Investigation on the Photosynthetic Membranes of Spruce Needles in Relation to the Occurrence of Novel Forest Decline
II. The Content of Q_B-Protein, Cytochrome *f*, and P-700

Aloysius Wild, Ute Flammersfeld, Iris Moors, Bernhard Dietz, and Wolfgang Rühle

Institut für Allgemeine Botanik der Johannes Gutenberg-Universität, Saarstraße 21, D-6500 Mainz, Bundesrepublik Deutschland

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In order to obtain an insight into the damage of thylakoid membranes of spruce *(Picea abies)* trees with damage of varying intensity, investigations were performed on the content of Q_B-protein, cytochrome *f*, and P-700 in chloroplasts of spruce needles from apparently healthy and from damaged trees. Needles from the second and third needle year and the seventh whorl were chosen. The investigations were carried out in 1986 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. In damaged trees an unequivocal decrease in the content of Q_B-protein, cytochrome *f*, and P-700 was found, even in needle groups that appear visibly green and healthy. The amount of cytochrome *f* decreased by 25% per dry weight (approximately to the same extent as chlorophyll); the content of Q_B-protein and P-700, however, were more drastically reduced compared to the control trees (about 40% and 50%, respectively). These results of measuring the photosynthetic electron transport components imply that the thylakoid membranes are sites of early injurious effects.

Introduction

In the previous paper (*cf.* [1]) we reported on the Hill-activity of chloroplasts from spruce trees showing different degrees of damage. Since a reduction of the photosynthetic electron transport rate and ultrastructural degradations of the thylakoid membranes [2] could be observed, it was interesting to measure the amounts of specific redox-components of the photosynthetic electron transport pathway in apparently healthy and in clearly damaged spruce trees. For that purpose, one specific component from each integral protein complex of the thylakoid membrane was examined: Q_B-protein (as an indicator of the PS II-complex), cytochrome *f* (as a representative of the cytochrome b_6/f-complex), and P-700 (as an element of the PS I-complex).

Abbreviations: Chl, chlorophyll; Cyt *f*, cytochrome *f*; EDTA, ethylenediaminetetraacetic acid disodium salt (dihydrate); HEPES, N-(2-Hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; P-700, reaction center of photosystem I; PS, photosystem.

Reprint requests to Prof. Dr. A. Wild.

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Materials and Methods

Description of the locations

For the description of the locations and the sampling of the material see the previous paper in this issue [1].

Chloroplast preparation

All investigations were performed with needle material (two- and three-year-old needles from branches of the 7th whorl) which was quick-frozen in liquid nitrogen on site, directly after harvesting, and stored at −80 °C. The chloroplast preparation was made, in accordance with the method described in the previous paper, up to the step of discontinuous sorbitol gradient centrifugation [1]. However, to prepare chloroplasts for the Q_B-protein determination, soluble Polyvinylpyrrolidone (final concentration: 5% w/v) was added to the resuspension medium. After the discontinuous sorbitol gradient centrifugation, the broken chloroplasts were collected and centrifuged at 26,900 × *g* for 5 min.

For the measuring of cytochrome *f* and P-700, the pellet was resuspended in storage medium (50 mM HEPES/KOH, pH 6.7; 2 mM EDTA; 1 mM $MgCl_2$; 0.5 mM K_2HPO_4; 20 mM NaCl; 2 mM $NaNO_3$).

For the determination of the Q_B-protein, the broken chloroplasts were resuspended in 40 ml of wash-

ing medium (50 mM HEPES/KOH, pH 6.7; 2 mM EDTA) and stirred carefully for 5 min. The suspension was centrifuged again at 26,900 × *g* for 5 min. The pellet was then suspended in storage medium and sucked through 4 layers of nylon mesh (22 μm pore diameter) in order to obtain a homogeneous chloroplast suspension.

Determination of the Q_B-protein

The measurements were performed according to the method of Tischer and Strotmann [3] except that the binding of the labelled inhibitor was carried out at 0 °C, the final concentrations of [^{14}C]atrazine were between 0.012 μM and 0.3 μM, and the final alcohol concentration was only 0.75%. The final chlorophyll concentration was 30–40 μg ml^{-1}. The concentration of the specific binding sites was calculated from the double reciprocal plots (mg Chl·nmol^{-1} bound inhibitor *vs.* μM^{-1} free inhibitor).

