EFFECTS OF OZONE ON
Mnium cuspidatum (L.) Leyss

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ABSTRACT

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By

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Controlled exposures to ozone at concentrations of 0.75 ppm or more for 3 hr caused permanent injury to leaves of the moss species, Mnium cuspidatum (Grout, 1965). Affected areas became distinct with a transparent appearance within 12 hr after exposure. Chloroplasts in ozone affected cells became shrunken and resembled distinct green dots in the injured cells. Gradually, the green chloroplast dots became smaller and less distinct. Eventually, affected areas became almost colorless. There was usually a sharp demarcation line between healthy and injured cells.

Four phases of cytological changes progressively developed in ozone injured cells of moss leaves. 1) Chloroplasts became distinctly shrunken soon after exposure. They were round or elliptical in shape, and were more sharply defined than were healthy ones. 2) Damaged chloroplasts continued to shrink to 1/3 - 1/2 the size of the normal ones. Nuclei disintegrated into a mass of membraneous fragments. 3) Plasmolysis was accompanied by shrinkage and breaking of plasmalemma. As affected chloroplasts further shrunk and/or split, they gradually broke down. 4) Plasmalemma and cytoplasmic contents, including chloroplasts, collapsed completely into a diffuse, jelly-like mass and injured cells ceased to function and did not recover.

Since Mnium cuspidatum does not possess stomata, its ozone sensitivity may be less affected by various environmental conditions.

It responded to ozone promptly and consistently. Hence, Mnium cuspidatum is believed to be potentially a good bioassay material for gross rapid detection of ozone toxicity.

EFFECTS OF OZONE ON *Mnium cuspidatum* (L.) Leyss

By

So-Yung Jane Chai

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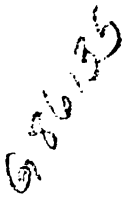

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I. INTRODUCTION

Since 1957, the year ozone was first recognized as a major phytotoxicant to crop plants, it has proved to be the most persistent, if not the most difficult, atmospheric phytotoxicant to control (Richards et al., 1965). Ozone damage has been reported on almost every type of ornamental and agronomic crop (Ledbetter, 1959; Hill et al., 1961). The problem has become increasingly serious in the U.S., during recent years.

Even though intensive research has been carried out to understand and solve the problems associated with ozone damage, many questions still remain unanswered. Among these is the role of stomata in controlling sensitivity of higher plants to ozone. Generally, it has been accepted that ozone injury to higher plants is regulated to a considerable degree by the action of stomata. Some investigators have shown that ozone sensitivity was positively related to opening of stomata (Rich, 1964; Lee, 1965; Macdowell, 1965; Engle and Gableman, 1966; Hill and Littlefield, 1969; Fletcher et al., 1972; Adedipe, et al., 1973). This was accomplished by regulating stomata openings with either environmental factors including light, temperature, water stress, or CO_2 (Heath, 1959; Zelitch, 1961; Ketellaper, 1963), or with chemicals such as phenylmercuric acetate (PMA) (Zelitch et al., 1962) or abscisic acid (Fletcher et al., 1972). However, other investigators failed to observe such a relationship with stomata (Dugger et al., 1962; Dugger et al., 1963; Menser et al., 1963a; Heck et al., 1965; Ting et al., 1968). These discrepancies may have been due to the several differences in techniques (porometer, silicone

rubber impressions, cellulose acetate impressions, transpiration, etc.) adopted by different investigators for studying stomata (Adedipe et al., 1973). It seemed plausible then that studying responses to ozone would be greatly simplified using plants which lack stomata. Since with such plants, the influence of stomatal activity could not be involved in the final response.

Moss leaves lack stomata and are but one cell in thickness, except at the midrib and occasionally around the leaf margin (Grout, 1965). Hence, photosynthetically active cells may be readily observed directly under a microscope. Moss plants have only rhizoids instead of true roots. Although most moss species possess central strands of narrow elongated cells to carry water lengthwise through the stems, they do not have the true vascular bundles of the higher plants in their stems. Furthermore, moss plants are fast-growing and easy to maintain in a small amount of space.

These distinct characteristics make the response of moss to ozone an interesting subject to study. Such a study may add knowledge to our understanding of phytotoxicant action since little is known concerning the response of lower green plants to ozone, environment relations influencing toxic action, and levels of toxicant required to injure moss.

In preliminary studies, several species of mosses were collected and tested for their sensitivities to ozone. Of the group, Mnium cuspidatum was chosen for the present study.

External injury symptoms and progressive cellular changes will be described in the present study. Where possible, responses of moss

to ozone will be compared to those of the higher plants. The possibility of using moss as a bioassay system for rapid gross detection of ozone toxicity will also be discussed.

II. LITERATURE REVIEW

Numerous reports have discussed ozone plant damage in relation to sources (Rich, 1964; Treshow, 1970), species susceptibility (Rich, 1964; Hill et al., 1970), symptoms (Hill et al., 1961; Rich, 1964; Treshow, 1970), growth suppressions (Ormrod, 1971; Adedipe et al., 1972), histological changes (Ledbetter, 1959; Hill et al., 1961; Rich, 1964), metabolic effects (Rich, 1964; Treshow, 1970), environmental predisposition (Heck, 1968), and synergisms with other gases (Menser and Heggestad, 1966; Heck, 1968) etc. (Above are listed the most important discussions selected from a much larger group of reports.) However, since the present study concerns mainly ozone damage observed on moss leaves, only closely related literature is reviewed.

A. Effects of Ozone on Higher Plants

General symptomatology

The degree of injury to susceptible plants is directly related to the concentration of ozone to which they are exposed and to the duration of exposure (Rich, 1964). Although symptoms of ozone injury vary with plant species, there are certain characteristics which appear to be typical of the ozone toxicity syndrome. However, not all of these symptoms are produced consistently on all susceptible plant species.

