Studies of Components of the Thylakoid Membrane of Undamaged and Damaged Spruce Trees at Different Mountain Sites

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During a five-year period, components of the thylakoid membrane in needles of the second generation of undamaged and damaged trees of Norway spruce were studied at three different mountain sites in West Germany. Visible signs of damage at these sites are a yellowing of the light-exposed sides of the needles as well as the loss of needles. The goal of this study was to determine damage-induced alterations in composition and physiological reactions of the thylakoid membranes in spruce needles. In order to meet this purpose, contents of chlorophyll *a* and *b*, electron transport rate of photosystem II, contents of the D1 protein, cytochrome *f*, as well as P-700 were measured.

The chlorophyll content in the needles of the damaged spruce trees was significantly lower than in the needles of the undamaged trees. In addition to this, the typical annual course of chlorophyll content was exclusively observed in the needles of the undamaged spruce trees. If related to dry weight, a drastic reduction of the electron transport rate and of the redox components of the thylakoid membrane was observed due to damage, indicating a degeneration of the photosynthetic membranes. The contents of D1 protein and the photosynthetic electron transport rates were also markedly reduced in the needles of the damaged trees, when related to chlorophyll content of thylakoids, suggesting an early and particular impairment of photosystem II. The comparison of spruce trees showing different signs of damage demonstrates that certain biochemical parameters concerning the photosynthetic membranes (chlorophyll, cytochrome *f*, ratio photosystem II/I) reflect the extent of damage and are suitable for an early indication of a beginning, but still invisible damage of spruce trees.

Introduction

The wide-spread occurrence of forest damage, which is called "novel forest decline", has been observed in Central Europe since the end of the seventies. This disease differs from the classical flue gas damage in its wide geographical spread, its appearance on all major woody tree species as well as a long lasting period of damage. In Germany the extent of damage increases with the age of the trees and the altitude of the sites [1]. Novel forest decline is regarded as a complex disease. Apart from climatic and edaphic factors, special attention is paid to air pollutants and the deficiency of nutrients, especially lack of magnesium [2, 3].

In the past few years the extent of damage observed on conifers has been ascertained predominantly by their loss of needles. Additionally, yellowing of needles has been taken into consideration [1]. These parameters allow a quick estimation. A reliable, early indication of a beginning damage, however, is almost impossible, because the loss of needles can either be caused by damage or originate from variable adaptation processes. Furthermore, the degree of yellowing is likely to be underestimated by assessment from the ground, as the loss of pigments predominantly occurs in the upper light-exposed parts of the needles.

From the physiological point of view the visible yellowing, as a typical feature of mountainous yellowing of spruce [4], is a consequence of damage to the photosynthetic membranes [5, 6]. In the present study, however, decreases in the content of certain components of the electron transport chain were not only found in thylakoids of trees showing

Abbreviations: DTE, dithioerythritol; EDTA, ethylenenitrilo tetraacetic acid disodium salt dihydrate (Titriplex III); HEPES, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); Polyclar AT, water-insoluble polyvinylpyrrolidone for binding phenols; DCPIP, 2,6-dichlorophenol-indophenol sodium salt; DW, dry weight; PS, photosystem.

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a visibly yellowing or a loss of needles but also in thylakoids of apparently healthy trees. Our results demonstrate that the analysis of the thylakoid membrane components is more sensitive to detect a beginning damage than the assessment of yellowing and loss of needles from the ground.

The aim of the presented study was to determine functional alterations and changes in the composition of the thylakoid membranes in the needles of trees showing various stages of damage, as well as of phenotypically undamaged trees. For this purpose contents of chlorophyll *a* and *b*, the quantity of photosystem II (D1 protein, photosynthetic electron transport rate), of cytochrome b_6f-complex (cytochrome *f*) and of photosystem I (P-700), were measured. Over a period of five years the tests were carried out on spruce trees grown at various sites. Doing so the role which environmental factors – like climate, season, and soil – play within the scope of this sort of studies can likewise be considered for the interpretation of the results.

Materials and Methods

Description of the sites

The presented studies were carried out at three natural habitats in Rheinland-Pfalz and Baden-Württemberg during a five-year period from 1987–1991. The damage classes of the respective spruce trees (*Picea abies* [L.] Karst.) were assessed according to the criteria of the forest damage report [1]. For reasons of clearity in the following the expression "undamaged" und "damaged" refers to the damage categories of the forest damage report [1]. In order to evaluate the state of the canopy, the percentage of needle loss is estimated. According to this the trees are divided in five damage categories (0–4). The second damage characteristic is discoloration of the needles. Both damage characteristics in combination yield the final damage class in damage inventory: 0 = undamaged, 1 = slightly damaged, 2 = moderately damaged, 3 = severely damaged, and 4 = dead.

Detailed descriptions of the sites have been presented by Wild *et al.* [6, 7], Tenter and Wild [8], and Tietz and Wild [9].

Wallmerod site

This site in Hoher Westerwald (Wallmerod Forestry Office, Höhn Forest District, Division 1C) is located on a plateau approximately 495 m above sea level. The soil is well supplied with nutrients and shows only a weak acidification (pH 4.3–4.5) of the upper layer.

