Survival of *Xanthomonas axonopodis* pv. *citri* in Leaf Lesions Under Tropical Environmental Conditions and Simulated Splash Dispersal of Inoculum

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ABSTRACT

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Asiatic citrus canker (ACC) is a severe disease of several citrus species and hybrids in many tropical and subtropical areas. Populations of *Xanthomonas axonopodis* pv. *citri* in leaf and twig lesions are the most important inoculum source for secondary infections. In areas with a marked winter season (e.g., Argentina and Japan), low temperatures induce a decrease of 10^2 to 10^4 in population sizes in lesions, thus creating a discontinuity in the *X. axonopodis* pv. *citri* life cycle. The purpose of this study was to evaluate the dynamics of *X. axonopodis* pv. *citri* populations in leaf lesions exposed to the mild winter temperatures prevailing in a tropical environment. Internal *X. axonopodis* pv. *citri* population levels in Mexican lime leaf lesions reached 10^6 to 10^7 CFU lesion^{-1} whatever the lesion size. These densities, however, were not strongly negatively affected by winter temperatures prevailing under experimental conditions. The estimated decrease in internal *X. axonopodis* pv. *citri* population sizes was approximately 10-fold. When exposed to 35 mm h^{-1} of simulated rainfall, internal population sizes decreased over time by ≈1 log unit for lesions 1 and 2 months old, but did not for older lesions. A microscopic examination indicated that lignin-like compounds are present in lesions more than 6 months old. The slow decrease over time of *X. axonopodis* pv. *citri* population sizes in leaf lesions may be the balanced result of defense reactions by the host at late stages of disease development, and the concomitant multiplication of the pathogen at the margin of old lesions. We conclude that the epidemiological significance of overwintered leaf lesions in the tropics is higher than that reported in other areas.

Additional keywords: histology, rainfall simulation device.

Asiatic citrus canker (ACC), caused by *Xanthomonas axonopodis* pv. *citri*, is an economically important bacterial disease of several citrus species (e.g., cultivars and hybrids of sweet orange [*Citrus sinensis* (L.) Osbeck], grapefruit [*C. paradisi* Macfad.], and lime [*C. aurantiifolia* (Christm.) Swingle]) in many tropical and subtropical areas where high temperatures and rainfall occur concomitantly (12). ACC decreases fruit marketability, can drastically decrease crop yields by causing premature fruit drop, and can induce severe defoliation on susceptible cultivars. ACC is endemic throughout Southeast Asia, in many countries bordering the Indian Ocean, and in some areas of South America (12).

Leaf infections occur mainly through wounds and stomata. The minimum levels of *X. axonopodis* pv. *citri* to induce the development of ACC lesions through wounds and stomata are 10^2 to 10^3 and 10^4 to 10^5 cells ml^{-1}, respectively (11,45). Leaves which are 50 to 80% expanded are most susceptible to stomatal infections (12). Wind-driven rains, with wind speeds in excess of 8 m s^{-1}, cause water congestion of leaf and green twig tissues and facilitate bacterial penetration (17,34).

Bacteria in leaf and twig lesions are probably the most epidemiologically significant inoculum for secondary infections (12). Internal population levels are not influenced by host genotype (37). Studies conducted in the Corrientes province (Argentina) and in Japan showed that internal *X. axonopodis* pv. *citri* population sizes reach 10^6 to 10^7 CFU/lesion whatever the lesion size throughout spring, summer, and fall, and decrease drastically to 10^2 to 10^4 CFU/lesion during the winter season (23,37). It also was emphasized that lesion age does not significantly affect bacterial population sizes in lesions outside the winter season (37). Thus, there is a discontinuity in the *X. axonopodis* pv. *citri* life cycle under such environmental conditions (low bacterial population sizes, no bacterial multiplication, lack of new susceptible host tissue). The climate prevailing in Argentina and Japan, especially during winter, is noticeably different from climates in tropical countries. Therefore, there is a need to know more about the epidemiology of ACC in tropical areas, where temperatures during the winter season do not decline markedly (7).

Citrus canker lesions consist of hyperplastic mesophyll tissue characterized by a rupture of the epidermis (20–22), allowing *X. axonopodis* pv. *citri* to be efficiently released onto leaf surfaces, in contrast to *X. axonopodis* pv. *citrumelo*, another bacterial pathogen of citrus which causes flat leaf lesions (24,40). Rainwater collected underneath *X. axonopodis* pv. *citri*-infected trees during the summer in Argentina contained 10^5 to 10^6 *X. axonopodis* pv. *citri* cells ml^{-1} (37). In the case of young (4- to 6-week-old) lesions, 10^4 to 10^5 *X. axonopodis* pv. *citri* cells ml^{-1} were released immediately after addition of water to the lesions. Release continued for 48 h, with cumulative population sizes over time reaching 10^5 to 10^6 cells ml^{-1} (40). By comparing these population sizes to internal populations in leaf lesions, it was concluded that not all cells of *X. axonopodis* pv.

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citri in lesions are available for rainwater-mediated release. Only very low population sizes of *X. axonopodis* pv. *citri* ($\approx 10^2$ ml^{-1}) were released from 4- to 6-month-old lesions and bacteria were released more slowly from old than from young lesions (40). Although internal population sizes of old lesions were not quantified (40), it was hypothesized that the low and slowly released population sizes from 4- to 6-month-old lesions are related to the structure of such lesions. Citrus canker lesions become highly suberized with age, and suberin was thought to act as a morphological barrier strongly affecting the release of *X. axonopodis* pv. *citri* in the external environment (40). In this study, lesions were placed in cell culture plate wells and soaked in water. The dynamics of internal and external populations of *X. axonopodis* pv. *citri* by a rainfall simulation approach similar to that developed for the study of the dispersal of fungal pathogens (25,31) should be evaluated. This is expected to provide a better understanding of the release and dispersal of *X. axonopodis* pv. *citri* from lesions under natural conditions.

Dispersal of *X. axonopodis* pv. *citri* in nurseries is due to rain or irrigation water splash facilitated by wind-driven rains. Studies in Argentina and Réunion Island nurseries showed that spread of *X. axonopodis* pv. *citri* has very little directionality, emphasizing the possibility that rain or overhead irrigation spread inoculum over short distances (14,16,29). Splash dispersal of *X. axonopodis* pv. *citri* at a tree-to-tree scale in groves is either inefficient (low-density groves) or row restricted (hedgerows). In groves, spread is predominantly associated with wind-driven rains with wind speed higher than 6 to 8 m s^{-1} (33,35). The dispersal efficiency of many plant pathogens is partly related to how much, how far away, and for how long inoculum is released from lesions (10). The purposes of this study were to evaluate (i) the behavior of *X. axonopodis* pv. *citri* in lesions produced in a tropical environment and differing in age and (ii) the characteristics of release and splash dispersal (over small areas) of *X. axonopodis* pv. *citri* originating from such lesions.

MATERIALS AND METHODS

Research site and climatic conditions. Experiments were performed from 1994 until 1996 at the CIRAD experimental station at Ligne Paradis, Saint Pierre, Réunion Island (elevation 140 m), except for population size measurements in lesions from commercial groves, which were performed in year 2000. Climatic data, including minimum, maximum, and average temperature; minimum, maximum, and average humidity; rainfall; and wind speed and direction were recorded hourly during the experiments. There are three main climatic seasons in Réunion Island. Cool, dry weather prevails from May to September. The temperatures increase in October and the weather remains mostly dry through December. Hot, humid weather occurs from January through April. Numerous rainstorms, tropical storms, and hurricanes occur during the latter season and ACC epidemics develop rapidly, as in many tropical areas, in association with such storms over the island, where the disease is now considered to be endemic. According to long-term averages calculated over a period of 20 years, temperatures on Réunion Island decrease by 0.7 to 0.8°C per 100 m of elevation (J. L. Chopart, M. Mézino, and L. Le Mézo, *unpublished data*).

