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Responses of spruce seedlings (*Picea abies*) to exhaust gas under laboratory conditions—II ultrastructural changes and stomatal behaviour

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Abstract

This study examines the effects of exhaust gas exposure on the epistomatal wax structure and mesophyll ultrastructure in needles of Norway spruce (*Picea abies* (L.) Karst.) seedlings. Stomatal diffusive resistance was also measured. Two independent exhaust gas fumigations were performed: 100 and 200 ppb measured as NO_x, for 10 days and 50, 100 and 200 ppb NO_x for 19 days. The obstructive effect of exhaust gas exposure on epistomatal wax tubules was apparent. The stomata became covered by flat and solid wax resulting from the structural degradation of the wax crystalloids. Increasing the exhaust gas concentration in the chamber atmosphere exacerbated the degradation of the wax structure. Exhaust gas exposure induced aggregation and electron translucence of plastoglobuli, swelling of thylakoids, increase of cytoplasmic lipids and slight increase of vesiculation of cytoplasm in mesophyll cells of current and previous year needles. These changes were exemplified in current year needles. Damage to the epicuticular waxes and mesophyll ultrastructure of spruce needles most likely reflects the NO_x and volatile hydrocarbon fraction. The alterations in epicuticular waxes and mesophyll ultrastructure can be related to accelerated senescence of the youngest, photosynthetically active, needle generation. The exhaust gas also resulted in decreased diffusive stomatal resistance at night which indicates that the exhaust gas exposure disturbed the gas exchange of spruce seedlings. The results show that even relatively short-term exposure to realistic concentrations of exhaust gas in the atmosphere can induce rather severe injuries to the needle surface structure as well as ultrastructure at the cellular level.

Keywords: Norway spruce; Exhaust gas; Epistomatal wax; Mesophyll ultrastucture; Diffusive resistance

1. Introduction

Spruce needles are covered with rod-like crystals concentrated in and around the epistomatal chamber. The needle surface, between the stomata, is covered with tubular crystals (Bermadinger-Stabentheiner, 1994; Huttunen, 1994; Turunen, 1996). The epicuticular wax layer on plant leaves is a barrier between the plant and its environment and is one of the first targets of atmospheric pollutants (Sauter et al., 1987; Bermadinger-Stabentheiner, 1994; Günthardt-Goerg et al., 1994). The wax crystals fuse and flatten with age, but in polluted air the erosion rate of the wax structures increases (Sauter and Voß, 1986; Günthardt-Goerg et al., 1994; Huttunen, 1994). At worst the epistomatal chamber of the needle may be plugged totally by the fused wax, inhibiting transpiration which could have far-reaching physiological consequences, such as prevention of gas exchange and photosynthesis (Sauter and Voß, 1986; Kammerbauer et al., 1987; Sauter et al., 1987). The accelerating effect of various air pollutants (SO_2, O_3, acid precipitation, dust, UV-B) on epicuticular wax erosion has been shown in many studies (e.g. Turunen and Huttunen, 1990; Günthardt-Goerg et al., 1994; Huttunen, 1994; Turunen, 1996). Exposure of spruce seedlings to motor vehicle emissions in the laboratory as well as along a highway, has revealed clear evidence of accelerated structural degradation of the epistomatal waxes (Sauter et al., 1987; Sauter and Pambor, 1989) and also decreases in transpiration and net photosynthesis rate (Kammerbauer et al., 1987).

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Air pollution has been found to disturb stomatal control. According to earlier studies, exposure of Norway spruce to polluted air caused loss of stomatal control which changed the drought avoidance abilities and thus induced premature needle abscission (Maier-Maercker and Koch, 1995). Exposure of Norway spruce at the roadside reduced the regulation capacity of the stomata, lowered the transpiration rate and reduced the net photosynthesis (Kammerbauer et al. 1987).

Gaseous pollutants penetrate needles through the stomata and spread along the intercellular spaces throughout the photosynthesizing mesophyll tissue. The structural injuries related to gaseous pollutants are, in most cases, restricted to mesophyll tissue and only very high pollution loads induce changes in conductive tissues in the inner parts of the needles (Fink, 1988; Holopainen et al., 1992). The ultrastructure of conifer mesophyll cells has proved to be a sensitive early phase indicator also revealing specific symptoms to different environmental stresses (Holopainen et al., 1992). The exhaust gas is a mixture of several components, the ultrastructural effects of which have been poorly studied so far.

In the present paper the effects of realistic exhaust gas exposure were studied in controlled environment chambers on Norway spruce (*Picea abies* (L.) Karst.) needle epicuticular waxes, mesophyll ultrastructure and stomatal behaviour. The results are discussed in relation to the air quality in the chambers (i.e. concentrations of NO_x and volatile organic hydrocarbons [VOCs] (Viskari et al., 1999).

