# Scanning Electron Microscopy of Virulent and Avirulent Strains of *Xanthomonas campestris* pv. *oryzae* on Rice Leaves

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## ABSTRACT

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Multiplication of strains of *Xanthomonas campestris* pv. *oryzae* on rice (*Oryza sativa*) cultivars and penetration of the bacteria through water pores of leaf blades were investigated with scanning electron microscopy. No marked difference in numbers of cells was noted on the leaf surface 1 hr after spray-inoculation on all cultivar-strain combinations. Twenty-four hours after inoculation, bacteria of strain PX061, which is virulent to cultivars TN1 and CAS 209, multiplied immediately outside the water pores and some bacteria had gained entrance through these pores. Cells of PX0101, a strain that has lost its virulence, did not multiply significantly on the leaf surface and appeared to be embedded in a thin layer of exudate secreted by the water pores. The exudate eventually sealed the opening of these pores. Bacterial cells of PX086, which is virulent to TN1 but avirulent to CAS 209, multiplied on the water pores of TN1 but were trapped in the exudate of CAS 209 48 hr after inoculation. Bacterial cells of all three strains were not observed to multiply on stomata of either cultivar. These results suggest that bacteria are immobilized and inhibited from dividing by excretions from water pores in incompatible host-bacteria combinations. Site specificity of bacterial multiplication on leaf surface was also observed.

*Additional key words*: epiphytes, resistance.

Phytopathogenic bacteria lacking active mechanisms for penetrating the protective barriers of a host can enter the host passively through wounds and other natural openings such as stomata and hydathodes (3). Although hydathodes appear to be easy portals of entry for bacteria, very few pathogenic bacteria are reported to enter through this pathway (1). *Xanthomonas campestris* pv. *campestris*, the black rot pathogen of crucifers, was the first bacterial plant pathogen reported to penetrate host plants through hydathodes (2).

Bacterial blight caused in rice by *X. campestris* pv. *oryzae* is primarily a vascular disease. Tabei (10,11) made extensive anatomical studies of rice plants infected by the bacterium. Generally, the causal bacterium enters the leaf tissues through hydathodes, multiplies in the epithem, and then invades the vessels through the "vascular pass," the tissue area where "the vessels connect with the epithem through two or three openings" (10). All of his investigations, however, were made with light microscopy of susceptible rice cultivars. Tabei (10) did not describe the behavior of the bacteria before entering the water pores.

The objective of the present research was to investigate the establishment of virulent and avirulent strains of *X. campestris* pv. *oryzae* on the leaf surface of resistant and susceptible rice cultivars. Special emphasis was placed on scanning electron microscopy of the interaction between the bacteria and the hydathodes of the leaf blades.

## MATERIALS AND METHODS

**Plant materials.** Plants of rice cultivars Taichung Native 1 (TN1, a susceptible cultivar) and CAS 209 (a cultivar with resistance conditioned by a single dominant gene [*Xa*-10] for bacterial blight resistance, and having differential resistance to strains of *X. campestris* pv. *oryzae* [5,12]) were used in the experiments. Seeds were treated with 0.5% sodium hypochlorite solution for 10 min, rinsed with tap water, and imbibed overnight. They were germinated in vermiculite in a greenhouse. Uniform and healthy seedlings were then transplanted to 15-cm-diameter pots containing steam-sterilized soil supplemented with complete fertilizer.

**Bacterial cultures and inoculation procedure.** Three strains of *X. campestris* pv. *oryzae* were used in the present study. PX061 is virulent to both cultivars, PX086 is virulent to TN1 but avirulent to CAS 209, and PX0101, a strain which has lost its virulence to both CAS 209 and TN1, was isolated from rice leaves at IRRI Farm. The bacteria were cultured on potato-sucrose semisynthetic agar slants for 2 days, washed twice with sterile distilled water, and centrifuged at 5,000 *g* for 10 min prior to inoculation. The bacterial inoculum was dispensed in sterile distilled water and then adjusted to $10^8$ colony-forming units (cfu) per milliliter.

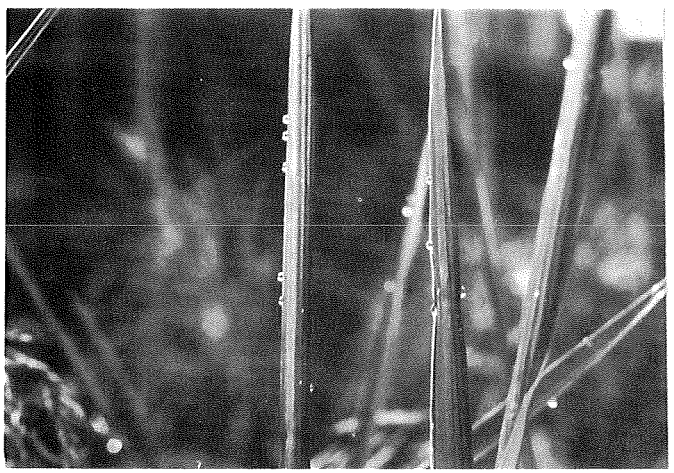

**Fig. 1.** Guttation water droplets on the edges of rice plant leaves.

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Prior to inoculation, 55-day-old rice plants were incubated overnight in a moisture chamber to induce guttation on the leaf blades. Bacterial suspension was then sprayed gently and uniformly onto the plants with an atomizer until fine droplets covered the leaf blades. The plants were incubated in the moisture chamber for 1 hr, then were kept in the greenhouse for sampling and lesion development.