Plant material and bacterial strains. Mexican lime (*C. aurantiifolia* (Christm.) Swingle) seedlings were used in all experiments. Plants were grown under shelter in a screenhouse in 20-cm-wide black plastic containers. Plants were 3 to 4 months old (i.e., 40 to 60 cm high) at the time of experiments. They were visually inspected individually for presence of ACC prior to each experiment and no visible infection was found.

Inoculum source plants were prepared by wound inoculation of mature leaves, using 5-µl droplets of a bacterial suspension containing $\approx 10^6$ CFU ml^{-1}, prepared from strain C40S, a virulent spontaneous mutant resistant to streptomycin sulfate (29). Each inoculated plant showed 10 lesions resulting from wound inoculation of five leaves. Inoculated plants were placed in a growth chamber at 30 ± 1°C and 95 ± 5% relative humidity for 2 weeks for optimal symptom expression. Plants were then transferred to the open air under a shelter.

Rainfall simulation device. Release dynamics from leaf lesions was studied using the EID330 rainfall simulation device developed by IRD (Institut de Recherche pour le Développement) and Deltalab (Voreppe, France) to study soil erosion (2). Briefly, water, whose pressure can be regulated, arrives at the top of the system to a nozzle swinging around an axis. A computerized device allows simulating rainfalls ranging from 5 to more than 200 mm h^{-1}, with various raindrop sizes depending on the selected rotation angle, the type of nozzle used, and the water pressure applied to the system. After calibration of each nozzle type (based on several replications of a 10-min simulated rain) (data not shown), two nozzle/pressure combinations were selected. The first nozzle/pressure combination (NP1) was used to simulate rainfalls of 8 mm h^{-1} with most drops having a diameter less than 1 mm. The second combination (NP2) simulated rainfalls of 35 mm h^{-1} with most drops having a diameter larger than 2 mm. The device was fixed onto a 4-m-high tower and was located in an experimental room, in order to avoid wind or natural rainfall during experiments. This physical constraint affected the terminal velocity of the drops (V_d). V_d of drops which was generated under NP1 conditions reached approximately maximal terminal velocity ($V_{d\max}$), but V_d of drops which were generated under NP2 conditions was only ≈85% of $V_{d\max}$ (25,31). Plants were placed perpendicular to the nozzle within a virtual square of 1 m^2, as recommended by the manufacturer.

Internal and external *X. axonopodis* pv. *citri* population sizes on inoculated leaves. Inoculations were performed at different periods, starting in November 1994, so that plants with lesions from 1 to 18 months old were available at the time when experiments were undertaken. Lesions which were 1, 2, 4, 6, 8, and 10 months old were analyzed in August and September 1995. Thus, lesions, which were at least 4 months old at the time of experiment were exposed to most (≈80%) of the winter temperatures (i.e., 80% of the duration of the winter). Eighteen-month-old lesions were further analyzed in a separate experiment performed in September 1996. For each set of inoculated plants, six individual lesions were used and internal population sizes were quantified as follows. Dry lesions were excised from the leaf blade and individually homogenized in 4.5 ml of 0.01 M, pH 7.2, Sigma 7-9 buffer (Sigma, Saint Quentin Fallavier, France) using an Ultraturax T25 homogenizer (Janke & Kunkel, IKA Labortechnik, Staufen, Germany). Aliquots of the homogenized suspensions and of 10-fold dilutions obtained from the suspensions were plated on YPGATS medium (yeast extract, 7 g; pastone [a pancreatic peptone of casein; Biorad, Marne la Coquette, France], 7 g; glucose, 7 g; agar, 18 g; distilled water, 1,000 ml; Tilt [propiconazole, 500 g liter^{-1}; Syngenta, Basel, Switzerland], 40 µl liter^{-1}; streptomycin sulfate [Sigma], 50 mg liter^{-1}, pH 7.2) using the Spiral System device (Interscience, Saint Nom la Bretèche, France) (19). The remaining parts of the leaves were individually homogenized in 20 ml of Sigma 7-9 buffer per gram of leaf, using a stomacher device (a lab homogenizer in which paddles apply pressure to contained samples, effectively washing out deep-seated organisms) (Seward, London, UK) at high speed for 2 min to recover surface populations of *X. axonopodis* pv. *citri* (8). Aliquots of the homogenized suspensions were plated as described above. Bacterial population sizes, based on enumeration of streptomycin-resistant colonies, whose morphology was identical to that of *X. axonopodis* pv. *citri*, were square root trans-

formed to stabilize sample variances. Variance homogeneity was checked with the Bartlett test (36). Data were subsequently analyzed with the nonparametric Kruskal & Wallis test and treatments were compared pairwise with the Mann-Whitney U test (Stat View 5; SAS Institute Inc., Cary, NC).

Influence of rain intensity on release and splash dispersal of *X. axonopodis* pv. *citri*. Inoculations were performed in May 1994. Inoculated plants with 3-month-old lesions were exposed in August 1994 to NP1 and NP2 rainfall regimes for 6 h. A device allowing the cessation of rainfall for brief times without any interfering drops was used for sampling. The experiment consisted of a set of two assays (one assay at each rainfall regime) and was replicated once using independently inoculated plants. Each assay consisted of sequential sampling of six rainwater aliquots (20 µl) from inoculated leaves near lesions, followed by the sampling of three diseased leaves (collected with maximum care using very sharp scissors, so that no disturbance was induced) at various times after rainfall induction (5, 10, 20, 45, 90, 180, and 360 min). Collected leaves were carefully dried and surface disinfested using 95% ethanol and lesions were taken from leaves, homogenized, and analyzed as described above to determine internal *X. axonopodis* pv. *citri* population sizes. Rainwater aliquots were diluted and plated as described above. The fitness of the log-transformed bacterial population data to a normal distribution was examined and variance homogeneity was checked with the Bartlett test (36). Data were subjected to the GLM procedure for repeated measures analysis of variance (ANOVA) (SAS Institute Inc.).

To evaluate splash dispersal of *X. axonopodis* pv. *citri*, microplots comprising 25 plants (5 by 5 within a 1 m^2 square) were used. One inoculated plant harboring 3-month-old leaf lesions was placed in the center of the plot and was surrounded by lesion-free plants with young shoots at a growth stage susceptible to ACC. Rainfall conditions as explained above were applied for times ranging from 20 to 360 min. Once a rainfall dose was applied, all of the available free water was collected individually from each plant and used to quantify *X. axonopodis* pv. *citri* population sizes. The experiment was replicated once.

Influence of lesion age on release and splash dispersal of *X. axonopodis* pv. *citri*. Inoculations were performed at different periods, starting in November 1994, so that plants with lesions from 1 to 18 months old were available at the time when experiments were undertaken. Lesions which were 1, 2, 4, 6, 8, and 10 months old were analyzed in August and September 1995. Inoculated plants were submitted to the NP2 rainfall regime for 6 h. The experiment consisted of a set of seven assays on plants bearing lesions of a single age (plants with lesions 1, 2, 4, 6, 8, 10, and 18 months old were used) and was replicated once using independently inoculated plants. Each assay consisted of sequential sampling of rainwater and leaf lesions after 5, 10, 20, 45, 90, 180, and 360 min. Subsequent enumeration of *X. axonopodis* pv. *citri* population sizes was as described above. The fitness of the log-transformed bacterial population data to a normal distribution was examined and variances homogeneity was checked with the Bartlett test (36). *X. axonopodis* pv. *citri* population sizes in lesions, measured just before the start of simulated rainfall, were compared among lesion ages using the Student-Newman-Keuls test. As time after beginning of rainfall was a repeated measure of bacterial population sizes, data were subsequently analyzed by the GLM procedure for MANOVA (SAS Institute Inc.). Pillai-Bartlett trace was used to test the effect of independent variables on *X. axonopodis* pv. *citri* population sizes. Linear elementary and polynomial contrasts of lesion age were made.

