Histopathological Characterization of Ozone Injury to Soybean Foliage

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ABSTRACT

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A histological study was conducted to determine the cells and structures in soybean leaves that were affected first by ozone. Primary leaf tissues were sampled 0, 4, and 24 hours after exposure to 590 $\mu g/m^3$ ozone and were examined by both light and electron microscopy to characterize the response. Paraveinal cells were affected first and more extensively than palisade or spongy parenchyma cells. Although more palisade parenchyma cells were injured than were spongy parenchyma cells, the percentage of each cell-type injured in relation to the total number of cells was equivalent. Characterization of symptom development at the ultrastructural level revealed two symptom types. In one case the protoplast collapsed and subsequently became totally disrupted; in the other, there was no cell collapse and membranes including the endoplasmic reticulum, mitochondrial membrane, and chloroplast limiting membrane retained a high affinity for stains. These symptoms may represent two different modes of ozone attack.

Additional key words: air pollution, *Glycine max.*

The phytotoxic nature of ozone has been described for a large number of plant species (6, 7). Hill et al. (6) used the light microscope and described histopathology of ozone injury in 34 plant species. Ultrastructural changes induced by ozone were studied in bean and tobacco (11, 14, 15, 16). Chloroplasts exhibited changes in size and shape, increased stromal density and, in the case of pinto bean, presence of crystalline bodies in the stroma. The changes in chloroplasts occurred prior to cell plasmolysis (14, 15, 16). Alteration of the pinto bean plasmalemma was not observed when altered chloroplasts were observed, but membrane function apparently was impaired since plasmolysis often was noted (14, 15).

The primary site of ozone attack has not been identified. Histological evidence supports the view that the chloroplast is injured first. There is physiological and biochemical evidence to support this hypothesis and other evidence to support an alternate hypothesis that the plasmalemma is the primary site of ozone attack (2, 8, 9, 12, 18). Since ozone must pass through the plasmalemma to reach the cytoplasm, it is plausible that this membrane could serve as the first site of ozone attack. Since electron microscopy studies to determine the sequence of ozone-induced injury have been conducted only with pinto bean and tobacco, the following study was conducted with soybean to define further the primary site of ozone action. Soybean was selected because of its sensitivity to ozone in the field and laboratory (3, 17). By using concentrations of ozone that would induce slow development of mild symptoms we hoped to identify the cell structure(s) first affected by ozone.

MATERIALS AND METHODS

Uniform seedlings of *Glycine max* (L.) Merr. 'Hark' were grown in a mixture (1:1:1, v/v/v) of peat:perlite (Pennsylvania Perlite Corporation, Lehigh Valley, Pennsylvania):Hagerstown silty clay loam soil. Two plants per 10.16 cm diameter pot were maintained in a chamber providing a 14-hour day at 25 ± 2 C, 70 ± 4% relative humidity (RH), fluorescent incandescent light energy with wavelengths between 400 nm and 700 nm of 160 microeinsteins/m^2/sec, and light intensity of 10,700 lux and a 10-hour night at 16 ± 2 C, and 70 ± 4% RH. Sixteen days after the seeds were sown, when the primary leaves were fully expanded and the trifoliolate leaves were beginning to expand, 12 pots with uniformly sized seedlings were exposed to 590 $\mu g/m^3$ (0.30 ppm) ozone for 3 hours. Ozone concentrations were measured as previously described (12). Ozone exposures were conducted in a modified growth chamber at 25 ± 0.5 C, 70 ± 1% RH, fluorescent incandescent light of 300 microeinsteins/m^2/sec, and light intensity of 21,000 lux. An equal number of control plants was placed under identical conditions in carbon-filtered air. Following the ozone exposure the experimental chamber was flushed with carbon filtered air; control and experimental plants remained in their respective chambers until symptoms developed and all samples were harvested.

