Biochemical Parameters as Biomarkers for the Early Recognition of Environmental Pollution on Scots Pine Trees. II. The Antioxidative Metabolites Ascorbic Acid, Glutathione, α-Tocopherol and the Enzymes Superoxide Dismutase and Glutathione Reductase

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Z. Naturforsch. **56c,** 767–780 (2001); received April 20/May 23, 2001

Pinus sylvestris L., Sulfur Dioxide, Antioxidants

Field investigations with Scots pine trees (*Pinus sylvestris* L.) were performed in eastern Germany, where ambient SO_2, NO_x and O_3 concentrations differed significantly in 1992–99 at three sites, namely Neuglobsow (yearly mean SO_2 in 1992: 9 µg m^{-3}), Taura (yearly mean SO_2 in 1992: 54 µg m^{-3}) and Rösa (yearly mean SO_2 in 1992: 73 µg m^{-3}). To investigate the effects of SO_2, NO_x and O_3 on antioxidants (superoxide dismutase, ascorbic acid, glutathione, glutathione reductase, α-tocopherol) and pigments including chlorophyll fluorescence as well as visible damage symptoms in the form of needle yellowing and tip necroses, needles of the 1st and 2nd age class from young and mature trees were collected at the sites every October. Eight years after the start of the field study in 1992, the ambient SO_2 concentrations had decreased significantly at Neuglobsow (yearly mean SO_2 in 1999: 4 µg m^{-3}), Taura (yearly mean SO_2 in 1999: 5 µg m^{-3}) and Rösa (yearly mean SO_2 in 1999: 5 µg m^{-3}). NO_x and O_3 differed less at the three sites and showed no temporal variations. Whole needle glutathione continuously decreased, although concentrations were higher in needles of the 1st and 2nd age class from the polluted sites Taura and Rösa than the unpolluted site Neuglobsow. The activities of glutathione reductase exhibited the same site-related differences and temporal variations and were correlated with concentrations of oxidized glutathione (GSSG). In contrast, the activities of the enzyme superoxide dismutase and the concentrations of whole needle ascorbic acid remained unchanged over the period. Only at the end of the investigation period did the concentrations of oxidized ascorbic acid (dehydroascorbate) increase in six-month-old needles at the polluted sites Taura and Rösa. Despite the clear decreases in SO_2, the visible symptoms of needle tip necroses remained unchanged, especially at the polluted sites Taura and Rösa, although the needles contained higher pigment concentrations than needles from the unpolluted sites. The results of measurements with antioxidants as biomarkers for SO_2-mediated stress in pine needles show that the adult Scots pine trees at the polluted sites suffered from greater oxidative stress than the needles from the less polluted site.

Introduction

Sulfur dioxide (SO_2), nitrogen oxide (NO_2) and ozone (O_3) are among the environmental pollutants that have had a lasting impact on forest ecosystems in the lowlands of northeast Germany for many years (Bellmann and Grote, 1998). Their uptake via the assimilation organs of for example Scots pines (*Pinus sylvestris* L.) results in changes to various metabolic processes in the whole plant (Weigel *et al.*, 1989). For example, O_3 attacks the allocation of carbon by inhibiting the saccharose transport from the leaves into the roots (Willenbrink and Schatten, 1993, Wellburn and Wellburn 1994), while parts of SO_2 and NO_2 are taken up by plants and included directly in the S and N metabolism by metabolization to sulfate or sulfide as well as nitrate and further assimilation into organic compounds (Rennenberg *et al.*, 1996). As a result, the nutrition balance of the plant can be influenced by its S/N ratio (Malcolm and Garforth, 1977). On the other hand, NO_2 and O_3 can themselves also be regarded as radicals and are subjected disproportionation reactions in the aqueous phase of the cell wall (Srivastava, 1999) or react with water-soluble and membrane-bound antioxi-

0939–5075/2001/0900–0767 $ 06.00

dants (e.g. ascorbic acid, α-tocopherol) in the apoplast or the symplast, forming nitrous acid as well as superoxide anions, hydroxyl radicals and hydrogen peroxide (Ramge *et al.*, 1993, Ranieri *et al.*, 1999). SO_2 or sulfite can be oxidized to sulfate under the action of light in the chloroplast (Asada and Takahashi, 1987), where oxygen radicals are developed, leading to damage of biomolecules including lipid peroxidation, inactivation of enzymes and subsequent chlorophyll bleaching (Hippeli and Elstner, 1996). Therefore, environmental pollutants are also responsible for triggering forest decline (Smith, 1990). However, it has not yet been clarified whether for instance the tip necroses occurring on needles of Scots pine trees are attributable to the irreversible damage of the cell membrane due to the effects of reactive oxidative species or are based on disturbances in the molar S/N ratio caused by the elevated accumulation of sulfate (Maninen *et al.*, 1998) and/or soluble non-protein-containing compounds (Huhn and Schulz, 1996; Schulz *et al.*, 1999).

Radical reactions may damage plants if the redox steady state of the cell is overloaded towards pro-oxidants (Pell *et al.*, 1997). To defend themselves against oxidative stress, most plants have effective decontamination systems and are equipped with various antioxidants. Ascorbic acid, glutathione and α-tocopherol have each been shown to act as antioxidants in the detoxification of reactive oxygen species in aerobic cells (Foyer, 1993; Hausladen and Alscher, 1993, Hess, 1993). Enzymes such as ascorbate peroxidase, glutathione reductase and superoxide dismutase are involved in the regeneration of ascorbic acid and glutathione (Foyer and Mullineaux, 1994; Allen, 1995). Superoxide dismutases (SOD) are the first line of defence against oxyradical-mediated injury and are involved in several aspects of the ascorbate-glutathione cycle (Foyer and Halliwell, 1976).

The concentration changes of individual antioxidants and/or its oxidized as well as reduced forms in conifers are generally interpreted as a response to oxidative stress and has been thought to provide an early indication of chlorophyll breakdown (Schmieden and Wild, 1994). On the other hand plants react very differently to environmental pollutants and changes in the redox status are not necessarily detectable before severe visible damage symptoms appear on the needles (Tausz *et al.*, 1999). Therefore, the objective of this work was to examine the sensitivity of the above-mentioned antioxidants in pines under drastic changes to the air pollution at different sites over a longer period, and to answer the following questions: 1. Which antioxidants in Scots pine needles react significantly to air pollutants causing oxidation? 2. Do the reactions give an indication of oxidative stress? 3. Is oxidative stress responsible for the damage symptoms to Scots pine needles still to be observed?

