# The Relation of Blocked Chloroplast Differentiation to Sugarcane Leaf Scald Disease

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Journal Series Paper 2740, College of Tropical Agriculture and Human Resources, Honolulu, HI 96822.

This work is a portion of a thesis to be submitted by the first author to the University of Hawaii in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

We are grateful to J. C. Comstock for help in obtaining sugarcane cultivars and *X. albilineans* and to R. E. Paull and A. A. Hinchee for training in electron microscopy.

Accepted for publication 5 April 1983.

## ABSTRACT

Birch, R. G., and Patil, S. S. 1983. The relation of blocked chloroplast differentiation to sugarcane leaf scald disease. Phytopathology 73:1368-1374.

Study of the ultrastructure of chlorotic leaf tissue from sugarcane and sweet corn with systemic leaf scald confirmed that the pathogen, *Xanthomonas albilineans*, was confined to the xylem during early disease development. Xylem vessels were sometimes blocked by tightly packed bacteria, but adjacent bundle sheath and mesophyll parenchyma were not invaded. Chloroplasts were absent from cells surrounding invaded vessels in narrow white leaf stripes, and from uninvaded white leaves emerging after invasion of sugarcane stalks by the pathogen. Proplastids, etioplasts, and vesicular forms, but no degenerating chloroplasts, were present in white leaf areas. The evidence suggests that *X. albilineans* in invaded xylem may produce a diffusible phytotoxin, which blocks chloroplast differentiation at the proplastid or etioplast stages.

*Additional key words*: electron microscopy, *Saccharum officinarum*, *Zea mays* var. *saccharata*.

Leaf scald disease caused in sugarcane (*Saccharum officinarum* L.) by the xylem-invading bacterium, *Xanthomonas albilineans* (Ashby) Dowson, presents symptoms ranging from chlorosis to rapid wilting and death of plants, frequently after prolonged latent infection (16). An early diagnostic symptom is the emergence of leaves with narrow, sharply defined white stripes ("white pencil lines") parallel to the main veins on leaves and leaf sheaths. As the stripes age, a diffuse chlorotic zone often develops around the sharply defined central white lines. Partial or complete white chlorosis, not restricted to leaves with narrow pencil lines, may occur in emerging leaves as the disease develops. The pathogen can be isolated from leaf pencil lines and from stalk tissue showing internal red streaks, but not from chlorotic tissue away from pencil lines. Several other grasses also can be infected by artificial inoculation, and sweet corn (*Zea mays* L. var. *saccharata* Bailey), is a useful indicator plant which rapidly develops the characteristic white pencil lines (18,19).

Early light-microscope studies (15,17) revealed the bacterium in leaf and stalk xylem elements, but not in chlorotic parenchyma adjacent to invaded vessels. In 1926, North (17) reported that chloroplasts were absent from the bundle sheath and surrounding

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parenchyma of the originally invaded bundle, whereas chloroplasts in the expanding diffuse chlorotic zone did not disappear, but were reduced in size and disorganized. In 1942, Orian (18) noted that white pencil lines and chlorosis always developed on leaves before they emerged from the spindle. He proposed that the effect was due to a toxic metabolite of the pathogen acting on plastids before chlorophyll developed, and that development of a diffuse chlorotic zone around stripes after emergence and greening of the leaf was due to slow disorganization of plastids and consequent chlorophyll destruction. Subsequently, workers have expressed similar conjecture, but the involvement of a phytotoxin in sugarcane leaf scald has not been experimentally demonstrated.

In this paper, the term phytotoxin is used in a broad sense to mean any nonenzymatic compound of microbial origin that adversely affects plant tissues at physiological concentration and is involved in disease development. Historically, such toxins have been detected by reproduction of disease symptoms in plants treated with extracellular products of the pathogen. However, exhaustive attempts to induce chlorosis in sugarcane with fractions from *X. albilineans* cultures have been unsuccessful (*unpublished*). We have therefore adopted alternative strategies to investigate the leaf scald phytotoxin hypothesis. Results of the ultrastructural study reported here demonstrate that the chlorosis of emerging leaves is due to blocked chloroplast differentiation at the proplastid or etioplast stages, in parenchyma at a distance from xylem vessels invaded by the pathogen.