The approximately 20-years-old spruce trees at Wallmerod site were apparently undamaged (damage class 0) and served as an external undamaged reference compared to the trees at Hattgenstein site. Studies were performed on five different trees each year.

Hattgenstein site

This spruce tree plantation is situated in the western part of the Hunsrück mountains at approximately 660 m above sea level (Idar-Oberstein Forestry Office, Hattgenstein Forest District, Division 257 b^2). The soil found at this site may be characterized as a podsolated, highly acidic brown earth (pH 2.7–3.8). The cation-exchange capacity and the nutrient supply are low.

The age of the spruce trees was 25–30 years. The studies here were carried out on pairs of trees: *e.g.* each time a tree showing relatively few symptoms (damage class 0 and 1) was compared to another adjacent one with clear signs of damage (damage class 2). Measurements were carried out on five different tree-pairs during the concerned five years.

Climatic and immission data for Wallmerod and Hattgenstein sites were registered by measurment stations of ZIMEN (Central Network for Measurement of Pollution in Rheinland-Pfalz). During the assay period the two sites showed high concentrations of ozone in summer months (70–110 $\mu g\ m^{-3}$, [10]). The spruce needles contain only low levels of Mg^{2+} (200–300 $\mu g/g$ DW, [7]).

Freudenstadt site

This site is located on a plateau based on sandstone, approximately 830–840 m above sea level, 4 km southwest of Freudenstadt in the northern Black Forest (Freudenstadt Forest Office, Vordersteinwald Forest District, Division III/12a^4, on Schöllkopf mountain).

Two different stands of spruce trees were examined in the years of 1989–1991. The age of the trees was 45–50 years. The trees of one stand were highly damaged and showed needle-losses of 10–30%, as well as 30–50% yellowing of the nee-

dles (damage classes 2 and 3). This stand was compared to an adjacent stand of apparently undamaged spruce trees, showing only 0–10% needle-loss and negligible yellowing of the needles (damage class 0) [4]. These studies were performed on the needles of six trees per stand combined to mixed samples.

Climatic and immission data were registered by the IVD (Institut für Verfahrenstechnik und Dampfkesselwesen) measuring station at the Schöllkopf mountain [11]. During the assay period the concentrations of SO_2 and NO_2 were rather low, while concentrations of ozone were high, especially in summer (100–120 μg m^{-3}). These concentrations of respective air pollutants are typical for a so-called clean-air region. The spruce needles contain only low levels of Mg^{2+} (130–270 μg/g DW, [12, 13]).

The studies at Freudenstadt site are part of a forest decline research project monitoring air pollution, constitution of the soil, genetic constitution of the trees, physiological and biochemical state of the trees and their mycorrhizas, and the supervising of biotic damages, which could possibly occur. The project is sponsored and coordinated by the Kernforschungszentrum Karlsruhe – Projekt Europäisches Forschungszentrum für Maßnahmen zur Luftreinhaltung [14].

Materials

The studies were performed on spruce needles of the second needle generation (*e.g.* vegetation period 1989, needle generation 1988) from the sixth to eighth whorl.

In order to measure chlorophyll content and redox components, the needles were removed from the twigs by stirring the latter in liquid nitrogen (−196 °C) immediately after harvesting. The needles were stored at −80 °C in the laboratory.

The photosynthetic electron transport was measured on twigs which had been stored on ice for two days at most.

Harvest dates

Sampling took place on the following dates at Wallmerod site: 12.5., 20.7., 7.9., 12.10.1987; 25.4., 20.6., 15.8., 3.10.1988; 1.2., 3.5., 19.6., 14.8., 9.10.1989; 20.2.1990; at Hattgenstein site: 27.4., 22.6., 10.8., 28.9., 14.12.1987; 26.4., 21.6., 16.8., 4.10.1988; 1.2., 2.5., 20.6., 15.8., 11.10.1989; 20.2.1990, and at Freudenstadt site: 19.4., 28.6., 30.8., 2.11.1989; 21.2., 25.4., 27.6., 26.8., 7.11.1990; 13.3., 29.4., 26.6., 28.8., 30.10.1991.

Thylakoid isolation

The isolation procedure was carried out at 0–4 °C. For the determination of the considered redox components 2.5–3.0 g frozen needles were homogenized in 60 ml isolation medium (50 mM Na-pyrophosphate/HCl, pH 7.0; 5 mM $MgCl_2$; 0.4 mM $KHSO_3$; 5 mM DTE; 20% w/v polyethylene glycol 6000) by means of an Ultraturrax 18 K (Janke & Kunkel) for 15 s at 17,000 rpm. The homogenate was filtered through four layers of gauze and then centrifuged for 5 min at 25,700 × *g*. After resuspending the pellet in 40 ml medium (50 mM HEPES/KOH, pH 6.7; 2 mM EDTA; 1 mM $MgCl_2$; 1 mM $MnCl_2$; 0.5 mM K_2HPO_4; 2 mM $NaNO_3$; 20 mM NaCl; 1 M sorbitol) centrifugation was repeated under the same conditions. The pellet was suspended in 5 ml shock medium (50 mM HEPES/KOH, pH 6.7; 50 mM $MgCl_2$) in order to break up the chloroplast envelopes by osmotic forces. After a reaction time of 5 min another centrifugation followed (25,700 × *g*, 10 min). Then 1.5 ml storage medium (resuspension medium without sorbitol) was added to the pellet and the chlorophyll content of the resulting thylakoid suspension was determined according to Ziegler & Egle [15]. For the tests focussing P-700 and cytochrome *f*, the suspension was frozen at −20 °C and used the following day. In order to determine D 1 protein, the suspension had to be used immediately.

