Changes in Levels of α-Tocopherol and Ascorbate in Spruce Needles at Three Low Mountain Sites Exposed to Mg^{2+}-Deficiency and Ozone

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The main objective of this study was the comparison of changes in levels of α-tocopherol and ascorbate in needles of spruce trees with various degrees of damage at three low mountain sites.

The ascorbate content in needles of spruce trees with various degrees of damage differs in the course of seasons as well as in the absolute level. The antioxidant status was affected mainly during summer. The content of ascorbate in needles of damaged trees was significantly increased compared to that of undamaged trees. Despite seasonal and daily fluctuations, the level of ascorbate seems to be a good indicator for the degree of damage in the case of symptoms described as montane yellowing. Together with an increasing content of α-tocopherol per chlorophyll, a rise of ascorbate content indicates enhanced oxidative stress in the needles of damaged trees, particularly in summer. Asc/αToc ratios were increased in needles of damaged trees. At the studied sites enhanced oxidative stress could be caused by the combined action of Mg^{2+}-deficiency, high ozone concentrations and high-light intensity, all inducing an increased production of radicals in combination with a reduced photosynthetic capacity.

Introduction

Declining natural forest stands are usually manifested by visual symptoms of foliage discolouration, thinning of the crowns, and excessive mortality. Needle chlorosis and premature needle shedding are the most commonly used quantitative indications of decline and its severity (Oren *et al.*, 1989; Hanisch and Kilz, 1990). These nonspecific indicators of a "stress-afflicted" tree can result from many biotic or abiotic factors (Ashmore, 1988). Especially unfavourable soil conditions together with different gaseous air pollutants like sulfur dioxide or photooxidants, with ozone as the main component, are thought to be involved in the decline of coniferous trees (Bosch *et al.*, 1983; Osswald and Elstner, 1987; Wild *et al.*, 1990). Studies on spruce trees at different natural habitats that showed the symptoms of montane yellowing, revealed that cellular membranes, especially the thylakoid membranes, are early sites of damage (Dietz *et al.*, 1988; Jung and Wild, 1988; Wild, 1988; Wild *et al.*, 1988; Flammersfeld and Wild, 1992; Wild *et al.*, 1993).

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Cellular antioxidants such as the synergistically acting α-tocopherol and ascorbate, or glutathione, are able to prevent an uncontrolled production of free radicals in cells, provided that optimal concentrations of antioxidants are available (Finckh and Kunert, 1985; Kunert, 1985). α-Tocopherol acts as a chain-breaking antioxidant in pollutant-initiated thylakoid lipid oxidation and can be regenerated by reduced glutathione and ascorbate (Fryer, 1992). In addition, ascorbate and glutathione are involved in the "Halliwell-Beck cycle", the function of which is the elimination of hydrogen peroxide in chloroplasts (Halliwell, 1981).

A multitude of exposure studies are dealing with dose-response relationships concerning the effect of sulfur dioxide and ozone on levels of antioxidants in needles of coniferous trees. The results regarding the ascorbate levels are inconsistent. After ozone and/or sulfur dioxide treatment content of ascorbate in needles has been shown to increase (Barnes, 1972; Mehlhorn *et al.*, 1986; Castillo and Greppin, 1988; Bermadinger *et al.*, 1990; Manderscheid *et al.*, 1991), to decrease (Bermadinger *et al.*, 1990; Messener and Berndt, 1990; Chen *et al.*, 1991), or no response has been found (Senger *et al.*, 1986; Hausladen *et al.*, 1990; Chen *et al.*, 1991). Only a few studies are concerned with the relationship between resistance to pollutant stress and the

antioxidant α-tocopherol of coniferous trees (Mehlhorn *et al.*, 1986; Kunert, 1987; Fryer, 1992).

The diversity of results complicates the transfer to the conditions at natural sites. Additionally, at a natural site there are multitude of factors which interact, *e.g.* climatic and soil conditions, air pollution, genetic diversity, nutrient and water supply, and the age structure of the population. However, deviations from well-known physiological patterns can be interpreted as expressions of chronic or periodic stress.

