

Ultrastructural Effects in Zinnia Leaves of a Chlorosis-Inducing Toxin from *Pseudomonas tagetis*

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ABSTRACT

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A partially purified toxin from *Pseudomonas tagetis* caused chlorosis in developing zinnia leaves about 3 days after introduction of the toxin into the stem. Ultrastructurally the toxic effect was confined to the chloroplast. Only 1 day after treatment, chloroplast grana and stroma lamellae showed signs of disorganization, usually at the periphery of the plastid. After 2 days, this internal disorganization was severe, lipid globules were more numerous, and starch grains were greatly reduced in number and size. Short fibrils (presumably of DNA) and vesicles were evident. By 4 days, the

chloroplast was scarcely recognizable. It was reduced in size and its outline was irregular, with invaginations that sometimes contained other organelles. The stroma lacked contrast because the ribosomes had disappeared and only remnants of the lamellae remained. In the more electronlucent stromal regions, the DNA fibrils had clumped and formed branched structures with fine tentacles. Lipid globules had increased in number and occurred in groups. No starch grains were present. The chloroplast envelope appeared to be intact.

Additional key word: *Zinnia elegans*.

Hellmers (4) originally described *Pseudomonas tagetis* in 1955 as the causal agent of a leaf spot disease of African marigold (*Tagetes erecta* L.). He also occasionally observed apical chlorosis, but did not include this symptom in his report because he was uncertain about its relationship with the leaf spots (10). Bakker (1) later showed that some isolates induced only the leaf spot phase whereas others induced both leaf spots and apical chlorosis. She attributed chlorosis to the action of a toxin since she was unable to isolate the bacterium from chlorotic tissues. However, other workers subsequently recovered the bacterium from the xylem of chlorotic tissue (9).

Recently the disease has become severe in eastern and southern coastal cities of Australia (9,10). Plants with leaf spots but no apical chlorosis were present as well as those with both phases of the disease. Some bacterial isolates that caused both leaf spots and apical chlorosis produced in culture a nonspecific toxin that induced apical chlorosis (7). To aid in determining its site of action, we have examined the ultrastructural effects of a semipurified toxin preparation on leaves of zinnia (*Zinnia elegans* Jacq.). A preliminary report was published (5).

MATERIALS AND METHODS

Pseudomonas tagetis Hellmers (isolate 26816, from P. C. Fahy) was grown with agitation in Woolley's glucose medium at 24 C for 6 days. The cells were removed by centrifugation, and the volume of the culture filtrate was reduced 10-fold in vacuo at 35 C. Aliquots of the crude concentrate were passed through a column of Biogel P-6 (2.5 × 36 cm) equilibrated with 0.04 M NaCl. A single peak which contained chlorosis-inducing activity was eluted at twice void volume (7). Twenty- μ liter droplets of this preparation were placed on stems of 14-day-old zinnia seedlings just above the first fully expanded leaves, and the stems were lightly pricked with a needle

through the droplets. The same technique was used to inoculate plants with a 4-day-old culture of the bacterium. After the droplets had dissipated, usually within 15–20 min, the plants were incubated in a growth chamber (28 C, 12-hr day length, 32,000 lux). Control plants were treated similarly with 0.04 M NaCl. Samples were taken at the end of each day and night period from the basal, middle, and top portions of the subapical leaves of both control and treated plants.

The samples were fixed in 3% glutaraldehyde for 18 hr; rinsed in either 0.2 M phosphate buffer, pH 7.4, or 0.08 M cacodylate buffer, pH 7.4; and postfixed in Palade's buffer for 2 hr at 4 C. The samples were dehydrated in a graded acetone series, stained in saturated uranyl acetate in 75% acetone for 16–18 hr, and embedded in Spurr's epoxy resin. Samples that had been treated with cacodylate buffer also were stained with uranyl acetate after sectioning. All samples were stained with lead citrate and viewed in a JEM 7 electron microscope.

RESULTS

The basal portion of the expanding, subapical leaves began to show chlorosis at the end of the second, or the beginning of the third, day after toxin application. One day later the basal portion was totally chlorotic. The visual transition between this region and the distal portion of the leaf which remained green throughout the experiment was very abrupt. Similar symptoms were noted in plants inoculated with *P. tagetis*.

