A Technique for Precise Inoculation of the Internal Phyllosphere of Cotton with *Xanthomonas campestris* pv. *malvacearum*

N. Kangatharalingam, Margaret L. Pierce, and Margaret Essenberg

Department of Biochemistry & Molecular Biology, Oklahoma State University, Stillwater 74078.

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ABSTRACT

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A technique was developed to inoculate uniformly and gently the internal phyllosphere from the upper surface of cotton leaves with the phytopathogenic bacterium *Xanthomonas campestris* pv. *malvacearum*. The inoculum consisted of 2 to 3×10^7 CFU/ml in CaCO_3 -saturated sterile distilled water containing 0.02%, vol/vol, of the wetting agent Silwet L-77. A custom-made inoculation apparatus was employed to immerse a

circular area of the adaxial surface of a leaf in inoculum for 90 s. This resulted in uniform, passive entry of bacteria into the substomatal chambers, producing an endophytic bacterial population of 2×10^4 CFU/cm². Microscopic signs of infection were visible 48 to 72 h after inoculation. In susceptible leaves, uniformly distributed water-soaked spots were observed 7 to 8 days after inoculation. When the technique was used on resistant leaves, the autofluorescence that is characteristic of hypersensitively necrotic cells developed in the guard cells and palisade cells lining substomatal chambers, but not in the underlying spongy mesophyll.

Additional keyword: adaxial epidermis.

For *Xanthomonas campestris* pv. *malvacearum*, as for many other bacterial plant pathogens, open stomata are important ports of entry into leaves (8). Phytopathogens survive and proliferate in the protected sites of the substomatal chambers, forming infection courts (9,16,18). We are interested in studying host responses to the developing microcolonies.

We have developed a set of near-isogenic cotton lines carrying major genes for resistance to bacterial blight (4). Each of these genes confers measurable resistance to races of *X. campestris* pv. *malvacearum* that carry the corresponding avirulence genes. One of our objectives was to make quantitative comparisons via light/fluorescence microscopy of the local cellular response of these lines to individual microcolonies of *X. campestris* pv. *malvacearum*. With cotton leaves, an entire microscope field can be brought into focus on an optical section of the palisade mesophyll cells through the adaxial (upper) epidermis. In contrast, the spongy mesophyll cells are irregular in shape, and the abaxial (lower) surface is uneven in contour; consequently, only a few mesophyll cells can be brought into focus at once. Hence the adaxial surface was preferred for our studies.

Spray infiltration and syringe-aided pressure infiltration are much less successful through the adaxial surface than the abaxial surface of cotton leaves, and these methods almost always cause injury to the foliar tissue. For studies of the hypersensitive response and phytoalexin production, tissue injury due to the inoculation process is unacceptable because phytoalexin elicitation can result from such injury (3,10,15). Vacuum infiltration methods are tedious and often nonuniform in the distribution of inoculum. Low-pressure misting with atomizers does not yield sufficient endophytic bacterial population for our purpose, apparently because of the failure of droplets to enter stomata. A method incorporating

a wetting agent (17) seemed most promising. The technique presented here provides a means of introducing bacterial cells uniformly and effectively into substomatal chambers on a selected surface of a leaf without tissue damage. The introduced bacterial cells rapidly formed microcolonies in the substomatal chambers, and disease symptoms followed. The method is simple, gentle, precise, and uniform.

MATERIALS AND METHODS

Inoculation apparatus. The inoculation apparatus (Fig. 1A and B) consists of a glass jar, with a mouth of 6-cm internal diameter and a shoulder 1 cm below the mouth (widely available in retail stores as a candle holder), and a custom-made plastic lid (diameter, 6.8 cm) with a smooth circular ridge on the bottom 6.5 mm high and 5.2-cm inner diameter. The jar has a capacity to hold 92 ml of liquid inoculum. The top of the lid carries a galvanized iron weight to raise its total weight to 150 g. The surface of the plastic lid was well polished to prevent any abrasion of the leaf surface.

