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Ultrastructure and Pigment Composition of Chloroplasts of Differently Damaged Green and Yellowed Needles of *Picea abies*

By

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With 8 Figures

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Summary

ZELLNIG G., GAILHOFER M., PFEIFHOFER H. W. & GRILL D. 1989. Ultrastructure and pigment composition of chloroplasts of differently damaged green and yellowed needles of *Picea abies.* – Phyton (Austria) 29 (2): 213–225, with 8 figures. – English with German summary.

Chloroplasts of mesophyll cells of one- to three-years-old green and yellowed spruce needles from a SO_2 damaged, a "new type" damaged and an area with low air pollution were examined by transmission electron microscopy (TEM). In addition pigment analyses were carried out.

For SO_2 stressed green needles a lightening of plastoglobuli and a deformation of the chloroplasts was characteristic. In green needles from "new type" damaged areas, changes in the thylakoid system and an increase in the number of plastoglobuli were found. Yellowed needles with "new type" damage show an accumulation of starch and a further reduction of the thylakoid system. These damages can be clearly distinguished from the so called "SO_2" damages. Even green needles from areas with low air pollution show changes in the chloroplasts, namely swellings of the thylakoids, accumulation of plastoglobuli and deformation of chloroplasts.

In the SO_2 and the "new type" damaged needles a decrease in the pigment content, in needles of areas with low air pollution a decrease of the chorophyll/carotinoid quotient was observed.

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Zusammenfassung

ZELLNIG G., GAILHOFER M., PFEIFHOFER H. W. & GRILL D. 1989. Ultrastruktur und Pigmentzusammensetzung in Chloroplasten unterschiedlich geschädigter, grüner und vergilbter Fichtennadeln *(Picea abies)*. – Phyton (Austria) 29 (2): 213–225, mit 8 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Chloroplasten von Mesophyllzellen ein- bis dreijähriger grüner und vergilbter Fichtennadeln aus einem „klassisch" SO_2, einem „neuartig" geschädigten und einem als weitgehend unbelastet angesehenen Gebiet wurden im Transmissionselektronenmikroskop (TEM) untersucht. Zusätzlich erfolgte eine Pigmentanalyse dieser Nadeln.

In SO_2-belasteten grünen Nadeln zeigt sich als Schädigungscharakteristikum eine Aufhellung der Plastoglobuli und Formveränderung der Chloroplasten. In den grünen Nadeln des „neuartig" geschädigten Gebietes sind Veränderungen im Thylakoidsystem und eine Erhöhung der Plastoglobuli-Zahl festzustellen. In den vergilbten Nadeln dieses Gebietes kommt es zusätzlich zu Stärkeakkumulationen und weiteren Reduktionen im Thylakoidsystem. Diese Schäden lassen sich deutlich von den SO_2-Schäden unterscheiden. Auch in den grünen Nadeln des weitgehend unbelasteten Gebietes zeigen sich schon Veränderungen in den Chloroplasten: Thylakoidanschwellungen, Plastoglobuli-Anhäufungen und Chloroplastendeformationen.

In den „klassisch" und „neuartig" geschädigten Nadeln ist eine Abnahme des Pigmentgehaltes, in den nahezu unbelasteten Nadeln eine Abnahme des Chlorophyll/Carotinoid-Quotienten festzustellen.

Introduction

The influence of air-pollutants (SO_2, NO_2, NO_x, HF, O_3) on the ultrastructure of various plants has been described repeatedly (GODZIK & KNABE 1973, SOIKKELI 1981a, HUTTUNEN & SOIKKELI 1984, MIYAKE & al. 1984, RUETZE & al. 1988). In connection with forest decline, investigations were carried out especially with conifer needles. Thereby green and yellowed needles were taken from differently exposed areas (PARAMESWARAN & al. 1985, SCHMITT & al. 1986, SUTINEN 1987b, JUNG & WILD 1988). Most data on ultrastructural changes in damaged conifers concern chloroplasts, which seem to be first to show damage. Nevertheless, it is quite difficult to make any comparisons as in these previous papers trees from the most diverse areas (North- and Central Europe respectively) were examined. Most of the time there is no information whatsoever about the nature and the quantity of the air-pollutants. Also the age of the trees and their location relative to the sea level differ and parasite influence can not always be excluded. These are the reasons, why in this paper different spruces of almost the same age and from exactly defined areas are examined. Thereby needles of SO_2 damaged trees, "new type" damaged trees and trees grown in regions with low air pollution were examined more closely to figure out the ultrastructure of the chloroplasts in mesophyll cells. The data were compared with previous results. Furthermore, the pigment composition of the needles was determined, such that in combination with the ultrastructure it was possible to make a complex statement about the condition of the needles.