The chloroplasts from leaves of control seedlings (Fig. 1) were characteristic of those described from other higher plants (2). The lamellae were evenly distributed throughout a dense stroma and ribosomes were evident, but smaller than those in the cytoplasm. Single, small osmiophilic globules were commonly present between the lamellae. Starch grains were common, especially in the samples taken following the period of exposure to light. Although it is known that DNA exists in chloroplasts as fine fibrils, these were not detected, probably because the stroma was too dense.

One day after treatment of the leaves with toxin, the chloroplasts

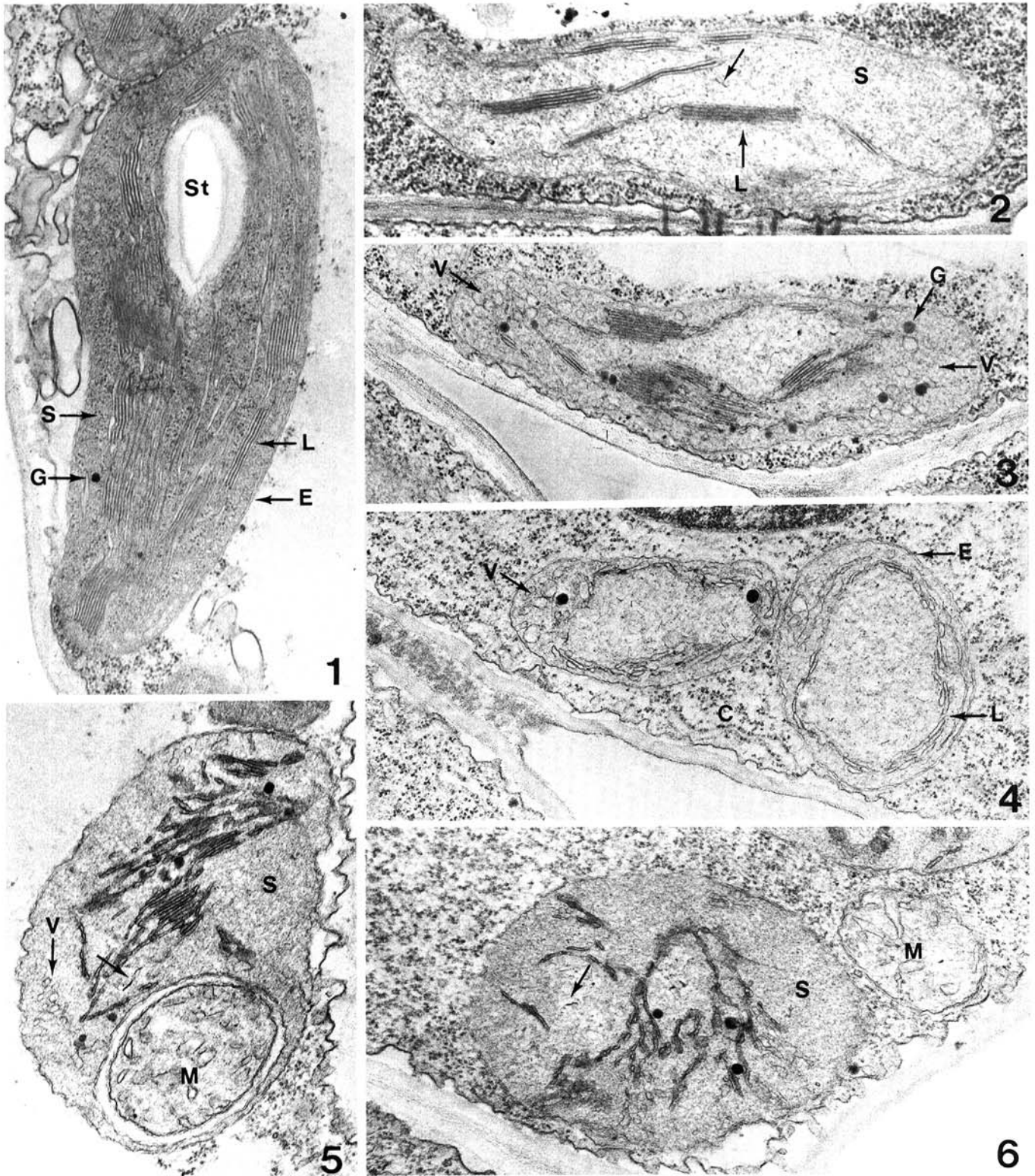


Fig. 1-6. Effects of *Pseudomonas tagetis* toxin on zinnia leaf chloroplasts. 1, Chloroplast from untreated plant showing regular distribution of intact lamellae (L), a large starch grain (St), dense stroma (S), osmiophilic globule (G), and an intact envelope (E). Fig. 2-6, Progressive degradation of chloroplast ultrastructural elements in toxin-treated leaves. All sections were fixed in cacodylate buffer ($\times 28,750$). 2, One day after treatment (dark period). L are less regularly arranged and few Gs and DNA fibrils (unlabeled arrows) are present. 3, Two days after treatment (dark period). L are grouped around a less-dense S, many Vs are observable, especially at the tips of the chloroplast, and increased numbers of Gs are present. 4, Two days after treatment (light period). L oriented in a ring with rounded Vs at the periphery of E. The chloroplast outline has started to appear abnormal. Fig. 5-6, Three days after treatment (dark period). Chloroplast is gradually assuming an inflated, irregular outline. 5, Chloroplast with an enclosed mitochondrion (M). Vs, DNA fibrils, and slightly more Gs are evident. 6, Additional view of disrupted L.