Determination of cytochrome f and P-700

Cytochrome *f* was determined by difference spectra in accordance with the method specified by Bendall *et al.* [4]. Triton X-100 was added (1%) to change the high-potential form of cytochrome b_{559} in the low-potential form. This form could not be reduced by hydroquinone. A chlorophyll concentration of 0.6–0.9 mg was used. Hydroquinone (2.5 mM) was added to the reference, and ferricyanide (0.25 mM) was added to the sample. The absorbance change of the α-peak at 554 nm (measured from a baseline drawn between the isosbestic points at 543.5 nm and 560 nm) was used to calculate the cytochrome *f* concentration ($\varepsilon = 17.7 \times 10^3$ l·mol^{-1}·cm^{-1}).

The P-700 concentration could also be investigated by means of the difference spectra in accordance with the methods specified by [5, 6]. After the addition of sodium ascorbate (4 mM) to the reference, and ferricyanide (1 mM) to the sample, the spectra were recorded. The difference between the absorbance values at 730 nm (isosbestic point) and at 698 nm was used to calculate the P-700 concentration ($\varepsilon = 64 \times 10^3$ l·mol^{-1}·cm^{-1} [7]).

Results

Hunsrück location

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.

Q_B-protein

On annual average, a remarkable decrease of the Q_B-protein in the more severely damaged spruce trees could be detected in both needle generations and irrespective of the reference parameter (Fig. 1). The extent of reduction varied depending on the reference parameter chosen. The decrease in relation to

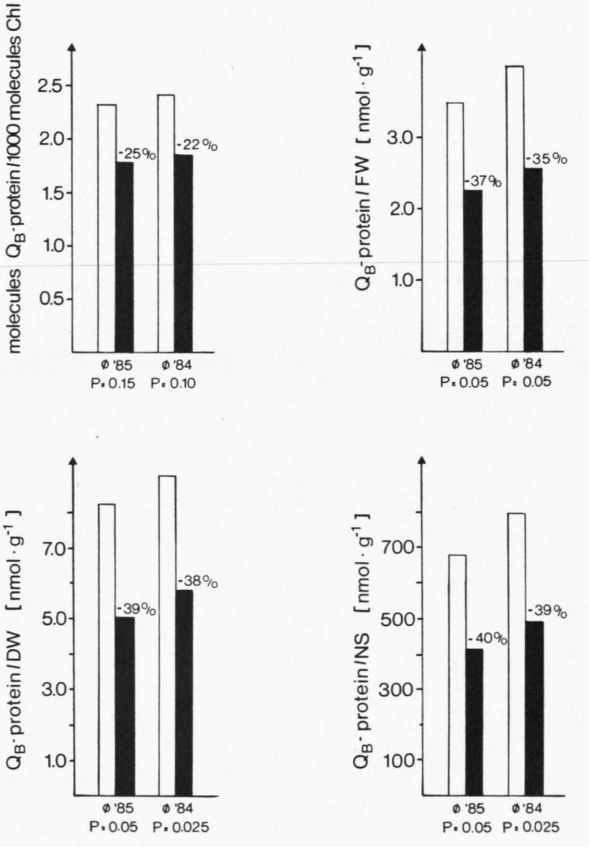

Fig. 1. Content of the redox-component Q_B-protein in relation to different parameters (Chl = chlorophyll *a* + *b*, FW = fresh weight, DW = dry weight, NS = needle surface area) at the Hunsrück location. Ø = mean value of three tree pairs over the investigation period 1986, '85 = the 1985 needle generation, '84 = the 1984 needle generation, □ = apparently healthy or only slightly damaged partners, ■ = more severely damaged partners. P = statistical probability (Student's T-test).

each, the fresh weight, dry weight, and needle surface area was greater than in relation to chlorophyll. When, for instance, the dry weight is chosen as reference parameter, it can be seen that the decrease was 39% in the 1985 needle generation and 38% in the 1984 needle generation.