Materials and Methods

One- to three-years-old needles of Norway spruce (*Picea abies* [L.] KARST.) were taken from the top of the trees (8.–9. branch whorl). The spruces are about 80 to 100 years old. The needle samples were collected during September 1986 from sites in the area of Judenburg-Pöls, a SO_2 polluted area (Table 1), Schöneben im Böhmerwald, a

Table 1.

Characteristics of the spruce-stands.

	Judenburg-Pöls	Schöneben	Murau
sea level (approximately)	800 m	1000 m	1130 m
geology	Muralpen Kristallin mica schist	Böhmische Masse granite	Muralpen Kristallin mica schist
climate: precipitation mm per year (approximately)	900	1000–1100	900
mean annual temperature in °C (approximately)	6	6	4–5
gaseous air-pollutants (monthly average)	SO_2 III–IX 10–40 μg/m₃	2–26 μg/m^3	region with low air pollution (BIN-map, Office of the Styrian Government 1987)
	X–II 30–70	3–70	
	NO_x III–IX 3– 6 ppb	1– 4 ppb	
	X–II 3–16	2– 6	
	NO_2 III–IX 3– 9 ppb	2– 8 ppb	
	X–II 3–20	4–12	
	O_3 III–IX 31–53 ppb	16–50 ppb	
	X–II 16–52	19–42	

site with "new type" forest decline (FÜHRER & NEUHUBER 1987) and as a reference area Murau in Styria, which is known to be a region with low air pollution (BIN-map, Office of the Styrian Government 1987). The sample trees grew at almost the same sea level. The needles from Judenburg-Pöls and Murau were green. Those from Schöneben were, depending on the tree, green or yellowed in the first and third year. It was always ensured, that there were no mycosis or parasite injuries.

Sections were taken from the front part of the needles and fixed for 2–14 h in 3% cacodylate buffered glutaraldehyde. After rinsing in buffer for 2 h the sections were post fixed for 2 h in 1% OsO_4, dehydrated and embedded in Polarbed or Spurr's epoxy resin. The ultrathin sections were post stained with lead citrate and uranyl acetate and viewed with a Philips CM 10.

The plastid pigments were extracted from 1 g of homogenised spruce needles with acetone. The chromatography separation and the quantitative analysis of the separated pigments were done by means of reversed-phase high performance liquid chromatography (HPLC) with a method described in detail elsewhere (PFEIFHOFER 1989). Chlorophyll a and b as well as the carotenoids present in spruce needles were determined. The quantitative evaluation of the chromatograms was done using an integrator in the external standard mode. For calibration, authentic standards were used. The pigments in the chromatograms were identified by cochromatography of the unknown mixture with a sufficient amount of the standard.

Results

SO_2 damaged needles:

The green needles with obvious SO_2 stress show changes in the mesophyll chloroplasts even in the first-year-needles. These chloroplasts frequently have irregular contours because of peripher stroma areas, and a normally developed membrane system (Fig. 1). Besides this, starch grains can be found. The number of plastoglobuli seems to be slightly increased and sometimes swellings of the intrathylakoidal space appear. At this stage a lightening of plastoglobuli is already visible, although not homogeneously. In second-year-needles no alterations can be observed in the thylakoid system, grana are normally developed. However, the number of plastoglobuli is clearly increased, either many small, or a few large plastoglobuli are found (Fig. 2). Most of them are lightened, some are also dark stained. Some vesicles occur at the periphery of the chloroplasts and sometimes membrane free stroma spaces can be observed.

"New Type" damaged needles:

Green needles: the chloroplasts of the first-year-needles contain starch and some plastoglobuli. The membrane system is normally developed; sometimes swellings of the intrathylakoidal space and membrane free stroma areas are visible (Fig. 3). In addition to this, vesicles sometimes appear near the chloroplast envelope.