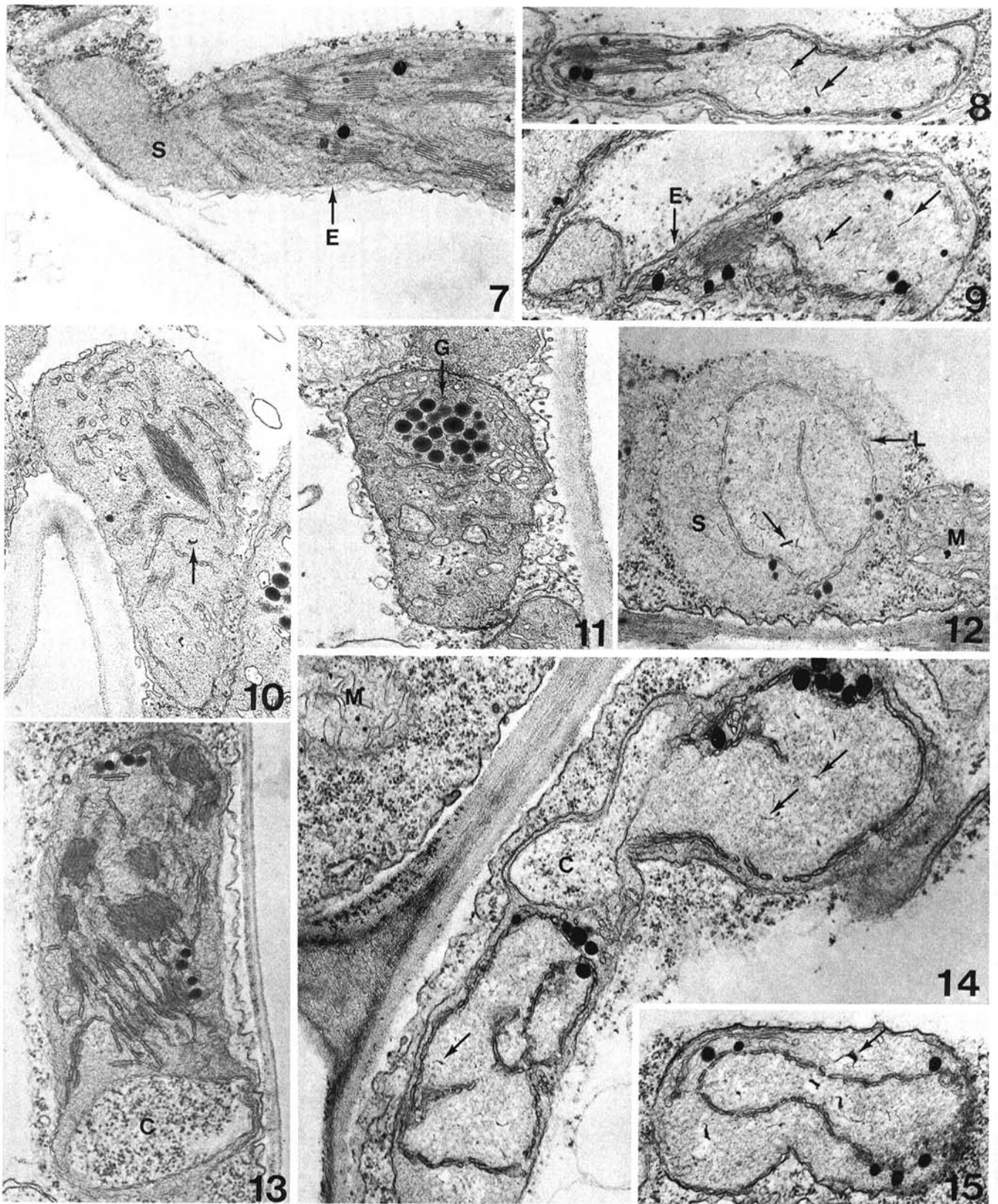


Fig. 7-15. Chloroplasts of zinnia leaves at advanced stages of degradation by *Pseudomonas tagetis* toxin. Leaf sections depicted in 10 and 11 were fixed in phosphate buffer. **7,** Chloroplast from distal portion of leaf three days after treatment (dark period) showing dense stroma (S) lacking lamellae (L) ($\times 23,000$). **Fig. 8-9,** Three days after treatment (light period). **8,** L are circularly arranged with DNA fibrils (arrows without labels) and numerous osmiophilic globules (G) present ($\times 23,000$). **9,** Chloroplast envelope appears corrugated and part of S is nearly pinched off ($\times 28,750$). **Fig. 10-11,** Chloroplasts from the leaf base four days after treatment (dark period). **10,** L are severely disrupted, S appears granular, and DNA fibrils and vesicles (Vs) are present. **11,** The chloroplast contains many Vs of different sizes, the large ones contain inclusions probably of cytoplasmic origin; note the high concentration of Gs. **12,** Leaf base four days after treatment (light period). Ribosomes are now absent from the chloroplast. Numerous DNA fibrils and Gs are visible ($\times 28,750$). **13,** Distal portion of leaf four days after treatment (dark period). L are disarranged and numerous Gs and Vs are present. The chloroplast envelope is indented by a cytoplasmic inclusion (C) ($\times 28,750$). **14,** Four days after treatment (light period). Chloroplast is at the point of collapse. It contains a large C and possibly has a ruptured envelope ($\times 23,000$). **15,** Additional view showing the circular shape of L. DNA fibrils are still present ($\times 23,000$).