Plants and bacterial inoculum. *Gossypium hirsutum* L. Ac44E, which has no known genes for resistance to bacterial blight, and near-isogenic lines *AcB₄*, *AcB₇*, and *AcB_{ln}*, which possess bacterial blight resistance genes *B₄*, *b₇*, or *B_{ln}* in the Ac44E genetic background (4), were grown in a controlled environment chamber (14). Two plants were grown per pot (16-cm-diameter). Four to seven weeks after planting, the youngest expanded leaves were used for inoculation. The bacterial pathogen *X. campestris* pv. *malvacearum* strain 3631 has a race 1 phenotype; it is virulent toward Ac44E and avirulent toward *AcB₄*, *AcB₇*, and *AcB_{ln}* (4). It was grown in *Xanthomonas* tryptone yeast glucose (XTYG) broth (2) at 30°C on a rotary shaker at 200 rpm for 4 h (mid-exponential phase) to a population density of 1 to 2×10^9 CFU/ml. The culture was diluted, without removal of the medium, to 2 to 3×10^7 CFU/ml in sterile distilled water amended with CaCO_3 (140 mg/liter) to maintain viability (6). Into this inoculum was added the wetting agent Silwet L-77 (Lehle Seeds, Round Rock, TX) to 0.02%, vol/vol. Other inoculum concentrations were used as indicated.

Corresponding author: M. Essenberg
E-mail address: margess@biochem.okstate.edu

The bulk inoculum was prepared in a large conical flask, which was swirled to resuspend the bacteria each time before pouring into the inoculation jar.

Inoculation method and incubation. To promote entry of bacterial cells through stomata, the selected leaves (still attached to plants and in the growth chamber) were placed in plastic reclosable bags containing a drop (0.2 to 0.3 ml) of sterile water and closed around the petiole for 45 min to maintain high humidity and thereby enhance stomatal opening. The sterile inoculation jar was placed on a horizontal surface and completely filled with the inoculum suspension. Bubbles on the surface were removed with a sterile spatula. The leaf was removed from the bag and turned upside down; the lamina was curled slightly upward to present a curved surface toward the inoculum. The curved center of the adaxial surface was carefully placed onto the inoculum, and the rest of the lamina was gradually laid down. This prevented formation of air bubbles that may otherwise be trapped under the leaf lamina. The lid was placed on the leaf and allowed to rest on the jar (Fig. 1C). The circular ridge on the inner surface of the lid depressed the middle of the leaf into the jar, displacing a small volume of the inoculum, which rose between the leaf and the jar and overflowed the rim of the jar. The liquid remaining between the leaf and the side of the jar was higher than the middle of the leaf and established a gentle hydrostatic pressure against the immersed leaf surface. The leaf surface was kept in contact with the inoculum for 90 s. After 90 s, the lid was carefully removed while holding onto the petiole of the leaf. The leaf then was returned to its normal orientation with the excess inoculum dripping off. At no time during or after the procedure was the abaxial surface exposed to inoculum. The leaf was allowed to air dry outside the growth chamber under room light, and the leaf surface was dry within 5 to 10 min. The inoculated plants were returned to the growth chamber, usually after about 1 h.

Estimation of initial population of viable bacterial cells. The initial endophytic bacterial population was estimated by sampling the inoculated region of the leaves within 1 h after inoculation. Leaf disks (diameter, 0.59 cm) were removed, one from each side of the midrib. The disks were placed in plastic bags or vials and placed on ice for at least 30 min to permit stomatal closure. They were thoroughly rinsed with two changes of CaCO_3 -saturated distilled water to remove surface microorganisms, particularly *X. campestris* pv. *malvacearum* remaining from the inoculum. The disks were then homogenized with a sterile mortar and pestle. The homogenate was diluted appropriately in CaCO_3 -saturated water, and aliquots were spread on XTYG or nutrient agar plates and incubated at 30°C. Yellow colonies of *X. campestris* pv. *malvacearum* were counted after 48 h.

Microscopic examination of infection sites. Starting 2 days after inoculation, leaf disks sampled from the inoculated region were examined under a microscope (Nikon Optiphot; Nikon, Lewisville, TX) by transmitted white light and by epifluorescence with blue (460 to 485 nm) excitation, a 510-nm dichroic mirror, and a >520-nm emission filter. The adaxial surface and hand-cut transverse sections of the sample disks were examined to locate the sites of inoculation and infection.

RESULTS AND DISCUSSION

The presence of the wetting agent Silwet L-77 at 0.02% (vol/vol) in the inoculum (XTYG culture diluted in CaCO_3 -saturated water) did not affect the viability of the bacteria for 2 h, which was the longest period we tested. Plating of the inoculum before and after inoculation on five occasions spanning a period of 1.5 to 2 h showed an average increase in colony forming units per plate of 1.5-fold. The wetting agent dispersed well in the liquid inoculum and produced very little frothing. As reported in previous studies (7,11), the organosilicone surfactant Silwet L-77 enhanced foliar penetration and stomatal infiltration of liquids by reducing the

surface tension and was an important aspect of the present technique. A concentration of 0.02% was most effective in our procedure and is in midrange of what other groups have used (1,12,17). In our preliminary studies, mock-inoculation with Silwet L-77 at 0.02% (vol/vol) did not produce any symptoms for 10 days in either susceptible (Ac44E) or resistant (Acb₇) lines. Others have found concentrations greater than 0.1% to be phytotoxic in *Arabidopsis* spp. (17).

