

Investigation on the Photosynthetic Membranes of Spruce Needles in Relation to the Occurrence of Novel Forest Decline

I. The Photosynthetic Electron Transport

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The investigations described here were carried out in the context of our research project on the physiological, biochemical, and cytomorphological characterization of spruce trees growing in natural habitats and showing damage of varying intensity. Here we report on specific aspects of the photosynthetic apparatus. The aim of the measurements was to analyze whether or not the activity of the photosynthetic electron transport pathway is affected in damaged trees. The investigations were carried out on a 20 to 25-year-old spruce plantation in the Hunsrück mountains and on an 80-year-old spruce plantation in the Westerwald mountains. The photosynthetic electron transport rate was determined by photoreduction of 2,6-dichlorophenolindophenol. A decrease of the electron transport rate was shown in the damaged spruce trees in comparison to the apparently healthy trees. The investigation of the water splitting enzyme system – determined in the Hill-reaction by feeding in electrons by means of diphenylcarbazide – indicates that the electron transport on the oxidizing side of photosystem II is impaired. The results imply that the photosynthetic electron transport chains in the thylakoid membranes of the spruce chloroplasts are sites of early injurious effects. This is in agreement with the electron microscopic analyses which show consistently that early damage occurs especially at the cellular membranes. This membrane damage is apparent even in the green needles of damaged spruce trees.

Introduction

For several years, “novel forest decline” has been occurring over wide areas in Central Europe. This class of forest diseases cannot be subsumed under the general category of classical smoke damage alone, because it is manifest also in areas far away from major polluters and industrial centres and even in so-called “clean air” regions with low SO₂ pollution. The damage has increased in intensity and extent over the past six years. Damage intensity increases

with the altitude of the mountains, although symptoms can now be observed in lower-elevation forests. The damage, which now affects all economically-important tree species of the forests and which is mainly visible in older plantations, already comprises about 52% (damage categories 1–4) of the entire forest area of West Germany today; about 17% – represented by the damage categories 2–4 – show medium to severe damage (*cf.* [1]).

There are different hypotheses concerning the causes of the new type of forest decline. The major factors discussed are natural factors or anthropogenic pollutants. According to the present state of research, this disease appears to be of multifactorial origin, *i.e.* the causes are very diverse and one stressor alone rarely plays the major role. Different stressors act on the trees simultaneously, and sometimes in a synergistic way, which leads to a complex disease with different causes and relationships. Involvement of climatic, edaphic, and silvicultural factors, of fungal and microbial diseases, and of insect infestations is undisputed and may be of crucial significance for damage in restricted areas. However,

Abbreviations: Carbowax, polyethyleneglycol; Chl, chlorophyll; Cyt *f*, cytochrome *f*; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; DPC, diphenylcarbazide; DTE, dithioerythritol (= erythro-1,4-dimercapto-2,3-butanediol); EDTA, ethylenediaminetetraacetic acid disodiumsalt (dihydrate); ETR, photosynthetic electron transport rate; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; PS, photosystem.

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these factors can definitely not be regarded as the only cause of such massive and widespread damage to the forest ecosystem. There is evidence that anthropogenic air pollution is likely to be responsible for the exceptional intensity of damage and for the spreading of forest injury over wide regions [2–9].

The investigations described here were carried out in the context of our research project on the physiological, biochemical, and cytomorphological characterization of spruce trees growing in natural habitats and showing damage of varying intensity [10–13]. Photosynthesis [14], the water status [15], the nitrogen assimilation [16], the content of chemical elements [17], and light- and electron-microscopic structure [18, 19] were previously examined. Here we report on some aspects of the photosynthetic apparatus.

Generally, photosynthesis is effected by damage at an early stage. It has been known that photosynthesis reacts very sensitively to different kinds of stress [20]. A direct influence of air pollutants on the photosynthetic apparatus is discussed by [21].

Fumigation experiments have already shown that the photosynthetic electron transport is exceptionally susceptible to air pollutants such as ozone [22, 23], sulphur dioxide [24–26] or PAN [27–29].

