Histopathology of Oxidant Injury and Winter Fleck Injury on Needles of Western Pines

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ABSTRACT

In comparison of ozone injury, total oxidant injury, and a winter fleck injury of pine needles, no histological difference was found between the two air pollutant injuries in ponderosa pine needles. In both, injury was confined to the mesophyll cells, especially those nearest the abaxial needle surface, and no other cell types were affected. Needles collected from remote areas free of oxidant air pollutants, and from areas where oxidant damage is known, both showed necrotic flecking of only the abaxial needle surface. In tissue with winter flecks, principally the mesophyll cells, but also endodermal, transfusion, and vascular cells were affected. Hyperplasia in the endodermis and transfusion area, was common only with winter fleck. No detailed account of the development of the winter fleck can be derived from histological evidence, but fleck symptoms are distinctly different from those of ozone, total oxidant, fungus infection, and sucking insects. The presence of winter fleck in needles exposed to snow suggests that the condition may be best characterized as winter weather injury.

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Additonal key words: ozone, peroxyacetyl nitrate, nitrogen dioxide.

The correct identification of the causes of various conifer needle symptoms is very difficult, unless the etiology of the disorder can be observed. If biotic agents are ruled out as a cause, an attempt to reconstruct the recent environmental history of the specimen is necessary to determine the role of air pollutants, temp extremes, or other environmental stresses. Histological examination of needle tissue is a useful complementary diagnostic tool (6, 8, 13), within certain limits (14.)

The need to properly identify the agents causing injury to pine needles comes into sharp focus where construction of new pollution sources, such as fossil-fuel-burning power plants in the arid west, requires environmental monitoring and preparation of environmental impact statements. A precise description and proper identification of existing needle symptoms must be available before the effects of pollutants can be evaluated.

This paper describes visual and histological symptoms of needle injury by ozone alone, and by total oxidant air pollutants including ozone, peroxyacetyl nitrate, its homologs and nitrogen dioxide, as distinguished from a winter fleck injury of pine needles of uncertain etiology. The winter fleck is common throughout the western United States in areas subject to frequent oxidant air pollution exposure, as well as vast areas remote from any pollutant source or roadside influence.

MATERIALS AND METHODS.—*Needle collections.*—In the field, 1-yr-old needles showing necrotic flecks were collected from areas subject to heavy, light, and no oxidant air pollution. Needles of *Pinus ponderosa* var. *scopulorum* Engelm. collected near Flagstaff, Arizona, where no pollutants were suspected, showed winter fleck injury on the abaxial leaf surface; additional samples were collected at several locations along the east slope of the Rocky Mountains in Colorado, by John M. Staley. Needles were obtained from *P. ponderosa* Laws. at Mount Palomar, California, and at Tiger Flat in the southern Sierra Nevada, where occasional incursions of oxidant air pollution may be expected. Needles of *P. lambertiana*, sugar pine, were also collected at Tiger Flat.

One-yr-old ponderosa pine needles displaying winter fleck injury alone, or both total oxidant injury (chlorotic mottle) and winter fleck, were obtained 6 and 26 August 1970 from saplings that had been enclosed in greenhouses provided either with carbon-filtered air or ambient total oxidant. The trees had received treatment for 4 yr during 8 mo annually (April to November), when heavy oxidant air pollution was present in the San Bernardino Mountains of southern California. Current-year and 1-yr-old needles were also collected from control saplings outside, but adjacent to, the two greenhouses.

Finally, current-year and 1-yr-old ponderosa pine needles were collected from container-grown seedlings which had produced secondary needles in carbon-filtered air, and were then fumigated with 882 $\mu g/m^3$ ozone, for 12 h daily for 4 wk, until prominent symptoms of ozone injury (chlorotic mottle) appeared. The fumigation chamber covered with Kreen plastic film, received natural sunlight, and refrigerated air did not allow midday temp to exceed 32 C. Samples were collected at the end of the fumigation period and compared with current-year needles of saplings exposed to total oxidants.

