A Device for Precise and Nondisruptive Stomatal Inoculation of Leaf Tissue with Bacterial Pathogens

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ABSTRACT

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A stomatal inoculation apparatus (SIA) was developed to produce water congestion of leaf tissues and provide a reproducible noninjurious means of introducing two *Xanthomonas campestris* pathovars of citrus into leaf tissues without wounding. The SIA consisted of a small inoculation chamber attached to an intact leaf. Water and inoculum were metered into an airstream and focused to impact on a 1-mm-diameter area of the leaf surface. Leaf tissues on the abaxial surface of Duncan grapefruit leaves expanded 50–75% were more susceptible to infection than were other growth stages. Inoculum concentrations of 10^6 cfu/ml consistently induced infection and resulted in discrete individual lesions. Airstream impact pressures of 6.28–8.04 kPa against the leaf surface consistently produced tissue congestion and infection without wounding. These same pressures were the minimum threshold for increasing water volume in the leaf. From calculations of volume versus concentration of inoculum that enters a leaf via SIA, it was determined that as few as 2 cfu were required to cause a single lesion.

Additional keywords: citrus bacterial spot, citrus canker, water-soaking, *Xanthomonas campestris* pv. *citri, X. c.* pv. *citrumelo*.

Phytopathogenic bacteria that cause leaf- and twig-spotting diseases commonly enter expanding leaf or twig tissues via stomata, where they multiply initially in the substomatal chamber (22). Intercellular spaces in leaf tissues normally contain air, but under certain conditions become filled with water. Such water congestion of leaf tissues favors infection by bacterial pathogens (15). Wounding also can play an important role in bacterial infection by tearing leaves and creating avenues through the cuticle for infection. Wounded plants are most vulnerable when damage and water congestion coincide with rainstorms, which simultaneously spread inoculum, aid in further water congestion, and thus promote conditions favorable for infection (2,3). Considerable numbers of infections have been achieved with several bacterial pathogens when foliar tissues were artificially congested with bacterial inoculum or water prior to spray or drench inoculation (1,8,14,25). This technique was thought to be primarily a stomatal inoculation and required inoculum concentrations of $\sim 10^5$ cfu/ml for infection to take place in absence of wounding (6,21,29).

Two leaf- and twig-spotting pathogens of citrus have recently caused considerable concern to the U.S. citrus industry. *Xanthomonas campestris* pv. *citri*, which causes Asiatic citrus canker, induces erumpent lesions on fruit, foliage, and young stems of susceptible cultivars of citrus (16). The disease can cause nominal to significant damage during seasons when spring and summer rains are combined with wind speeds in excess of 8 m/s (9, 24,26,27). In citrus nurseries infested with citrus canker, dissemination of bacteria is primarily by splash dispersal (4,11,26–28). *X. c.* pv. *citrumelo*, which causes citrus bacterial spot, forms flat lesions on foliage, has a more restricted host range than Asiatic citrus canker, and appears to be solely a nursery disease (7,10,12). Most strains of *X. c. citrumelo* gain entry into leaves via wounds; however, dissemination of and infection by more aggressive strains of the pathogen was associated with rainstorms and overhead sprinkler irrigation (7,8). Water-soaking (congestion) of tissues has been implicated in the epidemiology of both diseases and is often seen in young foliage immediately following rainstorms.

Susceptibility of citrus foliage to *X. c. citri* decreases as the leaf tissues mature (18,19,28). Susceptibility of citrus cultivars to citrus canker also has been directly related to the size and shape of stomatal pores (20). When bacteria were forced through stomatal openings, a resistant citrus cultivar of mandarin was reported to require higher pressures than susceptible grapefruit for infection to occur (21). Nearly all *Citrus* spp. and related rutaceous species can be successfully inoculated by pinprick inoculation that creates a wound through the cuticle and bypasses the route of stomatal entry into the mesophyll leaf tissues (18,23). The pinprick inoculation technique has been used to evaluate susceptibility of citrus cultivars to citrus canker in Japan (17) and to citrus bacterial spot in Florida (13). Pinprick inoculation is superior to injection infiltration, which apparently overwhelms the mesophyll tissues with bacteria and makes in situ population dynamics studies and comparisons of strain–cultivar interactions suspect (5).

The purpose of this study was to develop an apparatus that could be used under controlled conditions to reproducibly inoculate citrus foliage with bacterial pathogens without wounding the leaf tissues and to determine the effect of water congestion of tissues on infection. The apparatus was used to study bacterial penetration through stomatal openings, bacterial population dynamics in woundfree leaves, and the relationship of leaf development stage to cultivar susceptibility to citrus canker and citrus bacterial spot.