In the third-year-needles, a strong increase in the number of plastoglobuli is found (Fig. 4). They are often homogeneously dark stained, but some

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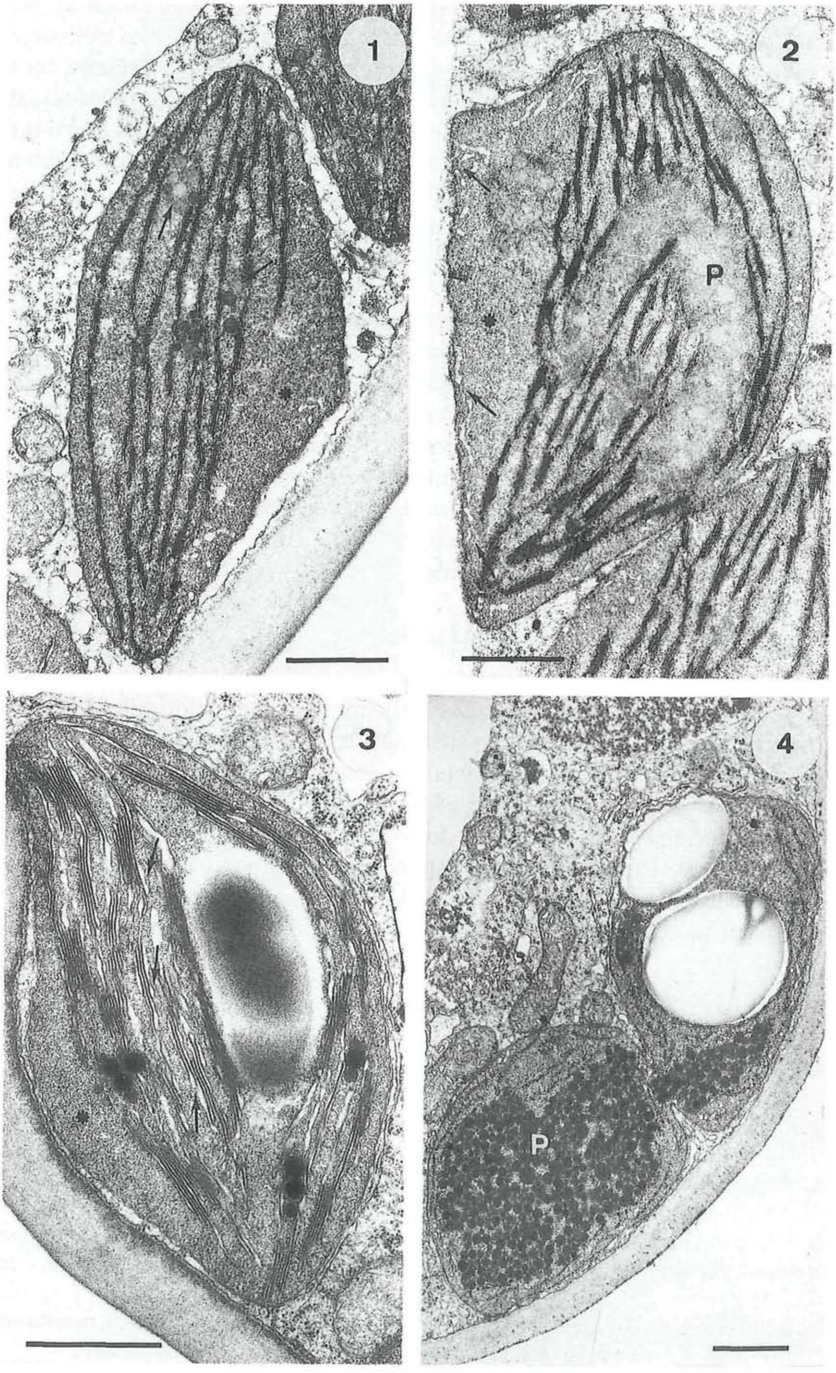
Figs. 1–4. *Picea abies,* cross-section of a needle, mesophyll-ceels; bar = 1 µm.

Fig. 1. One-year-old green needle from Judenburg-Pöls; the chloroplast shows a peripheral membrane free stroma space (star) and partially lightened plastoglobuli (arrows).

