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Structure and Function
Trees

Some effects of long-term ozone fumigation on Norway spruce

II. Epicuticular wax and stomata

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Summary. Potted cuttings of a 12-year-old *Picea abies* tree were fumigated with ozone, 100 or 300 μg $O_3 \cdot m^{-3}$ (50 or 150 ppb O_3) being added to charcoal-filtered air during the 1985 growing season for a total of 1215 h. The wax structure of ozone-fumigated needles was no different from that of controls. Because flattened wax structures and fused wax fibrils also occurred in controls, these phenomena could not serve as bioindications for the ozone concentrations applied. A smooth layer was found beneath the soluble wax layer and covered needle surface and stomatal openings of ozone-fumigated needles to a greater extent than in controls. Wax quantity was considerably reduced by fumigation with 300 μg $O_3 \cdot m^{-3}$. Leaf pigments (as extracted with the wax) were less abundant in needles treated with 300 μgO_3; the smooth layer probably contributed to the impeded extraction of pigments.

Key words: Ozone – *Picea abies* – Stomata – Wax

Introduction

The plant cuticle, with epicuticular wax as the outermost layer, is generally thought to protect the needle surface against desiccation, pollution and pathogens. It was called "the first line of defence" by Juniper and Cox (1973). Together with gas exchange and stomatal response (Keller and Häsler 1987) the sensitivity of epicuticular wax to long-term ozone fumigation was investigated.

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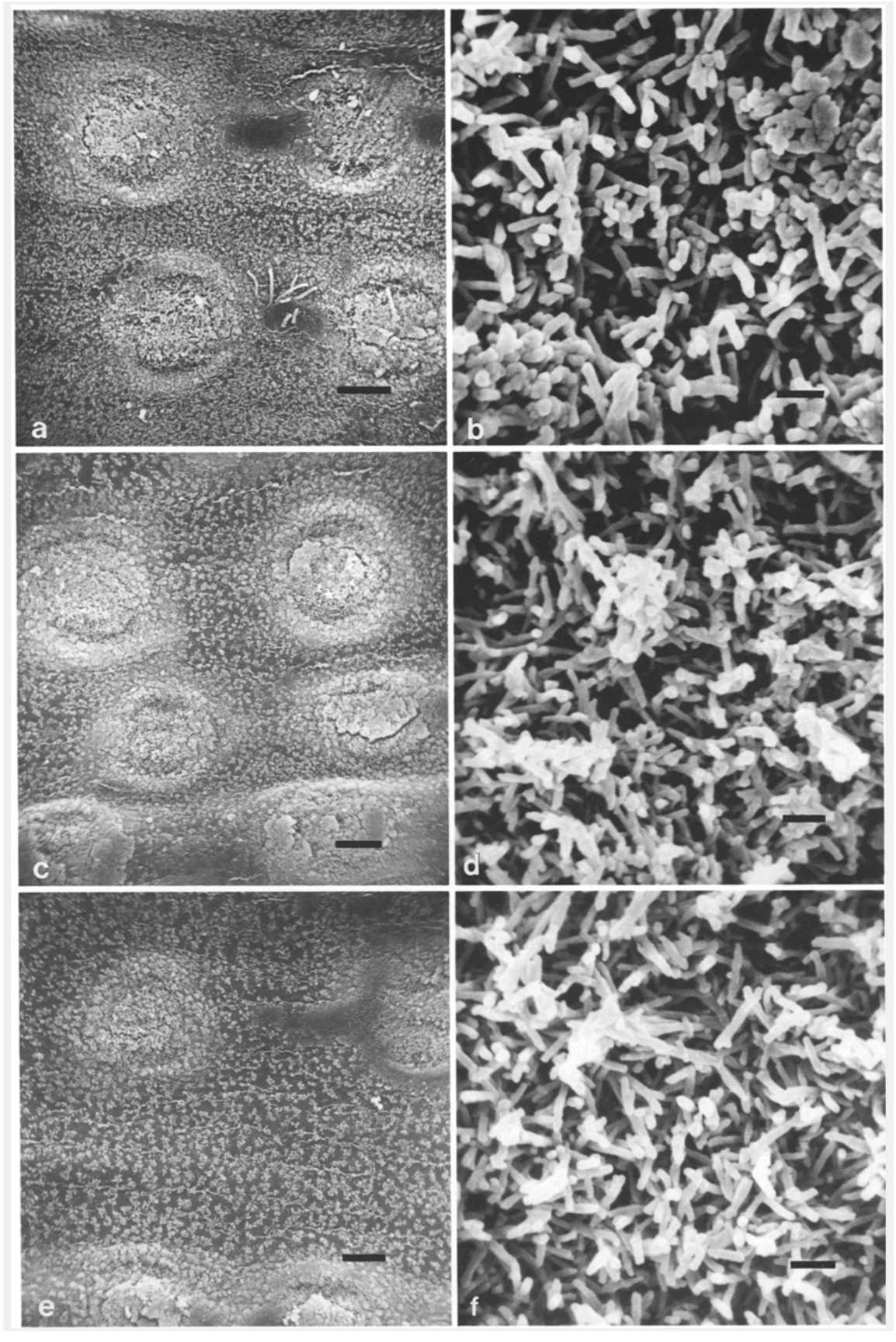
Conifer needles from polluted areas showed faster weathering, fusion, and erosion of the wax fibrils (Huttunen and Laine 1983; Crossley and Fowler 1986; Sauter and Voss 1986) than needles from clean-air sites. Wax became well known as the first target of injuries due to ozone in some theories which sought to explain the causes of forest decline in clean-air regions (Elstner and Osswald 1984). According to Schmitt et al. (1987), however, acid precipitation may be the main cause of changes in stomatal wax plugs. Nevertheless, ozone might be one factor in wax destruction. Therefore, its effects were investigated with genetically uniform plant material under controlled fumigation conditions.