## MATERIALS AND METHODS

Young plants of leaf scald-susceptible sugarcane cultivar H54-2508 and sweet corn cultivar Hawaiian Supersweet #9 were inoculated by decapitating plants above the shoot apex and applying a dense suspension (generally $\sim 10^{10}$ cells per milliliter) from a 3-day slant culture of *X. albilineans* strain LS2 to the freshly cut surface. Inoculated plants were maintained in the greenhouse until systemic symptoms appeared. Only sharply defined white pencil lines developed on uncut emerging leaves of Hawaiian Supersweet #9, usually 2–4 wk after inoculation. Systemic white pencil lines, and later general chlorosis of emerging leaves, appeared in H54-2408 3 wk to 6 mo after inoculation.

Sweet corn leaf material prepared for microscopy consisted of white pencil line segments and healthy green tissue from the first leaf below the emerging spindle leaf. White pencil line segments, chlorotic regions not associated with white pencil lines, and healthy green tissues from the first leaf below the spindle (leaf 1), as well as white pencil lines from the fourth leaf below the spindle (leaf 4), were examined from systemically invaded sugarcane. All samples were taken from plants in the greenhouse after 3 hr of morning sunlight.

Leaf segments were placed into a drop of Karnovsky's (11) formaldehyde-glutaraldehyde fixative, cut into pieces 1- to 2-mm square, transferred to fresh fixative at 4 C, allowed to stand for 2 hr, and then washed in three changes of 0.1 M phosphate buffer, pH 7.4 (30 min per wash). Tissues were postfixed in 1.3% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 2 hr, washed in three changes of fresh buffer, then dehydrated using a series of increasing acetone concentrations (10, 25, 40, 65, 80, 90, 95, 100, and 100%), 30 min at each concentration. Samples were returned to 24 C and infiltrated with Spurr's (21) low-viscosity epoxy resin, or with Epon 812 as described by Luft (14), infiltrating overnight in the final medium. Blocks were polymerized in three temperature steps for Epon (14), or at 65 C for 1 day for Spurr's resin.

Sections 1-$\mu$m thick were cut using glass knives on a Reichert OM-U2 ultramicrotome, stained with toluidine blue (6), and examined with a light microscope. Blocks were fine trimmed to selected areas and sectioned for electron microscopy using a diamond knife. Unsupported sections on 74- or 48-$\mu$m (200- or 300-mesh) grids were stained for 10 min with 3% aqueous uranyl acetate, and 1.5 min with Reynold's (20) lead citrate, then examined with a Hitachi HS8-1 electron microscope.

## RESULTS

By light microscopy, individual xylem vessels in invaded vascular bundles appeared occluded in transverse sections of systemic pencil lines on sweet corn leaves (Fig. 1). Parenchyma cells surrounding the invaded vascular bundle were almost completely devoid of chloroplasts for the width of the white pencil line, with a sharp transition to normal chloroplast content at the edge of the white line (Fig. 2). Sugarcane leaves with white pencil lines showed a similar marked reduction in chloroplast content of bundle sheath and mesophyll parenchyma cells adjacent to invaded vascular bundles. Chloroplasts also were absent from sections of chlorotic leaves not associated with white pencil lines, although bacterial cells and occlusion of xylem vessels were not observed.

By electron microscopy, healthy green tissues from sugarcane leaf 1 showed the ultrastructural features previously described for sugarcane leaves (13). Agranal bundle sheath chloroplasts with characteristic peripheral reticulum (Fig. 3), and granal mesophyll parenchyma chloroplasts (Fig. 4) were well developed with a few osmiophilic globules, often at the poles.

Sections from uninvaded chlorotic areas of leaf 1 were markedly different from the controls. No mature chloroplasts were present in bundle sheath or mesophyll parenchyma cells. Plastids resembled proplastids with very little internal structure (Fig. 5), etioplasts with noncrystalline prolamellar bodies and rudimentary lamellar development (Figs. 6 and 7), or highly vesiculated forms generally with more osmiophilic globules (Fig. 8). Serial sections revealed that single plastids could appear as etioplasts or vesicular forms in different sections. Plastid shapes ranged from spherical or ovoid to amoeboid or irregular and cytoplasmic invaginations were common (Figs. 9 and 10). Plastid sizes in chlorotic leaves ranged from 1.8–7.5 $\mu$m (mean = $4.4 \pm 1.27$ $\mu$m), compared with 4.3–11.2 $\mu$m (mean = $7.0 \pm 1.68$ $\mu$m) for chloroplasts from green leaves. Ribosomes were not apparent in plastids from chlorotic leaves, but other cell organelles appeared to be normal.