Fig. 2. Two-years-old green needle from Judenburg-Pöls; the chloroplast shows a large number of lightened plastoglobuli (P), peripheral membrane free stroma space (star) and many peripheral vesicles (arrows), which derive from the inner chloroplast envelope.

Fig. 3. One-year-old green needle from Schöneben; the chloroplast shows a swelling of the intrathylakoidal space (arrows) and a small peripheral stroma space (star).

Fig. 4. Three-years-old green needle from Schöneben; dividing chloroplast shows a lot of homogeneously dark stained plastoglobuli (P) and only a few thylakoids.


1
2
P
3
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P

are lightened. At the same time, a drastic decrease of the membrane system occurs. However, the increase of the plastoglobuli number does not correlate with the membrane decrease. Depending on the degree of damage, there are still normally developed grana, whereas the badly changed chloroplasts only contain membrane remains. However, in such chloroplasts a granum made up of five thylakoids was observed. Furthermore, the starch content and the number of vesicles are increased. The membrane free peripheral stroma areas, which are mentioned in the one-year-old needles, can also be found here. Occasionally dividing plastids are detected as well (Fig. 4).

Yellowed needles: In the first-year-needles the thylakoid system of the chloroplasts is reduced. Grana are rarely found; most of them consists only of few thylakoids. Besides a few plastoglobuli, which are differently stained, big starch grains and a lot of peripheral vesicles occur (Fig. 5). In the three-years-old needles a further reduction of the thylakoid system takes place. The number of the homogeneously dark stained plastoglobuli is strongly increased; together with huge starch grains these form the larger part of the strongly deformed chloroplasts (Fig. 6). Peripheral vesicles can also be found. In these needles, neither in the first- nor in the third-year membrane free stroma spaces visible.

Needles from an area with low air pollution:

Alterations in the ultrastructure of chloroplasts are already found in one-year-old green needles. Apparently undamaged chloroplasts with a normally developed thylakoid system and a few plastoglobuli (Fig. 7a), as well as visibly altered chloroplasts are found in the mesophyll. In these altered chloroplasts the number of thylakoids is decreased and even the grana are formed only out of four to five thylakoids (Fig. 7b).

Swellings of intrathylakoidal spaces are often visible. The number of homogeneously dark stained plastoglobuli is already increased; membrane free stroma spaces are sometimes detected. It is noticeable that in this case

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Figs. 5–8. *Picea abies,* cross-section of a needle, mesophyll-cells, bar = 1 µm.

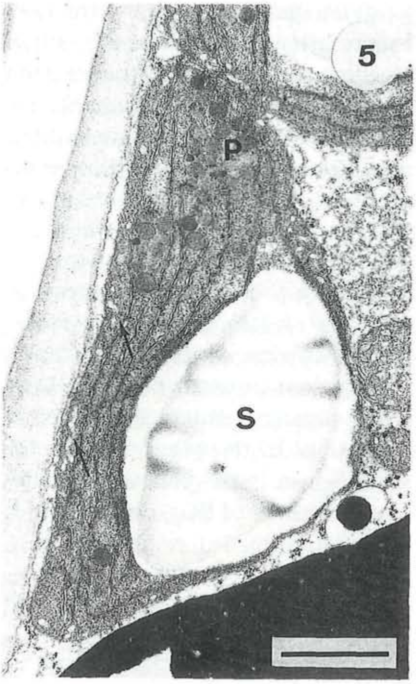
Fig. 5. One-year-old slightly yellowed needle from Schöneben; the chloroplast shows a large starch grain (S), a reduced membrane system, peripheral vesicles (arrows) and lightened plastoglobuli (P).

Fig. 6. Three-years-old yellowed needle from Schöneben; the chloroplast shows a huge starch grain (S) and a large number of plastoglobuli (P).

Fig. 7a. One-year-old green needle from Murau; the chloroplast shows a good developed thylakoid system and a few dark stained plastoglobuli.

Fig. 7b. One-year-old green needle from Murau; the chloroplast shows a poorly developed thylakoid system, intrathylakoidal enlargements (arrows) and homogeneously dark stained plastoglobuli (P).

