Bacterial Pustule Disease of Soybean: Microscopy of Pustule Development in a Susceptible Cultivar

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ABSTRACT

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Leaves of susceptible soybean cultivar Clark were spray-inoculated with either of two virulent strains of *Xanthomonas campestris* pv. *glycines*, the causative agent of bacterial pustule disease of soybean. After 7 days, leaf tissue bearing pustules was prepared for light microscopy and transmission or scanning electron microscopy. Pustules usually protruded from the abaxial surface of the leaf and were composed of enlarged, closely spaced parenchyma cells. Affected palisade parenchyma cells were longer and larger than control palisade cells. The increased number of cells and the presence of cross walls in some palisade cells indicated that cell division, as well as cell enlargement, was a mechanism of pustule development. Cells at the apices of developing pustules or throughout mature pustules contained scant cytoplasm with few organelles. In pustule cells closer to the midplane of the leaf, active wall synthesis was indicated by the presence of many dictyosomes and Golgi vesicles filled with fibrillar substance. The existing wall structure appeared to be loosened, with microfibrils fraying at the outer wall surfaces. Softening of the cell wall and increased wall synthesis may be involved in the enlargement of cells composing the pustules.

Additional key words: *Glycine max*, host cell organelles, ultrastructure, virulence.

Bacterial pustule disease of soybean is caused by *Xanthomonas campestris* pv. *glycines* (Nakano) Dye. The disease is characterized visually by small pustules surrounded by chlorotic halos on the surfaces of affected leaves. Pustules are formed by hypertrophy (6,13,20) or hypertrophy and hyperplasia (9) of parenchyma cells. Expanding pustular lesions often coalesce and later become necrotic, forming angular brown spots or patches on the leaf surface.

In an ultrastructural study comparing various virulent and avirulent strains of *X. c.* pv. *glycines* in their interactions with leaves of susceptible soybean cultivar Clark (10), we observed that pustule formation was suppressed by the use of heavy inocula, 3×10^9 colony-forming units (cfu) per milliliter, of virulent bacteria. Strong yellowing on the leaf was the primary visual symptom, with a few pustules appearing at the margins of yellowed areas. Virulent bacteria grew to high population densities in the host and remained free in the intercellular spaces of the leaf. Affected parenchyma cells of inoculated leaves displayed dilated cellular endomembranes

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and mild changes in chloroplast ultrastructure. In contrast, avirulent bacteria were restricted in population increase, tended to be immobilized in the intercellular spaces on host cell walls, and caused limited plasmolysis and/or hypersensitive collapse of host cells.

We now report the results of experiments employing a lower inoculum level (3×10^5 cfu per milliliter) of virulent bacteria in order to observe the equivalent of field symptomology, i. e., development of pustules in the mesophyll, and to compare the effects of high and low inocula of virulent bacteria on host cells.

