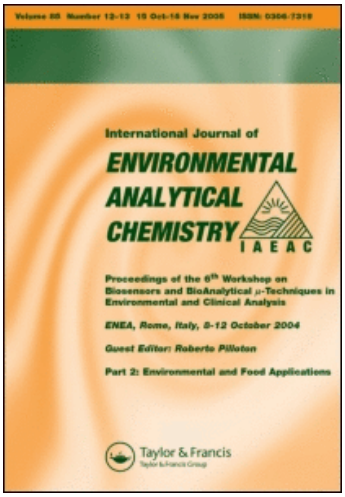

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Hydrogen Peroxide Dissolved in Acidic Fog as Air Pollutant—Effects on Spruce Needles

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HYDROGEN PEROXIDE DISSOLVED IN ACIDIC FOG AS AIR POLLUTANT—EFFECTS ON SPRUCE NEEDLES*

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In fog chamber experiments, 3-year-old spruce trees (*Picea abies* Karst.) were exposed to acidic fog (pH 4) containing 864 ± 250 ppb H_2O_2 4 days a week from 4 a.m. to 7 a.m. over a period of 8 weeks. During the remaining time, the chambers were opened and the trees were exposed to natural climatic conditions. This fumigation program was selected after the natural pollutant values of H_2O_2.

As effects of this nebulation experiment, we observed decreases in all histological parameters studied. An increased accumulation of phenols in the central vacuoles of the mesophyll cells of the needles was observed.

HPLC-measurements indicate that acidic fog containing H_2O_2 affects distinct intermediates of the phenolic pathway. The amounts of piceatannolglycoside, kaempferol-glycoside, catechin as well as quinic and shikimic acid increased.

KEY WORDS: Forest decline, fog chamber experiments, hydrogen peroxide, needle tissues, needle phenols.

INTRODUCTION

The causes of forest decline in the central part of Europe have not been understood satisfactorily until now. The original theory, which held acid deposition and subsequent acidification of the soil responsible, is only one part of the complex explanation.[1] Although the role of photo-oxidants, especially ozone, in plant damage is now widely accepted,[2–5] questions still remain. In some regions with high ozone concentrations, tree damage is absent.[6]

There are more oxidants in the air than ozone and PAN. Hydrogen peroxide is considered the most important oxidant of SO_2 in atmospheric water droplets, converting SO_2 to SO_4^{2-} at low pH (< 5.0).[7,8] So it has an important role in the acidification of rain-, cloud- and fog-water.[9] A dominant source of aqueous phase

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H_2O_2 is believed to be the dissolution of gaseous H_2O_2 from the air.[10] But the aqueous phase and solid surface generation of H_2O_2 may also be important sources of H_2O_2.[11–15]

Atmospheric H_2O_2 is generated by photochemical reactions involving radical species, i.e. $OH^{\cdot}$, $RH^{\cdot}$, $HO_2^{\cdot}$ and $RO_2^{\cdot}$.[16] The concentration of H_2O_2 in air is recognized to be relatively low, <4 or 5 ppb (v/v).[17–19] The seasonal variation of atmospheric H_2O_2 in Los Angeles was shown to exceed 1 ppb (a 4 h mean) in summer, and <0.3 ppb in winter.[20] High concentrations of H_2O_2 can occur in the liquid phase. Results of the determination of H_2O_2 in cloud water collected during several flights in July 1982 range from 850 ppb to 3000 ppb at 150–2000 m above sea level in Rotterdam, Den Helder (NL) and Hull (UK). H_2O_2 concentrations measured above the continent and at ground level indicate that its concentration is markedly reduced because of reactions with precursors (NO_x and SO_2).[21]

Trees in elevated areas are frequently exposed to clouds or fog. The occurrence of fog shows an increase with the height above sea level. The frequency is about 120 days per year at 60 m and goes up to more than 200 days per year at locations over 800 m.[22]

In the Egge Mountains, where we monitored the pH, anions and H_2O_2 content of rain and fog water, the frequency of fog occurrence at mountain tops and some valley areas is about 140 days per year. These observations have led to the suggestion that forest decline is connected to the frequency with which trees are in contact with fog or cloud water. One explanation could be that these trees are more often exposed to acidic water than those that are only wetted by rainfall. Fog- and cloud-water is more polluted than rain water and evaporation effects may lead to even higher concentrations of pollutants.[23]

The role of dissolved oxidants in fog- or cloud-water was not taken into account, mainly because the ozone concentration in water is very low, due to its poor solubility in water.

Remarkable amounts of H_2O_2 in rain-, cloud- and fog-water measured by Römer,[21] Klockow,[24] Kadleck[25] and our own group.[26] These results prompted us to investigate the effects of H_2O_2-containing fog on spruce trees, the most important forest trees in Europe.

In a former investigation we pointed out that H_2O_2 effects serious changes in the internal structure of beech leaves and spruce needles.[26] Since that time we have established new outdoor fog chambers which only close during the time of experimental nebulation, natural precipitation and fog episodes. All other times the chambers are open, and the young trees are exposed to natural environmental conditions. The question is if this simulation experiment with fog-containing H_2O_2 performed under outdoor conditions and realistic H_2O_2 concentrations, can validate our former results.

EXPERIMENTAL

Fumigation Experiments

Experimental fog chambers, consisting of 10 1-m^3 polycarbonate hood boxes were

placed on an outdoor substation near our university and sheltered from direct sunlight. Each of them contained between 12 and 20 plants. The plants were potted in 21 plastic boxes standing in a frame. Water was sucked by a glass fiber wick from a water reservoir. The plant parts above ground were standing most of the time in natural environmental conditions (temperature, light, humidity, wind, air pollutants). Only during experimental fog exposure, natural rainfall, fog or dew episodes, did the polycarbonate hood automatically pull over the plants. So it was ensured that the only wet deposition applied to the plants was artificial fog. As the hood pulled over, fresh unfiltered air was ventilated through the chambers at a rate of 15 m^3/h. During the artificial fog phase, the circulating air was additionally humidified to minimize evaporation and H_2O_2-concentration in the fog droplets. In the humidifier the air was first sprayed with water, then it passed through wet filters and a scrubbing tower.

In front of the air inlet, fog was created by a nebulizer, the original of which was performed by ECN, Petten in The Netherlands (Figure 1).

From each box, a fog water sample was taken using a small glass cyclon, and the ion composition was compared to that of the spray solution. The difference was less than 20%, which demonstrates the proper function of the humidification. The spray solution used includes the ions shown in Table 1. The composition was comparable to natural cloud water at pH 4.

Besides the fog chambers, a monitoring station was installed for measuring continuously all real air pollutants coming into contact with the plants.

For the experiment, 3–5-year-old Norway spruces (*Picea abies* Karst.) were obtained as homogenous seed material from a tree nursery (forest-district Paderborn) and exposed to the above-mentioned program (Figure 2) from 4/6/1987 to 5/8/1987. All trees were potted in the same soil.

The desired value of H_2O_2 concentration was 1000 ppb. For verification of these concentrations the stock solution was examined after each mist exposure using a fluorescent determination method, as shown in Figure 3. *P*-hydroxyphenyl acetic acid reacts in the presence of peroxidase and H_2O_2 to the corresponding dimer 2,2′-dihydroxy-biphenyl-4,4′ acetic acid. In contrast to the reacting products, the dimer is fluorescent using the wave length combintion 320 nm for excitation and 400 nm for emission. The sensitivity of this system is better than 1 ppb.[27,28] In this experiment, investigated H_2O_2-concentrations were set at 864 ± 250 ppb, in comparison to 1000 ppb, as desired value (Figure 2).

Biological Methodology

Preparation and fixation of spruce needles are included in earlier works.[27] For SEM preparation semi-thin sections were extracted from resin by amyl acetate, dried, mounted and coated with gold by sputtering. Quantitative analysis of tissues was made from 10 light microscopic pictures per experimental series, photographed from specimens of different needles taken from different spruce trees. A digitizer tablet, connected to a microcomputer was used for tissue area measuring.

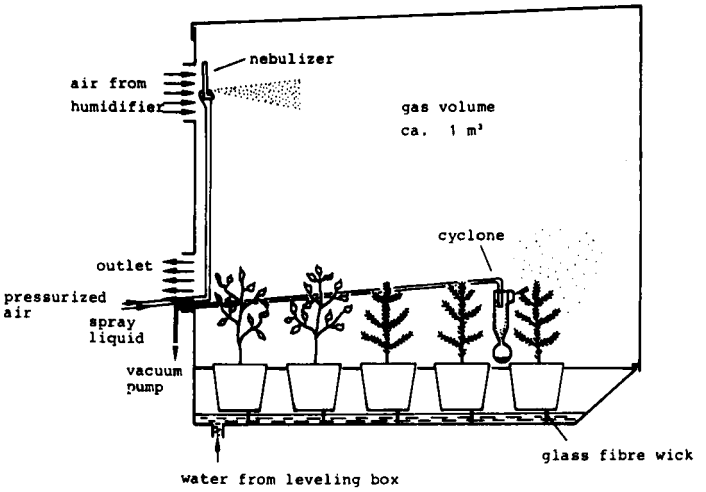

Figure 1 Schematic drawing of a fog chamber used to expose trees to fog.

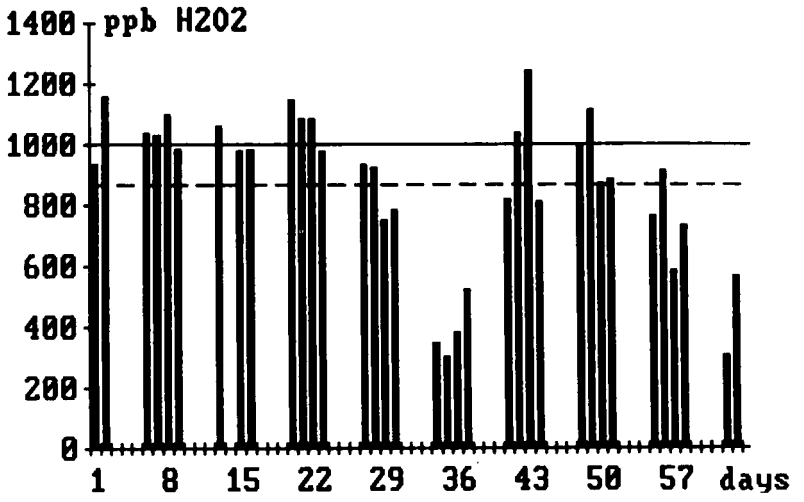

Figure 2 Columns showing the variation of the H_2O_2 concentration in the sampled fog water. Solid line: desired 1000 ppb level of H_2O_2; dotted line: average H_2O_2 level of the sampled fog water.