Fig. 8. Three-years-old green needle from Murau; the chloroplast shows a membrane free stroma space (star) and differently stained plastoglobuli (P).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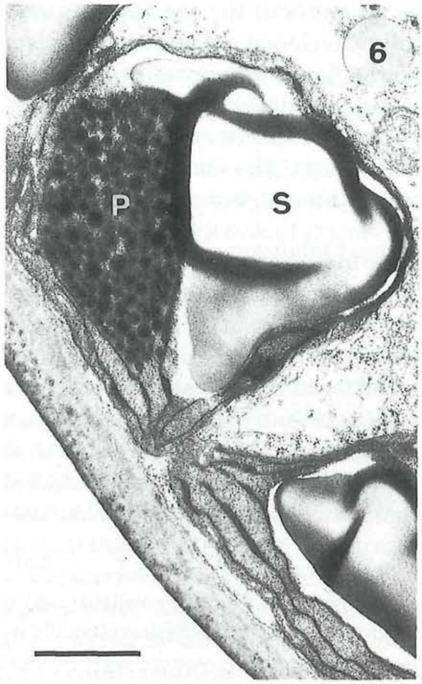
S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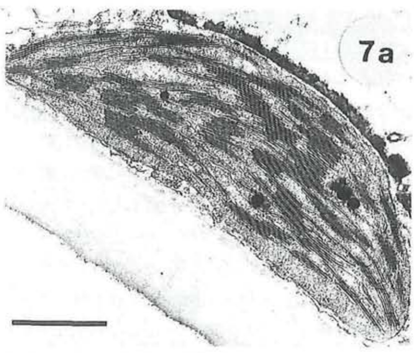
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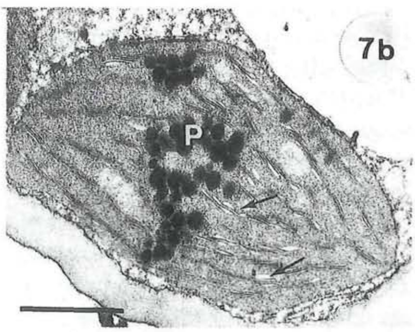
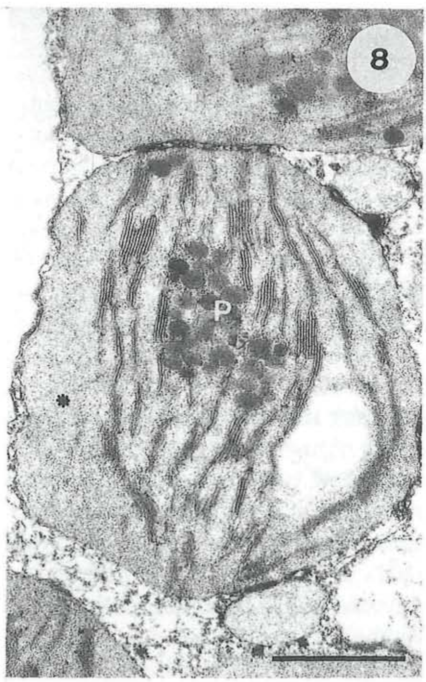

7b
P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the structure of the chloroplasts is very inhomogeneous, whereby the normally developed chloroplasts are found more often in the lower mesophyll layers. The chloroplasts of third-year-needles contain starch and a good developed thylakoid system with large grana (Fig. 8). Peripheral membrane free stroma spaces exist more often, which can lead to deformations of the chloroplasts. The number of plastoglobuli is strongly increased, they seem to be lightened, but not homogeneously stained.

Pigment analyses:

Using the HPLC method, all plastid pigments present in spruces can be detected: chlorophyll a and b, α- and β-carotene, lutein, neoxanthin, violaxanthin, antheraxanthin and zeaxanthin. The spruces of the three sites do not differ in a qualitative manner concerning their pigment pattern. However, quantitative differences are found. The pigment content is greatest in needles from the region with low air pollution. In the other spruces the pigment content is diminished up to 63%, the most in the yellowed needles from Schöneben (Table 2). Even under the influence of SO_2, pheophytin is

Table 2.