Based on these considerations, the main objectives of these studies are: (1) a comparison of α-tocopherol and ascorbate levels in needles of spruce trees with various degrees of damage at different low mountain sites; (2) a detection of a possible relationship between changes in antioxidant status and climatic, soil and air pollutant conditions as the natural sites; and (3) an examination of the studied parameters in regard to their suitability as indicators for damage.

Materials and Methods

Description of the sites

The studies presented here were carried out on needles of spruce trees (*Picea abies* [L.] *Karst.*) during the vegetation periods 1988 until 1991 at three habitats in Germany, two in Rheinland-Pfalz and another one in Baden-Württemberg. The selection of the trees was carried out according to the forest-damage-inquiry-criterias (Waldschadenserhebung, 1984–1993; Bundesministerium für Ernährung, Landwirtschaft und Forsten, Bonn).

Hattgenstein site

This site is located in the Hunsrück mountains at about 660 m above sea level. 1988–1989 the location was characterized by high ozone levels (monthly average of 70–110 μg m^{-3} during summer) while SO_2 and NO_x played minor roles (ZIMEN, 1985–1993). Typical features of the soil are its low cation-exchange capacity and the low nutrient supply.

The research was performed on 10 trees between 25 and 30 years old: 5 of this trees with damage class 0 and 1, and 5 trees with damage class 2. The damaged trees showed partial needle loss and yellowing of the upper surface of the older needles.

Wallmerod site

The spruce plantation is located on a plateau about 495 m above sea level at the Hoher Westerwald. Climatic and emission data are similar to those at Hattgenstein site. However, soil acidification is low (pH 4.3–4.5) and the site is well supplied with nutrients.

The five tested 20 years old spruce were apparently healthy (damage class 0) and served as an undamaged reference to the trees at Hattgenstein site.

Freudenstadt site

The site under research near Freudenstadt in the Northern Black Forest is located 820–830 m above sea level. The emissions of SO_2 and NO_2 were relatively low, while the ozone concentration was high with average monthly levels of 100–150 μg/m^3 during the summer. The test areas are located on permeable brown earth; Mg^{2+}-deficiency is existing (v. Wilpert and Hildebrand, 1992).

Two adjoining plantations were selected for the studies: (1) 6 spruce trees out of an undamaged area of 40–50 years old trees (damage class 0); (2) 6 spruce trees out of a clearly damaged area of 40–50 years old trees (damage class 2 and 3). The damaged trees showed symptoms of "montane yellowing", *i.e.* with ageing of the needles a significant yellowing on the upper needle surface can be observed. Mixed samples of needles from each group of trees were examined.

Further detailed descriptions of the sites are given in Wild *et al.* (1993) and KfK-PEF (1993).

Materials

The studies were performed on one-year-old (bud break in the previous-year) and two-year-old needles from the sixth to the eighth whorl. Right after harvesting the needles were removed from the twigs by stirring them in liquid nitrogen, then they were stored in plastic vials at −80 °C. Harvest always took place between 11 a.m. and 1 p.m. in order to avoid that the results are affected by diurnal fluctuations.

Methods

Total ascorbate

Extraction: 1 g of frozen needles was homogenized (cutter rod 18 N, Ultra Turrax) at 4 °C for 30 s

in 10 ml of 0.1 M sodium acetate buffer, pH 2.8. The homogenate was centrifuged for 25 min at 2 °C and 27000 × *g*. The supernatant was sucked through a cellulose nitrate filter (Sartorius, pore size 0.2 µm) and stored at −80 °C in plastic vials. Duplicates were run for each extract.