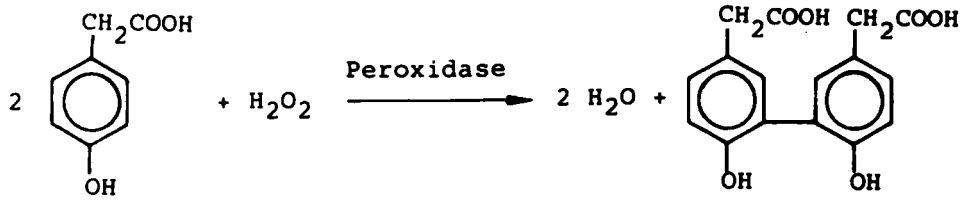

Figure 3 Reaction scheme of H_2O_2 determination in acidic fog by fluorescence method.

Table 1 Ion composition of the spray solution during fog exposure

Ion	Cl^-	SO_4^{2-}	NO_3^-	H^+	Na^+	NH_4^+	Ca^{2+}	Mg^{2+}	K^+
(μmol/l)	770	280	135	105	630	357	73	90	47

Electron microscopic studies were performed with a Hitachi H 3010 scanning electron microscope.

Sample Preparation for the Determination of Phenolic Acids in Needles

Needles from the reference series and needles from the H_2O_2 series were collected and frozen immediately in liquid nitrogen in order to avoid any biological change. During transport, the needles were transferred to Dewar vessels containing dry ice. The frozen liquid nitrogen samples, which were to be ground in a ball triturator the following day, were placed in a freezer overnight. All samples were ground within 1 day.

Crude needle extracts were obtained by extraction of 1 g of triturated and lyophilized needle material corresponding to our screening methodology.[29] Such extraction, even in favourable cases, will yield about 70% of the total needle phenols, except for the condensed phenolic compounds, because they are irreversibly bound to proteins within the cell and we can only discuss results received from a methanolic extract.[30]

Analytical Methodology of Needle Phenols

The HPLC system was comprised of a liquid chromatograph equipped with a photodiode-array detector, combined with an analytical workstation.[29]

RESULTS

1. Histological and Cytological Changes

Macroscopical investigations showed significant decreases in the length of primary needles and primary twigs. The primary needles of the reference series measure 10 ± 1.7 mm in length, those of the H_2O_2-series 8 ± 2 mm ($p < 0.05$). The same tendency can be seen in the length of primary twigs. While the primary twigs of the reference trees are 70 ± 13 mm long, the twigs nebulized with acidic fog containing H_2O_2 are only 46 ± 12 mm long ($p < 0.001$). In comparison to the reference series, the primary needles are a lighter yellow in colour.

In the needle transverse section, a longer, vertical ray axis can be distinguished from a shorter, horizontal cambium axis. Compared with the reference needles, the ray axis of the primary needles is reduced by 14%, the cambium axis by 12%. The

transverse diameter of the ray axis of reference needles is $1060 \pm 46\,\mu m$, that of the H_2O_2 series only $912 \pm 93\,\mu m$ ($p < 0.001$). The transverse diameter along the cambium axis of reference needles is $560 \pm 45\,\mu m$ long and $493 \pm 84\,\mu m$ in H_2O_2-nebulized needles ($p < 0.04$). As seen in the needle cross-cut, the needles of the H_2O_2-series look shorter in the vertical diameter and smaller in the horizontal diameter (Figures 4 and 5).

The tissue areas of the needle transverse sections are reduced in all needles exposed to H_2O_2 (Figure 6). The average transverse section area of primary needles decreased by 20.6% in comparison to the reference needles. The highest reduction rates were found in the vascular bundle (−29.1%), the hypodermis (−28%) and the mesophyll tissue (−17.6%). The area of the intercellular space also reduced by 22%.

The percentages of the tissue areas in relation to the total needle cross-cut did not change significantly, except in the epidermis, which increased from 8.5% to 9.5% ($p < 0.03$).

As a result of the reductions in needle length and tissue area in the needle cross-cut, the needle volume and tissue volume reduced. The total primary needle volume of the reference series amounts to $3.97 \pm 0.2\,mm^3$, that of the H_2O_2-series is reduced to $2.5 \pm 0.6\,mm^3$. The tissue volume of the mesophyll cells per needle decreased from $1.97 \pm 0.3\,mm^3$ in the reference series to $1.3 \pm 0.3\,mm^3$ in the needles exposed to H_2O_2.

The reduction of tissue area or tissue volume per needle is due to a reduced amount of cells per needle. The number of mesophyll cells and cells of the border layers, epidermis and endodermis, decreased significantly under the influence of H_2O_2 (Figure 7).

All the tissues of the vascular bundle decreased significantly after exposure to H_2O_2. The highest reduction rates we found for the xylem (41%), for the intact phloem (49%) and for the cambium area (44%). These tissues are not only reduced in direct comparison with the reference series but also in the percentages to the total transverse section areas belonging to them (Figures 8, 9). The cambial activity is reduced under the influence of H_2O_2, for we found less phloem and xylem elements produced by the cambial meristem (Figure 10).

The number of stomata increased by 13%. Primary or current-year needles polluted by H_2O_2 showed remarkable differences in the structural waxes, occluding the stomatal antechamber. In the primary needles of the reference group, structural wax outlines the stomatal openings and the antechamber (Figure 11). The borders of the guard cells can hardly be observed. Wax rods cover the guard cells and the outer vestibule. Fine wax rod-like tubes form crystalline wax. The epicuticular wax above the stomata forms a three-dimensional network of anastomosing wax rodlets (Figure 12). After treatment with acidic mist containing H_2O_2 cracks were built up in wax plugs (Figure 13). Exposure to acidic mist and H_2O_2 may damage the wax so severely as to cause melting of the fine stomatal wax structure shortly after the needles are formed. Structural waxes show that the rods were fused together (Figure 14).

Mesophyll cells of spruce needles usually contain variable amounts of phenolic droplets. In our reference series we occasionally found only some droplets in the

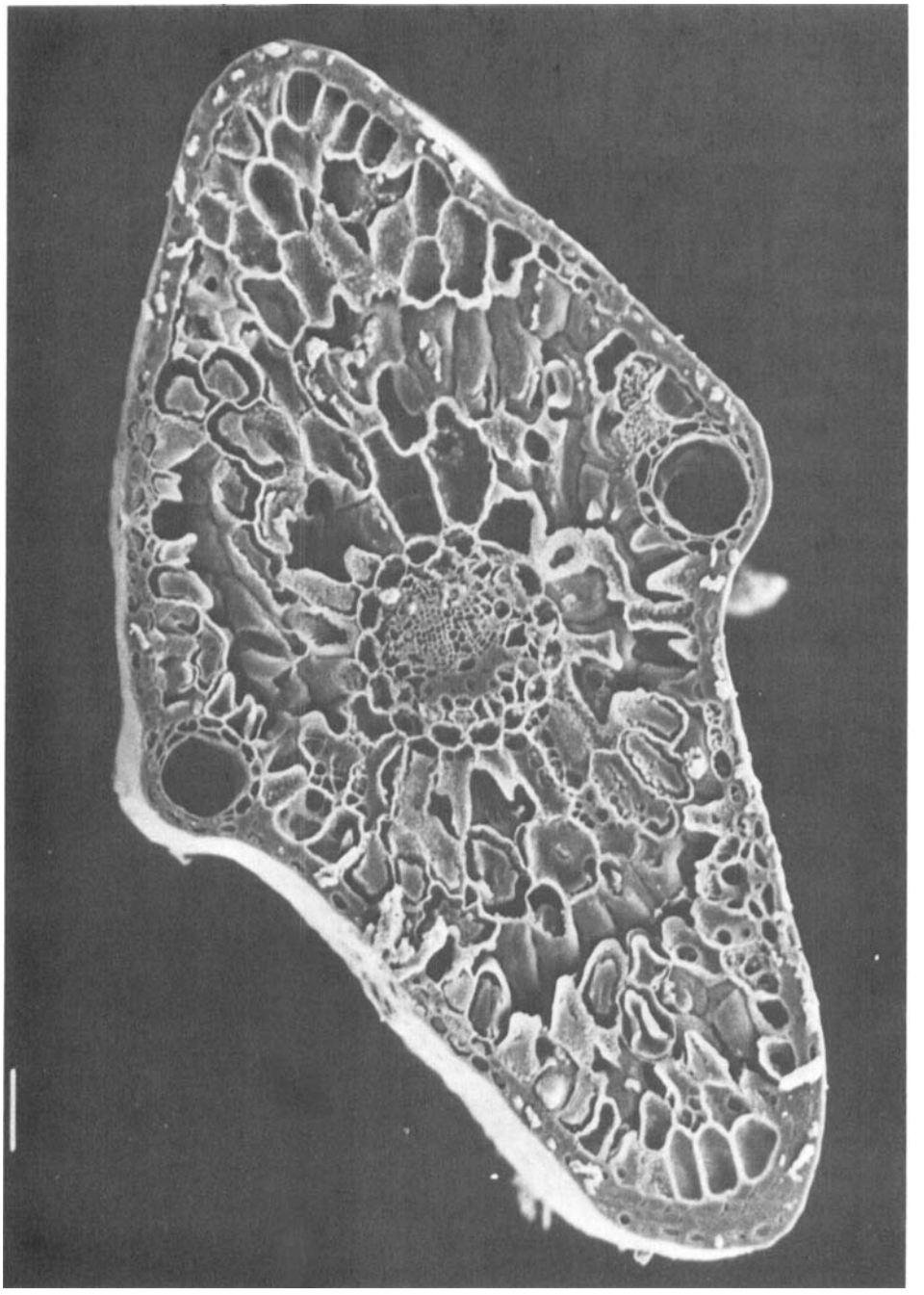

Figure 4 Transverse section of a primary needle of a Norway spruce from the reference series. Bar = 50 μm.