Exposure of adaxial leaf surfaces to the inoculum for durations of 30, 60, 90, and 120 s were tested. Infection frequencies were most variable with the 30-s exposure, less variable with the 60-s exposure, and most uniform with 90- and 120-s exposures. We set the exposure time at 90 s. The average time taken to set up and complete inoculation of a leaf by our method was 3.5 min. The large surface area of cotton leaves, compared with the top of the

Fig. 1. The inoculation apparatus: **A**, jar and inside surface of lid viewed separately; **B**, jar and lid with the washer-shaped metal weight mounted on it; and **C**, assembled inoculation apparatus, showing an inverted cotton leaf between jar and lid, with the lid holding the middle part of the adaxial side of the leaf immersed in inoculum.

inoculation jar (Fig. 1C), was an advantage in preventing exposure of the abaxial surface to the inoculum.

Simply spreading inoculum on the surface of susceptible leaves consistently produced a low frequency of infected substomatal chambers (only several chambers per square centimeter or fewer [Fig. 2]). In tests with leaves from resistant lines, yellow-green autofluorescence (Fig. 3A) and browning, which are signs of a resistance response, appeared mainly in the stomatal apparatus, evidently due to superficial or shallow infections. The inoculation apparatus, which aids in immersing the selected surface of the leaf in the inoculum, resolved the problem of superficial infections and infrequent infection centers. A full 90 s of repeated daubing of the leaf surface with inoculum was not equivalent to use of the inoculation apparatus. One would expect the results to be equal if time of exposure or active participation of the bacteria were responsible for the entry into the stomata. Hence, we conclude that in this method the entry is passive and is achieved by a small, uniform hydrostatic pressure due to the immersion of the desired portion of adaxial surface. There is a slight mechanical pressure on the part of the leaf contacted by the narrow circular ridge of the plastic lid. A central disk (4.8-cm-diameter) of the leaf lamina, however, experiences only the gentle hydrostatic pressure. No signs of injury were detected there. We know the pressure is uniform because the lid is horizontal in the apparatus and the resulting lesions are uniformly distributed (Fig. 4). In the susceptible Ac44E line, numerous water-soaked lesions appeared 7 to 8 days after inoculation (Fig. 4). In the resistant cotton lines that we tested using this method, palisade cells lining the substomatal chambers responded by developing the yellow-green autofluorescence (Fig. 3B and C) that is characteristic of the hypersensitively necrotic cells (6).

A bacterial inoculum of 2×10^7 CFU/ml achieved a mean endophytic bacterial population 1 h after inoculation of 1.8×10^4 CFU/cm² of leaf surface (Table 1). This corresponds to about 2 CFU per adaxial stoma. The efficiency of endophytic entry of viable bacterial cells in this method is less than 50% of that obtained by spray inoculation from the abaxial side (13; N. Kangatharalingam, M. L. Pierce, and M. Essenberg, *unpublished data*), reflecting the smaller volume of inoculum that enters the leaf with this technique compared with spray inoculation, which fills almost the entire intercellular space.

When the inoculum concentration was varied by 2 orders of magnitude, the resulting endophytic bacterial populations on day 0

Fig. 2. The abaxial surface of an inoculated leaf of susceptible cotton line Ac44E on day 10 after applying *Xanthomonas campestris* pv. *malvacearum* inoculum to the adaxial surface with a sterile cotton ball. The cotton ball was saturated with the bacterial suspension (2×10^7 CFU/ml) containing 0.02%, vol/vol, Silwet L-77. All lesions were visible on both surfaces but more clearly on the abaxial surface due to the light green background of the leaf surface. Arrowhead points to the nectary, which is on the abaxial side of the midrib.

and day 2 spanned slightly more than 2 orders of magnitude (Table 1). Seven days after inoculation, as macroscopic symptoms were beginning to develop, microscopic observation revealed circular areas that were bright due to the water-soaking characteristic of the disease (5). In leaves that received the two lower inoculum concentrations, the average number of water-soaked