**Tissue sampling and preparation for scanning electron microscopy.** Inoculated leaves were sampled 1, 24, 48, and 72 hr after inoculation and prepared for scanning electron microscopy. Leaves were detached at random, brought into the laboratory, and the tissues around the hydathodes were carefully cut out with a razor blade and fixed in 0.025 M potassium phosphate buffer containing 3% glutaraldehyde (pH 7.0) for 24 hr at 4 C and postfixed in 1% osmium tetroxide in the same buffer for 2 hr. The specimens were then washed three times with the phosphate buffer and subjected to serial dehydration with ethanol and freon and critical-point drying with Freon 113. The specimens were coated with platinum and examined with an ETEC Autoscan or a JEOL scanning electron microscope (SEM).

## RESULTS

**Features of hydathodes.** Droplets of water, secreted from hydathodes, were found on leaf blades after overnight incubation in the moisture chamber (Fig. 1). Hydathodes, 1–5 mm in length and 10–15 per leaf blade, were located near the edge of the rice leaf and appeared as "white streaks" to the naked eye (Fig. 2). These hydathodes predominately occurred toward the tip of a leaf and were densely distributed along the edge of a leaf blade, exclusively on the upper leaf surface. Hydathodes were made up of "water

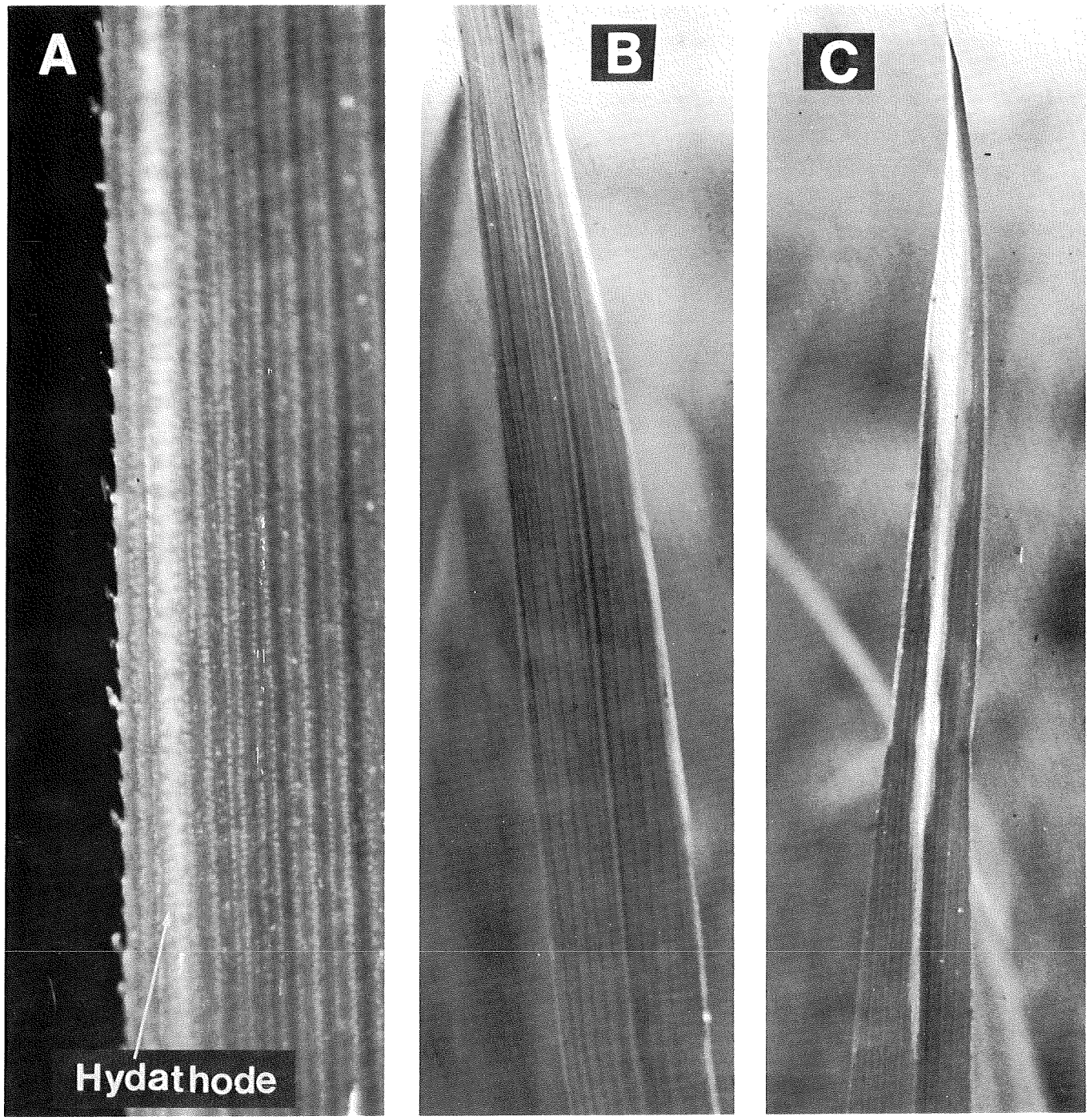


**Fig. 2.** Leaves of CAS 209 rice. **A,** The hydathode is seen as an elongate, lighter colored area near the edge of the blade. **B,** Natural infection by *Xanthomonas campestris* pv. *oryzae* at the edge of the blade. **C,** Expanded lesion.