Determination of total ascorbate by HPLC: Total ascorbate (ascorbate + dehydroascorbate) was determined through a reduction of dehydroascorbate to ascorbate by dithiothreitol (DTT). Respectively, 100 µl of the filtered homogenate were added to a mixture containing 800 µl of 0.1 M K-phosphate buffer, pH 7.0, and 100 µl of 0.05 M DTT. After vortex-mixing, samples were incubated 20 min at room temperature and then 40 min on ice. Extract stability was given for 3 h after incubation. For the chromatographic separation a WATERS-HPLC system controlled by an IBM AT personal computer was used. Ascorbate was separated at room temperature on a reversed-phase-C 18-column (250 × 4.6 mm, Hypersil ODS-5U; CS Chromatography Service Langewehe, Germany) using a solvent of 0.1 M sodium acetate buffer, pH 5.0, with an isocratic flow rate of 1.2 ml min^{-1}. The eluates were monitored by an UV-VIS detector at 264 nm.

For comparison the ascorbate content was also determined according to Law *et al.* (1983). This assay is a modification of the method of Okamura (1980) and is based on the reduction of Fe^{3+} to Fe^{2+} by ascorbate in acidic solution. The Fe^{2+} then forms complexes with bipyridyl, giving a yellow-orange colour that absorbs at 525 nm.

In each case of measurement the content of total ascorbate was calculated from a standard curve.

α-Tocopherol

Extraction: 1 g of frozen needles was homogenized (cutter rod 18N, Ultra Turrax) at 4 °C for 30 s in 10 ml ethanol containing 2% (w/v) soluble polyvinylpyrrolidone 25 and 20% (w/v) Na_2SO_4. The homogenate was centrifuged for 10 min at 2 °C and 3350 × *g*. The supernatant was sucked through a cellulose nitrate filter (Sartorius, pore size 0.2 µm), diluted 1:5 in ethanol, and stored at −80 °C in plastic vials. Duplicates were run for each extract.

Determination of α-tocopherol by HPLC: For the chromatographic separation the same HPLC system as described above was used. α-Tocopherol was separated at room temperature on a reversed-phase-C 18-column (250 × 4.6 mm, Sperisorb^R ODS-5; Bischoff Leonberg, Germany) using a solvent A 95% (v/v) methanol + 5% water and B 95% (v/v) methanol + 5% ethylacetate with a flow-rate of 1.2 ml min^{-1}. The gradient elution started at 0% A and 100% B increased up to 90% A within 1 min and up to 100% A in 15 min. The separation was finished after 20 min with 0% A and 100% B. The eluates were monitored by an UV-VIS detector at 292 nm.

The amount of α-tocopherol was calculated from a standard curve.

Chlorophyll a + b

Chlorophyll was extracted from needles with dimethyl sulfoxide (15 h, 65 °C) and measured spectrophotometrically according to Harborne (1973).

Statistics

Student's t-tests for independent random samples were used for statistical analysis. Before running the t-test variance homogeneity was checked by a f-test (Significance levels: $*\alpha \leq 0.05$; $**\alpha \leq 0.01$; $***\alpha \leq 0.001$).

Results

Ascorbate

The determination of the content of ascorbate by HPLC technique as well as by colorimetric tests led to similar qualitative and quantitative results. No matrix effect has been found; recovery rates of 92% for determination by HPLC and 89% for determination by the colorimetric assay were comparable. Therefore, it is possible to apply the methods alternatively.

The dry weight of the needles served as the main basic parameter. It was found out that the ratio between fresh weight and dry weight was not significantly different neither between needle generations nor between the damage categories (data not shown).

Mixed samples containing needles from 5 (Wallmerod) and 6 (Freudenstadt) trees were taken. Table I illustrates that the results gained from the investigation of the mixed samples at Freudenstadt site are representative for the chosen single

Table I. Content of ascorbate of needles from individual spruce trees which were constituents of the mixed samples at the Freudenstadt site; harvest date: 28th Aug. 1991. Significance level of the difference between undamaged and damaged trees: ***$\alpha < 0.001$.