Determination of P-700 and the oxidation speed of antennae chlorophylls

P-700 was quantitatively determined by the difference spectrum between P-700 kept in the reduced state by ascorbate and $P\text{-}700^+$ kept in the oxidized state by K-ferricyanide [16]. In order to start the test, the thawed chloroplasts were suspended in reaction medium (50 mM K-phosphate buffer, pH 7.0; 0.3% Triton X-100; 0.4 M saccharose; 1% w/v Polyclar AT; 0.025 mg chlorophyll a and b per ml medium). The mixture was kept in the dark for 30 min. Then it was centrifuged (10 min, 33,000 × *g*) and the supernatant was used

for measurements with a Shimadzu MPS-2000. As soon as a constant base line (670–730 nm) was obtained, the reaction was started by the addition of Na-ascorbate (final concentration 4 mM) to the test cuvette and K-ferricyanide (final concentration 1 mM) to the reference cuvette. The difference spectra were registered. Based upon the difference between the absorption change at 730 nm (isosbestic point) and the one at 696 nm the P-700 concentration was calculated ($\epsilon = 64$ cm^2 μmol^{-1} according to Hiyama & Ke [17]; chemical method). The tendency of the P-700 peaks towards shorter wavelengths is due to changes in the structure of the thylakoid membrane caused by action of detergents [18].

To check the reliability of the above described chemical method the content of P-700 was also determined from the light induced absorbance change at 696 nm (optical method according to Melis and Brown [19]) using an AMINCO DW-2 UV-VIS spectral photometer in the dual wavelength mode. The reference wavelength was 730 nm. The photooxidation of P-700 was induced by blue-green light of an intensity of 4 W/m^2. The photomultiplier was protected against exciting light by a red cut off filter (Schott RG 695, 2 mm). In order to minimize disturbance from chlorophyll fluorescence the cuvette was placed at a distance of 24 cm from the photomultiplier. Ascorbate (0.13 mM) was present to accelerate the reduction of P-700 in the dark.

By adding K-ferricyanide to the reference cuvette in the chemical method a prompt oxidation of P-700 occurs; with some delay, however, an oxidation of bulk chlorophylls follows. It proved to be relevant to calculate the relation of the oxidation peak of antennae chlorophylls (bulk-chlorophyll) to the relation of P-700 peak. We call this relation A/P. In order to determine A/P the absorbance both at 687 nm (= absorption maximum of the difference spectrum in the long-wave region of antennae chlorophylls) and at 696 nm (= P-700) 3.5 min after the start of the reaction are determined. In this way, a parameter that was independent of any reference quantity was obtained.

Determination of cytochrome f

Cytochrome *f* was also determined by recording difference spectra using a modified chemical method according to Bendall & Rolfe [20]. For studying cytochrome *f* the chloroplast suspension was treated as described for the P-700 test (50 mM K-phosphate buffer, pH 6.5; 1% Triton X-100; 0.3 M sorbitol; 1% w/v Polyclar AT; 0.075 mg chlorophyll *a* and *b* per ml medium). This time the incubation lasted only 20 min. As soon as a constant base line was obtained (530–570 nm Shimadzu MPS-2000) hydroquinone (0.25 mM) was added to the test cuvette and K-ferricyanide (0.03 mM) to the reference cuvette. A base line between the isosbestic points (543.5 nm and 560 nm) was constructed, and the extinction changes of the α-peaks at 554 nm in relation to this line were registered in order to calculate the concentration of cytochrome *f* ($\epsilon = 19.3$ cm^2 μmol^{-1}).

Determination of D 1 protein (Q_B protein)

D 1 protein was determined by [^{14}C]atrazine titration performed according to the method of Tischer and Strotmann [21] as modified by Wild *et al.* [6].

Photosynthetic electron transport rate

The isolation of thylakoids and the measurement of DCPIP-photoreduction were performed according to Dietz *et al.* [5].

Chlorophyll content of needles

Total chlorophyll content from spruce needles was determined according to Harborne [22]. This method provides a rapid and effective procedure for the serial determination of chlorophyll from a large quantity of needle samples. Whole needles were submersed in dimethyl sulfoxide (15 h, 65 °C) and the extracted chlorophyll was measured spectrophotometrically [22].

Statistics

Student's t-test for independent random samples was applied for statistical analysis. In advance, the variance homogeneity was checked by an f-test (Symbols for values: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

In the following text the term "significant" exclusively characterizes the results whose variance homogeneity p is equal to or greater than 0.05.