Our electron microscopic investigations have shown early damage to the various membrane systems of the cell organelles and the cytoplasm [18]. The chloroplasts are the sites where the damage is manifested most specifically. Therefore, the aim of the measurements described here was to analyze whether or not the activity of the photosynthetic electron transport pathway is affected in damaged trees. We were particularly interested in whether or not an inhibition of the photosynthetic electron transport can take place in apparently green needles of a damaged tree.

For this purpose, the Hill-activity of isolated thylakoids was determined by photoreduction of the artificial electron acceptor DCPIP. In order to investigate a possible impairment of the water splitting enzyme system, DPC was added to the test system. In addition, the chlorophyll content was determined.

In the following papers of the series, we will report on specific components of the photosynthetic electron transport chain – Q_B -protein, cytochrome *f*, and P-700 [30], in this issue) – and on the activity of the ribulosebisphosphate carboxylase (in preparation).

Materials and Methods

Description of the locations

Location in the Hunsrück mountains

This 20–25-year-old spruce (*Picea abies* (L.) KARST.) plantation is located at about 660 m to 674 m above sea level on a moderately steep south slope on the west side of the Hunsrück mountains (Idar-Oberstein Forestry Office, Hattgenstein Forest District, Division 257) in the southwest of the Federal Republic of Germany. The soil is based on quartzite, with significant water storage capacity. It mainly consists of slight podsolated brown-earth with a high level of acidity (pH 3.1–4.2) especially in the upper layers. The saturation of the soil with bases is very low. The average annual precipitation is 800–1100 mm · m⁻². The duration of the vegetation period is two to three weeks shorter in comparison to valley locations [31].

Climatic data and air pollution data were registered at the measurement station Leisel of ZIMEN (Central Network for the Measurement of Immission in Rheinland-Pfalz) in the vicinity of the Hunsrück plantation at about 650 m above sea level (cf. [32]). During the summer months of the 1986 vegetation period, a warm, dry, and sunny climate prevailed. The annual average concentration of the air pollutant SO₂ reached 23 µg m⁻³ air. An exceptionally high SO₂-concentration could be observed in February (monthly mean value: 114 µg m⁻³ air) with peaks (weekly mean values) of 140 µg m⁻³ air in the 7th week and 133 µg m⁻³ air in the 9th week of the year. In the summer months of July and August the SO₂-immission was relatively low (monthly mean values: 7–8 µg m⁻³ air).

The ozone-concentration increased parallel to the increase in temperature and the global solar radiation in the summer months. On annual average, the ozone-concentration reached values of 76 µg m⁻³ air with peaks (weekly mean values) of 162 µg m⁻³ air in the 21th week and 149 µg m⁻³ air in the 25th week of the year. On average, 110 µg m⁻³ air were reached from April to August in 1986, 49 µg m⁻³ air could be measured from January to March and from September to December.

On annual average, the NO₂-concentration reached 14 µg m⁻³ air with peaks (weekly mean values) of 47 µg m⁻³ air in the 7th and the 11th week of the year. On average, 5 µg m⁻³ air were reached from April to August, whereas from January to

March and from September to December $20 \mu\text{g m}^{-3}$ air could be measured.

The NO-immission was relatively constant (monthly mean values: $1\text{--}4 \mu\text{g m}^{-3}$ air) during the course of the year.

Location in the Westerwald mountains

This approximately 80-year-old spruce plantation is located at about 495 m above sea level on an only slight north-slope of the Westerwald mountains (Wallmerod Forestry Office, Höhn Forest District, growth area 9043) in the middle of the Federal Republic of Germany. The soil is based on basalt, with significant water storage capacity. It is very deep and consists of slight podsolated brown-earth well supplied with nutrients.