Tree no.	1	2	3	4	5	6	∅ ***	mixed sample
				[mg/g dw]				
Undamaged	4.5	2.9	3.7	2.9	3.0	3.3	3.4 ± 0.6	3.48
Damaged	5.8	5.9	5.8	5.2	5.1	4.3	5.4 ± 0.6	5.46

trees. The mean value of the measurements of individual trees reflects the results of the mixed sample of the sampling date. The difference between the ascorbate content of undamaged and damaged trees is statistically significant ($\alpha \leq = 0.001$).

In Fig. 1 A, B the seasonal changes of the ascorbate content in one-year-old and two-year-old needles at the Hattgenstein and Wallmerod sites are shown. Between the needle generations, there was no significant difference in the content of ascorbate. One-year-old needles as well as two-year-old needles of undamaged trees showed marked seasonal fluctuations in the ascorbate level. In each case the highest values were measured in winter and early spring. During the summer months the amounts of ascorbate decreased. In contrast to that the ascorbate content in needles of damaged trees increased continuously during the whole year. Only in 1988 a weak seasonal rhythm could be detected in two-year-old needles that could be compared to that of undamaged trees. Studies on the older trees at the Freudenstadt site (Fig. 2) led only partly to similar results. The seasonal changes in ascorbate content of apparently undamaged trees showed the typical feature with minimal values in summer. In 1990 there could be observed the same seasonal rhythm for the severely damaged trees. In contrast to that results that did not agree

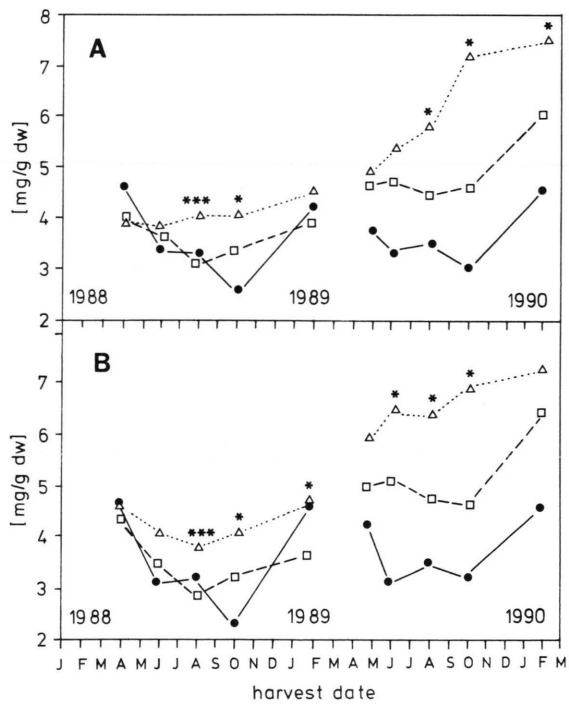

Fig. 1 A, B. Content of ascorbate per dry weight at the Hattgenstein site (□ = undamaged, △ = damaged trees) and the Wallmerod site (● = undamaged reference trees); test period 1988–1990. (A) One-year-old needles; (B) two-year-old needles; percentage standard deviation of the presented mean values, n = 5: (A) 17% for undamaged and 13% for damaged trees; (B) 17% for undamaged and damaged trees; significance levels of the difference between damaged and undamaged trees at the Hattgenstein site: *$\alpha \leq 0.05$, ***$\alpha \leq 0.001$.

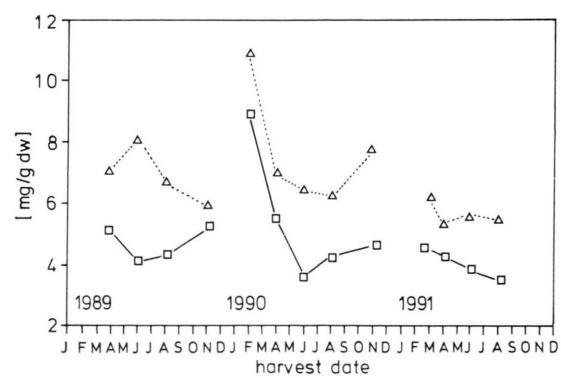

Fig. 2. Content of ascorbate per dry weight in one-year-old needles at the Freudenstadt site; test period 1989–1991; percentage standard deviation ≤ 5% (□ = undamaged trees, △ = damaged trees).

with the above mentioned findings where obtained in 1989 and 1991: there was no reference to a regular seasonal rhythm.