Histology.—The histological methods for ozone-injured needle samples have been previously outlined (6, 7); all tissue samples were obtained 10 mm from the needle tip, fixed in freshly prepared ethanol-acetic acid (3:1, v/v), dehydrated through a tertiary butanol series, and were paraplast infiltrated. Leaf sections were soaked in a 2% aerosol OT (American Cyanamid Co.) solution for at least 2 days before microtome sectioning at 30 μm and stained according to the method of Shellhorn and

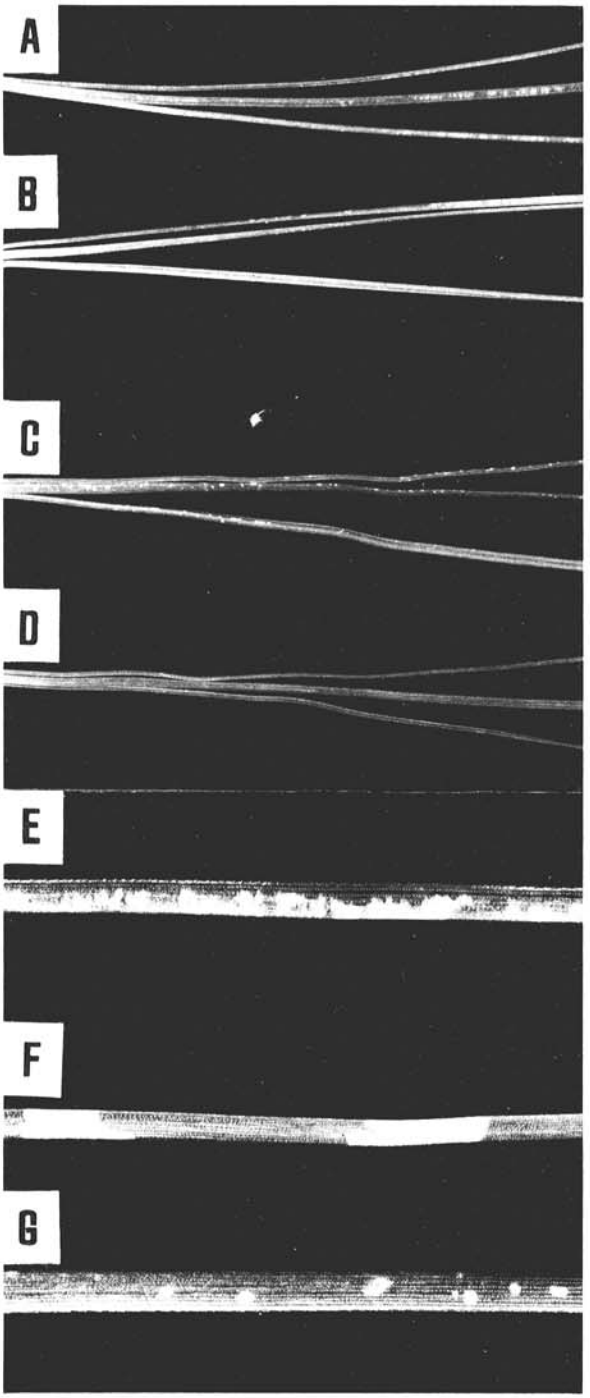


Fig. 1-(A to G). Needles of ponderosa pine. Chlorotic mottle from ozone injury is found on both abaxial **A)** and adaxial **B)** needle surfaces; necrotic flecks of uncertain origin are located on the abaxial surfaces exposed to the sky **C)** and are absent on both abaxial and adaxial surfaces not exposed to the sky **D)**. Necrotic lesions caused by chronic ozone injury **E)**, and by acute ozone injury **F)**, differ in appearance from the discrete necrotic fleck of uncertain origin **G)**.

Hull (12). During this schedule, some samples were stained in acid fuchin-erythrosin solution immediately after paraplast removal and hydration to 70% ethanol; the remainder of the procedure was unaltered. This shorter schedule avoided overstaining by methyl violet and Bismarck brown, which often obscured cellular details. Some sections were stained with thionin and orange G according to the method of Conn (3) to investigate the necrotic flecks for fungus hyphae.