Sampling

The Hunsrück plantation permitted pair comparison, because apparently healthy or only slightly damaged trees and trees with clearly visible damage were growing side by side. Thus, the data derived from a tree with clear symptoms of damage were always comparable with data obtained from an apparently healthy tree. The test trees were selected in accordance with the degree of visible phenotypic damage (*cf.* [33]). In general, mainly older needle generations of damaged trees showed symptoms of injury such as needle loss, whereas the 1985 and the 1984 shoots appeared visibly green and healthy.

All spruce trees of the Westerwald location were apparently healthy.

The investigations presented here were carried out between April and October in 1986. Five tree pairs of the Hunsrück spruce plantation and five trees of the Westerwald spruce plantation were chosen for the measurements in spring. On four harvest dates at the Hunsrück location (28 Apr, 16 Jun, 18 Aug, 20 Oct) and three harvest dates at the Westerwald location (20 May, 28 Jul, 22 Sep) twigs were cut off and stored on ice. Only needles of the second and third year (1985 and 1984 shoots) from the 7th whorl with comparable light-exposition were used.

Chlorophyll content

Chlorophyll was extracted from the needles with dimethylsulfoxide (DMSO) and measured spectrophotometrically according to [34].

Needle surface area

The needle surface area was determined by means of a planimetry device (Videoplan/Kontron). Mean values of the length and the cross-section perimeter (of a random sample of 20 needles) were determined and used to calculate the cylindrical surface area.

Chloroplast preparation

The operations were performed at 0°C .

5 g of washed spruce needles were homogenized with an Ultra Turrax T 25/18 K (Janke & Kunkel) for 10 s in 60 ml of isolation medium (50 mM sodium-pyrophosphate/HCl, pH 7.0; 5 mM MgCl_2 ; 4 mM KHSO_3 ; 5 mM DTE; 20% w/v Carbowax 6000; 1 M sorbitol) and filtered through 4 layers of gauze. The homogenate was centrifuged at $4350 \times g$ for 5 min. The pellet was resuspended in 40 ml of resuspension medium (50 mM HEPES/KOH, pH 6.7; 2 mM EDTA; 1 mM MnCl_2 ; 1 mM MgCl_2 ; 0.5 mM K_2HPO_4 ; 20 mM NaCl; 2 mM NaNO_3 ; 1 M sorbitol) and centrifuged again at $4350 \times g$ for 5 min. The pellet was taken up in 5 ml of H_2O to break the chloroplast envelope by osmotic shock. After 5 min, 5 ml of double concentrated resuspension medium were added to establish the previous buffer concentration. This chloroplast suspension was now cleared from cell debris and starch by a discontinuous sorbitol gradient centrifugation (lower phase: resuspension medium containing 2 M sorbitol, upper phase: chloroplast suspension containing 1 M sorbitol) at $300 \times g$ for 15 min without using the brake. The broken chloroplasts were located in the upper phase and in the boundary layer between the two phases. In order to obtain a homogeneous suspension of broken chloroplasts, the upper phase and the boundary layer were collected and sucked through 4 layers of nylon mesh ($22 \mu\text{m}$ pore diameter). According to the nomenclature of [35] such chloroplasts could be called type C–D.

The chlorophyll content (Chl $a + b$) of the chloroplast suspension was calculated according to [36] after pigment extraction with 80% v/v acetone.

Hill-reaction

The photosynthetic electron transport rate of the broken chloroplasts was measured as photoreduction of the electron acceptor DCPIP. The reaction medium contained (final concentrations) 50 mM HEPES/KOH, pH 6.5; 1 M sorbitol; $60 \mu\text{M}$ DCPIP;

2.5 µg Chl/ml; methanol 1% v/v; and, if required, 1 mM DPC. The reduction rate was determined spectrophotometrically (Ultrospec/LKB) at 605 nm in a darkened lab. For this purpose the previously mixed reaction medium was divided into two test-tubes. One test-tube was illuminated for 60 s (390 W m⁻², 25 °C), whereas the other one was stored in darkness for 60 s (25 °C) as a reference. For the calculation of the electron transport rate the molar extinction coefficient $\epsilon = 18.73 \times 10^3 \text{ l mol}^{-1} \cdot \text{cm}^{-1}$ (pH 6.5, 605 nm) was used. This procedure was repeated 5 times with each sample collection for each test tree and for each electron transport sequence which was tested.