MATERIALS AND METHODS

Description of apparatus. A device was designed and constructed for precise and nondisruptive stomatal inoculation of leaf tissue with suspensions containing bacterial pathogens. A schematic of the stomatal inoculation apparatus (SIA) is shown in Figure 1. Air from an air compressor at 551 kPa (80 lb/in^2)

enters the device (A). The compressed air then passes through an oil-water trap (B) to remove oil vapor and excess condensation from the system, and a pressure regulator maintains a constant pressure of 103 kPa (15 lb/in^2). Air then flows through a silica gel dryer (C) to remove any remaining oil and water vapor. Airflow is precisely controlled by a low-pressure regulator (D) and monitored via a flow meter (E), so that the final pressure of the airflow on the leaf surface can be adjusted. The air then passes through a copper tube and into the inoculation chamber (F), consisting of an 8- × 8- × 3-cm glass box. The end of the copper tube passes through one end of the inoculation chamber, which was drawn down to an inside diameter of 10 mm. The opposite end of the glass chamber is open. The copper tube terminates in a plastic pipette tip (G), with a terminal orifice diameter of 1.0 mm. The intact leaf (H) of a potted, greenhouse-grown plant is clamped to the open end of the glass chamber and is held in place by a Plexiglas backing plate (I) by means of two small springs (J), which are attached to both the glass chamber and the backing plate by means of small projections on each. The backing plate and the indented edge of the glass chamber are covered with 4-mm-thick foam rubber padding to avoid damaging the leaf tissues. The foam rubber, a closed-cell nonwettable type, was utilized to avoid retention of water or bacterial inoculum. When clamped in place, the leaf surface is held at a distance of 5.0 mm from the terminal opening of the plastic pipette tip. Sterile distilled water of a known quantity from a reservoir (K) can be injected into the airstream by means of a repeating pipette (L), the tip of which is inserted through a rubber stopper into a hole in the glass chamber wall. The tip of the micropipette is inserted through a 2-mm hole in the copper tube (M) inside the glass chamber and sealed to the tube by a small quantity of silicone caulk. Bacterial inoculum from a 100-ml bottle reservoir (N) is similarly injected into the airstream by means of a second repeating pipette (O), the tip of which is held by a second rubber stopper inserted into a second hole in the glass chamber wall. The tip of this second pipette also is inserted through a 2-mm hole in the copper tube (M) and sealed by silicone caulk. Thus, the impact pressure of both water and bacterial inoculum can be precisely controlled, as well as quantity and location of impact on the leaf surface. The backing plate does not fit tightly onto the end of the chamber, because of the rubber padding. The small gap, ~3 mm, between the chamber end and the backing plate serves as an air exhaust to keep pressure from building inside the chamber and as an escape for excess water and inoculum.

Impact pressure of the airstream, water, and inoculum was calibrated by means of a brass rod, 20 cm long and 2 mm in diameter, which was held fast at one end by a wooden block. The other end of the rod had a 1.0-cm-diameter brass plate welded to it. The center of the plate was held in front of the airstream at a distance of 5 mm from the pipette tip, and the deflection of the brass plate measured at various incremental settings of the flow meter (E) attained by adjusting the low-pressure regulator (D). The airstream, as it exited the orifice and impacted the brass plate, was ~1 mm in diameter. Calibration of the system was achieved by placing a small plastic weigh boat on the brass disk and filling it with grains of sand to achieve the same deflections (in millimeters) as caused by the impact of the airstream. The weight of the sand required to achieve the same deflection of the brass disk at each airflow setting was determined as grams of impact pressure per mm^2 of leaf surface and converted to kilopascals (kPa) for each airflow setting.

Range of SIA parameters capable of inducing infection. A series of experiments was conducted to determine optimum inoculum concentration, pressure of impact necessary to achieve congestion without tissue damage, and stage of leaf tissue development to maximize infection. Further studies were undertaken to examine the amount of inoculum which entered the leaf through the stomata and its effect on in situ bacterial populations over time and lesion development. All plant material consisted of potted, greenhouse-grown seedlings of Duncan grapefruit (*Citrus paradisi*

