. To determine whether epiphytic bacteria were able to multiply in water on the surface of leaves, wells as described above were placed on healthy leaves. To obtain epiphytic bacteria, 10 symptomatic leaves from canker-affected Pineapple sweet orange (*C. sinensis* (L.) Osb.) seedlings were washed in 25 ml of distilled water. One milliliter of this bacterial suspension was then placed in wells on healthy leaves. Ten replicate wells were used in each experiment. Samples (0.1 ml) were withdrawn at each sample time, plated on KCB medium, and incubated, and colonies were counted as described above.

RESULTS

Inoculum production with Asiatic canker. About 10^4–10^5 cfu/ml were detected in the zero-time samples from young lesions (Fig. 1A,B). Exudation at each sampling time was about 10^4–10^5 cfu/ml throughout the first 24 hr. Populations at each sample time were about the same even though the water in the wells was changed after each sampling and regardless of whether sampling was infrequent (Fig. 1A) or frequent (Fig. 1B). Cumulative bacterial exudation increased over time initially but eventually leveled off. Fewer than 10^2 cfu/ml were produced in samplings beyond 48 hr.

Fewer bacteria were exuded from old lesions than from young lesions (Fig. 1C). Wetting old lesions raised the bacterial concentrations, but levels were still much lower than those of younger lesions. Bacterial concentrations exuded at each sampling time were generally about 10^2–10^3 cfu/ml over the 48-hr period tested.

Bacterial exudation from individual lesions was quite variable. Occasionally, no bacteria at all were exuded from a lesion throughout the experiment; in other cases, no bacteria were detected at early sample times, but large amounts were detected later. Thus, the standard errors for most sample times and experiments were large.

When bacterial suspensions washed from symptomatic leaves from plants affected with Asiatic canker were placed in wells on leaves without lesions to determine the fate of these bacteria on the wetted leaves, populations declined gradually during the first 8 hr and were at low levels by 24 and 48 hr (Fig. 2). There was no evidence of multiplication under these conditions.

Inoculum production from CBS lesions. CBS lesions exuded much lower levels of bacteria than Asiatic canker lesions regardless of the strain, host plant, or age of the lesion (Fig. 3). Low levels of bacteria were exuded from lesions of the aggressive strain F1, even when younger lesions were used. Very few or no bacteria of the moderately and weakly aggressive strains (F6 and F100, respectively) were detected in water exposed to lesions from the field.