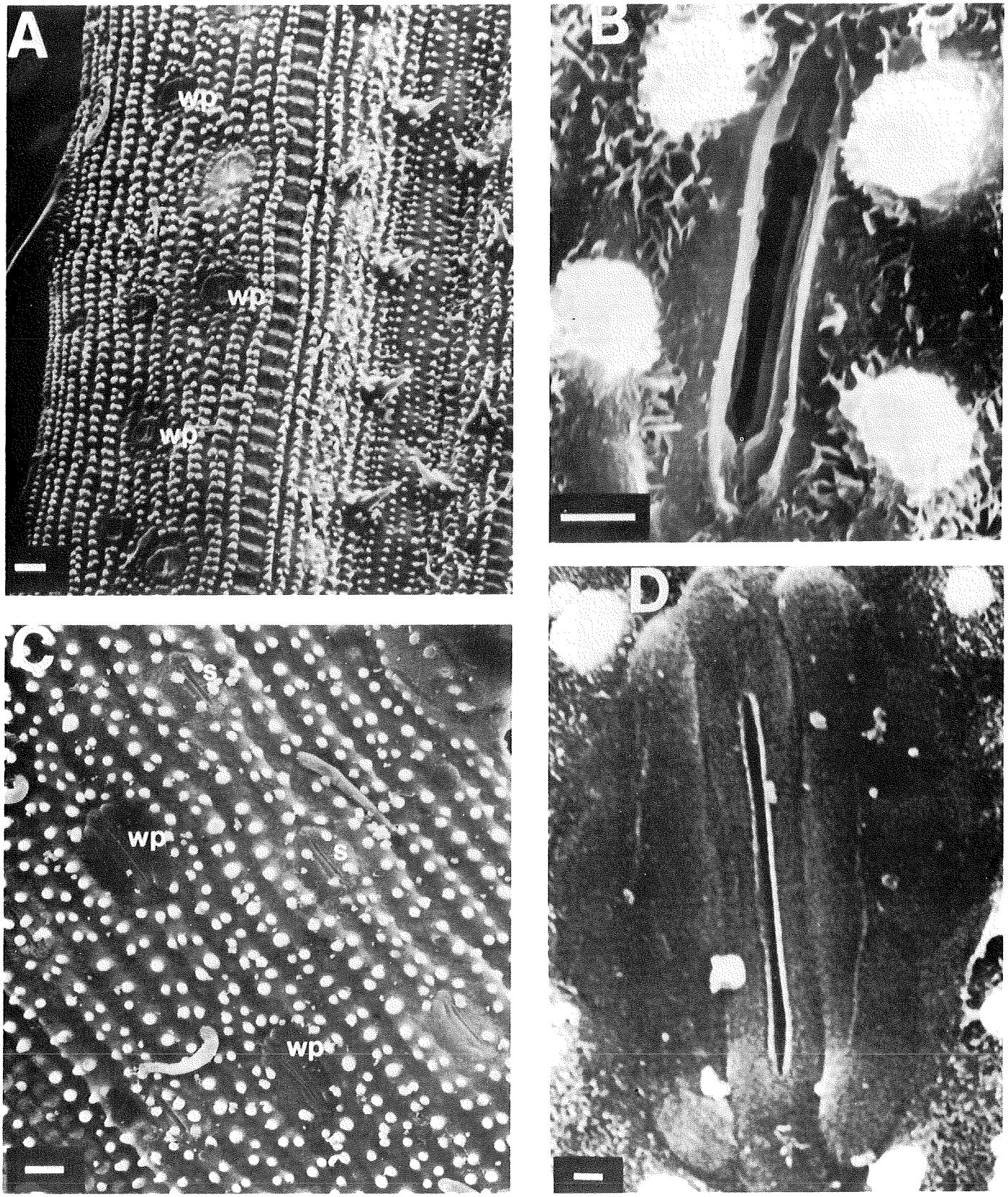


**Fig. 3.** Scanning electron micrographs of rice leaves. **A,** Portion of a hydathode showing several water pores (wp) in the hydathode. **B,** A stoma (note the four papillae and the waxy surface of the guard cells). **C,** A portion of a leaf showing the difference between water pores and stomates. **D,** A water pore. Note the absence of prominant papillae or waxy surface. Bars for A and C = 10 $\mu$m. Bars for B and D = 1 $\mu$m.

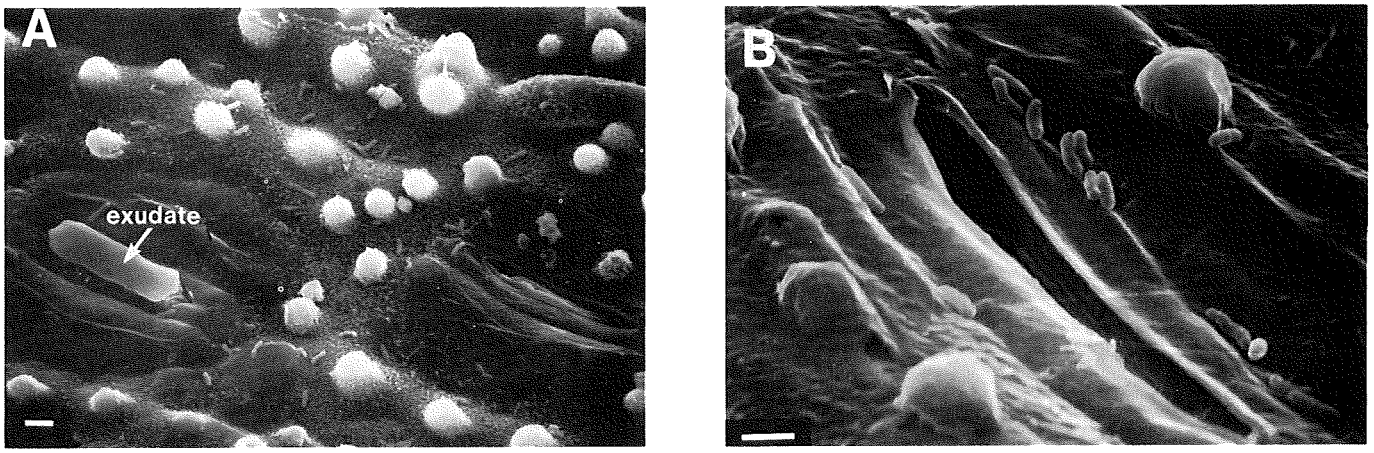


**Fig. 4.** Scanning electron micrographs of CAS 209 rice leaf surfaces 24 hr after inoculation with *Xanthomonas campestris* pv. *oryzae*. **A,** Strain PX0101, which is weakly virulent (note exudate in water pore) and **B,** strain PX086, which is virulent on CAS 209. Bars = 1 μm.