Results

The contents of D1 protein, cytochrome *f* and P-700 are expressed as the ratio of redox component divided by thousand molecules of chlorophyll *a* and *b* which is a measure for the content of the respective component in the thylakoid membrane. Information about the content of the same components in the needle can be obtained by calculation on a dry weight basis.

Dry weight

With respect to the ratio fresh weight per dry weight no significant differences are found between the needles of undamaged and damaged spruce trees on all harvesting dates (data not shown). Therefore, the dry weight (DW) is considered as a suitable reference quantity for our studied physiological parameters.

Chlorophyll content

Table I shows the annual average values of the chlorophyll content for samples of all sites studied. Compared to the needles of the undamaged trees in Hattgenstein and Freudenstadt, the needles of the respective damaged trees at the same sites are characterized by reduced chlorophyll contents. The reductions range about 19–33% in Hattgenstein and about 31–46% in Freudenstadt. Compared to the undamaged trees in Wallmerod, however, even the needles of the undamaged spruce trees in Hattgenstein contain less chlorophyll (approx. 10%).

Fig. 1 shows the annual courses of the chlorophyll content. A typical annual course of the chlorophyll content, with an increase to a maximum in summer can be established for the undamaged trees at Wallmerod and Hattgenstein. The undamaged trees at Freudenstadt and the damaged trees at Hattgenstein, however, only tend to follow this annual course, whereas the damaged trees at Freudenstadt show a marked decrease of the chlorophyll content during the vegetation period.

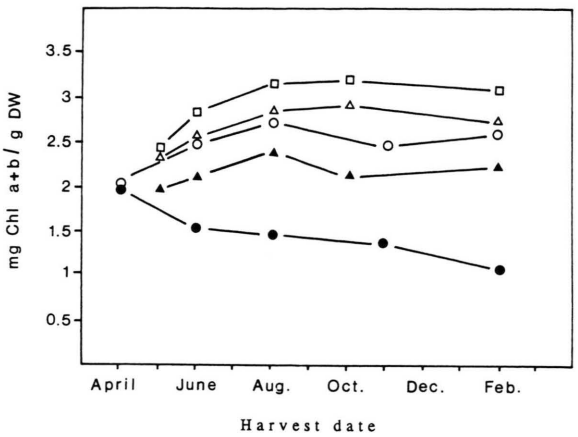

Fig. 1. Annual courses (1989) of the chlorophyll content per DW (mg/g) at Wallmerod, Hattgenstein and Freudenstadt sites. Comparison of spruce stands with different status of damage: (□) = Wallmerod site = undamaged trees (damage class 0), (△) = Hattgenstein site = undamaged trees (damage class 0 and 1), (○) = Freudenstadt site = undamaged trees (damage class 0), (▲) = Hattgenstein site = damaged trees (damage class 2), (●) = Freudenstadt site = damaged trees (damage class 2 and 3).

Table I. Annual average values of the chlorophyll *a* + *b* content per DW (mg/g) at Wallmerod, Hattgenstein and Freudenstadt sites.

Harvest period	1987	1988	1989	1990	1991
Wallmerod u	2.71	2.93	2.92	n.d.	n.d.
Hattgenstein u	2.44	2.68	2.66	n.d.	n.d.
Hattgenstein d	1.98*	1.80**	2.15**	n.d.	n.d.
Freudenstadt u	n.d.	n.d.	2.44	2.70	2.33
Freudenstadt d	n.d.	n.d.	1.40***	1.85**	1.26**

Comparison of spruce stands with different status of damage (u = undamaged trees, d = damaged trees). Annual values are calculated from five harvest dates. Significance levels between undamaged and damaged trees: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, student's t-test. n.d. = not determined.

Redox components of the thylakoid membrane

The determined redox components are presented in numbers of molecules per thousand molecules chlorophyll *a* and *b* and in nmol per g dry weight. The results of these redox components are listed in Tables II and III. The annual averages correspond mostly with the single results of every harvest date.

At Hattgenstein and Freudenstadt sites, the amount of D 1 protein is lower in the needles of the damaged spruce trees than in those of the corresponding undamaged ones. The reduction ranges about 18–40% at Hattgenstein site and about 30–37% at Freudenstadt site – if related to thousand molecules chlorophyll – and from 45–59% at Hattgenstein site and 52–66% at Freudenstadt site – if related to dry weight. At Freudenstadt, the photosynthetic electron transport rate *via* PS II is also distincly lower in the needles of the damaged spruce trees than in those of the undamaged ones (25–38% per thousand molecules chlorophyll and 56–59% per dry weight).

Depending on the years during which the samples were taken, concentrations of cytochrome *f* in numbers of molecules per thousand molecules chlorophyll in damaged trees at Hattgenstein site are either at the same level as in undamaged trees or lower. At Freudenstadt site, contents of cytochrome *f* per thousand molecules chlorophyll rath-

Table II. Annual average values of redox components per chlorophyll (molecules per 1000 molecules chlorophyll *a* + *b*) and per DW (nmol/g) at Wallmerod and Hattgenstein sites.