After ozone exposure the plants were divided into three groups of eight pots each (four exposed and four control), so that leaf tissue could be harvested 0, 4, and 24 hours after exposure. At each harvest 1 mm^2 sections of

interveinal primary leaf tissue were cut under 3% glutaraldehyde in 0.05 M phosphate buffer (pH 6.8), fixed for 1 hour, and then washed in phosphate buffer. Tissue was postfixed in 2% osmium tetroxide in 0.05 M phosphate buffer (pH 6.8) for 2 hours. Sections were dehydrated in acetone and subsequently washed twice with propylene oxide. The tissue then was embedded in epoxy resin as described by Spurr (13).

Tissue embedded in resin was sectioned (2.0 μm thick) with glass knives for examination with a light microscope; sections were heat-fixed onto glass slides and stained with 1% toluidine blue in 1% sodium borate. Single sections were collected at 40 μm intervals to insure that no cell was evaluated twice. Approximately 4,000 cells comprising spongy and palisade parenchyma, and paraveinal cells were evaluated for injury at each of the three harvest times.

Tissue for examination with an electron microscope was sectioned with a diamond knife and stained with 0.3% lead citrate. A Hitachi Model HU-11E-1 electron microscope was utilized to investigate changes in cell ultrastructure in response to ozone.

RESULTS

No macroscopic symptoms were apparent on the foliage until 24 hours after ozone exposure; the symptoms were a mild uniform reddish stipple across the primary leaves of all exposed plants.

Light microscope.—Three cell types in soybean foliage were sensitive to ozone, viz. palisade parenchyma, spongy parenchyma, and paraveinal cells (Fig. 1). Paraveinal cells were the most susceptible to ozone injury. Complete collapse of the protoplast of many paraveinal cells was apparent immediately after exposure to ozone, and the percentage of collapsed cells was higher than for parenchyma cells at all harvest times (Table 1). A 24-hour period was required before the collapse of the protoplast became apparent on greater than 1 percent of the parenchyma cells, whereas 18.7 percent of the paraveinal cells collapsed immediately after the exposure. By 24 hours, 46.0 percent of the paraveinal cells had collapsed but only 17.3 and 13.0 percent of the palisade and spongy parenchyma, respectively, were severely injured. The absolute number of cells injured was different although the percentage of each cell type collapsed was similar (Table 1).

Electron microscopy.—Figures 2 and 3 illustrate a representative spongy parenchyma cell and chloroplast taken from a control leaf. Note the orientation of the chloroplasts within the cell; the chloroplasts float in a thin layer of cytoplasm bounded by the plasmalemma and tonoplast. The chloroplasts are similar in shape and structural orientation to those described by Ballantine et al. (1). The grana are highly developed, and the trilamellar image of the limiting membrane is readily apparent.

Three symptoms were observed (Fig. 4-7). The spongy parenchyma cell illustrated in Fig. 4 exhibited one symptom type. Although the cell showed no sign of collapse, the endoplasmic reticulum, both mitochondrial membranes, and the limiting membrane of the chloroplast have all been intensely stained by lead. This symptom was observed most frequently 24 hours after exposure. Another form of injury expression was total protoplast collapse (Fig. 5). The vacuole was absent and the protoplast was aggregated to one side of the cell. Although some of the chloroplasts and mitochondria became distorted, others remained normal in appearance. The only discernible difference between these chloroplasts and control chloroplasts occurred at the limiting membrane where deposition of lead was apparent (Fig. 6). Similar deposition of the heavy metal stain was observed on the outer membrane of the mitochondria.

Eventually, total protoplast disruption occurred (Fig. 7). The frequency with which total protoplast disruption was observed increased over the 24-hour period. At × 120,000 magnification grana were still apparent, but limiting membranes were no longer discernible. Accumulations of lead stain were apparent where limits of the chloroplast once were.

Ozone injury often was limited to single cells (Fig. 8). Figure 9 illustrates ultrastructural differences between a disrupted paraveinal cell and an adjacent palisade parenchyma cell which apparently was intact. Note the increased cytoplasmic and stromal density of the disrupted protoplast; although grana were still present they were not stacked regularly in the disrupted cell. The limiting membrane was well defined in the chloroplast of the uninjured cell; it was not apparent in the disrupted cell.