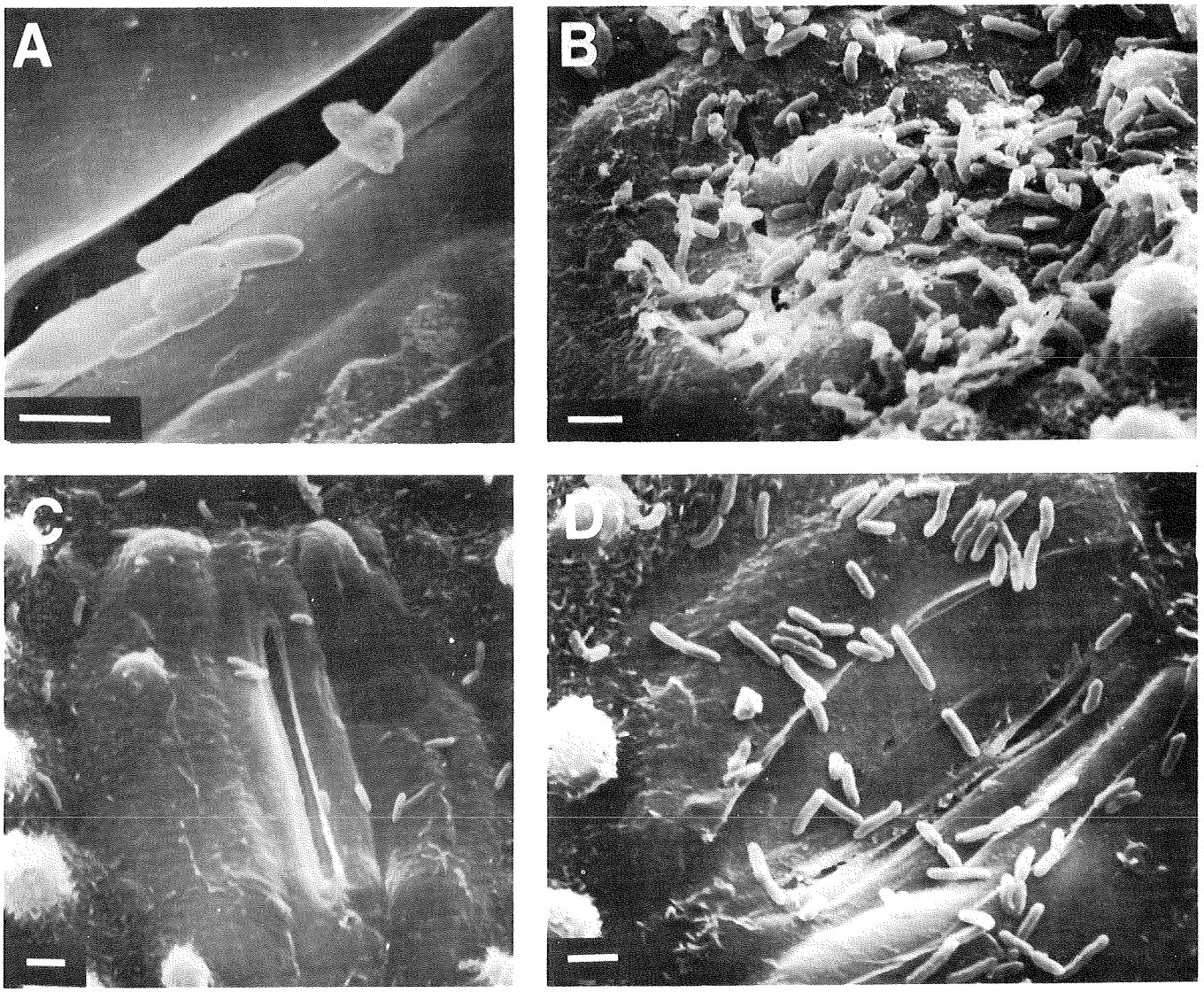


**Fig. 5.** Scanning electron micrographs of water pore areas of leaves of CAS 209 rice inoculated with virulent strain PX061 of *Xanthomonas campestris* pv. *oryzae* for **A,** 1 hr or **B,** 24 hr and avirulent strain PX086 for **C,** 1 hr or **D,** 24 hr. Strain PX061 increased in cell number by 24 hr, and some cells entered the opening of the water pore. The number of cells of strain PX086 did not increase substantially between 1 and 24 hr. Bars = 1 μm.

pores" (Fig. 3). The number of water pores per hydathode varied from 10 to 20 on TN1 and CAS 209. No differences in diameters of water pore openings between the two rice cultivars were observed.

Each water pore resembled a stoma in having a pair of guard cells, but was not covered with wax and had no papillae. Four papillae of a stoma, one at each end of the two guard cells, were noted. The guard cells of a stoma were covered with wax. The size of a water pore was about two to four times that of a stoma (Fig. 3). In contrast to stomata, water pores remain open without regard to the photoperiod.

**Scanning electron microscopy of inoculated rice leaves.** About 50–100 water pores from 10 pieces of leaf specimens per treatment were examined. At 1 hr after inoculation, bacteria were found on the leaf surfaces of many specimens. The bacterial cells were not evenly distributed on the leaf surface; some were found on the water pores, others around the trichomes. No marked differences in bacterial numbers were noted between TN1 and CAS 209 inoculated with PX061, PX086, or PX0101 1 hr after inoculation. The bacterial cells were all rod-shaped and single-celled. There were signs of bacterial division on all host-parasite combinations.

Because CAS 209 is resistant to PX086 and PX0101 and susceptible to PX061, leaves inoculated with the three strains were examined in detail 24 and 48 hr after inoculation. Many water pores of both TN1 and CAS 209 were found to have bacteria on the leaf surface. No bacteria, however, were found on stomata in the present study. Among all the CAS 209 leaves inoculated with PX0101, exudate from the water pores completely sealed all openings (Fig. 4A). Increase in the number of bacterial cells at 2 and 24 hr was nominal compared to that 1 hr after inoculation. Bacterial cells remained single and rod-shaped. When CAS 209 was

TABLE 1. The number of cells of *Xanthomonas campestris* pv. *oryzae* strains PX061, PX086 and PX0101 on the water pores of CAS 209, a rice cultivar resistant to PX086 and PX0101, but susceptible to PX061

| | Bacterial cells per water pore at postinoculation time:[a] | | | |
|---|---|---|---|---|
| Cultivar-strain | 1 hr | 24 hr | 48 hr | 72 hr |
| CAS 209-PX061 | 5–20 | 100–200 | 100–200 | 100–200 |
| CAS 209-PX086 | 5–20 | 10–50 | 50–80[b] | ...[c] |
| CAS 209-PX0101 | 5–10 | 5–20[b] | 10–60[b] | 10–80[b] |

[a] Mean of 50–100 pores observed to have bacterial cells.
[b] Bacterial cells were observed to be embedded in the exudates on water pores or at pore openings.
[c] No observation.

