Scanning Electron Microscopy of the Ingress and Establishment of *Pseudomonas alboprecipitans* in Sweet Corn Leaves

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ABSTRACT

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Leaf tissues from whorl areas of sweet corn plants inoculated with *Pseudomonas alboprecipitans* were prepared for scanning electron microscopy. The bacterium was detected in stomata and substomatal cavities of corn leaves after corn-plant whorls were exposed to inocula for 160–320 min.

Although stomata were manipulated successfully with 10^{-4} M abscisic acid, the partial closure of stomata prior to inoculation did not affect significantly the amount of leaf damage due to the bacterium. Bacteria were observed frequently in the basinlike depressions surrounding "closed" stomata. It was concluded that "closed" as well as open stomata in whorl areas could serve as portals of ingress for the small (0.57×1.5 μm) rod-shaped cells.

Bacterial leaf blight and stalk rot of sweet corn (*Zea mays saccharata* [Sturtevant] Bailey) caused by *Pseudomonas alboprecipitans* Rosen occurs commonly in Florida (7). The pathogen causes a variety of symptoms throughout the development of the plant, the most striking of which are long, narrow stripes on the leaves and eventually leaf shredding.

There is no evidence that leaves or other plant surfaces can be penetrated directly by plant pathogenic bacteria. Consequently, in this and other bacterial diseases, natural openings and wounds are important for bacterial ingress into plants (3,10,14). Bacterial ingress could be a critical event in the development of bacterial plant diseases. Haas and Rotem (8) suggested that factors controlling ingress were important to the progress of epidemics of angular leafspot of cucumber. Once *Pseudomonas lachrymans* was present in the crop, inoculum remained available but disease outbreaks were sporadic. Therefore, factors controlling ingress, such as wounding, may have controlled disease progress. Rich (12) recognized the importance of ingress for disease progress, and he speculated that stomatal-inhibiting chemicals, which induced stomatal closure, protected plants from bacterial pathogens.

Evidence of stomatal ingress usually has been through histological studies which traced the origin of infection in early colonized tissues (3,13). Therefore, the purpose of this study was to determine the mode(s) of ingress of *P. alboprecipitans* into corn leaves by scanning electron microscopy. In addition, closure of stomata with abscisic acid was examined as a potential procedure for the prevention of bacterial ingress.

MATERIALS AND METHODS

Inoculum. Isolate PA 78-5 of *P. alboprecipitans* isolated from sweet corn at Zellwood, FL, was used in these experiments. A 24-hr nutrient broth culture of PA 78-5 was centrifuged to a pellet at 3,000 *g* for 10 min, and resuspended in 0.85% saline. The suspension of bacteria was adjusted to OD 600 nm = 0.3 (approximately 1×10^8 bacterial cells per milliliter) in a spectronic 20 spectrophotometer. The bacteria were concentrated to a pellet by low-speed centrifugation and resuspended in 1/10 the original volume of 0.85% saline, which provided an inoculum density of 10^9 bacterial cells per milliliter.

Test plants. Sweet corn plants (cultivar Gold Cup) 2–3 wk of age (postemergence) were used in these experiments. Plants employed for scanning electron microscopy and stomatal manipulations were grown in clay pots (127 mm in diameter) containing steam-pasteurized soil. These plants were subjected to continuous light (6,500 lux) and constant temperature (28C) in a controlled environmental chamber. Conditions favorable for open stomata were created by the enclosure of plants in glass chromatography tanks ($30 \times 30 \times 60$ cm) to each of which was added 1.0 L of 2.0 M potassium hydroxide. Plants were positioned on inverted petri dishes to avoid absorption of the potassium hydroxide and were subjected to the subsequent low carbon dioxide atmosphere for 48 hr.

Inoculation and sampling. Plants were inoculated with 1.0 ml of a 1×10^9 cells per milliliter suspension of PA 78-5. Inoculum was added to whorls of corn plants with a pasteur pipet. Blocks of tissue (5×5 mm) were sampled for scanning electron microscopy from whorl areas of each plant at 20, 40, 80, 160, and 320 min following inoculation. Prior to fixation, samples were exposed to an undetermined but elevated concentration of carbon dioxide in order to close the stomata and consequently retain any bacteria at that time. Four blocks of tissue from four replicates of inoculated and uninoculated plants as well as from plants with lesions were sampled for examination. Control plants were not exposed to an elevated concentration of carbon dioxide.