The earliest macroscopic indication of ozone injury on broad-

leaved, herbaceous species is the appearance of shiny, oily spots due to intercellular water congestion (Taylor et al., 1960; Hill et al., 1970). These water soaked spots are usually visible on the upper leaf surface, and may or may not become permanent lesions. If the damage is not too severe, injured cells may recover.

When tissue is irreversibly damaged, affected areas become water-soaked and dull, gray-green in appearance (Hill et al., 1961). Gradually the water-soaked areas become chlorotic to bleached. Isolated groups of palisade cells between the smallest veinlets are affected first, forming the characteristic punctate or flecked pattern. Flecks are usually white or light tan in color, but on some plants, they are brown to black (Richards et al., 1958). It is generally agreed among investigators that leaves with adaxial palisade parenchyma show the flecking symptoms chiefly on the upper surface of the leaf (Heggestad and Middleton, 1959; Hill et al., 1961; Rich, 1964; Hill et al., 1970). With more severe injury, chlorotic or discolored areas may: 1) become necrotic and collapse; 2) extend completely through the leaf; and/or 3) form bifacial necrotic lesions (Hill et al., 1961; Rich, 1964). Sometimes the entire leaf was killed.

Another symptom of ozone injury is yellowing or premature senescence of older leaves, accompanied by abscission (Ledbetter, 1959; Menser and Street, 1962). The outgrowth of enations on the under surface of broccoli leaves subjected to ozone is an unusual response not seen on other plants (Hill et al., 1961).

The pattern of injury on plants with undifferentiated mesophyll (i.e. grasses) varies only slightly. Damage usually extends

completely through the leaf (Ledbetter, 1959; Hill et al., 1961). Symptoms develop randomly on both upper and lower leaf surfaces. The mild symptom on such parellel-veined monocotyledonous plants as corn, wheat, etc, is a fine chlorotic stippling between the largest veins. When injury is more severe, the bleached flecks coalesce into elongated, chlorotic, interveinal streaks often extending through the leaf blade. Lesions are characteristically pale tan to bleached in color.

Sensitive conifer species may develop pinkish spots in response to ozone. The spots coalesce into bands of dead tissue, and cause a chlorotic or necrotic banding. Tips of the needles beyond this zone generally die, producing a needle tip necrosis (Berry and Ripperton, 1963; Costonis and Sinclair, 1969).

Young plants are usually more sensitive to ozone and mature plants are relatively resistant (Hill et al., 1961; Rich, 1964). In general, on a single plant, the very youngest leaves are the most resistant (Ledbetter, 1959; Menser et al., 1963b). As the leaves begin to expand, they become sensitive at their tips. With continuing expansion, more and more of the leaf area becomes susceptible. The most sensitive stage is when the leaves are about three-quarters fully expanded (Ting and Dugger, 1968). Expanded mature leaves again become resistant, with many of the older leaves sensitive only at their bases (Ledbetter, 1959; Menser et al., 1963b).

Histopathological effects

Usually, palisade tissue comprising the upper leaf mesophyll is most sensitive to ozone and the first injured; next are the interior spongy mesophyll cells and finally, the lower mesophyll cells (Hill

et al., 1961; Rich, 1964). When 2-3 palisade layers were present as in spinach, the outermost was usually, but not invariably, the first to show injury. On leaves without palisade cells, the outer spongy mesophyll cells were most readily affected. Epidermal cells overlaying ozone-injured tissues may or may not show damage, depending on the type and severity of injury. Vascular tissues are most tolerant of ozone, becoming damaged only when the associated tissues are killed (Ledbetter et al., 1959; Hill et al., 1961; Rich, 1964).

Cellular responses to ozone vary with different types of symptoms. The only apparent changes with chlorotic symptoms were disrupted chloroplasts and reduced chlorophyll content of affected cells (Hill et al., 1961).

Dark punctate stipples were caused by thickening and pigmentation of the walls in groups of palisade cells before their collapse (Ledbetter et al., 1959; Richards et al., 1958). The overlaying epidermal and surrounding mesophyll cells appeared to be unaffected. There was a sharp demarcation, with no gradation, between affected and adjacent unaffected cells.

Light-colored upper-surface flecks were produced by collapse of islands of palisade cells (Ledbetter et al., 1959; Hill et al., 1961; Rich, 1964). Epidermal tissues and spongy mesophyll adjacent to injured cells generally remained normal unless injury was severe. Breakdown of chloroplasts was accompanied by plasmolysis of the protoplast. However, plasmodesmatal connections were retained. Cell wall collapse followed plasmolysis and produced large intercellular spaces. This probably gave the lesions the bleached appearance.

Bifacial necrotic lesions were formed because of simultaneous collapse of cells through the affected areas -- including the epidermal layers. The adaxial and abaxial surfaces were drawn close together by the desiccating mesophyll, resulting in thin, papery areas (Hill et al., 1961).

In ozone-injured tobacco palisade cells, Povilaitis (1962) found that nuclei were greatly shrunken and distorted. The nuclear membrane became clearly discernible in some injured cells. Some chloroplasts were disrupted apparently because of excessive swelling. He believed that nuclei and cytoplasm were damaged before the chloroplasts showed abnormalities.

Thomson et al. (1966) reported that effects of ozone on fine structure of palisade cells in bean leaves were of two phases. The first phase involved changes in the chloroplast stroma, which was almost entirely granulated. In some cells, ordered arrays of granules and fibrils also developed in the stroma. In the second phase, nuclei of the damaged palisade cells became shrunken to spherical or irregularly ellipsoid shapes. The plasmalemma was pulled away from the wall and the cellular contents aggregated in the center of the cells. Most of the membrane system, including membranes of plasmalemma, tonoplast, and limiting membranes of chloroplasts and mitochondria, gradually disintegrated into small fragments. A general disruption of cell organelles followed and remnants of the degenerated organelles scattered throughout the cytoplasmic mass. Thomson (1966) also indicated that these changes were probably related to the oxidation properties of ozone.

