Ultrastructure of Chloroplasts of Phaseolus vulgaris Leaves Treated with Benomyl and Ozone

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ABSTRACT

The total chlorophyll content of the primary leaves of pinto bean plants treated with benomyl and exposed to ozone was significantly higher than that of leaves from control plants. The ultrastructure of chloroplasts in the palisade parenchyma cells of primary leaves from control plants consisted of a grana-fretwork system which comprised the greater part of the organelle. The stroma appeared as a fine granular matrix and the grana, for the most part, were regular in shape. Each granum appeared to be a compartmented structure. The other cellular membranes were intact. Palisade leaf cells of plants not treated with benomyl, but exposed to ozone, exhibited striking alterations in the chloroplast and cellular structure. There was almost complete disruption of the chloroplast membranes, the plasmalemma, and tonoplast. The chloroplasts of bean leaves treated with benomyl and exposed to ozone remained intact and were similar to those of the control plants. The results demonstrate that benomyl protects pinto bean leaf chloroplasts from ozone damage.

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Studies concerning the influence of ozone on plant tissue demonstrate that the damaging effects of this air pollutant are associated with a broad spectrum of adverse cytological and biochemical changes (2, 8, 10). The cytological effects are well illustrated by the work of Thomson et al. (20) who observed changes in the stroma of chloroplasts in the palisade parenchyma of bean leaves exposed to ozone. Such changes involve either granulation of the stroma and an increase in electron opacity or the formation of granules and fibrils or plates. Cellular damage is also characterized by membrane disintegration, distortion of mitochondria, and the aggregation of cellular components in the center of the cell.

Benomyl [(methyl-1-(butylcarbamoyl)-2-benzimidazolecarbamate)], a systemic fungicide, has been reported to protect tobacco leaves (18) and pinto bean leaves (13) from the damaging effects of ozone. Further, benzimidazole-type compounds appear to be involved in the retention of chlorophyll in detached leaves (14, 25), the inhibition of the NAD-induced acceleration of chlorosis in detached leaves (4), and the organizational enhancement of chloroplast lamellae (22, 26). Therefore, it was of interest to study the ultrastructure of chloroplasts of pinto bean leaves subjected to both benomyl treatment and ozone exposure.

MATERIALS AND METHODS.—*Growth of plants.*—Seeds of *Phaseolus vulgaris* L. 'Pinto 111' were surface-sterilized in 10% Clorox for 30 minutes and subsequently washed in distilled water. They were planted at a depth of 0.5 cm in 500 ml pots (4 seeds per pot) each containing 450 g of air-dried, loam-sand (1:1) soil.

During germination and seedling growth, the pots were maintained in a growth chamber set at a photoperiod of 14 hours. The relative humidity (RH) and temperature, respectively, were maintained at 70% and 26 C with light (intensity 25,834 lux), and 65% and 21 C during darkness. After emergence of the hypocotyls, the seedlings were thinned to two plants per pot and fertilized by subirrigation with Hoagland's nutrient solution (6).

TABLE 1. Influence of benomyl and/or ozone on the total chlorophyll content of primary leaves of Pinto bean

Treatment		Total chlorophyll content (mg/g tissue, fresh wt.)[a]	
Benomyl (μg/g dry wt. soil)	Ozone (μg/m^3)	Exp. 1	Exp. 2
0	0	23.8 A[b]	25.0 A[b]
0	495	19.9 B	20.8 B
160	0	25.5 AC	27.3 AC
160	495	26.1 C	28.3 C

[a]Values are the mean of five replications.
[b]Means not followed by the same letter are significantly different (Duncan modified least significant difference test. $P = 0.05$).

Benomyl and ozone treatments and chlorophyll determination.—A water suspension of the 50% wettable powder formulation of benomyl, DuPont Benlate Fungicide 1991, was sprayed on the air-dry soil (160 μg benomyl per gram dry weight of soil) while the soil was tossed in a tumbling mixer. Bean seedlings (11-days old) were exposed to ozone in a modified controlled environment chamber. The ozone was produced by the passage of dry, purified oxygen through an ozonator utilizing an ultraviolet light. The plants were exposed for 4 hours at an ozone level of 495 μg/m^3, as measured by a coulometric ozone meter, manually calibrated during exposure according to the neutralized 1% buffered KI method (21). Further, the treatment and control areas of the chamber were maintained at 70% RH, 28 C, and light intensity of approximately 25,834 lux.

For the experiments, plants were grouped according to the following conditions: (i) Plants grown in soil not containing benomyl and not exposed to ozone (nontreated, nonexposed); (ii) Plants grown in nontreated soil but exposed to ozone (nontreated, exposed); (iii) Plants grown in soil containing benomyl but not exposed to ozone (treated, nonexposed); (iv) Plants grown in soil containing benomyl and exposed to ozone (treated, exposed).