Fig. 3. **A**, Fluorescence micrograph showing the hypersensitive response by guard cells and accessory cells alone in a transverse section of a resistant *Acb7* leaf 8 days after inoculation with *Xanthomonas campestris* pv. *malvacearum*. A cotton ball was used to spread the inoculum. Only the guard cells and accessory cells emitted bright yellow-green fluorescence, indicating that the bacteria had scarcely penetrated the stoma. **B**, Fluorescence micrograph showing the hypersensitive response visible in a transverse section of a resistant *AcBln* leaf 8 days after inoculation with *X. campestris* pv. *malvacearum*. The inoculation apparatus shown in Figure 1 was used. Bright yellow-green fluorescence was emitted by palisade cells lining substomatal chambers. **C**, Fluorescence micrograph showing the hypersensitive response visible in a transverse section of a resistant *Acb7* leaf 8 days after inoculation with *X. campestris* pv. *malvacearum*. The inoculation apparatus shown in Figure 1 was used. Bright yellow-green fluorescence was emitted by guard cells and by at least two ranks of palisade cells on each side of substomatal chambers. In these black and white images, yellow-green autofluorescence appears white amid the paler autofluorescence due to chlorophyll. Inoculum concentration was 2×10^7 CFU/ml. Scale bar = 100 μ m.

substomatal chambers per square centimeter was about 75% of the colony forming units per square centimeter on day 0 (Table 1). This success rate of endophytic bacteria in causing infections matches that previously observed with vacuum infiltration (5). At the two lower inoculum concentrations, a 10-fold increase in inoculum concentration resulted in a 13-fold increase in density of water-soaked chambers—a nearly proportional increase (Table 1). Although the initial density of endophytic bacteria at the highest inoculum concentration exceeded the density of stomata about twofold, only about half of the substomatal chambers exhibited water-soaking at 7 days (Table 1). This lesion density probably represents the maximum attainable by inoculation of cotton leaves by the method we employed. An inoculum concentration of 2×10^7 CFU/cm² was used for the work illustrated in the Figures.

Microscopic examination of the inoculated adaxial surface of leaves as early as 48 and 72 h after inoculation revealed a uniform distribution of infected substomatal chambers with developing microcolonies. The microcolonies of *X. campestris* pv. *malvacearum* looked like dew droplets, possibly due to their production of extracellular polysaccharide. Such dew formation on the walls of cells lining the substomatal chambers was the first visible sign of infection (Fig. 5). Mock-inoculated leaves rarely showed the presence of fluid droplets in the substomatal chambers, whereas

droplets were seen at a much higher frequency in inoculated leaves (Table 2). These droplets were obvious in approximately 40% as many substomatal chambers as later developed into water-soaked lesions (Table 2). The dew was less prominent or barely visible in resistant cotton lines after 72 h as the hypersensitive response developed.

In leaves of all three resistant lines, the bright yellow-green autofluorescence of hypersensitive necrosis appeared in palisade cells surrounding the substomatal chambers and sometimes in guard cells and accessory cells (Fig. 3B and C; *AcB₄* not shown), but not in spongy mesophyll cells. In *AcB_{1n}* and *AcB₄*, the hypersensitive response generally involved only the palisade cells lining the substomatal chamber (Fig. 3B), whereas in *AcB₇*, the responding zone was larger, including more than one rank of palisade cells surrounding the chamber (Fig. 3C).

This technique is superior to existing inoculation methods, such as spray inoculation or vacuum infiltration, because it precisely deposits the inoculum into substomatal chambers of the selected leaf surface, avoids leaf injury, requires only a small inoculum volume, and achieves a uniform distribution of infection sites. In previous studies, leaves were inoculated by spraying the abaxial surface or by vacuum infiltration (5,6,13), and we counted the numbers of hypersensitively necrotic cells that could be seen in optical sections of the palisade mesophyll as viewed through the adaxial epidermis (13). Infection sites that originated in the spongy mesophyll were observed only if palisade cells became

Fig. 4. The abaxial surface of a leaf of cotton line Ac44E 8 days after inoculation with the apparatus shown in Figure 1. Water-soaked lesions (dark spots) are uniformly distributed in the central inoculated area of the leaf. Although the leaf was inoculated on the adaxial surface, water-soaking on that surface is less visible due to masking by the dark green pigmentation. Water-soaking viewed from the abaxial surface illustrates that the lesions extend all the way from the substomatal chambers of the adaxial surface through the intercellular space of the leaf to the abaxial surface. Inset shows individual water-soaked lesions in a portion of the inoculated area magnified 3.6-fold. Inoculum concentration was 2×10^7 CFU/ml.