DISCUSSION

Paraveinal cells are the most ozone-sensitive cells in primary leaves of soybean. This unusual layer of cells may function in the transport of photosynthate from the palisade parenchyma to the phloem (4). If transport is the function of the paraveinal cells, then ozone sensitivity of this cell type may be significant to other functions of the soybean plant. It has been reported that plants displaying mild ozone-induced chlorosis continued to

TABLE 1. Comparison between percent plasmolysis of palisade parenchyma, spongy parenchyma, and paraveinal cells of soybean leaves at 0, 4, and 24 hours after exposure to 590 $\mu g/m^3$ ozone for 3 hours

Time (hr)	Cell Type					
	Palisade		Spongy		Paraveinal	
	No. observed	No. collapsed	No. observed	No. collapsed	No. observed	No. collapsed
0	2950	2	920	0	315	59
4	2879	40	802	46	292	21
24	2838	493	888	116	297	138

photosynthesize at rates similar to control plants (9). Even if primary productivity is not reduced, diminished transport of photosynthate can be expected to result in a decrease in yield. The reason for sensitivity of the paraveinal cells is open to speculation. They are large cells with diameters as great as 100 μm. Perhaps the large surface area of paraveinal cells increases the probability that ozone will interact with this cell type.

We have found that the spongy and palisade parenchyma were equally sensitive to ozone. Most