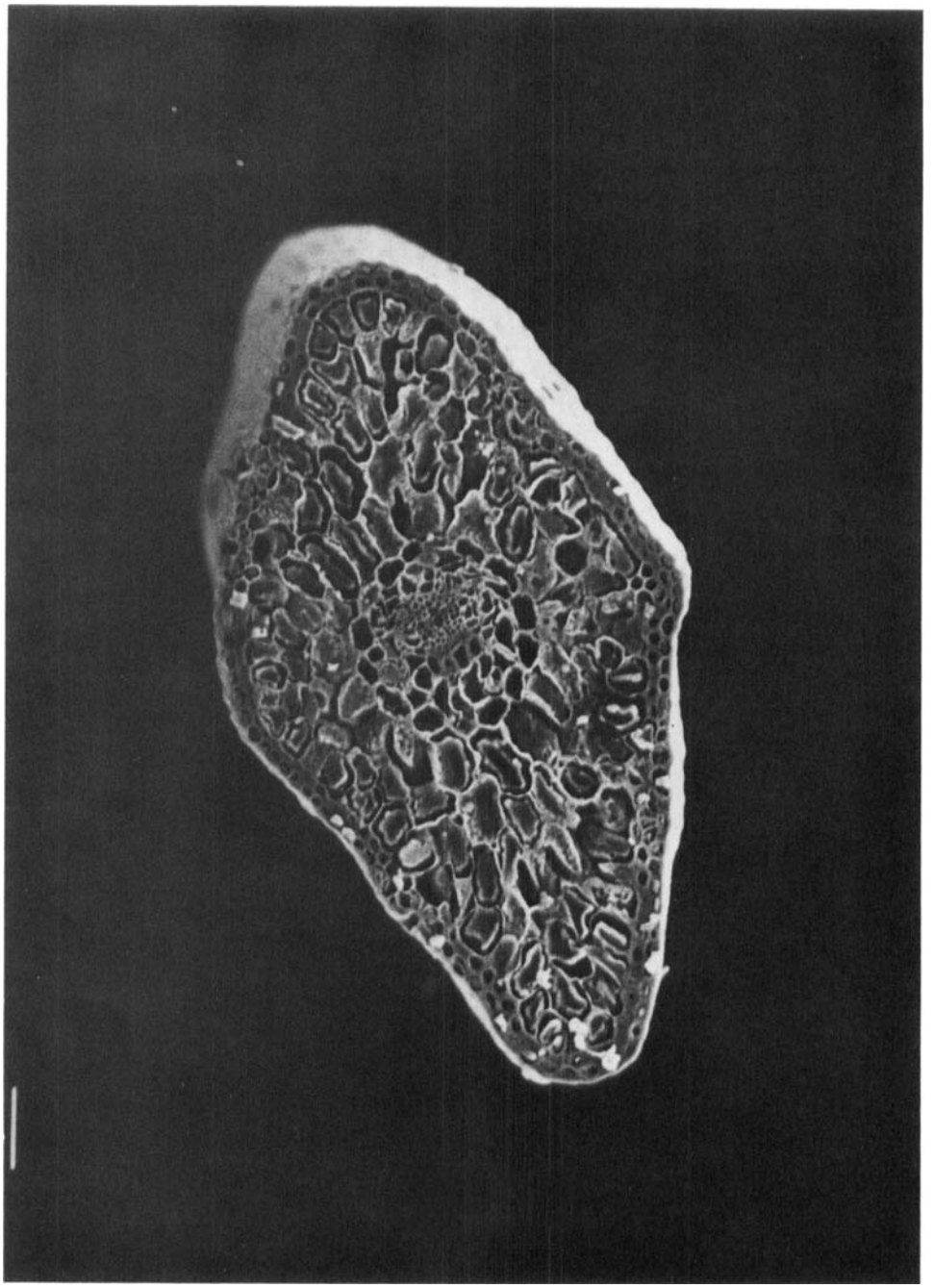

Figure 5 Transverse section of a primary needle taken from a Norway spruce exposed to acidic fog (pH 4) containing 864 ± 250 ppb H_2O_2. Bar = 50 μm.

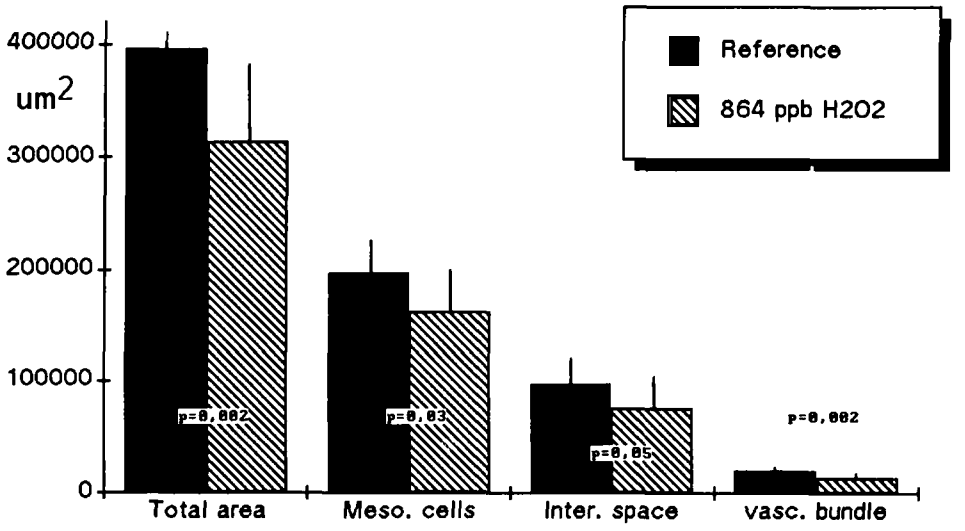

Figure 6 Tissue areas of transverse sections of primary needles of Norway spruce.

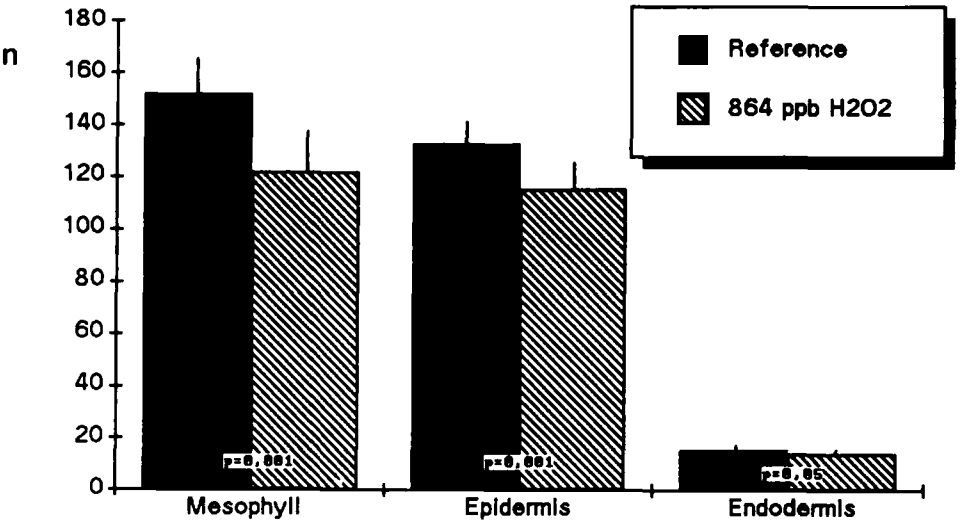

Figure 7 Number of cells per transverse section area of primary needles of Norway spruce.

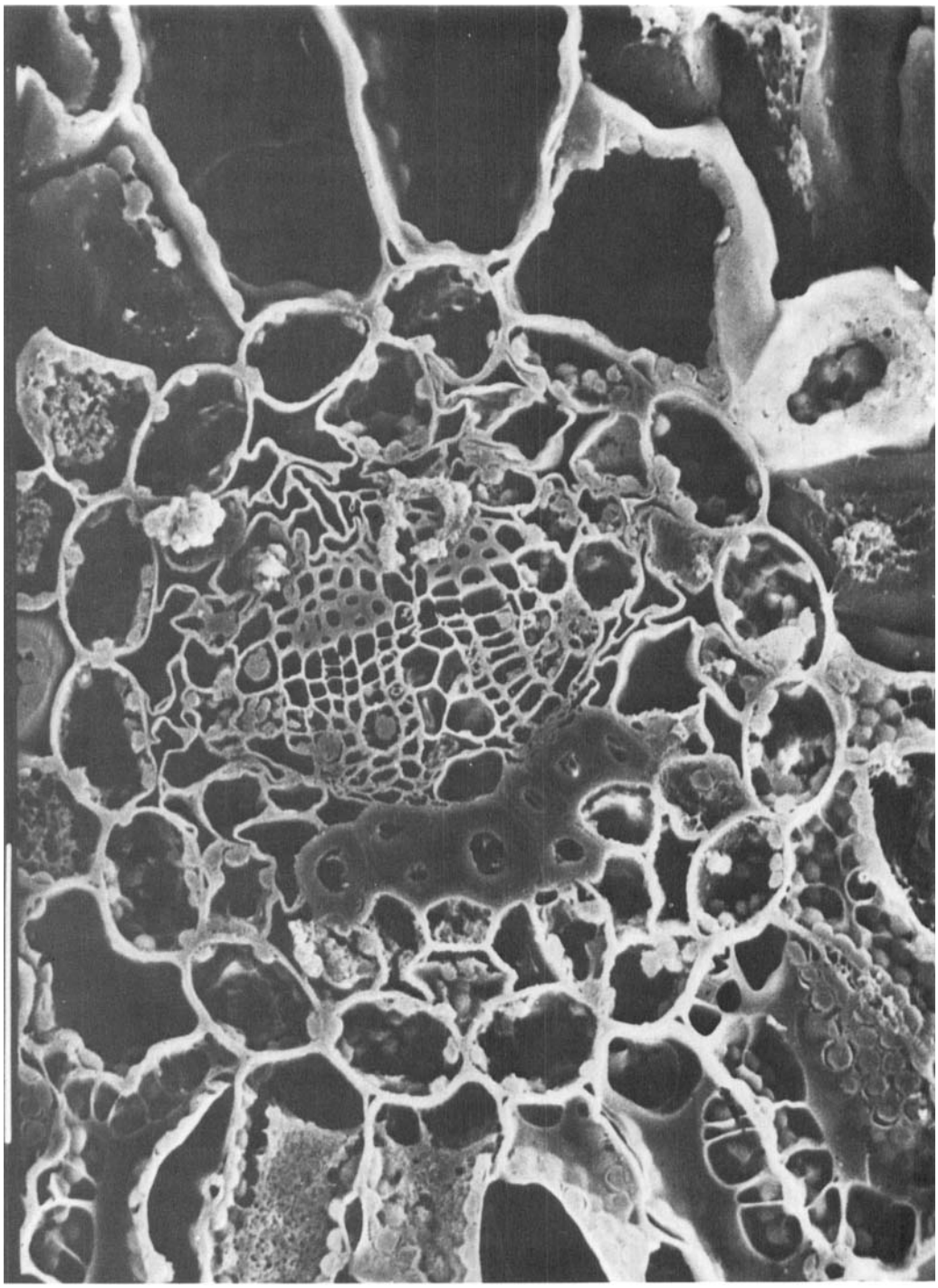

Figure 8 Transverse section of a vascular bundle taken from a Norway spruce primary needle from reference series. Bar = 50 μm.