Harvest site		Wallmerod u		Hattgenstein u		Hattgenstein d	
Harvest period		1987	1988	1987	1988	1987	1988
per 1000 molecules chlorophyll	D 1 protein	2.27	2.27	1.59	2.55	1.33	1.54*
	Cyt *f*	1.20	1.70	1.26	1.54	1.21	1.34
	P-700	n.d.	2.07	n.d.	2.48	n.d.	2.00*
per DW	D 1 protein	7.15	7.60	5.33	7.58	2.93**	3.07*
	Cyt *f*	2.58	5.27	2.73	4.60	2.40	2.63*
	P-700	n.d.	6.94	n.d.	7.29	n.d.	4.08**

Comparison of spruce stands with different status of damage (u = undamaged trees, d = damaged trees). Annual values are calculated from five harvest dates. Significance levels between undamaged and damaged trees: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, student's t-test. n.d. = not determined.

Table III. Annual average values of the electron transport rate (ET) with DCPIP (μmol e^-/mg Chl · h) and redox components per chlorophyll (molecules per 1000 molecules chlorophyll *a* + *b*) and per DW (nmol/g) at Freudenstadt site.

Harvest period		1989		1990		1991	
Damage		u	d	u	d	u	d
per 1000 molecules chlorophyll	ET	148	111	163	101*	208	150
	D 1 protein	2.22	1.40**	2.70	1.88*	2.95	1.97
	Cyt *f*	1.15	1.22	1.22	1.36	1.26	1.52
	P-700	2.72	2.73	3.03	2.99	3.11	3.09
per DW	ET	357	148***	428	190***	477	194**
	D 1 protein	6.02	2.19***	8.13	3.93**	7.88	2.69*
	Cyt *f*	3.08	1.90***	3.60	2.79*	3.26	2.12*
	P-700	7.31	4.28***	8.99	6.10***	8.01	4.32**

Comparison of two spruce stands with different damage status (u = undamaged trees, d = damaged trees). Annual values are calculated from five harvest dates. Significance levels between undamaged and damaged trees: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, student's t-test.

er show an elevation with an increasing damage of the trees [6–21%). In relation to dry weight, cytochrome *f* is distincly lower due to damage at all sites (22–38%).

The damaged spruce trees in Hattgenstein exhibit lower contents of P-700 than the undamaged ones, both per thousand molecules chlorophyll and related to dry weight. In Freudenstadt, however, a reduction can only be observed in relation to dry weight (32–46%).

The annual courses of various redox components of the thylakoid membrane for the years 1989–1991 are presented in Fig. 2–4. The values originate from samples taken at Freudenstadt site. Amounts of D 1 protein, cytochrome *f*, and P-700 are illustrated in their relation to thousand molecules chlorophyll and in their relation to dry weight.

The D 1 protein shows an annual characteristic – both in numbers of molecules per thousand molecules chlorophyll and in nmol per g dry weight – with increasing concentrations from spring to summer and lower concentrations during winter. The concentration of cytochrome *f* does not follow a clear annual course. For P-700 per thousand molecules chlorophyll, a pronounced annual course cannot be observed. Related to dry weight, however, the annual course approximately corresponds to the annual course of the chlorophyll content.

Oxidation speed of antennae chlorophylls

The damaged spruce trees at Freudenstadt site show a marked increase in the oxidation speed of the antennae chlorophylls *in vitro* during the vegetation periods 1989–1990 (Table IV).

Comparison of the different sites and stands

Fig. 5 A–C shows the annual average levels of the chlorophyll contents (harvesting year 1989), the contents of cytochrome *f* per dry weight, and the PS II/PS I-ratio (results of 1988 for Hattgenstein and Wallmerod sites, results of 1989 for