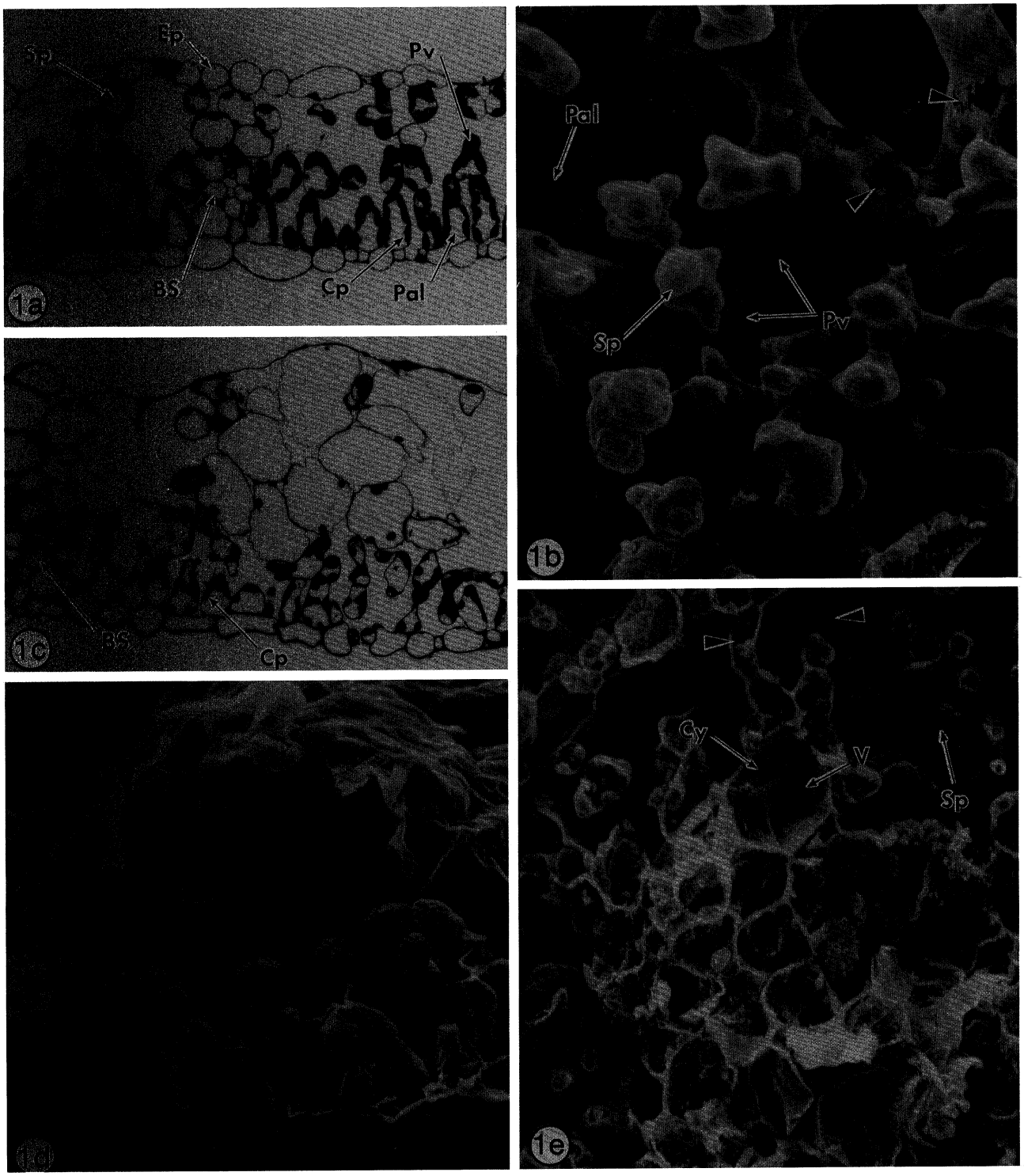

Fig. 1. A, Control leaf of soybean cultivar Clark, 2 days after spray-infiltration with water, was not yet fully expanded (×400). BS = bundle sheath, Ep = epidermis, Cp = chloroplast, Pal = palisade parenchyma, Pv = paraveinal parenchyma, Sp = spongy parenchyma. **B,** SEM of fully expanded, fractured control leaf 7 days after water infiltration. Paraveinal parenchyma (Pv) cells were randomly connected with the abaxial epidermis (not present) through some of the small spongy parenchyma (Sp) cells in the foreground. Arrowheads indicate scars where connecting cells broke away with the epidermis (×500). Pal = palisade parenchyma. **C and D,** Pustules caused by *Xanthomonas campestris* pv. *glycines* strain XP175 examined 7 days after spray-inoculation with bacterial cell suspension containing 3×10^5 cfu per milliliter (×400, ×500). BS = bundle sheath, Cp = chloroplast. **E,** Paradermal fracture through a pustule 7 days after inoculation with *X. c.* pv. *glycines* strain A at 3×10^5 cfu per milliliter revealed confluent cells containing abundant cytoplasm (Cy). Spongy parenchyma (Sp) cells in the background covered the inside of the abaxial epidermis. Thus, the pustule fractured in the paraveinal mesophyll. Connections of paraveinal cell with the bundle sheath are indicated by arrowheads (×450). V = cell vacuole. (In all micrographs, the abaxial surface of leaf is uppermost unless otherwise noted.)

MATERIALS AND METHODS

X. c. pv. *glycines* strain XP175 (= NCPPB 554) was obtained from the collection of M. P. Starr. *X. c.* pv. *glycines* strain A was from our own culture collection. Both strains were virulent on soybean cultivar Clark.

Bacteria were grown on nutrient agar (Difco) for 24 hr at 28 C. Cells were washed from the agar, suspended in sterile water, and washed three times by centrifugation. Bacterial suspensions were adjusted to A_{600nm} = 1.0 and used undiluted, or diluted 1:10,000 with sterile water, to give final concentrations of 3×10^9 or 3×10^5 cfu per milliliter, respectively.

Seeds of cultivar Clark were supplied by Dr. R. L. Bernard, U. S. Regional Soybean Laboratory, Urbana, IL. Seeds were cultivated in Baccto potting soil (Michigan Peat Co., Houston, TX) in clay pots in a growth chamber at 24 C day, 20 C night, 75% relative humidity. Fluorescent and incandescent bulbs provided 1.1×10^4 lx on a 13-hr photoperiod.

Plants were 21 days old at the time of inoculation and had one fully opened, but not yet fully expanded, trifoliate leaf per plant. Inoculum was sprayed with force by means of a chromatographic spray bottle onto the abaxial side of leaflets until water-soaking appeared. Leaves were then rinsed thoroughly under running tap water. After all evidence of water-soaking disappeared (approximately 2 hr), inoculated plants were returned to the growth chamber. During the incubation period, growth chamber temperatures were raised to 28 C day, 24 C night.

For microscopy, leaf tissue (1–2 cm^2) was excised 2 days or 7 days after spray inoculation and vacuum-infiltrated with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Water-sprayed tissue was processed as a control. Tissue was fixed 3–4 hr at 4 C, rinsed overnight at 4 C in the same buffer plus 1.5% sucrose, minced into 2-mm^2 pieces, and post-fixed for 4 hr in 2% osmium tetroxide in 0.05 M veronal-acetate-HCl buffer, pH 7.0 (8). Fixed tissue was dehydrated by a graded water/acetone series (50%, 70%, 2× 90%, 3× 100% acetone, 10 min each) and embedded in Spurr's low viscosity resin. Plastic sections (1 μm) for light microscopy were stained with aqueous 1% methylene blue in 1% sodium borate (14) and observed with a Leitz Orthoplan light microscope. Images were recorded on Kodak Panatomic-X film. Ultrathin (60–70 nm) sections for transmission electron microscopy (TEM) were stained with aqueous 5% uranyl acetate for 1 hr and Reynold's lead citrate (16) for 30 min. Sections were observed in a Zeiss EM 10B TEM at an accelerating voltage of 60 kV. Electron images were recorded on Kodak Electron Microscope Film 4489.

Leaf samples for scanning electron microscopy (SEM) were prepared as above through acetone dehydration and then dried in a Denton critical point drying apparatus (Denton Vacuum, Cherry Hill, NJ), using carbon dioxide as transition fluid. Paradermal fractures were achieved by sandwiching a dried leaf segment between two SEM stubs with cyanoacrylate adhesive ("super glue") and snapping the stubs apart. The two stubs then possessed complementary leaf halves. Tissue on SEM stubs was sputter-coated with 15 nm of gold/palladium and observed in a JEOL 50A SEM at an accelerating voltage of 15 kV. Images were recorded on Polaroid 55 P/N film.

Cell areas in 1-μm plastic sections were estimated by superposition of a grid (10 lines to the half inch) on a light micrograph at ×400 magnification and counting squares that wholly or partially covered cells.

