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Stress-physiological Investigations and Chromosomal Analysis on Norway spruce (*Picea abies* (L.) KARST.) – A Field Study

By

Michael TAUSZ, Maria MÜLLER, Edith STABENTHEINER & Dieter GRILL

With 3 figures

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Summary

TAUSZ M., MÜLLER M., STABENTHEINER E. & GRILL D. 1994. Stress-physiological investigations and chromosomal analysis on Norway spruce (*Picea abies* (L.) KARST.) – A field study. - *Phyton* (Austria) 34 (2): 291–308, 3 figures. - English with German summary.

The antioxidative defence system and chloroplast pigments in needles as well as chromosomal aberrations in root tip meristem cells of Norway spruce trees (*Picea abies* (L.) KARST.) were investigated at four natural sites in one region of Austria. At the two higher elevated sites the sampled trees exhibited increased contents of ascorbic acid and glutathione together with an elevated level of chromosomal damages and alterations in chloroplast pigment ratios and concentrations. Elevated antioxidant contents pointed to an increased level of oxidative stress the respective trees were exposed to. Pigment depressions and an increased number of chromosomal aberrations indicated reduced vitality of these trees, that were not visibly damaged. An evaluation of possible stress factors lead to the conclusion that in particular ozone impact was to be suspected of playing an important role in influencing these sites. The combination of the methods applied in this case study may provide a valuable tool for the bioindication of non-accumulating, oxidative stresses.

Zusammenfassung

TAUSZ M., MÜLLER M., STABENTHEINER E. & GRILL D. 1994. Streßphysiologische Untersuchungen und Chromosomenanalysen an Fichten (*Picea abies* (L.) KARST.) –

Mag. M. TAUSZ, Dr. M. MÜLLER, Dr. E. STABENTHEINER & Dr. D. GRILL, Institute of Plant Physiology, University of Graz, Schubertstraße 51, 8010 Graz.

Eine Freilandstudie. - *Phyton (Austria)* 34 (2): 291-308, 3 Abbildungen. - Englisch mit deutscher Zusammenfassung.

In dieser Studie wurde das antioxidative Schutzsystem und die Chloroplastenpigmente in den Nadeln sowie die Chromosomenaberrationen in den Wurzelspitzenmeristemen von Fichten (*Picea abies* (L.) KARST.) an vier natürlichen, benachbarten Standorten in Österreich untersucht. Fichten der zwei höhergelegenen Standorte zeigten höhere Ascorbinsäure- und Glutathiongehalte gemeinsam mit erhöhter Anzahl von Chromosomenaberrationen und Veränderungen der Pigmentgehalte und -relationen. Erhöhte Antioxidantiengehalte wiesen auf ein erhöhtes oxidatives Streßniveau hin, dem die Bäume an diesen Standorten ausgesetzt waren. Pigmentverminderungen und vermehrte Chromosomenaberrationen zeigten Vitalitätsverminderungen dieser Bäume, die kaum sichtbare Schäden zeigten, an. Eine Abschätzung vieler möglicher Streßfaktoren ergab, daß die Einwirkung von Ozon eine wichtige Rolle an diesen Standorten spielt. Die Kombination der hier angewendeten Methoden wird als ein Bioindikationssystem für nichtakkumulierende, oxidative Stressoren vorgeschlagen.

Introduction

Since the beginning of the eighties a novel form of forest decline has been observed in both Europe (BLANK 1985) and North America (McLAUGHLIN 1985). The syndrom was called "mountainous yellowing" or "novel forest decline" and occurred in so-called clean air areas far from pollution sources mainly at mountainous sites. The phenomenon is commonly thought to be caused by multiple factors. Among others, there is strong evidence for a participation of air pollutants in inducing forest damages (SCHULZE 1989). In this connection especially ozone is paid much interest, probably in combination with other photochemically formed compounds (GUDERIAN 1985). In contrast to accumulating agents such as SO₂, fluoride, or chloride, the indication of these non-accumulating substances is difficult.