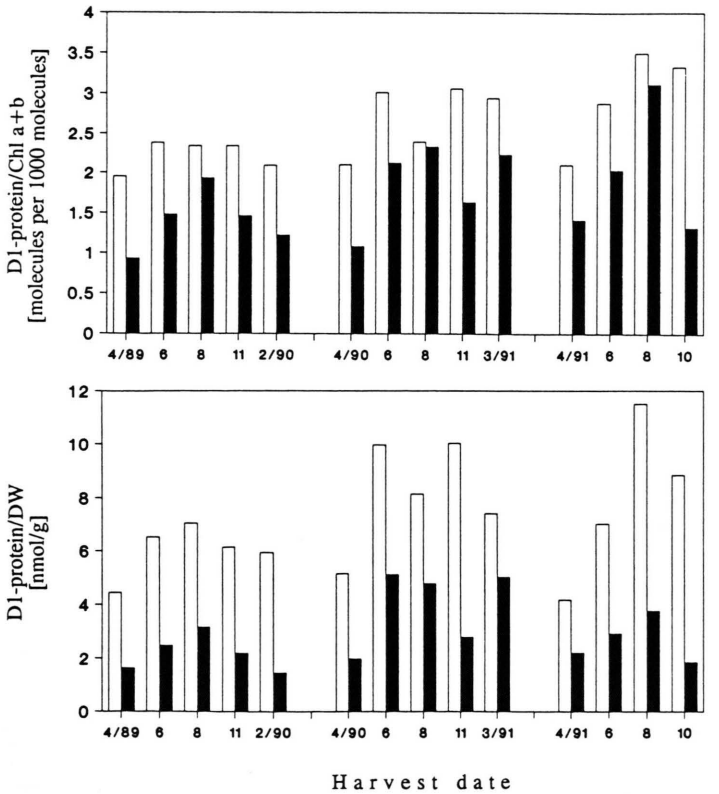

Fig. 2. Annual courses of D 1 protein per 1000 molecules chlorophyll *a* + *b* (molecules/1000 molecules) and per DW (nmol/g) at the Freudenstadt site. Values were measured in the vegetation periods 1989/90, 1990/91 and 1991. (□) = Freudenstadt site = undamaged trees (damage class 0), (■) = Freudenstadt site = damaged trees (damage class 2 and 3).

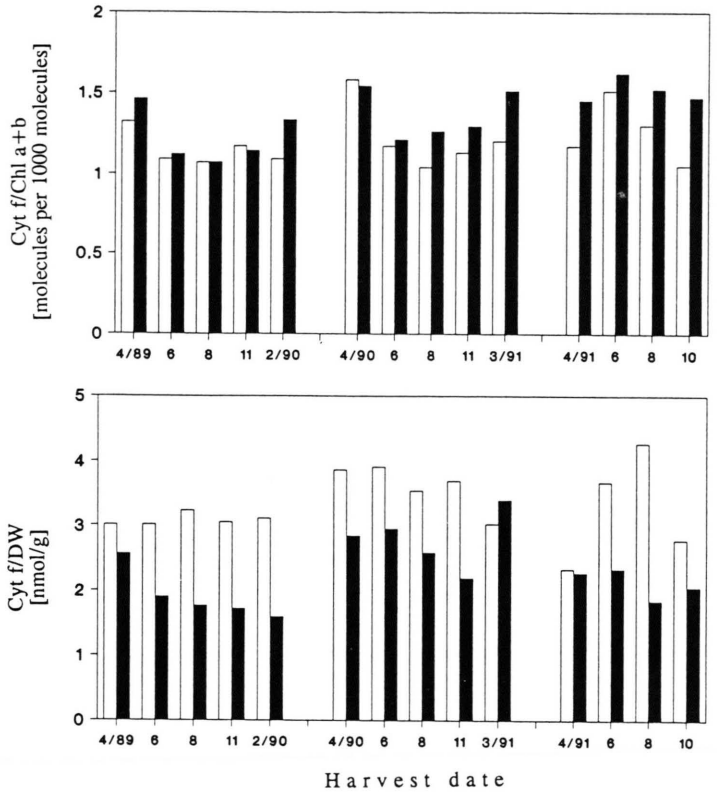

Fig. 3. Annual courses of P-700 per 1000 molecules chlorophyll $a+b$ (molecules/1000 molecules) and per DW (nmol/g) at Freudenstadt site. Values were measured in the vegetation periods 1989/90, 1990/91 and 1991. (□) = Freudenstadt site = undamaged trees (damage class 0), (■) = Freudenstadt site = damaged trees (damage class 2 and 3).

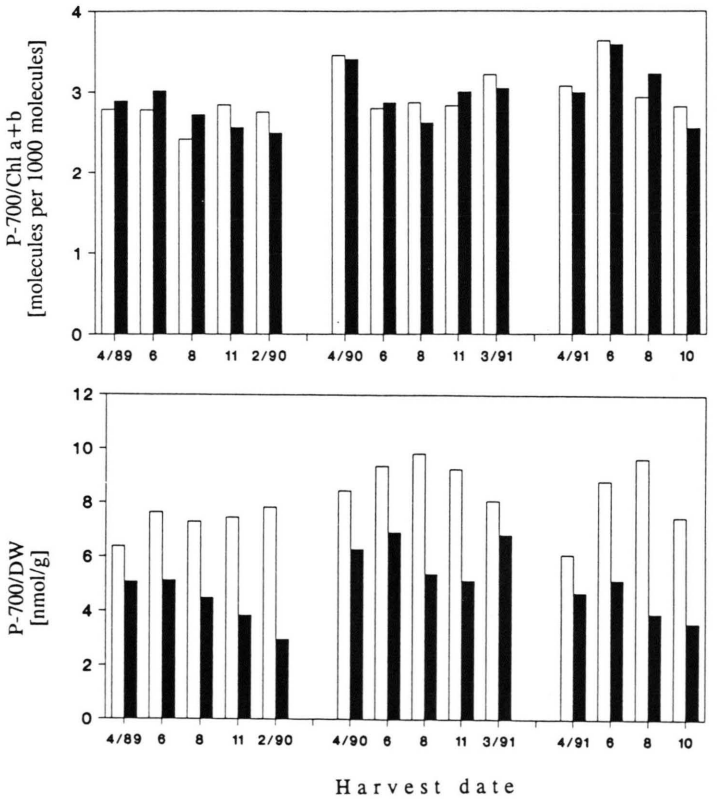

Fig. 4. Annual courses of cytochrome *f* per 1000 molecules chlorophyll $a+b$ (molecules/1000 molecules) and per DW (nmol/g) at Freudenstadt site. Values were measured in the vegetation periods 1989/90, 1990/91 and 1991. (□) = Freudenstadt site = undamaged trees (damage class 0), (■) = Freudenstadt site = damage trees (damage class 2 and 3).