TABLE 1. Variation in chloroplast numbers in soybean cultivar Clark leaf cells in response to virulent strains of *Xanthomonas campestris* pv. *glycines*

Inoculum	Time after inoculation	Inoculum concentration (cfu/ml)	Chloroplasts per leaf cell section: Spongy mesophyll	Chloroplasts per leaf cell section: Paraveinal mesophyll
Water	24 hr	···	6.2 (1.8)[a]	6.6 (1.9)
	48 hr	···	5.2 (1.9)	5.1 (1.5)
	11 days	···	6.2 (1.8)	7.6 (2.9)
Strain XP175	7 days	3×10^5	1.0 (1.2)[b]	4.5 (1.1)
	48 hr	3×10^9	4.0 (1.5)[c,d]	3.9 (2.0)[d]
Strain A	7 days	3×10^5	1.3 (1.1)[b]	3.5 (1.4)
	48 hr	3×10^9	3.4 (1.4)[c]	4.0 (1.3)

[a] Mean +/− S.E.; $n = 50$.
[b] Unilateral pustules.
[c] No pustules.
[d] Extensive cell collapse in these regions.

RESULTS

The histology of a young (not fully expanded) control leaf of soybean cultivar Clark 2 days after infiltration with water is shown in Figure 1A. Soybean leaves possess a characteristic cell layer between the palisade and spongy parenchyma cell layers, which is designated paraveinal mesophyll (3,18). SEM of a paradermal fracture of control tissue (Fig. 1B) 7 days after infiltration with water (fully expanded leaf) revealed large, interconnecting paraveinal mesophyll cells and smaller, spongy parenchyma cells, also interconnecting, which attached the mesophyll at random to abaxial epidermal cells.

Seven days after inoculation with bacterial cell suspension containing 3×10^5 cfu per milliliter, leaves exhibited yellow-green chlorosis and widespread pustules. The primary histological characteristic of pustules was the appearance of very large, rounded parenchyma cells that produced swellings in the leaf (Fig. 1C and D) and eventually caused collapse of epidermal cells and rupture of the cuticle. Enlarged cells in pustules had few chloroplasts (Fig. 1C, Table 1). Size and shape of neighboring palisade parenchyma cells were often not affected, but their chloroplasts were rounded (Fig. 1C) rather than lens shaped as in the control (Fig. 1A). Paradermal fracture through the base of a pustule caused by *X. c.* pv. *glycines* strain A disclosed confluent cells with abundant cytoplasm (Fig. 1E).

After inoculation with the more concentrated bacterial cell suspension (3×10^9 cfu per milliliter), the primary visual symptom at 2–3 days was leaf yellowing. Abscission occurred in 3–4 days. A section from a yellowed area of a leaf inoculated with *X. c.* pv. *glycines* strain A (Fig. 2) displayed a 30% increase in leaf thickness compared with a control of similar maturity (Fig. 1A) because of cell enlargement in the palisade cell layer and the contiguous paraveinal mesophyll. The average section area of affected palisade cells was approximately 30% more, and paraveinal cells, 100% more, than corresponding cells of the control. Large nuclei with prominent nucleoli were observed in many cells (Fig. 2). Changes in chloroplast shape were less evident than at the lower inoculum level.

Most pustules that had developed by 7 days after inoculation with 3×10^5 cfu per milliliter of *X. c.* pv. *glycines* strain A or XP175

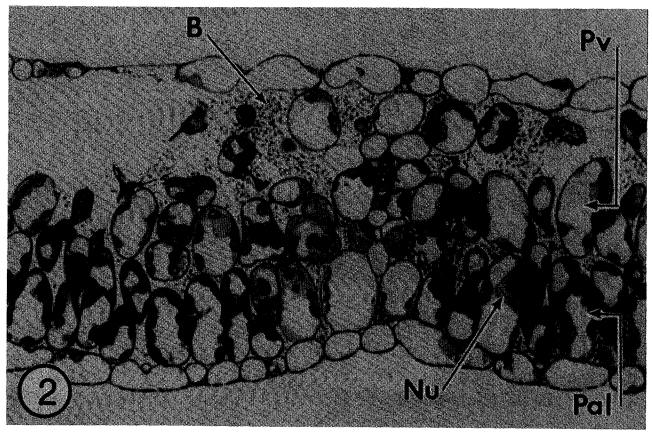

Fig. 2. Cell enlargement in the palisade (Pal) and paraveinal (Pv) parenchyma 2 days after inoculation with *Xanthomonas campestris* pv. *glycines* strain A cells at 10^9 cfu per milliliter (×400). B = bacteria, Nu = nucleus.

protruded from the abaxial side of the leaflet, but some involved palisade parenchyma cells as well and protruded from both sides of the leaflet (Fig. 3A and B). In large, bilateral pustules, an increase in cell number was evident. The pustule in Figure 3A exhibited approximately 60% more cell profiles per unit length of section than a typical control (Fig. 1A). Affected palisade cells (Fig. 3B) were elongated, confluent, and some exhibited cross walls, which suggested cell division activity. Nuclear duplication was not observed.

Inspection of host cell wall surfaces in pustules caused by *X. c.* pv. *glycines* strain XP175 and strain A disclosed colonies of bacteria associated with enlarged cells (Fig. 4A–C), often at cell junctions. Bacteria left imprints on cell walls, suggesting wall degradation or secretion of surface materials by host or bacterial cells, and also indicating that bacteria formed a real association with host walls and were not simply deposited there during critical point drying, which must be considered as a possibility in other locations (Fig. 4D). Strands of material, possibly bacterial extracellular polysaccharide (EPS), were often present near bacteria (Fig. 4C and D).