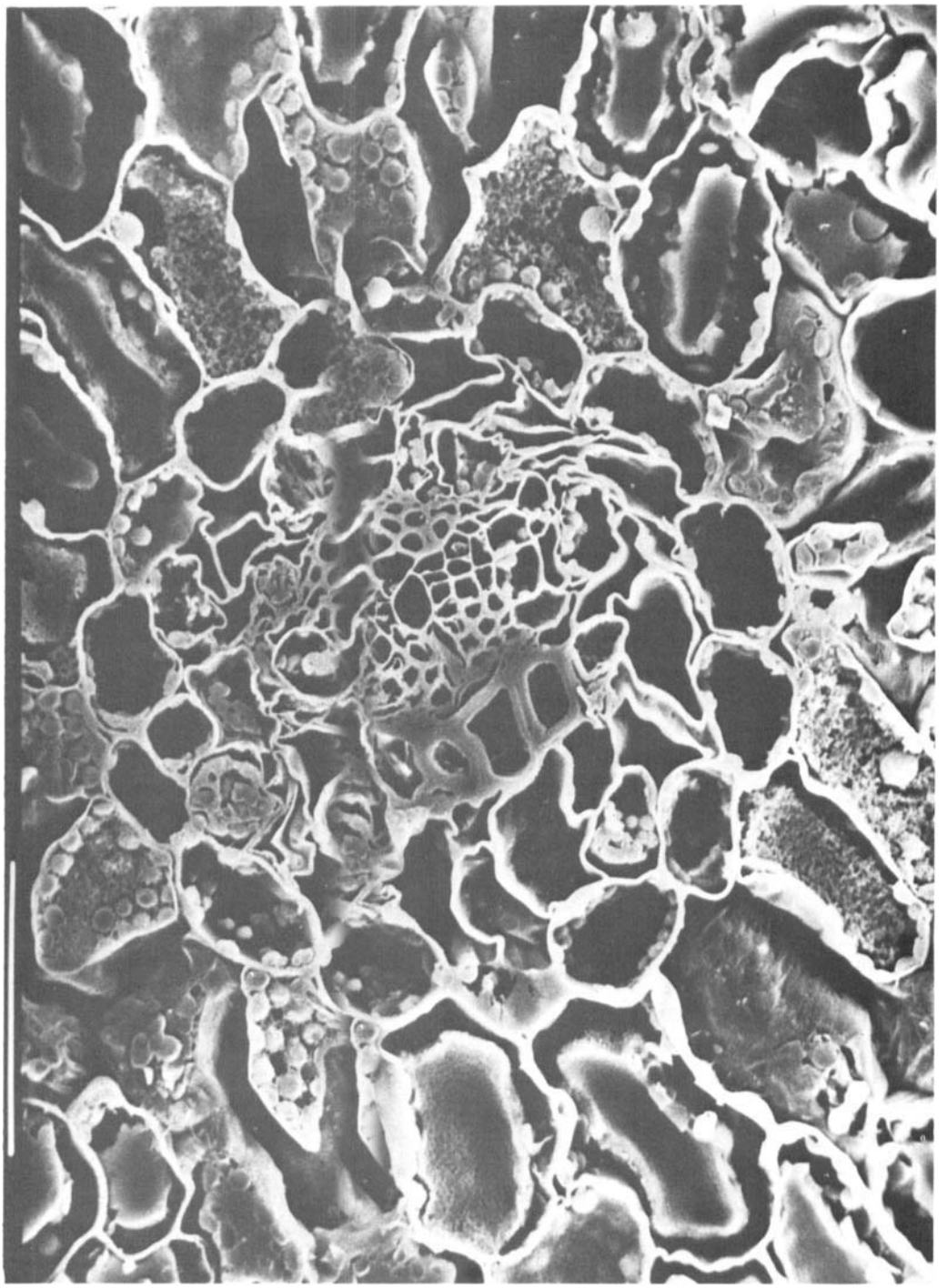

Figure 9 Transverse section of a vascular bundle taken from a Norway spruce primary needle exposed to acidic fog (pH 4) containing 864 ± 250 ppb H_2O_2. Bar = 50 μm.

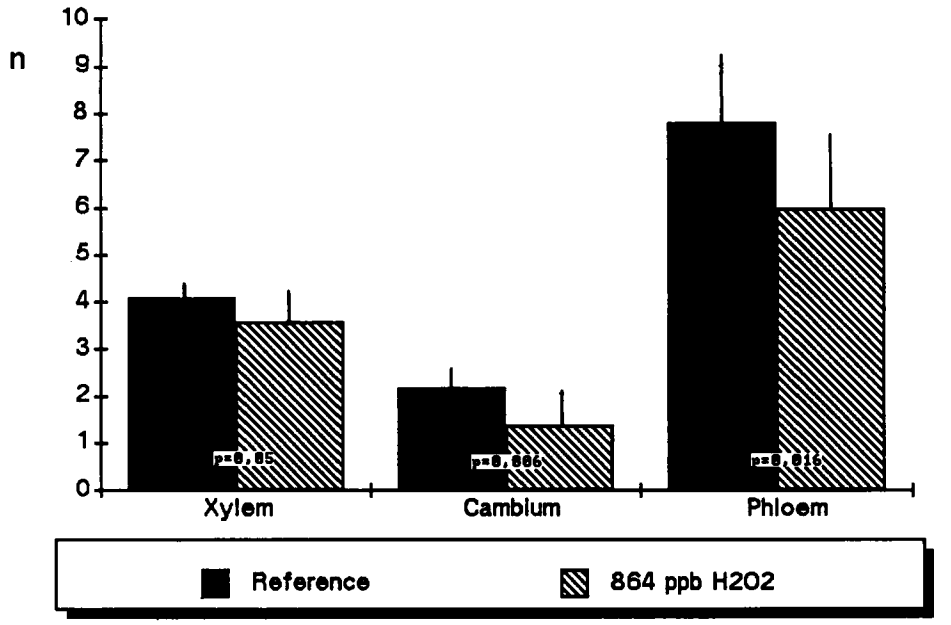

Figure 10 Average number of cells per descendent cell row in the vascular bundle of primary needles of Norway spruce.

central vacuole. The large background bodies are packed chloroplasts (Figure 15). But even in some of the mesophyll cells of the reference needles a higher content of phenolic droplets, smaller in size, can be found in the central vacuole (Figure 16). Size and arrangement of phenolic compounds are quite different in needles treated with acidic mist containing H_2O_2 (Figure 17). The extremely small phenolic droplets are knotted like a string of pearls. In the peripheral mesophyll cells of needles exposed to acidic mist and H_2O_2, phenolic droplets are packed close together; the central vacuole is nearly filled with phenolic material (Figure 18).

The evaporation rate of spruce needles exposed to H_2O_2-containing acidic mist is reduced (Figure 19).

2. *Metabolic Changes*

Among the classes of phenols presented are shikimic acid, some derivatives of benzoic acid, *o*-coumaric acid and *p*-hydroxyacetophenone, the 3-glycosides of the flavonoids kaempferol and quercetin, the stilbenes piceatannolglucoside and isorhapontin as well as several catechins (catechin, epicatechin, dimers and trimers of catechin and catechin/epicatechin).

After exposure to acidic fog containing hydrogen peroxide, the amount of most quantified needle phenols did not change significantly, but the amounts of piceatannolglucoside, kaempferol-glucoside, catechin, as well as quinic and shiki-

