Cytology and Histology

Role of Bacterial Immobilization in Race-Specific Resistance of Soybean to *Pseudomonas syringae* pv. *glycinea*

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ABSTRACT

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Electron micrographs of leaf intercellular spaces of the soybean (*Glycine max*) differential cultivars Flambeau and Harosoy spray inoculated with *Pseudomonas syringae* pv. *glycinea* showed bacterial cells of both homologous and heterologous strains occasionally enveloped by electron-dense material at 4 and 24 hr after inoculation. However, there was no evidence that this was an active or specific host response. Structures similar to those enveloping bacteria were found cross-bridging adjacent leaf mesophyll cells at cell junctions in water-infiltrated and, to a lesser extent, uninfiltrated leaf tissue. Often, a distinct layer of fibrillar material was found in contact with plant cell walls and appeared contiguous with structures enveloping bacteria or with cross-bridges in uninoculated leaf tissue. Bacterial envelopment was not seen in several heterologous *P. syringae* pv. *glycinea*-soybean combinations, even though a typical hypersensitive response was induced, indicating that bacterial attachment per se is not a prerequisite for induction of the hypersensitive response in soybean. From experiments with rifampicin, induction time for the hypersensitive response was determined to be between 2 and 4 hr.

Additional key words: bacterial blight.

Several investigators have concluded, on the basis of ultrastructural studies, that immobilization or attachment of heterologous pathogenic bacteria and saprophytic bacteria by fibrillar and granular material at the plant cell wall is an active and specific defense mechanism and/or facilitates movement of a factor(s) that induces a hypersensitive response or of other bacterial products to their sites of action (1,7,9,10,15,17,19,22). Plant hosts that were used in these studies include apples (10), beans (17,22), cotton (1), rice (9), and tobacco (7,15,19).

Investigations of several homologous or heterologous pathogenic bacteria and saprophytic bacteria on bean (*Phaseolus vulgaris* L.) have yielded contradictory findings. Sigee and Epton (20,21) initially reported the occurrence of a "surface-membrane" over bacterial cells of both heterologous and homologous strains of *Pseudomonas syringae* pv. *phaseolicola* in the intercellular space of

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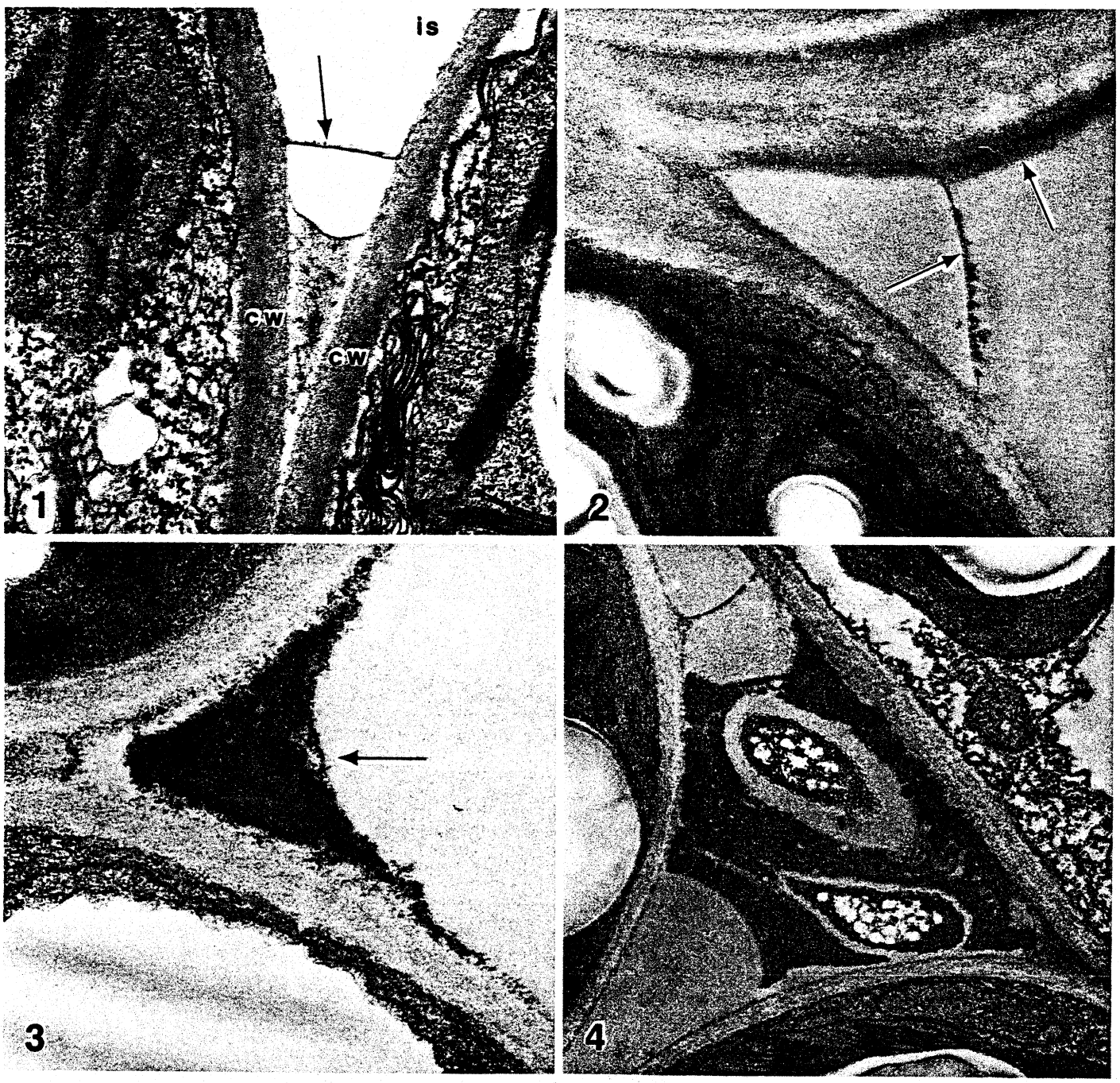
bean. Subsequently, Sing and Schroth (22) and Roebuck et al (17) reported attachment of a saprophytic bacterium and of a heterologous strain of *P. syringae* pv. *phaseolicola*, respectively, to bean cell walls. However, Daub and Hagedorn (3) found no evidence of bacterial attachment as a mechanism of resistance against *P. syringae* pv. *syringae* in bean leaf and pod tissues.