X. c. pv. *glycines* strain A induced the more severe effects on host cells. A typical enlarged parenchyma cell in a pustule elicited by strain A is shown in Figure 5A and can be compared to a similar region of a control in Figure 5B. The smaller spongy parenchyma cells in the pustule (Fig. 5A) at higher magnification (Fig. 5C) displayed ultrastructural features characteristic of strain A-affected cells in cultivar Clark, including thickening and apparent loosening of the wall matrix, and abundant dictyosomes, Golgi

TABLE 2. Organelles of soybean cultivar Clark mesophyll cells that exhibited changes in response to leaf inoculation with virulent *Xanthomonas campestris* pv. *glycines*

		Pathological appearance resulting from:			
Organelle[a]	Normal appearance[b]	Strain XP175 low dose[c]	Strain A, low dose[c]	Strain XP175, high dose[c,d]	Strain A, high dose[c,d]
Golgi	inconspicuous	stimulated; vesicles contained visible material	severe hypertrophy	stimulated; lucent vesicles	same[e]
Endoplasmic reticulum	scant; lightly studded with ribosomes	abundant, heavily studded; slight dilation	same[e], and cisternae with content	slight dilation; no visible content	same
Ribosomes	mostly cytoplasmic	large increase in ER-bound	same, and curved arrays of polysomes	no change	no change
Cell wall	firm or fuzzy outer surface	thickened	thickened, microfibrils loosened	possible thickening; EPS obscured changes	same
Tonoplast	firm, slightly irregular profile	no change	very irregular profile; possible coat	no change	some puckering
Mitochondria	elliptical; cristae sparse and poorly defined	often autolysing	often small with dilated cristae	normal or elongated, dividing	some small with dilated cristae
Chloroplasts	lens-shaped; starch plump and lucent	eventually degenerate	same	rounded, recessed from plasma membrane	same
Nuclei	elongate or lobed	no change	possible increase in size and lobosity	no change	no change

[a] Listed in approximate order of most affected to least affected organelles.
[b] Generalized from observations of controls.
[c] Low dose = 10^5 cfu per milliliter of inoculum; examined 7 days after inoculation. High dose = 10^9 cfu per milliliter of inoculum; examined 2 days after inoculation. Inoculum sprayed onto leaf abaxial surface.
[d] Observations taken from (10).
[e] Same = same as previous column.

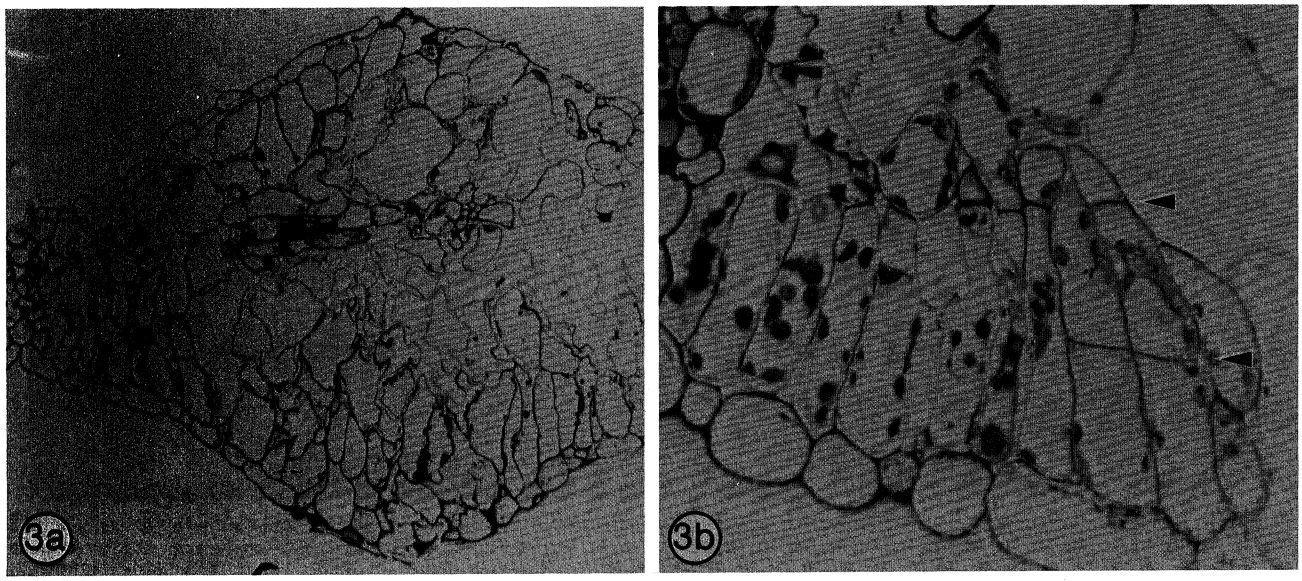

Fig. 3. A, Section through a bilateral pustule caused by *Xanthomonas campestris* pv. *glycines* strain A 7 days after inoculation (×160). **B,** On the flank of the pustule, palisade parenchyma cells were elongated and confluent. Cross walls (arrowheads) suggested that cell division occurred (×640).

vesicles, and endoplasmic reticulum (ER). ER was heavily studded with ribosomes and frequently spread to form a pool of material possessing no evident bilayer membrane. These areas were interpreted as oblique sections through protein-filled cisternae having linear or curving arrays of polysomes on their surfaces (7).

In Figure 6, ultrastructure of a normal paraveinal parenchyma cell is compared with that of enlarged cells toward the base of a developing pustule induced by *X. c.* pv. *glycines* strain A. In the normal cell (Fig. 6A), most of the cytoplasmic volume was occupied by chloroplasts. In the small expanses of cytoplasm visible, ribosomes were mostly cytoplasmic, endoplasmic reticulum was poorly developed, cytoplasmic vesicles were small and inconspicuous, and in general, a quiescent mesophyll cell cytoplasm was observed. In contrast, cytoplasm and cytoplasmic organelles were abundant in cells near the base of a pustule (Fig. 6B and C). Hypertrophy of dictyosomes was common and some Golgi vesicles contained fibrous material. The ultrastructure of these cells, apparently in the process of enlarging, indicated very active macromolecular processing, while energy-processing organelles (mitochondria, chloroplasts) were minimally affected at this stage. Tonoplasts were typically irregular in profile and appeared to be coated. Golgi vesicles and smooth ER bearing a content of amorphous material sometimes appeared to form a continuum with coated regions of the tonoplast (Fig. 6B).