According to Dugger and Ting (2), 11-day-old (after sowing) bean seedlings are especially sensitive to ozone treatment and exhibit effects two days after exposure. Therefore, random samples of primary leaves were collected for chlorophyll analysis and electron microscopy 48 hours after the ozone exposure.

Total chlorophyll extraction and determinations were performed according to the method of Arnon (1) and in accordance with the absorption coefficients of MacKinney (9).

Preparation of samples for electron microscopy.—Primary bean leaf samples were obtained from an interveinal area 1 cm from the base of the midrib. The leaf sections (4 × 4 mm) were cut on dental wax in glutaraldehyde fixative and trimmed to 1 × 1 mm sections. The tissue from the designated areas on the leaf did not always exhibit easily observable ozone damage. However, there were no attempts to select tissue only from plants which exhibited visible damage, since physiological and cell structural changes occur prior to the visible effects of the gas (5). Furthermore, sections were taken from at least 25 plants per treatment for each exposure, and there were at least five separate complete experiments performed.

Sections of tissue 1 × 1 mm were cut and subsequently incubated in the glutaraldehyde fixative (3%, glutaraldehyde in 0.05 M potassium phophate buffer, pH 6.8) for 1.5 hours at room temperature, and then overnight at approximately 4 C. Following incubation the samples were washed at room temperature for approximately 1.25 hours in five to seven changes of buffer. For each sample, the washing was discontinued when no glutaraldehyde odor could be discerned. Samples were finally fixed for 2 hours at room temperature in 2% osmium tetroxide, and buffered to pH 6.8 with 0.05 M potassium phosphate buffer. Fixed tissue was dehydrated in acetone and embedded in the "hard" formula of a low-viscosity epoxy resin according to Spurr (17) and as modified by Williams (24).

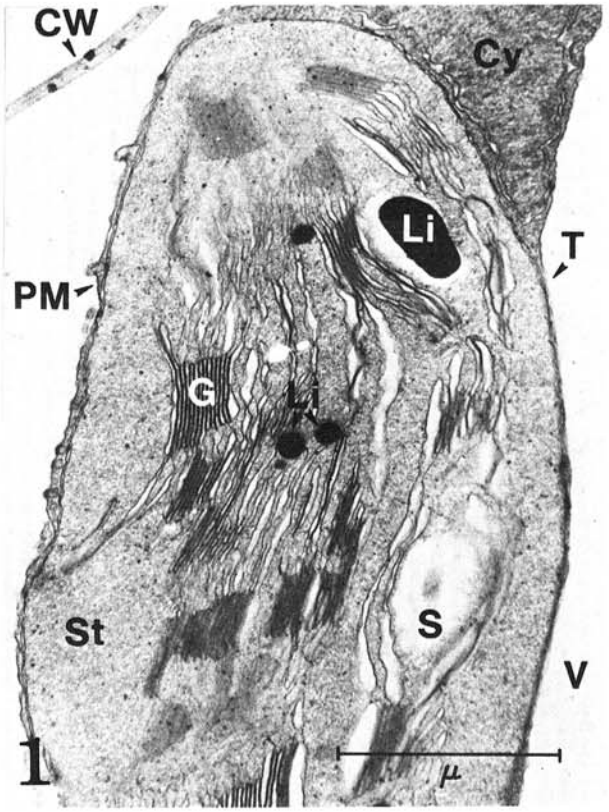
Silver-gray to pale gold sections were cut with glass knives mounted on an LKB 880 A ultramicrotome. The sections were picked up on 48- and 38-μm (300- and 400-mesh) naked copper grids which had been cleansed within 48 hours with glacial acetic acid. The sections were stained with 5% (w/v) aqueous uranyl acetate for 25 minutes, washed in distilled water, and immediately incubated in 0.1% (w/v) lead citrate (15) for 20 minutes. The stained sections were immediately examined with a Hitachi Hu-11c-1 electron microscope at 50 KV as outlined by Sjostrand (16) and Pease (11). Standard photographic methods were employed to develop and print the electron micrographs.

RESULTS.—The primary leaves of nontreated, exposed plants exhibited ozone injury, characterized by chlorosis and necrosis. No visible injury was observed following ozone exposure of benomyl treated plants.

The total chlorophyll content of the primary leaves of nontreated, exposed plants was significantly lower than that of leaves from plants treated with benomyl prior to ozone exposure (Table 1). The data also illustrate the fact that the chlorophyll content of the primary leaves of treated, exposed plants was significantly higher than that of the primary leaves from nontreated, nonexposed plants.