RESULTS.—*External needle symptoms.*—Important distinctions exist between the necrosis attributed to chronic and acute ozone injury and the necrotic flecks of unknown cause. Chronic ozone injury is present on both abaxial and adaxial surfaces (Fig. 1-A, B); it has relatively indefinite boundaries, with small islands of green or yellow tissue often present (Fig. 1-E). Acute injury (Fig. 1-F) has well-defined boundaries and all tissue is necrotic.

Comparisons of external needle symptoms caused by total oxidant in the field with those caused by 0.45 ppm (882 $\mu g/m^3$) ozone during controlled fumigation of ponderosa pines, revealed no important differences. The symptom of chronic ozone injury (chlorotic mottle) on current-year and older needles was identical with that of total oxidant injury, even though it had developed more rapidly during fumigation (20-30 days) than under field conditions (40-60 days).

The appearance and intensification of total oxidant injury symptoms on current-year needles of sapling ponderosa pines enclosed in the greenhouse and provided with ambient total oxidant from April through November each year, was slightly less advanced than on outdoor trees nearby. No symptoms appeared on needles of trees in the companion greenhouse provided with carbon-filtered air.

The winter fleck injury present on all needle samples collected from the field, were generally smaller and more nearly circular than the lesions attributed to ozone or total oxidant injury. Their boundaries were well defined. Flecks were present only on the abaxial surface of 1-yr-old or older needles, directly exposed to the sky (Fig. 1-C, D, G). In all the needles observed, necrotic flecks were randomly distributed along the length of the needle. They became more frequent with increasing age of the needle whorls retained on the stems. No flecks were visible on current-year needles which had not been exposed to winter weather.

The 1-yr-old needles collected from the enclosed sapling ponderosa pines showed necrotic flecks on the abaxial surface, whether the trees received total oxidant or carbon-filtered air during the summer period of enclosure. The trees in both houses had been exposed to winter weather, including intermittent snow loads.

On two occasions, a heavy snow caused the collapse of several roof sections of another plastic-covered greenhouse enclosing container-grown stock which had been continuously maintained in carbon-filtered air for 2 yr. Necrotic flecks were observed the following spring, only on those plants which had contact with snow and had been buried for several weeks.

Needle samples from sugar pine collected at the same location as a ponderosa pine sample at Tiger Flat in the southern Sierra Nevada of California, had abundant necrotic flecks on each needle, as did nearby ponderosa

pines. Because sugar pine is known to be much more tolerant to total oxidant and ozone than other ponderosa pine (9), this offers additional evidence that oxidant air pollutants are not involved in the winter fleck injury syndrome.

Histology of uninjured control needles.—Current-year, uninjured secondary needles of ponderosa pine, stained by the method of Shellhorn and Hull (12), have been described in detail (6). Plicate parenchyma (mesophyll) cells showed a large number of red-staining plastids with green cytoplasm. In sections fixed with ethanol-acetic acid, the cell wall stained a dark green that was easily distinguished from the lighter-colored cytoplasm. Nuclei were usually centrally located and occasionally present in a cell branch (Fig. 2-A).

In mesophyll cells of uninjured 1-yr-old needles, staining differed from that of current-year needles; i.e., the cell cytoplasm in the older tissue retained a darker cytoplasmic green stain approaching that of the cell walls. Nuclei in 1-yr-old sections were larger and accumulated more violet color than in current-year tissues. Two-year-old uninjured needles had thicker mesophyll cell walls that generally accumulated more stain.

Histopathology of needles injured by ozone and total oxidant.—In mature needles, within 5 days of fumigation with 0.45 ppm ozone for 12 h per day, chloroplasts and cytoplasm aggregated in the peripheral portions of mesophyll cells. Intracellular injury was present before cell wall deformations or macroscopically visible needle symptoms appeared (Fig. 2-B).