2. Materials and methods

Two independent exhaust gas fumigations were performed. In experiment 1 2-year-old Norway spruce (*P. abies*) seedlings were exposed to two exhaust gas concentrations of 100 and 200 ppb measured as NO_x for 10 days. In experiment 2 the seedlings were exposed to three exhaust gas concentrations of 50, 100 and 200 ppb NO_x for 19 days. The growing conditions and experimental treatments of the seedlings are presented in detail in Viskari et al. (1999).

For studying the epistomatal waxes on the surface of the needle, sampling was performed after 5 days of exposure and at the end of the exposure in both experiments. Two needles from eight (experiment 1) or 10 (experiment 2) different seedlings per treatment were sampled and a total of 40 stomatal areas were analysed per needle. The needle samples were air-dried in room temperature and the middle part of the needle was mounted on aluminium stubs with double-sided tape. Samples were sputtered with a 60-nm layer of gold-palladium under vacuum (Polaron Equipment Limited, UK, SEM Coating Unit E5100) and needle surface structures were studied with scanning electron microscope (SEM; JEOL JSM-35). The structural degradation of the epistomatal wax was classified to four stages as described earlier by Sauter et al. (1987); stage I, healthy, epistomatal wax crystalloids without visible degradation; stage II, slightly damaged, beginning structural degradation and aggregation of the wax rodlets; stage III, moderately damaged, advanced structural degradation and aggregation of wax rodlets on more than 30% up to 80% of the surface of the epistomatal chamber and beginning formation of a flat layer of wax; and stage IV, severely damaged, most severe aggregation and degradation of wax and formation of continuous layer on 90% or more of the epistomatal chamber leading to their structural occlusion.

For light and transmission electron microscopic studies five needles, each from different seedling, were collected from the middle part of the leading shoot in all treatments of experiment 2. An approximately 1 mm piece was cut from the middle region of the needles and prefixed in 2% glutaraldehyde in phosphate buffer (0.1 M, pH 7.0) overnight and postfixed in buffered OsO_4 solution at 6°C for 6 h, dehydrated in graded ethanol series and embedded in epon LX-112 (Soikkeli, 1980). Sections for light microscopy were stained with toluidine blue and sections for electron microscopy (JEOL 1200 EX) with uranyl acetate and lead citrate.

The diffusive resistance of water vapour from the needles was measured in the first experiment. The measurements were carried out using a steady state porometer (LI-COR LI-1600) equipped with a cylindrical chamber (1600-07), which is specifically designed to measure stomatal resistance of irregularly shaped leaves, such as conifer needles. Diffusive resistance was measured from 10 seedlings per treatment, two branches from current-year needles and three branches from 1-year-old needles. The measurements were carried out three times during the day: in the morning, before starting the exposure (0:800), in the afternoon just after the exposure had stopped (16:00) and during the night, when it was dark (23:00–02:00).

2.1. Statistical analyses

The statistical analyses were carried out using the SPSS/PC+ package. Analysis of variance (ANOVA) and Tukey's multiple range tests were used for comparing the group means between treatments. The normal distribution of the data was examined with Kolmogorov-Smirnov distribution test. The qualitative classification of epistomatal wax injuries was changed into numerical variables by calculating the number of epistomatal wax in each injury class. The statistical analyses were then performed from data where tree-specific average values were used. The diffusive resistance measurement data from eight different days was combined into one datum.

3. Results

3.1. Epistomatal wax structure

No visible injuries were found in current-year needles of spruce seedlings after the exposure in experiment 1. However, SEM revealed that the epistomatal waxes had degraded during 10-day exposure. Electron micrographs of some of the characterizing injuries are presented (Fig. 1). Fig. 1b–d also shows indications of particle deposition in the epistomatal area of exhaust gas-exposed needles. After 5 days of exposure there were some observable degradation of the epistomatal wax in current-year needles of treated seedlings. About 65% of needle wax structures were still undegraded in both 100 and 200 ppb NO_x treatments (Fig. 2a). After 10 days of exposure the wax degradation was more apparent. In both treatments, 60% of the needles had slightly or moderately degraded epistomatal wax when compared to the control. In addition, treatment with 100 and 200 ppb NO_x severely damaged epistomatal wax structures in 10 and 13% of needles, respectively. Some severe damage (2.5% of needles) was also observed in control seedlings (Fig. 2b). The differences in the proportion of needles in the respective damage classes between control and exhaust gas treatments were also statistically significant.