Plants used for stomatal manipulation were treated with an aqueous solution of 10^{-4} M abscisic acid. Abscisic acid was applied with an aerosol chromatography sprayer until leaves were visibly wet. After 1 hr of exposure to abscisic acid, leaf impressions were made with Sears household vinyl cement (Sears, Roebuck and Co., Chicago, IL 60607). One hundred stomatal apertures were measured per plant in four replicates. Plants treated with a 1-hr exposure to abscisic acid and nontreated plants were inoculated with *P. alboprecipitans*. After a 96-hr incubation period, plants were scored for percent leaf damage due to bacterial leaf blight.

Scanning electron microscopy. Tissues were fixed for 3 hr at 22 C in a buffered (0.2 M sodium cacodylate, pH 7.3) solution containing 3% glutaraldehyde. Samples were dehydrated in a series

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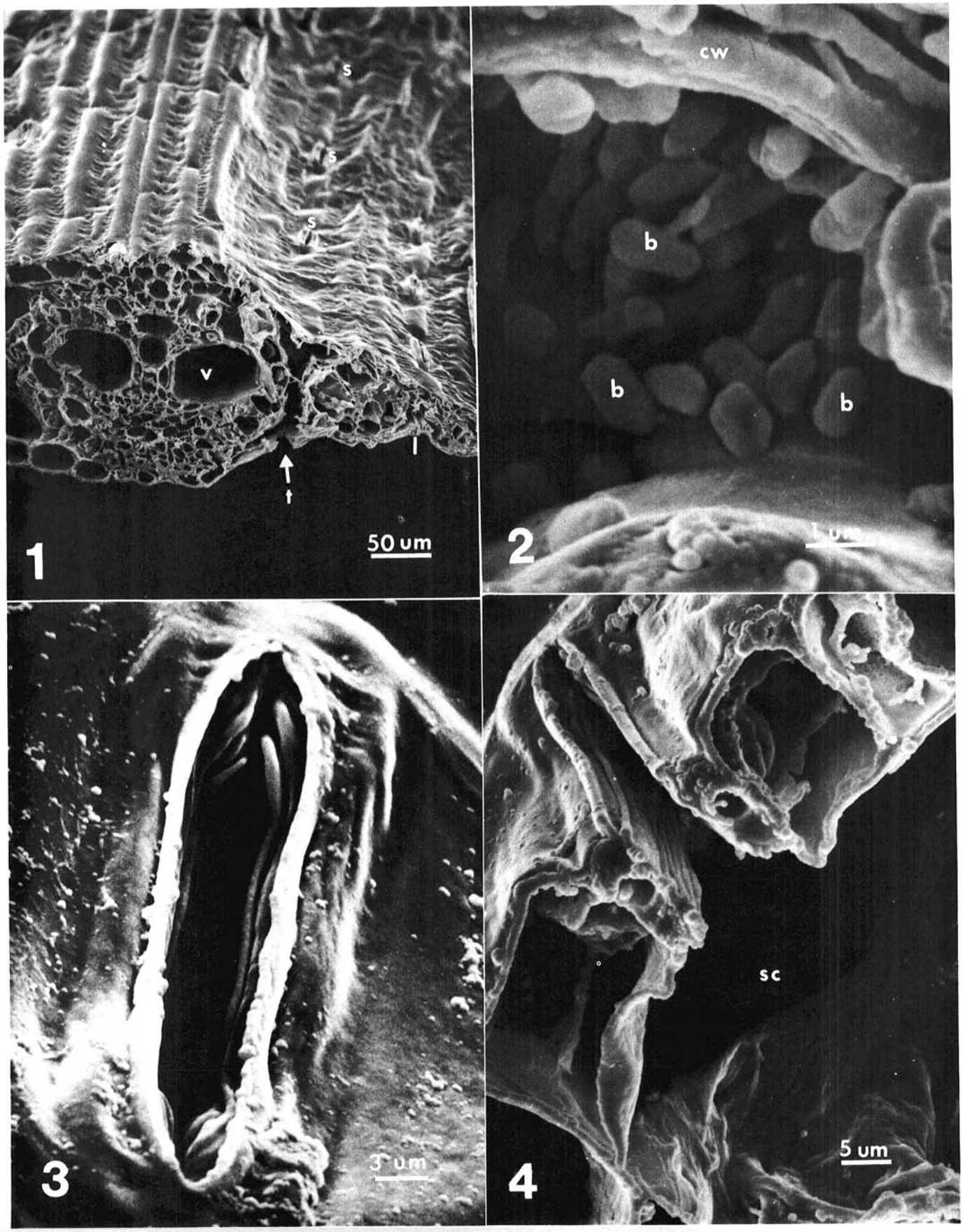
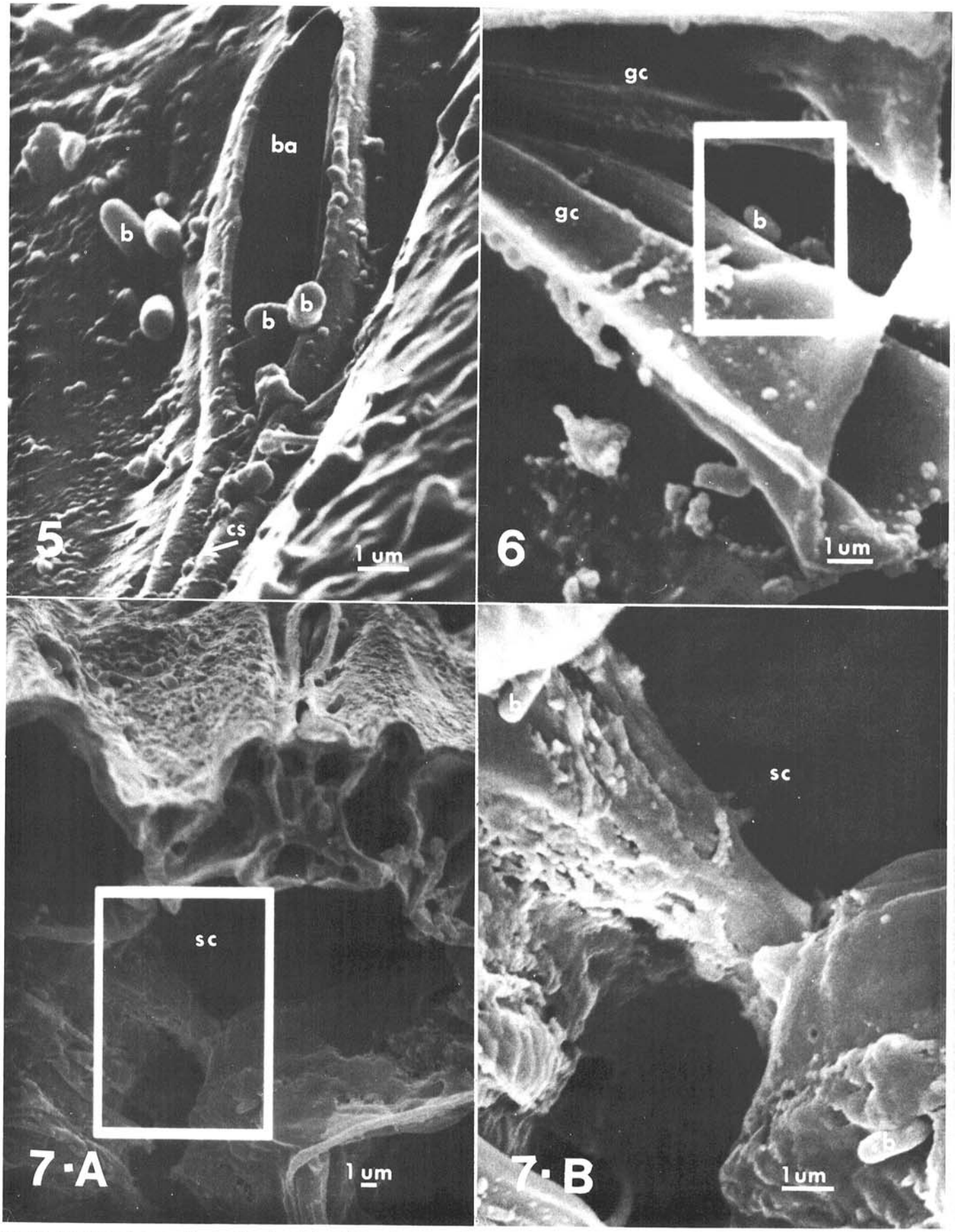