Fig. 5. Microscopic view of fluid droplets in substomatal chambers below the adaxial epidermis of a bacterial blight-susceptible Ac44E leaf 48 h after inoculation with *Xanthomonas campestris* pv. *malvacearum*. Arrowheads point to some of these droplets. Variation in focal plane across this micrograph allows localization of the fluid droplets to the substomatal chambers: on the left, the focus is on the epidermis; on the right, the palisade cells are in focus; and in the middle, where the focus is between the epidermal and the palisade layers, is where the fluid droplets are in focus. Scale bar = 100 μ m.

TABLE 1. The effect of inoculum concentration on infection of bacterial blight-susceptible cotton line Ac44E by *Xanthomonas campestris* pv. *malvacearum* using the described inoculation apparatus and technique^a

Inoculum concentration group	Inoculum concentration (CFU/ml)	CFU/cm ² of lamina (day 0)	Stomata/cm ²	Maximum possible fraction of stomata infected ^b	CFU/cm ² of lamina (day 2)	Water-soaked substomatal chambers/cm ² (day 7) ^c	Fraction of stomata that are water-soaked ^d
I	2.0×10^5	1.3×10^2	7.3×10^3	0.018	6.3×10^3	1.2×10^2	0.016
I	2.1×10^5	0.8×10^2	7.2×10^3	0.011	4.5×10^3	0.4×10^2	0.006
II	2.0×10^6	1.5×10^3	9.9×10^3	0.15	1.2×10^5	1.3×10^3	0.13
II	2.1×10^6	1.2×10^3	5.6×10^3	0.21	1.0×10^5	0.7×10^3	0.13
III	1.9×10^7	2.4×10^4	6.6×10^3	1.0	1.1×10^6	2.8×10^3	0.42
III	2.2×10^7	1.4×10^4	7.2×10^3	1.0	1.3×10^6	3.4×10^3	0.47
III	2.3×10^7	1.6×10^4	9.9×10^3	1.0	1.4×10^6	5.3×10^3	0.54
Averages (I:II:III)	1:10:104	1:13:170	1:1.1:1.1	1:12:69	1:20:235	1:13:48	1:12:43

^a Each line of the table represents a different leaf; all inoculations were performed on the same day.
^b Colony forming units per square centimeter on day 0/stomata per square centimeter, limited to 1.0.
^c Determined by microscope examination of 15 fields of 0.0196 cm² on each leaf using a $\times 4$ objective lens.
^d Water-soaked substomatal chambers per square centimeter on day 7/stomata per square centimeter of leaf lamina.

TABLE 2. Microscopical evaluation of *Xanthomonas campestris* pv. *malvacearum*-inoculated and mock-inoculated leaves for the presence of droplets of fluid in substomatal chambers on day 3 and of water-soaked spots on day 7 after inoculation of bacterial blight-susceptible cotton line Ac44E

Treatment ^a	Droplet-positive chambers ^b	Frequency of positives	99% CI ^c	Water-soaked chambers/cm ^{2d}	Stomata/cm ²	Frequency of positives	99% CI ^c
Mock	5 of 480	0.01	0 – 0.02	0	8,500	0.00	...
<i>X. campestris</i> pv. <i>malvacearum</i>	90 of 480	0.19	0.14 – 0.23	4,100	8,600	0.47	0.46 – 0.49

^a The bacterial inoculum concentration of *X. campestris* pv. *malvacearum* was 2.2×10^7 CFU/ml.

^b A $\times 40$ objective lens was used; 160 adaxial stomatal chambers were examined on each of three leaves per treatment.

^c Ninety-nine percent confidence intervals were estimated from the binomial distribution: $N = 480$ for column 4; $N = 7.6 \times 10^3$ for column 8.

^d A $\times 4$ objective lens was used; 15 fields of 0.0196 cm^2 were examined on each of the same three leaves per treatment.

part of the hypersensitive response. With this new inoculation technique, the first cells to respond to the inoculum will be visible through the adaxial epidermis. Infection sites will be well distributed because they are determined by the regular arrangement of adaxial stomata, and thus, no closer to one another than the distance between two stomata. This technique is applicable to studies of bacterial interactions with leaf mesophyll tissue. Because it places the bacteria directly into leaves, this method will not provide information on any process involved in natural entry. However, by placing the bacteria into substomatal chambers within a 90-s period, it will permit quantitative as well as qualitative time-course studies of the events that follow that entry.

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