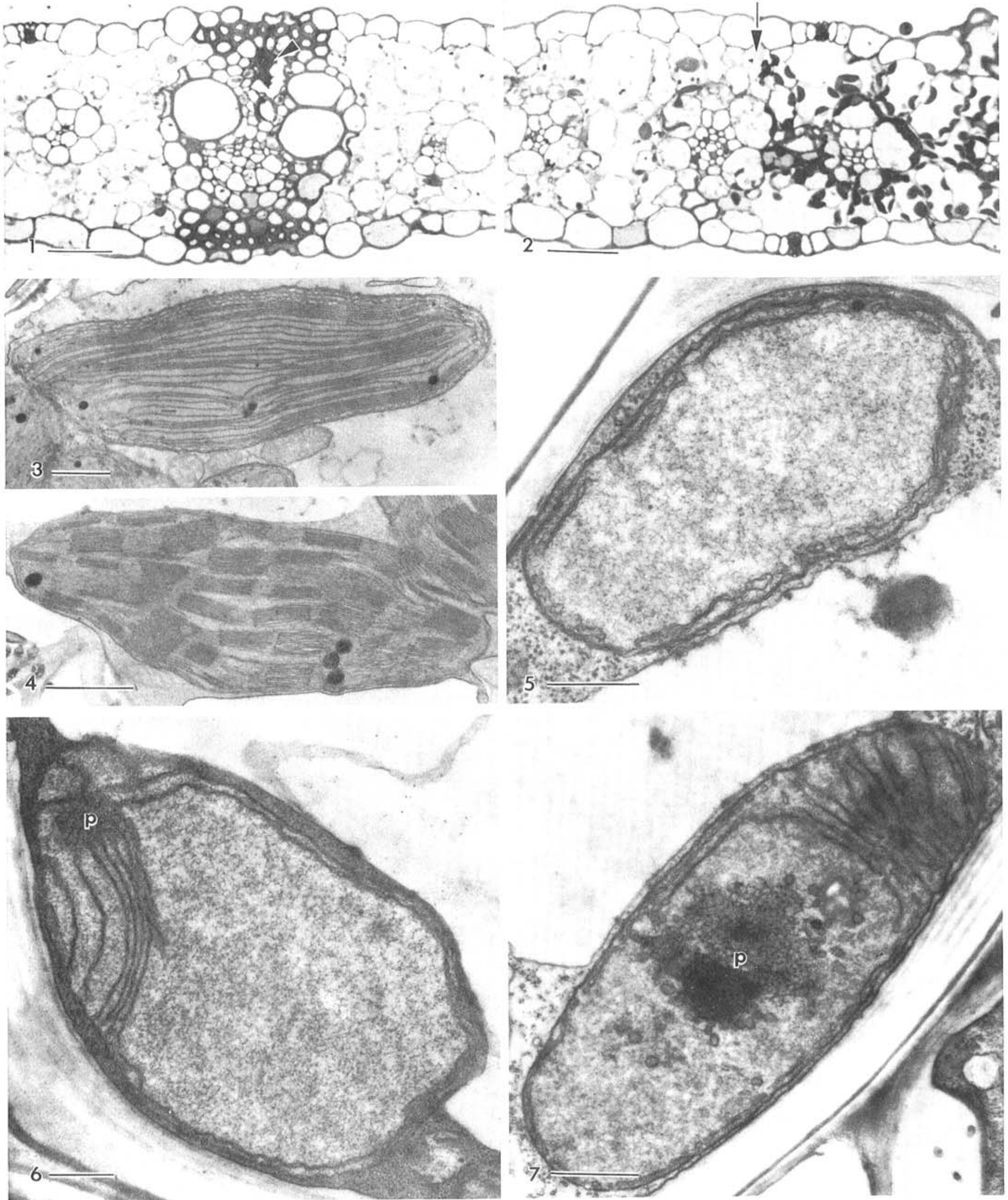
Bacteria in systemic white pencil lines on sugarcane leaf 1 were restricted to the xylem and intercellular spaces of xylem tissue only. In many sections, they were scattered around the edges of xylem vessels (Fig. 11). However, in some vessels, the bacteria were packed so tightly that their cells were compressed to polygonal shapes in cross section (Fig. 12). Plastids in bundle sheath and mesophyll parenchyma cells adjacent to invaded vascular bundles resembled those described above from uninvaded chlorotic leaves. Some sections showed plastids with more advanced development near the margins of white pencil lines (Figs. 13 and 14). Bacteria were occasionally observed in xylem elements at the margins of pencil lines, accompanied by swelling of mature chloroplasts in adjacent bundle sheath cells (Fig. 15), clearly different in appearance to the effects within the pencil lines.