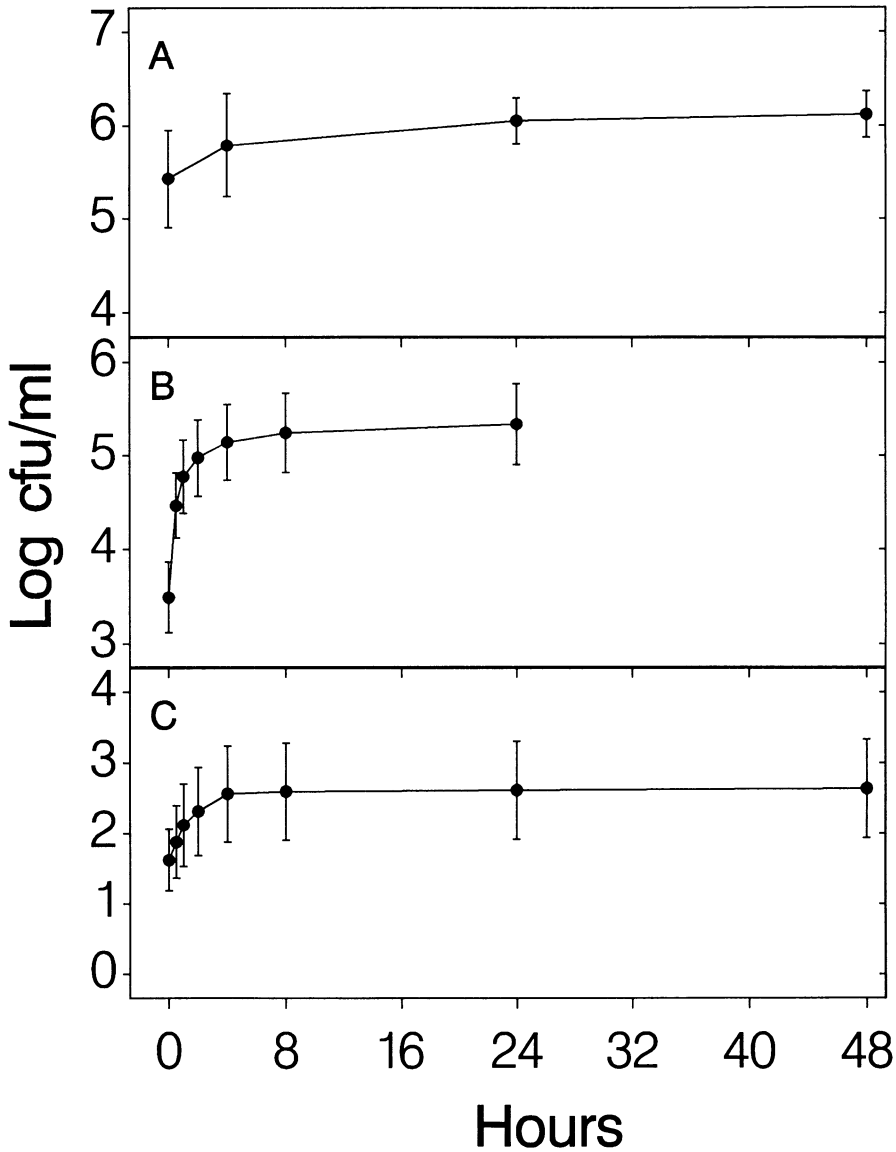

Fig. 1. Cumulative populations of *Xanthomonas campestris* pv. *citri* exuded into water placed in wells surrounding individual lesions of Asiatic citrus canker. Water in wells was completely replaced at each sampling time. **A and B,** Data from experiments with young lesions (4–6 wk old). **C,** Data from experiment with old lesions (4–6 mo old).

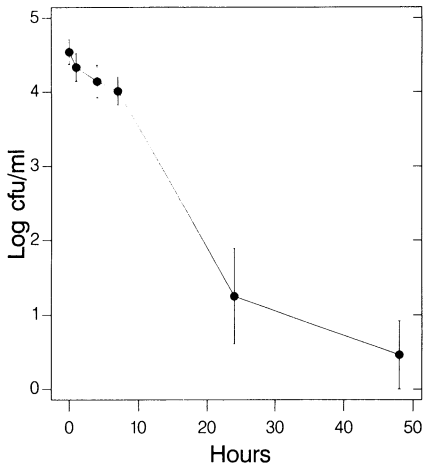

Fig. 2. Populations of *Xanthomonas campestris* pv. *citri* in bacterial suspensions prepared by washing canker-affected leaves of Pineapple sweet orange seedlings with distilled water and placed in wells affixed to asymptomatic leaves in the laboratory and sampled over time.

A few lesions exuded most of the bacteria from CBS lesions, and many lesions produced few or no bacteria.

Analysis of variance of the total numbers of bacteria exuded indicated that strain F1 produced significantly more bacteria than strain F6 or F100, and 14-day-old lesions produced more bacteria than 49-day-old lesions. There was no significant difference between grapefruit and Swingle citrumelo in the number of bacteria exuded from the lesions.

Growth chamber comparison of Asiatic citrus canker and CBS. In contrast to CBS lesions from field plots, newly formed lesions that developed under growth chamber conditions yielded high populations of bacteria (Fig. 4). With most host-strain combinations, bacterial levels were about 10^5–10^6 cfu/ml initially, rose somewhat during 8 hr of wetting, and continued high through 48 hr. Lesions induced by the F100 strain on grapefruit yielded low levels of inoculum initially, but by 48 hr of wetting, cumulative totals were comparable to those of the other strains.