A comparison of the ascorbate content in needles of trees with various degrees of damage led to the following results: at the Hattgenstein site the amounts of ascorbate in needles of damaged trees (Fig. 1 A, B) were significantly increased compared to the healthy trees. In 1988 ascorbate contents of the healthy reference trees at the Wallmerod site were in the range of those in needles of undamaged trees at the Hattgenstein site, in 1989 the measured values were clearly smaller than at the Hattgenstein site. The difference between undamaged and damaged trees becomes still more evident at the Freudenstadt site (Fig. 2). At each harvest date the content of ascorbate in needles of damaged trees was significantly increased compared to the needles of undamaged trees. By far the highest ascorbate values have been measured in needles of severely damaged spruce trees at the Freudenstadt site.

In the case of montane yellowing it is quite possible to take the chlorophyll $a+b$ content as a measure for existing damage. In Fig. 3 the content of chlorophyll has been plotted in relation to the ascorbate content; as a result we get a relatively good correlation ($r_{0.001} = -0.6382$). The ascorbate content of the needles increased continuously with decreasing amounts of chlorophyll independent of age and location of the studied trees.

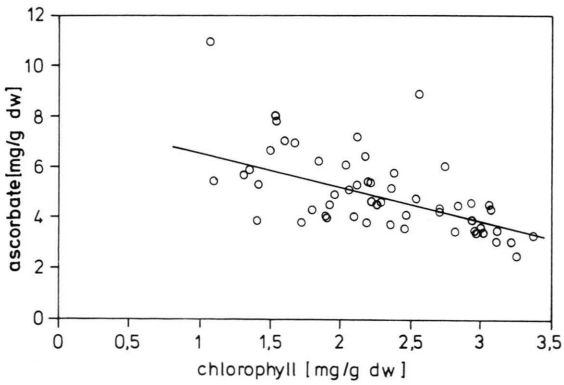

Fig. 3. Correlation between content of ascorbate and content of chlorophyll $a+b$ per dry weight in one-year-old needles at the Hattgenstein and the Freudenstadt site during the test period 1988–1990; $r_{0.01} = -0.638$.

α-Tocopherol

The content of α-tocopherol related to dry weight increased constantly from April at all sites studied (Fig. 4A, B and Fig. 5A). Only at the Wallmerod site in February an additional increase could be noticed. As a result, the two-year-old needles contained higher amounts of α-tocopherol than the one-year-old needles (Fig. 4A, B).

A comparison between trees with various degrees of damage led to the following results: The contents of α-tocopherol per dry weight of both damaged and undamaged trees at Hattgenstein site did not differ significantly from each other (Fig. 4A, B). In contrast, in needles of the severely damaged trees at the Freudenstadt site pronounced higher amounts of α-tocopherol were measured compared to the undamaged trees