Plastids in the white pencil lines of sugarcane leaf 4 remained in proplastid, etioplast, or vesiculated forms (Figs. 16–18) but frequently contained large osmiophilic globules (Fig. 19). As in younger leaves, organelles other than plastids appeared to be normal.

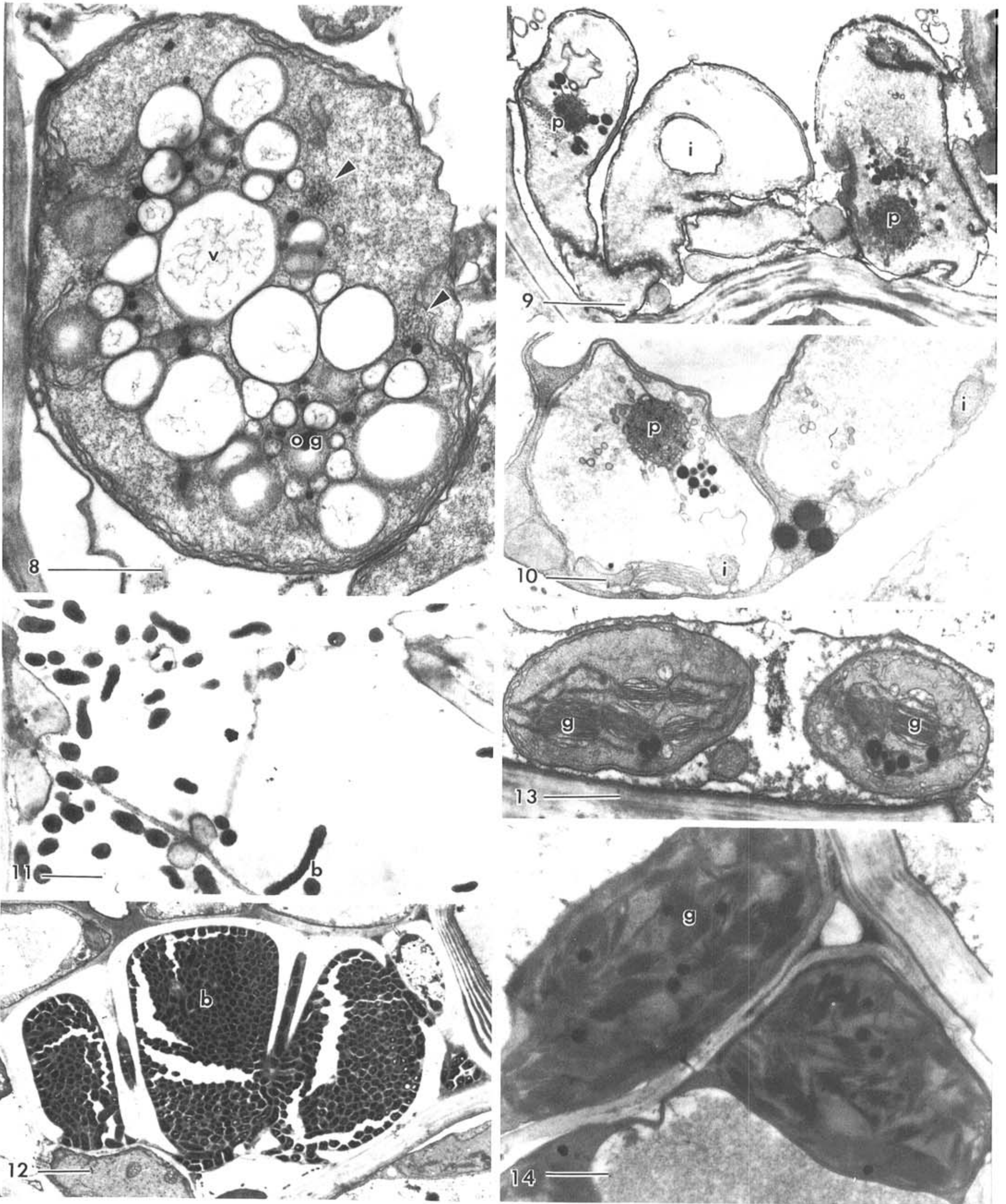
Plastids in systemic white pencil lines on sweet corn leaves measured 1.8 to 4.4 $\mu$m (mean = $3.2 \pm 0.96$ $\mu$m). Many contained tubular vesicles, although proplastid and etioplastlike stages were also present, as in sugarcane (Figs. 20–22).