Table IV. Oxidation speed (ratio A/P) of antennae chlorophylls of undamaged (u) and damaged (d) spruce trees at the Freudenstadt site.

Harvest period	1989		1990	
Damage	(u)	(d)	(u)	(d)
April	1.45	2.06	1.45	1.84
June	2.00	1.86	1.65	2.03
August	1.63	1.79	1.91	2.31
November	1.85	2.07	2.02	2.12
February	1.62	1.89	1.03	2.09
Mean value	1.71	1.93	1.61	2.08

The significance level between undamaged and damaged trees is 90% in both years (student's t-test).

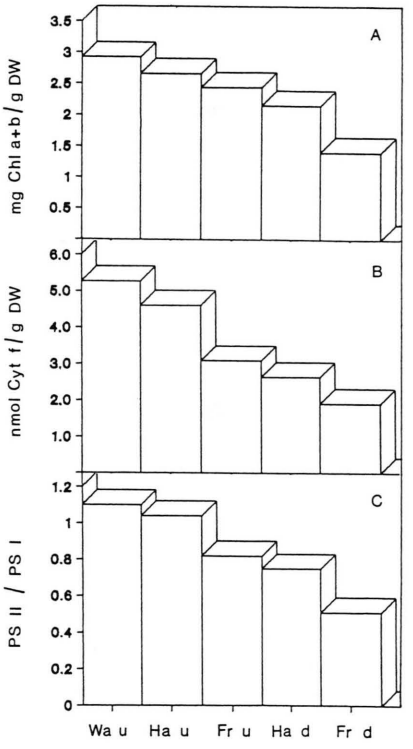

Fig. 5. Annual values of the chlorophyll content per DW (A), the content of cytochrome *f* per DW (B) and the ratio of PS II/PS I (C). Comparison of the different spruce stands: Wa u = Wallmerod site = undamaged trees (damage class 0), Ha u = Hattgenstein site = undamaged trees (damage class 0 and 1), Fr u = Freudenstadt site = undamaged trees (damage class 0), Ha d = Hattgenstein site = damaged trees (damage class 2), Fr d = Freudenstadt site = damaged trees (damage class 2 and 3).

Freudenstadt site). With increasing damage a decrease concerning all considered parameters can be observed. Noteworthy the healthy trees at Freudenstadt site (previously rated at damage class 0 according to the forest damage survey) show lower levels concerning all three parameters than the healthy trees at Wallmerod and Hattgenstein sites.

Discussion

Determination of P-700

When comparing publications concerning the concentration of P-700 per chlorophyll [6, 23, 24] it can be noticed, that the levels for spruce trees and other conifers are definitely lower than those for the leaves of angiosperms. We were able to prove that the large amount of phenolic compounds occurring in conifers disturbs the determination of P-700 in spruce needles when the chemical method is applied; it does not occur if the optical method is used. Adding Polyclar AT to remove the phenolic compounds from the extract the chemical method leads to results comparable to those of the optical method. Due to the remarkable amount of samples, however, the optical method, which demands far more equipment and time, was not used during the serial measurements.

Chlorophyll content

The needles of the undamaged trees at Wallmerod and Hattgenstein sites show a typical annual course in the chlorophyll content with an increase towards summer. There is a close connection between the annual change of the chlorophyll content and the structural organization and metabolite composition in the spruce chloroplasts. At bud break the needles of the second generation store large quantities of starch in their chloroplasts that the latter could almost function as amyloplasts. At the same time the levels of chlorophyll are very low. The growth of the newly emerged needles consumes these reserves of starch. Simultaneously to the building up of the inner membrane system (grana and stroma thylakoids), the content of chlorophyll increases [25–30].

In the needles of the undamaged trees at Freudenstadt and of the damaged trees at Hattgenstein site, however, the described annual course in the chlorophyll content is less pronounced. Further-

more, this particular annual course cannot be observed at all in the damaged trees at Freudenstadt site. These alterations of the typical annual course may be interpreted as signs for the beginning or the advance of damage to the trees.

Without any exception the needles of the more severely damaged spruce trees at Hattgenstein and Freudenstadt sites show significantly lower chlorophyll contents during the entire test period. Therefore, the decrease of chlorophyll may be considered as an indicator for a change of or a general damage to the photosynthetic system [5, 31] including pigment bleaching processes. This pigment bleaching can be caused by oxidation processes in which reactive oxygen species take part [32–34]. This explanation is supported by the fact that the oxidation speed of the antennae chlorophylls *in vitro* is distinctly increased when the needles of damaged trees are compared to those of undamaged trees. Experiments in open-top chambers, for example, demonstrated an increased sensitivity about the oxidation of antennae chlorophyll *in vitro* especially after periods of high ozone concentrations [35]. Another indication for an increased instability of the pigment protein complexes in the needles of the damaged trees is provided by the fact, that the light-harvesting complex II in the needles of the damaged trees at Freudenstadt site lost its photo- and acid-stability, before yellowing occurred [4, 13, 36].