Materials and Methods

Field sites and sampling conditions

Pine needles were sampled from adult (60–80-year-old) and young (25–30-year-old) Scots pine stands (*Pinus sylvestris* L.) growing at three differently polluted field sites in eastern Germany. Neuglobsow (13°02′25″E, 53°09′07″N, Brandenburg) is located in a rural area near Lake Stechlin about 70 km north of Berlin. Taura (13°00′28″E, 51°28′25″N, Dahlener Heide, Saxony) is situated 40 km northeast and downwind of Leipzig. Rösa (12°29′15″E, 51°37′03″N, Dübener Heide, Saxony-Anhalt) is located 9 km west of Bitterfeld, to the northeast of the industrial district of Halle.

Air concentrations of SO_2, NO_x and O_3 recorded at meteorological stations located next to the sites are given in Figure 1. The concentrations of the air pollutants reflect the different deposition load of the sites and the temporal variations in the period from 1988 to 1999.

At each field site, 5 test plots were selected in which 15 trees were randomly chosen. Needles were always sampled between 10 and 20 October. One twig was cut from each tree in the sun-crown using a mobile elevator platform. First- and second-generation needles were collected. Mixed samples were prepared by mixing equal amounts of collected needles from 15 branches (i.e. 15 different trees). The mixed needles were immediately deep-frozen in liquid nitrogen and stored at –80 °C until analysis.

Visible symptoms of foliage damage

Visible damage symptoms for the first and second age classes were estimated by assessing of chlorotic and necrotic needle tips (chloroses, ne-

croses) according to the following key: 0 = green need-les, no signs of chlorophyll loss in the needle tips, 0.5 = green needles with partially chlorotic tips, 1 = all needles with chlorotic tips, 1.5 = some needles with 1–5 mm necrotic tips, 2 = all needles with 1–5 mm necrotic tips, 2.5 = some needles with 5–10 mm necrotic tips, 3 = all needles with 5–10 mm necrotic tips. Between a chlorotic or a necrotic needle tip and the undamaged green part of the needle exists a very distinct, dark band. Chloroses and necroses for each class were summed so that mean values (indexes) are based on totals of 15 twigs per test site.

The chlorotic and necrotic needle area (mm^2 $needle^{-1}$) was determined with a scanalyzer using image processing (LemnaTec GmbH, Würselen, Germany). Using morphologic filters the different needle areas were separated from each other, then counted and classified. This classification was used to calculate the average needle area and the distribution of the individual needle areas. The colours of the needle areas were reduced to different colour classes representing for example healthy (green), chlorotic (yellow) and necrotic (brown) needle areas. 500 measurements with 50 needles each randomly selected from the 1st and 2nd age class from 15 branches were taken per test site.

Chlorophyll fluorescence

The chlorophyll fluorescence ratio (F_v/F_m) was determined from the needle surface of 15 min dark-adapted needles using a portable chlorophyll fluorometer (PAM 2000, H. Walz, Effeltrich, Germany). After measuring F_o, F_m was measured by exciting the needle surface with white light in the saturation pulse mode. 20 measurements with 4 needles each randomly selected from the 1st and 2nd age class from 15 branches were taken per test site.

Pulverized needle material

All biochemical analyses were performed with pulverized needle material. Frozen pine needles were pulverized in liquid nitrogen using a micro dismembrator (Braun, Melsungen, Germany).

Total chlorophyll

Pulverized needle material was extracted with 80% acetone. The chlorophyll concentration was calculated using extinction coefficients by Lichtenthaler and Wellburn (1983).

Total glutathione (GSH+GSSG)

The extraction, reduction and derivatization of the thiols with monobromobimane were carried out as previously described by Schupp and Rennenberg (1988). The separation of the thiol derivatives with HPLC took place as published previously (Härtling and Schulz, 1995).

GSSG

Determination of GSSG took place by high-performance liquid chromatography with electrochemical detection (ED 40, Dionex fitted with a gold electrode and a silver-AgCl electrode) using a modified method of Vandeberg and Johnson (1993). After the extraction of the pulverized needle material with 0.001 M EDTA (1 g/5 ml), the solution was subjected to HPLC in a column filled with LiChrospher RP 18 (5 μm). The mobile phase consisted of 0.3% acetonitrile/0.05M NaH_2PO_4, pH 3.0

Total ascorbic acid and simultaneous determination of ascorbic acid (AA) and dehydroascorbate (DHA)

The extraction and determination of total ascorbic acid was carried out as described by Polle *et al.* (1990). Ascorbic acid is completely oxidized to dehydroascorbate with ascorbate oxidase. Dehydroascorbate was derivatized with o-phenyldiamine. The fluorescent derivatives were separated by HPLC.

To determine the pool sizes of AA and DHA in the total ascorbic acid of the needle extracts, the method specified by Tausz *et al.* (1996) was used with the HPLC technique being modified. The stationary phase was LiChrospher 100 RP 18 (250 × 4 mm; particle size 5 μm; Merck) The mobile phase was methanol:water (1:4; v/v) containing 1 mmol hexadecyltrimethylammonium bromide and 0.05% (w/v) sodium dihydrogen phosphate monohydrate. The pH was adjusted to 3.6 by adding 85% (w/v) o-phosphoric acid.

α-Tocopherol

The concentrations of α-tocopherol in the needles were measured using the method published by

Schmieden and Wild (1994). Powdered needles were extracted with ethanol. The extracts were filtered and α-tocopherol was separated by HPLC.