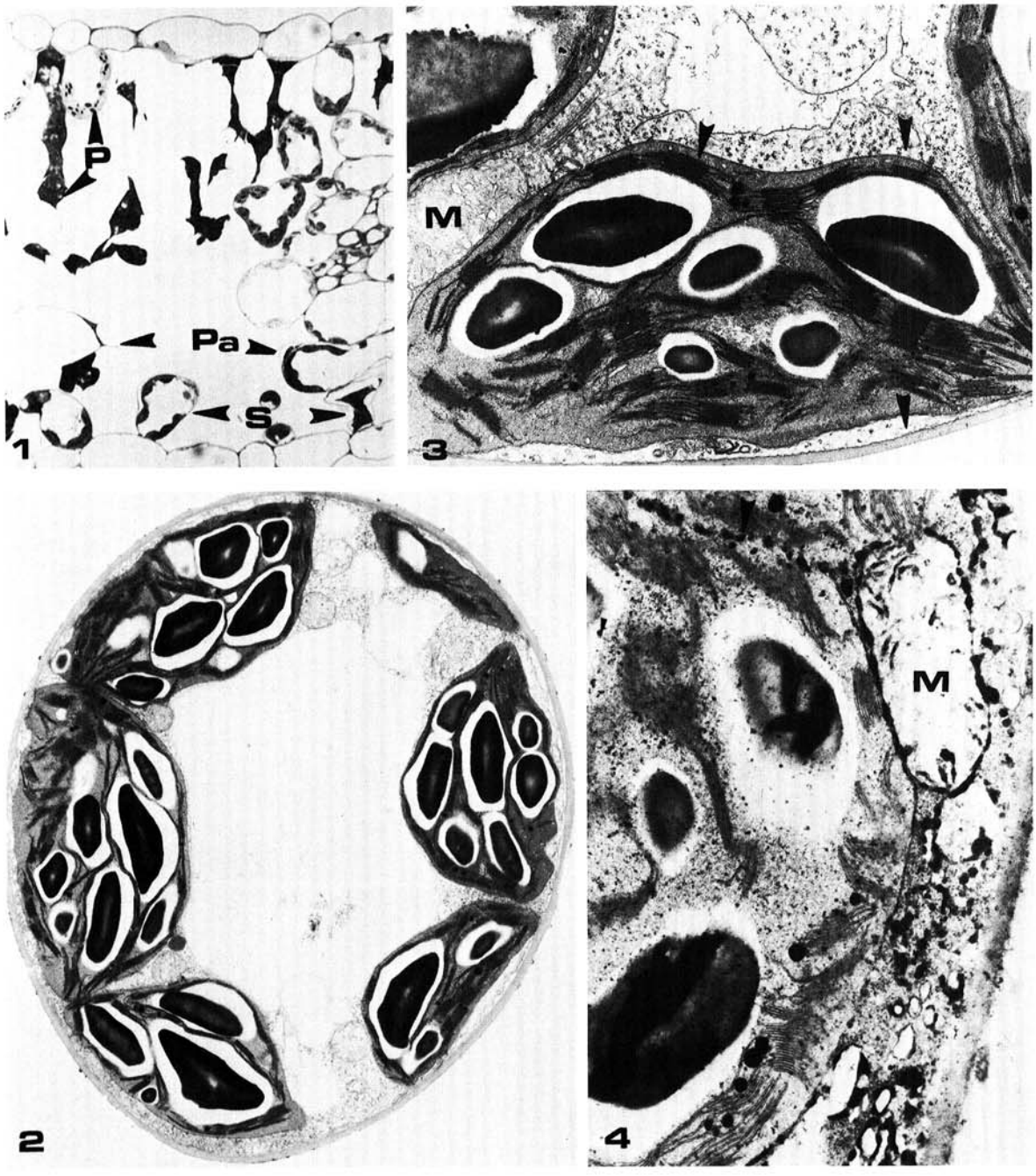

Fig. 1-4. 1) Cross-section of ozone-treated primary leaf of *Glycine max* 'Hark'. Note the collapsed and healthy palisade (P) and spongy (S) parenchyma and paraveinal (Pa) cells (× 337). (2-4) Electron micrographs of primary leaf cells of *Glycine max* 'Hark'. 2) Spongy parenchyma cell of an untreated primary leaf (× 5,800). 3) Chloroplast of a spongy parenchyma cell from an untreated plant. Note the limiting membrane (arrow) and grana (arrow) of the chloroplast, the plasmalemma (arrow) and mitochondrion (M) (× 16,100). 4) A portion of a cell injured by ozone. Note the lead deposition on the endoplasmic reticulum (arrow), the limiting membrane of the chloroplast (arrow) and the inner and outer membranes of the mitochondrion (M) (× 13,000).

reports in the literature state that the palisade parenchyma cells were preferentially injured by ozone (6, 7). Visually, ozone appeared to injure the adaxial surface of soybean. Upon examining cross sections of foliar tissue, however, we found injury to both types of mesophyll at the two leaf surfaces. Although greater numbers of palisade parenchyma were injured than spongy parenchyma, this was directly related to the numbers of cells of each type present; this became apparent when percentages of each cell type were compared and found equivalent. Whether these relationships are unique to the Hark cultivar of soybean or true for other cultivars and species awaits similar comparisons. We observed three basic ultrastructural changes: (i) intensely stained membranes, (ii) protoplast collapse, and (iii) total protoplast disruption. Symptoms (ii) and (iii) may well be sequential; we did not observe an intermediate step leading to collapse or plasmolysis. It was possible that the cell collapse occurred when either the plasmalemma or tonoplast membrane was injured. Subsequent collapse might occur rapidly; if this were the case, the probability of observing intermediate steps in the collapse process would be remote.

Analysis of the micrographs did not provide conclusive evidence concerning the origin of protoplast collapse. Lead accumulation was not observed along the membranes of the plasmalemma or tonoplast. Even in a totally disrupted protoplast (Fig. 7 and 8) lead accumulated only at the periphery of chloroplasts, not along the cell border. To ascertain the integrity of the plasmalemma or tonoplast in response to ozone, alternate methods such as "freeze-fracture," would have to be employed. It was also possible that protoplast collapse did not result directly from plasmalemma or tonoplast destruction but rather from ion imbalances due to alteration of internal membranes of cell organelles.