The ultrastructure of the chloroplasts in the palisade parenchyma cells of primary leaves from nontreated, nonexposed plants was similar to that reported by Thomson (19). The single plastid in the micrograph (Fig. 1), which is representative of the sections observed, is

Fig. 1-4. 1) Chloroplast of the palisade parenchyma of a *Phaseolus vulgaris* primary leaf from a nontreated plant. 2) Palisade parenchyma cell of a *Phaseolus vulgaris* primary leaf from a nontreated plant exposed to ozone. 3) Chloroplast of a *Phaseolus vulgaris* primary leaf from a benomyl treated plant. 4) Chloroplast of a *Phaseolus vulgaris* primary leaf from a plant treated with benomyl and exposed to ozone. The structures indicated in the micrographs are: cell wall (CW), plasma membrane (PM), cytoplasm (Cy), vacuole (V), tonoplast (T), stroma (St), granum (G), starch granule (S), fibrils (Fi) and osmiophilic lipid globule (Li). Solitary arrows indicate some end compartments.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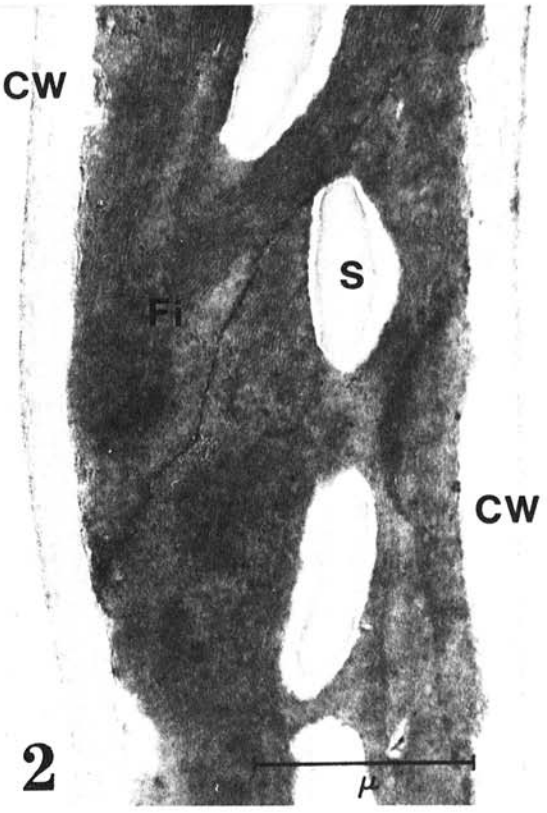
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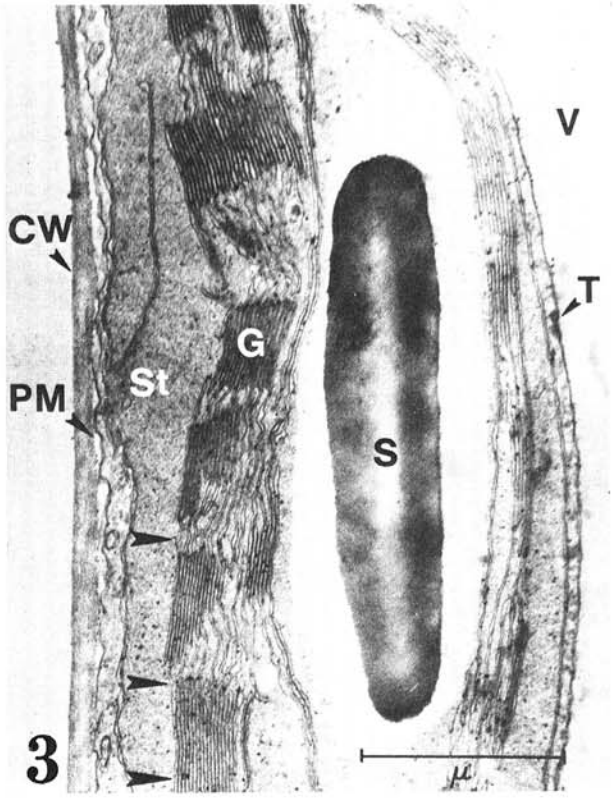
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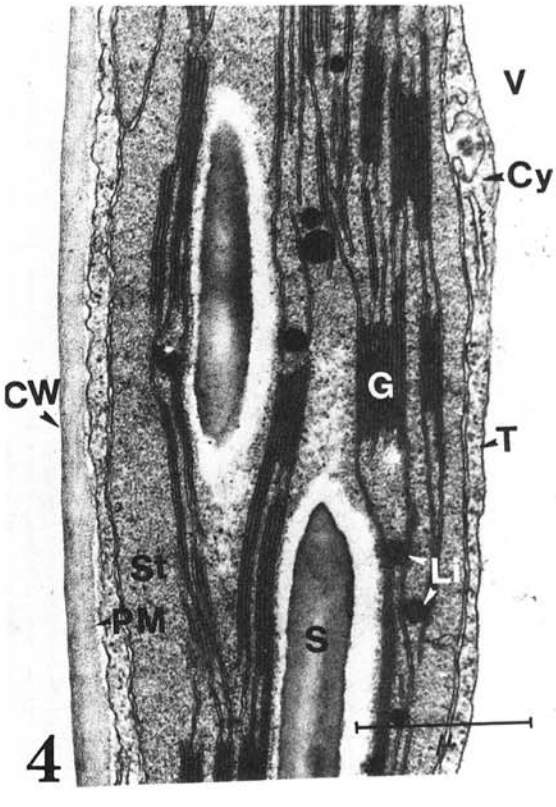
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surrounded by a thin layer of cytoplasm, limited on one side by the plasma membrane and on the other by the tonoplast.