Recently, Hildebrand et al (8) reported that homologous and heterologous pathogenic bacteria and saprophytic bacteria were associated with electron-dense material in the intercellular spaces of bean leaf tissue within 3 hr after bacterial infiltration. Similar fibrillar material was also found in uninfiltrated and water-infiltrated tissues.

The study reported here was undertaken to determine if active immobilization of heterologous strains of *P. syringae* pv. *glycinea* by resistant soybean (*Glycine max* (L.) Merrill) cultivars is a race-specific mechanism of defense.

MATERIALS AND METHODS

The strains of *P. syringae* pv. *glycinea* used were NCPPB 1134, NCPPB 1137, NCPPB 2159 (from the National Collection of Plant Pathogenic Bacteria, Harpenden, England), K1 (obtained from B. W. Kennedy, University of Minnesota), and J3-17-2 (obtained from a Wisconsin soybean field). Strains K1 and NCPPB 2159 belong to Race 1, strain J3-17-2 to Race 5, and NCPPB strains 1134 and 1137 to Race 9 (2,6). Members of Race 1 are pathogenic on the differential soybean cultivar Flambeau, but cause a hypersensitive response (HR) on the cultivar Harosoy (2). Members of Race 5 cause an HR on Flambeau and a susceptible response on Harosoy (2). Members of Race 9 cause an HR on both soybean cultivars (6). Seeds of the differential soybean cultivars Flambeau and Harosoy (2) were obtained from R. L. Bernard, United States Regional Soybean Laboratory, Urbana, IL. Seed was sown in vermiculite