Effects of ozone on duckweed (Lemna spp.)

Feder and Sullivan (1969) reported that ozone caused depression of frond multiplication and floral production in duckweed, Lemna perpusilla. Feder (personal communication) indicated that stomata of Lemna perpusilla were non-functional. Plants grown in an environment charged daily with a low concentration of ozone (10 pphm) for over 2 weeks were slower to begin multiplying. They had a significantly lower rate of frond doubling and required longer to produce fewer flowers than control plants. Treated plants produced smaller, slightly yellow fronds but had no symptoms of acute injury. Control plants produced four times as many fronds and six times as many flowers as plants continuously exposed to ozone.

Later, Craker (1971) studied effects of ozone on Lemna minor, another duckweed species, and found significant loss of chlorophyll. He suggested that chlorophyll loss could be used as an indicator of injury to Lemna plants following ozone fumigation.

In further studies, Craker (1972) investigated the influence of ozone on RNA and protein content of Lemna minor plants. He found immediate decrease in the amount of RNA and protein in the ozone treated plants, as compared with those not treated. Differences remained detectable throughout a 24-hr sampling period.

B. Effects of Ozone on Fungi

For a long time ozone has been known to have fungicidal activity and has been used in meat and fruit storage to retard fungal development (Richards, 1949). Fungicidal effects of high ozone

concentrations have also been noted both by Magie (1963) and Ridley et al. (1966). However, not until very recently were the effects of ozone determined on additional types of fungi.

Some fungi are as sensitive to ozone as are higher plants. Rich and Thomlinson (1968) first reported effects of ozone on conidiophores and conidia of Alternaria solani. They found that 10 pphm ozone for 4 hr or 100-pphm for 2 hr stopped the elongation of conidiophores, caused apical cells to swell, and cell walls often collapsed at their tips. If removed from ozone and exposed to light to prevent sporulation, injured conidiophores usually resumed elongation. However, the collapsed end wall was sloughed and the new cells often grew at an angle to the original axis of the conidiophores. Sometimes breaks appeared in the pigmented layers of walls of the swollen cells. Given the necessary dark period for sporulation, ozone-damaged conidiophores sporulated normally. If sporulating cultures were exposed to 100 pphm ozone for 30 min, conidia began to germinate while still attached to the conidiophores.

Hibben (1969) exposed detached spores of 14 fungi on agar to 1-100 pphm ozone for 1 to 6 hr and found considerable variation in germination. Larger pigmented spores of Chaetomium sp., Stemphylium sarcinae-forme, S. loti and Alternaria sp. were insensitive to 100 pphm. Spores of Trichoderma viride, Aspergillus terreus, A. niger, Penicillium egyptiacum, Botrytis allii and Rhizopus stolonifer were reduced in germination by 50 and 100 pphm ozone over longer exposures. Small hyaline spores of Fusarium oxysporum, Collectotrichum lagenarium, Verticillium albo-atrum and V. dahliae were the most sensitive, as

germination was prevented or reduced by most exposures at 50 and 100 pphm and were occasionally reduced by doses as low as 25 pphm for 4 and 6 hr. Lower doses sometimes stimulated spore germination. They also reported abnormal growth characteristics of fungus colonies maintained in an ozone atmosphere. However, on air dried spores or spores in a liquid medium, ozone had little inhibitory effect.

Somov et al. (1972) also reported that Verticillium dahliae, Fusarium sp., Penicillium purpurogenum and Alternaria tenuis died in media exposed to certain doses of ozone while Escherichia coli and Candida tropicalis were stable to ozone.

Kormelink (1967) found that the refractive globules in ozone-fumigated mycelia were larger and more numerous than in the controls. The same was also detected by Treshow et al. (1969). Furthermore, they also found that radial growth and spore production of Collecto-trichum lindemuthianum were suppressed when fumigated daily for 4 days with ozone, 10 pphm or more for 4 hr. Macroscopic differences included loss of the characteristic violet color of the cultures; formation of growth rings (or annulations) which resulted from the alternation of aerial and "subterranean" growth, coinciding with fumigation periods. The other two species tested, Helminthosporium sativum and Alternaria oleraceae, were more tolerant to ozone. Neither radial growth nor mass growth was inhibited by 60 pphm ozone. While sporulation of the former was slightly suppressed, that of the latter was greatly stimulated. In all cases, ozonated cultures had a lower lipid content than controls. However, no distinctive differences were noted in fatty acid composition.

Heagle (1970) exposed ten crown rust differential varieties of oats (Avena spp.), infected with Puccinia coronata, to 10 pphm ozone for 6 hr in the light for 10 days. On all varieties tested, rust pustles were significantly smaller on leaves exposed to ozone than on nonexposed leaves. This suggested that ozone treatment significantly reduced the growth of uredial sori. However, urediospores produced on the ozone-exposed plants germinated as well as those produced on nonexposed leaves. The former also produced as many appressoria as did the latter. Exposure of dry leaves at 20 pphm for 3 hr for 1-5 days did not affect urediospore germination, appressoria formation, or penetration.