The grana-fretwork system, proposed by Weier (23) comprised the greater part of the chloroplasts of nontreated, nonexposed plants (Fig. 1). The grana, for the most part, were regular in shape and each granum appeared to be a compartmented structure. The margins of the grana completely enclosed all loculi and separated the internal regions from the stroma. The frets were separated from the stroma and connected the grana of the chloroplasts. The partition complex was composed of two electron-dense layers, separated by an electron-translucent region which seldom extended across the grana. The stroma appeared as a fine, granular matrix.

Starch granules, measuring 0.5 μ to 1.0 μ in length were apparent within the chloroplast stroma and were consistently located in a marginal zone of low electron density. The marginal zones were probably due to shrinkage which occurred during the dehydration procedure (7). Osmiophilic lipid globules were present and varied in size and number per plastid.

The organization of chloroplasts from leaves of nontreated, exposed plants were markedly altered (Fig. 2). The stroma was entirely granulated with an increase in the electron opacity. Some fibrils were evident, but there was almost complete degeneration of the limiting membranes of the chloroplasts. Degeneration of the other cellular membranes, plasmalemma, and tonoplast was also evident.

Bean leaf chloroplasts from treated, nonexposed plants (Fig. 3) seemed to be more compartmentalized than those of nontreated, nonexposed plants. The most noticeable distinction observed in the plastids of leaves from treated, nonexposed plants was the increased size of the starch granules which were 2-3 μ in length. These measurements were based upon observation of approximately 100 sections. The low-density marginal zone surrounding the starch granules also appeared larger in the plastids of leaves of treated, nonexposed plants.

The grana-fretwork system was not evenly distributed throughout the chloroplasts of benomyl treated plants and the osmiophilic lipid globules appeared to be reduced in size and in number when compared with chloroplasts of leaves of nontreated, nonexposed plants (Fig. 1).

Chloroplasts of bean leaves from benomyl treated plants exposed to ozone (Fig. 4) remained intact, and the stroma, which appeared as a fine granular matrix, was similar to that of chloroplasts of nontreated nonexposed plants (Fig. 1). The well-defined grana fretwork system (Fig. 4) comprised the larger portion of the plastids; and the starch granules, which measured 2.0-2.5 μ in length were very pronounced. Although smaller than in the chloroplasts of benomyl treated, nonexposed plants (Fig. 3), the starch granules of chloroplasts from leaves of treated, exposed plants (Fig. 4) were larger than those of chloroplasts of nontreated, nonexposed plants (Fig. 1).

The micrographs illustrate that benomyl and/or ozone influence the structure of plastids; and that benomyl does, indeed, protect Pinto bean leaf chloroplasts from ozone damage.

DISCUSSION.—The results obtained in this study demonstrated that the primary leaves from bean plants treated with benomyl contained a higher chlorophyll content than leaves from nontreated plants whether exposed to ozone or not. The increased chlorophyll content of leaves from plants treated with benomyl is similar to that of Waygood's findings with benzimidazole (22).

The ultrastructure of chloroplasts of the primary leaves of plants nontreated and nonexposed (Fig. 1) corresponded to the structure of Pinto bean chloroplasts studied by Thomson (19). The chloroplasts of *Phaseolus vulgaris* leaves of nontreated, exposed plants were markedly altered (Fig. 2). These observations support the findings of Thomson et al. (20) that severe ozone damage results in a general disruption of the cellular components with an aggregation of the cellular components in the center of the cell. Similarly, the characteristic components of the aggregates are believed to be remains of chloroplast membranes and numerous ordered arrays of granules and fibrils (20). This disruption of the cells is very likely due to the damaging influence of ozone on membranes in general (3, 20).

Pellissier et al. (12, 13) have shown that benomyl is a protective agent against ozone damage to vegetation. Further, the results reported herein show that benomyl inhibits ozone damage to vegetation, and maintains the structural integrity of *Phaseolus vulgaris* leaf chloroplasts.

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