With respect to this question research focussed on mechanisms of damage and defence in plant cells during the last years. Under the influence of air pollutants the formation of toxic free oxygen radicals within the cells is enhanced, thus causing an increased level of oxidative strain (ELSTNER 1982, GUDERIAN 1985, JÄGER & al. 1986, ELSTNER & OSSWALD 1994). Radical reactions will eventually lead to lipidperoxidations, protein oxidations as well as oxidative damages in plastid pigments, if the capacity of radical scavenging systems of the cells is exceeded. The antioxidative defence system of plant cells involves the presence of carotenoids and antioxidants such as ascorbic acid or glutathione (FOYER & HALLIWELL 1976, ALSCHER 1989) as well as the action of radical scavenging enzymes (ALSCHER & AMTHOR 1988, SALIN 1988). Fumigation experiments showed that plants react to the impact of air pollutants by an activation of their antioxidative defence system: Exposure of conifer trees to ozone and/or SO₂ resulted in elevated concentrations of glutathione (DOHMEN &

al. 1990, BERMADINGER & al. 1990) and/or ascorbic acid (MEHLHORN & al. 1986, BERMADINGER & al. 1990) and in increased activities of scavenging enzymes such as peroxidase (CASTILLO & al. 1987, KLUMPP & al. 1989). In field studies some authors reported increased antioxidant levels in visibly damaged spruce trees compared to apparently healthy ones (OSSWALD & al. 1987, 1992, POLLE & al. 1992a, LUCAS & al. 1993, SCHMIEDEN & al. 1993). However, in order to predict occurring damages the study of physiological reactions, repair mechanisms, and internal damages at apparently healthy trees will be of great interest. Investigations at altitude profiles in the Alps showed that trees exposed to increased stress influences at high altitudes (in areas, where no "classical" pollutant impact, for instance by SO₂, occurred) exhibited elevated levels of antioxidants (PFEIFHOFER & al. 1987, GRILL & al. 1988, MADAMACHI & al. 1991, POLLE & RENNENBERG 1992) together with depressed levels and altered ratios of chloroplast pigments, thus indicating biochemical damages (BERMADINGER & al. 1989). Visible damage of the spruce trees was not notable in some of these cases. These results correspond well with findings about increasing ozone concentrations with increasing sea level (SMIDT 1993), suggesting that ozone plays an important role in imposing oxidative strain on these trees.

So, antioxidant analyses may provide a suitable tool for bioindication of non-accumulating, oxidative compounds that act upon trees. These influences are not detectable by classical bioindication methods on conifers, such as element analysis of needles or bark analysis (GODBOLD & al. 1993, TAUSZ & al. 1994).

Responses of the antioxidative defence systems have also been reported due to various other stress factors, such as drought stress (SMIRNOFF 1993) or nutrient deficiencies (CAKMAK & MARSCHNER 1992). Responses of single metabolites are more or less unspecific. For example, glutathione concentrations may be increased due to oxidative stress but also due to activated sulphur metabolism in the course of SO₂-impact (GRILL & ESTERBAUER 1973, GRILL & al. 1982). Pathogen or animal attack may cause enhanced activities of detoxifying enzymes. In order to assess specific stresses it will be necessary to study patterns of physiological reactions including antioxidants, chloroplast pigments and enzyme activities in combination with classical bioindication methods. Some of the possible reasons for radical strain mentioned above can be ruled out by sulphur analyses, nutrient concentration determinations, or measurements of water status of the needles.

Recently, another new bioindication method with spruce trees, that is based on the classification of chromosomal aberrations in cells of the root tip meristems, was developed (MÜLLER & al. 1991). The use of chromosomal aberration tests with plants is commonly accepted for toxicity monitoring (RANK & NIELSEN 1993) and has turned out practicable to determine the vitality of spruce trees at natural sites (MÜLLER & al. 1991). This method responds to classical pollutant impacts (MÜLLER & al. 1994) as well

as to non-accumulating agents such as ozone (MÜLLER & al. 1994, MÜLLER & GRILL 1994).

For this paper, biochemical stress parameters as well as chloroplast pigments were determined in needles of Norway spruce trees (*Picea abies* (L.) KARST.) at four natural stands in an area partly affected by "novel forest decline". Simultaneously, chromosomal analyses were carried out at these sites. The objective of this study is to attempt an assessment of the specific stress situation to which the trees were exposed by combined application of stress-physiological methods and chromosomal analyses.

Materials and Methods

Site description

Sites: The present study was carried out at four natural spruce stands near Schöneben, Upper Austria. These sites were included in a national research program on forest decline (Forschungsinitiative gegen das Waldsterben = FIW). In this region uniform spruce forests cover large areas of the silicate mountains of the Bohemian Mass. Soil types at the investigated sites were brown earth and podsol brown earth with pH 2.6 to 4.0. Symptoms of "novel forest decline" in form of chlorosis and needle shed especially of the older needles have been observed since the early eighties. In spite of gradually reduced import of air pollutants, in particular SO₂, from Czechia, the damage symptoms did not improve.