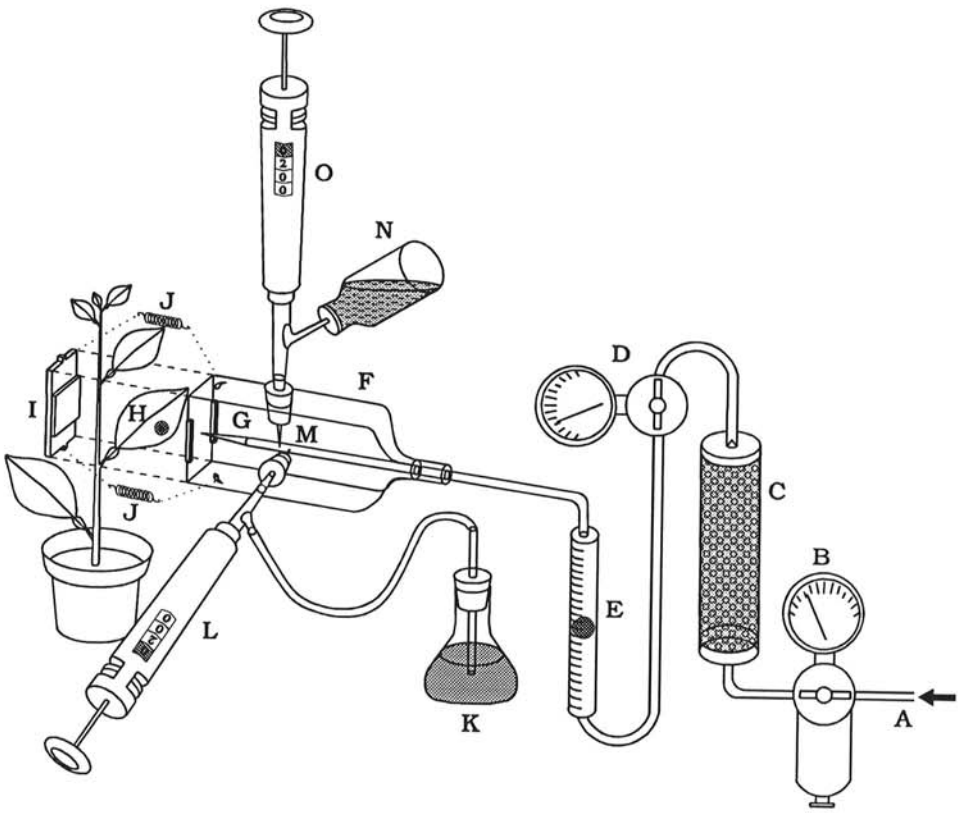

Fig. 1. Schematic diagram of the stomatal inoculation apparatus (SIA). **A,** Entrance of compressed air. **B,** Oil-water trap to remove excess condensation and initial pressure regulator. **C,** Silica gel dryer. **D,** Low pressure regulator. **E,** Flow meter. **F,** Glass inoculation chamber. **G,** Plastic pipette tip with orifice diameter of 1.0 mm. **H,** Intact leaf of a potted, greenhouse-grown plant. **I,** Plexiglas backing plate. **J,** Retaining springs. **K,** Sterile distilled water reservoir. **L,** Micropipette for injection of water into airstream. **M,** Pipette tip of the micropipette is inserted through a 2-mm hole in the copper tube. **N,** 100-ml bottle reservoir of bacterial inoculum. **O,** Second micropipette for injection of inoculum into airstream.

Macfady.). Because both pathogens were under strict quarantine, the experiments were conducted at the USDA-ARS disease containment facility at Plymouth, Florida. Following inoculation with the SIA, all plants were held in a growth chamber (model I-35DL, Percival Manufacturing, Boone, Iowa) at 30 C with a 14-h photoperiod for 14 days to allow symptoms to develop.

For each experiment, *X. c. citri* strain MF23P and *X. c. citrumelo* strain F1 were grown on nutrient glucose agar for 48 h at 28 C, harvested, suspended in sterile 0.075 M phosphate buffer (pH 7.2, containing 3.5 g of KH_2PO_4 and 5.8 g of Na_2HPO_4 per liter of water), and the suspension adjusted to the desired concentration spectrophotometrically. Inoculum was serially diluted and plated on nutrient glucose agar to confirm concentration.

Prior to inoculation, all plants were placed in the dew chamber at 30 C and 100% relative humidity for at least 2 h to promote opening of the stomatal apertures. Intact leaves were inoculated on the abaxial surface (unless otherwise stated). Up to 10 such individual inoculations could be made on each expanding leaf, with five on each side of the midrib. Inoculated plants were placed in the dew chamber with a 12 h light–12 h dark regime at 30 C without dew formation, and the number of individual lesions that developed in each inoculated area was recorded 7–10 days after inoculation.

Experiment 1 was conducted to determine the range of pressure necessary for inoculum in droplets to impact the leaf surface, infiltrate the leaf, and induce lesion formation without wounding the tissue. This was conducted across a series of leaf expansion stages to determine the optimum expansion stage for infection by both bacterial strains. Individual inoculations were performed by pipetting five 200-μl aliquots of inoculum at 10^6 cfu/ml of either strain F1 or MF23P into the airstream, which impacted the leaf surface in an area ~1.0 mm in diameter. During this and all succeeding experiments, excess inoculum not entering the leaf was quickly blown off the leaf surface by the airstream. Each pressure–leaf expansion stage combination was repeated on two treatment sites per leaf for each of five different leaves per treatment combination. Inoculated plants were held in the dew chamber for 14 days and number of lesions recorded as explained above. The experiment was repeated once, and results from the second trial are presented.

Experiment 2 was conducted to determine the effect of bacterial concentration on infection of expanding leaf tissues. Each inoculation site was predisposed to inoculum penetration by congestion (water-soaking). Congestion of leaf tissues was achieved by introducing five bursts of 200 μl of sterile distilled water into the airstream. This was immediately followed by a single burst of 200 μl of inoculum at 10^2, 10^3, 10^4, 10^5, or 10^6 cfu/ml. The impact pressure was set at 8.04 kPa and the leaf expansion stage was 50–75% of full expansion (optimum from experiment 1). This same range of leaf expansion was used for all succeeding experiments. Treatment with each inoculum concentration was repeated on five different leaves at two sites per leaf for each bacterial strain.