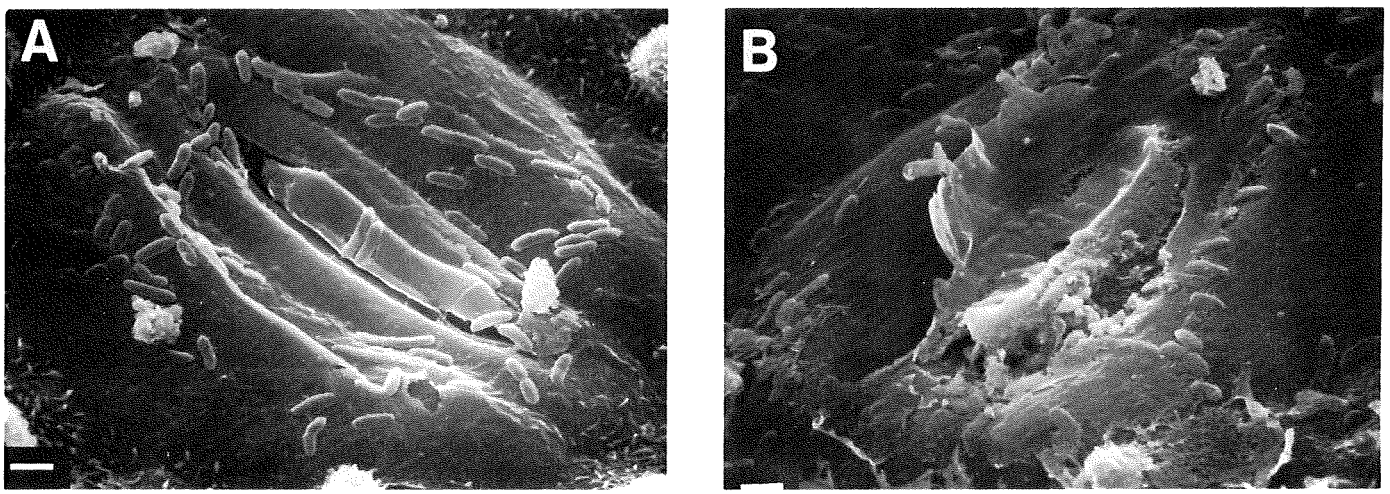


**Fig. 6.** Scanning electron micrographs of leaf surface of cultivar CAS 209 rice 24 hr after inoculation with weakly virulent strain PX0101 of *Xanthomonas campestris* pv. *oryzae*, showing **A,** exudate plugging the water pore opening and **B,** bacterial cells embedded in the exudate. Bars = 1 μm.