Heagle and Strickland (1972) also studied effects of ozone on all stages of the asexual growth cycle of Erysiphe graminis f. sp. hordei. Barley plants infected with powdery mildew were exposed to low levels of ozone. Germination of exposed conidia was not significantly affected. However, the number of normal infections was significantly reduced when maturing or germinating spores were exposed to ozone. Of conidia that failed to infect, the germ tube protoplasm appeared to be released at tips of the germ tubes which indicated germ tube rupture. After two exposures, colonies on exposed plants were significantly smaller than on those not exposed. However, after 4, 6 and 8 exposures, colony lengths on ozone exposed leaves were usually significantly larger than those on the controls. Ozone did not significantly affect the number of conidiophores after 4 exposures. Once infection had been established, vegetative growth of Erysiphe graminis hordei was tolerant to ozone or was even slightly stimulated

by ozone treatments. Heagle and Strickland (1972) suggested that either a lack of penetration of hyphae by ozone or a physiological resistance of hyphae protoplasm to ozone may account for this tolerance. They felt that ozone may have reacted with the hyphal cell walls; decomposed; and hence failed to penetrate the hyphae. Otherwise, had ozone penetrated hyphae of powdery mildew, injury should be anticipated.

III. MATERIALS AND METHODS

The apparatus for exposing plants to ozone was prepared as illustrated (Figure 1). Ambient laboratory air was drawn through a variable speed Masterflex Tubing Pump (Figure 1a) with a suitable head (Catalogue No. 7018) to give proper air flow. A Lab-Crest Century Flowmeter (Figure 1b) was connected to the air pump for measuring and adjusting the air flow rate. Air was then hydrated, (possibly "saturated" (Treshow et al., 1969)), by bubbling it through distilled water (Figure 1c). Various concentrations of ozone were produced by a Pen-Ray SOG-2 ozonator (Figure 1d) by varying the rate of airflow and the length of the tubular bulb exposed for irradiation of the air passing over the tube. The latter was accomplished by an adjustable aluminum sleeve which fitted over the bulb. The ozonated air was then passed into a glass chamber, 10 cm in diameter and 43 cm long, which served as a fumigation chamber (Figure 1e). Another glass jar was fitted inside the tubing to support the plants for fumigation. The inlet of the fumigation chamber was maintained about 20 cm above the plants so that exposure of plants would be as uniform as possible.

Estimation of ozone concentration exposed to plants

The relationships between ozone concentration (in ppm) and length of bulb exposed at a flow rate of 1 L/min are given in Figure 2. Theoretical concentrations of ozone produced from the ozonator were estimated. The actual ozone concentrations within the fumigation chamber were not determined due to the lack of necessary quantitative analyzing equipment.

Figure 1.-- Apparatus for fumigating Mnium cuspidatum to ozone

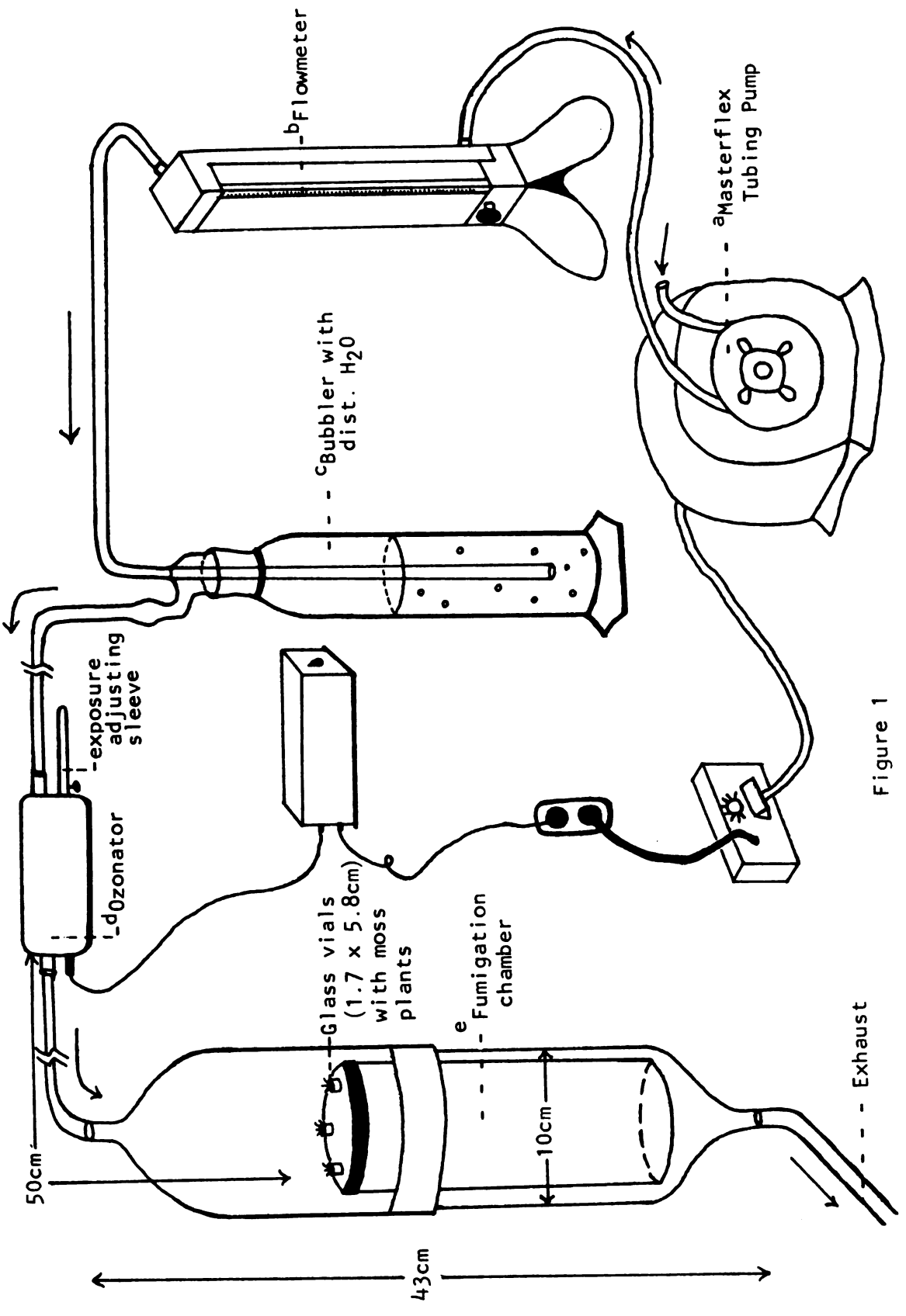

[b]Flowmeter
[a]Masterflex Tubing Pump
[c]Bubbler with dist. H_2O
-exposure adjusting sleeve
[d]Ozonator
Glass vials (1.7 x 5.8cm) with moss plants
[e]Fumigation chamber
Exhaust
50cm
10cm
43cm