Figs. 1–4. Scanning electron micrographs of sweet corn leaves infected with *Pseudomonas alboprecipitans*. **1,** Cross section of corn leaf with a bacterial lesion. The lesion is limited by a vascular bundle with a sclerenchymatous sheath. Tissues along the edge of the lesion were subject to tearing (arrow). l = lesion, s = row of stomata, t = tear, v = vascular bundle. **2,** Cross section within a bacterial lesion on a corn leaf. Intercellular spaces are congested with rod-shaped *P. alboprecipitans*. b = bacteria, cw = plant cell wall. **3,** Stoma on the surface of a corn leaf. Tissue sampled from uninoculated control plants had open stomata even though the whorl contained free water. **4,** Cross section of corn leaf from control plant. Bacteria were not detected in the substomatal chamber (sc).



Figs. 5-7. Scanning electron micrographs of rod-shaped *Pseudomonas alboprecipitans* in stomata of sweet corn leaves. **5,** Surface of a stoma in tissue sampled from the whorl area after a 320-min exposure to *P. alboprecipitans.* Rod-shaped bacteria (b) surround and are in a basin (ba) formed by the closed stoma (cs). **6,** Surface of corn leaf which had been exposed to inoculum for 160 min. Bacteria are in the basin of a closed stoma. b = bacteria, gc = guard cell. **7,** Cross section of corn leaf which had been exposed to *Pseudomonas alboprecipitans* for 320 min. **A,** Substomatal chamber below guard cells which were closed after inoculation. **B,** Higher magnification of highlighted box in 7-A. Note the presence of rod-shaped bacteria (b) lodged against cell walls in the substomatal chamber (sc).

of 25, 50, 70, 90, 95, and 100% ethanol, followed by a series of 33.3, 50, 66.7, and 100% amyl acetate (diluent was 100% ethanol). Tissues were placed in a pressure chamber of a critical-point drier (DCP-1, Denton Vacuum, Inc., Cherry Hill, NJ 08034) and infiltrated with liquid carbon dioxide until the amyl acetate was purged. Tissues were then critical-point dried. Samples were mounted on edge on aluminum stubs and were coated with gold-palladium in a Hummer I sputtering unit (Technics, Inc., Alexandria, VA 22313). Stomata, substomatal cavities, and leaf surfaces were examined at 20 KV with a scanning electron microscope (ETEC Omniscan, Hayward, CA 94551).

RESULTS

Scanning electron microscopy. Lesions caused by *P. alboprecipitans* were vein-limited and consisted of severely collapsed tissue (Fig. 1). Both the parallel arrangement of vascular bundles in corn and the collapse of cells in the lesion between vascular bundles account for the striped leaf symptoms and the shredding of leaves. The lesion area adjacent to the vascular bundle contained a large percentage of intercellular space as a row of substomatal cavities existed parallel to the lesion's edge. The lesion area along the edge was further weakened by the collapse of cells which caused the area to tear away from the vascular bundle.

Intercellular spaces of the lesion area were congested with numerous rod-shaped bacteria and microcolonies of *P. alboprecipitans* (Fig. 2). Uninoculated control plants, which were not exposed to carbon dioxide, were free of bacteria and had stomata from whorl tissue open at the time of inoculation even when the whorl contained free water (Figs. 3 and 4).

Corn plants inoculated with *P. alboprecipitans*, but sampled before symptom expression (lesion development) had bacteria (measuring 0.57×1.5 μm) associated frequently with stomata in four replicates when whorls were exposed to inoculum for 320 min. Stomata never closed fully as they bulged at the ends, which created basins where bacteria were observed (Fig. 5). Bacteria were detected in stomata of plants exposed to inoculum for only 160 min on one occasion in one replicate (Fig. 6) and never were observed in samples that had been exposed to inoculum for less than 160 min.