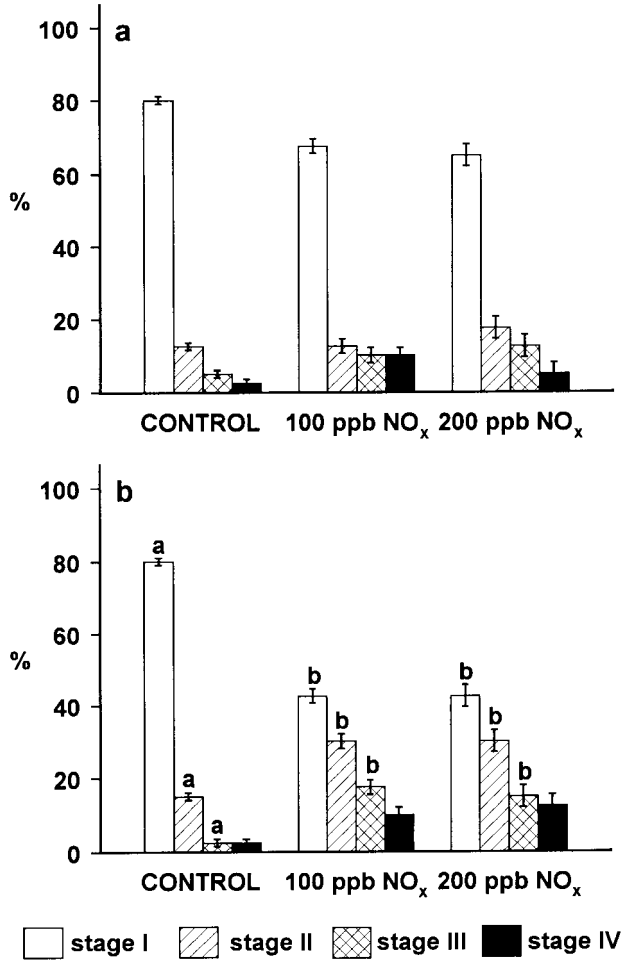

Fig. 1. The amount of needles in different stages of epistomatal wax injury in current-year needles of exhaust gas-exposed Norway spruce seedlings: (a) after 5 days of exposure; and (b) after 10 days of exposure. Bars (SE) with different letters are statistically significantly different between exposure treatments at $p<0.05$ level (ANOVA, Tukey's multiple range test), $n=32$ in each treatment. For stages of injury see Sauter et al. (1987).

In 1-year-old needles the effects of the exposure were more obvious. After 5 days of exposure the amount of healthy needles decreased and the amount of moderately damaged needles increased significantly in the treatment 200 ppb NO_x compared to the control treatment. In the 100 ppb treatment, 52.5% and in the 200 ppb NO_x treatment only 32.5%, of the needles were healthy while in the control the amount of healthy needles was 68% (Fig. 3a). After 10 days of exposure the amounts of healthy needles were 25, 12.5 and 72.5%, respectively. After 10 days of exposure the epistomatal wax was severely degraded in 2.5% of needles in the control treatment and in the 100 and 200 ppb NO_x treatments the amounts were 13 and 18%, respectively (Fig. 3b). The differences between control and exhaust gas treatments in injury stages I–III were also statistically significant.

In experiment 2 a similar pattern was observed as in experiment 1. No visible injuries were observed in exposed seedlings. Fig. 4a shows that at the end of the experiment, i.e. after 19 days of exposure, epistomatal waxes were healthy in 85% of the current-year needles in the control treatment, while in the treatment 200 ppb NO_x only 40% of the needles were healthy. In the control treatment the epistomatal wax was severely degraded in 7.5% of needles, while in treatment 200 ppb NO_x the epistomatal wax was severely degraded in 20% of needles. The differences were also statistically significant. The longer exposure period increased the amount of severely degraded epistomatal wax in treatment 200 ppb NO_x when compared to experiment 1, but this was the only noticeable effect of increasing the exposure period.

In the control treatment 45% of the 1-year-old needles were healthy, and the proportion of healthy needles decreased gradually with increasing exhaust exposure. In the 100 ppb NO_x treatment 15% and in the 200 ppb NO_x only 5% of the needles were healthy. In the control treatment the epistomatal waxes were moderately degraded in 15% of needles, while in treatments 100 and 200 ppb NO_x the epistomatal waxes were moderately degraded in 40 and 45% of needles, respectively (Fig. 4b). The differences were also statistically significant. The longer duration of the exposure increased the proportion of moderately and severely degraded epistomatal wax of 1-year-old needles in treatments 100 and 200 ppb NO_x when compared to experiment 1.