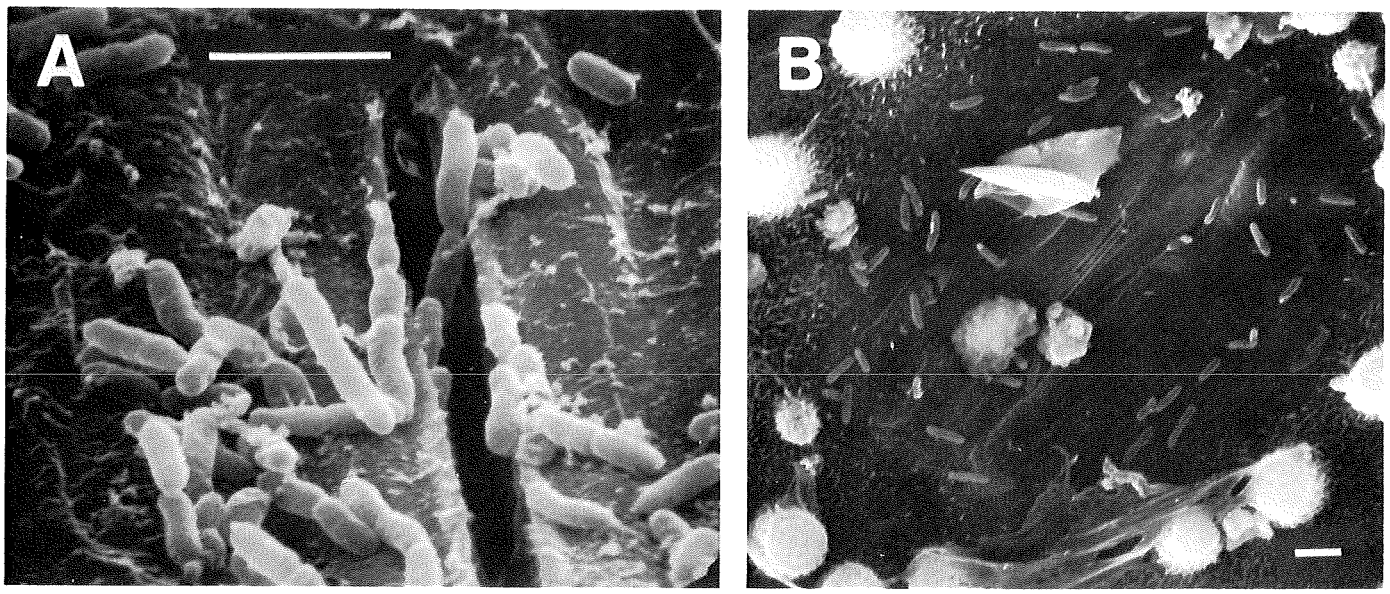


**Fig. 7.** Scanning electron micrographs of leaf surface of cultivar CAS 209 rice 48 hr after inoculation with *Xanthomonas campestris* pv. *oryzae*. **A,** Virulent strain PX061 (note bacterial cells entering the stomatal opening and the absence of exudate) and **B,** avirulent strain PX086. Bars = 1 μm.

inoculated with PX086, there was no evidence of exudation from the water pores, consequently the water pores remained accessible to bacterial penetration (Fig. 4B). When CAS 209 was spray-inoculated with PX061, the number of bacterial cells initially was low, but increased significantly 24 hr after inoculation (Table 1, Fig. 1). Many PX061 cells were dividing and massed around or entering the water pore openings (Fig. 5B). On the other hand, the number of bacterial cells of strain PX086, which is avirulent to CAS 209, did not increase substantially from 1 to 24 hr after inoculation.

On CAS 209, more PX0101 cells were embedded in substances secreted from the water pores at 48 hr than at 24 hr after inoculation. The openings of water pores were totally covered by this exudate (Fig. 6). The water pores of CAS 209 inoculated with PX086 cells were similarly sealed off with the exudate (Fig. 7). PX0101 and PX086 cells were embedded, and their numbers were considerably lower than the numbers of PX061 cells (Figs. 6 and 7A). PX061 cells increased on CAS 209 and were densely distributed on the water pores. Many bacterial cells were in the openings of the water pores (Fig. 7A).