The fit of several generalized nonlinear models to the rainwater population data was examined (S-plus 2000; Mathsoft Inc., Seattle, WA). Several response surface models (exponential, rectangular hyperbole) were compared (27). The overall most appropriate model (a three-parameter rectangular hyperbole model, whose equation was $y = \alpha + [\beta/(x - \gamma)]$) and was chosen for further analysis. The S function used was *gnls* with nonlinear least squares algorithm (Gauss-Newton method) for parameter estimation. In this model y, x, α, β, and γ represented the log-transformed population size, duration of rainfall, position of the y hyperbole asymptote, pattern of curvature, and position of the x hyperbole asymptote, respectively (27). The amount of time for a half-decrease of the concentration of released inoculum (x_{50}) was estimated for each data set. Confidence intervals for parameters were calculated from the asymptotic variance-covariance matrix. The Delta method was used to calculate confidence intervals for x_{50}.

The evaluation of the splash dispersal of *X. axonopodis* pv. *citri* was performed in microplots consisting of 25 plants (5 by 5 within a 1 m^2 square). One inoculated plant was placed in the center of the plot and was surrounded by lesion-free plants with young shoots at a growth stage susceptible to ACC. Focal plants, with lesions which were 1, 4, 6, 8, and 10 months old, were

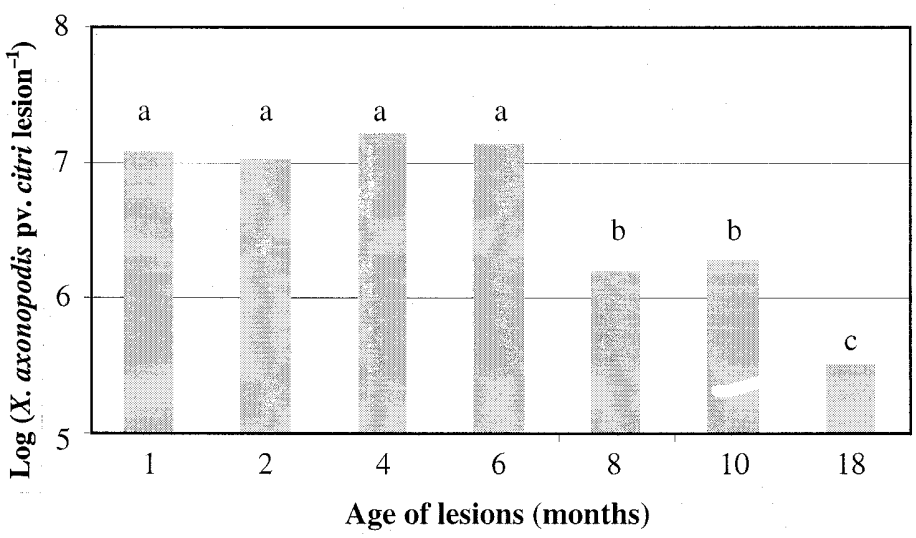

Fig. 1. Culturable population sizes of *Xanthomonas axonopodis* pv. *citri* in 1- to 18-month-old leaf lesions from wound-inoculated Mexican lime plants. Values are means of two replicates with six individual leaf lesions per replicate. Means were compared pairwise with the Mann-Whitney U test. Means with the same letter are not statistically different ($P = 0.05$).

used in separate experiments. Simulated rainfalls (35 mm h^{-1}) were applied for 20 min. Each experiment was replicated once. Once a rainfall dose was applied, all of the free water available was individually collected from each plant and used to quantify *X. axonopodis* pv. *citri* population sizes.

Internal *X. axonopodis* pv. *citri* population sizes in leaf lesions collected from commercial groves. Diseased leaves were sampled randomly from trees in commercial groves, all located in Réunion Island, at altitudes ranging from sea level to 900 m and, therefore, exposed to various environmental conditions. For each location, 30 natural leaf lesions were collected at the end of winter (October) 2000 from Meyer (*C. meyeri* Y. Tan.) and combava (*C. hystrix* D. C.). They included 20 lesions 5 to 6 months old and 10 lesions 3 to 4 weeks old. For a given leaf age, each assayed leaf lesion originated from different trees. Internal *X. axonopodis* pv. *citri* population sizes in these lesions were estimated as described above using a semiselective medium (YPGAKCT). This medium, adjusted to pH 7.2, contained yeast extract (7 g), pastone (7 g), glucose (7 g), agar (18 g), distilled water (1,000 ml), Tilt (propiconazole, 500 g $liter^{-1}$; Syngenta) (40 µl), kasugamycin hydrochloride (20 mg), and cephalexin (40 mg). Antibiotics were purchased from Sigma. Kasugamycin was dissolved in 0.01 M sterile Sigma 7-9 buffer (pH 7.2) and cephalexin in 0.1 N sodium hydroxide. Antibiotics were filter sterilized prior to addition to the agar media at 42°C.

Microscopic examination of ACC leaf lesions over time. Fragments of inoculated leaf tissues, with 1- to 12-month-old lesions, were fixed at room temperature for 4 h in 0.1 M sodium cacodylate buffer, pH 7.2, supplemented with 0.5% glutaraldehyde and 4% paraformaldehyde. Samples were rinsed in buffer, dehydrated in increasing concentrations of ethanol, and embedded in LR White medium grade resin (London Resin Co., Reading, UK) as recommended by the manufacturer. Sections 1 to 1.5 µm thick were obtained using a diamond knife on an Ultracut E microtome (Reichert, Vienna, Austria). Sections were further dyed in 2.5% sodium carbonate buffer, pH 8.0, containing 0.5% toluidine blue. Sections were examined with a Leitz Diaplan photonic microscope (Leitz, Wetzlar, Germany). Identification of the bacteria as a *Xanthomonas* sp. was performed by indirect immunofluorescence microscopy using the BOR9H8 monoclonal antibody raised against *X. axonopodis* pv. *manihotis* LPS (5), which crossreacts with *X. axonopodis* pvs. *citri*, *euphorbiae*, and *vasculorum*. Autofluorescence was examined under UV light (type I autofluorescence) and blue light (type II autofluorescence). Sections for which autofluorescence was detected were further treated with phloroglucinol (a phloroglucinol-saturated solution in 20% HCl) for detecting the presence of lignin or suberin-like compounds.

RESULTS

Internal and external *X. axonopodis* pv. *citri* population sizes on inoculated leaves. *X. axonopodis* pv. *citri* population sizes in 1- to 6-month-old lesions were statistically different ($P < 0.0001$) from those in lesions 8 to 18 months old. Lesions 1 to 6 months old contained approximately 10^7 culturable *X. axonopodis* pv. *citri* cells. Population sizes decreased by 1 log unit in 8- and 10-month-old lesions, and by 1.5 log units in 18-month-old lesions (Fig. 1). Generally, no *X. axonopodis* pv. *citri* cells were detected from the surface of these leaves (threshold 10^2 CFU g^{-1}). When detected, *X. axonopodis* pv. *citri* levels were always less than 10^3 CFU g^{-1}.

Influence of rain intensity on release and splash dispersal of *X. axonopodis* pv. *citri*. Internal *X. axonopodis* pv. *citri* population sizes in leaf lesions ranged from 0.6 to 6×10^7 CFU $lesion^{-1}$, with means remaining stable ($\approx 2 \times 10^7$ CFU $lesion^{-1}$) whatever the length of rainfall (Fig. 2A and B). Rain intensity did not affect internal population levels ($P = 0.219$). Released population sizes ranged from $\approx 3 \times 10^6$ to $\approx 9 \times 10^6$ CFU ml^{-1} when exposed to 5 min of rainfall. The number of *X. axonopodis* pv. *citri* cells released decreased with increasing length of rainfall, regardless of rain intensity ($P = 0.0001$). The reduction was ≈1 log unit at rainfall rates of 8 mm h^{-1} (Fig. 2A) and ≈2 log units at rainfall rates of 35 mm h^{-1} (Fig. 2B).