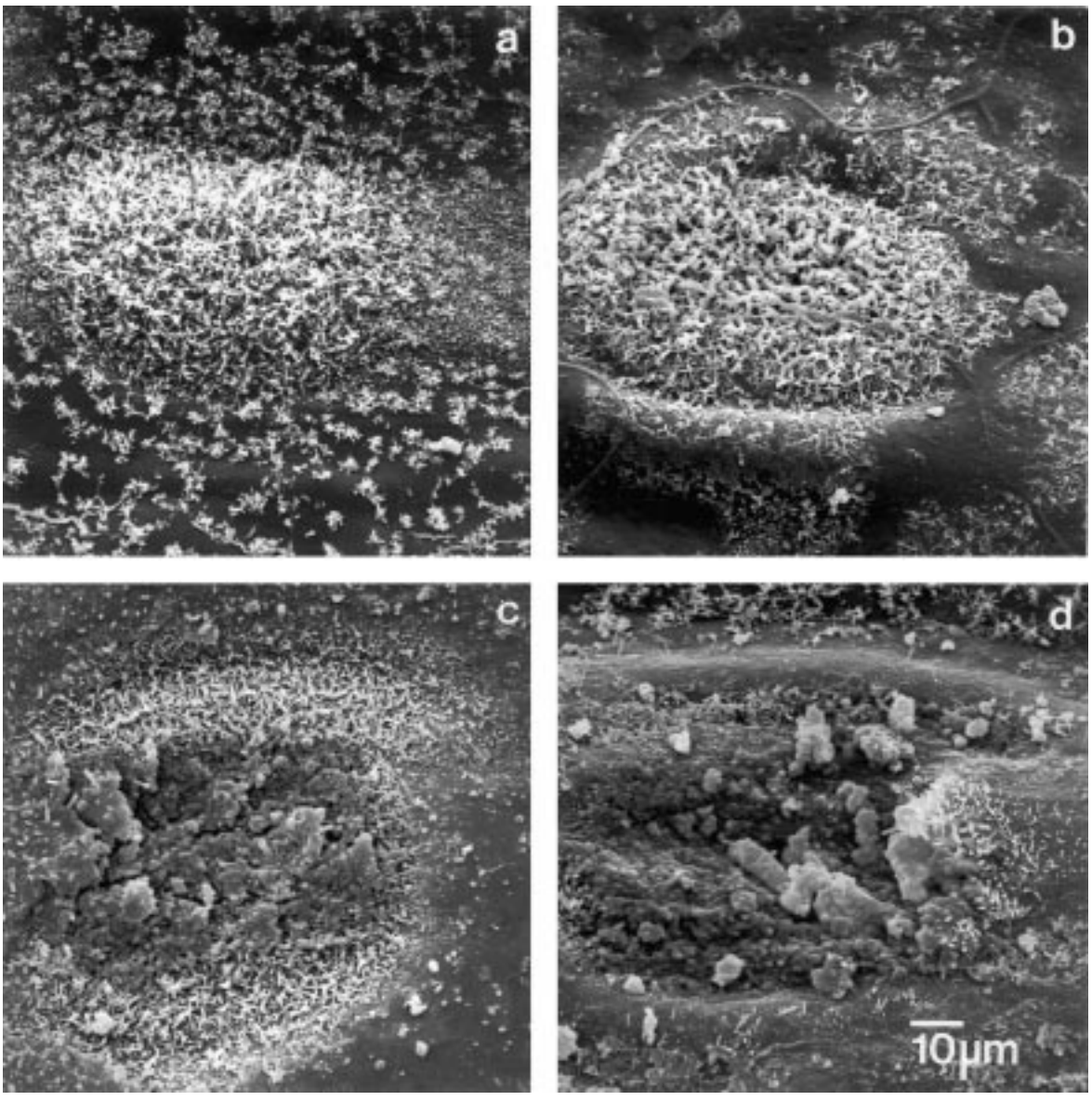

Fig. 2. Electron micrographs of different injury stages observed in exhaust gas-exposed Norway spruce needles: (a) healthy epistomatal wax from control treatment, current-year needle (stage I) (×780); (b) slightly injured epistomatal wax from treatment 100 ppb NO_x, 1-year-old needle (stage II) (×780); (c) moderately injured epistomatal wax from treatment 100 ppb NO_x, 1-year-old needle (stage III) (×780); and (d) severely injured epistomatal wax from treatment 200 ppb NO_x, current-year needle (stage IV) (×780). For stages of injury see Sauter et al. (1987). Scale = 10 µm.

3.2. Ultrastructure of mesophyll cells

The ultrastructural analysis of the mesophyll cells of current- (Fig. 5a) and 1-year-old (Fig. 5b) needles, collected at the end of experiment 2 (19 days exposure), revealed several qualitative changes that are summarized in Table 1. The early signs of the changes were observable at the lowest exposure level (50 ppb NO_x) and the severity of the symptoms increased with increasing NO_x concentration. The most obvious symptoms in both needle generations was aggregation and/or electron translucence of plastoglobuli (Fig. 5c and d), but no significant changes in the number of plastoglobuli were found. Swelling of stroma thylakoids (Fig. 5c) was evident in both needle generations. A clear increase in cytoplasmic and vacuolar lipid accumulations (Fig. 5c) was observable only in current-year needles. The highest exhaust gas level (200 ppb NO_x) induced an increase in vesiculation of the cytoplasm and reticulation of the central vacuole (Fig. 5d) in both needle generations. The measured dimensions of chloroplasts and starch grains did not show significant changes related to the treatments. No changes were observed in the structure of conductive tissues by light or electron microscopy.