Cytochrome f

There was no difference in the cytochrome *f* content between apparently healthy and damaged trees per chlorophyll, neither in the 1985 needle generation nor in the 1984 needle generation (Fig. 2). On the other hand, in relation to other parameters (FW, DW, needle surface area), there was a clear decrease in cytochrome *f* in the more severely damaged partner. This decrease appeared to be even stronger in the older needles (Fig. 2). When the dry weight was chosen as reference parameter, the decrease was 22% in the 1985 needle generation and 27% in the 1984 needle generation. Incidentally, the tree pair, which displayed the greatest damage difference on the basis of the ranking list established (see previous paper), showed a reduction of this redox component, even when chlorophyll was taken as the reference parameter (values not shown).

P-700

In relation to chlorophyll, this redox component was drastically reduced in concentration in the more severely damaged trees (Fig. 3). The difference be-

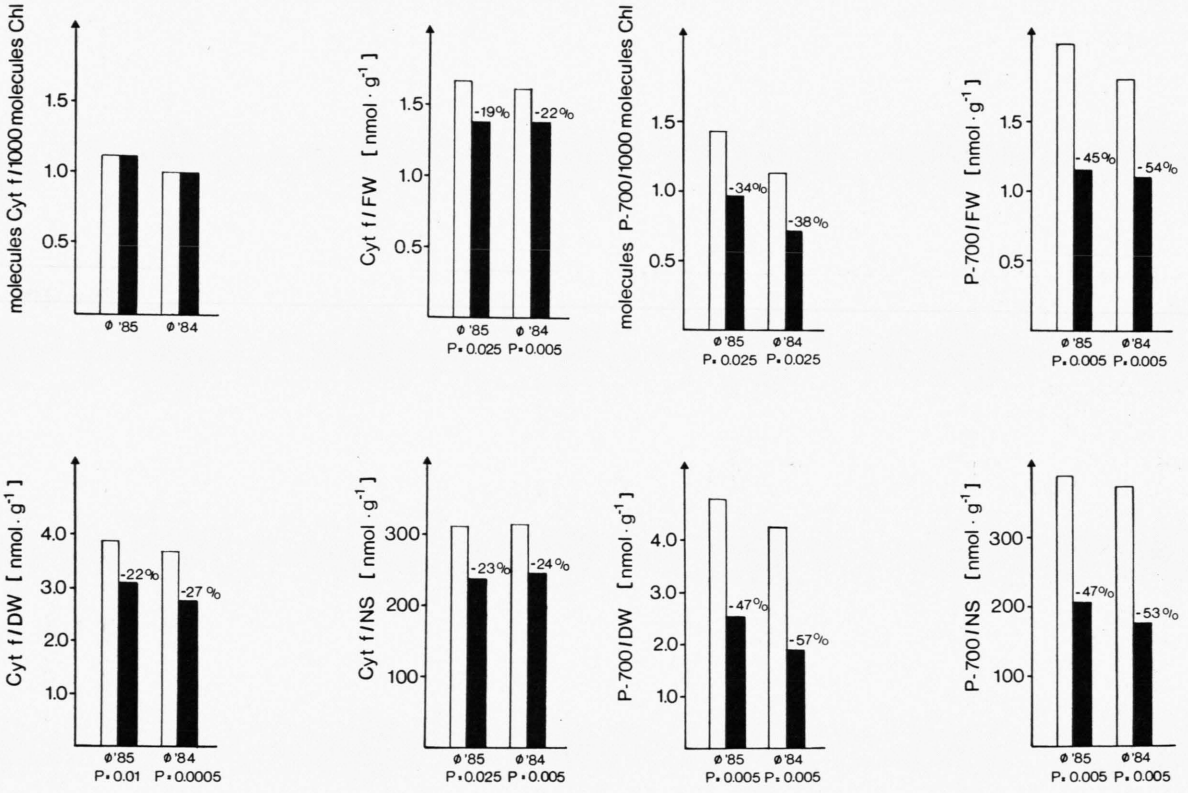

Fig. 2. Content of the redox-component cytochrome *f* (Cyt *f*) in relation to different parameters (Chl = chlorophyll *a* + *b*, FW = fresh weight, DW = dry weight, NS = needle surface area) at the Hunsrück location. Ø = mean value of four tree pairs over the investigation period 1986, '85 = the 1985 needle generation, '84 = the 1984 needle generation, □ = apparently healthy or only slightly damaged partners, ■ = more severely damaged partners, P = statistical probability (Student's T-test).