Splash dispersal of *X. axonopodis* pv. *citri* was very limited at rainfall rates of 8 mm h^{-1}, whatever the duration (Fig. 3A and B). When rainfalls of 35 mm h^{-1} were applied, *X. axonopodis* pv. *citri* population sizes, which were detected in rainwater from the focal plant, were $\approx 6 \times 10^6$ CFU ml^{-1} after 20 min of simulated rainfall, and then decreased by ≈2 log. units over time (Table 1). Splash dispersal of the inoculum was

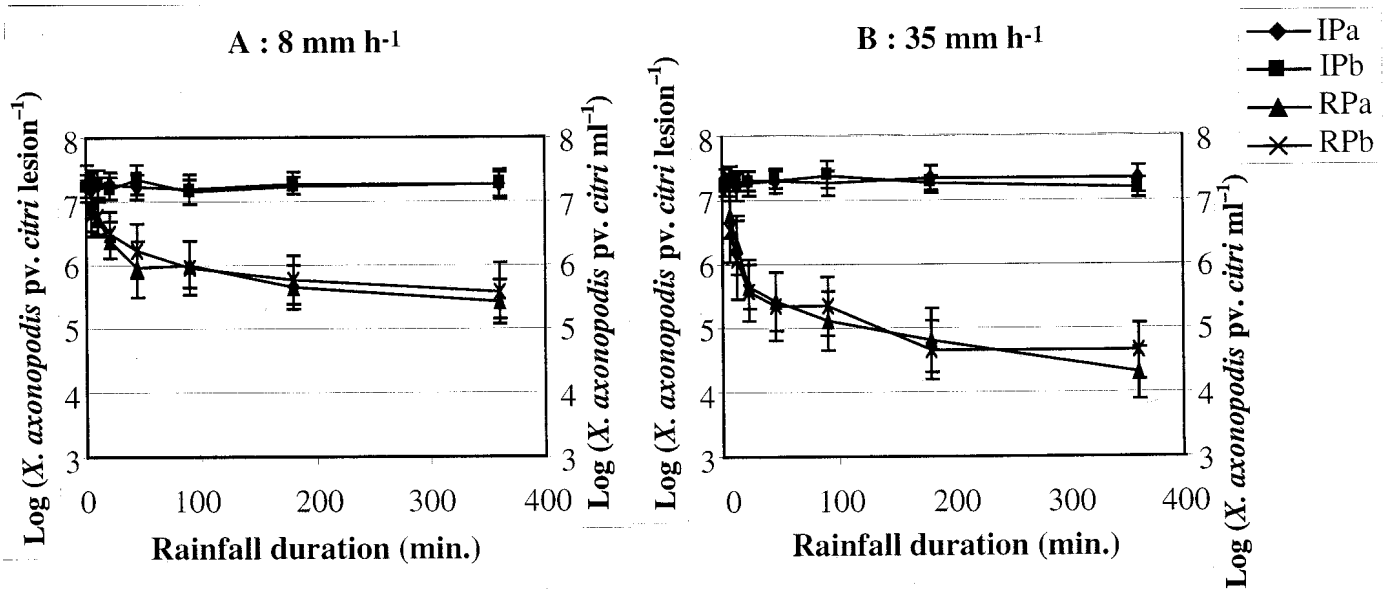

Fig. 2. Culturable population sizes of *Xanthomonas axonopodis* pv. *citri* detected in 3-month-old leaf lesions and rainwater collected from wound-inoculated Mexican lime plants. Log-transformed bacterial population sizes are presented as means (six samples) and error bars represent 95% confidence intervals. IPa = internal *X. axonopodis* pv. *citri* population sizes experiment 1, IPb = internal *X. axonopodis* pv. *citri* population sizes experiment 2, RPa = released *X. axonopodis* pv. *citri* population sizes experiment 1, RPb = internal *X. axonopodis* pv. *citri* population sizes experiment 2.

highly heterogeneous. Detectable *X. axonopodis* pv. *citri* levels on plants ranged from 1×10^1 to 4×10^3 CFU ml^{-1}. Short rain durations were more conducive to efficient establishment of *X. axonopodis* pv. *citri* on leaves (Table 1; Fig. 3C and D).

Influence of lesion age on release and splash dispersal of *X. axonopodis* pv. *citri*. At the beginning of simulated rains, *X. axonopodis* pv. *citri* levels in the lesions (from 1 to 3 × 10^7 CFU lesion^{-1}) (Fig. 4A) were not different among 1- to 6-month-old lesions, and were significantly lower (by ≈1 log unit) for 8- to 10-month-old lesions. The water pressure applied to the plants induced the defoliation of most leaves with 18-month-old lesions. When submitted to 35 mm h^{-1} rainfalls, *X. axonopodis* pv. *citri* internal population sizes decreased by 0.6 to 1.0 log unit ($P < 0.05$) in 1- and 2-month-old lesions (Fig. 4A). Rainfalls of 20 and 10 min were necessary to induce a significant decrease of internal *X. axonopodis* pv. *citri* population sizes in 1- and 2-month-old lesions, respectively. The internal population sizes remained stable whatever the duration of rainfall ($P > 0.05$) for lesions which were 4 to 10 months old (Fig. 4A).

When submitted to 5 min of rainfall, the released *X. axonopodis* pv. *citri* population sizes ranged from ≈0.2 to 8 × 10^6 CFU ml^{-1}, according to the age of the lesions (Fig. 4B). The values decreased with the duration of rainfall by ≈2 log units irrespective of lesion age. Estimated *y* hyperbole asymptotes (α) ranged from 4×10^3 to 5×10^4 CFU ml^{-1} (Table 2). Young lesions (i.e., 1 or 2-months old) were slightly more conducive to inoculum release for secondary infections. The