Effect of pressure of impact of bacterial inoculum on lesion formation was examined in experiment 3. Each inoculation site was predisposed with five bursts of 200 μl of sterile distilled water at an impact pressure of 0, 6.28, 8.04, 9.81, 10.79, or 11.57 kPa. This was followed immediately by a single burst of 200 μl of inoculum adjusted to 10^6 cfu/ml applied at 6.28 kPa. All preinoculation water congestion pressure treatments were applied on different areas of each leaf, and five such leaves were used per bacterial strain.

In experiment 4, congestion of leaf tissues was achieved by introducing five bursts of 200 μl of sterile distilled water into the airstream at a pressure of 9.81 kPa, followed by a single 200-μl burst of inoculum at 10^6 cfu/ml at an impact pressure of 0, 6.28, 8.04, 9.81, 10.79, or 11.57 kPa. All six inoculation pressures were applied on different areas of each leaf, and five such leaves were used per bacterial strain.

Experiment 5 was designed to test the effect of pressure of impact of bacterial inoculum on in situ population growth of *X. c. citrumelo* and *X. c. citri*, respectively, in young Duncan grapefruit foliage. Congestion was induced by five bursts of 200 μl of sterile distilled water at an impact pressure of 0, 6.28, 8.04, 9.81, 10.79, or 11.57 kPa. Inoculation consisted of one burst of 200 μl of inoculum at 10^6 cfu/ml at the given pressure. In situ bacterial population growth was determined at each of five inoculation sites at 5, 24, 48, and 72 h postinoculation. The leaf surface was swabbed with 70% ethanol to remove residual leaf surface bacteria. Following inoculation treatments, 0.5-cm^2 leaf disks were removed from the treated areas of inoculated leaves with a cork borer, macerated in 2 ml of 0.075 M sterile phosphate buffer, dilution-plated on semiselective KCB medium (consisting of 23 g of nutrient agar, 16 mg of kasugamycin, 35 mg of cephalexin, and 12 mg of chlorothalonil per liter of distilled water), and incubated for 4 days at 30 C. Internal leaf bacterial populations were expressed as log cfu of bacteria per inoculation site.

Experiment 6 was designed to determine volume of water introduced into a leaf through stomatal openings by different impact pressures. Individual treatment sites were congested by introducing five bursts of 200 μl of sterile distilled water into the airstream at an impact pressure of 0, 6.28, 8.04, 9.81, 10.79, or 11.57 kPa. All six inoculation pressures were applied to single leaves and replicated on 10 different leaves. A 0.5-cm^2 leaf disk from each site was then immediately excised with a cork borer and weighed with a microbalance. Simultaneously, a noncongested leaf disk of the same size was taken from the same leaf on the opposite side of the midvein and weighed as a control. Leaf disks were then dried in an oven at 70 C for 24 h and the disks reweighed. Weight increases of the SIA-water–congested leaf disks at various impact pressures were converted to increases in volume of water within the leaf disks and plotted.

In experiment 7, bacterial penetration of the adaxial versus abaxial sides of leaves was determined. For this test, each inoculation site was predisposed by five bursts of 200 μl of sterile distilled water at an impact pressure of 0, 4.61, 6.28, 8.04, 9.81, 10.79, or 11.57 kPa. This was immediately followed by a single burst of 200 μl of *X. c. citri* inoculum adjusted to 10^6 cfu/ml applied at 6.28 kPa. In situ bacterial populations were determined at 5, 24, 48, 72, and 168 h as in experiment 5 above. Each inoculation pressure–bacterial strain–leaf surface combination at each sampling time was replicated eight times.

Data were averaged over replications and summary statistics generated by the SAS MEANS procedure (SAS Institute Inc., Cary, NC). Graphics were prepared by use of a combination of AutoCAD (Release 11c, Autodesk, Inc., Sausalito, CA), Freelance Graphics (Lotus Corporation, Cambridge, MA), CorelDraw (Corel Systems Corporation, Ottawa, Ontario, Canada), and Surfer (Golden Software, Inc., Golden, CO).

RESULTS

The seven experiments were conducted sequentially, with the optimal ranges for individual parameters from one experiment often used as a basis for succeeding experiments. Leaves less than 50% of full expansion were too tender and small to use on a routine basis for inoculation by SIA. Furthermore, stomatal pores were not yet open at leaf stages of <50% expansion, as revealed by scanning electron microscopy (Graham and D. Achor, *unpublished*). From experiment 1, optimum susceptibility of leaf tissue to both citrus bacterial spot and citrus canker occurred when leaves were between 50 and 75% expanded, regardless of inoculation pressure (Fig. 2). Impact pressures greater than 8.04 kPa caused cellular collapse or small tears in the epidermis of tender leaf tissues, but those under 8.04 kPa did not, as revealed by transmission electron microscopy and visual examination of treated tissues by stereomicroscope over time (*data not shown*). Also, pressures greater than 8.04 kPa did not result in increased infection. Thus, in all succeeding experiments, leaves 75% expanded (compared to the size of mature leaves on the same plant) and a pressure of 8.04 kPa were adopted as optimum for nondestructive inoculation via SIA. Occasionally, the next lower pressure, 6.28 kPa, was used if tissues were particularly tender, but both pressures were capable of consistently causing noninjuri-