Results

Hunsrück location

Ranking list of the test trees

The test trees were appraised separately before the first harvest date in accordance with the degree of visible phenotypic damage (*cf.* [33]). In addition, on each harvest date the branches and twigs, which were used for the measurements, and a random sample of 100 needles were classified using a special list of criteria (see also [14]). The colour of the needles, needle loss, dot necroses, brown banding, gold tips, etc. were taken as the criteria for evaluation. A list ranking the degree of damage to the test trees was compiled at the end of the vegetation period (Table I).

Chlorophyll content

At the Hunsrück location a significant decrease of the chlorophyll content in the severely damaged spruce trees was found in pair comparison on each harvest date (*cf.* Fig. 1, see also [37]). It is obvious

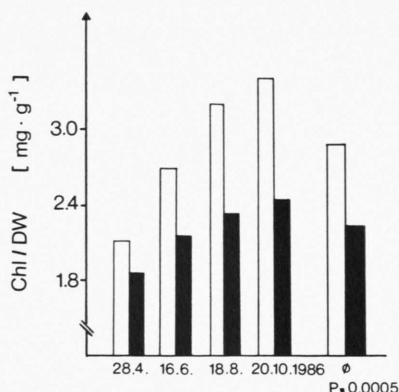


Fig. 1. Chlorophyll (Chl) content of the spruce needle generation 1985 at the Hunsrück location. Mean values of five tree pairs on different harvest dates. □ = apparently healthy or only slightly damaged, ■ = more severely damaged, Ø = mean value over the investigation period 1986, P = statistical probability (Student's T-test).

that the chlorophyll content of the test trees increased as summer approached. The difference in the chlorophyll content between apparently healthy and more severely damaged trees became more obvious with increasing needle age: three-year-old needles showed a greater reduction in chlorophyll content, with an annual average decrease of 31%, than two-year-old needles, with an annual average decrease of 23% (Table II). It is also obvious that the chlorophyll content in the needles of the 1984 generation was higher than in those of the 1985 generation of the apparently healthy trees.

The chlorophyll a to chlorophyll b ratios appeared to be lower in the more severely damaged trees compared to the less damaged trees (data not shown).

Table I. Ranking list of the test trees at the Hunsrück location for the investigation period 1986. Optically discernible damage according to a special list of criteria. Position 1 represents the least damage, and position 10 the greatest damage. The tree pairs were numbered consecutively (1 to 5). The second digit of the tree number stands for apparently healthy (1) and more severely damaged (2). '84 = the 1984 needle generation, '85 = the 1985 needle generation.

Tree No.	1.1	1.2	2.1	2.2	3.1	3.2	4.1	4.2	5.1	5.2
'85	2	6	5	7	1	7	4	9	3	9
'84	1	5	4	7	2	8	6	9	3	9
Altogether	1	6	4	7	1	8	5	9	3	9
Damage interval	5		3		7		4		6	

Table II. Chlorophyll content of the needles per dry weight. $\bar{\varnothing}$ = mean value over the investigation period 1986, '85 = the 1985 needle generation, '84 = the 1984 needle generation.

Chl <i>a</i> + <i>b</i> [mg · g ⁻¹]	$\bar{\varnothing}$ '85	$\bar{\varnothing}$ '84
Westerwald location		
Apparently healthy trees	2.79 ± 0.38	3.04 ± 0.32
Hunsrück location		
Apparently healthy trees	2.87 ± 0.60	3.28 ± 0.44
Hunsrück location		
Clearly damaged trees	2.20 ± 0.47	2.25 ± 0.44

Hill-activity

A decrease in the electron transport rate in the more severely damaged spruce trees was shown irrespective of the reference parameter (Fig. 2). Nevertheless, the reduction was much more drastic in relation to the dry weight, the fresh weight, and