Fig. 3. Content of the redox-component P-700 in relation to different parameters (Chl = chlorophyll *a* + *b*, FW = fresh weight, DW = dry weight, NS = needle surface area) at the Hunsrück location. Ø = mean value of four tree pairs over the investigation period 1986, '85 = the 1985 needle generation, '84 = the 1984 needle generation, □ = apparently healthy or only slightly damaged partners, ■ = more severely damaged partners.

tween the content of P-700 in trees with symptoms of damage and less damaged trees was even stronger when other reference parameters were used, and it was especially drastic in relation to the dry weight (Fig. 3). This reduction was more marked in the 1984 needle generation (57%) than in the 1985 needle generation (47%). Fig. 4 shows the content of P-700 in relation to the dry weight on the different harvest dates.

Westerwald location

All spruce trees of the Westerwald location were apparently healthy.

The amount of cytochrome *f* and P-700 in the needles of the trees at the Westerwald location was about the same as that determined at the Hunsrück location for the apparently healthy or only slightly damaged spruce trees (Table I). In contrast, the

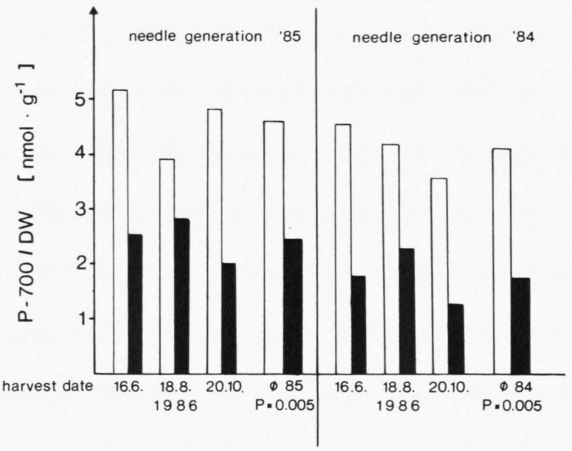

Fig. 4. Content of the redox-component P-700 in relation to the dry weight at the Hunsrück location. Mean values of four tree pairs on different harvest dates. ∅ = mean value over the investigation period 1986, needle generation 1984 and 1985, □ = apparently healthy or only slightly damaged, ■ = more severely damaged.

Table I. Contents of the redox-components Q_B-protein, cytochrome *f* (Cyt *f*), and P-700 in relation to different parameters (Chl = chlorophyll *a*+*b*, FW = fresh weight, DW = dry weight, NS = needle surface area). ∅ = mean value of four tree pairs from the Hunsrück location and five 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 partners, tree group 2 = more severely damaged partners.

Redox-component	Tree group	Hunsrück ∅ '85	Hunsrück ∅ '84	Westerwald ∅ '85	Westerwald ∅ '84
Molecules Q_B-protein/ 1000 molecules Chl	1	2.36	2.39	1.90	1.61
	2	1.77	1.86		
Q_B-protein/FW [nmol g^{-1}]	1	3.56	4.01	2.73	2.50
	2	2.25	2.59		
Q_B-protein/DW [nmol g^{-1}]	1	8.14	8.85	6.11	5.37
	2	4.93	5.52		
Q_B-protein/NS [nmol m^{-2}]	1	661	782	398	458
	2	397	476		
Molecules Cyt *f*/ 1000 molecules Chl	1	1.13	0.99	1.13	1.02
	2	1.13	1.00		
Cyt *f*/FW [nmol g^{-1}]	1	1.68	1.63	1.59	1.60
	2	1.37	1.28		
Cyt *f*/DW [nmol g^{-1}]	1	3.80	3.66	3.49	3.34
	2	2.97	2.67		
Cyt *f*/NS [nmol m^{-2}]	1	308	316	299	337
	2	236	241		
Molecules P-700/ 1000 molecules Chl	1	1.40	1.11	1.40	1.04
	2	0.93	0.69		
P-700/FW [nmol g^{-1}]	1	2.06	1.86	2.10	1.67
	2	1.14	0.85		
P-700/DW [nmol g^{-1}]	1	4.67	4.13	4.80	3.62
	2	2.46	1.78		
P-700/NS [nmol m^{-2}]	1	378	362	347	313
	2	200	170		