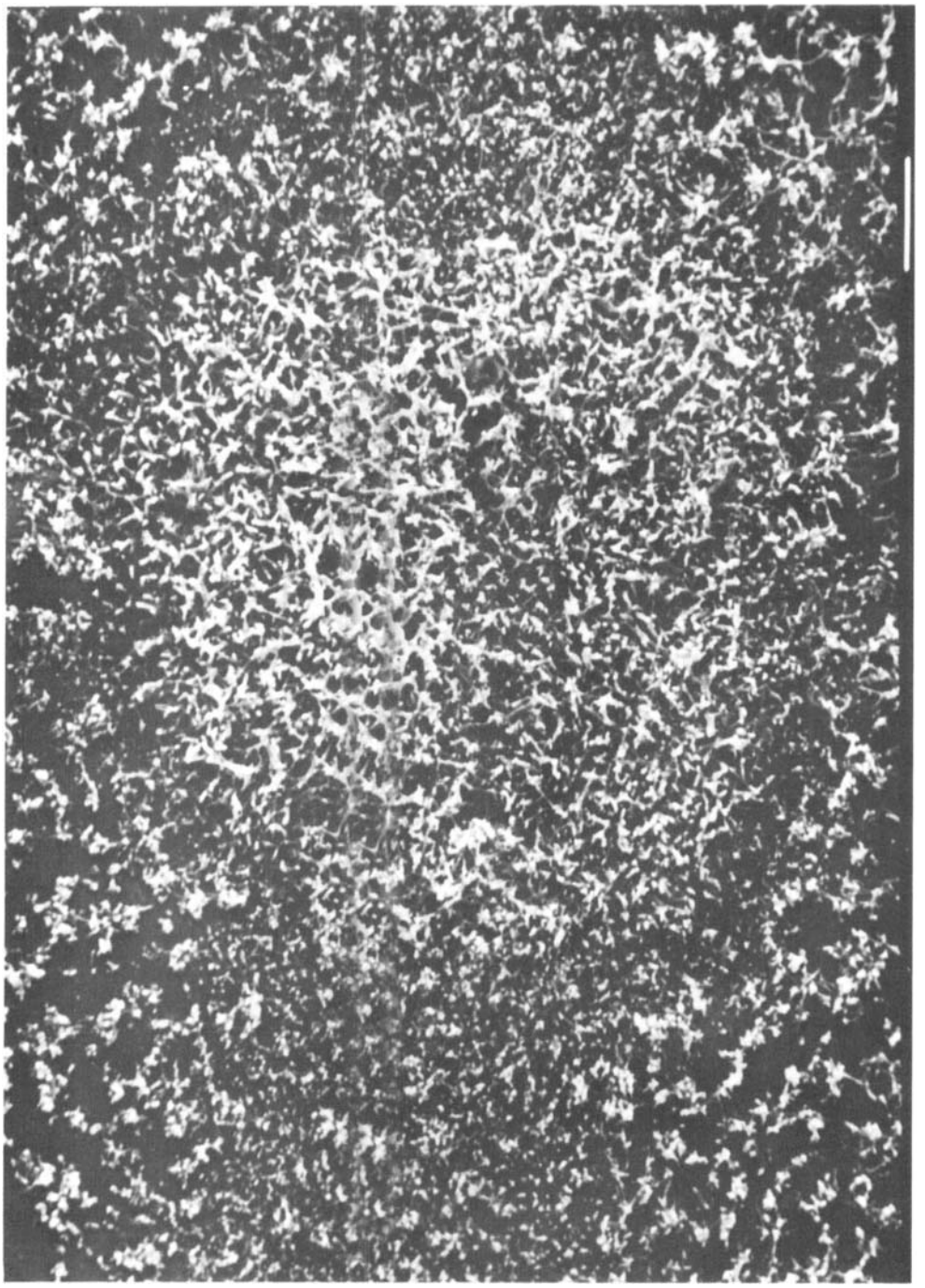

Figure 11 Coarse crystalline wax on a stomate of a primary spruce needle of the reference series. Bar = 5 μm.

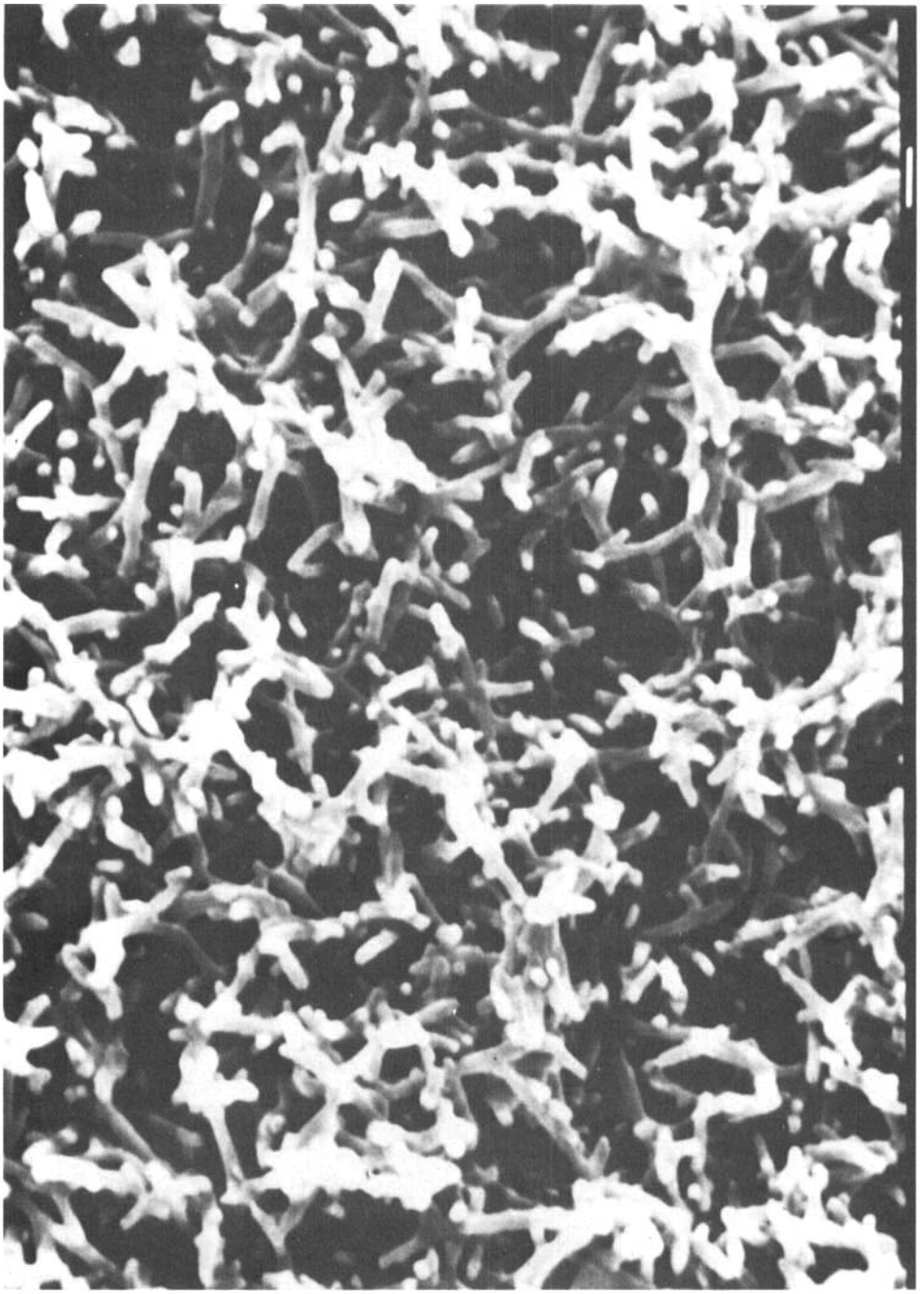

Figure 12 Three-dimensional network of crystalline wax in the stomatal alveoli of the reference primary spruce needles. Bar = 0.5 μm.

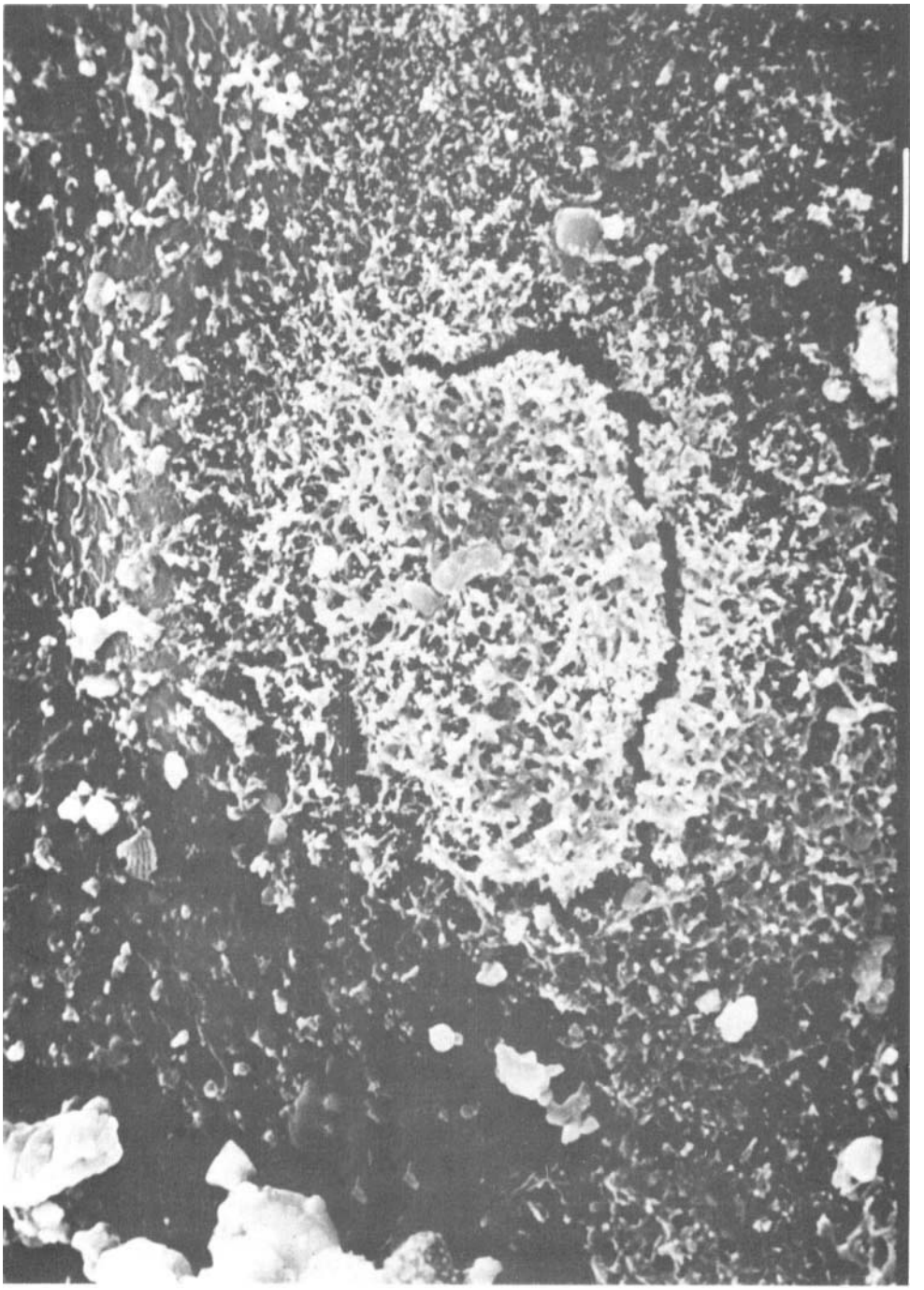

Figure 13 Melted wax covering the stoma of a primary needle of Norway spruce exposed to acidic fog (pH 4) containing 864 ± 250 ppb H_2O_2. Bar = 5 μm.