Materials and methods

Four-year-old cuttings of a 12-year-old *Picea abies* (L.) Karsten tree (clone 31-4) were utilized (five for each treatment).

Fumigation was carried out in the Birmensdorf open-air installation from 23 April to 28 October 1985 with: (1) charcoal-filtered air, (2) charcoal-filtered air + 100 μg $O_3 \cdot m^{-3}$ (50 ppb), 9 h/day on 135 workdays, and (3) charcoal-filtered air + 300 μg $O_3 \cdot m^{-3}$ (150 ppb), 9 h/day on 135 workdays. Details are described by Keller and Häsler (1987).

Sample treatment. All the youngest twigs (6 months old) of a tree were cut immediately after fumigation. Needles of each tree were handled separately. Twenty needles per sample were taken randomly with forceps and freeze dried. Freeze-dried needles were coated with 80% Au/20% Pd for scanning electron microscopy (SEM). Needles were harvested by immersing the twigs for 4 s in liquid nitrogen, after which the needles were easily stripped off; needles were stored in a deep freeze. Wax was extracted by shaking an aliquot of 50 g (fresh weight) needles in a separatory funnel with three volumes (2.5 times the needle volume) of fresh chloroform. Extraction was quantitative as judged by the clean surface observed by SEM. No waxy granules or impurities were present as known from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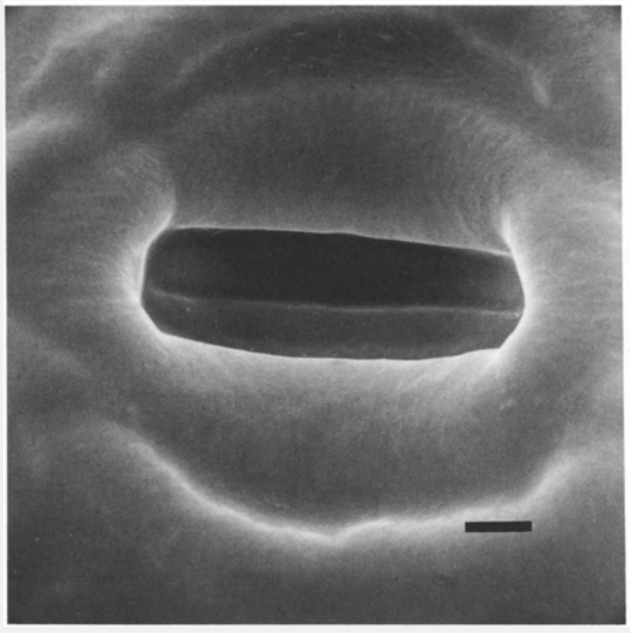