Site number	altitude [m a. s. l.]	exposition	slope
1	860	NE	5-20°
2	990	SE	0-20°
3	880	SW	10-40°
4	1350	SE	0-10°

Climatic situation and pollution data: Climatic and pollution data were monitored at one permanent registering station in Schöneben from 1986 to 1991. Mean summer temperature in summer 1991 was 10 °C, mean winter temperature in winter 1991/1992 was just below 0 °C. Total precipitation amounted to 300 mm in summer 1991, and to 230 mm in winter 1990/1991. One late frost event (defined as a day in May to September with minimum temperature below -2 °C) was registered in May 1991 (STOHL & KROMP-KOLB 1992).

Pollutant concentrations seemed to be low for SO₂ and NO₂. Mean SO₂ concentrations amounted to 7 µg m⁻³ in summer 1991, and to 14 µg m⁻³ in winter 1990/1991. Half hour's means above 70 µg m⁻³ (legally limiting value in Austria for april-october according to BUNDESGESETZ 1984) occurred only in 1% of cases (including winter 1990/1991). Mean NO₂ concentration was 6 µg m⁻³ in summer 1991. Exceedings of half hour's limiting value of 200 µg m⁻³ (ÖSTERREICHISCHE AKADEMIE DER WISSENSCHAFTEN 1987) did not occur. Highest pollutant concentrations were observed for ozone with mean concentrations of 85 µg m⁻³ in summer 1991. This figure exceeds vegetation period's limit of 60 µg m⁻³ (WORLD HEALTH ORGANISATION 1987) pronouncedly. Half hour's means for ozone exceeded 100 µg m⁻³, which was taken as a limiting value by STOHL & KROMP-KOLB 1992, in 30% of cases in summer 1991. This resulted in 140 days with at least one half hour's mean greater than this value. However, limiting

half hour's value according to VEREIN DEUTSCHER INGENIEURE 1989 ($300 \mu\text{g m}^{-3}$) was exceeded in fewer than 5% of cases. Meteorological and pollution data were taken from STOHL & KROMP-KOLB 1992.

Nutrient status and sulphur contents: In order to identify main components of the stress situation which the sampled trees are exposed to, it is necessary to rule out some possible stress factors by classical indication methods. Sulphur contents in the needles revealed no (at sites 2, 3 and 4) or only mild burden (at site 1) and suggested that direct damage by SO_2 impact is unlikely at the investigated sites (following the classification of BUNDESGESETZ 1984, see GLATTES 1989). Generally, mineral nutrient concentrations are just below optimum ranges, except for nitrogen, thus indicating mild deficiency at all four sites for this element. Phosphorus and potassium levels were adequate at all four sites (using the ranges according to FOERST & al. 1987). Magnesium concentrations were around deficiency limit (according to FOERST & al. 1987), but did not much underrange this threshold. Magnesium contents below yellowing thresholds of 0.35 mg g^{-1} dry weight (cp. LANGE & al. 1989: 265, fig. 14) were not observed. Furthermore, nutrient analyses did not reveal pronounced differences between the investigated sites (Data from KATZENSTEINER by personal communication).

Biochemical analyses

Plant material: Five to six Norway spruce trees were sampled per site. The trees were dominant in the stand and about 100 years old. The state of crown was class 1 or 2 according to forest damage inquiry criteria in Austria (WALDZUSTANDSINVENTUR 1990). Signs of pathogen damage or insect attack were apparently not manifest at the sampled plants. Apparently severely damaged trees were not included in the analysis. Southward branches were taken with the help of a tree climber from the 7th whorl. The sampling date was the end of august 1991 according to the suggestion of BERMADINGER & al. 1989.

Sample preparation: Diurnal rhythms mainly caused by illumination were reported for antioxidant levels (SCHUPP & RENNENBERG 1988) and chloroplast pigments (SIEFERMANN-HARMS 1977). In order to use these parameters as stress indicators the sampling conditions must be standardized with respect to these short time changes (cp. POLLE & RENNENBERG 1992). For this purpose the harvested branches were kept cool and dark overnight. Needles were removed from the branches afterwards, separated in needle age classes (current, 1, 2 and 3 year old needles), portioned in 1 g fractions, and shock-frozen in liquid nitrogen. The frozen needles were stored at -25°C until analysing. For determination of fresh weight/dry weight ratio 1 g needles were dried at 105°C for 24 hours.

Pigments: The determination of chloroplast pigments was conducted using the HPLC gradient-method previously described by PFEIFHOFER 1989 (column Spherisorb S5 ODS2 25×4.6 mm with precolumn S5 ODS2 5×4.6 mm, solvent A: acetonitril:methanol:water = 100:10:5 (v/v/v), solvent B: acetone:ethylacetate = 2:1 (v/v), linear gradient from 90% (v) A to 30% (v) A in 17 min, run time = 30 min, flow = 1 ml min^{-1} , photometric detection at 440 nm). Acetone extracts of the needles were injected by a cooled (0°C) autosampler.