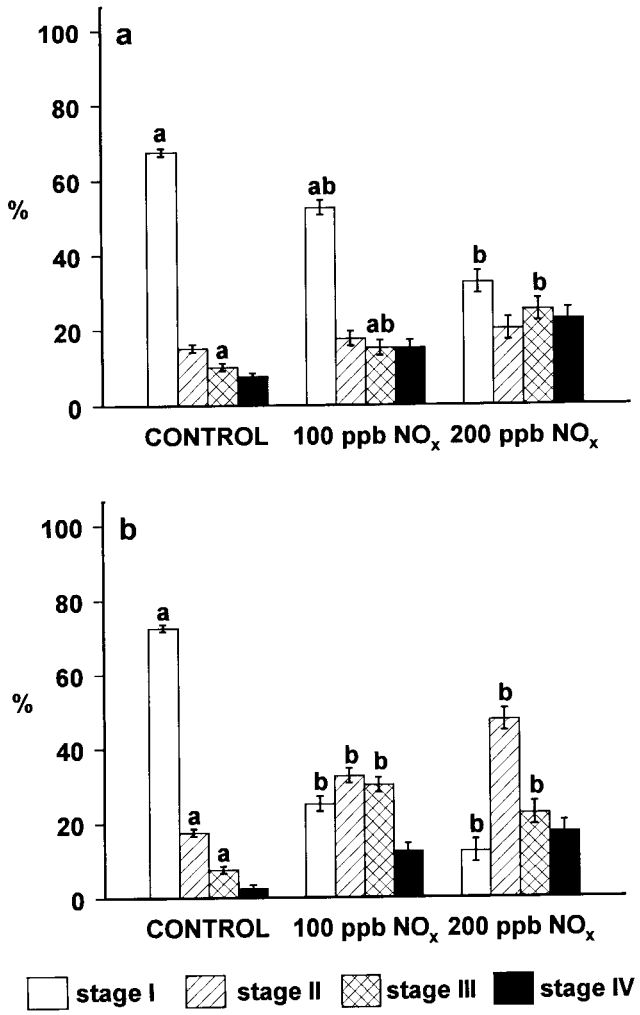

Fig. 3. The amount of needles in different stages of epistomatal wax injury in 1-year-old needles of exhaust gas-exposed Norway spruce seedlings: (a) after 5 days of exposure; and (b) after 10 days of exposure. Bars (SE) with different letters are statistically significantly different between exposure treatments at $p < 0.05$ level (ANOVA, Tukey's multiple range test), $n = 32$ in each treatment. For stages of injury see Sauter et al. (1987).

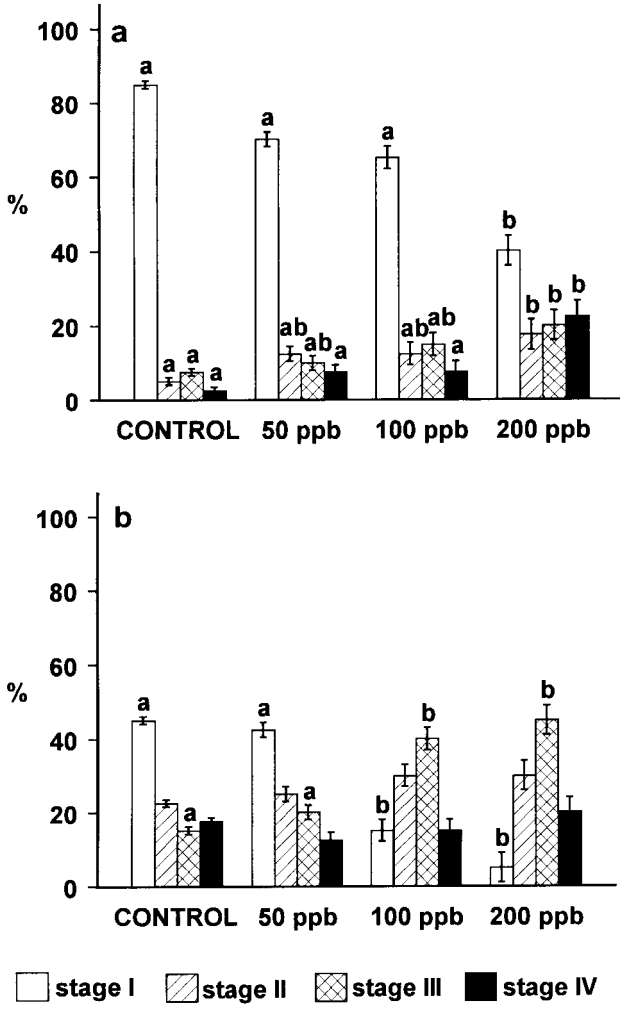

Fig. 4. The amount of needles in different stages of epistomatal wax injury in (a) current- and (b) 1-year-old needles of exhaust gas-exposed Norway spruce seedlings at the end of the 19 days exposure. Bars (SE) with different letters are statistically significantly different between exposure treatments at $p < 0.05$ level (ANOVA, Tukey's multiple range test), $n = 40$ in each treatment. For stages of injury see Sauter et al. (1987).