Fig. 2. Free guard cell, smooth layer degree 0, fumigation with charcoal-filtered air (tree no. 19); *scale marker* = 5 μm

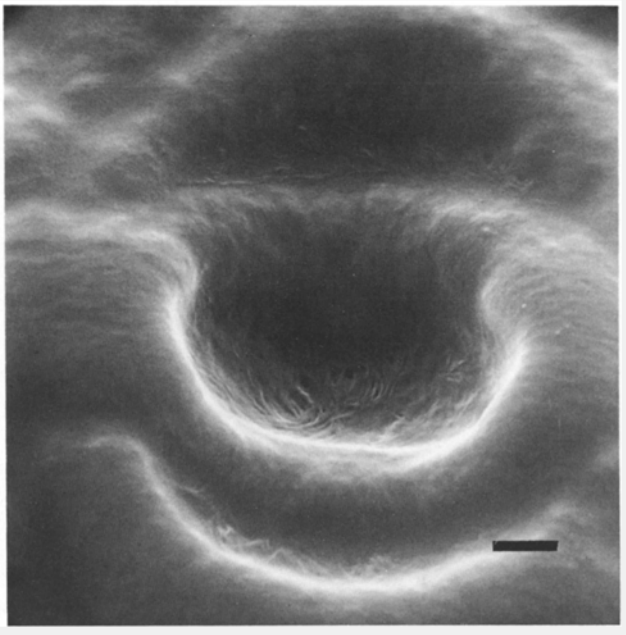

Fig. 4. Covered guard cell, smooth layer degree II, fumigation with charcoal-filtered air + 300 μg $O_3 \cdot m^{-3}$ (tree no. 9); *scale marker* = 5 μm

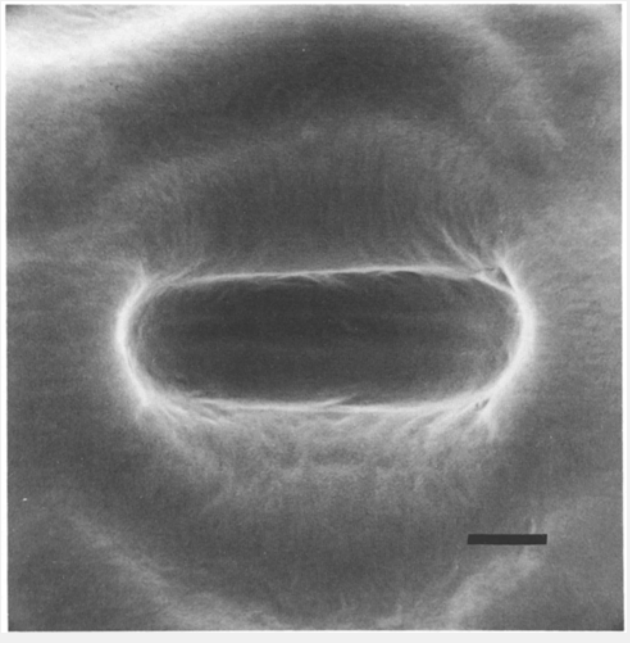

Fig. 3. Veiled guard cell, smooth layer degree I, fumigation with charcoal-filtered air + 100 μg $O_3 \cdot m^{-3}$ (tree no. 14); *scale marker* = 5 μm

Fig. 1. a–f: Wax structure on stomatal openings. *Left row:* scale marker = 20 μm; *right row:* scale marker = 1 μm. *Top:* fumigation with 300 μg $O_3 \cdot m^{-3}$; *middle:* fumigation with 100 μg $O_3 \cdot m^{-3}$; *bottom:* fumigation with charcoal-filtered air. The figures in the right row were taken from the corresponding needles of the left row

incomplete extraction of soluble epicuticular wax. After filtering the solvent was evaporated under vacuum and the extract and the needles were dried (80° C) to constant weight. Needle length and dry weight were each determined on the basis of 50 randomly selected needles. Twenty extracted and dried needles were taken out randomly for another SEM observation. Some leaf pigments, extracted together with the wax, were removed by means of their insolubility in boiling hexane. Extracted leaf pigments were checked by gas chromatography for any loss of wax. Mean values and standard deviations were calculated and the treatments were compared using the *t*-test.