Superoxide dismutase and glutathione reductase

The SOD1 isozyme activity was assayed after the enzyme extraction of acetone dry powder (Schulz, 1981) with 20 mmol l^{-1} KH_2PO_4/EDTA, pH 8.0, and by separating them on a 7% polyacrylamide gel according to Schulz (1983). The activity of glutathione reductase (GR) was determined analogously to the modified method of Wingsle and Hällgren (1993). Needle powder was mixed with potassium phosphate buffer at pH 7.0 containing 4% PVP, 2 mmol EDTA and 5 mmol DTE, homogenized and centrifuged. The crude enzyme extract was used for enzymatic analyses. The assay mixture consisted of 1.35 ml 50 mM potassium phosphate buffer at pH 7.8, 0.2 ml 10 mM GSSG, 0.2 ml 1.7 mM NADPH*Na_4 , 0.2 ml 10 mM EDTA and 0.05 ml enzyme extract. Adding enzyme extract started the reaction. Blank included 1.95 ml phosphate buffer and 0.05 ml crude enzyme. To assess recoveries of SOD and GR activity in pine samples, enzyme dilutions with a known activity of purchasable enzymes were added for the extraction of needle samples. The recoveries amounted to 83±23% (n = 10) for SOD from horseradish (Boehringer) and 89±18% (n = 10) for GR from yeast (Boehringer).

Reference parameter

The dry weights were taken after drying the pulverized needle material for 96 h at 80 °C.

Statistics

Statistical evaluations were completed using SPSS/PC+4.0 (SPSS Inc., Chicago, USA). At least 3 aliquots of individual needle sample were measured. Tables were used to calculate site means (n = 5 test plots ± S. D.). The non-parametric Mann Whitney U-Test was used to test differences between sites pair by pair. Details are given in the table legends.

Results

The results on the reaction of different antioxidants in pine needles on air pollutants causing oxidation at three test areas in the lowlands of northeast Germany presented here follow on from the investigations by Härtling and Schulz (1995) and Schulz *et al.* (1998) to ascertain ecotoxicological effects in forest ecosystems. In addition, we determined the enzyme glutathione reductase as well as the metabolites ascorbic acid and α-tocopherol, including the redox status of ascorbic acid and glutathione (reduced and oxidized forms).

Needle dry weights served exclusively as base parameters, because in contrast to soluble protein concentrations they exhibited neither site-related nor temporal variations (data not shown). Preced-

Table I. Degrees of needle damage symptoms (mean values ± S. D.) at adult Scots pines. Significant differences were determined according to the Mann Whitney U-test. Different small letters (a, b, c) within the rows indicate significant differences between the test sites. Different large letters (A, B) within the columns indicate significant temporal differences at $P < 0.05$ (reference year 1992).

Year	Neuglobsow	Taura	Rösa
Needles of the 2nd age class class		Necroses (Index)	
1992	1.08 ± 0.21 a; A	1.14 ± 0.05 a; A	1.90 ± 0.17 b; A
1993	1.13 ± 0.12 a; A	1.33 ± 0.11 b; B	2.18 ± 0.12 c; B
1994	1.07 ± 0.13 a; A	1.51 ± 0.11 b; B	2.16 ± 0.16 c; B
1995	1.07 ± 0.04 a; A	1.29 ± 0.23 a; A	1.87 ± 0.26 b; A
1996	0.84 ± 0.16 a; A	0.95 ± 0.17 a; A	1.42 ± 0.14 b; B
1997	1.16 ± 0.19 a; A	1.37 ± 0.29 a; A	1.75 ± 0.17 b; A
1999	0.87 ± 0.19 a; A	1.37 ± 0.25 b; A	1.72 ± 0.42 b; A
Needles of the 2nd age class		Necroses (mm^2 $needle^{-1}$)	
1999	0.32 ± 0.15 a	0.62 ± 0.31 b	0.90 ± 0.46 b
Needles of the 1st age class		*Chloroses* (mm^2 $needle^{-1}$)	
1999	2.52 ± 1.77 a	1.02 ± 0.89 b	0.56 ± 0.41 c
Needles of the 2nd age class			
1999	0.92 ± 0.46 a	1.05 ± 0.54 a;b	1.50 ± 0.81 b

ing investigations by Schulz *et al.* (1998) proved the significant influence of the N-supply on concentrations of soluble nitrogen-containing compounds. Consequently, the needle dry weights were more suitable as neutral basis parameters than the protein concentrations.

The results of needle assessment including quantitative measurements for the needle tip chloroses and necroses are summarized in Table I. Clear damage symptoms in the form of needle tip necroses were only observed on needles of the 2nd age class. With increasing ambient SO_2 and NO_x concentrations (Fig. 1), the degree of needle tip necroses rose significantly. When the investigations were completed in 1999, the older pine needles still showed further tip necroses and a decreasing trend in needle necroses could not be seen, even though the SO_2 concentrations no longer varied between the sites (Fig. 1). To verify the needle assessments, the areas of chloroses and necroses were exactly determined on needles of the 1998 and 1999 age classes. A non-linear relationship was identified between the quantitatively determined necrotic needle areas and the estimated degree of needle tip necroses with the regression function: $y = 0.0729*\exp(1.51*x)$; regression coefficient: $r^2 = 0.65$. Consequently, the method of needle assessment gave reproducible results. However, the results engage that the needle yellowing in the 1st age class of the low polluted site Neuglobsow was significantly higher at both the strongly polluted sites Taura and Rösa. By contrast, the chlorotic needle areas were increased in needles of the 2nd age class with increasing tip necroses and air load (Table I).

The pigment concentrations in needles of both age classes were always significantly lower in the lower polluted site Neuglobsow than in Taura or Rösa and exhibited no temporal variations between 1992 and 1999. The level of total chlorophyll in needles of the 1st age class in Neuglobsow lay in the range of 1.86 ± 0.10 to 2.39 ± 0.15, in Taura 2.26 ± 0.15 to 2.87 ± 0.07 and in Rösa 2.31 ± 0.10 to 2.97 ± 0.23 mg g^{-1} dry weight (dwt.). Significantly higher pigment concentrations were measured for needles of the 2nd age class (Neuglobsow: 2.50 ± 0.3 to 2.70 ± 0.30, Taura: 2.97 ± 0.09 to 3.41 ± 0.21 and Rösa: 2.82 ± 0.38 to 3.23 ± 0.23 mg g^{-1} dwt). In contrast to the lower pigment concentrations in needles from Neuglobsow, the measure-