Ascorbic acid: Content of ascorbic acid was determined by an isocratic HPLC method according to KNEIFEL & SOMMER 1985. (Column: Spherisorb S5 ODS2 25×4.6 mm, precolumn Spherisorb S5 ODS2 5×4.6 mm, solvent: 1 mmol/l

hexadecylammoniumbromide and 0.05% (w/v) NaH_2PO_4 in methanol:water = 3:7 (v/v), run time = 20 min, flow = 1 ml min^{-1} , photometric detection at 248 nm). Extracts of needles were prepared in 3% (w/v) citric acid.

Low molecular thiols: Low molecular thiols were measured according to GRILL & ESTERBAUER 1973, using a photometric method involving Ellman's reagents (DTNB). Determination wavelength was 412 nm. Glutathione represents about 95% of water soluble thiols in spruce needles.

Peroxidase activity: Peroxidase activity was measured according to KELLER & SCHWAGER 1971, following the increase in absorbance at 485 nm after reaction with 1,4-diamino-benzene. Enzyme activities are given in units (1 unit = 1 $\mu\text{mol min}^{-1}$).

Chromosomal analysis (according to MÜLLER & al. 1991)

Plant material: Three to six five-year-old on-site Norway spruce plants were used per site. They were potted in forest soil from the site in 12-cm-diameter clay pots in May 1990. Root tips were harvested at the end of August 1991 and prepared the same day.

Sample preparation: Root tips were treated with 1-bromonaphthalene, and fixed in ethanol:acetic acid = 3:1 (v/v). Root tips were then hydrolysed in HCl (3 mol l^{-1}) for 3 min at 63°C, stained in freshly prepared Schiff's reagent, and squashed in a few drops of carmine acetic acid.

Classification and evaluation: The cells in metaphase were classified in following categories: metaphase, ring, break/fragment, connection, clumping, and amorphous chromatin mass. Each affected metaphase was counted as one aberration and the percentage of abnormalities in total metaphases was calculated. In order to assess the stress situation which the trees at one specific site are exposed to MÜLLER & al. 1991 (according to DRUSKOVIC 1988) established a classification system for sites: Mean values of percentages of chromosomal aberrations of all sampled trees at one site were divided by the aberration rate of trees from a control site, thus resulting in the so-called cytogenetic site index. These cytogenetic site indices were classified in four classes (in 10 steps) with class 1 representing lowest percentages of chromosomal damages (indicating vital trees) and class four representing highest aberration rates.

Data presentation and statistics

Physiological data are presented on dry weight basis. Chromosomal aberrations are presented as described above. Statistical calculations were carried out with NCSS[®] computer program. Mean values and standard errors allow an evaluation of occurring trends in data. These trends could not be statistically verified in all cases, as due to small sample sizes ($n = 4$ to 6) comparably strict non-parametric analyses of variance methods (KRUSKAL-WALLIS H-test, CONOVER cross comparisons between group's means according to BORTZ & al. 1990) had to be used to compare sites. For both physiological analyses and cytogenetic investigations each individual tree was counted as one replicate. For physiological analyses needle age classes were treated separately.

Results and discussion

Oxidant impact was one of the main suspects of causing severe strain in the investigated trees, as air pollution data (cp. site description) sho-

wed. Slight nutrient deficiencies concerned all the sites included in this study, and revealed no differences between them (cp. site description). The plant responses described in the following chapter must be evaluated under these conditions.

Stress-physiological investigations showed pronounced differences in patterns of measured parameters between the sampled sites. The spruce trees at site 4 exhibited lower contents of all chloroplast pigments in the older needles (Table 1). Especially total chlorophyll contents of spruce needles from site 4 are lower than the corresponding figures from the other sites (Fig. 1). The figures range between 1.5 and over 2.5 mg g⁻¹ dry weight. Reported values for total chlorophyll contents of one-year-old needles from apparently healthy spruce trees at comparably elevated sites, which were sampled in late summer, range from about 4.0 mg g⁻¹ dry weight (BERMADINGER & al. 1989, LANGE & al. 1989) to only 1.5 mg g⁻¹ dry weight (POLLE & al. 1992b). However, the spruce trees analysed in the study mentioned last showed visually notable discolorations. Investigations of chlorophyll concentrations are a widely accepted tool for assessing the vitality of spruce trees (MIES & ZÖTTL 1985, PFEIFHOFFER & GRILL 1987, OREN & al. 1993). Depressions of chloroplast pigments (in samples from site 4) were observable especially in 3 and 4-year-old needles (Table 1), thus resulting in an alteration of the age dependent pigment contents. Healthy spruce trees show an increase of total chlorophyll concentrations with increasing needle age at least up to two-year-old needles (Tree A in Fig. 2), whereas spruce trees affected by "novel forest decline" exhibit equal levels or even decreasing contents (Tree B in Fig. 2) with increasing needle age (PFEIFHOFFER & GRILL 1987, KÖSTNER & al. 1990). The mean values of total chlorophyll concentrations from site 1, site 2 and site 3 indicated an increase in pigment concentrations with increasing needle age, the trees at site four exhibited a pronounced depression of pigment concentra-