in the basal leaf tissue still appeared normal in shape and starch grains were present. However, the stroma was less dense and the lamellae were less densely packed (Fig. 2). Fibrils, presumably of DNA, occasionally were faintly visible.

Two days after treatment, several degenerative changes generally had become evident. Towards the chloroplast's periphery, vesicles occurred in clusters, and the number of globules had increased slightly (Fig. 3, 4). After the light period, the lamellae in many chloroplasts were arranged circularly in both cross and longitudinal sections (Fig. 4). The stroma inside the lamellar ring was less dense and contained fibrils. Starch grains were common at the end of the light period, but were rare and quite small after the dark period; usually they lay inside the lamellar ring. Some chloroplasts had irregular outlines, and occasionally they contained cytoplasm-filled cavities. Few ribosomes were visible.

After 3 days, the chloroplasts had become quite distorted. The membranes had a corrugated appearance and invaginations were common. Other organelles, especially mitochondria, often were "caught" in these invaginations (Fig. 5). The clusters of vesicles were smaller and less numerous, and between the disorganized lamellae, in areas with less dense stroma, fibrils were present (Fig. 6). Frequently, parts of the stroma were nearly pinched off (Fig. 9) and sometimes they contained small vesicles and remnants of lamellae (Fig. 9). The lamellae continued to be in circular arrangements (Fig. 8, 9). Sometimes remnants of grana were present. There was a slight increase in the number and size of the globules. No starch grains were seen and ribosomes were difficult to visualize.