amount of Q_B-protein in the needles of the apparently healthy trees at the Westerwald location was comparable rather with that in the needles of the more severely damaged trees at the Hunsrück location.

Discussion

A remarkable decrease in the content of the Q_B-protein between the apparently healthy and the damaged trees was found on the last harvest date, the 20th of October. This appears to be of particular interest in connection with the prevailing climatic conditions in September and October 1986. Before the last harvest date there had been a period (31 days) of sunny days without any rainfall. As it is known, the photosynthetic capacity of an organism can be severely reduced following exposure to light intensities in excess of that required to saturate photosynthesis. This phenomenon is referred to as photoinhibition. The primary site of the light-induced loss of photosynthetic activity appears to be at the position of the Q_B-protein. Photoinhibition results when the rate of degradation of the Q_B-protein exceeds the rate of *de novo* synthesis [8]. Activated oxygen species may be involved in the genesis of the photodamage to the thylakoid membranes, because the damage can be minimized by the addition of antioxidants (*cf.* [9]). Thus, the lowering of the amount of Q_B-protein, especially on the last harvest date, may indicate an increased photosensitivity of the thylakoid membranes [10, 11].

In relation to the fresh weight, dry weight, and needle surface area, a significant decrease of cytochrome *f*, between 20–30%, was found in the severely damaged trees. On the other hand, there was no difference in the content of cytochrome *f* in relation to chlorophyll. This is a reflection of the parallel decrease of the chlorophyll content in the thylakoid membranes [1]. The amount of cytochrome *f* in the needles correlated well with the damage difference between the two partners of a tree pair. Only in extremely damaged trees was the amount of cytochrome *f* per chlorophyll reduced as well. Cytochrome *f* is an integral component of the cytochrome b_6/f-complex, which mediates the electron transport between the two photosystems. The concentration of cytochrome *f* is taken as a representative of this complex. The reoxidation of reduced plastoquinone is the limiting step in the photosynthetic electron transport pathway. Therefore, the concentration of the cytochrome b_6/f-complex limits the electron transport capacity in the thylakoid membrane [12, 14]. The loss of cytochrome *f* corresponds to a real loss of photosynthetic electron transport capacity under light saturation conditions.

In the needles of the more damaged trees a drastic decrease in the concentration of P-700 is manifested. Even in relation to chlorophyll, an unequivocal reduction was found in each individual pair comparison, as well as on annual average of all tree pairs. In damaged spruce trees the concentration of P-700 is even lower than that of cytochrome *f* (Fig. 5). This indicates that P-700 is an early and sensitive indicator of damage to the photosynthetic membranes of spruce trees. In the apparently healthy spruce trees the ratio of Chl/P-700 is approximately two times higher than that usually observed in herbaceous angiosperm plants [15–18]. This agrees with results of Alberte *et al.* [19] who found for various conifers Chl/P-700 ratios 1.6 to 3.8 times larger than that typically found in crop plants. A high sensitivity of P-700 under winter-stress-conditions was found by Öquist *et al.* [20], Martin *et al.* [21] and Öquist and Martin [22].