Pigment content (μg/g dry weight) and quotients of chlorophyll a/b, xanthophyll/carothene, chlorophyll/carotenoids in spruce needles from the three stands.

spruce-stand	Judenburg-Pöls		Schöneben				Murau		
			green needles		yellowed needles				
age of the needles	1	2	1	3	1	3	1	2	3
chlorophyll	2446	2125	2758	2868	1928	1585	3224	4042	4299
carotene	137	106	149	182	153	109	197	245	252
xanthophyll	471	374	381	383	244	280	478	500	555
chlorophyll a/b	2.6	2.6	2.3	2.4	2.3	2.6	2.8	2.6	2.7
xanthophyll/ carotene	3.4	3.5	2.6	2.1	1.6	2.6	2.4	2.0	2.8
chlorophyll/ carotenoids	4.0	4.4	5.2	5.1	4.9	4.1	4.8	5.4	5.3

detectable only in not quantifiable small amounts. Depending on the sites of the trees the needles differ concerning the ratio between the green and yellow pigments. In green needles from the area with low air pollution and from "new type" regions the quotient is usually above five (except in the one-year-old needles from the region with low air pollution it is 4.8). Trees under the influence of SO_2 and yellowed trees from the area with "new type" damages show a quotient which is below five. This implies a strong decrease in chlorophylls compared with carotenoids. Among the carotenoids, the ratio changes in favour of xanthophyll, but only under the influence of SO_2. The chlorophyll a/b quotient does not admit any conclusions.

Discussion

The ultrastructural changes in the obviously SO_2 stressed samples from Judenburg-Pöls are comparable with several previously described damages caused by SO_2. The particular combination of the symptoms covered here, like swelling of the intrathylakoidal space, changes in the shape, plastoglobuli lightenings, has not yet been described with sulphur stress. According to a paper on *Picea* and *Pinus* by SOIKKELI 1981 b, a reduction of grana and/or plastoglobuli lightening are a sign of SO_2 stress. MALHOTRA 1976 mentions a swelling of the thylakoids in *Pinus* with SO_2. In a comprehensive study of the consequences of air-pollutants SOIKKELI 1981 a also writes about changes of the chloroplast shape and swellings of thylakoids under the influence of SO_2. In spinach leaves fumigation experiments with SO_2 lead first to a chloroplast deformation and later to a swelling of thylakoids (MIYAKE & al. 1984). These findings show that the discovered damages do not coincide with each other, altough the air-pollutants are of the same kind. As mentioned before, the situation of the specific stands was not always considered. That is why differences in the outside grown samples are probably due to slightly varying stress. Furthermore, it is possible that the chloroplasts of deciduous trees and conifers react differently to pollutants. A fairly exact interpretation of damages caused by air-pollutants is only practicable when several specific environmental conditions are considered.