ous congestion of tissues. Although water congestion of tissues resulted from SIA inoculation on a ~1.0-mm-diameter area of leaf surface, water congestion was often seen extending 2–3 mm out into the leaf tissue from the point of inoculation. Such congestion was short-lived, and it dissipated within a few minutes. Several discrete lesions often developed, but only within the same area of the leaf blade as was congested by inoculation, and lesions were visually counted by stereomicroscope at this early stage. When symptoms were allowed to develop further, these discrete lesions usually coalesced.

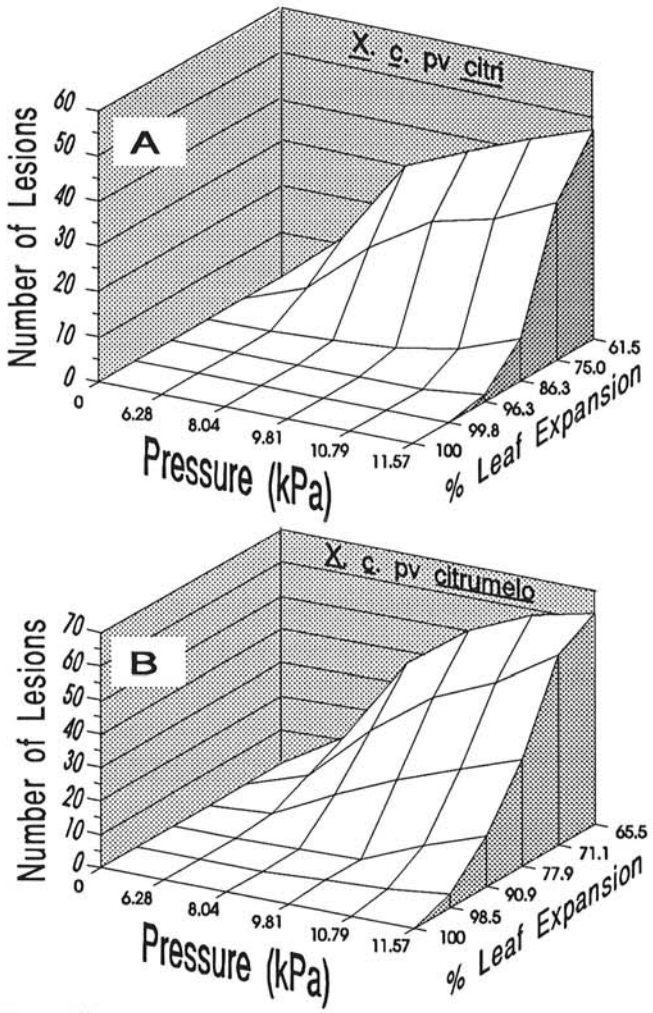

Fig. 2. Effect of leaf expansion stage of Duncan grapefruit versus impact pressure of inoculum on lesion formation by **A,** *Xanthomonas campestris* pv. *citri* strain MF23P, and **B,** *X. c.* pv. *citrumelo* strain F1 (experiment 1). Five 200-μl aliquots of inoculum were introduced into the airstream at 10^6 cfu/ml.

TABLE 1. Effect of inoculum concentration of *Xanthomonas campestris* pv. *citrumelo* and *X. c.* pv. *citri* on infection of expanding Duncan grapefruit leaves[a]

Inoculum concentration (cfu/ml)	Number of lesions ± standard error	
	X. c. citrumelo (strain F1)	*X. c. citri* (strain MF23P)
10^2	0	0
10^3	0	0
10^4	0	0.1 ± 0.1
10^5	1.0 ± 0.3	1.7 ± 0.4
10^6	13.6 ± 0.8	14.0 ± 0.8

[a]Experiment 2, with the following conditions: impact pressure at 8.04 kPa; leaf expansion of 50–75%; congestion caused by five preinoculation bursts of 200 μl of distilled water; and inoculum consisting of one burst of 200 μl.

In experiment 2, minimum concentration of inoculum necessary to cause infection at 8.04 kPa was 10^5 cfu/ml for both *X. c. citrumelo* and *X. c. citri*; however, considerably higher lesion numbers occurred at 10^6 cfu/ml for *X. c. citri* strain MF23P (Table 1). Therefore, in subsequent experiments, 10^6 cfu/ml was selected as a standard inoculum concentration for repeatable lesion development. In experiment 3, the 10^6 cfu/ml inoculum concentration was tested across a range of impact pressures for congestion of tissues with water followed by a burst of inoculum at 6.28 kPa. The number of lesions increased as the impact pressure increased up to ~9.81 kPa, after which there was no further increase in lesion numbers (Table 2). When the reverse was examined, in experiment 4 (tissues were congested with water at a single impact pressure of 9.81 kPa, followed by a range of pressures for the application of inoculum), no discernible difference occurred among any of the impact pressures used for inoculum delivery (Table 3).