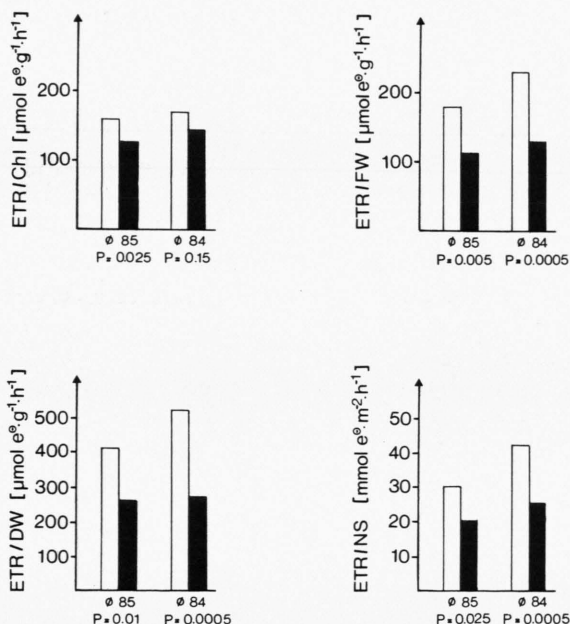


Fig. 2. Photosynthetic electron transport rate (ETR) in relation to different parameters (Chl = chlorophyll *a* + *b*, FW = fresh weight, DW = dry weight, NS = needle surface area). DCPIP-Hill-activity with broken chloroplasts of spruce needles from the Hunsrück location. □ = apparently healthy or only slightly damaged trees, ■ = more severely damaged trees, $\bar{\varnothing}$ = mean value of two tree pairs over the investigation period 1986, '85 = the 1985 needle generation, '84 = the 1984 needle generation, P = statistical probability (Student's T-test).

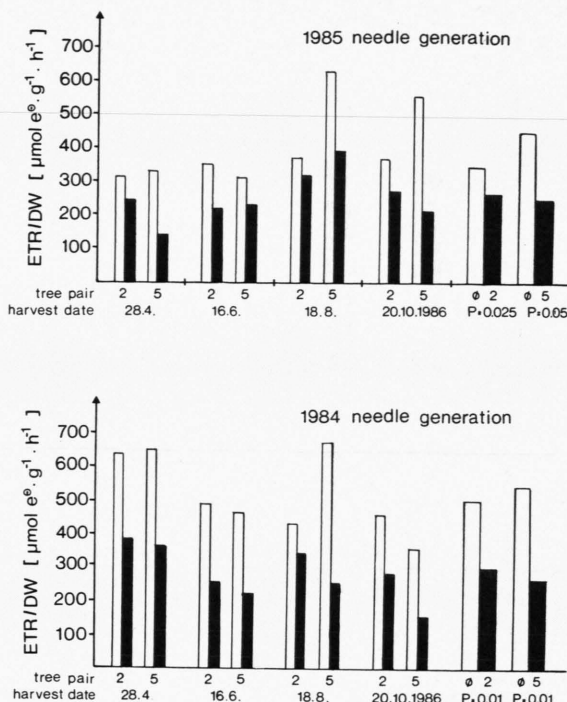


Fig. 3. Photosynthetic electron transport rate (ETR) in relation to the dry weight. DCPIP-Hill-activity with broken chloroplasts of spruce needles from the Hunsrück location. Pair comparison of two tree pairs on different harvest dates. □ = apparently healthy or only slightly damaged, ■ = more severely damaged, $\bar{\varnothing}$ = mean value over the investigation period 1986.

the needle surface area than in relation to the chlorophyll content. Fig. 3 shows that this reduction in the Hill-activity could be observed on each harvest date and for both spruce pairs. The decrease in the electron transport rates correlated well with the damage (*cf.* Table I): the tree pair with the greater damage difference between the tree partners (tree pair no. 5) also shows a greater decrease of the Hill-activity on most harvest days and on annual average (Fig. 3).