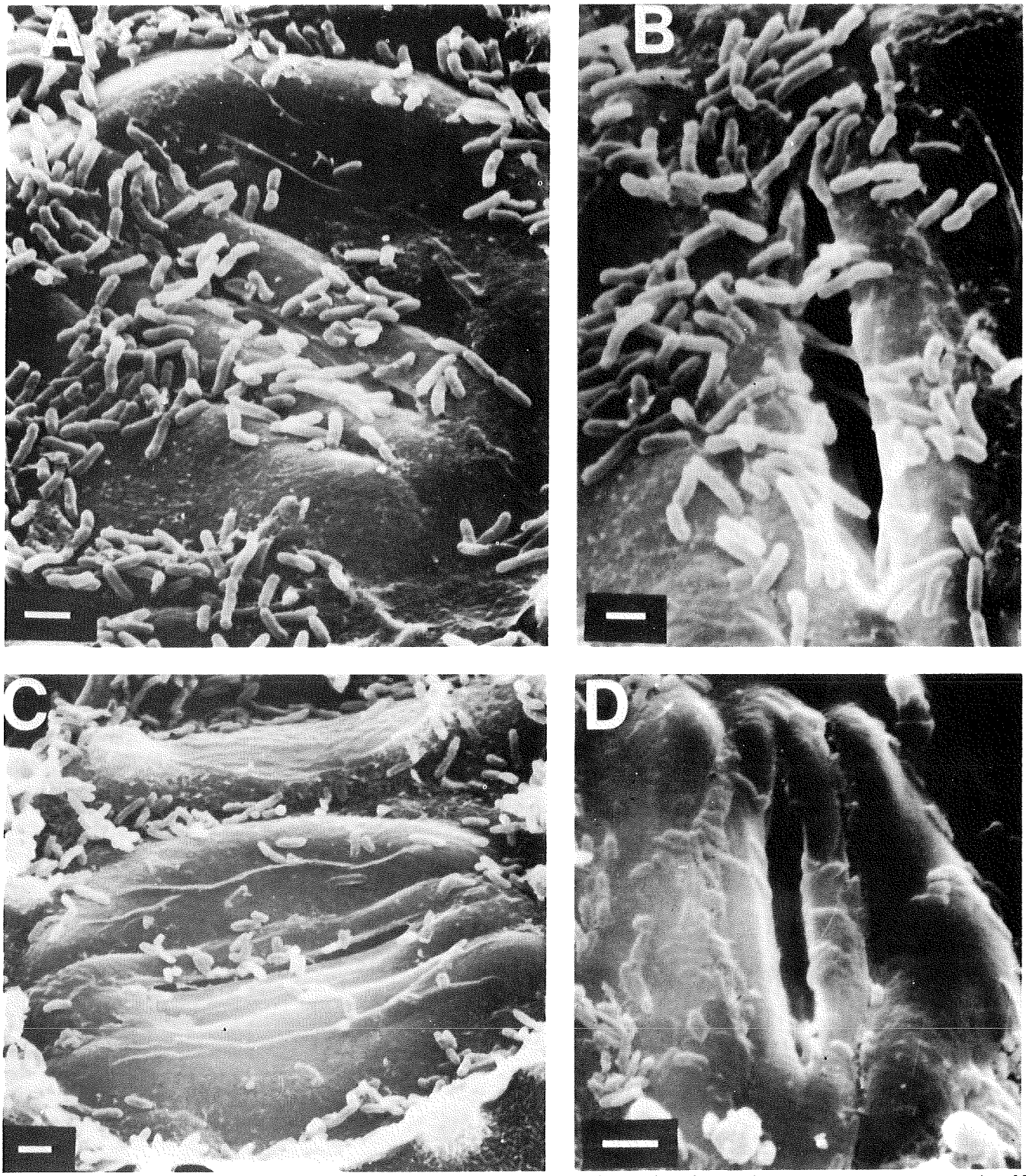


**Fig. 8.** Scanning electron micrographs of leaf surface of cultivar CAS 209 rice 72 hr after inoculation with *Xanthomonas campestris* pv. *oryzae*. **A and B,** Virulent strain PX061 was fully established at the water pores. **C,** Avirulent strain PX086 and **D,** weakly virulent strain PX0101 showed embedded cells and lesser cell numbers. Bars = 1 $\mu$m.

Bacteria of PX0101 remained embedded 72 hr after inoculation (Fig. 8D). The water pores reopened at this time. The PX061 strain on CAS 209 remained high in number (Fig. 8A and B), while some of the bacterial cells of PX086 appeared to be lysed and empty (Fig. 8C).

## DISCUSSION

The hydathode secretes excess water from leaf interiors. In the tropics, guttation water droplets are often observed in the morning. The guttation water secreted by the hydathodes may also be drawn back into the leaf (2). Our results indicated that hydathodes of rice plants are portals of entry for the bacterial blight pathogen and are involved in the specificity of the host-parasite interaction.

A hydathode is composed of a series of water pores. Tabei (10,11) indicated that the bacterial cells of *X. campestris* pv. *oryzae* enter rice leaf blades through hydathodes and multiply in the epithem into which the xylem vessels open. Upon bacterial multiplication, sufficient number of cells enter the vascular system to cause infection. Our results indicated that in a compatible rice cultivar-bacterial strain combination, bacterial multiplication occurred on the water pores. In an incompatible host-parasite system, bacterial multiplication was considerably reduced. The presence of avirulent or weakly virulent bacteria at the water pore triggered the production of exudate by the host that sealed off the pore and perhaps immobilized the bacterial cells. The water pore appeared to reopen after the bacteria had lost mobility ( Fig. 8).