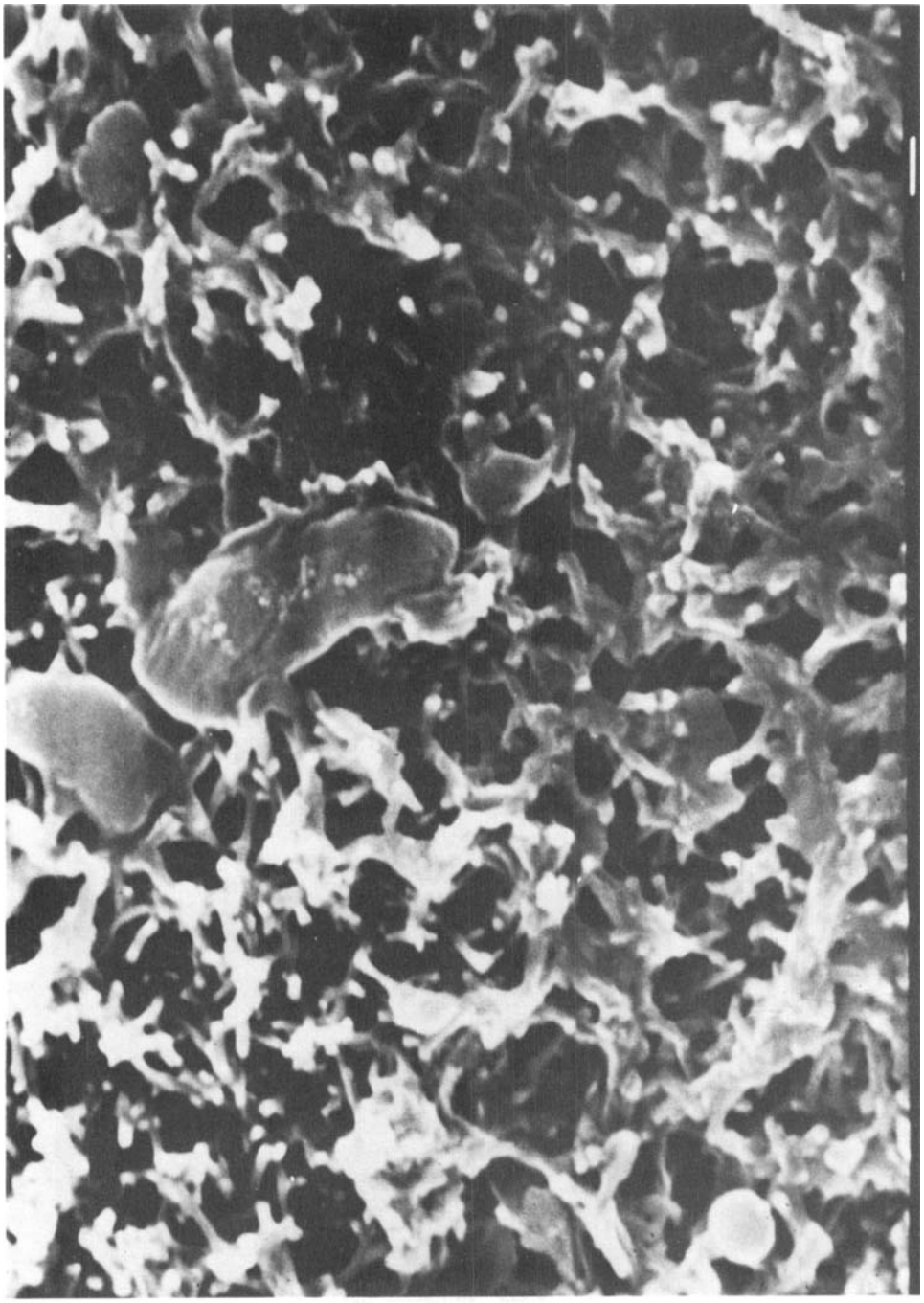

Figure 14 Fused wax rodlets covering the stoma of a primary needle of Norway spruce exposed to acidic fog (pH 4) containing 864 ± 250 ppb H_2O_2. Bar = 0.5 μm.

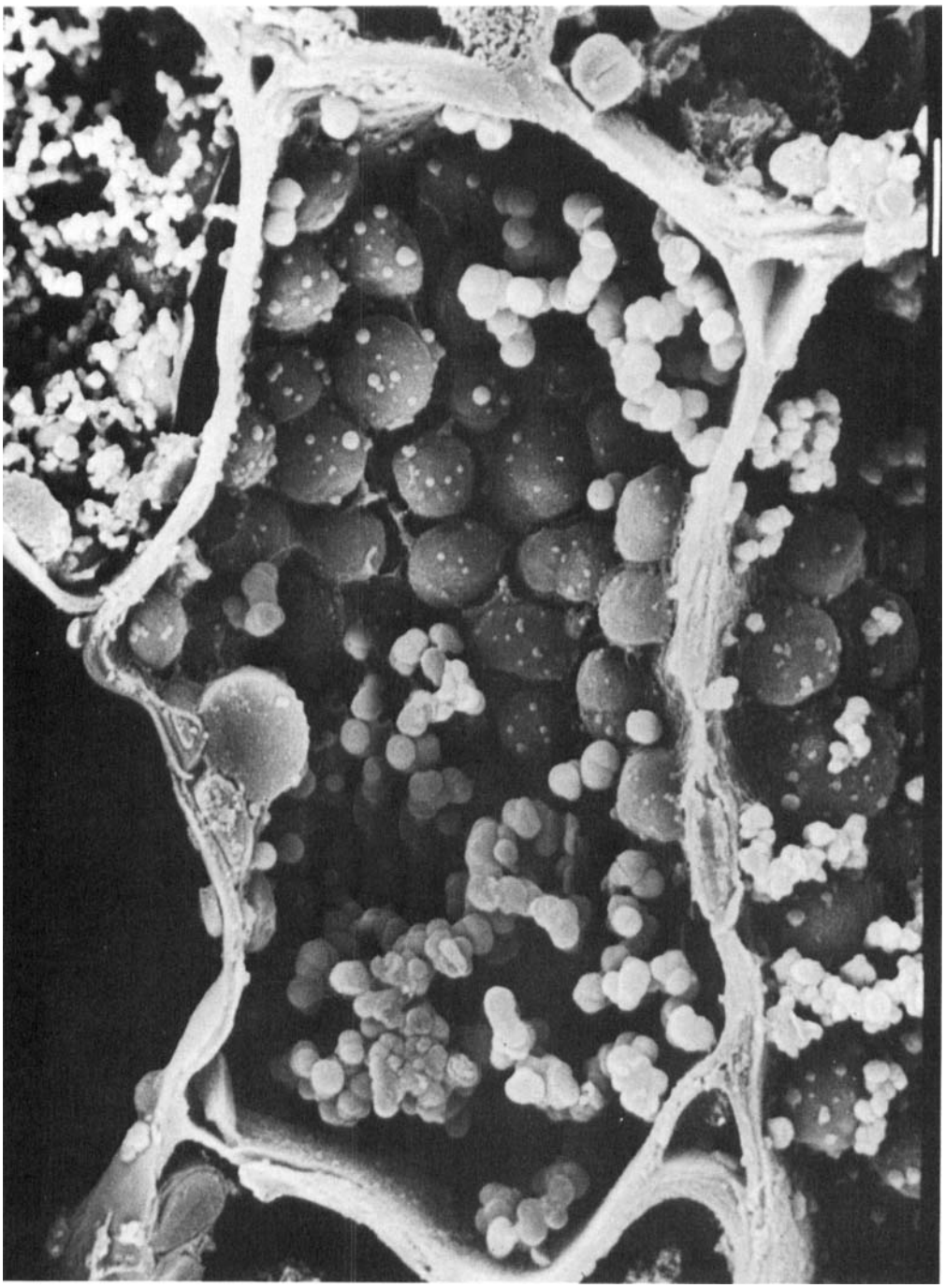

Figure 15 Mesophyll cell of Norway spruce primary needle taken from the reference series showing packed chloroplasts in the background and some phenolic droplets in the central vacuole. Bar = 5 μm.