Whether symptom (i), membrane staining, was related developmentally to the other two symptoms is open to speculation. The fate of the cells with accumulations of lead on the membranes was questionable. These cells may have ultimately collapsed and died or they could have retained some integrity and functioned in a limited way. This membrane staining symptom was observed most frequently 24 hours after exposure to ozone when much protoplast disruption and macroscopic symptoms were apparent. Since we did not assess increased levels of necrosis after this period, we are unable to comment on whether continued symptom development was likely in these cells. It is possible that these cells did not die, but that they survived as injured cells with some functions impaired. Ozone-induced "premature senescence" (5, 10) may be the result of injury which is severe enough to impair function but not induce cell death.

We have found the grana to be extremely resistant. Even the disrupted cells often had perceivable granal structures. This observation was consistent with those of Swanson et al. (14). The change in granal orientation (Fig. 5) may result from fixation stress since we observed this type of change in control plastids as well. We never observed crystal formation reported by Thomson et al. (15, 16) in pinto bean. Since Swanson et al. (14) did not observe crystals in tobacco either, this formation may be characteristic of pinto bean.

A major objective of this study was to determine the sequence in development of ozone injury. We have observed several types of cellular injury. Whether these symptoms are different stages leading to cell death, or distinct types of cellular injury to ozone, is difficult to determine. One problem which confounded subsequent interpretation was the low frequency with which injured cells were observed in the electron microscopy study. For studies such as this one, there may be merit in exposing

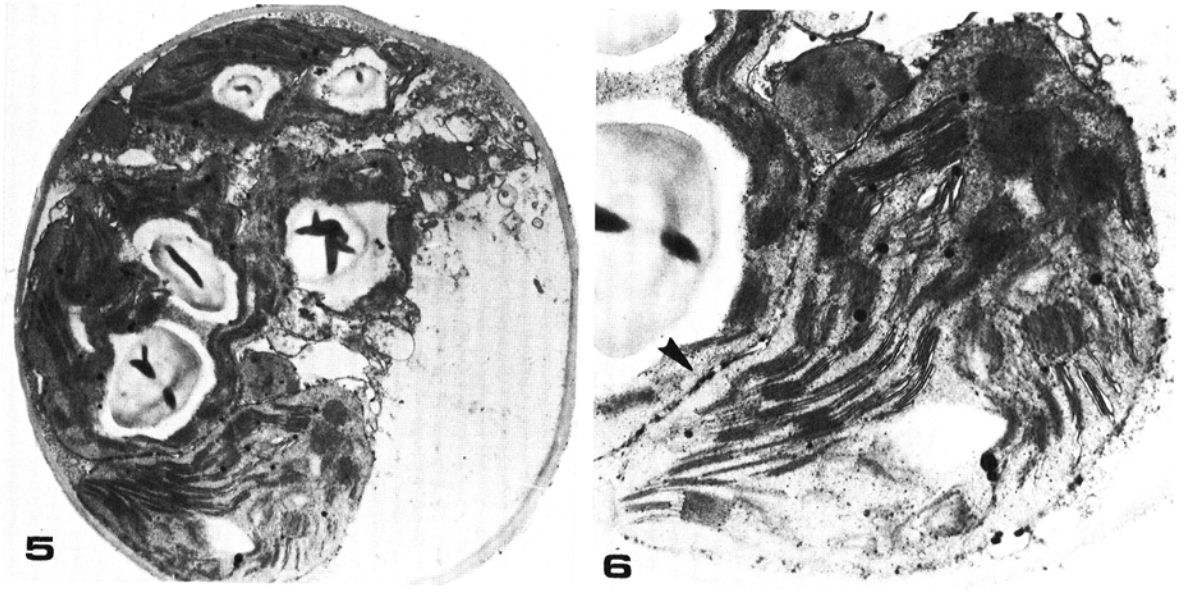

Fig. 5-6. Electron micrographs of spongy parenchyma cells of *Glycine max* 'Hark' injured by ozone. **5)** The protoplast is collapsed but the chloroplasts retain their integrity (× 9,000). **6)** High magnification view of chloroplast in figure 5. Note lead accumulation along the limiting membrane (arrow). Grana are regularly stacked as in control cells (× 18,000).

plants to higher doses of ozone and harvesting tissue at early times. In that manner it might be possible to observe early events in symptom development at higher frequencies.