Results

Wax structure

Six-month-old needles showed variations in wax structure, independent of O_3 concentrations (Fig. 1 a, c, e) and under strong magnification (Fig. 1 b, d, f) areas were readily found with the same structure. Wax structures varied in all treatments between entirely crystalline wax with fine or stout wax fibrils and entirely flattened wax with fused wax fibrils and lines of amorphous wax covering the stomatal openings. Sometimes clotted wax or isolated long wax fibrils were observed. Even within single needles, great local differences were present. The two trees with the most flattened wax on the needles investigated had been fumigated with charcoal-filtered air. Cracks in the wax layer occurred randomly in all samples: they are artefacts formed by freeze-drying. Because of the great variety in the wax structure between individual needles and even within one

Table 1. Estimate of the qualitative degree of stomatal covering observed in 6-month-old needles

Treatment	Degree 0 Tree no.	Degree I Tree no.	Degree II Tree no.	Degree III Tree no.
Filtered air	3, 7, 11, 15, 19	7	11	–
100 μg $O_3 \cdot m^{-3}$	–	6, 10, 14	2, 10, 18	–
300 μg $O_3 \cdot m^{-3}$	17	1, 5, 13	5, 9, 13	13

Table 2. Mean dry weight (dw) per needle, mean needle length of 6-month-old needles

Treatment	dw/needle (mg)	Needle length (cm)
Filtered air	1.74±0.01	1.34±0.07
100 μg $O_3 \cdot m^{-3}$	1.83±0.25	1.34±0.07
300 μg $O_3 \cdot m^{-3}$	1.62±0.20	1.30±0.07

single needle, we did not venture to estimate percentages of fused wax areas.

Stomatal surface

Threefold extraction with chloroform usually removes the wax layer and renders the guard cells distinctly visible (Fig. 2, degree 0). In this study, however, the needles exhibited a phenomenon previously observed in needles from the alpine timberline (Günthardt and Wanner 1982) viz. the covering of needles and guard cells beneath the soluble epicuticular wax with an insoluble smooth layer. This smooth layer was classified into four qualitatively judged degrees: (1) degree 0 = no smooth layer, visible guard cells (Fig. 2); (2) degree I = veiled guard cells (Fig. 3); (3) degree II = thin smooth layer (Fig. 4); and (4) degree III = more pronounced smooth layer.

Of ten trees fumigated with 100 or 300 μg $O_3 \cdot m^{-3}$, five had needles with very thin smooth layers, that is only veiled guard cells (Fig. 3, degree I), and five had thin smooth layers belonging to degree II (Fig. 4) or in some cases degree III on the investigated needles (Table 1). Needles fumigated with charcoal-filtered air had, with a few exceptions, free guard cells (Fig. 2, degree 0). Within most samples the smooth layer was homogeneous. The needles in themselves were also homogeneous, which prompted us to omit a statistical treatment. Therefore, trees 5 and 10 had on the investigated needles smooth layers belonging to degree I or II. Needles of tree 13, however, were partly exceptional in that three different degrees could be found on the same needle.

Within the samples fumigated with 100 μg $O_3 \cdot m^{-3}$, those with a continuous thin smooth layer of degree II (trees 2 and 18, Table 1) had less leaf pigment in the crude wax extract. In the samples fumigated with 300 μg O_3 this distinction could not be found, because all samples contained less leaf pigment (Table 4). No relationship was found between the degree of the smooth layer and the needle dry weight or the wax content within the five trees of the same treatment.

Wax quantity

No visible differences were found between the different treatments. Growth parameters of the needles (Table 2) showed no statistically significant differences, although 300 μg O_3-treated needles had the lowest mean dry weight per needle and were shortest.

The filtered chloroform extract of entire needles comprises mainly epicuticular wax together with some pigments and perhaps other lipids exuded from wounded needles. It is normally used to measure wax amount. Table 3 reveals that amounts of chloroform extracts (top) and of wax (bottom) were diminished by ozone, but only the results of the fumigation with 300 μg O_3 showed a statistically significant difference from controls. Wax itself is soluble in a large volume of boiling hexane, whereas the pigments form removable clots. The difference shows that for *P. abies* these leaf pigments in the crude extract are not negligible and may mask the real wax quantity, although ozone-fumigated needles contain less leaf pigment in the extract (Table 4).