Figure 1

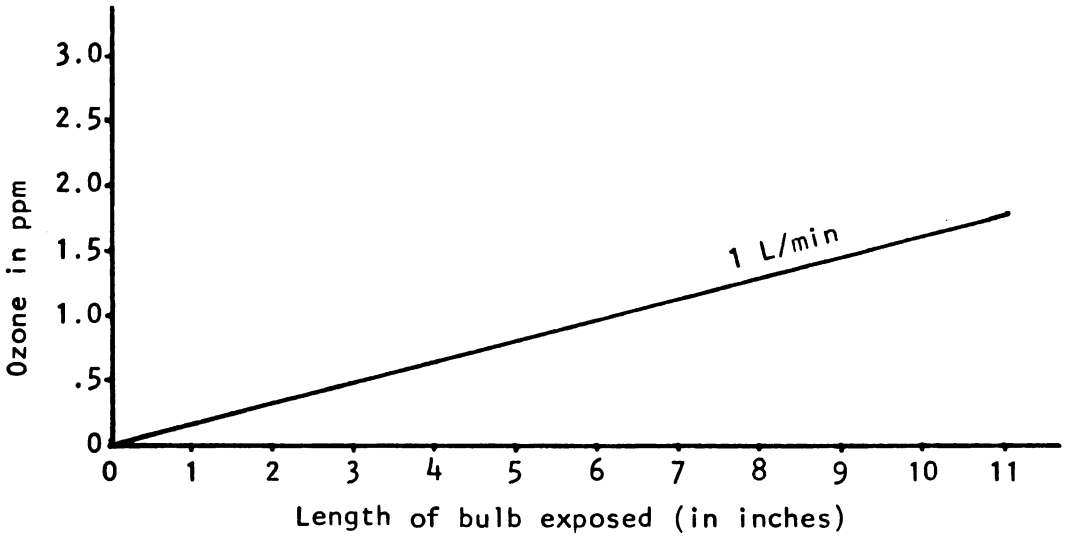


Figure 2.-- Estimation of ozone concentration produced by the ozonator from data supplied by the manufacturer.

Portions of Mnium cuspidatum gametophytes, bearing rhizoids and/or leaves, were planted in glass dishes (diam. 95 x 72 mm) containing vermiculite and covered with a glass plate. They were grown in a growth chamber with ambient laboratory air which was apparently ozone-free. Growth conditions were 18°C, light intensity of 1500 ft-candle at plant surface, and relative humidity of 95-100%. Distilled water was added to the dishes when needed. Some dishes were supplemented with a diluted nutrient solution (Plant Marvel Laboratories, Chicago 28, Illinois; 12, 31 and 14 percent of N, P and K respectively.)

Prior to fumigation, uniform branches of moss leafy gametophytes (hereafter referred to as "moss plants"), with 6-7 leaves, were selected. They were kept in petri dishes filled with water, and examined under a dissecting microscope for any pre-existed injury. Healthy plants were then transferred carefully with forceps to glass vials (diam. 1.7 x 5.8 cm), standing vertically in water moistened vermiculite and immediately placed into the fumigating chamber.

Control plants were exposed either in the light or in the dark to non-ozonated ambient laboratory air in the fumigation chamber for 2 to 4 hours at an air flow of 1 L/min. Before and after exposure, plants in the fumigating chamber were sprayed with water vapor from an atomizer to prevent dehydration.

After control plants were prepared, similar moss plants were exposed to an atmosphere containing ozone, following the same procedure. The bulb of ozonator was usually exposed at lengths of 4-8 inches so that 0.7 - 1.3 ppm of ozone was introduced at the same flow rate as for control plants, i.e. 1 L/min.

After exposure, plants were returned to the water-filled petri dishes and maintained in the laboratory. Injury was observed under an American Optical Co. Spencer Cycloptic Stereoscopic microscope (25x) and a Wild-Heerbrugg microscope at 100x, 400x and 1000x. Sequential comparisons were made between treated and untreated plants up to 30 days.

IV. RESULTS

Moss identification

The moss plant used for fumigation in this study was identified according to the key and descriptions in MOSSES With Hand-Lens and Microscope by Grout (1965), as Mnium cuspidatum (L.) Leyss. Because no capsules were produced from the leafy gametophytes when grown under artificial conditions, identification of the moss species was based chiefly on the characteristics of the sterile shoots. The following are general descriptions of this moss plant:

1. Leaves broadly oblong to obovate, acute, decurrent. Strongly bordered and serrate in the upper half with one-celled single teeth.
2. Costa stout, vanishing in or just below the cuspidate apex, sometimes confluent with the border and appearing excurrent.
3. Leaf cells hexagonal, smaller near the apex, larger near the middle and the base of the leaf, somewhat collenchymatous, not papillose.
4. Leaves strongly shriveled when dry.
5. Sterile shoots prostrate or suberect.
6. Synoicous, capsules single. (This was not confirmed due to the previously mentioned reason.)