## DISCUSSION

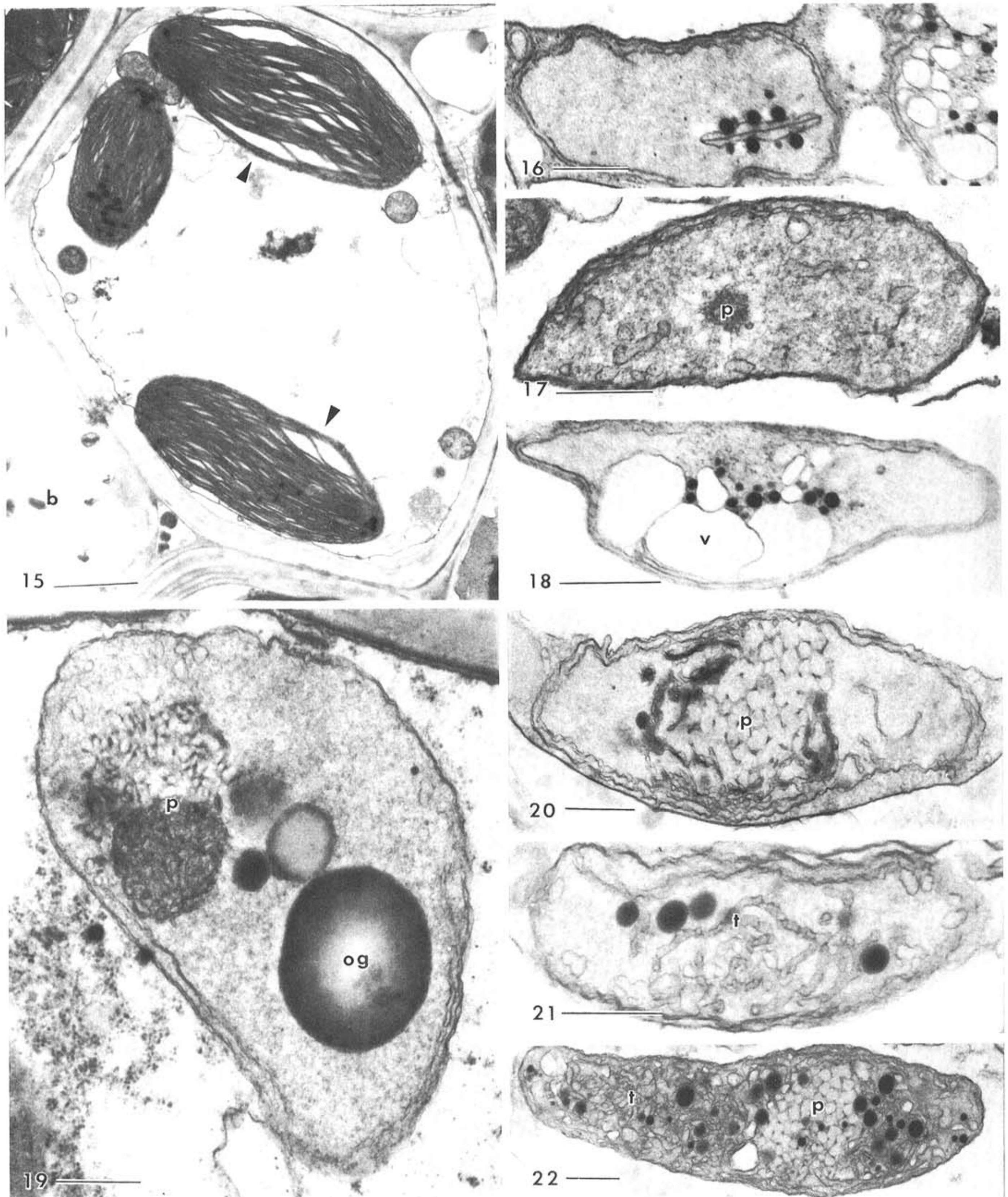
Two observations made at the light microscope level by earlier workers (15,17,18) have been confirmed in this ultrastructural study. First, the leaf scald pathogen was confined to xylem vessels or adjacent intercellular spaces during the early stages of disease development. No bacteria were observed in chlorotic parenchyma surrounding invaded vascular bundles in narrow white leaf stripes, or in broad chlorotic areas on leaves emerging after invasion of sugarcane stalks by the pathogen. Second, mature chloroplasts were absent from white leaf tissue. In addition, electron microscopy revealed that plastids were present in these tissues as proplastids, etioplasts, and vesicular forms smaller than chloroplasts.