Experiment 5 was conducted to determine the number of bacteria that entered the leaf via SIA inoculation resulting from different impact pressures of water followed by an application of 10^6 cfu/ml of inoculum at 6.28 kPa, and the effect of these regimes on in situ bacterial population growth over time. For *X. c. citrumelo*, lower impact pressures (4.61 and 6.28 kPa) of water to congest the leaf tissues prior to inoculation resulted in little or no infection (Fig. 3B), whereas these same congestion-impact pressures eventually resulted in bacterial growth for *X. c. citri* (Fig. 3A). Higher impact pressures for congestion of tissues with water (8.04 and 11.59 kPa) resulted in typical bacterial growth curves over time for both bacterial strains (Fig. 3A and B).

TABLE 2. Effect of varying impact pressures of preinoculation water causing congestion of tissues followed by inoculum of *Xanthomonas campestris* pv. *citrumelo* or *X. c.* pv. *citri* at a standardized pressure on infection of expanding Duncan grapefruit leaves[a]

Impact pressure of preinoculation water (kPa)[b]	Number of lesions ± standard error	
	X. c. citrumelo	*X. c. citri*
0	0	0
6.28	1.2 ± 0.4	1.0 ± 1.0
8.04	3.6 ± 1.1	11.0 ± 0.4
9.81	7.6 ± 1.5	12.6 ± 1.4
10.79	13.2 ± 2.4	14.0 ± 4.2
11.57	14.8 ± 1.9	10.8 ± 4.5

[a]All six impact pressures were applied to each leaf at different locations. Five leaves were used per strain. Inoculum was adjusted to 10^6 cfu/ml.
[b]Results of experiment 3. Each inoculation location was subjected to congestion by five applications of 200 μl of distilled water at the indicated pressure of impact followed by one application of 200 μl of inoculum at 6.28 kPa. An impact pressure of 0 indicates that the inoculum was simply placed on the leaf surface.

TABLE 3. Effect of varying impact pressures of inoculum of *Xanthomonas campestris* pv. *citrumelo* and *X. c.* pv. *citri* on infection of expanding Duncan grapefruit leaves[a]

Impact pressure of inoculum (kPa)[b]	Number of lesions ± standard error	
	X. c. citrumelo	*X. c. citri*
0	2.8 ± 1.8	4.8 ± 1.5
6.28	14.8 ± 2.7	15.6 ± 3.9
8.04	15.0 ± 4.0	20.8 ± 4.2
9.81	16.4 ± 3.9	17.0 ± 3.4
10.79	16.8 ± 5.4	18.0 ± 3.2
11.57	15.4 ± 3.7	17.4 ± 3.1

[a]All six impact pressures were applied to each leaf at different locations. Five leaves were used per strain. Inoculum was adjusted to 10^6 cfu/ml.
[b]Results of experiment 4. Each inoculation location was subjected to congestion by five applications of 200 μl of distilled water at an impact pressure of 9.81 kPa followed by one application 200 μl of inoculum at the indicated pressure. An impact pressure of 0 indicates that the inoculum was simply placed on the leaf surface.

The volume of water that enters the leaf and causes congestion at each impact pressure was examined in experiment 6. No appreciable change in volume of water within leaf was detected at the lower impact pressures of 4.61 and 6.28 kPa from ingress of water through the stomata, whereas the higher impact pressures of 8.04–11.57 kPa forced increasingly greater volumes of fluid through the stomata (Fig. 3C).

In experiment 7, the effect of increasing impact pressures was again examined over time with an additional impact pressure of 11.59 kPa, to determine the effect on bacterial population dynamics in situ and eventual lesion formation. However, this time both the upper and lower leaf surfaces were examined for bacterial penetration through stomatal openings. Bacterial growth in situ and lesion development from inoculations on the lower leaf surface were as expected. Initial bacterial populations increased with increasing impact pressure and over time (Fig. 4A and B). Lesion number, assayed at 168 h (7 days) postinoculation, increased with impact pressure (Fig. 4C); however, very little penetration of inoculum occurred through the top leaf surface, and only at the greatest impact pressures (Fig. 4B), where some damage to tissue was noted by transmission electron microscopy and resulted in the development of very few lesions, and only at the highest pressure, 11.59 (Fig. 4C).

Given the results from experiments 1 and 7, five bursts of 200 μl of inoculum at a concentration of 10^6 cfu/ml, applied at an impact pressure of 6.28 kPa, resulted in an increase in leaf water volume of $\sim 6.0 \times 10^{-5}$ ml. Thus, 60 cfu ($[6.0 \times 10^{-5}$ ml$] \times [10^6$ cfu/ml]) of bacteria potentially entered the leaf through stomatal openings. This resulted in an average of 8.6 lesions (over a range of 0–20) and 9.0 lesions (over a range of 0–25) for strains *X. c. citri* MF23P and *X. c. citrumelo* F1, respectively. Therefore, an average of ~7 cfu (over a range of 2.4–60 cfu) was required to produce a single lesion.