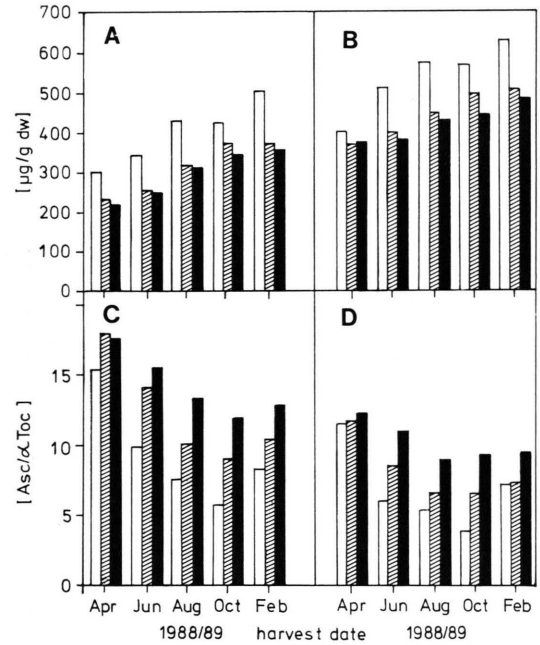

Fig. 4A–D. Content of α-tocopherol and Asc/αToc ratios at the Hattgenstein (▨ = undamaged, ■ = damaged trees) and the Wallmerod site (□ = undamaged reference trees); test period 1988/89. (A) Content of α-tocopherol per dry weight in one-year-old needles, and (B) in two-year-old needles. (C) Asc/αToc ratio in one-year-old needles, and (D) in two-year-old needles; percentage standard deviation of the presented mean values, n = 5: (A) 9.7% for undamaged and 8.8% for damaged trees; (B) 8.5% for undamaged and 10.1% for damaged trees.

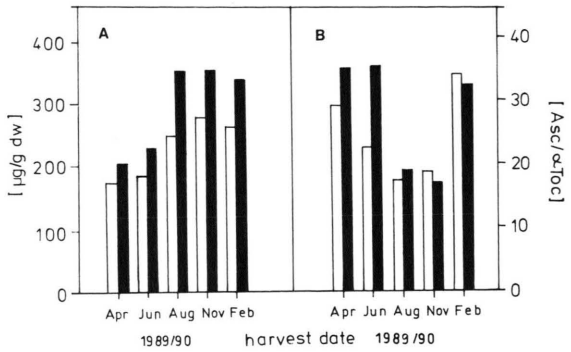

Fig. 5A, B. (A) Content of α-tocopherol per dry weight, and (B) Asc/αToc ratio in one-year-old needles at the Freudenstadt site; test period 1989/90; percentage standard deviation ≤ 5% (□ = undamaged trees, ■ = damaged trees).

(Fig. 5A). The highest levels of α-tocopherol were obtained in needles of trees at the reference site in Wallmerod (Fig. 4). The relation of α-tocopherol to the content of chlorophyll was less differentiated (Table II). At the Hattgenstein site as well as the Freudenstadt site the needles of damaged trees contained significantly higher contents of α-tocopherol ($\alpha \leq 0.001$ and $\alpha \leq 0.01$, respectively). The levels of α-tocopherol of undamaged trees from all studied sites did not differ significantly from each other. The highest amounts of α-tocopherol per chlorophyll were determined in needles classified in the highest damage category.

Ascorbate/α-Tocopherol ratio (Asc/αToc ratio)

The Asc/αToc ratio decreased with needle age (Fig. 4C, D). In each case the highest ratios were calculated in April (Fig. 4C, D and Fig. 5B). Seasonal changes were characterized by a clearly visible decrease of the ratio in summer and an increase during winter months. At the Hattgenstein site, damaged trees showed a less significant decrease during summer. Furthermore, the needles of damaged trees at Hattgenstein site showed higher Asc/αToc ratios (annual ∅ 14.6 for one-year-old needles and 10.5 for two-year-old needles) than the needles of the corresponding healthy trees (annual ∅ 12.6 for one-year-old needles and 8.4 for two-year-old needles). The smallest ratios were found in needles of healthy trees at Wallmerod site (annual ∅ 9.7 for one-year-old needles and 7.0 for two-year-old needles). In the one-year-old needles of damaged trees at the Freudenstadt site (Fig. 5B) only in April and June a marked increase of Asc/αToc ratio could be observed. Altogether, at Freudenstadt site the Asc/αToc ratios were higher (annual ∅ 24 for undamaged and 28 for damaged trees) than at the other studied sites.