**Figs. 1-7. 1-2,** Cross section of a systemic "white pencil line" lesion on a young sweet corn leaf. **1,** Major vein at the center of the white line showing protoxylem lacuna partly occluded by cells (arrowhead) of *Xanthomonas albilineans* and absence of chloroplasts from surrounding mesophyll. Bar = 20 $\mu$m. **2,** Transition to normal chloroplast content at the sharply defined lesion margin (arrow). Bar = 20 $\mu$m. **3-4,** Chloroplasts from the youngest leaf outside the emerging spindle (leaf one) of healthy sugarcane. **3,** Agranal bundle sheath chloroplast with peripheral reticulum. Bar = 1 $\mu$m. **4,** Granal mesophyll parenchyma chloroplast. Bar = 1 $\mu$m. **5-7,** Plastids from an uninvaded chlorotic area of leaf one of sugarcane with stem invaded by *X. albilineans.* **5,** Proplastid with little internal structure. Ribosomes are apparent in the cytoplasm but not in the plastid. Bar = 0.5 $\mu$m. **6,** Etioplast with prolamellar body (p) and a few radiating lamellae. Bar = 0.5 $\mu$m. **7,** Etioplast with partly dispersed prolamellar body (p), small vesicles and few lamellae. Bar = 1 $\mu$m.




**Figs. 8–14. 8–10,** Plastids from an uninvaded chlorotic area of leaf one of sugarcane with stem invaded by *Xanthomonas albilineans*. **8,** Plastid with large vesicles (v), small osmiophilic globules (og) and small prolamellar centers (arrowheads). Bar = 1 μm. **9,10,** Plastids of irregular shape showing prolamellar bodies (p), osmiophilic globules, small vesicles, few lamellae and vacuolar or cytoplasmic invaginations (i). Bar (Fig. 9) = 2 μm, Bar (Fig. 10) = 1 μm. **11–12,** Xylem vessels invaded by *X. albilineans* in a "white pencil line" lesion on sugarcane leaf one. **11,** Bacteria (b) scattered near vessel walls. Bar = 2 μm. **12,** Bacteria (b) packed tightly in xylem vessels, pit cavities, and a space between xylem and bundle sheath walls. Bar = 2 μm. **13–14,** Plastids near the margin of a "white pencil line" lesion of sugarcane leaf one. **13,** Plastids with rudimentary granal development (g). Bar = 1 μm. **14,** Plastids with advanced granal development (g), but disorganized granal orientation. Bar = 1 μm.




**Figs. 15–22. 15,** Bundle sheath cell from the expanding diffuse chlorotic zone at the margin of a "white pencil line" lesion on sugarcane leaf one showing swollen mature chloroplasts (arrowheads). The adjacent xylem vessel contains *Xanthomonas albilineans* (b). Bar = 2 μm. **16–19,** Plastids from a "white pencil line" lesion on the fourth leaf outside the emerging spindle of diseased sugarcane. **16,** Proplastidlike form. Bar = 0.5 μm. **17,** Etioplastlike form. Bar = 1 μm. **18,** Vesicular form. Bar = 1 μm. **19,** Etioplast with large osmiophilic globule (og). Bar = 0.5 μm. **20–22,** Plastids from a "white pencil line" lesion on a young sweet corn leaf, showing partly dispersed prolamellar bodies (p) and tubular vesicles (t). Bars (Figs. 20–22) = 0.5 μm.

Proplastids in sugarcane normally proceed through an etioplast stage while the leaf is in the spindle, surrounded by older leaves and sheaths, then develop into mature chloroplasts on emergence of the leaf and exposure to light (13). Proplastids and etioplasts in chlorotic tissue resembled normal developmental stages, and not degenerative forms characteristic of senescence (5). Vesicle formation in some plastids is probably a secondary effect when normal differentiation is blocked, rather than a degenerative change, since more advanced degenerative effects were not apparent in plastids of older chlorotic leaves.