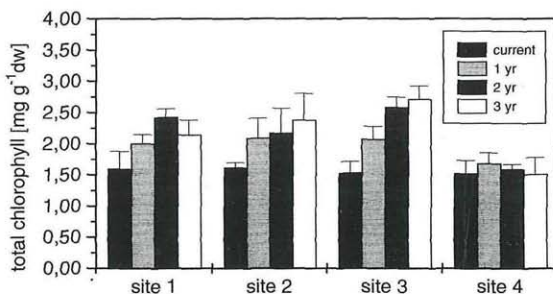


Fig. 1: Mean total chlorophyll (chlorophyll a+b) contents in four needle age classes of Norway spruce at the investigated sites. Error bars represent standard errors. Different letters indicate significant differences at the level $P < 0.05$.

Table 1
Chloroplast pigment contents in four needle-age-classes of Norway spruce trees at the investigated sites.

pigment contents [µg g ⁻¹ dw]	site 1	site 2	site 3	site 4
<i>neoxanthin</i>				
current	39±15	48±8	27±8	24±3
1 yr old	33±12	36±7	38±6	21±2
2 yr old	32±2ab	37±9ab	51±10a	
3 yr old	28±6ab	36±12ab	51±12b	24±6a
<i>v+a+z</i>				
current	31±1	33±4	27±4	36±10
1 yr old	35±7	42±10	51±12	41± 8
2 yr old	60±9	53±8	63±7	43± 6
3 yr old	52±3	48±11	63±7	41± 4
<i>lutein</i>				
current	154±24	181±9	134±22	160±12
1 yr old	153±16	172±12	166±8	137±12
2 yr old	180±10ab	163±22ab	201±15a	132±7 b
3 yr old	157±25	176±26	212±23	128±16
<i>chlorophyll b</i>				
current	434±76	494±46	405±70	513±40
1 yr old	540±40	615±81	592±40	495±40
2 yr old	635±45	583±115	710±72	472±20
3 yr old	572±96	654±117	782±73	462±91
<i>chlorophyll a</i>				
current	1168±204	1129±92	1090±173	1011±173
1 yr old	1471±104	1483±247	1481±197	1193±132
2 yr old	1795±89 ab	1592±281ab	1880±115a	1120± 67b
3 yr old	1576±139ab	1727±117a	1934±143a	1054±180b
<i>α-carotene</i>				
current	73±15	91±9	83±6	67±8
1 yr old	92±10ab	108±16ab	116±10b	66±8b
2 yr old	88±12ab	84±18ab	115±9b	61±6a
3 yr old	79±19ab	89±15ab	106±16b	53±13a
<i>β-carotene</i>				
current	63±7	72±9	70±3	80± 5
1 yr old	75±6	83±7	89±6	82±10
2 yr old	78±7	74±12	94±7	74± 5
3 yr old	74±9	77±13	94±5	66± 7

v+a+z=violaxanthin+antheraxanthin+zeaxanthin. Different letters indicate significant differences on the level P < 0.05.

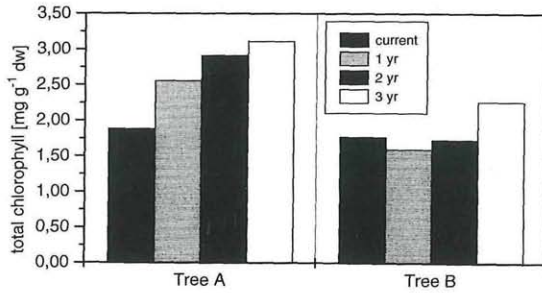


Fig. 2: Total chlorophyll (chlorophyll a+b) contents in four needle age classes of two representative Norway spruce trees from the investigated sites. Tree A exhibits normal increase of chlorophyll contents, tree B shows the typical symptom of "novel forest decline" with chlorophyll depressions in older needles.