Discussion

At the different sites, the known seasonal rhythm of the ascorbate content with a relatively high level in winter and spring and a low level dur-

Table II. Content of α-tocopherol in one-year-old (bud break in the previous-year) needles of undamaged and damaged spruce trees at the Wallmerod, Hattgenstein, and Freudenstadt sites during the test period 1988–1990; significance levels: $**\alpha \leq 0.01$, $***\alpha \leq 0.001$.

Site:	Wallmerod	Hattgenstein		Freudenstadt	
Damage categorie:	Undamaged 0	Undamaged **0–1**	Damaged **2**	Undamaged 0–**1**	Damaged 2–**3**
Sampling		[µg/mg Chl a + b]			
Apr 88	132	120 ± 18	163 ± 40		
Jun 88	111	103 ± 10	144 ± 15		
Aug 88	125	100 ± 10	168 ± 38		
Oct 88	128	123 ± 13	169 ± 36		
Feb 89	182	124 ± 11	191 ± 45		
Apr 89				84	127
Jun 89				74	149
Aug 89				92	236
Nov 89				117	259
Feb 90				100	317
∅	136 ± 27	114 ± 17	167 ± 17 (***)	93 ± 16	218 ± 79 (**)

ing summer (*cf.* Esterbauer *et al.*, 1980; Hausladen *et al.*, 1990; Osswald *et al.*, 1990) has been observed in the needles of undamaged spruce trees. The increase of the ascorbate level in winter seems to be connected with frost hardening and dormancy (Levitt, 1980; Anderson *et al.*, 1992). This can be compared to the increase of the amount of glutathione and the activity of glutathione reductase during the dormancy induction period of conifer needles Esterbauer and Grill, 1978; Schmieden *et al.*, 1993). Each of these antioxidants is involved in a cycle for removal of hydrogen peroxide mainly in illuminated chloroplasts (Halliwell, 1981).

The yearly fluctuation of the ascorbate content of severely damaged spruce trees either differs in several cases from the above mentioned findings or is not strongly developed. This was especially noticeable in 1989. Besides, a significant increase of the ascorbate content was measured in the needles of damaged trees (on each harvest date at the Freudenstadt site; during summer and autumn at the Hattgenstein site). Moreover compared to needles of healthy trees, a significant increase in the content of glutathione and the activity of glutathione reductase was measured in the needles of these damaged trees (Schmieden *et al.*, 1993). All results point to an increase of capacity of the cycle for removal of hydrogen peroxide.

At the sites in question the cause for the increased need of antioxidants can be seen in the heightened concentration of ozone and Mg^{2+}-defenciency in combination with enhanced light intensities. The highest concentrations of ozone have been registered in May and June 1989 (monthly average: 113 µg/m^3 in May and 120 µg/m^3 in June 1989 in contrast to 86 µg/m^3 in May and 77 µg/m^3 in June 1988). At the same time the greatest deviations of seasonal rhythm of the level of ascorbate from normal has been noticed in needles of damaged trees. Besides, Mg^{2+}-deficiency in the tested needles was determined, with the exception of Wallmerod (data not shown). Especially the needles of damaged trees at the Freudenstadt site showed extremely high deficiencies with Mg^{2+}-contents below 0.2 mg/g dry weight which are correlated with needle yellowing (Siefermann-Harms, 1993). Studies on bean leaves have shown that both light intensity and Mg nutritional status control the regulation of O_2^- and H_2O_2 scavenging enzymes in chloroplasts (Cakmak and Marschner, 1992). Thus, it was demonstrated that Mg-deficient bean leaves are highly photosensitive. The chlorosis due to an increase in light intensity has been attributed to Mg-deficiency-induced photooxidation of thylakoid components by photogenerated toxic O_2-species (Marschner and Cakmak, 1989). The results lead to the assumption, that especially during summer months needles of damaged trees are exposed to an increased oxidative stress, caused by the impact of the combined action of Mg^{2+}-deficiency, ozone and high light intensity, all inducing an enhanced production of radicals in combination with a reduced photosynthetic capacity. It is impossible to differentiate between these causal factors at the natural site because of their simultaneous occurrence.