Pustules often abutted or surrounded minor leaf veins. A comparison of perivascular cells in control versus pustular tissue (Fig. 7A and B) indicated that bundle sheath cells in affected tissue contained more cytoplasm than usual with many small vacuoles, small chloroplasts, and large pleiomorphic nuclei and vascular parenchyma appeared to be unusually rich in cytoplasm. No alterations were evident in companion cells and no bacteria were found in xylem or phloem elements.

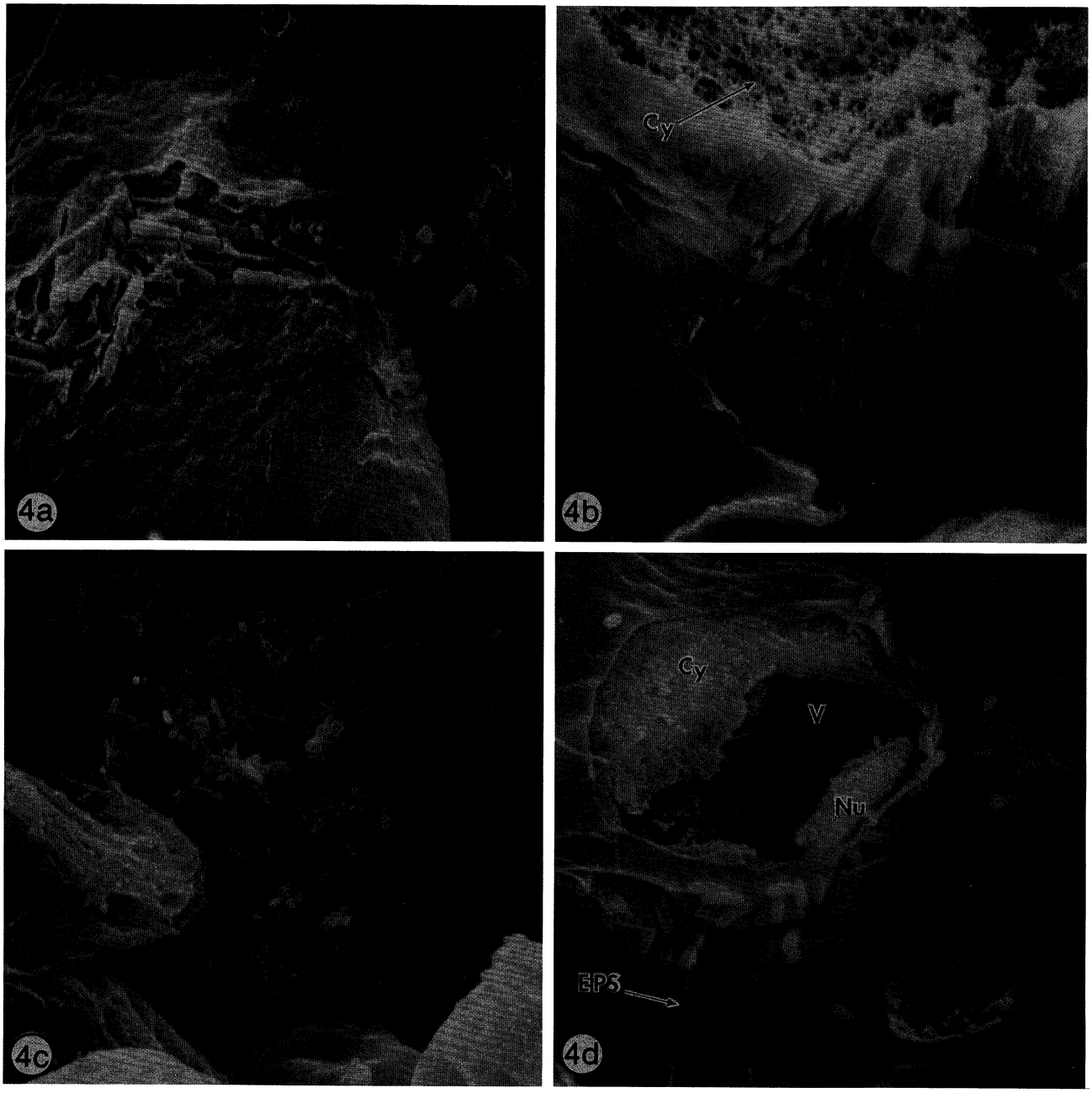

Fig. 4. Bacteria on host cell surfaces in or near pustules caused by *Xanthomonas campestris* pv. *glycines* strain XP175. **A and B,** Imprints of bacteria on walls of enlarged host cells suggested wall degradation or accumulation of host- or bacterial-secreted substances (×6,000). Cy = cytoplasm. **C,** Matted material with the bacteria on enlarged host cell may be degraded wall substances or bacterial extracellular polysaccharide (×3,000). **D,** Bacteria on this spongy parenchyma cell showed no evident interaction with host walls. Bacteria may have been deposited on host cells during critical point drying (×5,000). Cy = cytoplasm, EPS = bacterial extracellular polysaccharide, Nu = nucleus, V = cell vacuole.