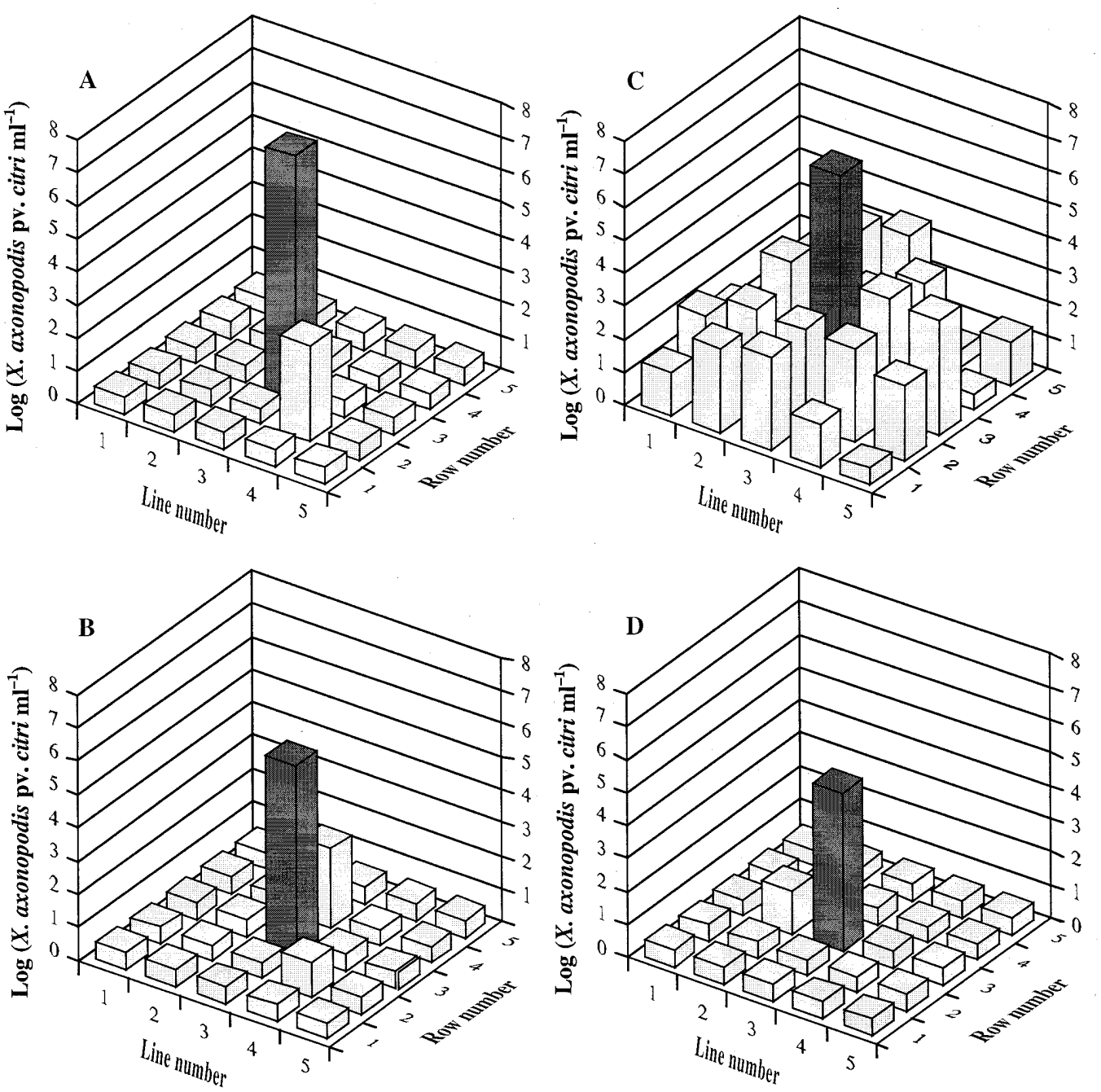

Fig. 3. Splash dispersal of *Xanthomonas axonopodis* pv. *citri* from inoculated Mexican lime plants under different simulated rainfall regimes. Bars represent log-transformed bacterial population sizes (means of the two replicates) in rainwater collected on each plant. Distance between plants along lines and rows was 0.2 m. **A,** Simulated rainfall of 8 mm h^{-1} for 20 min, **B,** simulated rainfall of 8 mm h^{-1} for 360 min, **C,** simulated rainfall of 35 mm h^{-1} for 20 min, and **D,** simulated rainfall of 35 mm h^{-1} for 360 min. The dark bar represents the focal plant.

time for a 50% decrease of released population sizes in rainwater (x_{50}) was ≈65 min, in contrast to 10 to 30 min for older lesions (i.e., 4 to 10 months old) (Table 2).

Splash dispersal of *X. axonopodis* pv. *citri* is presented in Table 3. *X. axonopodis* pv. *citri* population sizes, which were released from lesions on the focal plant, were ≈2×10^6 CFU ml^{-1} on plants whose lesions were 1 month old and were 2 to 40 times lower on plants with older lesions (Table 3). Splashed inoculum was highly heterogeneous on the neighboring plants. *X. axonopodis* pv. *citri* population sizes on plants that had detectable populations ranged from 1×10^1 to 1×10^4 CFU ml^{-1}. Although the highest splashed *X. axonopodis* pv. *citri* population sizes were recovered using focal plants with 1-month-old lesions, there was no distinct decrease in these population sizes when older (from 4- to 10-month-old) lesions were used as the inoculum source (Table 3).

Internal *X. axonopodis* pv. *citri* population sizes in leaf lesions collected in commercial groves. Internal *X. axonopodis* pv. *citri* population sizes ranged from 6×10^6 to 1×10^7 and from 1×10^6 to 2×10^7 CFU $lesion^{-1}$ in lesions which were 3 to 4 weeks old and 5 to 6 months old, respectively (Table 4). There was no relationship between winter temperatures on sites from which the samples were collected and internal *X. axonopodis* pv. *citri* population sizes in overwintered lesions.

Microscopic examination of ACC leaf lesions. Hypertrophy and hyperplasy of parenchyma cells were observed in all lesions, irrespective of lesion age. Large amounts of bacterial cells embedded into a pink-stained material were observed in lesions that were 1 or 2 months old (Fig. 5A and B), primarily in intercellular spaces of the paravascular parenchyma and spongy parenchyma. Bacteria-embedded matrix material was especially abundant in areas with hypertrophy and hyperplasy of parenchyma cells. Bacteria did not colonize metaxylem vessels, but some bacterial cells were observed near protoxylem vessels. Rupture of the epidermis was frequently (Fig. 5A), but not always, observed. A dark blue staining of middle lamella occurred occasionally. A limited autofluorescence was observed under UV and blue light (Fig. 5C).

In lesions more than 4 months old, rupture of the epidermis occurred in association with cell wall lysis. Tissues which had been highly colonized by bacterial cells displayed a necrotic aspect with dark blue staining of cells and walls (Fig. 5E and F). The development of fungal mycelium was often seen. Some bacterial cells reacting with the BOR9H8 monoclonal antibody adhered to fungal spores. Some areas showed an important autofluorescence of host cells and walls under UV (Fig. 5D) and blue light (Fig. 5H), in contrast with healthy tissue (Fig. 5G). At the margin of lesions, large amounts of bacterial cells embedded into a pink-stained material were observed in intercellular spaces of the paravascular parenchyma and spongy parenchyma.

DISCUSSION

The current knowledge on the biology and epidemiology of *X. axonopodis* pv. *citri* is based primarily on studies conducted in Argentina, Florida, and Japan (12–16), where bacterial population sizes decrease substantially during winter. No data on this subject is available for tropical areas, where winter temperatures remain rather high (7). This might cause inoculum to remain at high levels year round and epidemics to be more continuous, because of year-round (e.g., young trees) or at least regular (e.g., older trees) availability of susceptible host tissue.

Winter temperatures of a tropical environment, such as those of Réunion Island, did not induce an important reduction of *X. axonopodis* pv. *citri* population sizes in ACC leaf lesions in contrast to those observed in previous reports in Argentina and Japan (23,37). A limited decrease in *X. axonopodis* pv. *citri* internal population sizes was only recorded for lesions that were initiated more than 2 months before the beginning of winter. In the present study, the estimated decrease of internal *X. axonopodis* pv. *citri* population sizes in young versus old lesions was approximately 10-fold (from ≈10^7 to ≈10^6 CFU $lesion^{-1}$). Thus, in a tropical environment such as that of Réunion Island, *X. axonopodis* pv. *citri* population sizes in lesions more than 8 months old were 100 to 10,000 times higher than population sizes in overwintered lesions from more temperate environments (23,37). The highest decrease in internal population sizes occurred in lesions that were 18 months old and this decrease was only ≈35-fold. In our experiments, the 6- to 10-month-old lesions were exposed to only ≈80 to 90% of the time of winter temperatures, and this could account for the moderate decrease in *X. axonopodis* pv. *citri* population sizes in old lesions. However, the population size decrease in 18-month-old lesions, which were subjected to winter temperatures for almost two successive years, was still less pronounced than those recorded in Japan and Argentina. Although a strain effect (i.e., strain C40S has a better ability than strains from Argentina and Japan to survive in canker lesions) cannot fully be excluded, the survival of *X. axonopodis* pv. *citri* strain C40S in sweet orange leaf lesions throughout the growing season under field conditions in Réunion Island (42) was not strikingly different from that of strains from Japan (23) and Argentina (37). Strain C40S was used previously in an epidemiological study performed in Réunion Island (29) and the estimated daily rates of disease increase were very similar to those calculated for similar experiments conducted in Argentina (16).