The timing of the appearance of histological ozone injury was dependent on needle age. In the greenhouse samples, ozone injury in 1-yr-old needles of ponderosa pine was identical to that of current-year needles, but cytoplasmic and organelle destruction occurred sooner during fumigation in the 1-yr-old needles. Chloroplasts of ozone-injured cells of 1-yr-old tissues were aggregated adjacent to the plasmalemma before complete chloroplast aggregation, in direct contrast with the sequence in younger tissues, where chloroplasts aggregated first in the lumen. Also, cell wall deformations were rarely present in mesophyll cells of 1-yr-old needles, though these were frequent in those of current-year needles.

Needle injury from total oxidant in two different environments was observable in needles collected from saplings in the unfiltered-air house and from adjacent trees growing outside. Greenhouse samples showed less extensive intracellular destruction than outside samples at both sampling dates. In both, however, total oxidant injury (Fig. 2-C) was histologically indistinguishable from ozone injury, consisting of an aggregation of chloroplasts concomitant with complete destruction of the cytoplasm. In greenhouse samples, injury was usually limited to the abaxial leaf portions after shorter exposure. Cell wall deformations were seen only in the first, and occasionally the second, abaxial mesophyll cell layer within the hypodermis. Although total oxidant injury was more advanced in outside trees on the first sampling date, it was histologically identical in tissues from both environments on the second sampling date, after 20 additional days of exposure.

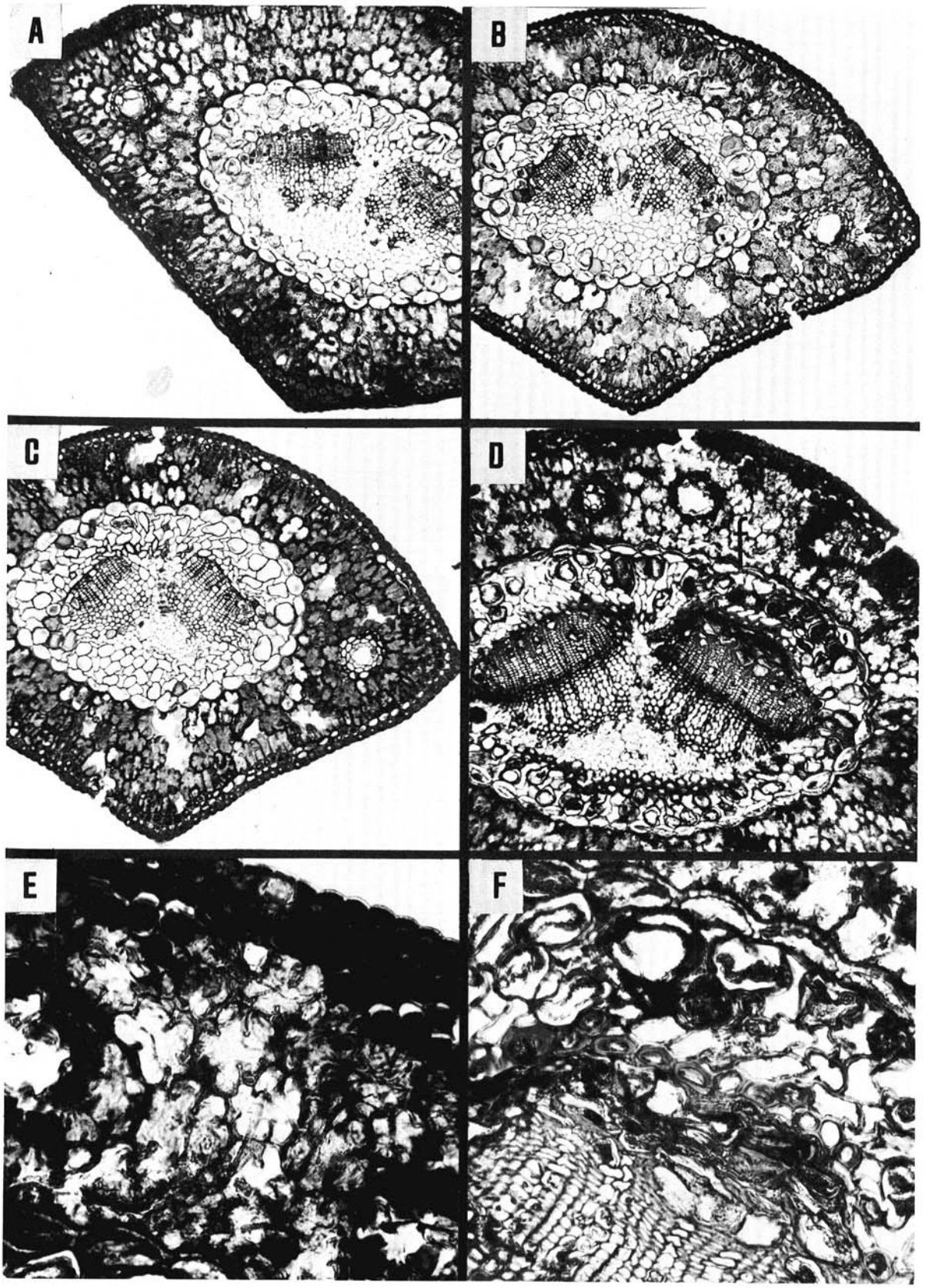
Initially, total oxidant injury in 1-yr-old needles was similar to that of current-year needles. The cytoplasm collapsed as chloroplasts aggregated in the peripheral cell portions. In extremely damaged cells, the nucleus was positioned adjacent to the cell wall with little cytoplasm remaining. Cell walls in 1-yr-old needles were only slightly deformed after complete cytoplasmic dissolution. In sections from 1-yr-old tissue, severely injured cells showed only a skeleton of cell walls without cytoplasm or nuclei. It is possible that thicker cell walls of the older needle remained more rigid following oxidant injury than did the thinner walls of younger tissues. In general, histological evidence of total oxidant injury to 1-yr-old tissues was of similar intensity in greenhouse and outdoor samples. Thus, the initial histologically evident increased sensitivity in current-year tissues of trees growing outside, was obscured in needles exposed for nearly two full summers.