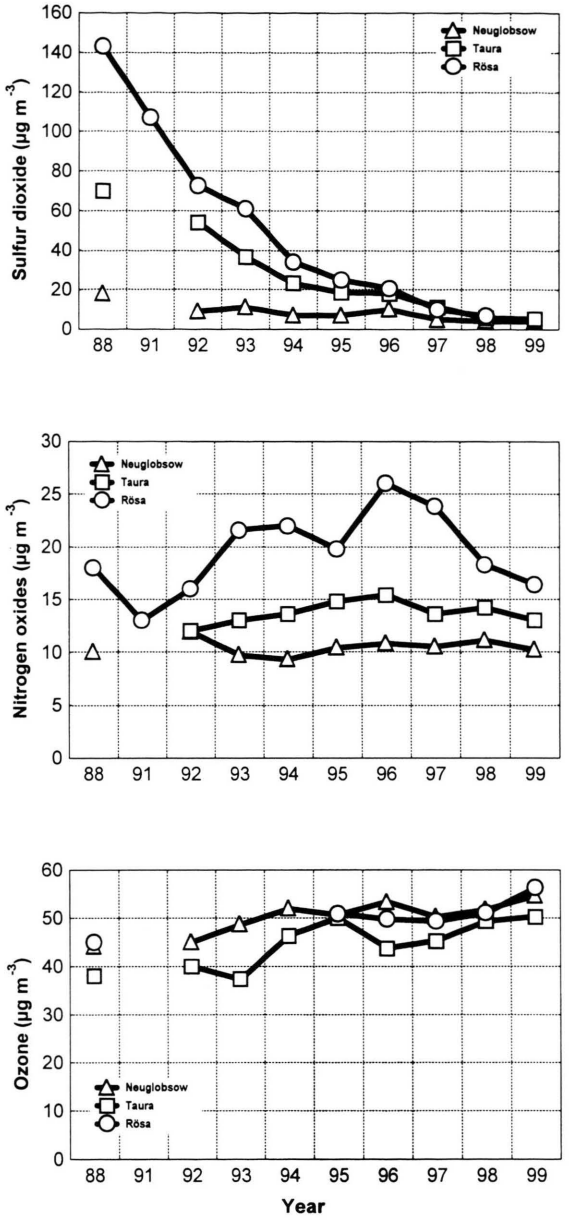

Fig. 1. Temporal variations of ambient sulfur dioxide (SO_2), nitrogen oxides (NO_x) and ozone (O_3) concentrations (annual mean values) measured at meteorological stations (Neuglobsow, Melpitz and Pouch) close to the sites. Neuglobsow Station and Melpitz (Taura) Station of the German Department of the Environment (distance approx. 5 km); Pouch Station (Rösa) of the Saxony-Anhalt Department of Environmental Protection (distance approx. 3 km).

ments of chlorophyll fluorescence showed no site-related differences. The mean F_v/F_m ratios for needles of the 1st and 2nd age classes were between 1996 and 1999 in the range of 0.83±0.006 to 0.84±0.006 in Neuglobsow, 0.82±0.008 to 0.83±0.007 in Taura, and 0.81±0.007 to 0.83±0.007 in Rösa.

Significant site-dependent differences were detected in the needle levels for the sulfur-containing inorganic and organic compounds. As already reported by Schulz *et al.* (1998), the whole needle sulfur and sulfate as well as glutathione increased significantly with rising SO_2 concentrations at the sites and decreased continuously with falling oxidative load until 1995. This trend was continued until 1999. Table II lists the time series of total glutathione acting for all soluble sulfur-containing compounds analyzed (sulfate, cysteine, γ-glutamylcysteine) in Scots pine needles. Considering total glutathione, the activity of the enzyme glutathione reductase (GR) also decreased from 1992 to 1999 (Table III). At Taura and Rösa with higher SO_2 pollution, the GR activity was significantly higher in needles of the 1st and 2nd age classes. As expected, a site-dependent and time-related effect was also proved for the oxidized glutathione (GSSG). However, the concentrations of GSSG in six-month-old needles refer by themselves to less than 5% of total glutathione owing to the high SO_2 load in Taura and Rösa. In contrast to this, no significant temporal variations between 1993 and 1995 were found for reduced glutathione (GSH). Correspondingly, the ratios of GSH/GSSG increased significantly in needles of the 1st age class with decreasing ambient SO_2 concentrations at all sites, varying between 1993 and 1995 in Neuglobsow from 27.8±13.7 to 65.6±28.7, in Taura from 22.1±9.8 to 37.5±13.4 and in Rösa from 18.0±8.6 to 28.6±12.8.

The activities of the superoxide dismutase isozyme (SOD1) proved to be less relevant bioindications. The results of enzyme activity measurements are summarized in Table III. Although always significantly higher SOD1 activities were determined at the higher polluted sites Taura and Rösa, no temporal variations were detected in needles of the 1st and 2nd age classes.

One especially remarkable observation is that the levels of all antioxidants did not vary in six-month-old needles of either young (Table IV) or adult Scots pine trees. The exception here was ascorbic acid. Higher ascorbic acid concentrations were measured in needles of adult than young Scots pine trees. However, no dependency on the SO_2 load and no variation in the needle levels over time were observed for total ascorbic acid in both needle age classes (Table V). By contrast, significant concentration differences were detected for

Table II. Concentrations of total glutathione, GSH and GSSG (mean values ± S. D.) in needles from adult Scots pines. Significant differences were determined according to the Mann Whitney U-test. Different small letters (a, b, c) within the rows indicate significant differences between the test sites. Different large letters (A, B) within the columns indicate significant temporal differences at $P < 0.05$ (reference year 1992, 1993 or 1995).