Moreover, the decrease in maximum population size in overwintered versus nonoverwintered lesions collected from commercial groves under various environmental conditions in Réunion Island was only fivefold. This was observed even for samples taken from highland groves, where mean winter temperatures are ≈6 to 7°C lower than in the lowlands. Mean winter temperatures recorded in the highlands of Réunion Island are not

TABLE 1. Splash-dispersed log-transformed *Xanthomonas axonopodis* pv. *citri* population sizes detected on lesion-free Mexican lime plants exposed to simulated rainfall at 35 mm h^{-1} for various durations

Distance (m)[a]	Samples[b]	Rainfall duration (min)[c]				
		20	45	90	180	360
0.2	8	2.34–3.64	2.30–2.38	2.00–3.05	1.00–2.67	ND–1.30
0.3	8	2.84–3.29	2.32–2.40	2.00–2.63	ND–2.51	ND
0.4	8	1.70–3.44	ND–2.15	1.30–2.28	ND–1.60	ND
0.5	16	ND–3.04	ND–2.38	ND–2.65	ND–2.15	ND
0.6	8	ND–1.30	ND–2.15	ND–1.00	ND	ND
Focal plant	2	6.58–6.95	5.45–5.98	4.97–5.46	4.54–5.00	4.33–4.97

[a] Distance from focal plant. In each experiment, the focal plant bore 3-month-old leaf lesions and was placed at the center.
[b] Total number of samples.
[c] Values are log-transformed *X. axonopodis* pv. *citri* population sizes expressed per milliliter of rainwater; range indicates minimum to maximum population size detected; ND = not detected (minimum threshold of the assay was 10^1 cells ml^{-1}).

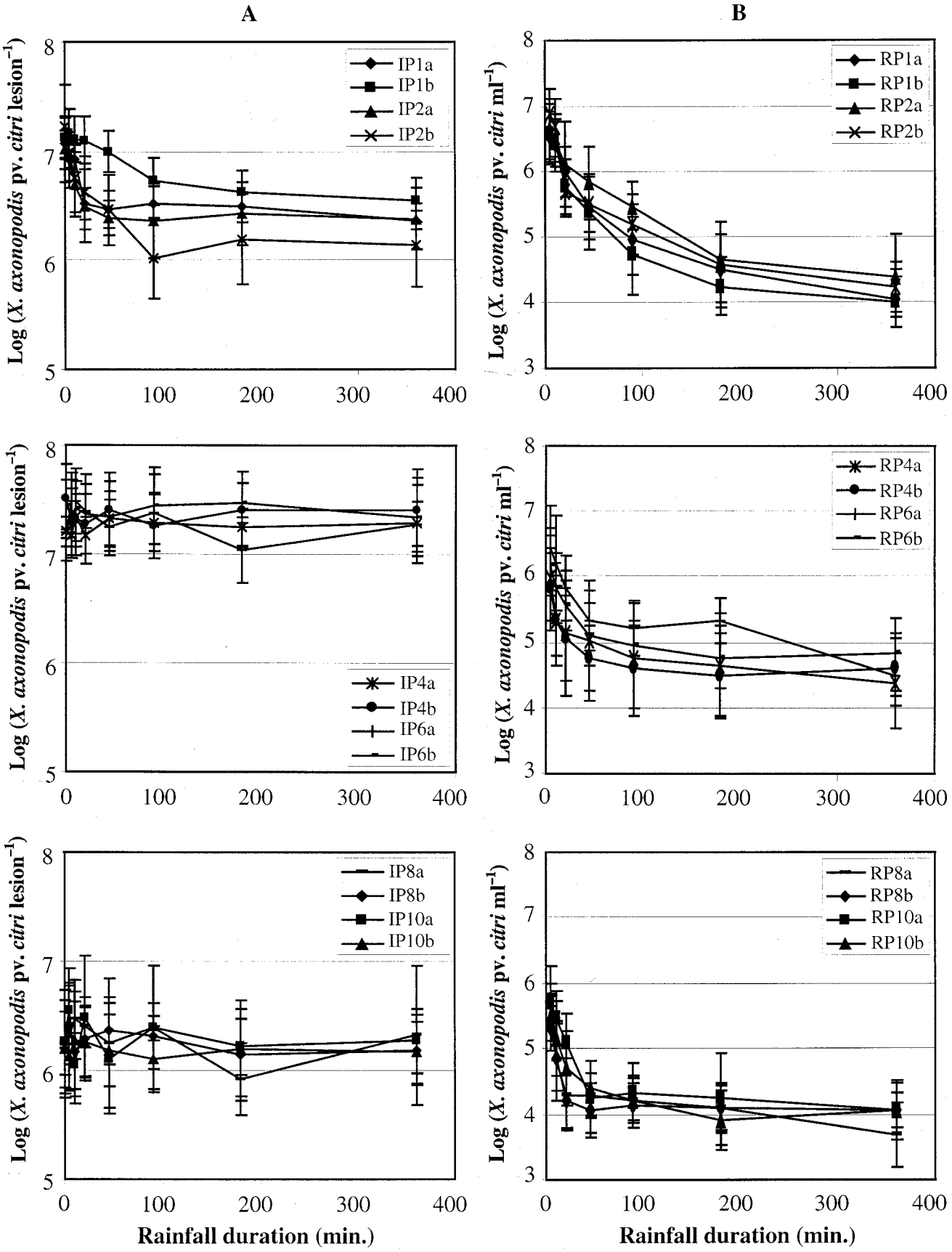

Fig. 4. Influence of canker lesion age on culturable population sizes of *Xanthomonas axonopodis* pv. *citri* in leaf lesions and rainwater collected from wound-inoculated Mexican lime plants. Log-transformed bacterial population sizes are presented as means (six samples) and error bars represent 95% confidence intervals. IP = internal *X. axonopodis* pv. *citri* population sizes, RP = released *X. axonopodis* pv. *citri* population sizes. Lesion age ranged from 1 (e.g., IP1, RP1) to 10 months (e.g., IP10, RP10). Two separate experiments (indicated as **A and B**) were performed.

notably different from those of the Entre Rios province of Argentina. The differences in *X. axonopodis* pv. *citri* survival during winter between the two areas could be explained by the range of temperature variations, which is much more moderate in Réunion Island than in Concordia, where occasional temperatures below 0°C occur in winter.

A relationship was observed between the age of ACC leaf lesions and the way internal *X. axonopodis* pv. *citri* population levels behaved when exposed to simulated rainfall. Internal population sizes decreased significantly (by ≈1 log unit) for lesions that were 1 and 2 months old. In the case of older lesions, internal population sizes remained stable and were mostly dependent on lesion age.

Based on *X. axonopodis* pv. *citri* population dynamics in rainwater collected from diseased leaves, *X. axonopodis* pv. *citri* release decreased over time (reaching an asymptote at $\approx 10^4$ CFU ml^{-1}), irrespective of lesion age. Such a decrease could be explained by (i) a decrease in inoculum availability with time, as previously suggested (40), or (ii) an increase in the adherence of *X. axonopodis* pv. *citri* cells on citrus leaf surfaces over time, minimizing population sizes in rainwater. Takahashi and Doke (38) showed that a host glycoprotein binds to the exopolysaccharide of the cell wall of *X. axonopodis* pv. *citri*; this might result in the formation on citrus leaves of biofilm-like structures containing *X. axonopodis* pv. *citri* cells (28). Although the adherence rate to unwounded citrus surfaces was $\approx 2 \times 10^3$ CFU cm^{-2} (39), it is unlikely that observed decreases of *X. axonopodis* pv. *citri* population sizes in rainwater are related to an adherence phenomenon. We further confirmed (data not shown) that *X. axonopodis* pv. *citri* population sizes on citrus leaves from diseased plants under simulated rainfall and collected just after natural drying were very low (less than 10^2 CFU cm^{-2}). When comparing external to internal population sizes of leaf lesions, we conclude that not all cells of *X. axonopodis* pv. *citri* in lesions are available for rainwater-mediated release. This is consistent with previous work (40).