Histopathology of winter fleck injury.—Necrotic fleck symptoms of needles occurred in two areas of the needle: the plicate parenchyma (mesophyll) and vascular tissues (Fig. 2-D, F, and 3), whereas ozone injured only the parenchyma. Within the mesophyll, injury began in cells nearest the hypodermis, progressed internally, and eventually cells were damaged adjacent to the endodermis. There was vertical (Fig. 2-D) and lateral (Fig. 2-F) destruction of mesophyll cells, resulting in an expanding area of necrotic tissue. Like ozone injury, cell destruction from necrotic fleck was not confined to substomatal mesophyll cells, although these cells were usually extensively damaged. Thus, a positive association between the incidence of necrotic fleck and stomatal location could not be made.

Necrosis usually developed in distinct zones of the mesophyll on the abaxial surface. The epidermis and hypodermis remained unaffected until all underlying cells were injured. In an early stage of disease development, necrosis was limited to four or five damaged cells in the outer mesophyll layer. In more advanced stages, necrosis was evident in more internally and laterally adjacent cells (Fig. 2-E, F).

Initial histological evidence of injury in mesophyll cells was characterized by an increase in cytoplasmic staining, followed by complete loss of all cytoplasmic contents (Fig. 2-E). This initial increase in cytoplasmic staining obscured the events preceding cell destruction. The cytoplasm of healthy cells was medium green, while recently injured cells showed increasing amounts of red or reddish brown. During the development of necrosis in mesophyll cells, some cytoplasmic constituents were aggregated in the central lumen of the cell initially, but eventually the entire cytoplasm accumulated there. This aggregation was not considered to be plasmolysis. Concurrently, mesophyll cell wall abnormalities appeared. In longitudinal view (Fig. 3), cell walls showed gradual collapse, but in cross section, little cell wall injury was evident (Fig. 2-D). This description of intracellular injury is incomplete because the various stages of injury intermediate between healthy and recently damaged mesophyll cells were not observed.