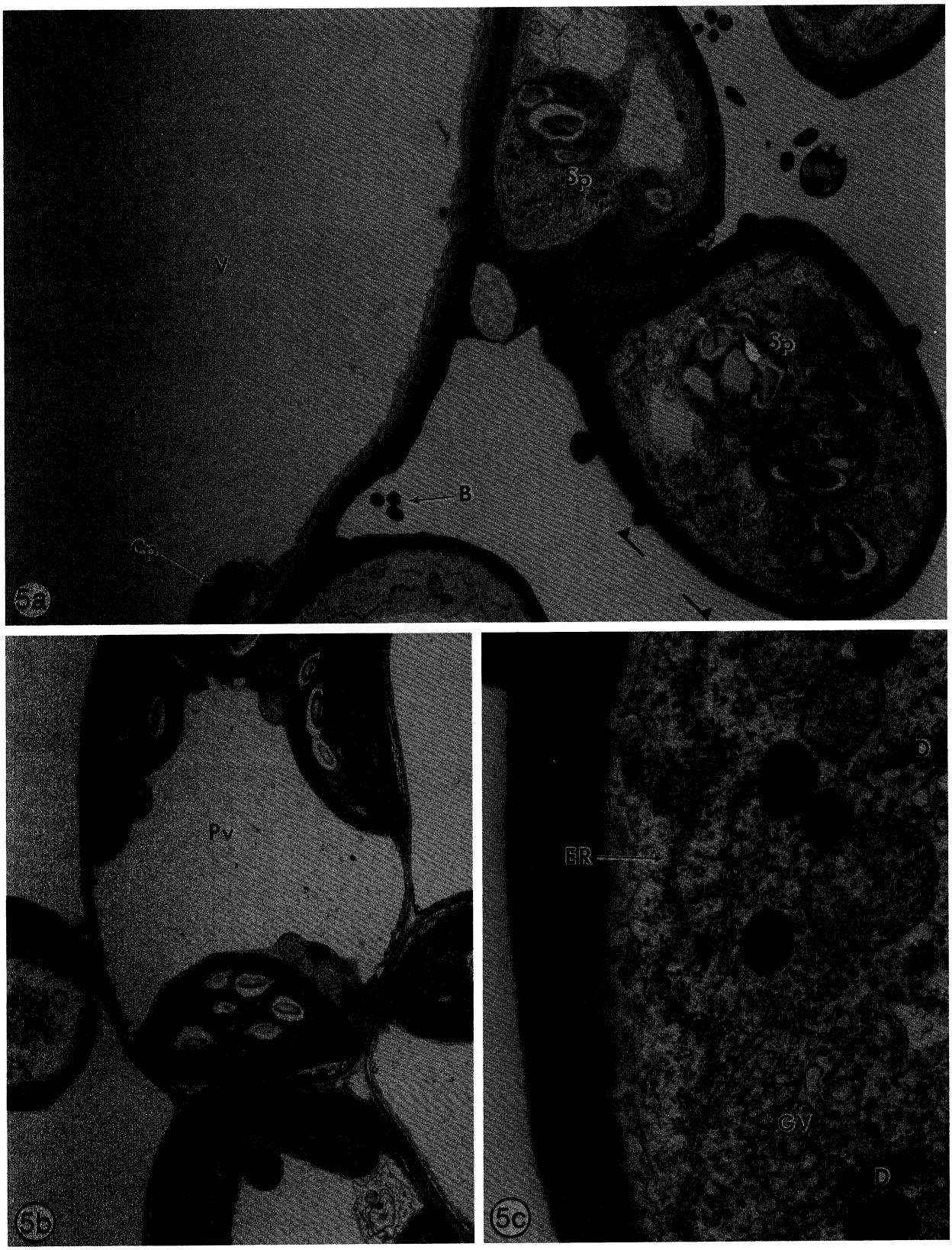

Fig. 5. Transmission electron micrographs showing enlarged cells in a pustule caused by *Xanthomonas campestris* pv. *glycines* strain A, compared with a control. **A,** In a pustule, the enlarged cell on the left had a thin shell of cytoplasm and a few rudimentary chloroplasts (Cp). Bacteria (B) were within the intercellular space. Spongy parenchyma (Sp) cells exhibited cytoplasmic features that typified strain A-affected cells (bracketed area enlarged in Fig. 5C) (×6,000). V = cell vacuole. **B,** A comparable area of control tissue (×6,000). Pv = paraveinal parenchyma. **C,** Subcellular morphology commonly observed in affected host cells in pustules induced by strain A included abundant dictyosomes (D), Golgi vesicles (GV), and rough endoplasmic reticulum (ER) and loosening of host wall microfibrils. Cytoplasm contained osmiphilic droplets (×35,000).