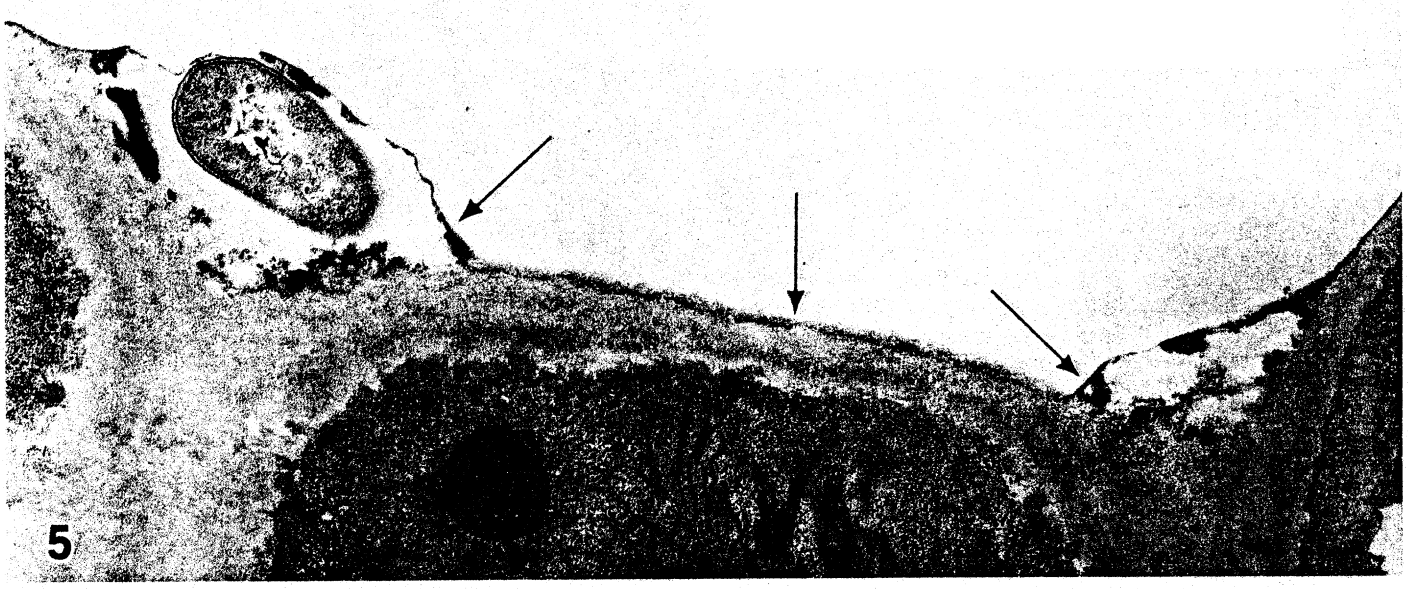
Figs. 1–4. Soybean leaf mesophyll intercellular spaces (is) outlined by host cell wall (cw), uninfiltrated (Fig. 1), infiltrated with water (Figs. 2 and 3), or water plus *Pseudomonas syringae* pv. *glycinea* (Fig. 4). **1 and 2,** Cross-bridging material at cell junctions in cultivar Harosoy at 4 hr (×24,000 and ×43,000, respectively.) Note distinct layer of fibrillar material covering the host cell wall in Fig. 2. **3,** Cell junction in Harosoy completely filled with dense material at 4 hr (×32,000). **4,** Cells of homologous strain K1 enveloped by electron-dense material in an intercellular space of cultivar Flambeau at 4 hr (×24,000).