DISCUSSION

The epidemiological significance of the combined effect of wind and rain in producing water-soaking of foliage tissues and

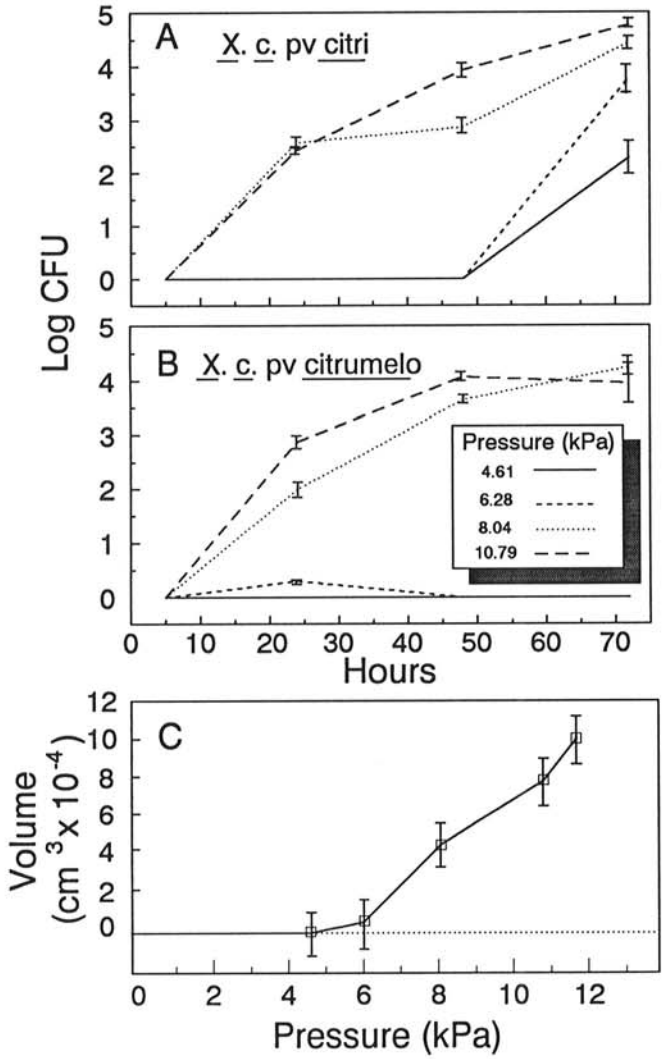

Fig. 3. Effect of pressure of impact of inoculum on in situ bacterial population growth in young Duncan grapefruit foliage (experiment 5). **A,** *Xanthomonas campestris* pv. *citrumelo* strain F1. **B,** *X. c.* pv. *citri* strain MF23P. Experimental conditions were as follows: leaves were expanded 50–75%; congestion was caused by five preinoculation bursts of 200 μl of sterile distilled water; inoculum consisted of one burst of 200 μl of inoculum at 10^6 cfu/ml; all inoculations were replicated five times. **C,** Determination of the volume of water entering the leaf through stomatal openings at various impact pressures after treatment with five preinoculation bursts of 200 μl of sterile distilled water (experiment 6). Bars represent standard errors.