In addition, certain abnormalities also occurred in needle vascular tissues. Various vascular cells commonly showed hypertrophy and/or hyperplasia (Fig. 2-D, F). Hyperplasia occurred more frequently than hypertrophy


A
B
C
D
E
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in all vascular tissues except possibly the xylem, but only occasionally in the plicate parenchyma. Hyperplasia was more frequently evident in the phloem and closely adjacent conjunctive tissues (Fig. 2-F). These abnormal cell divisions occurred infrequently in the endodermis and rarely in the plicate parenchyma. When hyperplasia developed in the latter tissues, proliferation began in the closely adjoining phloem and conjunctive tissues.

Hyperplasia and hypertrophy in the vascular tissues resulted in a crushing of adjacent cells. Because these abnormalities usually began in the conjunctive tissue adjoining the phloem, the phloem and endodermal cells were frequently distorted in shape or completely destroyed (Fig. 2-F). Alterations within the phloem destroyed the distinct pattern of cell rows, except in the region of the youngest derivatives next to the vascular cambium.

The development of these early vascular abnormalities, relative to the appearance of mesophyll injury, is unknown. Usually, a small degree of mesophyll cell injury was observed in a cross section from a stoma toward the endodermis either slightly before, or concomitant with, that in the transfusion tissue. In several instances, however, vascular abnormalities were quite extensive, although there was no mesophyll injury. In general, increased mesophyll cell destruction was simultaneous with transfusion and phloem cell hypertrophy, and subsequent proliferation.

Histological evidence of necrotic fleck was identical in *Pinus ponderosa* and *P. lambertiana*, at all needle developmental stages from current-year needles through 4-yr-old needles, from a common collection site.

No fungus hyphae or fruiting structures were evident in the central portion of the fleck, or the parenchyma, or vascular tissue which bordered the fleck, in needle transections stained with thionin and orange G.

DISCUSSION.—Ambient total oxidant measured by the colorimetric potassium iodide or coulombmetric (Mast Development Co., Davenport, Iowa) method, is usually 85-90% ozone; the remaining oxidizing potential is attributed to peroxyacetyl nitrate (PAN), its homologs, and nitrogen dioxide (15). The interaction of oxidants in ambient smog, to change the type or amount of pine needle injury, has not been thoroughly investigated; however, two investigators have previously shown that ozone alone causes external needle symptoms identical to those observed on smog-injured ponderosa pines in the forest (10, 11).The ozone injury symptoms described by Davis (5), on Virginia pine, were identical with those observed on ponderosa pine (Fig. 1-E, F). Further, the histopathological similarity of ponderosa pine needle injury caused by ozone alone and ambient total oxidant,

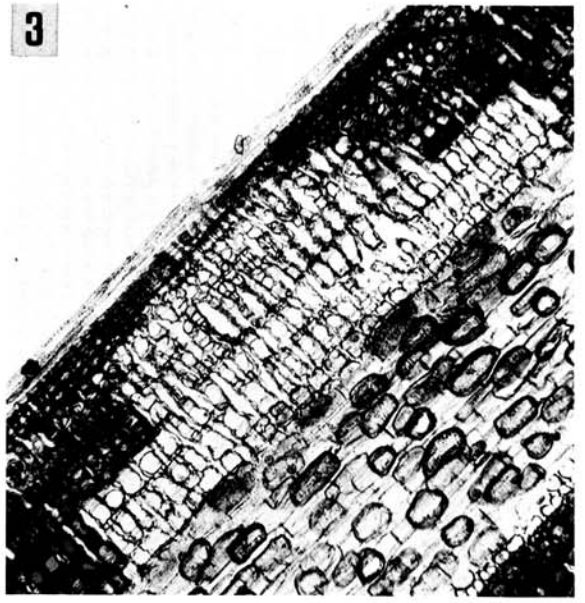

Fig. 3. A longitudinal section through a necrotic fleck complements the cross-sectional views (Fig. 2-D, E, F) and shows that injury is most extensive in the two inner mesophyll layers adjacent to the endodermis, and less extensive externally. Within the fleck, there is considerable cell collapse after loss of all intracellular materials. Vascular tissue abnormalities are not positively seen in this view (× 120).

serves as additional evidence that ozone is the primary toxicant involved, and that the participation of other components of the oxidant complex, if any, is not histologically distinguishable at present ambient concns.