Chloroplasts of spruces with "new type" damages are clearly distinguishable from the ones of SO_2 damaged trees. A coherent trend with regard to plastoglobuli lightenings and intrathylakoidal swellings does not exist. Nevertheless, severe reductions of membranes and accumulations of starch and plastoglobuli are found. SUTINEN 1987 b mentions similar changes with green and yellowed spruce needles from the Taunus area. According to her, these effects are due to longer term influence of air-pollutants e.g. $SO_2 + O_3$. KÜPPERS & BLIND 1987 described an accumulation of starch in *Picea* after fumigation experiments with $SO_2 + O_3$. However, the same gasing experiment did not lead to starch accumulation in spinach (MIYAKE & al. 1984). In damaged outside grown fir trees from the Black-forest, an accumulation of starch grains was also found in the mesophyll chloroplasts. However, this was not the case with spruces (PARAMESWARAN & al. 1985). The starch accumulation seems to be an unspecific symptom. It indicates a change of the carbohydrate-metabolism in the chloroplast and/or in the cytoplasm. Thus, no mobilization or transport is possible. Chloroplasts of one- and three-years-old needles analysed for this study seem to become senescent early and to develop into gerontoplasts because of reductions in the membrane system and plastoglobuli accumulations. The increase of the plastoglobuli number and size is frequently considered to be characteristic of aging (LICHTENTHALER 1969, THOMSON & WHATLEY 1980, STAEHELIN 1986), whereas electron opacity depends on the composition, especially on the quantity of carotenoids (LICHTENTHALER 1970). According to SUTINEN 1987 a naturally aged chloroplasts of spruces are characterized e.g. by plastoglobuli lightenings and stroma reductions. Aging, however, does not start before a needle age of four years. No membrane reductions or plastoglobuli accumulations were observed. RUETZE & SCHMITT 1988 observed a slight swelling of thylakoid membranes, a decreasing of grana thylakoids and an increase in the number of plastoglobuli during aging, but distinct degeneration symptoms occurred exclusively in 7 and 8 years old spruce needles. GRILLI 1965 a, b shows with *Cucurbita* that globular chromoplasts can develop from amyloplasts and proplastids. As they do not have any available membrane lipids for plastoglobuli synthesis, there must be another way of lipidsynthesis. In the chloroplasts of „new type" damaged needles, which were analysed within this study, no correlation whatsoever could be found between membrane reduction and plastoglobuli increase. The gerontoplast development does not seem to be irreversible, as with such plastids dividing-states and apparently new formed grana were found in the third-year-needles. A naturally occurring reversible transformation from chloroplasts into chromoplasts was described with *Buxus* (KOIWA & al. 1986) and *Abies* (CHABOT & CHABOT 1975). SCHMITT & al. 1986 found damage only in the chloroplasts of the transfusion parenchyma and the endoderm of green and obviously damaged needles from the Black-forest. Therefore, the previously named authors suppose that these damages are caused by pollutants, which get into the plant by penetrating the root system. As in the "new type" damaged green needles ultrastructural changes occur in the chloroplasts of the first and second mesophyll layers, it is possible to assume that the reason for these damages is the influence of air-pollutants on the needle surface. The results of the pigment analyses are in conformity with the TEM data, since the pigment content of needles from polluted areas is reduced. There are also differences between SO_2 and "new type" damaged needles concerning their chlorophyll and carotenoid content in the two- and three-years-old needles. In comparative research on spruce needles, further characteristics of damage were found besides a pigment decrease (PFEIFHOFER & GRILL 1987 and unpublished data). Out of these characteristics the increase of the chlorophyll a/b quotient will be mentioned here.

The chloroplasts of green needles from areas with low air pollution show structural modifications in the first- and third-year needles which are not in accordance with the seasonal type (SENSER & al. 1975). The combination of thylakoid swellings, plastoglobuli lightenings and deformation of chloroplasts had not yet been reported for just one singular pollutant (cf. HUTTUNEN & SOIKKELI 1984). Similar symptoms were discovered after O_3 fumigation experiments with bean leaves (FISCHER 1987) and spruce needles (ZELLNIG & al. 1987). In the needles from areas with low air pollution undamaged chloroplasts were found in addition to the damaged ones. Yet, the data about the structure of such chloroplasts are not consistent. Chloroplasts which are considered to be undamaged, often show differences in their structure (SENSER & al. 1975, SUTINEN 1987 b).

Micromorphological modifications of the plastids include changes in the pigment content of the needles, but not in the qualitative composition. As the trees grew in comparable habitats and the needles were gathered at the same time of year, the differences were probably caused by differences in the quality of the air (GRILL & al. 1983, GRILL & PFEIFHOFER 1985). The strong destruction of plastids caused by SO_2 stress and by „new type" air-pollutants, coincide with a strong decrease in chlorophyll and a lesser decrease in the yellow components. This distinct change in the structur of plastids because of SO_2 stress has to be viewed critically because of the pigment pattern; for although pheophytin was detected only in not quantifiable small amounts, the xanthophyll/carotene quotient being above 3 (Table 2) indicates an increase of xanthophyll at the cost of carotene. This has to be regarded as a severe reaction, perhaps even in the subletal range. Micromorphological alterations in green needles from trees which show "new type" symptoms, do not lead to detectable consequences concerning the pigment pattern, except a general diminuation of pigments. Such an interpretation is complicated, because the "reference" samples from the region with low air pollution with the highest pigment amount are exposed to air-pollutants as well. This is indicated by electron microscopical pictures and the chlorophyll/carotenoid quotient being below five in the first-year-needles.

Pigment analysis is advantageous for quantitative assessment, whereas TEM-analyses are more suitable for the qualitative differentiation of pollutants. Thus, both kinds of analysis are complementary to each other and offer a good possibility to make an early diagnosis of damage.

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