Transmission electron microscopy by Horino (6,7) revealed that bacterial cells were irregular in shape and enveloped by abundant fibrillar material on leaves of resistant rice cultivars infected with *X. campestris* pv. *oryzae*. A similar phenomenon was demonstrated with other host-bacteria systems (8,9). Our results indicate that immobilization of the bacteria, shown by embedment of bacterial cells in exudates, occurs at the infection court on the leaf surfaces. There was no difference in growth rate on potato-sucrose semisynthetic medium of the three strains used in the present study. Differences in bacterial cell numbers at 24 and 48 hr as compared to 1 hr after inoculation became apparent when the bacteria came in contact with water pores of compatible and incompatible rice cultivars. Mew and Kennedy (4) indicated the cell number of *Pseudomonas glycinea* on resistant and susceptible soybean cultivars varied significantly. More bacterial cells were detected on a susceptible cultivar than on a resistant cultivar. The present study is the first to demonstrate that in incompatible host-parasite combinations, bacterial multiplication may be inhibited and bacterial cells may be immobilized at the water pore.

Furthermore, in the present study, no bacteria colonized stomata when bacterial suspension was sprayed onto rice leaves. Tabei (11) introduced the bacterial cells of *X. campestris* pv. *oryzae* to the stomata and found no external symptoms. Anatomical study showed that the bacterial cells were apparently localized in the aperture of the stomata. It appears, therefore, that there is site specificity for penetration through natural openings.

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