Large populations of *X. axonopodis* pv. *citri* were released on diseased leaves shortly after the beginning of simulated rainfall. Our results show that, under a tropical environment such as that of Réunion Island, *X. axonopodis* pv. *citri* displays a good ability to be released from old lesions. Under such conditions, the amount of released *X. axonopodis* pv. *citri* is related to internal population sizes. This is in contrast with previous work in Argentina using naturally occurring citrus canker diseased material (40). Timmer et al. (40) hypothesized that the very low and slowly released population sizes from 4- to 6-month-old lesions probably could be explained by differences in the structure of young versus old ACC lesions. However, internal popu-

TABLE 4. Differences in *Xanthomonas axonopodis* pv. *citri* population sizes per lesion detected in overwintered (5- to 6-month-old) versus non-overwintered (3- to 4-week-old) leaf lesions collected from combava (*Citrus hystrix* D. C.) and Meyer lemon (*C. meyeri* Y. Tan.) commercially grown under various environmental conditions

Location	Altitude (m)	Host	Δ[a]
Étang Salé	10	Combava	–0.01
Étang Salé	10	Meyer	–0.47
Saint André	80	Combava	0.89
La Bretagne	600	Combava	0.23
Mare à citrons	640	Combava	–0.60
Palmiste Rouge	850	Combava	0.17
Petite Ile	900	Meyer	0.61

[a] Differences between log-transformed *X. axonopodis* pv. *citri* population sizes present in 3- to -4 week-old lesions versus 5- to 6-month-old lesions (negative values indicate higher population sizes in 5- to 6-month-old lesions).

TABLE 2. Values of parameters for the selected three-parameter rectangular hyperbole model and the time (min) necessary to reach a half-decrease of released log-transformed *Xanthomonas axonopodis* pv. *citri* population sizes (x_{50})[a]

	Parameters for the selected rectangular hyperbole model[b]			
Age of lesions (months)	α	β	γ	Time to x_{50} (min)[c]
1	3.623 (0.297)	174.047 (73.867)	–53.803 (21.289)	64.7 (52.3)
2	3.913 (0.872)	182.404 (235.241)	–60.494 (71.08)	66.8 (38.2)
4	4.503 (0.166)	14.953 (10.982)	–6.014 (7.913)	15.4 (5.0)
6	4.700 (0.294)	37.788 (36.056)	–18.655 (21.170)	28.4 (13.3)
8	3.974 (0.199)	8.165 (7.225)	–0.135 (4.451)	10.2 (2.7)
10	3.940 (0.190)	29.384 (18.252)	–12.393 (10.359)	23.1 (6.2)

[a] The selected model was a three parameter-rectangular hyperbole model, whose equation was $y = \alpha + [\beta/(x - \gamma)]$. In this model y, x, α, β, and γ represented the log-transformed population size, duration of rainfall, position of the y hyperbole asymptote, pattern of curvature, and position of the x hyperbole asymptote, respectively; x_{50} represented the estimated amount of time for a half-decrease of the concentration of released inoculum.
[b] Values given in parentheses are asymptotic 95% confidence interval bounds.
[c] Values given in parentheses are 95% confidence interval bounds calculated by the delta method.

TABLE 3. Influence of leaf lesion age on splash-dispersed *Xanthomonas axonopodis* pv. *citri* population sizes detected on lesion-free Mexican lime plants submitted to simulated rainfall at 35 mm h^{-1} for various durations

		Age of lesions on focal plant (months)[c]				
Distance (m)[a]	Samples[b]	1	4	6	8	10
0.2	8	1.71–4.02	ND–2.61	1.48–3.09	ND–2.24	ND–3.23
0.3	8	1.61–3.37	ND–2.31	1.01–3.09	ND–2.44	ND–2.85
0.4	8	ND–2.72	ND–2.50	ND–2.40	ND–1.01	ND–2.31
0.5	16	ND–3.18	ND–2.01	ND–3.03	ND–1.61	ND–2.44
0.6	8	ND–1.79	ND	ND–1.71	ND–1.71	ND–2.01
Mean (experiment 1)[d]	…	3.13 (3.43)	1.70 (1.90)	2.01 (2.41)	1.57 (1.78)	1.77 (2.00)
Mean (experiment 2)[d]	…	2.25 (2.59)	1.49 (1.91)	2.52 (2.56)	1.16 (1.55)	2.17 (2.56)
Focal plant	2	6.33–6.36	4.85–5.09	5.71–5.96	4.69–4.91	5.18–5.50

[a] Distance from focal plant.
[b] Total number of samples.
[c] Log-transformed *X. axonopodis* pv. *citri* population sizes are expressed per milliliter of rainwater; range indicates minimum and maximum population size detected; ND = not detected (minimum threshold of the assay was 10^1 cells ml^{-1}).
[d] Standard errors in parentheses. Values are means of *X. axonopodis* pv. *citri* population sizes on uninoculated plants.

lation sizes, which would have been an estimate of the culturable *X. axonopodis* pv. *citri* in assayed old lesions, were not determined in their study.

Discrepancies between results obtained in Argentina and Réunion Island also may be explained by our inoculation technique. Wounds might have modified the kinetics of release. To test this hypothesis, control experiments using stomatally infected Carrizo citrange (*C. sinensis* × *Poncirus trifoliata*) plants with overwintered lesions were performed (data not shown). The dynamics of internal and external *X. axonopodis* pv. *citri*