Comparisons between dark and light fumigation

In preliminary studies, two sets of experiments were initially conducted, one with light (500 ft-candle at plant surface), the other without. Results were compared to evaluate qualitative differences in injury between light and dark fumigation. However, desiccation

injury was sometimes found on moss plants exposed to ozone in the light. In order to eliminate possible complication of light and its effect upon humidity, temperature, etc., subsequent experiments were carried out in the dark.

Symptoms of external injury

Degree of injury was directly related to concentration of ozone and duration of the exposure to which M. cuspidatum gametophytes were exposed. Due to the lack of appropriate analytical equipment for determining the actual ozone concentrations applied to moss plants, the "threshold" concentration necessary for causing evident and permanent ozone injury on moss leaves could not be critically determined. An exposure of approximately 0.75 ppm ozone (theoretical value estimated according to Figure 2) for 3 hr was required.

The first indication of ozone injury on moss was evident immediately after exposures as chlorotic, water-soaked areas on both the upper and the lower leaf surfaces. If moss leaves were permanently injured, affected cells were conspicuous with a transparent appearance. Chloroplasts became distinctly shrunken and resembled well defined green dots. Irregularly shaped affected areas gradually became more clearly defined with very clear-cut boundaries. Within the damaged areas, almost every cell was injured. As the chloroplasts were disrupted and cells degenerated, both the upper and lower surfaces of affected areas were slightly depressed below the normal surface of the leaf. The dot-like injured chloroplasts progressively became smaller and less distinct. Eventually, damaged areas became completely bleached (Figure 3). However, there was no apparent rupture of

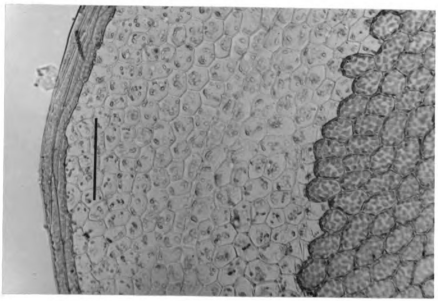


Figure 3.-- Leaf of Mnium cuspidatum, 3 weeks after exposure to 1 ppm ozone for 3 hr. Damaged areas were almost completely bleached while border remained apparently unaffected. (Bar equals 100 μ).

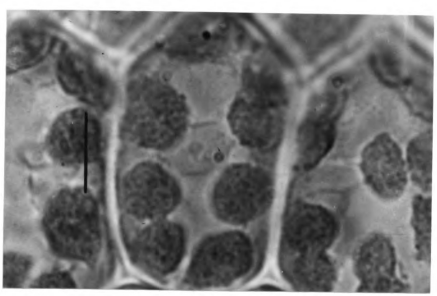


Figure 4.-- Normal cells in non-ozonated leaf of M. cuspidatum, shows the nucleus with nucleolus. Protoplasmic streaming was evident. (Bar equals 10 μ.)

cell walls nor dissolution of middle lamella. Cells around lesions did not develop the reddish brown pigment as was usually observed in desiccation-injured moss leaves.

Usually, desiccation injury could be distinguished from the typical ozone injury by the second or the third day after treatment. It was characterized by the rapid break down of chloroplasts and complete collapse of cells including the cell walls. Injured areas gradually became papery and finally holes appeared. The cells surrounding the holes were usually reddish-brown.

When moss leaves were exposed to sub-lethal concentration, cells occasionally recovered from injury 12 to 24 hrs after fumigation and the leaf returned to normal appearance.

Since only one layer of cells is present in most of the leaf area of Mnium cuspidatum, injury was visible from both sides. The third to fifth leaves from the shoot apex were most prone to injury. In a single leaf, cells adjacent to and interior to the elongate, marginal cells (borders) were most sensitive and were evidentally the first injured. Next in sensitivity were the cells close to the tip and at the base of the leaves. And finally, the more mature cells between the midribs and the edges of the leaves. To some extent this order of sensitivity varied due possibly to uneven exposure to ozone, and the age of the tissues. Borders of the leaves usually remained normal unless injury was severe. Midribs and stems were most tolerant of ozone, and were damaged only when injury was very severe.

Cytological changes

Chloroplasts in the younger cells of healthy moss leaves were somewhat square shaped with the margins rounded. Some were constricted in the middle and were dumb-bell shaped. Cytoplasm appeared as a turgid, fine granular matrix. Under high magnifications (1000x), numerous granules and small spheruoles were present, apparently within the chloroplasts (Figure 4). The plasmalemma, apparently unaffected, was usually not detectable. In most cells, nuclei and nucleoli (Figure 4), vacuoles, cytoplasmic strands and protoplasmic streaming were readily apparent when focused properly.

As the leaf matured, chloroplasts enlarged and filled the space within the cell. Limited by one another, they became irregularly shaped. In very old leaves, the sides of chloroplasts almost touched each other. Dumb-bell shaped chloroplasts (Figure 4) constricted in the middle were commonly found in most cells. The uninjured control plants normally remained healthy and exhibited no apparent changes for a period of time until they degenerated naturally.

Sequential microscopic observations of ozone-injured moss plants suggested that at least four stages of cellular changes were involved. Because both the dosage (exposure time and concentration) of ozone and the age of tissues affected the response patterns of moss, injured cells did not necessarily go through all four stages. For instance, if cells were only slightly damaged, chloroplasts might temporarily shrink into round or elliptical shapes, and spread less evenly in the cytoplasm. But with time, they gradually expanded again and regained their normal appearance. In contrast, cells

exposed to extremely high concentrations of ozone might be irreversibly injured. In these cells, chloroplasts quickly disintegrated into fragments, nuclei disappeared, plasmolysis occurred, plasmalemma broke down, and cells began to degenerate.