It is apparent from this study and others (2, 12) that membranes do undergo changes in response to ozone. From our studies it would appear that ozone does not always react with the same membranes. The ultimate fate of the cell may well be related to the membranes which are attacked. Attack of the plasmalemma and/or tonoplast may lead to cell death, whereas injury to other cell membranes (e.g., mitochondrial, chloroplast or endoplasmic reticulum) may only result in limited cell function.

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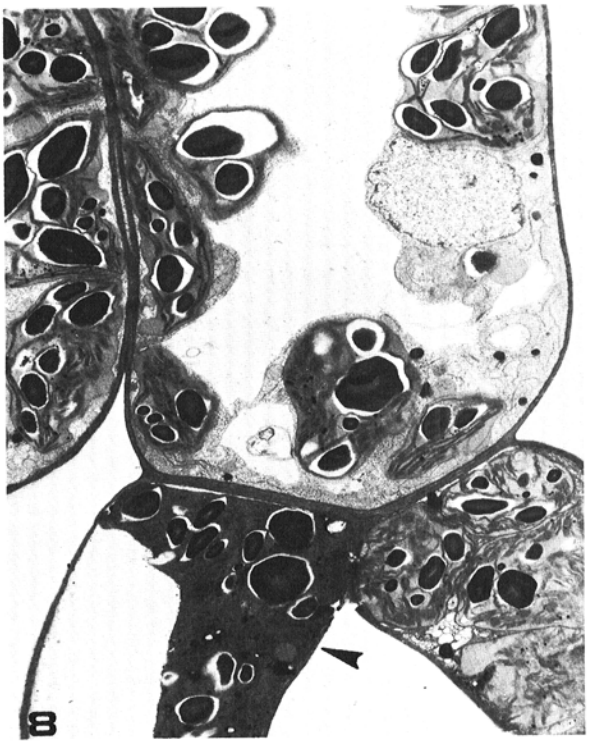

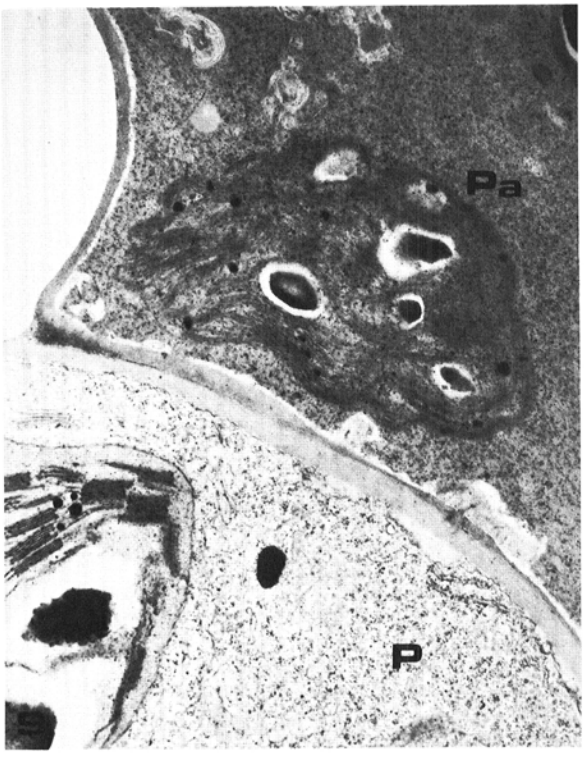

Fig. 7-9. Electron micrographs of cells of *Glycine max* 'Hark' injured by ozone. **7)** Palisade parenchyma cell totally disrupted by ozone. Note the concentration of lead at the junctions between disorganized chloroplasts (arrow) (× 4,000). **8)** Micrograph of palisade parenchyma cells exposed to ozone. Note the cell with collapsed protoplast adjacent to apparently uninjured cells (× 3,500) (arrow). **9)** Micrograph of disrupted paraveinal cell (Pa) adjacent to an uninjured palisade parenchyma (P) (× 15,000).

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