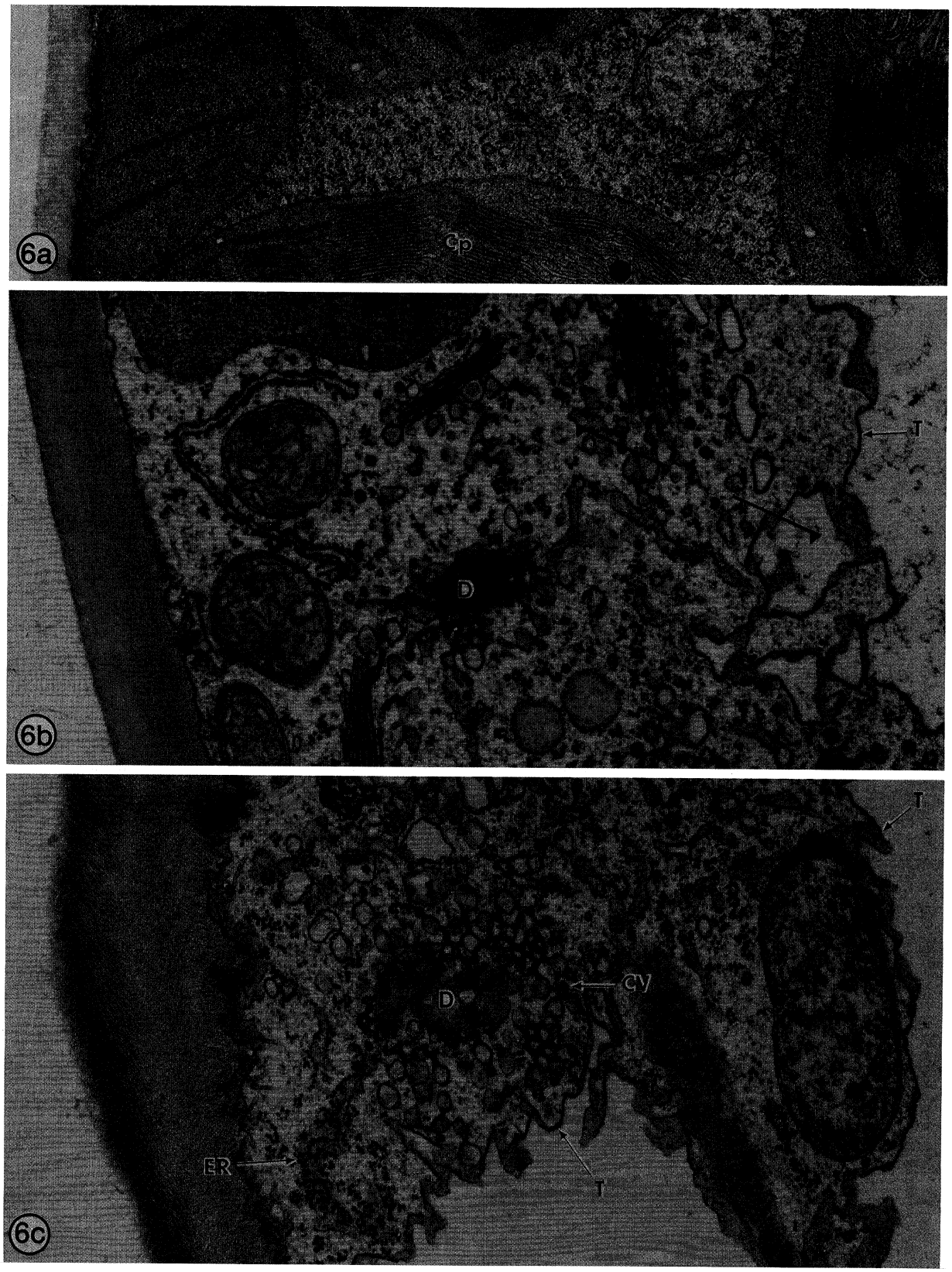

Fig. 6. Ultrastructure of severely affected host cells in a pustule induced by strain A, compared with a control. **A,** In a normal paraveinal parenchyma cell, most of the cytoplasmic volume was occupied by chloroplasts (Cp). **B and C,** Host cells near the base of a pustule exhibited loosened wall matrix and increased cytoplasm with abundant dictyosomes (D), Golgi vesicles, and coated vesicles (CV). Some Golgi vesicles contained fibrous material. Curved arrays of polysomes on swollen endoplasmic reticulum (ER) were common. Tonoplasts (T) were convoluted and did not give a clear profile in section. The presence of a coat on the tonoplast is suggested. Golgi vesicles, ER, and tonoplast sometimes appeared to form a continuum (bracketed region, 6B) (×35,000).

X. c. pv. *glycines* strain XP175 produced similar but less severe subcellular changes than did strain A. Table 2 presents a summary of comparative ultrastructural features of mesophyll cells affected by strains A and XP175 at high and low inoculum levels.

DISCUSSION

Information from micrographs suggested that pustule development began with stimulation of paraveinal mesophyll cells near the leaf midplane. The prolific activity observed in stimulated cells appeared to be directed initially toward production of cytoplasm and organelles and then into cell wall synthesis. The amount of cytoplasm relative to cell size first increased and then decreased as affected cells grew larger. Nascent vacuolization frequently observed in affected cells was probably the mechanism for increasing the proportion of vacuole to cytoplasm. Several observations pointed to erosion or partial disassembly of cell walls of affected cells. Chloroplasts were diminished in size and number in pustules, in general agreement with recent findings of Groth and Braun (6).

A frequently addressed question in the study of plant pathogenic bacteria is the relationship of bacterial cells to host cell walls. In a TEM study of sectioned soybean leaves (10), we observed avirulent strains of *X. c.* pv. *glycines* immobilized at mesophyll cell surfaces, i.e., covered by material that appeared capable of restricting movement of the bacteria. Virulent strains A and XP175 were not immobilized, but were free in the intercellular space, occasionally in contact with a host cell wall. Those studies were performed with bacterial inocula containing 3×10^9 cfu per milliliter. The present study has confirmed these results for strains A and XP175 at a lower inoculum level, i.e., the bacteria appeared to be freely movable in the intercellular spaces when examined in sections. Bacterial cells were not observed in vascular elements. SEM images, in contrast, showed colonization of host cell surfaces by virulent bacteria.

Consideration of the technique should be made when interpreting results. In the sample preparation for TEM and SEM, bacteria that are not anchored to cell walls by EPS or by other immobilizing materials may be moved about in the intercellular space or washed out altogether by the multiple fluid exchanges in fixation and dehydration. Infiltration with plastic for TEM may trap some bacteria in the middle of the intercellular space, while the drying procedure for SEM deposits suspended particles back onto the host cells. Neither technique gives straightforward information about the location of "free" bacteria. Moreover, TEM does not see a single layer of bacteria on a cell wall as a colony unless there is another distinguishing feature, such as a colony border of EPS.

Single-layer bacterial colonies were consistently found on the surfaces of enlarged host cells when critical point dried material was examined by SEM. Bacteria were often partially embedded in host cell walls, so that artifacts of drying could be discounted. These bacteria were neither free in the intercellular space nor immobilized as defined above. The results indicated that virulent bacteria were more closely associated with stimulated cells than was indicated in TEM micrographs.