Year	Neuglobsow	Taura	Rösa
Needles of the 1st age class		Total glutathione (μg g^{-1} dwt.)	
1992	329.07 ± 56.88 a; A	483.90 ± 51.40 b; A	503.62 ± 50.61 b; A
1993	314.05 ± 27.18 a; A	468.63 ± 27.72 b; A	441.11 ± 52.63 b; A
1994	338.51 ± 22.62 a; A	547.43 ± 20.62 b; A	488.50 ± 60.06 b; A
1995	284.20 ± 39.99 a; A	443.65 ± 33.42 b; A	430.63 ± 29.02 b; B
1996	321.98 ± 24.04 a; A	389.14 ± 20.11 b; B	350.28 ± 21.73 a; B
1997	268.21 ± 15.62 a; B	382.17 ± 32.97 b; B	331.31 ± 38.70 b; B
1999	272.43 ± 10.21 a; A	305.70 ± 17.34 b; B	247.69 ± 28.27 a; B
Needles of the 2nd age class			
1995	262.19 ± 38.89 a; A	430.31 ± 15.84 b; A	365.67 ± 13.91 c; A
1999	236.27 ± 19.58 a; A	269.41 ± 26.07 b; B	190.63 ± 50.26 a; B
Needles of the 1st age class		GSH (μg g^{-1} dwt.)	
1993	249.39 ± 30.10 a; A	355.75 ± 21.24 b; A	355.10 ± 14.30 b; A
1995	205.18 ± 13.20 a; A	323.24 ± 20.90 b; A	329.97 ± 32.90 b; A
Needles of the 1st age class		GSSG (μg g^{-1} dwt.)	
1993	8.96 ± 2.20 a; A	6.13 ± 2.16 b; A	19.75 ± 1.67 b; A
1995	3.13 ± 0.46 a; B	8.62 ± 1.56 b; B	11.54 ± 0.69 b; B

Table III. Activities of glutathione reductase and superoxide dismutase isozyme SOD1 (mean values ± S. D.) in needles from adult Scots pines. Significant differences were determined according to the Mann Whitney U-test. Different small letters (a, b) within the rows indicate significant differences between the test sites. Different large letters (A, B) within the columns indicate significant temporal differences at $P < 0.05$ (reference year 1992 or 1996).

Year	Neuglobsow	Taura	Rösa
Needles of the 1st age class class	GR (units g^{-1} dwt.)		
1992	5.70 ± 0.76 a; A	10.24 ± 0.52 b; A	10.35 ± 0.61 b; A
1993	5.53 ± 0.58 a; A	10.82 ± 0.92 b; A	11.25 ± 2.01 b; A
1994	4.61 ± 0.82 a; A	8.23 ± 0.47 b; B	8.80 ± 0.55 b; B
1995	5.08 ± 0.91 a; A	8.32 ± 0.73 b; B	8.61 ± 1.71 b; A
1996	4.27 ± 0.28 a; B	5.33 ± 0.64 b; B	5.90 ± 0.67 b; B
1997	4.69 ± 0.67 a; B	7.74 ± 1.36 b; B	8.84 ± 1.04 b; B
1999	3.53 ± 0.58 a; B	6.21 ± 1.45 b; B	7.07 ± 0.63 b; B
Needles of the 2nd age class			
1996	6.94 ± 0.67 a; A	9.56 ± 0.34 b; A	8.58 ± 1.40 a,b; A
1999	6.27 ± 0.76 a; A	5.99 ± 1.21 a; B	6.51 ± 1.22 a; B
Needles of the 1st age class class		SOD1 activity (units mg^{-1} dwt.)	
1992	0.94 ± 0.32 a; A	1.07 ± 0.08 a; A	1.48 ± 0.22 b; A
1993	0.71 ± 0.14 a; A	1.04 ± 0.19 b; A	1.14 ± 0.28 a,b; A
1994	1.34 ± 0.18 a; A	1.90 ± 0.26 b; B	2.13 ± 0.22 b; B
1995	0.92 ± 0.15 a; A	1.37 ± 0.25 b; A	1.50 ± 0.31 b; A
1996	0.89 ± 0.16 a; A	0.98 ± 0.09 a; A	1.26 ± 0.27 a; A
1997	1.06 ± 0.31 a; A	1.64 ± 0.26 b; A	1.57 ± 0.08 b; A
1999	0.78 ± 0.13 a; A	1.02 ± 0.21 a; A	1.03 ± 0.32 a; A
Needles of the 2nd age class			
1995	0.89 ± 0.18 a; A	1.02 ± 0.16 a,b; A	1.28 ± 0.15 b; A
1997	0.96 ± 0.38 a; A	1.29 ± 0.18 a; A	1.36 ± 0.18 a; A

Table IV. Activities and concentrations of superoxide dismutase isozyme SOD1, glutathione reductase, total ascorbic acid, total glutathione as well as degrees of necroses (mean values ± S. D.) in needles from young Scots pines. Significant differences were determined according to the Mann Whitney U-test. Different small letters (a, b) within the rows indicate significant differences between the sites. Different large letters (A, B) within the columns indicate significant temporal differences at $P<0.05$ (reference year 1993 or 1995).

Year	Neuglobsow	Taura	Rösa
Needles of the 1st age class		SOD1 activity (units mg^{-1} dwt.)	
1993	0.56 ± 0.10 a; A	1.22 ± 0.38 b; A	1.10 ± 0.28 b; A
1995	0.76 ± 0.13 a; A	1.17 ± 0.23 b; A	1.31 ± 0.33 b; A
		GR activity (units mg^{-1} dwt.)	
1993	5.99 ± 1.04 a; A	9.46 ± 1.42 b; A	11.12 ± 0.57 c; A
1995	4.31 ± 0.77 a; A	6.39 ± 1.36 b; B	6.27 ± 0.61 b; B
		Total glutathione ($\mu g\ g^{-1}$ dwt.)	
1993	321.86 ± 26.88 a; A	423.32 ± 45.29 b; A	474.52 ± 56.86 b; A
1995	279.10 ± 24.72 a; B	428.61 ± 37.06 b; A	409.36 ± 27.61 b; B
		Ascorbic acid (mg g^{-1} dwt.)	
1993	2.73 ± 0.42 a; A	2.73 ± 0.50 a; A	2.75 ± 0.37 a; A
1995	2.74 ± 0.67 a; A	2.81 ± 0.50 a; A	2.98 ± 0.57 a; A
Needles of the 2nd age class		Necroses (Index)	
1993	0.91 ± 0.17 a; A	1.12 ± 0.29 a; A	1.63 ± 0.27 b; A
1995	0.63 ± 0.24 a; A	1.08 ± 0.37 a, b; A	1.49 ± 0.16 b; A

the oxidized and reduced ascorbic acid forms. The concentrations of dehydroascorbate in needles of both age classes rose depending on the site as well as in time, taking into account decreased concentrations of the reduced ascorbic acid form in the same ratio. This is remarkable, because it was assumed that a decreasing oxidative load at the sites would be accompanied by an increase in ascorbic acid or a decrease in the oxidized form (dehydroascorbate). This effect appeared for the first

Table V. Concentrations (mean values ± S. D.) of total ascorbic acid, dehydroascorbate (ΔHA) and ascorbic acid (AA) in needles from adult Scots pines. Significant differences were determined according to the Mann Whitney U-test. Different small letters (a, b) within the rows indicate significant differences between the test sites. Different large letters (A, B) within the columns indicate significant temporal differences at $P < 0.05$ (reference year 1992 or 1995).