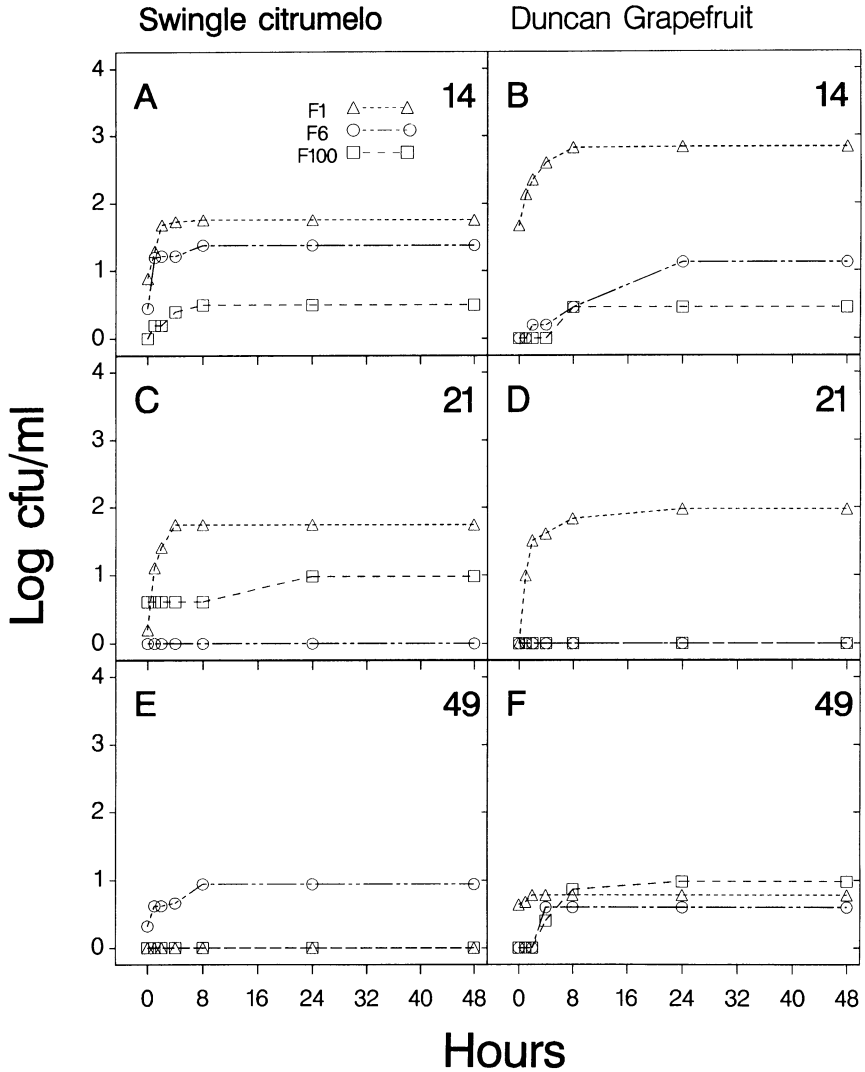

Fig. 3. Cumulative populations of *Xanthomonas campestris* pv. *citrumelo* strains F1, F6, and F100 exuded into water placed in wells surrounding individual lesions of citrus bacterial spot. Water in wells was completely replaced at each sampling time. Lesions were collected from Swingle citrumelo **(A, C, and E)** and Duncan grapefruit **(B, D, and F)** 14 (A and B), 21 (C and D), and 49 (E and F) days after inoculation. The average standard errors across all sampling times were 0.49, 0.33, and 0.08 for strains F1, F6, and F100, respectively, on Swingle citrumelo and 0.77, 0.09, and 0.33 for the same respective strains on Duncan grapefruit.

DISCUSSION

Stall et al (14) found that Asiatic citrus canker lesions under field conditions have similar numbers of viable cells despite differences in the size and age of the lesions and the cultivar on which the lesions were produced. The only difference found in that study was a marked decline in bacterial populations in lesions that had overwintered. That is, lesion age did not affect populations of bacteria in lesions unless the lesions had overwintered (14). Although older lesions may have populations of bacteria similar to those of young lesions, inoculum is released from these lesions more slowly, and total amounts produced are lower than from younger lesions. As lesions age, they become highly suberized, and thus bacteria are released only with difficulty, if at all. Virtually all of the inoculum produced is the result of exudation of bacteria already existing in the lesions; little or no multiplication appears to occur in water on the surface of leaves.

In our studies, young lesions of Asiatic canker yielded large numbers of bacteria almost instantaneously upon wetting. Thus, the pathogen does not require long periods of wetting to produce inoculum for further spread.

Cumulative populations exuded from lesions were in the range of 10^5–10^6, whereas grinding and plating lesions yielded 10^6–10^7 bacteria per lesion (14). Thus, not all bacteria in lesions become available for spread upon wetting.

Graham et al (9) found that the number of bacteria in lesions of CBS was initially high but declined rapidly as the lesions aged. In that study, only very young CBS lesions produced under conditions of high temperature and humidity exuded large numbers of bacteria. In the field, the ability of even the aggressive strain to produce large numbers of bacteria was greatly reduced.

Differences in the morphology of Asiatic canker lesions and CBS lesions may account for some of the differences in amounts of inoculum released. Surfaces of CBS lesions are flat, with few openings through the necrotic leaf epidermis, whereas Asiatic canker lesions consist of hyperplastic mesophyll tissue that erupts through the epidermis (1,11,12). Thus, when CBS lesions are wetted, few pathways exist for release of bacteria to the surface. The erumpent, irregular Asiatic canker lesions allow wetting of internal tissue and thus more rapid and prolific inoculum production.