3.3. Diffusive resistance

There were no differences in diffusive resistance between treatments during the daytime (Fig. 6a and b). In treatment 200 ppb NO_x at night the diffusive resistance was significantly lower, both in current- and 1-year-old needles. In current-year needles the diffusive resistance was still significantly lower in treatment 200 ppb NO_x in the morning (Fig. 6a), indicating that the mechanism of stomatal closure (guard cells) was perturbed.

4. Discussion

After 2- and 3-week exposure to exhaust gas current- and 1-year-old needles of Norway spruce seedlings exhibited a clear accelerated epistomatal wax degradation. Ultrastructural evaluation of the mesophyll cells revealed clear effects on the photosynthetic tissues. When the duration of the exposure was increased from 10 to 19 days the amount of severely degraded epistomatal wax increased slightly. In both needle age classes, but especially in the 1-year-old needles, increasing the exposure time increased the amount of severely degraded epistomatal wax, which indicates accelerated senescence in exposed needles (Sauter and Voß, 1986; Günthardt-Goerg et al., 1994; Huttunen, 1994). By comparison the changes in mesophyll ultrastructure were more clear in the current-year needles than in the 1-year-old needles.

The causes of the epistomatal wax injuries in needles are most likely connected to the organic hydrocarbons and NO_x in the exhaust gas. The major VOCs found in