Year	Neuglobsow	Taura	Rösa
Needles of the 1st age class		Total ascorbic acid (mg g^{-1} dwt.)	
1992	3.28 ± 0.13 a; A	3.35 ± 0.45 a; A	3.82 ± 0.45 a; A
1993	2.90 ± 0.18 a, b; B	2.80 ± 0.17 a; B	3.13 ± 0.18 b; B
1994	4.01 ± 0.21 a, b; B	4.08 ± 0.21 a; A	3.71 ± 0.18 b; A
1995	2.82 ± 0.21 a; B	3.26 ± 0.29 b; A	3.19 ± 0.26 a,b; A
1996	3.10 ± 0.15 a; A	3.05 ± 0.16 a; A	3.37 ± 0.20 b; A
Needles of the 2nd age class			
1995	3.66 ± 0.39 a; A	3.64 ± 0.11 a; A	3.75 ± 0.12 a; A
1996	3.73 ± 0.14 a; A	3.97 ± 0.23 a,b; B	4.27 ± 0.22 b; B
Needles of the first age class		Dehydroascorbate (mg g^{-1} dwt.)	
1992	0.87 ± 0.10 a; A	1.17 ± 0.13 b; A	1.36 ± 0.07 b; A
1993	0.99 ± 0.06 a; A	1.10 ± 0.09 a; A	1.21 ± 0.07 a; B
1995	0.77 ± 0.02 a; A	0.86 ± 0.07 b; B	0.99 ± 0.12 b; B
1996	1.37 ± 0.10 a; B	1.32 ± 0.19 a, b; A	1.14 ± 0.08 b; B
1999	2.22 ± 0.38 a; B	2.30 ± 0.42 a; B	2.80 ± 0.38 a; B
Needles of the 2nd age class			
1995	1.40 ± 0.09 a; A	–	1.59 ± 0.20 a; A
1999	3.64 ± 0.63 a; B	2.61 ± 0.82 a,b	2.75 ± 0.18 b; B
Needles of the 1st age class		Ascorbic acid (mg g^{-1} dwt.)	
1992	2.28 ± 0.32 a; A	2.46 ± 0.16 a; A	3.05 ± 0.26 b; A
1993	2.25 ± 0.04 a; A	2.10 ± 0.16 a; B	2.10 ± 0.22 a; B
1995	1.91 ± 0.18 a; A	2.08 ± 0.04 a,b; B	2.34 ± 0.25 b; B
1996	2.61 ± 0.07 a; B	2.13 ± 0.10 b; B	2.56 ± 0.11 a; B
1999	1.82 ± 0.52 a, b; A	1.82 ± 0.32 a; B	1.29 ± 0.25 b; B
Needles of the 2nd age class			
1995	2.43 ± 0.36 a; A	–	2.34 ± 0.32 a; A
1999	1.57 ± 0.30 a; B	2.27 ± 0.53 b	1.76 ± 0.35 a, b; B

Table VI. Concentration of α-tocopherol (mean values ± S. D.) in needles from adult Scots pines. Significant differences were determined according to the Mann Whitney U-test. Different small letters (a, b) within the rows indicate significant differences between the test sites. Different large letters (A, B) within the columns indicate significant temporal differences at $P<0.05$ (reference year 1993).

Year	Neuglobsow	Taura	Rösa
Needles of the 1st age class		α-Tocopherol (μg g^{-1} dwt.)	
1993	332.04 ± 31.12 a; A	369.91 ± 37.28 a; A	351.56 ± 10.56 a; A
1994	371.06 ± 15.62 a; A	286.25 ± 16.63 b; B	275.58 ± 15.66 b; A
1996	261.02 ± 6.01 a; B	300.65 ± 16.47 b; B	267.76 ± 19.38 a; B
Needles of the 2nd age class			
1996	364.37 ± 14.71 a	493.53 ± 49.38 b	489.86 ± 37.39 b

time in 1996 and was proved significantly at the higher SO_2-polluted sites in 1999, where the total glutathione concentrations in Scots pine needles were reduced by more than half compared to 1992 (Table II).

The only findings, which were not reproducible results, were found for α-tocopherol (Table VI). Whereas in six-month-old needles the concentration differences between the sites were very small and statistical significances were found by sheer chance, the needles of the 2nd age class indicated significantly site-dependent differences with clearly higher needle levels.

Discussion

The results reported here suggest spatial and temporal variations of visible symptoms on foliage and of antioxidants in Scots pine needles after fundamental changes in the atmospheric chemistry at three sites (Neuglobsow, Taura, Rösa) in the northeast German lowlands along a deposition gradient of SO_2, NO_x and O_3 between 1992 and 1999 (Fig. 1). As a result of these changing effects on forest ecosystems, as already reported by Schulz *et al.* (1998) the amounts of sulfate and total glutathione decreased significantly in needles of adult Scots pine trees and it was concluded that SO_2 is the dominant air contaminant. Therefore, we assumed that the dose-effect relationships and temporal variations in the antioxidant level of Scots pine needles observed ought also to be mainly influenced by SO_2 and less by NO_x and O_3, even though high O_3 concentrations were measured at all sites.