Observations of outbreaks of CBS in citrus nurseries in Florida and epidemiological studies indicate that the pathogen has only a limited capacity for spread

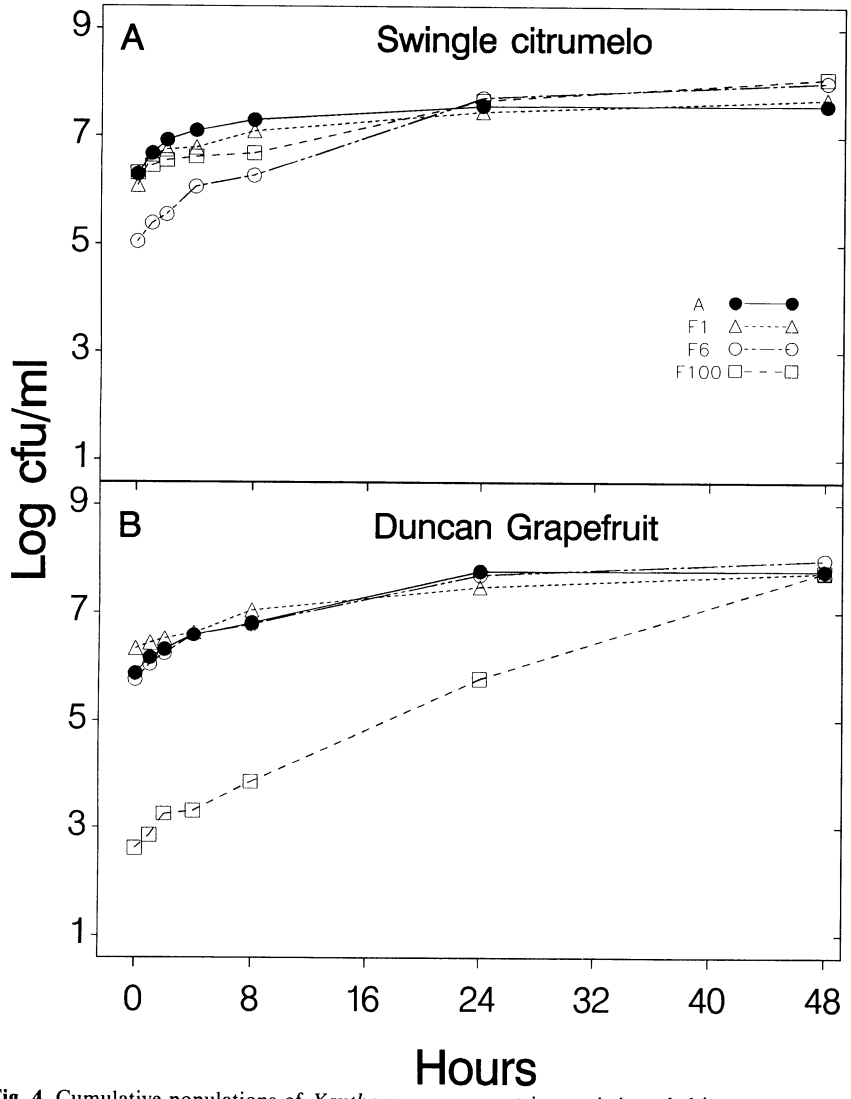

Fig. 4. Cumulative populations of *Xanthomonas campestris* pv. *citri* exuded into water placed in wells surrounding individual lesions of Asiatic citrus canker and of *X. c.* pv. *citrumelo* strains F1, F6, and F100 exuded into water placed in wells surrounding individual lesions of citrus bacterial spot (CBS) on Swingle citrumelo **(A)** and Duncan grapefruit **(B).** Leaves were wounded, inoculated, and placed in a dew chamber at 30 C. The average standard errors across all sampling times were 0.32, 0.09, 0.18, and 0.52 for the Asiatic strain and CBS strains F1, F6, and F100, respectively, on Swingle citrumelo and 0.19, 0.19, 0.51, and 0.13 for the same respective strains on Duncan grapefruit.

in the field (3–5,8). Given the low potential of CBS strains for producing large quantities of inoculum and for surviving long periods in old lesions, the disease represents a minimal threat to the citrus industry. Outbreaks may occur occasionally under highly favorable conditions but are unlikely to be sustained, especially when weakly or moderately aggressive strains of the pathogen are involved.

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