Discussion

Wax structure

Wax covering the stomatal openings showed in its structure some flattened areas with fused wax fibrils (in the literature often termed erosion) after fumigation with O_3. Because the same flattened

Table 3. Mean quantity of chloroform extract (*top*) and of wax (*bottom*) (after extraction of leaf pigments) in 6-month-old needles

	Treatment	Extract/needle (μg)	Differs from	Extract/g dw (mg)	Differs from
a	Control	35.7 ± 3.9	c***	20.6 ± 2.4	c**
b	100 μg $O_3 \cdot m^{-3}$	34.7 ± 3.7	c**	19.2 ± 2.5	c*
c	300 μg $O_3 \cdot m^{-3}$	23.0 ± 2.2	a***, b**	14.3 ± 0.9	a**, b*
a	Control	24.0 ± 1.3	c**	13.8 ± 0.8	c*
b	100 μg $O_3 \cdot m^{-3}$	25.8 ± 2.6	c**	14.2 ± 0.6	c***
c	300 μg $O_3 \cdot m^{-3}$	19.0 ± 1.5	a**, b**	11.8 ± 0.5	a*, b***

Significantly different with $P < 0.01$ (***), $P < 0.02$ (**) and $P < 0.05$ (*)

Table 4. Percentage non-wax components in the crude wax extract

	Treatment	% Leaf pigments	Differs from
a	Control	31.1 ± 4.6	c***
b	100 μg $O_3 \cdot m^{-3}$	24.1 ± 6.6	–
c	300 μg $O_3 \cdot m^{-3}$	16.9 ± 2.6	a***

Significantly different with $P < 0.01$ (***)

structures were present in the controls (fumigated with charcoal-filtered air, Fig. 1 e) we could not attribute such "erosion" to ozone. This result leads to the opinion that such fused wax fibrils should not be considered as a bioindication of latent or direct injury by low concentrations of gaseous O_3. The flattened wax areas found in control needles raise the question as to whether fine crystalline wax fibrils over the stomatal openings of 6-month-old needles of *P. abies* are alone to be taken as a sign of health. The fact that long-term low concentration fumigation with O_3 alone did not directly affect the wax structures agrees with the findings of Trimble et al. 1982, Skeffington and Roberts 1985, and Riding and Percy 1985. Magel and Ziegler (1986), on the other hand, detected fused wax fibrils in 4- to 6-week-old spruce needles fumigated with O_3 but not in the controls.

Stomatal surface

In the first weeks after flushing the guard cells begin to sink beneath the surface; the subsidiary cells form the outer stomatal cavity, which is filled with epicuticular wax (Günthardt 1985). Chloroform extraction removes the epicuticular wax and usually renders the guard cells visible (observed by SEM). In 5-month-old extracted needles of healthy *P. abies* from the alpine timberline, we observed a thin smooth layer covering the needle surface, which was striking because the guard cells were veiled. This layer increased with needle age (Günthardt and Wanner 1982). In the present study, such a smooth layer was found on needles treated with ozone, whereas in needles under charcoal-filtered air guard cells were visible as usual. This suggests that the smooth layer is a stress phenomenon. Thus it may be due to ozone, although the needles taken from healthy trees in the forests have perhaps formed the smooth layer due to other stress factors, e.g., drought. The formation of the smooth layer is probably an additional natural mechanism for the regulation of gas exchange. It may even be a disadvantage for the needles to have free stomata, but the question as to whether the presence and the degree of the smooth layer may serve as a bioindication for stress situations cannot yet be answered.

Wax quantity

The concentration of 300 μg $O_3 \cdot m^{-3}$ during the first growth period tended to reduce needle growth in general, but differences were not significant. On the other hand, the wax quantity built up during this period was considerably reduced. This agrees with several reports indicating that needles on sites with stress situations contain less wax. In needles fumigated with 300 μg $O_3 \cdot m^{-3}$ the presence of a smooth layer and reduced wax quantity coincided, but this was not the case in the fumigation with 100 μg $O_3 \cdot m^{-3}$.

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