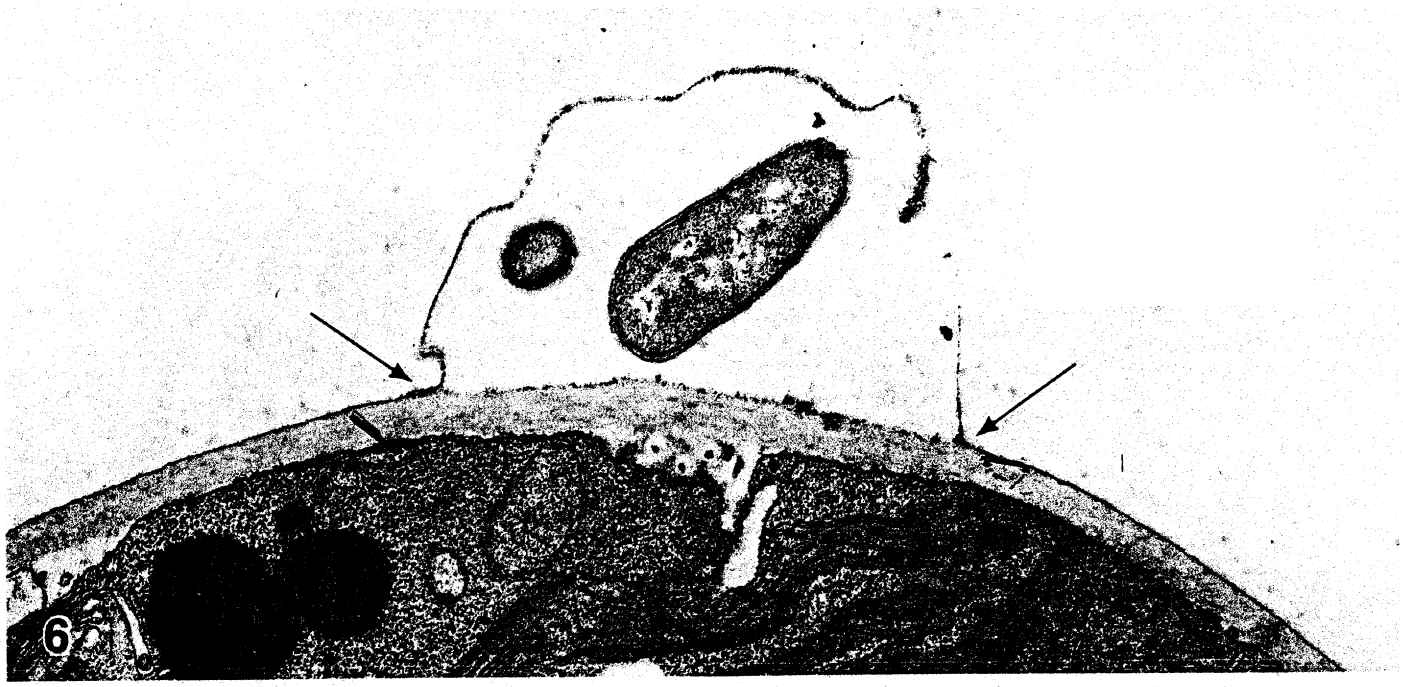
contained in clay pots and then placed in a growth chamber maintained at 23 C day, 19 C night, 75% relative humidity, with fluorescent and incandescent bulbs providing 1.1×10^4 lux during a 13-hr photoperiod.

To prepare inocula, bacterial cells grown on King's medium B agar (12) for 24–48 hr at approximately 24 C were suspended in sterile water and adjusted turbidometrically to a final concentration of approximately 10^9 colony-forming units per milliliter. Inocula were sprayed on the abaxial side of fully opened, partially expanded unifoliolate or trifoliolate leaves with a Paasche airbrush (Paasche Airbrush Company, Chicago, IL 60614) until they were water-soaked (11). Inoculated plants were left uncovered on a laboratory bench for 4 hr, then were either returned to the growth chamber or processed for electron microscopy as described below. All evidence of water-soaking was gone within 60–90 min.

At 4 or 24 hr after bacterial inoculation, leaf tissue was excised and vacuum infiltrated with 5% glutaraldehyde in 0.08 M cacodylate buffer, pH 7.4. Tissue was fixed for 2 hr at 4 C and rinsed in cacodylate buffer containing 1.5% sucrose. Postfixation was in Palade's fixative (4,14). Fixed tissue segments were dehydrated through a graded ethanol series followed by propylene oxide, and embedded in Epon epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and were observed in a Zeiss EM 10 B electron microscope operated at 60 kV. For controls, uninfiltrated or water-infiltrated leaf tissue was also processed and examined by electron microscopy.

The induction period for the HR (13,18) was determined by applying rifampicin (Boehringer, Mannheim, West Germany) (25 μg/ml H_2O), a specific inhibitor of procaryotic RNA synthesis, to inoculated leaves at 2, 4, 6, 8, and 10 hr after inoculation. Unifoliolate leaves of cultivar Flambeau were inoculated with heterologous *P. syringae* pv. *glycinea* strains NCPPB 1134 and J3-17-2, and leaves of cultivar Harosoy were inoculated with strain NCPPB 2159 as stated above except that bacterial inocula





Figs. 5 and 6. *Pseudomonas syringae* pv. *glycinea* cells enveloped by electron-dense materials in intercellular spaces of soybean cultivar Flambeau 24 hr after inoculation. **5,** Cell of heterologous strain NCPPB 1137 at host cell junction and distinct layer of fibrillar material lying on the host cell wall (×24,000). **6,** Cell of heterologous strain NCPPB 1134 not at a host cell junction (×26,000).

contained approximately 10^8 colony-forming units/ml. Inoculated plants were kept in high humidity under plastic bags. Inoculated areas were reinfiltrated with a rifampicin spray, and the plants were returned to the growth chamber after the last treatment with rifampicin. Development of the HR was followed by visual inspection.