tions from 2-year-old to 3- and 4-year-old needles (Table 1, Fig. 1). Nevertheless, at 40% of the trees at site 2 the pattern of "novel forest decline" (cp. tree B in Fig. 2) was observable indicating a worse situation at this site than mean values (Fig. 1) might suggest. Pigment depressions included all investigated xanthophylls, chlorophylls, and carotenes, but different pigments were affected to a different extent. Concentrations of chlorophyll a and α -carotene were most depressed (see significant changes indicated in table 1), whereas β -carotene seemed less affected, thus resulting in a shift of α -carotene/ β -carotene-ratio (Table 2). The trees at site 4 showed significantly lower α -carotene/ β -carotene-ratios in needles of most investigated age classes (Table 2). Shifts in α -carotene/ β -carotene ratios to lower values were observed at spruce trees from high altitudes in field studies (BERMADINGER & al. 1989, SMIDT & al. 1994) and were generally put as a plant response to an oxidative stress situation. SIEFERMANN-HARMS 1994 reported that α -carotene/ β -carotene-ratios were closely linked to chlorophyll content in spruce needles. Additionally, in this study shaded needles exhibited higher α -carotene/ β -carotene-ratios than sun-exposed ones. Investigations at green algae showed that illumination lead to oxidation of α -carotene to its corresponding xanthophyll (=lutein), whereas β -carotene remained unaffected (SENGER & al. 1993). However, lutein concentrations in spruce needles are much higher than α -carotene concentrations, so shifts in lutein contents are hard to find. But α -carotene/ β -carotene ratios in needle samples may serve as indicator for an oxidative strain within the cells or chloroplasts, assuming that lower values for this ratio point to an increased strain level. Lower values of this ratio together with low total pigment contents in samples from site 4 lead to the assumption that oxidation processes are heavily involved in pigment degradations in these trees. In literature, some more pigment ratios have been reported as indica-

Table 2:
Chloroplast pigment ratios in four needle-age-classes of Norway spruce at the investigated sites.

pigment ratios	site 1	site 2	site 3	site 4
<i>α-carotene/β-carotene</i>				
current	1.13±0.15a	1.32±0.11a	1.19±0.12a	0.83±0.07b
1 yr old	1.22±0.04ab	1.28±0.14ab	1.31±0.10a	0.81±0.07b
2 yr old	1.13±0.13a	1.11±0.15a	1.25±0.10a	0.79±0.05b
3 yr old	1.04±0.11	1.15±0.10	1.10±0.12	0.78±0.10
<i>xanthophylls/carotenoids</i>				
current	1.54±0.08	1.66±0.18	1.40±0.12	1.47±0.07
1 yr old	1.30±0.04	1.32±0.07	1.25±0.11	1.35±0.06
2 yr old	1.65±0.16	1.65±0.11	1.49±0.07	1.77±0.35
3 yr old	1.55±0.13	1.58±0.15	1.59±0.06	1.53±0.04
<i>chlorophylls/carotenoids</i>				
current	4.60±0.44	3.86±0.16	3.98±0.33	4.19±0.38
1 yr old	5.40±0.63	4.74±0.39	4.55±0.41	4.86±0.13
2 yr old	5.62±0.14	5.25±0.28	4.99±0.18	5.15±0.36
3 yr old	5.73±0.40	5.57±0.31	5.36±0.28	5.00±0.14

Figures represent mean values ± standard errors. Different letters indicate significant differences on the level $P < 0.05$.

tor tools. Elevated ratios of xanthophylls/carotenoids may point to oxidative damages and beginning needle yellowing (BERMADINGER & al. 1989). LICHTENTHALER & BUSCHMANN 1984 defined figures above 2.5 as pathological symptoms. In this study, no differences between the sites could be found for xanthophylls/carotenoids-ratios and the figures remained distinctly below 2 (Table 2). Decreases of chlorophylls/carotenoids-ratios are often combined with needle chlorosis and losses of chlorophylls (cp. OREN & al. 1993). Values below 4 for one-year-old and older needles have been considered a sign for needle yellowing (KANDLER & al. 1987). Carotenoids protect the chlorophylls from oxidative damages (cp. SIEFERMANN-HARMS 1977, SMIRNOFF 1993), on the other hand chlorophylls seem more sensitive to degradation than carotenoids (KANDLER & al. 1987). In this case study we found neither lower values for chlorophylls/carotenoids-ratios nor pronounced differences between the sites (Table 2). In this connection one must take into account that the needles investigated in the present study did not show visible discolorations or chlorosis. Chlorophylls/carotenoids-ratios and chlorophyll a/chlorophyll b-ratios were tested for their indicator value too. Especially chlorophyll a/chlorophyll b-ratios were often used as an indicator for oncoming needle damage. However, recent work

resulted in the conclusion that these ratios are not suitable for indication purposes (BRACHER & MURTHA 1993). In the present study we did not observe significant differences between the sites (data not shown). These results for the chloroplast pigment ratios indicate that chlorosis symptoms are not manifested in the investigated trees yet. Degradation of chlorophylls by acid agents leads to pheophytine formation. High amounts of this degradation product of chlorophyll may also point to cell necroses, possibly caused by animal attack. In samples of this study pheophytine was hardly detectable. This corresponded with low sulphur contents in the needles indicating no massive SO₂ impact. However, responses of chloroplast pigments are somewhat unspecific, because they occur in connection with responds to mineral deficiencies as well as in connection with air pollutant impact (cp. OREN & al. 1993). Thus, it is necessary to include more parameters in the analysis and establish patterns of physiological reactions as bioindicators.