The outline of the chloroplasts was severely distorted after 4 days. In the cacodylate-buffered samples, the main orientation of lamellae was circular; also they had disintegrated into short fragments and sometimes were absent (Fig. 12, 14, 15). Globules continued to increase in number and tended to form clusters, especially in samples taken at the end of the light period. Only a few small vesicles were visible near the periphery. The number of fibrils had increased and ribosomes could not be visualized. The phosphate-buffered samples showed profound lamellar disorganization (Fig. 10, 11) and the stroma was somewhat granular. Many vesicles and large inclusions, sometimes bound by a membrane, could be seen (Fig. 11). Fibrils were obvious. In this tissue the globules were concentrated in one region and were much larger and more numerous than in the control tissue. No starch grains were observed. The chloroplasts of treated plants usually displayed a double-layered envelope, although sometimes not all of it was readily visible because of oblique sectioning. However, the envelope sometimes seemed to be locally ruptured or dissolved where interior degradation had been severe (Fig. 14).

The green apical portions of the affected leaves did not show ultrastructural aberrations until 3 days after treatment. Then, the edges of many chloroplasts became devoid of lamellae and more or less pinched off from the rest of the plastid (Fig. 7). One day later, most of the chloroplasts had abnormal shapes with indentations enclosing cytoplasm, as did those in the chlorotic tissue 3 days after treatment (Fig. 13). Also, large vesicles occurred along the periphery. Some fibrils and ribosomes could be observed, the lamellae had become more disorganized, and the grana were irregularly organized. Starch was present at all stages except in samples taken at the end of the dark period on the 4th day after treatment. Leaf chloroplasts from control plants exhibited no abnormalities during the course of the experiment.

DISCUSSION

The effects of the toxin from *Pseudomonas tagetis* appear to be confined to the chloroplast. Additional results from our observations and measurements of other organelles and internal structures which show no differences between chlorotic and healthy cells support this contention. These results suggest, but do not

prove, that the chloroplast is the primary site of action of the toxin. The relatively short lag time before chloroplast alterations appear in the green apical tissue further suggests that the toxin affects some structure that is metabolically active after chloroplast maturation. The sequence of observed changes points to the lamellar membrane as the primary site of toxin action. However, it cannot be determined from our studies whether this represents target specificity or simply reflects a general inhibition in which some crucial lamellar component is rapidly turning over so that it appears to be selectively affected. A definitive conclusion must await metabolic studies and purification of the toxin.

The increase in osmiophilic globules may be causally related to the disappearance of the lamellar membranes. Lichtenthaler (6) found a close relationship between the content of osmiophilic globules, which he called "plastoglobuli", and the development of the lamellar fraction. He found that if lamellar synthesis was prevented or the lamellae broke down, the number and volume of globules increased. Susalla and Mahlberg (8) showed that extensions of lamellae-free stroma and clusters of osmiophilic globules occur in chloroplasts from phenotypically green leaves of a genetically albino strain of *Nicotiana*. The globules were seen especially in the green-albino plastids in which the lamellae were reduced and were loosely and irregularly scattered in the stroma, thereby confirming the findings of Lichtenthaler (6).

The presence of fibrils which we have interpreted as being composed of DNA is of special interest. Normally DNA fibrils form a fine network in the stroma (2). Susalla and Mahlberg (8) also found similar network-like structures in the stroma of the green-albino plastids of *Nicotiana*. However, under certain circumstances, such as preparation for ultrastructural studies, such structures may clump together, as has been shown to occur with mitochondrial DNA (3). The fibrils may, therefore, simply be an artifact of preparation. In any event, toxin treatment does not permanently affect chloroplast biogenesis, for chloroplasts of plantlets derived from protoplasts isolated from chlorotic tissue of *Nicotiana tabacum* L., induced by treatment with the toxin, are normal (R. D. Durbin, unpublished). Effects of the toxin appear to be specific in that other types of chlorosis-inducing agents do not cause the same ultrastructural changes.

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