The investigations on the photosynthetic apparatus of spruce trees show that all components and

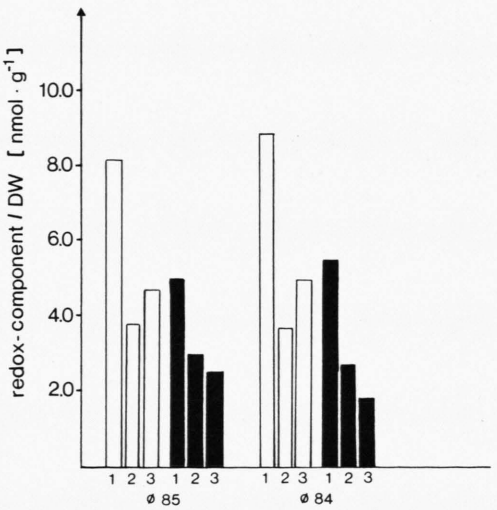

Fig. 5. Comparison of the redox-components Q_B-protein (1), cytochrome *f* (2), and P-700 (3). ∅ = mean value over the investigation period 1986 of four and three tree pairs, respectively, '85 = the 1985 needle generation, '84 = the 1984 needle generation, □ = apparently healthy or only slightly damaged partners, ■ = more severely damaged partners.

processes investigated are impaired by damage (see also [1, 23]). As shown by Dietz *et al.* [1], the loss of chlorophyll points to the fact that the damage extends to the pigment-protein-complexes. The function of the water splitting enzyme system on the oxidizing side of PS II and the PS II-catalyzed electron transport were obviously impaired, too [1]. In addition, the content of Q_B-protein, which is located on the reducing side of PS II, was distinctly reduced in the damaged trees. Even the reaction center of PS I (P-700), which is known as a particularly stable component of the thylakoid membrane in angiosperms, was drastically decreased by more severe damage to the spruce trees. Finally, the concentration of cytochrome *f* is reduced in damaged spruce trees as well.

In parallel to our physiological investigations, electron microscopic studies of the spruce needles were performed. They show that early damage occurs at the cellular membranes. This membrane damage already occurs in those needles of damaged trees which were visibly green. Particularly the thylakoid membranes in the chloroplasts of the mesophyll cells are sites of early injurious effects. Yellowing of the needles on the top side of the twigs indicates an increased photosensitivity [10, 24].

At the Hunsrück location high concentrations of ozone were measured during the vegetation period 1986 [1]. Therefore, it appears that air pollutants – ozone probably in combination with further photooxidants, nitrogen oxides, sulphur dioxide, and different organic compounds – are directly involved in the early damage to the biomembranes. Activated oxygen species appear to play a major role in the genesis of the membrane damage. There is *e.g.* an increase in the activity of different scavenging systems for active oxygen. Early damage was also found on the organelles and cytoplasmic membranes in the cells of the vascular bundle sheath (endodermis). Finally, collapse of the sieve cells occurs even at an early stage, which leads to an impairment of the transport of assimilate.

According to recent studies, novel forest decline seems to be a complex disease. Particularly, the combined action of different pollutants [25] in combination with extreme climatic events, such as drought (*cf.* [26]), and soil conditions (*e.g.* the supply with nutrients [27]), are noticeable. The combined action of anthropogenic pollutants and cellular noxae, as well as of natural stressors (for example water stress, Mg-deficiency), can disturb the equilibrium between the processes of membrane degradation and repair syntheses, leading to irreparable cell damage, although a diverse system of protective mechanisms is present in the cells [28]. The altered membrane structure might for instance impair the compartmentalization in cells, the transport processes, photosynthesis, and the function of stomata and thus lead to loss of function not only of the cells, but also of the needles, and lead ultimately to the death of the whole tree (*cf.* [11, 29]).