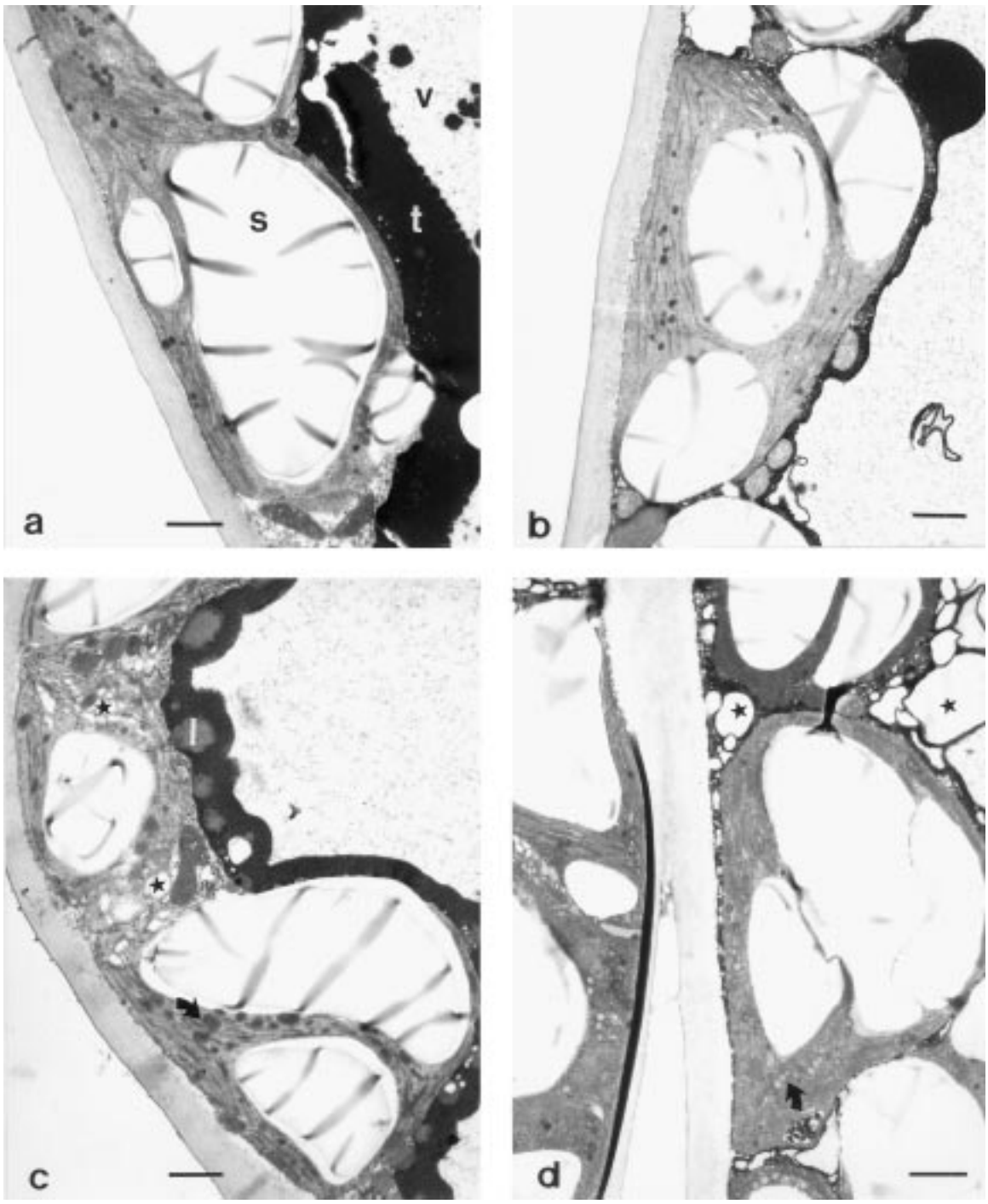

Fig. 5. Electron micrographs (TEM) from the mesophyll cells of Norway spruce needles. (a) First- and (b) second-year needles from the control treatment; s, starch grain; t, tannin deposit; v, vacuole. (c) First-year needle from the 100 ppb NO_x treatment showing partially aggregated plastoglobuli (arrow), swollen thylakoids (stars) and abundant lipid droplets (l) in cytoplasm and vacuole. (d) Second-year needle from the 200 ppb NO_x treatment showing electron translucent of plastoglobuli (arrow) and vesiculation (stars) of cytoplasm and the edge of the central vacuoles. Scale = 1 μm in all figures.

the chamber atmosphere were methyl-ethylbenzenes, different xylenes and toluene. Also significant amounts of unidentified VOCs were found (see Viskari et al., 1999, for details). The concentrations of the VOCs increased with increasing exhaust gas concentration together with the increasing symptoms of epicuticular waxes. Sauter and Pambor (1989) similarly found accelerated degradation of spruce needle waxes with road traffic emission exposure, and exposure to benzene and xylene. They concluded that it is the lipophilic

Table 1
Summary of the observed ultrastructural changes in mesophyll cells of current- (C) and 1-year-old (C+1) needles of Norway spruce in experiment 2[a]

	Aggregation of plastoglobuli	Swelling of thylakoids	Increase of cytoplasmic lipids	Vesiculation of cytoplasm
C				
Control	–	–	–	–
50 ppb	+	+	+	–
100 ppb	++	++	++	–
200 ppb	++	++	++	+
C+1				
Control	–	–	–	–
50 ppb	+	–	–	–
100 ppb	++	+	–	–
200 ppb	++	+	–	+

[a] –, no change; +, slight change; ++, clear change.

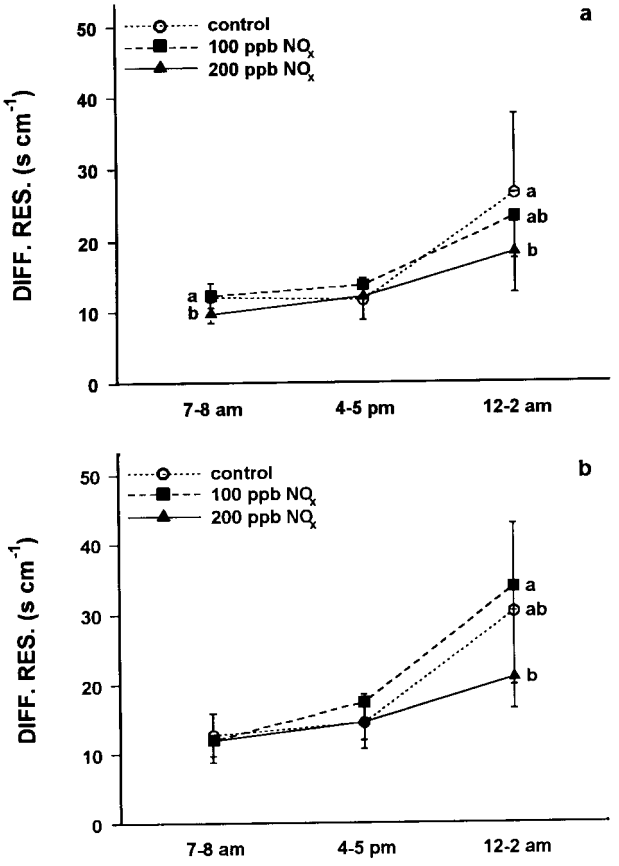

Fig. 6. Diffusive resistance of exhaust gas-exposed spruce seedlings measured at different times of the day (SE): (a) current-year needles, (b) 1-year-old needles. Different letters mean statistically significant difference between treatments (ANOVA, Tukey's multiple range test, $p < 0.05$, $n = 80$) in each treatment.

aromatic hydrocarbons in vehicle emissions (eg. benzene and xylene families) which are responsible for the wax degradation. Excluding these lipohilic exhaust gas compounds may serve to concentrate them, amplifying the damage to the wax structure at the surface of the needle. There are indications of deposited particles in the epistomatal area of exposed needles, which is probably caused by the fine particles in the exhaust gas. The fine particles $D_p < 1$ μm were the most abundant and present in significantly greater amounts in exposure chambers than in control chamber (Viskari et al., 1999). The fine particles, which preferentially deposit in the stomatal regions of the needles (Burkhardt et al., 1995), may also contribute to the degradation of epistomatal wax in needles (Grill and Golop, 1983; Simmleit et al., 1986).