The following are general descriptions of the progressive pattern of ozone injury. They were determined by comparative studies of the cellular structures of slightly, moderately, and severely damaged moss leaves.

Phases of ozone injury:

Phase 1.--Microscopic examination immediately after exposure to 1 ppm ozone revealed that chloroplasts seemed to be the first organelle to respond to ozone, becoming markedly shrunken, round or elliptical (Figure 5). Some of the dumb-bell shaped chloroplasts formed deep furrows transversely in the middle. Affected chloroplasts became more sharply defined and less granulated than in healthy cells, and were less evenly distributed in the cytoplasm. Injured cells were more transluscent than the normal cells possibly because shrinking of chloroplasts exposed greater space between plastids.

Under higher magnifications (1000x), nuclei as well as cytoplasmic strands appeared to be normal. Protoplasmic streaming was very active and distinct. Plasmalemma and tonoplasts also appeared to be normal. The spheruoles within chloroplasts seemed to be smaller and less distinct, possibly in a process of disappearing.

Phase 2. -- With time (12 hr after fumigation with 1 ppm ozone for 3 hr), damaged chloroplasts continued to shrink to about 1/3 - 1/2 the size of the normal ones (Figure 6). They aggregated in groups,

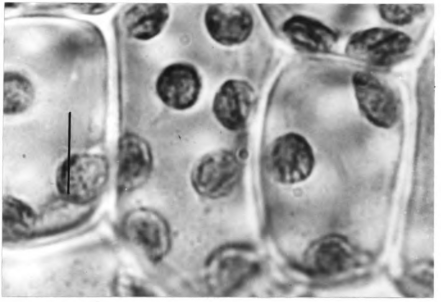


Figure 5.-- Ozone injury -- phase 1. Leaf cells of M. cuspidatum immediately after exposure to 1 ppm ozone for 3 hr. Chloroplasts have shrunk noticeably. (Bar equals 10 μ.)

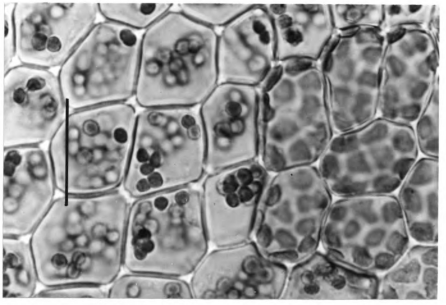


Figure 6.-- Ozone injury -- phase 2. 12 hr after exposure of M. cuspidatum leaf to 1 ppm ozone for 3 hr. Chloroplasts aggregated in groups and were about 1/3 - 1/2 of the normal size. (Bar equals 30 μ.)

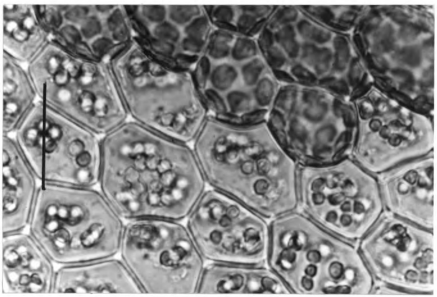
either in the center or near the edge of cells. Short connections were sometimes detectable between adjacent chloroplasts before they separated completely. This suggests that the dumb-bell shaped chloroplasts might have split along the constriction, and formed the abnomal appearance. Another noticeable change within the chloroplasts was that spheruoles were no longer visible. Instead, short dark lines were prominent.

Meanwhile, nuclei underwent a series of changes, and eventually some of them disintegrated into a mass of membraneous fragments.

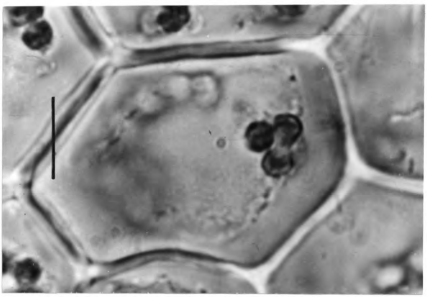
The plasmalemma and tonoplast showed no sign of breaking down at this stage. Cytoplasmic strands remained clearly visible, although strands seemed to be shortened by the grouping together of shrunken chloroplasts. Protoplasmic streaming seemed to be less active.

Phase 3. -- Plasmolysis was evident in the injured cells as early as one day after exposure to 1 ppm ozone for 3 hr (Figure 7a). With breaks developing, the plasmalemma pulled away from the cell wall. Highly granulated cytoplasmic contents clumped in the center of the cells. Tonoplasts broke down and vacuoles collapsed.

As a result of further shrinkage, affected chloroplasts became even smaller, about 1/4 the size of the normal ones. Some chloroplasts superficially resembled dividing animal cells in late-telophase (Figure 7b), and might have been splitting into two halves. Occasionally, tiny empty vesicles were attached to the chloroplasts. Since the numbers of total existing chloroplasts in the affected cells did not significantly exceed those in the healthy cells, what actually happened to those chloroplasts is still in speculation. One possible



(a)

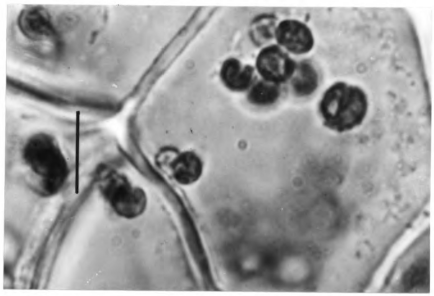


(b)

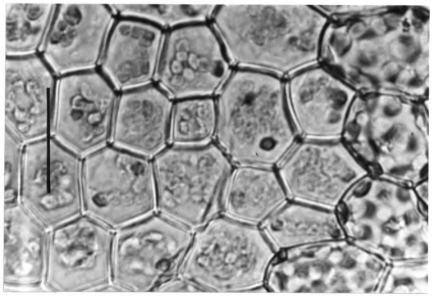
Figure 7.-- Ozone injury -- phase 3. Leaf of M. cuspidatum, 1 day after exposure to 1 ppm ozone for 3 hr. a) Plasmolysis with disintegration of plasmalemma. Cytoplasmic contents clumped in the center of cells. (Bar equals 30 μ.) b) Chloroplasts may be splitting into two halves (Bar equals 10 μ.)

explanation is that, after splitting, only some of the divided chloroplasts retained their integrity and turgidity. The others may have disintegrated. Protoplasmic streaming, though still detectable, was very slow.