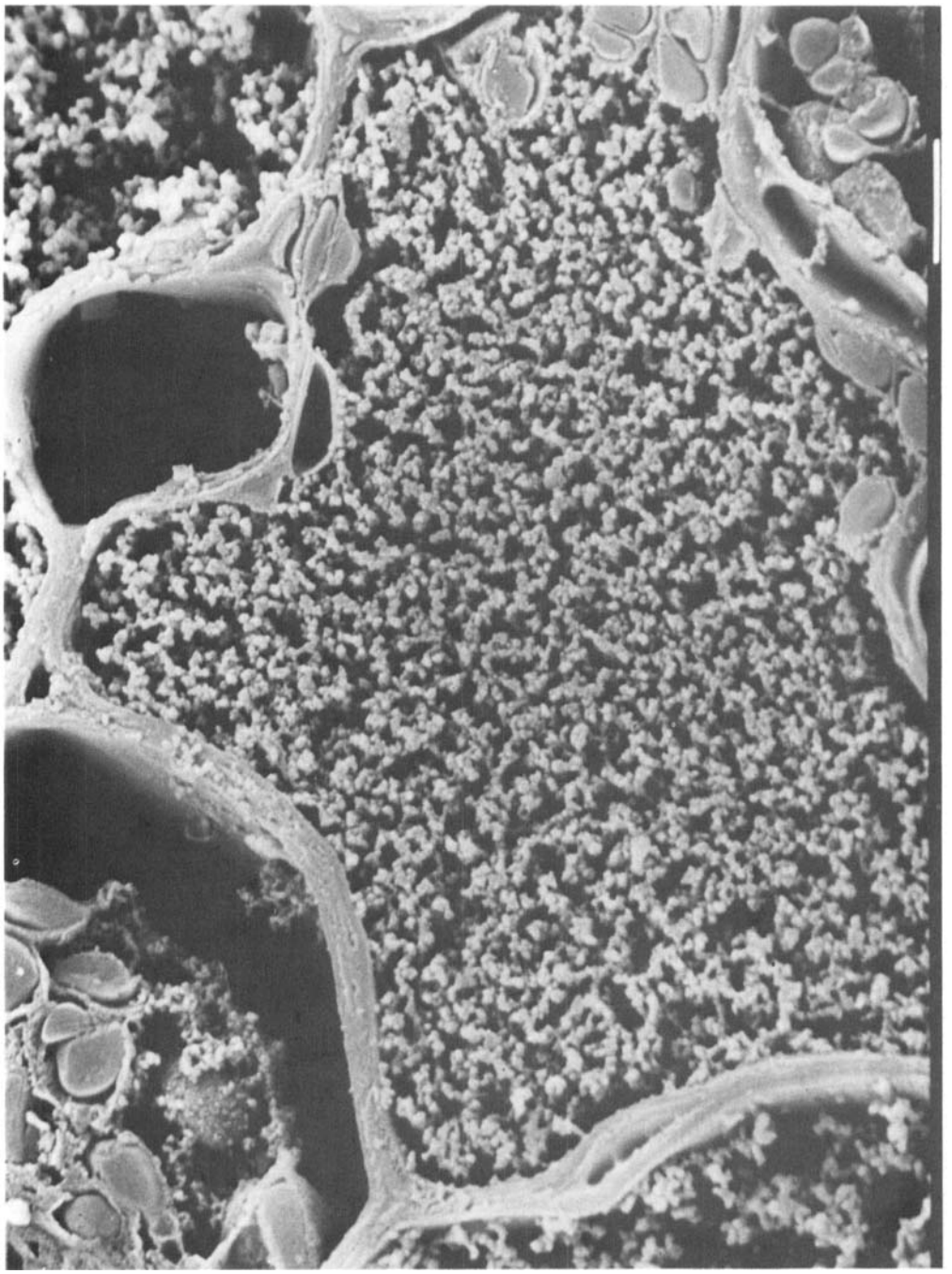

Figure 16 Mesophyll cell of Norway spruce primary needle taken from the reference series showing a higher content of phenolic droplets, smaller in size. Bar = 5 μm.

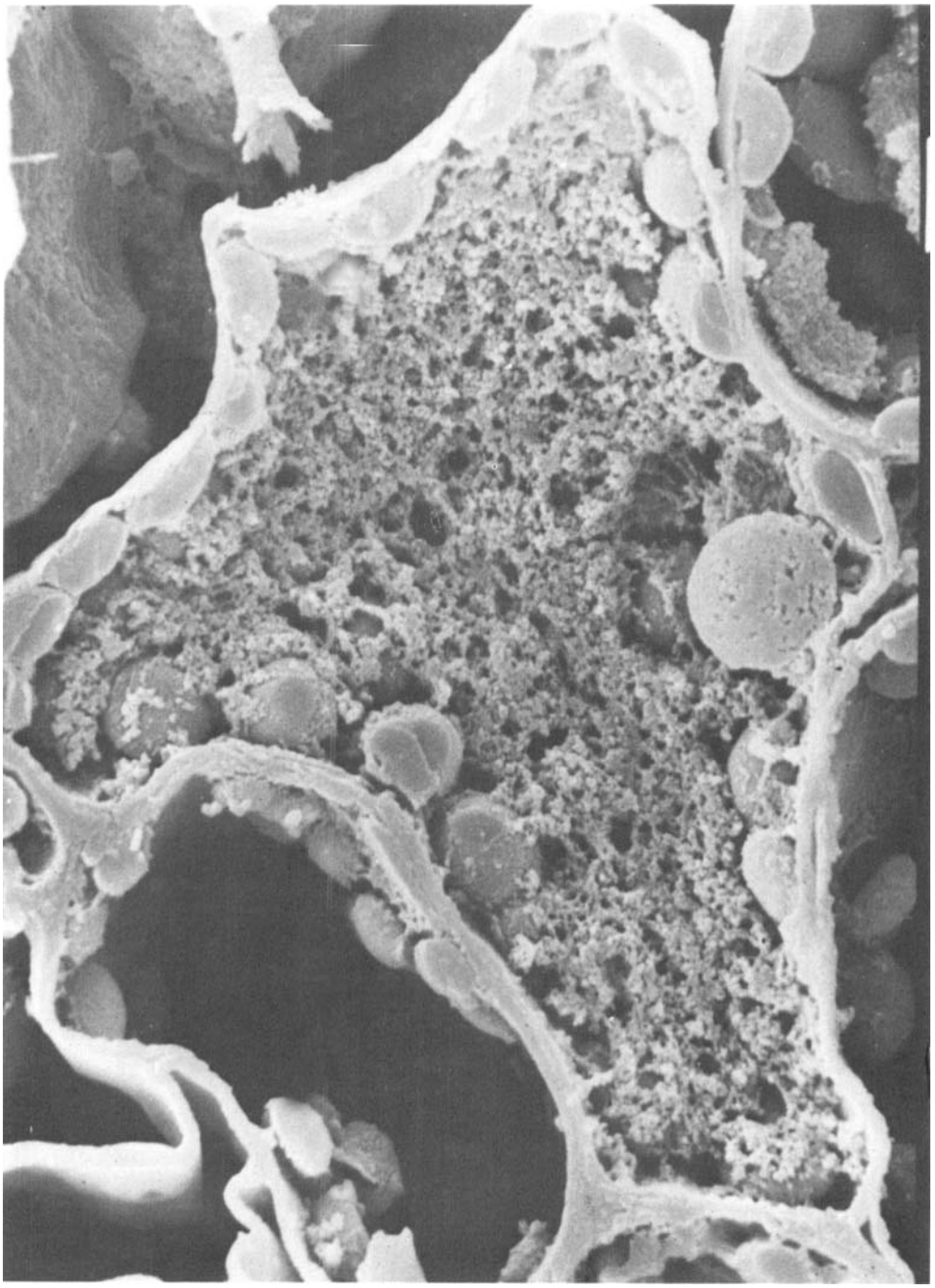

Figure 17 Mesophyll cell of a primary needle of Norway spruce exposed to acidic fog (pH 4) containing 864 ± 250 ppb H_2O_2. Small phenolic droplets are knotted like a string of pearls. Bar = 5 μm.

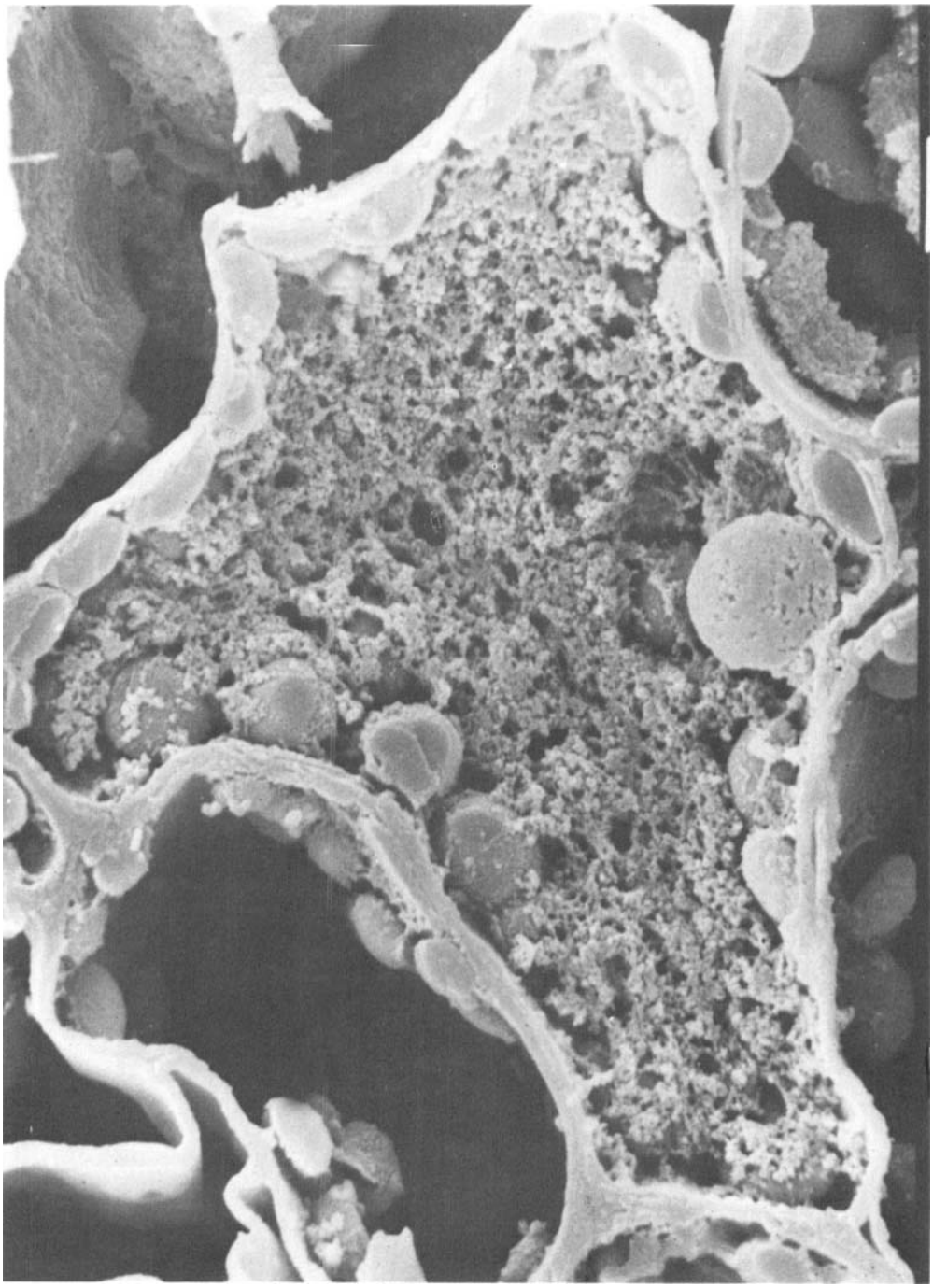

Figure 18 Mesophyll cell of a primary needle of Norway spruce exposed to acidic fog (pH 4) containing 864 ± 250 ppb H_2O_2. Densely located small phenolic droplets fill the central vacuole. The thick-walled cells on the left belong to the hypodermal sclerenchyma. Bar = 5 μm.