The cuticular permeances of intact conifer needles are linearly correlated with the lipophilicity of the compound (Riederer et al., 1995). According to Lerdau et al. (1997), the monoterpene emissions can be adequately predicted from the monoterpene concentration in the foliage. The monoterpenes emitted by needles may be an extra stress to the seedlings as the monoterpenes can perturb the plant (Tollsten and Müller, 1996) possibly causing injuries to epistomatal waxes as well. However, this was not the case in this study, since our earlier results showed no differences in monoterpene concentrations in needles as a consequence of exhaust gas exposure (Viskari et al., 1999) and therefore the symptoms caused to the epistomatal waxes are obviously caused by the exhaust gas components.

The observed changes in mesophyll ultrastructure also point to disturbances in lipid metabolism and can be partly interpreted as symptoms of accelerated senescence process of cells, especially in the current-year needles. The increase in plastoglobuli numbers and cytoplasmic and vacuolar lipids have been related to natural senescence of spruce needles (Wulff et al., 1996), and these symptoms have also been observed in accelerated form in industrial areas where conifers are exposed to SO_2 and NO_x (Soikkeli, 1981; Anttonen, 1992; Wulff and Kärenlampi, 1996). In the present material the aggregation of plastoglobuli, without increase in their number, was especially clear and could indicate chemical changes in lipids resulting from exposure to some lipophilic agents in the exhaust gas.

Nitrogen oxides also are lipid soluble and may react directly with the cuticle after long-term exposure to high concentrations (Cape, 1994). It is possible that gaseous air pollutants change the sorptive and transport properties of plant cuticles resulting in an increase in permeability to water, gases and inorganic ions (Lendzian and Kerstiens, 1991). When absorbed into the needle NO_x (Saxe, 1986; Thoene et al., 1991) cause acidification of cell surfaces that may be reflected to the ultrastructure of cells. The swelling of thylakoid membranes in chloroplasts have been earlier observed in conifer needles exposed to acidifying gaseous pollutants like SO_2 (Saastamoinen and Holopainen, 1989) or NO_2 (Schiffgens-Gruber and Lütz, 1992). In addition, vesiculation

of cytoplasm, that in present material was observed after exposure to highest NO_x-level, has been related to effects of acid rain in earlier studies (Holopainen and Nygren, 1989; Bäck and Huttunen, 1992).

Our results indicate perturbation of stomatal function by the exhaust gas especially in the night. It appears that the stomata lose their ability to close and with it their regulating capability. This is consistent with other studies (Maier-Maercker and Koch, 1995; Robinson et al., 1998). According to their results, air pollution causes loss of stomatal control which changes, for example, the drought avoidance abilities or increases the dose of the pollutant entering the mesophyll. Schenone et al. (1994) also found in their studies with bean (*Phaseolus vulgaris*) that the stomatal conductance increased after exposure to air pollution (mixture of NO_2, SO_2 and O_3). Kammerbauer et al. (1987) concluded that exposure of Norway spruce near a highway reduced the regulation capacity of the stomata. In spite of the significant degradation of epistomatal wax, no differences in stomatal resistance occurred during the day. Apparently the degree of injury in epistomatal wax of needles was not severe enough to cause more obvious changes in stomatal behaviour.

The exhaust gas concentrations (expressed as NO_x concentrations) used in this study were realistic based on studies where urban air quality has been measured (Derwent et al., 1995; Kukkonen et al., 1996; Palmgren et al., 1996). The NO/NO_2 ratio was 9:1 (Viskari et al., 1999) which is also found in urban air (Palmgren et al., 1996). Urban background levels of NO_x around 20 ppb have been measured (Palmgren et al., 1996) and in our study the concentrations in the control chamber were below this value, varying between 10 and 18 ppb. Alterations in the epistomatal wax structure were observed even after 10 days of exposure and at rather low concentrations of exhaust gas (50 ppb NO_x). Also, the mesophyll ultrastructural changes were observed even at the lowest exhaust gas concentrations. The changes in both epistomatal wax structure and the mesophyll ultrastructure appear to be sensitive, though non-specific indicators for exhaust gas pollution.

5. Conclusions

The exposure of Norway spruce seedlings with exhaust gas in controlled environment chambers for 10–19 days caused:

1. aggregation and fusion of epistomatal wax crystalloids; the epistomatal wax degradation became more severe with increasing atmospheric exhaust gas concentration and needle age;
2. aggregation of plastoglobuli, swelling of thylakoids, increase of cytoplasmic lipids and slight increase of vesiculation of cytoplasm in needle mesophyll cells; these changes were most apparent in the current-year needles;
3. Inhibition of the normal stomatal closure at night; and
4. accelerated senescence of the tissues exposed to exhaust gas.

The epicuticular wax layer, mesophyll ultrastructure and diffusive resistance of the needles showed significant changes to realistic concentrations from low to significantly polluted atmosphere with exhaust gas (50–200 ppb NO_x, respectively). They are thus sensitive indicators for exhaust gas pollution.

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