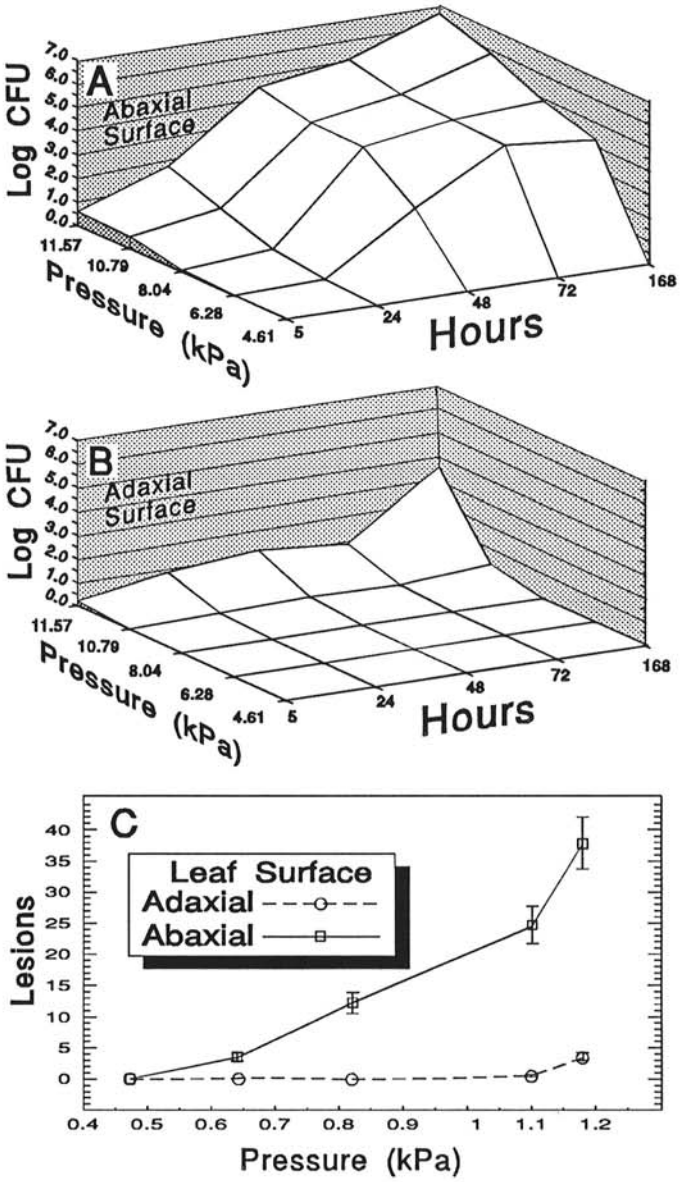

Fig. 4. Effect of impact pressure of inoculum on **A,** abaxial, and **B,** adaxial leaf surface, on in situ growth of *Xanthomonas campestris* pv. *citri* strain MF23P and **C,** lesion development following inoculation of adaxial and abaxial leaf surfaces of Duncan grapefruit leaves (experiment 7). Individual inoculations were carried out by introducing five bursts of 200-μl aliquots of sterile water at the pressure indicated, followed by inoculum at 10^6 cfu/ml at a pressure of 6.28 kPa into the airstream. Lesion number was determined at 168 h (4 days) postinoculation. Bars represent standard errors.

introduction of bacterial pathogens through the stomata of such congested tissues has long been recognized (15,22,24,26). The SIA described in this study was developed to produce water congestion of foliage tissues and provide a repeatable means of introducing inoculum into these tissues without wounding. Through the series of preliminary experiments, ranges and optimums of pressure required for congestion of tissues; inoculum impact pressure; host tissue stage; and inoculum concentration parameters were determined. Leaf tissues on the abaxial surface of leaves expanded 50–75% were the most susceptible to stomatal penetration by the bacterial pathogens. This supports previous findings that indicated that expanding juvenile tissues are more susceptible than fully expanded leaves (19,28). In such tissues, the difference in susceptibility between adaxial and abaxial surfaces was dramatic, presumably because numerous stomata are located on the abaxial surface but few occur on the adaxial surface of citrus (20,21; Graham and Achor, *unpublished*). Inoculum concentrations of 10^6 cfu/ml consistently yielded discrete individual lesions when applied at airstream impact pressures of 6.28–8.04 kPa. This range of pressure consistently and effectively produced tissue congestion and thus infection without causing wounding. It was determined that these same pressures were the lowest threshold for causing a detectable increase in water volume in the leaf mesophyll. Thus, concentration of inoculum versus volume of bacterial inoculum, which probably entered the leaf tissues via SIA, indicates that as few as 2.4 bacteria were necessary to cause a single lesion.

Further studies have shown that occasionally a 1:1 ratio exists between number of bacteria entering the leaf and number of lesions formed (Gottwald and Graham, *unpublished*). This was demonstrated by isolation from SIA-treated tissues, which allowed us to follow bacterial growth in situ, from very low initial levels of bacteria (2–20 cfu per inoculation site). Such controlled inoculation with bacterial pathogens is extremely difficult to repeatably achieve by other means of inoculation (5). The available volume of free intracellular spaces in citrus mesophyll tissues was previously calculated from injection-infiltration studies to be 7.2 μl/cm^2 (28). The volume of water that the stomatal inoculation apparatus was capable of forcing into these intracellular mesophyll spaces was ~1.0 μl/cm^2. With very precise dilutions, Stall et al (6) demonstrated that a single bacterium of *X. c. citri* could produce a single lesion by injection-infiltration. Injection-infiltration potentially delivers bacteria to many more susceptible sites in the mesophyll per unit volume than realistically occurs with water congestion by wind-driven rain. The injection-infiltration method relies on wounding for introduction of the pathogen; therefore, it is not possible to study host resistance and dynamics of infection of unwounded leaves by this method. For instance, Egel et al (5) found that no differences in population growth among *X. c. citri* and two strains of *X. c. citrumelo* in resistant and susceptible hosts were discernible by injection-infiltration, but differences of host × strain interaction were apparent with pinprick inoculations. Presumably, pinprick inoculations introduced fewer bacteria into the mesophyll, did not overwhelm the tissues, and allowed for expression of tissue resistance to bacterial multiplication (5). The SIA method, much like the pinprick inoculation method, introduces only a few bacteria into mesophyll tissues. However, unlike the pinprick inoculation method, the SIA method takes advantage of natural openings in the plant surface for bacterial infiltration and allows the study of in situ population and lesion development without the disadvantage of wounding caused by either injection infiltration or pinprick inoculation.

The SIA is presently being used to investigate effects of stomatal aperture morphology, cuticular development on bacterial penetration and infection, and susceptibility of citrus cultivars to quarantined bacterial pathogens under containment facility conditions.

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