Previous experiences suggest that the study of the antioxidative defence system of the cells may provide these additionally needed parameters. Elevated concentrations of cellular antioxidants, such as ascorbic acid and glutathione, were found in spruce trees showing symptoms of mountainous yellowing (OSSWALD & al. 1987, SCHMIEDEN & al. 1993) as well as in spruce trees treated with ozone and/or SO₂ (MEHLHORN & al. 1986, BERMADINGER & al. 1990, DOHMEN & al. 1990). Besides their general antioxidant function (SALIN 1989) ascorbic acid and glutathione detoxify oxygen radicals in a cyclic reaction involving NADPH and the action of glutathione reductase (FOYER & HALLIWELL 1976). An increase of antioxidant concentrations indicates a successful plant response to oxidative strain (ELSTNER & OSSWALD 1994). Thus, their levels in the needles may serve as marker substances for oxidative strain caused among others by oxidizing air pollutants (cp. JÄGER & al. 1986). Due to needle development current year's needles seem less suitable for indication purposes (cp. BERMADINGER & al. 1989) than 1-year-old needles, because their physiological development status is still unstable. However, in the present study ascorbic acid levels turned out to be generally higher in 1-year-old needles than in current year's needles (Table 3). For glutathione, results of comparisons between needle age classes are not uniform (Table 3). 1-year-old needles of spruce trees at site 2 and site 4 contained higher concentrations of ascorbic acid and glutathione than those at site 1 and site 3 (Fig. 3). These results, which indicated an active plant response to an oxidative influence pointed to an elevated level of stress the trees at the sites 2 and 4 were exposed to. These facts confirmed the conclusion drawn from pigment investigations assuming that oxidation processes played an important role in stressing the spruce trees especially at these sites.

Elevated peroxidase activities may occur due to acute stresses, such as animal attack as well as due to oxidative stress (cp. CASTILLO & al. 1987).

Table 3

Radical scavengers in two needle-age-classes of Norway spruce at the investigated sites.

	site 1	site 2	site 3	site 4
<i>ascorbic acid</i>				
[mg g ⁻¹ dw]				
current	2.39±0.28	2.93±0.40	2.70±0.22	3.07±0.10
1 yr old	3.36±0.33	4.24±0.69	3.16±0.40	4.62±0.22
<i>water soluble thiols</i>				
[nmol g ⁻¹ dw]				
current	462±126	347± 46	442± 36	422± 64
1 yr old	405± 74	452±117	337± 31	438± 55
<i>peroxidase activity</i>				
[units g ⁻¹ dw]				
current	24 ± 7	26 ± 2	17 ± 4	26 ± 3
1 yr old	34 ±10	25 ± 6	20 ± 4	29 ± 6

The figures represent mean values ± standard errors.

Acute needle damages were not observed at the trees in question. Activities of leaf peroxidases showed little differences between sites, except a slight trend towards lower activities in samples from site 3 (Table 3), thus confirming the absence of severe needle damages.

Summing up the combined results of the biochemical investigations we constated that the spruce trees at site 2 and site 4 suffered from an elevated oxidative stress level. This conclusion could be drawn from the pattern of stress-physiological reactions observed at these trees that pointed to an activation of the antioxidative defence system, i. e. simultaneous increase of ascorbic acid and glutathione in one-year-old needles. The trees from site 4 additionally showed distinct symptoms of a beginning reduction of vitality in form of pigment depressions and alterations in needle-age dependent chlorophyll concentrations. At site 2, some trees showed such symptoms, too, but for this site the results were not uniform.