Phase 4. -- As injury progressed, more and more breaks appeared in the limiting membrane of chloroplasts (Figure 8a). Losing their integrity, the chloroplasts began to break down. Eventually, plastids were partially or completely bleached, and only a few plastids retained color. The plasmalemma and cytoplasmic contents collapsed completely into a diffuse, jelly like mass. The remnants of various degenerated organelles and membranous fragments could hardly be distinguished (Figure 8b). However, no apparent changes were observed in thickness of cell walls nor in size of intercellular spaces between ozone injured cells.



(a)



(b)

Figure 8.-- Ozone injury -- phase 4. Leaf of M. cuspidatum, 2-3 weeks after exposure to 1 ppm ozone for 3 hr. a) Breaks developed in the limiting membrane of chloroplasts. (Bar equals 10 μ.) b) Cytoplasmic contents have broken down and collapsed, and cells have degenerated. (Bar equals 30 μ.)

V. DISCUSSION

Mnium cuspidatum is potentially a useful bioassay plant for detection of ozone toxicity. Due to the fact that Mnium plants do not have stomata, sensitivity to ozone may be less affected by various environmental conditions, such as light, water stress, temperature, etc. In my trials, similar responses were obtained under different light conditions, provided that moisture was maintained throughout the experiment. The relatively low sensitivity to variations in light intensity suggests that moss may be useful in a bioassay system. In these trials, presence or absence of light seemed to have minor influence on symptom development and sensitivity of plants. In addition, Mnium cuspidatum responded to ozone promptly, and injury was usually manifested within 24 hr after fumigation. Severity of injury could be assessed directly by microscopic examination of both size of affected areas and damage to cell organelles.

The main disadvantage of such a bioassay system is the difficulty in maintaining high moisture throughout the experiment so as not to desiccate moss plants during exposure.

Practicability of moss as a bioassay plant could not be established with certainty because the actual threshold concentration for ozone injury could not be determined critically due to lack of suitable analytical equipment. However, the threshold exposure for injury of moss was approximately 75 pphm for 3 hrs (as estimated according to Figure 2). This intensity of exposure to ozone is considerably higher than that required to injure sensitive Bel W-3 tobacco (5 pphm for 4 hrs) or to injure eastern white pine (7 pphm

for 4 hrs) (Hill et al., 1970). The possibility exists that the actual ozone concentration to which moss plants were exposed may have been somewhat less than that estimated (Figure 2) due to the relatively slow rate of introduction of ozone into the system (1 L/min) and sorption of ozone by the plants or by spontaneous reversion to O_2.

The presence of a leaf like structure a single cell in thickness permits sequential microscopic observation of cellular responses. Furthermore, lack of stomata permits responses apparently less complicated by environmental variation than is possible with the higher plants. This advantage may more than offset the disadvantage of relative insensitivity to ozone.

Even though the various cells of the moss leaf were apparently equally exposed to ozone during exposure, differences in tolerance to ozone toxicity were apparent. Cells at the leaf border were markedly more tolerant to ozone than were those in the central portion of the lamina. Cells of the midribs and stems were also more tolerant than those of the central leaf lamina. Tolerance in the midrib and stem might well be associated with multiple layers of cells because the midribs and stems are more than one cell in thickness. Further work is necessary to explain these cellular differences.

Craker (1971) suggested that chlorophyll loss could be used as an indicator of ozone injury to Lemna plants. Perhaps this might also be true for moss plants, and possibly ozone toxicity could be measured quantitatively and correlated to ozone concentration.

VI. SUMMARY

A study was made of the effects of ozone on leaves of the moss plant, Mnium cuspidatum. If exposed to 0.75 ppm ozone (or higher) for 3 hr, moss leaves suffered permanent injury. Affected areas were extensive and evident, with a transparent appearance. Chloroplasts following substantial shrinkage were visible as numerous small green dots from both sides of the injured leaves. As injury progressed, those green dots gradually became smaller and less distinct. Eventually most of them became colorless, giving the affected area a bleached appearance.

Cytological changes induced by ozone were in four successive phases. 1) Immediately after ozone exposure at 1 ppm for 3 hr, chloroplasts became markedly shrunken. Injured cells were more transluscent than the normal cells. 2) Within 12 hr after ozone exposure, damaged chloroplasts shrunk to about 1/3 - 1/2 the size of normal chloroplasts and they were often aggregated in groups. Meanwhile, nuclei gradually disintegrated, resembled a mass of membraneous fragments. 3) Plasmolysis began in the injured cells one day after exposure. Plasmalemma pulled away from the cell wall and disintegrated. Cytoplasmic contents clumped in the center of the cells. Chloroplasts continued to shrink. Splitting of chloroplasts into two parts was also suspected. 4) Chloroplasts disintegrated into fragments and the debris and remnants of other disintegrated cell organelles were surrounded by a diffuse, jelly-like mass. Eventually, the degenerated cells were almost completely colorless.

Usually, the third to fifth leaves from the shoot apex were

most prone to injury. In a single leaf, cells adjacent to and interior to the borders were most sensitive. Mature cells near the midribs were more tolerant, borders and midribs of the leaves, and stems were most tolerant of ozone.

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