RESULTS

Rifampicin completely prevented the HR (characterized by browning of leaf lamina sometimes accompanied by reddening of the veins [2]) only when applied 2 hr after inoculation of the soybean cultivars Flambeau and Harosoy with heterologous *P. syringae* pv. *glycinea* strains. Infiltration of rifampicin at 4 and 6 hr greatly reduced the intensity of the HR, and infiltration at 8 and 10 hr had no inhibitory effect. Thus, an HR induction time of 2–4 hr was consistent with findings for other heterologous bacteria-plant host interactions (13,17).

All *P. syringae* pv. *glycinea* strains gave the expected reactions on cultivars Flambeau and Harosoy. For incompatible combinations, the HR was visible within 24 hr after inoculation. For compatible interactions, the water-soaking symptom was evident within 3 days.

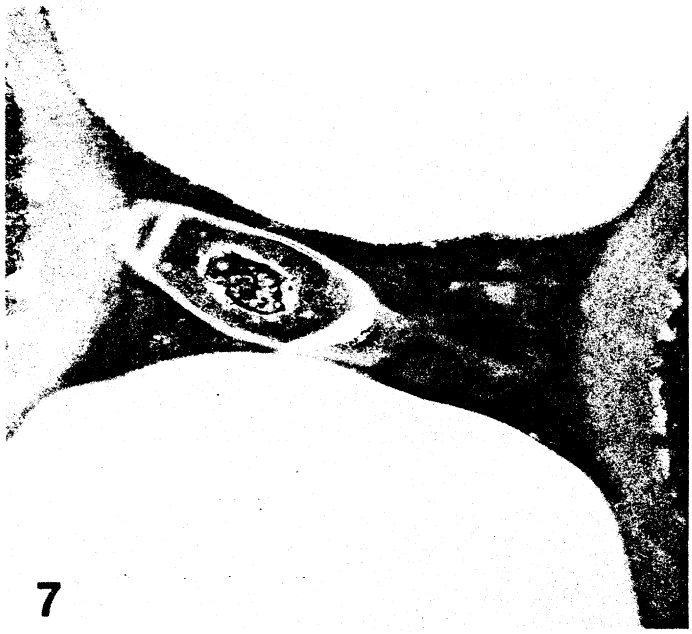
Electron microscopic observation of non-water-infiltrated leaf tissue of cultivars Flambeau and Harosoy showed occasional thin films, possibly intercellular cuticle (16) that had pulled away during processing of the tissue, bridging some mesophyll cells at cell junctions (Fig. 1). Water-infiltrated leaf tissue of both cultivars also contained similar cross-bridging material present at both 4 hr (Fig. 2) and 24 hr (*unpublished figure*). Water-infiltrated tissue occasionally had cell junctions filled with electron-dense material (Fig. 3), similar to that seen enveloping some bacterial cells (Fig. 7).

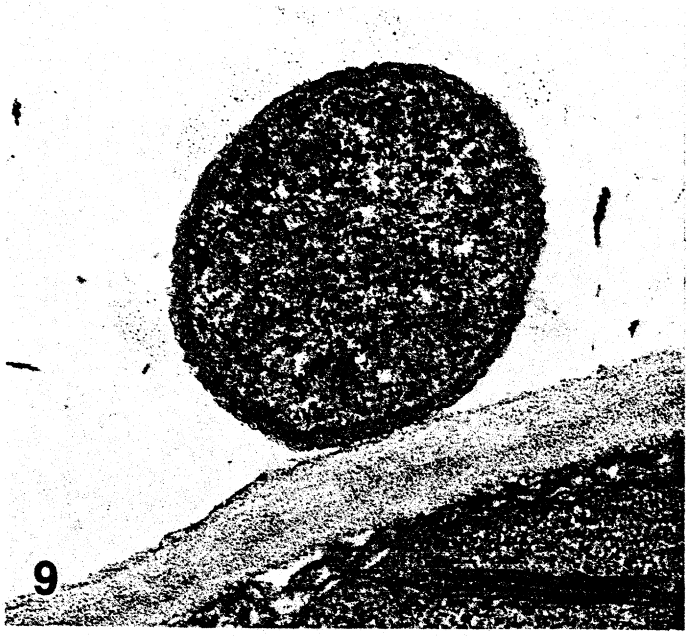
At 4 hr after inoculation, only the interaction between *P. syringae* pv. *glycinea* strain K1 and plants of cultivars Flambeau (susceptible) and Harosoy (resistant) was studied. Few bacteria were found in the intercellular spaces in either combination. Bacterial cells present were either free in the intercellular spaces (not shown) or enveloped by electron-dense material (Fig. 4). The bacterial cells of homologous strain K1 in Fig. 4 appear to be dead, but the great majority of K1 cells in both host cultivars appeared to be viable. No apparent disruption of the host plasmalemma, accumulation of vesicles, or host cell wall dissolution occurred near the enveloped bacteria.