Chromosomal analysis, which serves as a very sensitive tool for assessing the vitality of spruce trees at natural sites (MÜLLER & al. 1991) provided results that corresponded strictly with the results derived from physiological analyses. The investigations of chromosomal abnormalities revealed different types of chromosomal aberrations (Table 4). The total number of chromosomal abnormalities was higher in root tip meristems of the trees from site 2 and site 4 compared to those from site 1 and 3 (Table 4, Fig. 3). This indicated a reduced vitality of these trees. According to the

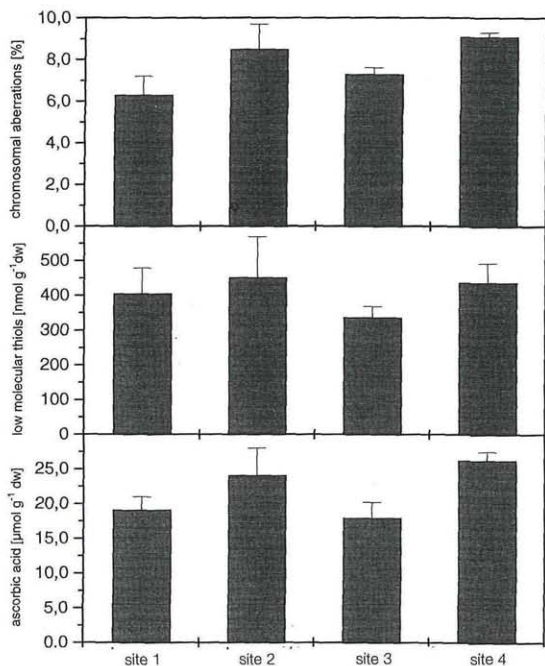


Fig. 3: Mean antioxidant concentrations in one-year-old needles and chromosomal aberrations in root tip meristems of Norway spruce at the investigated sites. Error bars represent standard errors.

classification system by MÜLLER & al. 1991 the trees at sites 2 and 4 were calculated as class -3, thus showing the severely affected vitality of those plants. Nevertheless, the results for samples from site 1 and 3 (classes +3 and -2, respectively) did not indicate optimal vitality, which would be represented by class 1, either (Table 4).

Oxidative stress, which could be clearly detected at the spruce trees from site 2 and 4, can be imposed on plants by pollutant influence, including among others ozone. Air pollution data showed that ozone levels in this region were constantly high compared to threshold values. On the other hand, various other possible reasons for oxidative strain within the plants could be ruled out (for instance by nutrient analyses and meteorological data - cp. site description). Therefore, ozone is certainly suspicious of being one of the main culprits in this case. Generally, ozone levels increase with increasing elevation of the sites (SMIDT 1993). Transferring this fact to the present study, it may explain the worse situation of the spruce trees at the sites 2 and 4, which was indicated by both stress-physiological and cytogenetic vitality parameters. The assumption of prominent ozone impact harmonizes well with the results of our combined investigations, taking

Table 4

Chromosomal aberrations in root tip meristems of Norway spruce at the investigated sites.

	site 1	site 2	site 3	site 4
metaphases	476	528	768	678
breaks/fragments [%]	4.0±2.0	0.7±0.7	0.5±0.5	2.9±1.2
rings [%]	0.0	0.0	0.0	0.3±0.3
connections [%]	0.3±0.3	0.0	0.0	0.0
clumping [%]	1.7±1.2	5.1±2.2	5.7±0.8	4.3±1.1
amorphous mass [%]	0.3±0.3	2.7±1.3	1.1±0.4	1.6±0.4
total [%]	6.3±0.9	8.5±1.2	7.3±0.3	9.1±0.2
cytogenetic site index	1.58	2.13	1.83	2.28
cytogenetic class	-2	-3	+3	-3

Percentages represent percent aberrations of total metaphases. Figures represent mean values ± standard errors. Cytogenetic site indices and cytogenetic classes as described in text.

into account that several studies reported the responds of antioxidants to experimental ozone exposure (MEHLHORN & al. 1986, BERMADINGER & al. 1990). Negative effects of ozone on pigment contents in conifers were found repeatedly (BERMADINGER & al. 1990, HAVRANEK & al. 1990, ROBINSON & WELLBURN 1991, LUCAS & al. 1993), but mostly already accompanied by visible damages. In the present study the observed symptoms concerning patterns of biochemical parameters (increased ascorbic acid and glutathione contents, depressed pigment contents, decreased α -carotene/ β -carotene-ratios) were detectable in green, apparently healthy needles. As far as chromosomal damages are concerned, MÜLLER & GRILL 1994 proved that exposure to such levels of ozone as are present in environment are able to cause a significantly increased number of chromosomal aberrations in root tip meristem cells. These alterations were observable even when visible damages of the trees did not occur.

Nevertheless, it will always be difficult to detect and evaluate the influence of single stresses in field studies. In order to arrive to valid conclusions about specific stresses it is necessary to apply various indication methods in combination. The results of the case study presented in this paper suggest that studying patterns of diverse biochemical reactions and chromosomal aberrations may provide a valuable tool to enhance the possibilities of classical bioindication methods. These methods may render good services in particular for early diagnosis purposes at apparently vital trees.

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