Hill-activity after the addition of DPC

After the addition of DPC to the assay system, the electron transport rates rose in the more severely damaged, and even in the less damaged trees (Fig. 4). However, this increase in the Hill-activity is much more marked in the severely damaged trees (about 40%) than in the apparently healthy or only slightly damaged trees (about 20%). There is again a

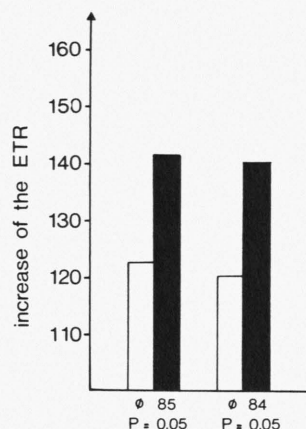


Fig. 4. Increase of the photosynthetic electron transport rate (ETR) at the Hunsrück location after the addition of diphenylcarbazine (test system: DPC \rightarrow PS II \rightarrow DCPIP). Control (test system: $H_2O \rightarrow$ PS II \rightarrow DCPIP) = 100%. \square = apparently healthy or only slightly damaged trees, \blacksquare = more severely damaged trees, \varnothing = mean value of two tree pairs over the investigation period 1986, '85 = the 1985 needle generation, '84 = the 1984 needle generation.

correlation between the damage interval of the two partners of both tree pairs and the electron transport rate after DPC addition: a low damage difference between the tree partners is also reflected in a smaller difference in the increase of the electron transport rates. These results were found to be similar for both needle generations.

DCMU-inhibition

The DCPIP-photoreduction could be inhibited to more than 95% by the addition of DCMU (10 μM) to the test system, irrespective of whether or not DPC was added.

Westerwald location

The amount of chlorophyll in the needles of the apparently healthy trees at the Westerwald location was about the same as in the needles of the Hunsrück location for the undamaged or only slightly damaged spruce trees (Table II). The enhancement of the Hill-activity after the addition of DPC was also the same as for the undamaged trees of the Hunsrück location. The electron transport rates of the apparently healthy spruce trees at the Westerwald location were even higher (on average about 24% in relation to the chlorophyll content) than those of the undamaged or only slightly damaged trees of the Hunsrück location (Table III).

Discussion

The amount of chlorophyll in the needles of the more severely damaged trees was always distinctly lower compared to that in the apparently healthy trees, even in needle groups with a green and healthy appearance. The chlorophyll content of the needles

Table III. Photosynthetic electron transport rate (ETR) in relation to different parameters (DW = dry weight, FW = fresh weight, NS = needle surface area, Chl = chlorophyll $a + b$). DCPIP-Hill-activity with broken chloroplasts of spruce needles. \varnothing = mean value of two tree pairs from the Hunsrück location and four trees from the Westerwald location over the investigation period 1986, '85 = the 1985 needle generation, '84 = the 1984 needle generation, tree group 1 = apparently healthy or only slightly damaged, tree group 2 = more severely damaged.

Hill-activity	Parameter	Tree group	Hunsrück location		Westerwald location	
			\varnothing '85	\varnothing '84	\varnothing '85	\varnothing '84
- DPC	ETR/DW	1	406 \pm 123	508 \pm 118	498 \pm 97	586 \pm 70
	[$\mu mol e^- g^{-1} h^{-1}$]	2	254 \pm 80	267 \pm 72		
	ETR/FW	1	176 \pm 43	230 \pm 59	223 \pm 35	272 \pm 35
	[$\mu mol e^- g^{-1} h^{-1}$]	2	117 \pm 35	128 \pm 36		
	ETR/NS	1	30 \pm 9	42 \pm 8	41 \pm 7	52 \pm 9
	[$mmol e^- m^{-2} h^{-1}$]	2	21 \pm 5	24 \pm 6		
	ETR/Chl	1	160 \pm 27	171 \pm 52	197 \pm 24	213 \pm 40
	[$\mu mol e^- mg^{-1} h^{-1}$]	2	132 \pm 24	144 \pm 44		
+ DPC	ETR/Chl	1	195 \pm 35	206 \pm 62	242 \pm 39	265 \pm 71
	[$\mu mol e^- mg^{-1} h^{-1}$]	2	187 \pm 37	202 \pm 41		
	Percent increase	1	122	120	123	124
	(- DPC value = 100%)	2	142	140		