The ultrastructural evidence that chlorosis is due to blocked chloroplast differentiation rather than disruption of organelle structure, and the occurrence of this blocked differentiation in parenchyma at a distance from xylem vessels invaded by the pathogen, suggest several alternative mechanisms which cannot be resolved by the ultrastructural approach alone. For example, bacteria in invaded xylem may act as a sink for an immobile mineral nutrient, resulting in chlorosis of adjacent parenchyma in emerging leaves. However, the ultrastructural features observed in the present study do not closely resemble those described for any mineral deficiency (23), and leaf scald chlorosis cannot be reversed by application of foliar fertilizer. Alternatively, differentiating xylem parenchyma may be stimulated by invading bacteria to produce a hormone blocking chloroplast photomorphogenesis in emerging leaves. This possibility is difficult to evaluate because the control of normal chloroplast differentiation is not yet clearly defined, but attempts to induce chlorosis in sugarcane with extracts from leaf scald diseased plants have not been successful. A more tenable possibility is that chloroplast differentiation is prevented in tissues exposed to a diffusible phytotoxin produced by *X. albilineans* in invaded xylem vessels, resulting in narrow white stripes around vessels invaded early in leaf development, or in more extensive chlorosis when developing leaves are exposed to toxin translocated from invaded stem vessels even when the leaves themselves are not invaded.

Lateral spread of *X. albilineans* from the central invaded bundle has previously been shown to occur through lateral connecting bundles, and to coincide with the development of a diffuse chlorotic zone around the sharply defined white pencil lines (17). It seems likely that the swelling of mature bundle sheath chloroplasts observed at the stripe margin is an effect of toxic metabolites diffusing from xylem invaded by lateral spread after leaf emergence. However, we cannot be certain from the ultrastructural evidence whether one or several toxic substances are involved in blocked chloroplast differentiation, vesiculation of plastids, and swelling of mature chloroplasts. Blockage of xylem vessels by tightly packed bacteria possibly accounts for withering of leaves along pencil lines, and wilting of systemically invaded plants as the disease progresses.

Phytotoxins inducing apical chlorosis have previously been reported from *Rhizobium japonicum* (9) and *Pseudomonas syringae* pv. *tagetis* (22). Ultrastructural effects of rhizobitoxine have not been reported. Ultrastructural effects of tagetitoxin, although restricted to chloroplasts of emerging leaves, have been interpreted as degenerative changes (10). Tentoxin from *Alternaria alternata* induces chlorosis in germinating seedlings, causing starch accumulation and reduced lamellar development in chloroplasts (7). Other phytotoxins may cause chlorosis of both green and emerging leaves, but none are known to block chloroplast differentiation (8).

Herbicides blocking carotenoid biosynthesis, antibiotics blocking the 70 S ribosome, and several plastid mutations also cause chlorosis by blocking normal chloroplast development (1,12). However, blockage at the etioplast stage with persistence of prolamellar bodies in light is unusual. To our knowledge, the only previous reports of light-stable prolamellar bodies are from an $F_1$ heterozygote between albino and pastel mutants of maize (2), and from wheat seedlings treated with the herbicide amitrole (3). The maize mutant has a partial block in carotenoid biosynthesis resulting in loss of most chlorophyll by photooxidation (2), and amitrole also acts by blocking carotenoid synthesis (4). In contrast with the maize mutant and amitrole-treated wheat seedlings, chlorotic sugarcane leaves do not accumulate carotenoid precursors (*unpublished*) and further investigation is required to establish the molecular basis of leaf scald-induced chlorosis.

Expression of leaf scald symptoms is erratic, even in systemically infected susceptible sugarcane cultivars (16). Failure to induce chlorosis in sugarcane with fractions from cultures of *X. albilineans* may reflect low toxin production in vitro, difficulty in exposing differentiating plastids to toxin in sufficient dose or duration, or temporal variation in susceptibility of plants to the toxin. Further investigation of the proposed phytotoxin would be difficult without a reliable assay. However, we have observed that chlorosis-inducing isolates of *X. albilineans* produce a microbial inhibitor in culture (*unpublished*), and we are currently using genetic and biochemical approaches to investigate the relationship between the microbial inhibitor and the putative inhibitor of chloroplast differentiation in leaf scald diseased sugarcane.

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