Not only air pollutants, such as ozone, may be the cause for the loss of chlorophyll, but also nutrient deficiency, *e.g.* the lack of magnesium can be responsible for it [7, 37, 38]. At Freudenstadt and Hattgenstein the stress situation includes magnesium deficiency, high ozone pollution and strong global irradiance.

Redox components of the thylakoid membrane

Over a period of five years the redox components D 1 protein, cytochrome *f*, and P-700 were determined. If calculated on a chlorophyll basis the mentioned redox components show a marked difference in their reaction towards membrane damage. The D 1 protein is significantly decreased in damaged trees, while cytochrome *f* and P-700 show no significant differences between undamaged and damaged trees at Hattgenstein and Freudenstadt. Related to dry weight all three parameters are distinctly reduced in the needles of the damaged spruce trees at both sites. These results demonstrate a particular sensitivity of the D 1 protein while both cytochrome *f* and P-700 seem to decline within a general membrane damage process including loss of chlorophyll. The PS II and, above all, the D 1 protein is in current discussion to be the possible target of photoinhibitory processes [39–41]. Light intensities in the habitats attain the range of light saturation of photosynthesis on sunny days and are occassionally even higher (data not shown), so that photoinhibition may take place if additional stress factors occur. Especially during summer, when low precipitation and high temperatures prevailed, greater differences in the concentrations of thylakoid components between spruce trees classified as undamaged and damaged can be observed. In damaged trees the D 1 protein (PS II) is much more affected than P-700 (PS I). This is obvious in the ratio of PS II/PS I, which decreases distinctly in damaged spruce trees (Fig. 5).

The content of the D 1 protein was determined in order to get information about the structural integrity of PS II. In addition to this the DCPIP mediated electron transport was taken as a measure for the fraction of photochemically active PS II. Especially the severely damaged spruce trees at Freudenstadt show a drastically reduced electron transport rate, when related to both the chlorophyll content and the dry weight. Thus, it has to be assumed that the electron transport chains themselves – in addition to the pigments – are effected. Altogether these results demonstrate that distinct alterations in the thylakoid membranes of spruce trees occur within the development of damage. Alterations in the ultrastructure of the chloroplasts in needles of damaged spruce trees also indicate a distinct progression of damage to the thylakoid membranes [28, 30].

Comparison of the different sites and stands

A comparison of trees from various habitats and various damage classes reveals decreased levels of chlorophyll with an increase of damage. The trees at Wallmerod site, which are considered as healthy, show the highest average content followed by the not severely damaged trees at Hattgenstein. Consequently the lowest chlorophyll contents are found for the strongly damaged trees

at Freudenstadt. A decrease of the chlorophyll content can even be measured in needles from the undamaged trees at Hattgenstein and Freudenstadt, which show no visible signs of chlorophyll loss. So the determination of the chlorophyll content seems to be useful in the detection of a beginning damage. Furthermore, from these results it appears that the increase of damage to the thylakoid membranes proceeds continuously from weak to more severe damage.

If in addition results from determinations of cytochrome *f* are included an even more detailed evaluation of the extent of damage is possible. Although care has to be taken in interpreting of values from different sites and different sampling periods the presented results show a clear tendency of decreased levels of cytochrome *f* with increased damage. The needles of the healthy spruce trees at Hattgenstein and Freudenstadt sites contain less of cytochrome *f* than the reference trees at Wallmerod site, although most of these trees had been classified damage class 0. In the course of the disease, the concentration decreases again and – in case of severe damage – falls to 36% of the level measured in the needles of the healthy spruce trees at Wallmerod site.

The PS II/PS I ratio, represented by the quotient D1 protein/P-700, decreases continuously with increasing damage. Undamaged spruce trees at Wallmerod site show a ratio of about 1.1. In damaged trees, this ratio distinctly falls below 1, and in the damaged spruce trees at Freudenstadt site (damage class 2–3) it even drops below 0.6. As previously mentioned, this demonstrates the higher vulnerability of PS II compared to PS I.

The results demonstrate, that these physiological parameters concerning the photosynthetic membrane reflect the extent of damage of the studied trees. In contrast to the criteria which form the basis of the forest damage report they give, however, a more detailed and precise information. They show a beginning, not yet visible damage of spruce trees. A comparison between the undamaged trees at Wallmerod, Hattgenstein and Freudenstadt sites, for example, points out that the undamaged spruce trees at Freudenstadt contain the lowest levels of chlorophyll, of cytochrome *f*/DW and the lowest PS II/PS I ratio. This indicates that these spruce trees are already affected although they are classified as damage class 0 according to the criteria of the forest damage report.

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