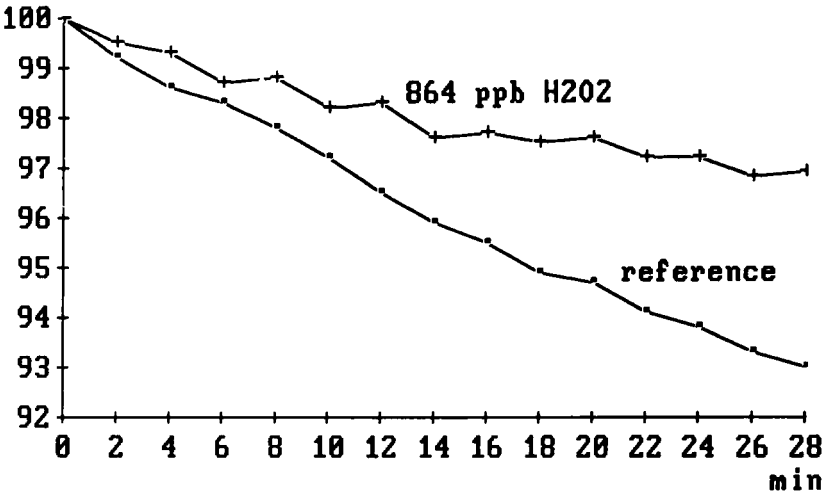

Figure 19 Evaporation rates of primary spruce needles in % of fresh weight.

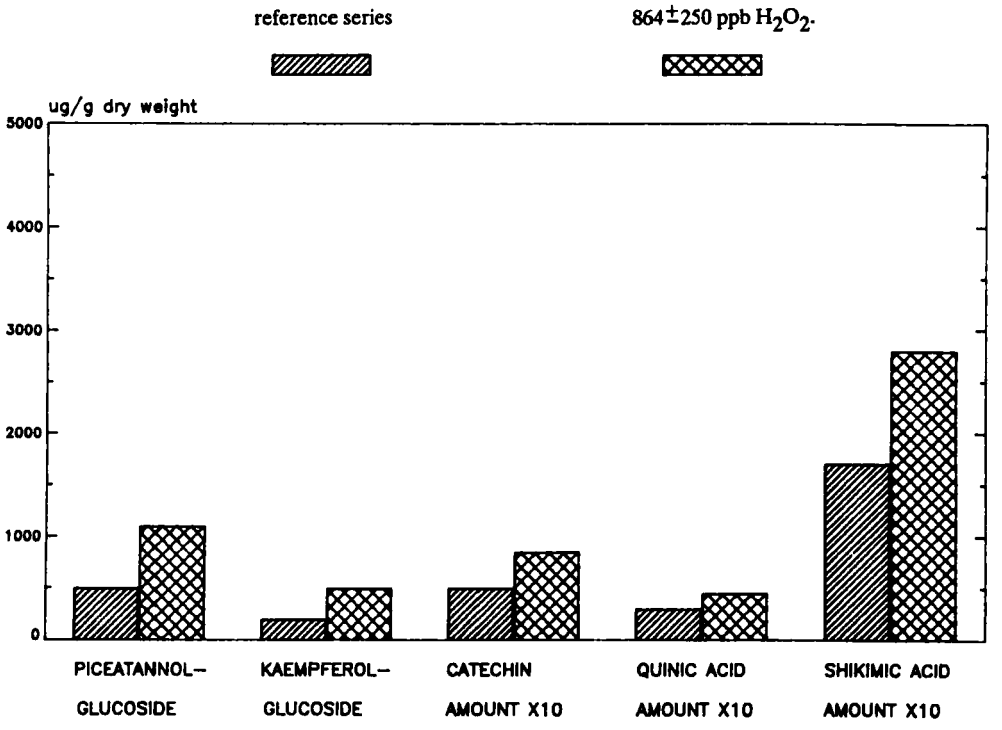

Figure 20 Amount of phenols in primary Norway spruce needles exposed to acidic fog (pH 4) containing 864 ± 250 ppb H_2O_2.

mic acid, increased (Figure 20). Block 1 illustrates the increasing amount of piceatannolglucoside, block 2 that of kaempferol-3-glucoside, which increases approximately two-fold.

The amount of quinic acid and shikimic acid (blocks 4 and 5 in Figure 20) were determined by the ion chromatographic separation technique and conductivity detection. The high absorption rate and the noisy baseline of the purified extracts below 220 nm did not allow quantitative UV measurements of these phenols in RP-HPLC.

Although the amounts of primer phenols are high in young reference needles, they increased under the influence of additional H_2O_2; the rise of shikimic acid was most distinct.

DISCUSSION

In former investigations we pointed out that tissue areas of primary spruce needles as well as secondary spruce needles, were decreased by H_2O_2.[27,31] Because of the higher concentration of H_2O_2 (1–5 ppm) dissolved in acidic fog, as we have reported in an earlier work,[27] the mesophyll cells showed deformations and were collapsed if adjacent to the intercellular space. Thus the area of the intercellular space increased. We did not find this result in our recent investigation, when we used 864 ± 250 ppb H_2O_2. The area of the intercellular space was also reduced by 22% in comparison to the reference needles, but mesophyll cells were hardly deformed. Reductions in leaf tissues, nebulized with H_2O_2-containing acidic fog, were also observed in growing beech leaves[32] and leaves of agricultural grasses.[33] Tissue reduction may be a main effect of H_2O_2 pollution due to a reduced frequency of mitoses.

Comparing these results to those of geobiological studies, the primary needles of damaged trees in the Fichtel Gebirge (FRG) showed the same tendency; reduced areas of needle transverse section, mesophyll cells and intercellular spaces. But it must be said that pollution with SO_2 exhibits similar reducing effects on needle tissues.[34]

The role of acid misting acidified with nitric and sulfuric acid at different pH levels was studied in eastern white pine (*Pinus strobus*) seedlings. Statistically significant increases in area were detected in needle transections with the increased acidity of treatment, down until pH 2.6. This trend was attributed to nutritional effects of nitrogen and sulfur components in the acid mists.[35]

Though the composition of fog water used in our experiment contained NO_3^- and SO_4^{2-} anions, a decrease in tissue area was observed due to H_2O_2.

One very illustrative symptom observed in the spruce needles is the increase of phenols in the vacuoles of the mesophyll cells, as a function of H_2O_2 pollution. Though the total amounts of quantified needle phenols, as detected from methanolic extracts by an HPLC system, with a photodiode-array detector, did not change significantly, the SEM-pictures clearly indicate an increase of phenolic droplets (Figures 17 and 18). Thus we can conclude that a special amount of phenols is not soluble in methanol. Such extraction, even in favourable cases, will

yield only 60 to 70% of the total needle phenols, except for the condensed phenolic compounds that are irreversibly bound to other polymers, like proteins within the cell.[30]

The dominant pathway for Norway spruce phenols is the shikimic acid pathway. Shikimic acid and dehydroshikimic acid are intermediates of this biochemically important pathway and they are the precursors of aromatic compounds, which are often found in free acid state or in bound forms with one of their hydroxyl function esterified to a mono or oligo sugar compound.[36]

The large accumulation of piceatannolglucoside, kaempferol-glucoside, catechin as well as quinic and shikimic acid in primary spruce needles affected by H_2O_2, seems to be a reaction of spruce trees to stress. The function of these compounds in diseased needles is not known yet. It is discussed that piceatannolglucoside may negatively affect the osmotic system.[37]

Production of phenols increases the plant's resistance to transpiration.[38] This finding is confirmed by the reduced evaporation rate of spruce needles in our experiment (Figure 19). The increased density of stomates is also a symptom of water stress.[39,40]

A possible cause of water-stress may be the observed reduction of xylem cells, originating from reduced divisions of cambium cells. The rate of mitosis as well as the growth of cells can be hindered by water-stress.[41,42] When the water content in a needle is minimized and its turgor is reduced, the downward transportation of assimilates in the phloem may stop.[43]

At the end of the first vegetation period, the rows of stomata on the spruce needles are covered with wax-pearls. As revealed by SEM the stomatal alveoli are nearly filled with tubular wax crystals.[44,45] Several investigations have shown damage to the wax layer of conifer needles due to air-borne industrial pollution. Long-distance air-borne pollution may damage the wax so severely as to cause melting of the fine stomatal wax structure shortly after the needles are formed.[46] Structural wax showed fused rodlets after ozone fumigation; cracks were found in the wax plugs of spruce needles treated with acid mist (pH 3).[47] Our experiments with acidic fog (pH 4) without H_2O_2 did not show severely-melted wax and cracks within the plug.

Fused wax rods were observed in the lighter yellow coloured spruce needles from the Black Forest (FRG). This damage was attributed to photochemical oxidants.[48]

Formaldehyde and H_2O_2 are considered to be the cause of degradation in epicuticular waxes.[49] Epicuticular wax damage, caused by ozone,[47] looks similar to those changes we observed under the influence of H_2O_2. We also observed H_2O_2-induced damage of epicuticular wax on needles of Douglas firs.[50] Fused epicuticular wax and cracks in wax plugs may be causes for uncontrolled diffusion of water vapours[51,52] and thus increased water stress in conifer needles.

CONCLUSIONS

Exposure of spruce trees to acidic fog containing H_2O_2 shows significant effects on

primary needles which were still growing during the fumigation experiment. Histological and cytological findings correlate with subcellular changes in secondary plant products. Although the amount of needle phenols dissolved in a methanolic extract did not change significantly in quantities, some compounds of the phenols changed quantitatively after exposure to H_2O_2, especially piceatannolglucoside, kaempferol-glucoside, catechin, quinic acid and shikimic acid. Furthermore, our investigations point to a disturbed water balance as the physiological consequence of the exposure to H_2O_2. Water deficit causes changes in the metabolism of spruce needles, which leads to premature senescence with increasing catabolic and decreasing anabolic metabolisms. These can be observed in increased synthesis of phenolic compounds. There are some accompanying phenomena to water deficit in needles: structural degeneration of chloroplasts, decay of chlorophylls. Some histological changes refer to chronic water deficit: reductions in the number and area of xylem vessels and sieve tubes, caused by reduced divisions of the cambial cells. The rate of mitosis as well as the growth of cells may be hindered by water stress. When the water content of needle cells is minimized and their turgor is reduced, the downward transportation of assimilates in the phloem may stop.

The incorporation of phenols into the central vacuoles of mesophyll cells is suited to prevent dehydration of the spruce needles. It has been discussed that the phenolic compound piceatannolglucoside may play an important role in the water threshold of needles. As a consequence, the evaporation rate of primary needles exposed to acidic fog containing H_2O_2 is reduced. As our experiments with acidic fog containing H_2O_2 were performed in natural growing conditions, the reported results make it obvious that H_2O_2 cannot be ruled out as an important factor in forest decline.

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