Bacteria also were observed in substomatal cavities of corn leaves which were exposed to the inoculum for 320 min (Fig. 7). Bacteria were not observed in any area of leaf cross sections other than in substomatal cavities.

Stomatal manipulation. The use of a fast-drying (15–30 sec) cement for the taking of leaf impressions had the advantage of replicating the stomatal aperture before there was a change in size. Plants exposed to potassium hydroxide had a mean stomatal aperture of 4.2 μm ($n = 400$) with a range between 2.0 to 6.8 μm. Similar plants with a 1-hr treatment of abscisic acid exhibited a significant decrease in stomatal aperture with a mean of 0.4 μm ($P = 0.01$). However, the range was 0.0 to 1.6 μm, with at least 40% of the stomata open to some degree. All measurements were made across the center of the stoma. Stomata remained closed for the duration of the experiment (4 days). Although treatment with abscisic acid reduced stomatal openings, plants which were inoculated when stomata were closed sustained nearly as much leaf damage as did plants which were inoculated with stomata open. Plants treated with abscisic acid prior to inoculation had 20.7% damaged leaf area compared to 35.1% for plants with open stomata, a difference that was not statistically significant ($P = 0.05$).

DISCUSSION

Corn stomata are extremely sensitive to carbon dioxide. Open stomata were difficult to find unless the plants were grown in an atmosphere with low levels of carbon dioxide. However, the closure of stomata facilitated the detection of bacteria in the stoma. Although the basins formed by closed stomata provided a site for bacteria to collect, the stoma appeared to be open below the basin in some instances. Such an opening could create an avenue of entry for bacteria even when stomata were "closed." In any case, fully opened stomata were not necessary for ingress to occur. Inhibition of stomatal opening prior to inoculation did not reduce substantially the degree of disease severity. Daub and Hagedorn (4) reported that stomatal structure and distribution did not affect the resistance of *Phaseolus* line WRB 133 to *P. syringae*. In addition, they thought that stomatal apertures on bean leaves were so large that small partial openings would have been sufficient for penetration. Our work with corn verifies that stomata need not be fully opened for bacterial ingress to occur. In fact, the structure of grass stomata allowed for apparent stomatal opening at the ends even when the stomata were closed tightly across the center of the aperture. Therefore, control of bacterial ingress into corn by stomatal manipulations would not be a practical disease control procedure.

Panopoulos and Schroth (11) calculated that ingress of *P. phaseolicola* into bean leaves reached equilibrium in 1 hr. We observed bacteria in corn leaves only after a 160–320 min exposure. Ingress of *P. alboprecipitans* into corn leaves may be slower than ingress of *P. phaseolicola* into bean leaves. Alternatively, the time required for ingress may vary depending on the condition of the plant. Clayton (2) and Johnson (9) documented the importance of water congestion for infection of tobacco by *P. tabaci*. Diachun et al (5) demonstrated that bacteria, india ink, toxic chemicals, and tobacco mosaic virus entered the intercellular spaces of water-congested tissues. The retraction of stomatal droplets into leaves also has been implicated as a means of bacterial ingress (1). The role of water congestion for ingress of *P. alboprecipitans* has not been established. However, induced congestion of corn leaves with a dye resulted in stained areas similar in size and shape to the striped lesions caused by the bacterium (6). Because water congestion and other plant conditions were not monitored, an interaction between the time required for ingress and plant-water relations was not established. However, under our experimental conditions, a 160–320 min exposure was necessary before *P. alboprecipitans* were detected in stomata and intercellular spaces.

Although we used a high level of inoculum (10^9 cells per milliliter) in this study, stomatal ingress by *P. alboprecipitans* is a possibility under natural conditions. Corn leaves are capable of developing typical lesions even when exposed to *P. alboprecipitans* at inoculum levels as low as 10^2 bacterial cells per milliliter (6). Although high inoculum doses could alter disease reactions, it is unlikely that it would affect the mode of penetration. High inoculum levels were used in this study to increase the probability of observing ingress by the bacterium.

Because bacteria were observed only in substomatal cavities of leaf cross sections from plants exposed to inoculum for 320 min, it was considered highly unlikely that bacteria were relocated randomly to the substomatal cavities during the sampling and processing of the specimens. Our observations by scanning electron microscopy substantiated stomatal ingress for this disease.

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