The results show that not all of the biomarkers examined in pine reacted significantly to the site-dependent differences and temporal variations in the oxidative loads under field conditions. In contrast to previous field investigations (Schulz *et al.*, 1995) with young Scots pine trees under stronger SO_2 loads (differences 140 µg SO_2 m^{-3}), no clear dose-effect relationships were determined for the Cu- and Zn-containing isozyme SOD1 of superoxide dismutase. Significant site-dependent differences were detected in the enzyme activity, without any changes to the temporal variations of the SO_2 concentrations at the sites (Table III). Also Polle *et al.* (1994) found no variations of SOD activity in pine needles under comparable conditions at the three sites. Therefore we conclude that the oxidative load (10–60 µg SO_2 m^{-3}) at the sites between 1992 and 1999 was probably already too small to prove significant dose-effect relationships. Kurepa *et al.* (1997) discusses that oxidative stress causes higher transcript levels of SOD genes but doe not affect SOD activity. According to more recent results by Wingsle and Karpinski (1996), the transcript level of cytosolic Cu,Zn-SOD was decreased by the glutathione pool as the sum of the reduced and oxidized forms (GSH, GSSG) without any significant change in enzyme activity. Our findings are supported by theses investigations. The activities of SOD1 change only negligibly depending on the total glutathione concentrations in six-month-old pine needles (Fig. 2). However, as already described by Schulz *et al.* (1998), the significant changes in the glutathione pool (Table II) cannot be attributed to direct interactions between glutathione and O_2-radicals, but are the result of enhanced and uncontrolled sulfur assimilation at the sites. The same conclusion was also reached by Foyer and Rennenberg (2000). The authors rule out the direct involvement of GSH in the decontamination of active oxygen compounds in the symplast of plant cells, because both the content and the affinity of GSH to O_2-radicals are by far smaller than those from ascorbic acid (Polle and Rennenberg, 1994). In the ascorbate-glutathione-cycle, GSH plays an important role in its reduced form during the regeneration of ascorbic acid. Tausz *et al.* (1999) accept that in this case reduced GSH is decreased. This working hypothesis cannot be confirmed by our investi-

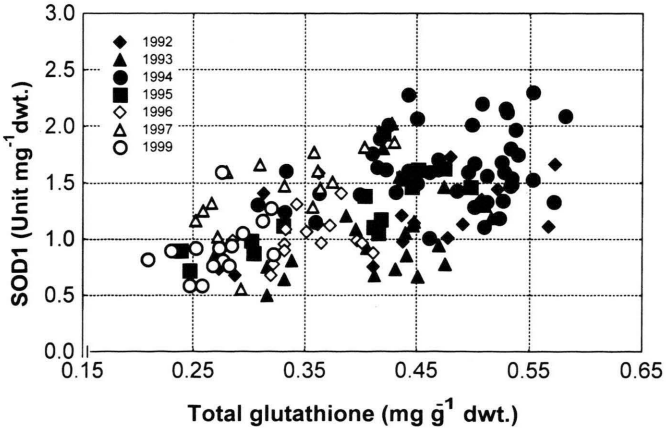

Fig. 2. Correlation between activity of isozyme SOD1 and total glutathione concentration in six-month-old pine needles at three sites from 1992 to 1999.

gations with Scots pine needles. According to the data for total glutathione as well as redox status (Table II), no significant concentration differences were proved for reduced GSH in the case of varying oxidative loads. However, significant site-related and temporal variations appeared for the oxidized form GSSG. The concentration changes of oxidized glutathione positively correlate with the activity of the GR (Fig. 3), which means that the concentrations of GSSG and the activities of GR were increased or decreased by varying oxidative loads at the sites (Table III, Fig. 1). In accordance with the previous statement, the GSH/GSSG ratio negatively correlates with the GR activity (data not shown). Rao and Dubey (1992) found in investigations with wheat exposed to SO_2 that both the GR activity and the GSH/GSSG ratio were also enhanced significantly. In addition, Foyer and Rennenberg (2000) report in a current work that the transcript level of GR is influenced by GSH and GSSG. This information is of special interest for the interpretation of our results, because we assume that glutathione played a key role in the antioxidative defence system under decreasing SO_2 loads on Scots pine trees at the sites. A decreased glutathione level in pine needles could mean that the regeneration of ascorbic acid is increasingly restricted by a delimited amount of whole needle glutathione. In this case, the dearth of total glutathione becomes a limiting factor in the detoxification of reactive oxygen species in pine needles. Precisely this condition was proved in the antioxidative system of Scots pine needles. The concentrations of total glutathione in six-month-old needles decreased by up to a level of 0.25 mg g^{-1} dwt. at the sites Taura and Rösa in 1999 (Fig. 4). Finally, it can be assumed that the redox status of glutathione also influenced the in vivo regulation of the Cu,Zn-SOD1 gene expression in pine needles, which would explain the relatively unchanged enzyme activities described above.

In contrast to total glutathione, in the present study no significant variations were observed for the whole needle ascorbic acid (Table V). However, in the case of decreasing SO_2 concentrations in the ambient air at the higher polluted sites, the levels of dehydroascorbate rose significantly in six-month-old pine needles (Table V). Apparently, the changes in the redox status of ascorbic acid in the antioxidative system of pine needles reflect the special multiple exposure at the investigation sites. Hausladen *et al.* (1990) and Luwe (1996) found an accumulation of GSSG in O_3-polluted spruce needles and a decrease in the GSH/GSSG ratio, respectively (Schmieden *et al.* 1993). An accumulation of dehydroascorbate in O_3-polluted Spruce needles has never been detected (Ranieri *et al.* 1999, Plöchl *et al.* 2000) because ozone cannot penetrate the plasma membrane (Polle *et al.*, 1999) and reacts exclusively with cell wall bound ascorbic acid by the formation of dehydroascorbate, which is immediately transported into the cytosol and is regenerated by GSH. Since ascorbic acid is transported back again in the apoplast after its regeneration, no significant concentration changes between the reduced and oxidized form are found in either the apoplast or the symplast (Turcsányi *et al.*, 2000). Therefore, it is concluded that the accumulations determined here of GSSG and DHA

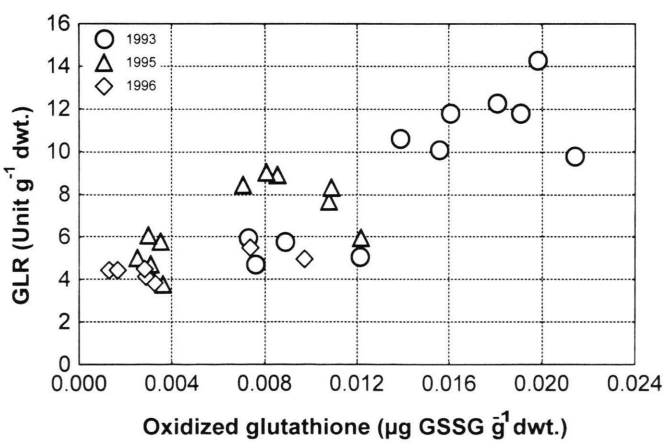

Fig. 3. Correlation between activity of glutathione reductase (GR) and concentration of oxidized glutathione (GSSG) in six-month-old pine needles at three sites from 1993 to 1996.