thus proves to be a reliable indicator of the overall damage condition of a tree as well as a tree collective. This, however, applies only to needle material of similar age, growing in the same environment, and harvested on the same date. It is particularly important to take care that the light exposition of the selected twigs is comparable, because of the possible adaptation of the photosynthetic apparatus to different light conditions [38, 39]. The chlorophyll content of the apparently healthy trees is obviously higher in the 1984 needle generation than in the 1985 needle generation. This could indicate that the chlorophyll content of the needles rises with increasing age (*cf.* [40]). However, this does not apply to the more severely damaged trees, explaining the greater difference in the chlorophyll content between the apparently healthy trees and the more severely damaged trees in the 1984 needle generation. This leads to the suggestion that the symptoms of damage could be found to be stronger with increasing age of the needles and longer exposition to the damage factors.

The increase of the chlorophyll content during the vegetation period corresponds to the changes in the structural organization and the alterations in the metabolite composition of conifer chloroplasts during the year (*cf.* [18, 41, 42]).

In each individual pair comparison, as well as on annual average of all tree pairs, the more severely damaged trees at the Hunsrück location showed a distinctly lower photosynthetic electron transport rate compared to the apparently healthy spruce trees. This reduction was especially marked in relation to the fresh weight, the dry weight, and the needle surface area. A lower photosynthetic electron transport rate in the needles of the more severely damaged trees is also found in relation to the chlorophyll content. Then the decrease of the Hill-activity is less drastic, although still distinct (about 18%). Thus, the photosynthetic electron transport pathways in the thylakoid membranes are even more affected by damage than the photosynthetic pigments.

In order to study this in detail, several membrane components were examined quantitatively (see the following paper in this issue [30]). In addition, the water splitting enzyme system, which is known to be sensitive to stress, was investigated. For this purpose, the artificial electron donor DPC was added to the test system ($\text{H}_2\text{O} \rightarrow \text{PS II} \rightarrow \text{DCPIP}$), because

DPC is able to donate electrons to the electron transport pathway after the water splitting enzyme system on the oxidizing side of PS II [43]. Thus, DPC is able to compensate for possible disturbances in the photosynthetic electron transport caused by an impairment of the function of the water splitting enzyme system. Consequently, the photosynthetic electron transport rate should show an enhancement after the addition of DPC to the assay if the water splitting enzyme system is damaged. Both the apparently healthy and the more severely damaged spruce trees showed an enhancement of the Hill-activity after the addition of DPC to the test system. The enhancement occurring in the apparently healthy trees could be explained either by a partial injury to the water splitting enzyme during the preparation, or by latent damage to the system. Nevertheless, the Hill-activity of the severely damaged trees could be raised to, on average, twice as much as the activity of the less damaged partners. These differences between the apparently healthy and the more severely damaged trees were found to be similar in both needle generations. This is strong evidence showing that the function of the water splitting enzyme system is especially impaired when the electron transport chain in the thylakoid membranes is damaged.

Our electron microscopic investigations indicate a general membrane damage in the cell, especially in the chloroplasts of the mesophyll cells [18]. This implies that not only the photosynthetic pigments, the photosynthetic electron transport, and the water splitting enzyme system are affected by the membrane damage, but also other components of the thylakoid membrane. In order to see whether the damage also affects other components of the thylakoid membrane, one specific redox-component from each of the three integral protein complexes, PS II-complex, cytochrome b_6/f -complex, and PS I-complex, was examined quantitatively. The results will be presented in the following paper in this issue [30].

Acknowledgements

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