Strain A produced more extreme changes in host cell ultrastructure than strain XP175. Because the rate of growth of strain XP175 was about the same as that of strain A in cultivar Clark (4,10), the difference in effect on host cells appeared to be an intrinsic difference between the two strains in their ability to cause metabolic and morphologic alterations in the host cells. There were strain-related differences apparent even at the high inoculum level (10), which induced minor changes in host cells, and some of the strain A-induced changes at the high inoculum level were clearly a mild form of the host responses observed with the low inoculum level (Table 2).

The effect of the virulent bacteria on the leaf cells was suggestive of auxin-induced cell stimulation (12,17). Indoleacetic acid (IAA) and cytokinins of host and/or bacterial origin are known to be involved in pathogenesis by *Agrobacterium tumefaciens*, *A. rhizogenes*, *Pseudomonas syringae* pv. *savastanoi*, and *Corynebacterium fasciens* and are considered to be necessary but

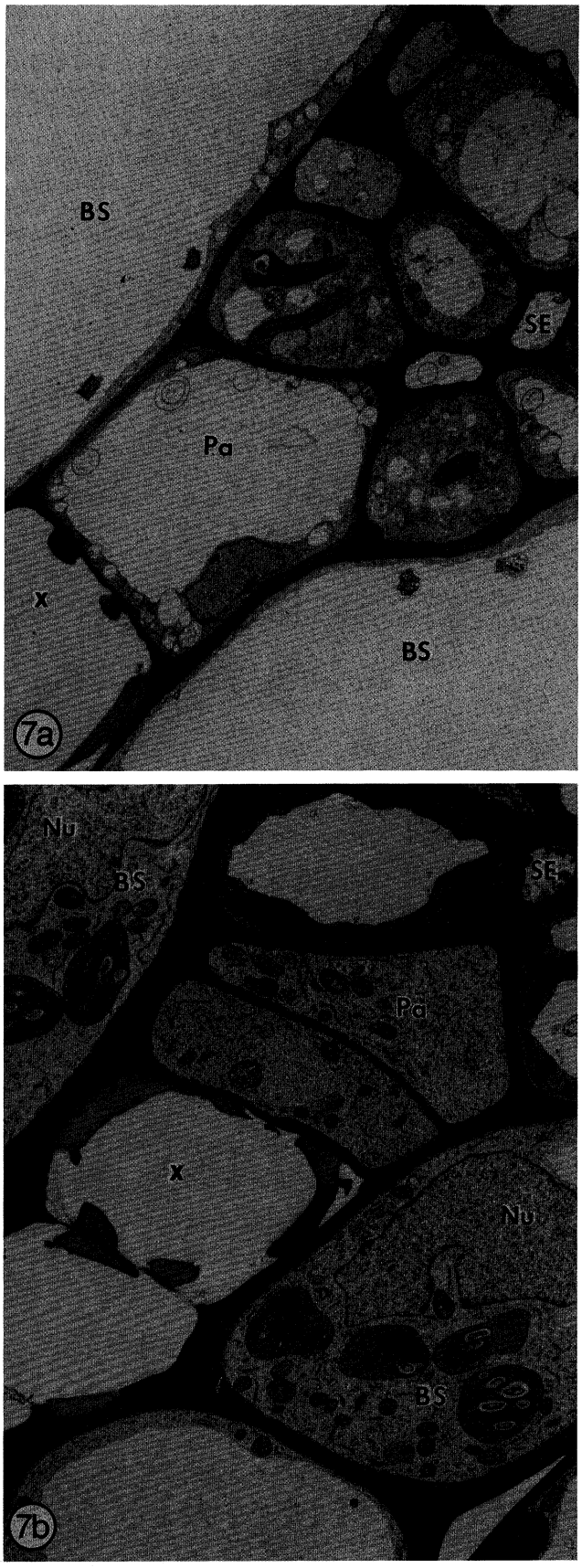

Fig. 7. Comparison of perivascular cells in control versus pustular tissue. **A,** In the control, cells flanking the vascular complex were highly vacuolated. **B,** Vascular parenchyma and bundle sheath cells in diseased tissue contained more cytoplasm than comparable cells in control, many small chloroplasts and large irregularly shaped nuclei (Nu) (×4,500). BS = bundle sheath cell, Pa = vascular parenchyma cell, SE = sieve element, X = xylem.

not sufficient to produce the tumors, galls, knots, or other tissue distortions caused by these organisms (11). A primary role for IAA in crown gall is believed to be nutrient accumulation in affected areas by induction of a high electrochemical proton gradient across the cell membrane (15). IAA causes cell elongation and multiple other effects on cellular metabolism (2) and may be involved in the cell stimulation and cell enlargement in bacterial pustule disease. *X. campestris* pv. *glycines* can produce IAA in vitro (Fett, Osman, and Dunn, *unpublished*). The absence of pustule formation at high levels of bacteria may be related to a hyperauxinic condition in which IAA-mediated cell elongation is inhibited and lateral expansion increases (1). This symptomology is compatible with our observations.

The primary effect of *X. c.* pv. *glycines* on soybean leaf histology was cell enlargement; whether a mitogenic effect was also present was less certain. However, the clear increase in cell number in some pustules and the presence of cross walls indicated that, at least in some instances, cell division occurred. If cytokinins are involved, their role appears to be secondary. The effects of *X. c.* pv. *glycines* are clearly not like the case of oleander tumors caused by *P. s.* pv. *savastanoi* where light microscopy studies showed nuclear duplication and multiple cell walls developing to produce the primary lesion (19). It should be noted that wounding is not implicated in bacterial pustule disease but wounds are the primary site of infection by *A. tumefaciens* and *P. s. savastanoi* (11). Overgrowths develop at the wound site because of stimulation of mitotic activity in cells around the wound (5,11), indicating a primary role for cytokinins in symptom development.

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