The ascorbate content rose continuously with decreasing contents of chlorophyll. The results are confirmed by studies of Osswald *et al.* (1987; 1990); they found the highest levels of ascorbate in bleached needles and in bleached needle segments. Therefore, the content of ascorbate seems to be a good indicator for the degree of damage in case of montane yellowing.

α-Tocopherol undergoes seasonal changes contrary to the changes found for ascorbate, where the content of α-tocopherol increases from spring on and reaches a steady state level in August. On one hand this seasonal course corresponds to the typical annual course of the content of chlorophyll, indicating a close connection between structural organization and metabolite composition of the spruce chloroplasts (Senser *et al.*, 1975; Wild *et al.*, 1993). On the other hand α-tocopherol has been identified as an endogenous free radical scavenger which slows down the ageing process in plants (Dhindsa *et al.*, 1982; Thompson *et al.*, 1987). In a study conducted on senescent beach leaves and fir needles, the levels of α-tocopherol rose with the increasing age of both systems (Kunert and Ederer, 1985).

The content of α-tocopherol per dry weight was only increased in needles of severely damaged trees at the Freudenstadt site. α-Tocopherol seems to be localized almost solely in chloroplasts (Wise and Naylor, 1987; Fryer, 1992), therefore a relation to chlorophyll content gives the strongest evidence of the effectiveness of α-tocopherol as a protective compound of membranes against free radicals. All needles of damaged spruce trees were provided

with higher amounts of α-tocopherol per chlorophyll in comparison with the undamaged trees. However, an evaluation of this is difficult, because no distinction between functional and storage α-tocopherol is given. It is well known that α-tocopherol is contained in large amounts in the osmiophilic plastoglobuli of chloroplasts (Lichtenthaler *et al.*, 1981). Since plastoglobuli serve as a storage site for excess chloroplast lipids, their prenylquinones do not seem to play a physiological role (Lichtenthaler *et al.*, 1981). In needles of damaged trees chloroplasts with higher amounts of plastoglobuli were found (Jung and Wild, 1988; Wild, 1987). Accordingly, at Freudenstadt site the thylakoidal system in the chloroplasts of needles of damaged trees was reduced (Siefermann-Harms, 1992; Wild *et al.*, 1993). Thus, one cannot suggest that at the Freudenstadt site the obviously enhanced levels of α-tocopherol in needles of severely damaged trees lead to an increased protection of membranes.

The opposite seasonal rhythm of the content of ascorbate and α-tocopherol is expressed by the seasonal changes in Asc/αToc ratio. Healthy trees showed seasonal changes in Asc/αToc ratio corresponding to the rhythm of ascorbate. According to Finckh and Kunert (1985), the antioxidative effectiveness of α-tocopherol depends on the capacity of regeneration by ascorbate and glutathione. Based on this consideration, the above mentioned results on seasonal changes of the levels of antioxidants are highly consistent in case of Asc/αToc ratio. The highest Asc/αToc ratios were calculated during winter until sprouting in spring. Chilling temperatures combined with enhanced light intensities lead to increased lipid peroxidations and radical scavengers will be expected to increase (Schöner and Krause, 1990; Fryer, 1992).

At the Hattgenstein site higher Asc/αToc ratios (between 12 and 18) have been observed in needles of damaged trees than in those of undamaged trees; the same is evident for damaged trees at the Freudenstadt site in April and June (ratio about 35). According to Finckh and Kunert (1985), optimal ratios belong to values in the range of 10–15, higher as well as smaller ratios will lead to increased lipid peroxidation. Asc/αToc ratios that are not whitin the optimal range, however, may not guarantee a maximal protection of membranes from free radicals.

Acknowledgements

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