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[1] B. Dietz, I. Moors, U. Flammersfeld, W. Rühle, and A. Wild, Biosciences **43c,** 581–588 (1988).
[2] G. Jung and A. Wild, J. Phytopath, in press (1988).
[3] W. Tischer and H. Strotmann, Biochim. Biophys. Acta **460,** 113–125 (1977).
[4] D. S. Bendall, H. E. Davenport, and R. Hill, in: Methods in Enzymology (A. San Pietro, ed.), **Vol. 23,** pp. 327–344, Academic Press, New York 1971.
[5] T. V. Marsho and B. Kok, in: Methods in Enzymology (A. San Pietro, ed.), **Vol. 23,** pp. 515–522, Academic Press, New York, 1971.
[6] A. Wild, B. Ke, and E. R. Shaw, Zeitschrift f. Pflanzenphys. **69,** 344–350 (1973).
[7] T. Hiyama and B. Ke, Biochim. Biophys. Acta **267,** 160–171 (1972).
[8] D. J. Kyle and I. Ohad, in: Photosynthesis III, Encyclopedia of Plant Physiology, New Series (L. A. Staehelin and C. J. Arntzen, eds.), **Vol. 19,** pp. 468–475, Springer Verlag, Berlin, Heidelberg, New York, Tokyo 1986.
[9] K. Asada and M. Takahashi, in: Photoinhibition, Topics in Photosynthesis (D. J. Kyle, C. B. Osmond, and C. J. Arntzen, eds.), **Vol. 9,** pp. 227–287, Elsevier, Amsterdam, New York, Oxford 1987.
[10] A. Wild, in: Klima und Witterung in Zusammenhang mit den neuartigen Waldschäden (Projektgruppe Bayern zur Erforschung der Wirkung von Umweltschadstoffen, ed.), GSF-Bericht **10,** pp. 100–113, München/Neuherberg 1987.
[11] A. Wild, Allg. Forst Zeitschr. **27/28/29,** 734–737 (1987).
[12] A. Wild, E. Nies, and J. Ewen, in: Advances in Photosynthesis Research (C. Sybesma, ed.), **Vol. 4,** pp. 333–336, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague, Boston, Lancaster 1984.
[13] C. Wilhelm and A. Wild, J. Plant Physiol. **115,** 115–135 (1984).
[14] W. Rühle and A. Wild, Naturwissenschaften **72,** 10–16 (1985).
[15] A. Wild, Ber. Deutsch. Bot. Ges. **92,** 341–364 (1979).
[16] O. Björkman, in: Physiological Plant Ecology I, Encyclopedia of Plant Physiology, New Series (O. L. Lange, P. S. Nobel, C. B. Osmond, and H. Ziegler, eds.), **Vol. 12A,** pp. 76–95, Springer Verlag, Berlin, Heidelberg, New York 1981.
[17] A. Wild, M. Höpfner, W. Rühle, and M. Richter, Z. Naturforsch. **41c,** 597–603 (1986).
[18] A. Wild, M. Höpfner, W. Rühle, and M. Richter, in: Progress in Photosynthesis Research (J. Biggins, ed.), **Vol. 2,** pp. 363–366, Martinus Nijhoff Publishers, Dordrecht, Boston, Lancaster 1987.
[19] R. S. Alberte, P. R. McClure, and J. P. Thornber, Plant Physiol. **58,** 341–344 (1976).
[20] G. Öquist, O. Martensson, B. Martin, and G. Malmberg, Physiol. Plant. **44,** 187–192 (1978).
[21] B. Martin, O. Martensson, and G. Öquist, Physiol. Plant **43,** 297–305 (1978).
[22] G. Öquist and B. Martin, Physiol. Plant. **48,** 33–38 (1980).
[23] A. Wild, B. Dietz, U. Flammersfeld, and I. Moors, in: Effects of Air Pollution on Terrestrial and Aquatic Ecosystems, Proceedings Symposium from 18th to 22nd May in Grenoble, Commission of the European Communities, Brussels 1988.
[24] A. Wild, Naturwiss. Rundschau **41,** 93–96 (1988).
[25] V. C. Runeckles, in: Air Pollution and Plant Life (M. Treshow, ed.), pp. 239–258, John Wiley & Sons, Chichester, New York, Brisbane, Toronto, Singapure 1984.
[26] T. Rosenkranz, C. Bareis, J. Bode, and A. Wild, J. Plant Physiol., in press.
[27] W. Forschner and A. Wild, in: Effects of Air Pollution on Terrestrial and Aquatic Ecosystems, Proceedings Symposium from 18th to 22nd May in Grenoble, Commission of the European Communities, Brussels 1988.
[28] A. D. Dodge, in: INPAC Pesticide Chemistry (J. Miyamoto, ed.), pp. 59–66, Pergamon Press, Oxford, New York, Toronto, Sydney, Paris, Frankfurt 1983.
[29] A. Wild and G. Hasemann, Forschungsendbericht 10607046/16, Umweltbundesamt Berlin 1986.