Necrotic flecks of the upper needle surface were distinguishable histologically from ozone injury in two important ways. First, injured mesophyll cells were confined to well-defined areas, having an abrupt boundary with surrounding healthy cells. The intracellular contents of affected cells were absent. Ozone-injured mesophyll cells are more randomly scattered, and chloroplasts and other cell contents are aggregated in the peripheral portions of the cells. Second, the vascular tissues in needles with necrotic fleck were also affected when adjacent mesophyll cell injury was present, whereas in ozone injury they were not.

The hypertrophy and hyperplasia observed in the phloem and adjacent conjunctive tissues suggested that sucking insects might be the cause of winter fleck symptoms. External evidence of scale and aphid feeding

Fig. 2-(A to F). In a cross section **A)** of an untreated current-year needle of ponderosa pine exposed to carbon filtered air, nuclei are centrally located and there are many distinct chloroplasts throughout the mesophyll cells (× 75). A cross section **B)** of an ozone-treated needle shows a more or less random distribution of mesophyll cell injury. Intracellular aggregations are present in the abaxial mesophyll, whereas some needle portions show complete cell dissolution (× 69). A cross section **C)** of a smog-injured needle shows intracellular aggregations and complete cell disruption similar to that in an ozone-damaged needle (× 71). A cross section **D)** shows necrotic fleck injury (f) to mesophyll and vascular tissues (× 94). A detail view **E)** shows mesophyll damage attributed to necrotic fleck. An initial increase in cytoplasmic staining was observed; however, with more extensive lesion development, only cell walls remained (× 301). A cross section **F)** of vascular tissues showing hyperplasia and hypertrophy in phloem and conjunctive tissues adjacent to the endodermis (× 301).

(2), however, does not resemble the discrete necrotic flecks in question. In this study, and in similar observations of ponderosa pine in Colorado (J. Staley, *personal communication*), fungus hyphae have been present only occasionally in the lesions. The needle surface symptoms do not resemble those caused by the needle disease fungi *Davisomycella ponderosae* (Staley) Dubin in Colorado, or those caused by *Elytroderma deformans* (Weir) Darker in California. Necrotic fleck appearance is essentially the same on samples collected from California, Colorado, and Arizona. It is difficult to believe that a biotic agent could be so ubiquitous and so consistently active over this broad area, with its diverse climatic conditions.

The following observations made in California were also made in Colorado by Staley (*personal communication*): (i) flecks are more abundant on older needles and always on the exposed needle surfaces that have over-wintered, (ii) flecks are less abundant on needles sheltered by branches above, (iii) flecks are common on trees of different species growing side by side, and (iv) flecks are evident on trees growing at elevations ranging from 1,550 to over 2,750 m, where winter snows often accumulate to considerable depths.

Neither sulfur dioxide, fluoride, nor oxidant air pollution can be implicated because the winter fleck condition is observed hundres of miles from a pollution source. Oxidant-tolerant sugar pine (9) is equally affected in areas exposed to heavy oxidant and areas with no oxidant. Also, the histological symptoms in sugar pine are identical with those in ponderosa pine. The necrotic fleck condition was recognized as being distinct from the chlorotic mottle symptom of total oxidant injury, by G. F. Edmunds and B. L. Richards in the San Bernardino Mountains (*unpublished*).

Clements (2) observed needles with necrotic spots or dead tips on ponderosa pine exposed to severely low winter temp in Washington, on sites where whole trees were occasionally killed. Daniker (4) described frost injury to spruce needle mesophyll, which is strikingly similar histologically to that observed in the pine tissues in this study. Stewart, et al. (14) found that winter injury, salt toxicity, poor soil aeration, and sulfur dioxide sometimes caused hypertrophy of phloem cells in the needles of several conifer species. Finally, in the course of our work, the fleck was observed to develop on the youngest needles of ponderosa pines buried by snow, when a small section of greenhouse roof collapsed under the snow load. Immediately adjacent trees not covered by snow did not develop fleck symptoms. These necrotic flecks on the abaxial needle surface appear to be associated with winter weather conditions, particularly exposure to snow.

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