At 24 hr after inoculation, 44 and 48% of the cells of heterologous *P. syringae* pv. *glycinea* strains NCPPB 1134 and 1137, respectively, and no cells of heterologous strain J3-17-2, were enveloped by electron-dense material in the intercellular spaces of Flambeau. Enveloped bacteria were usually near cell junctions (Fig. 5), but occasionally were observed at other locations along mesophyll cell walls (Fig. 6). In one electron micrograph, there appeared to be a slight host cell wall thickening or cell wall apposition (15) opposite enveloped bacteria (Fig. 6).

Electron-dense material, where seen in the intercellular spaces, often appeared contiguous with a similar-appearing layer of loose fibrillar material (Figs. 2, 5, 8) or a thin, more compact layer (Fig. 6) on an adjacent host cell wall.

Most cells of the homologous *P. syringae* pv. *glycinea* strains K1 and NCPPB 2159 (78 and 100%, respectively) were free in the intercellular spaces of Flambeau at 24 hr, but occasional cells of strain K1 were enveloped by electron-dense material (Fig. 7). Again, after 24 hr incubation, there was no evidence of disruption of host plasmalemma, migration of vesicles, or dissolution of plant cell walls at locations adjacent to enveloped bacteria. Almost all the enveloped bacteria observed after 4 or 24 hr incubation appeared viable with no cell wall distortion or condensation of cytoplasmic





Figs. 7–9. Leaf intercellular spaces of soybean cultivars Flambeau (Fig. 7) and Harasoy (Figs. 8 and 9) 24 hr after infiltration with *Pseudomonas syringae* pv. *glycinea*. **7,** Cell of homologous strain K1 surrounded by dense material (×34,000). **8,** Unenveloped cell of heterologous strain NCPPB 2159 (×21,000). **9,** Cell of heterologous strain K1 in close contact with leaf mesophyll cell wall (×64,000).

contents.

No enveloped bacteria of homologous *P. syringae* pv. *glycinea* strain J3-17-2 or heterologous strains NCPPB 2159 and K1 were seen in the intercellular spaces of Harosoy at 24 hr, although cross-bridging material was present at host cell junctions (Fig. 8). However, some bacterial cells were closely aligned with the host cell wall (Fig. 9).

DISCUSSION

In our experiments, at the approximate time of induction of the HR (4 hr) and after 24 hr, we observed instances of bacterial envelopment in both compatible and incompatible interactions of *P. syringae* pv. *glycinea* with soybean cultivars. The enveloping structures appeared similar to those that envelop heterologous and saprophytic bacteria in other hosts (7,9,15,19). This electron-dense material is of uncertain origin. The enveloping material may have been composed of host intercellular cuticle (16) and/or dissolved host cell wall material (8).

We found no evidence that envelopment was an active host response. Disruption of the host plasmalemma and accumulation of vesicles at the site of bacterial envelopment (15) were not observed. Almost all enveloped cells of *P. syringae* pv. *glycinea* appeared viable with no bacterial cell wall distortion or condensation of cytoplasm in contrast to the reaction of heterologous *Xanthomonas campestris* pv. *oryzae* in rice (9).

No attachment of heterologous strain J3-17-2 in Flambeau or strains NCPPB 2159 and K1 in Harosoy to leaf mesophyll cell walls was found, even though these strains elicited an HR in the host cultivars. However, heterologous bacteria were sometimes in close contact with soybean cell walls. This indicates that bacterial attachment per se is not a prerequisite for induction of the HR in soybean and confirms earlier results with another bacterial soybean leaf pathogen, *X. campestris* pv. *glycines* (5), but does not rule out the possibility that bacterial contact with soybean cell walls is necessary for induction of the HR (23).

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