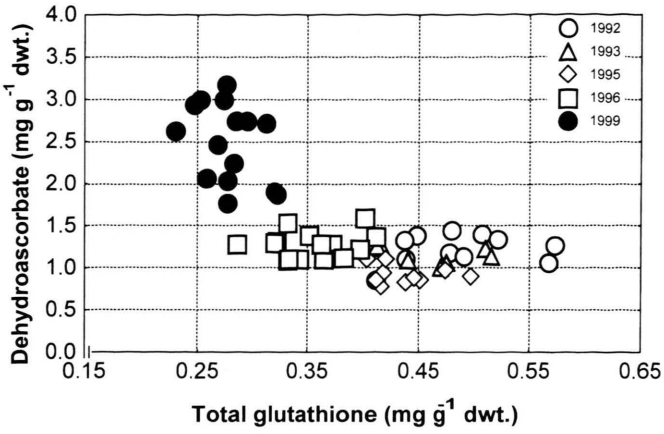

Fig. 4. Correlation between concentrations of total glutathione and oxidized ascorbic acid (dehydroascorbate) in six-month-old pine needles at three sites from 1992 to 1999.

in pine needles at the sites are to be attributed to oxidative SO_2 effects. Although ozone concentrations with an annual mean of 49 ± 3 µg m^{-3} among all the sites do represent an oxidative damage potential (Srivastava, 1999), owing to their sporadic temporal variations (Fig. 1) they can hardly be responsible for the significant differences in the redox status of glutathione and ascorbic acid. We cannot rule out the possibility of a stimulating effect on the accumulation of the oxidized forms of glutathione and ascorbic acid by NO_x, since the annual means showed not only significant site-related differences, but also a slightly increasing trend in the investigation period (Fig. 1).

Finally, it should be discussed whether the reactions ascertained in the antioxidative system of pine needles led to irreversible changes on the cellular and organism levels. In conclusion, both the direct and the indirect accumulations of GSSG and DHA in six-month-old pine needles need to be evaluated as a sensitive early indication of oxidative stress, because any condition in which cellular redox homeostasis is disrupted can be defined as oxidative stress (Alscher *et al.*, 1997). However, there is no evidence that the oxidative stress on the cellular level is causally related to the observed tip necroses on needles of the 2nd age class (Table I). Although decreasing levels of α-tocopherol are described as an early indication of chlorophyll destruction (Ribarič-Lasnik *et al.*, 1999), we did not observe any changes in the needle level of α-tocopherol in either six-month-old or older pine needles (Table VI). Moreover, the ratio of total ascorbic acid to α-tocopherol was never smaller than the critical level of 10:1 in pine needles with marked necroses. Thus, sufficient protection against cell damage was always ensured (Ribarič-Lasnik *et al.*, 1999). Furthermore, the photochemical efficiency of photosystem II as indicated by the ratio of variable to maximum fluorescence (F_v/F_m) was not lower for needles of trees at the more highly polluted sites. The mean F_v/F_m ratio of 0.82 or higher for all needles indicated a healthy photosynthetic apparatus (Saarinen and Liski, 1993. Mohamed *et al.*, 1995), regardless of the significantly different degrees of necroses. The chlorophyll concentrations did not decrease either in necrotic needles from Taura and Rösa. We observed in needles with clear tip necroses increased pigment concentrations. In contrast to this, increased chloroses were observed in needles of the 1st age class in Neuglobsow, where the lowest pigment level was also measured (Fig. 5). These observations confirm our assumption that the tip necroses in needles of the 2nd age class were not caused by oxidative stress. The damage symptoms might result from a vitality reduction caused by an accumulation of Scots pines with soluble non-protein compounds as result of an oversupply of ammonium and nitrate in the humus layers at the sites (Schulz *et al.*, 1998). Another factor arguing for these findings is that during reduced SO_2 loads between 1992 and 1999 at the more polluted sites Taura and Rösa, no significant variations were observed in the degree of necroses (Table I). Needle yellowing among the 1st age class of pines in Neuglobsow did not lead to tip necroses on the same needles in the following year (Table I). Probably two pro-

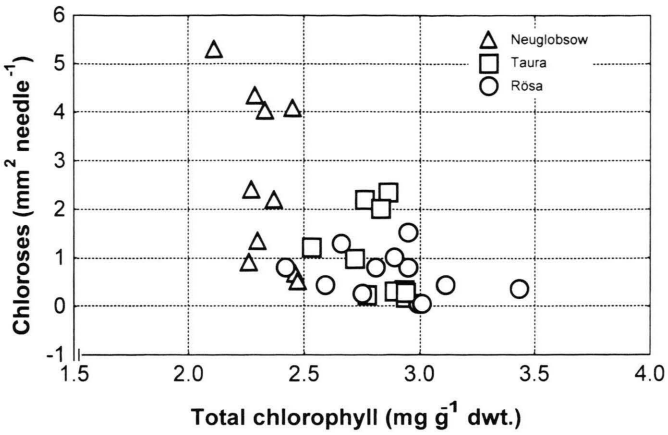

Fig. 5. Correlation between chloroses (chlorotic needle area) and total chlorophyll concentration in ix-month-old pine needles at three sites in 1999.

cesses are taking place independently of each other on the cellular level in Scots pines, which on the one hand are influenced by an insufficient and on the other hand by an increased supply of nutrients, and which need to described in detail elsewhere.

Acknowledgements

We would like to thank M. Herrmann, G. Pelzl and R. Rudloff for their excellent technical support during antioxidant analyses, and M. Jungnitsch and S. Illek for their help in image analyses.

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