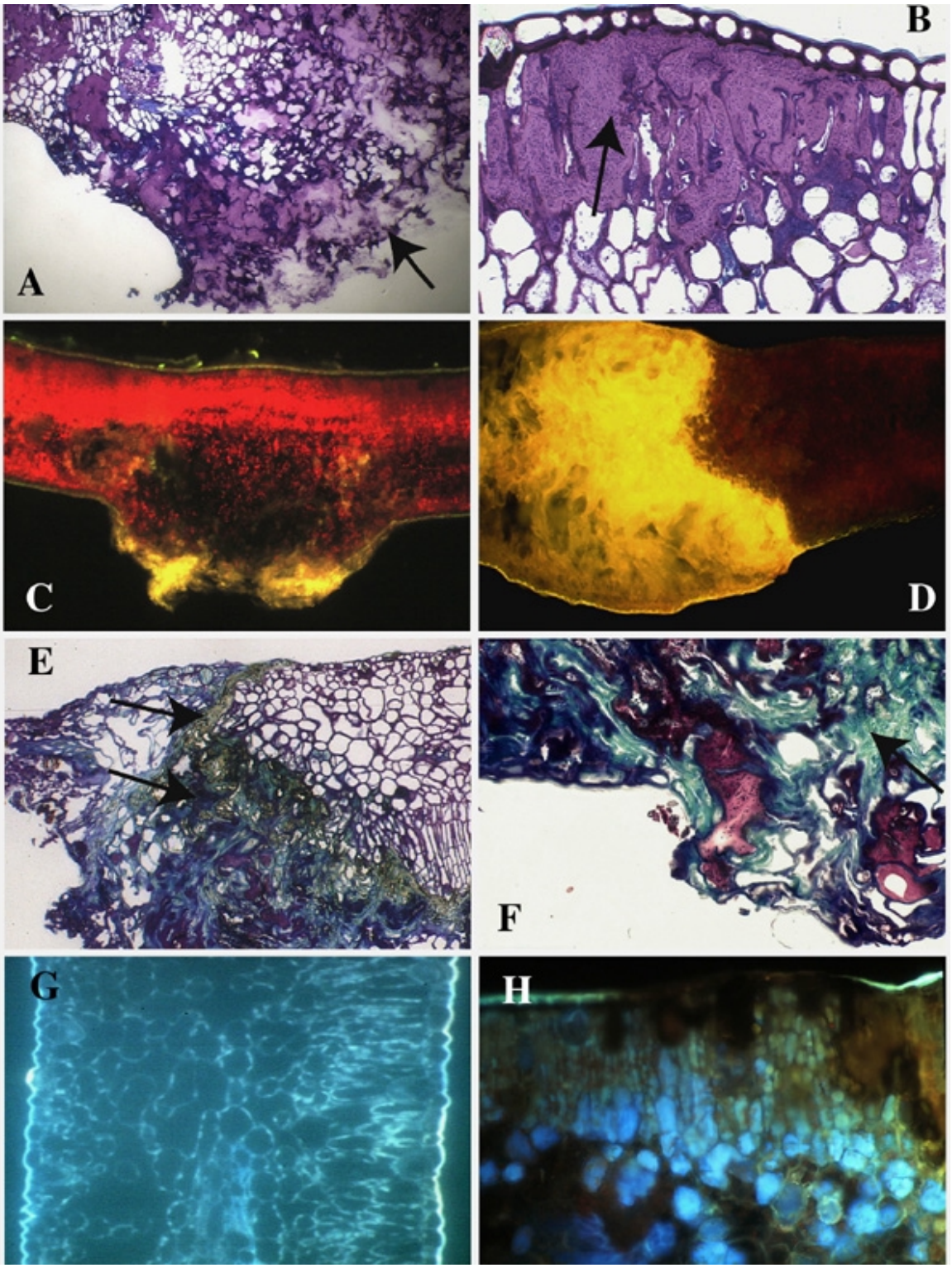

Fig. 5. Mexican lime (*Citrus aurantifolia* (Christm.) Swing.) leaf sections observed by light microscopy. **A,** Two-month-old lesion stained with toluidine blue (×100). Arrow indicates epidermis rupture. **B,** Two-month-old lesion stained with toluidine blue (×500). Arrow indicates the *Xanthomonas axonopodis* pv. *citri* containing pink-stained, polysaccharidic material. **C,** Limited yellow (type II) autofluorescence in a 2-month-old lesion (×100). **D,** Extensive yellow (type II) autofluorescence in a 6-month-old lesion (×100). **E,** Ten-month-old lesion stained with toluidine blue (×100). Arrows indicate dark blue and green phenolic-impregnated material. **F,** Ten-month-old lesion stained with toluidine blue (×500). Arrows indicate dark blue and green phenolic-impregnated material. **G,** Type I autofluorescence of healthy plant material (×250). **H,** Blue (type I) autofluorescence in a 10-month-old lesion section (×250).

populations were very similar to those presented for wound-inoculated Mexican lime lesions of the same age.

The development of hypertrophy and hyperplasy in the parenchyma, and associated ruptures of epidermis, are major features for the release of *X. axonopodis* pv. *citri* in rainwater (20–22). Microscopic examinations of leaf lesion sections from material collected in Réunion Island were made in an attempt to explain differences in the behavior of *X. axonopodis* pv. *citri* in old lesions submitted to different environments (40). Hypertrophy and hyperplasy of parenchyma cells were observed in all lesions, irrespective of age. Rupture of the epidermis was observed frequently. In lesions that were 1 or 2 months old, a large number of bacterial cells were embedded into a pink-stained material located primarily in intercellular spaces of the hypertrophied and hyperplastic parenchyma. Toluidine blue is known to stain polysaccharides a pink to red color (26). However, this stained material may be of plant origin, as reported for the rice–*X. oryzae* pv. *oryzae* pathosystem (44), or of bacterial origin, as shown for *X. axonopodis* pvs. *manihotis* and *vesicatoria* (4,6). It is likely that the gradual disorganization of parenchyma cells and walls is related to the ability of *X. axonopodis* pv. *citri* to produce cellulolytic, pectinolytic, and proteolytic enzymes (43). An important cell wall lysis and dark blue staining of cells and walls by toluidine blue, indicating a possible accumulation of phenolic compounds by the host (3), were observed after staining 4-month-old and older lesions. Autofluorescence was often observed in altered cells of 6-month-old and older lesions. The inhibition of autofluorescence with phloroglucinol indicated that lignin-like compounds were present (data not shown). The polysaccharidic matrix embedding bacterial cells in autofluorescent areas also stained green with toluidine blue, suggesting that the matrix became impregnated with phenolic compounds. This observation led us to hypothesize that *X. axonopodis* pv. *citri* may be trapped in this structure. However, large amounts of bacterial cells embedded into a polysaccharidic pink-stained material at the margins of old lesions indicated that bacteria were still active in 6- to 10-month-old lesions, and this likely explains why *X. axonopodis* pv. *citri* release kinetics were not directly related to lesion age.

In several pathosystems, increased peroxidase activity is correlated with host resistance (32). Reimers and Leach (30) showed that lignin-like phenolic compounds were synthesized by peroxidases in rice resistant lines infected by *X. oryzae* pv. *oryzae*. However, it is unknown if lignin directly affects xanthomonad pathogenesis, specifically by restricting bacterial multiplication in intercellular spaces (3,30), although some precursors of lignin have an antibacterial activity (32).

The splash dispersal of *X. axonopodis* pv. *citri* was studied using simulated rains delivered by a spray nozzle containing a mixture of water droplets of various diameters. The splashing efficiency of plant pathogens is directly related to the size of water droplets (10). We used simulated rainfalls of 35 mm h^{-1} in most experiments, which produce a distribution of drop sizes close to that of a natural rain, rather than a single drop size approach (2,10,25). The selected nozzle type–water pressure combination produced mostly drops with a diameter larger than 2 mm, but the percentage of very large drops (4 to 5 mm in diameter; i.e., drops which are the most efficient for splash dispersal of propagules) was low. For this reason, the splashing efficiency of *X. axonopodis* pv. *citri* was limited but probably reflected the situation under natural rain conditions (25).

Higher *X. axonopodis* pv. *citri* splashed population sizes were detected (i) on plants directly neighboring the focal plant 20 cm away and (ii) after short-duration rainfalls. The short splashing distance of *X. axonopodis* pv. *citri* is consistent with results previously obtained for *Erwinia carotovora*, which indicated that the half-distance of splashing induced by 3- to 5-mm-diameter droplets was only 5.8 cm, with a maximum distance of 125 cm from the point of impact (9). Due to the small working area imposed by the experimental device, our data may not reflect all of the secondary splash dispersal. Our results further confirm that rains of short duration are the most efficient ones for dispersal of *X. axonopodis* pv. *citri* (29,33), probably because inoculum is washed off by long rainfall. However, the effect of lesion age on levels of splashed *X. axonopodis* pv. *citri* populations was unclear. The epidemiological significance of splashed *X. axonopodis* pv. *citri* populations may be low, because (i) there is no evidence of multiplication of *X. axonopodis* pv. *citri* on wet or dry citrus surfaces and persistence on the phyllosphere in the absence of symptoms (41) and (ii) population sizes just reached the inoculum threshold necessary for stomatal infections (10^3 CFU ml^{-1} for Mexican lime) (data not shown), slightly less than what was obtained for other host species (11,45), This confirms that efficient bacterial ingress through stomata is likely associated with (i) simultaneous occurrence of rains and winds, which causes water congestion of leaf tissues resulting in a continuous layer of water from the leaf mesophyll through the stomata to the leaf surface, or (ii) the presence of surfactants, associated with pesticide use, on leaf surfaces (17,18).

The development of fungal mycelium was often recorded on lesions more than 4 months old. Some bacterial cells reacting with the BOR9H8 monoclonal antibody adhered to fungal spores. The question of dissemination ability of *X. axonopodis* pv. *citri* cells adhering to fungal spores compared with planktonic bacterial cells remains unanswered.

We conclude that, under environmental conditions prevailing in the lowland tropics, there is no drastic decrease in *X. axonopodis* pv. *citri* population sizes in canker leaf lesions subjected to winter temperatures. A slow decrease of *X. axonopodis* pv. *citri* population sizes associated with leaf lesions occurred over time. It may be the balanced result of defense reactions (e.g., accumulation of phenolic compounds) developed by the host at late stages of disease development (1) and the concomitant multiplication of the pathogen at the margin of old lesions. The epidemiological significance of overwintered leaf lesions in a tropical environment such as those of Réunion Island is therefore higher than was previously reported in other locations.

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