Biochemical Parameters as Biomarkers for the Early Recognition of Environmental Pollution in Scots Pine Trees. I. Phenolic Compounds

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*Pinus sylvestris*L., Catechin, Ferulic Acid, *p*-Coumaric Acid, Picein

Within a series of regular projects investigating the suitability of biochemical parameters as biomarkers for the early recognition of environmental pollution, the levels of selected phenolic compounds were determined in Scots pine needles (*Pinus sylvestris* L.) from young and adult trees at three field sites with different SO_2 pollution (Rösa>Taura>Neuglobsow). Young trees showed no differences in the levels of soluble phenolics in case of increasing loads of SO_2, whereas the concentration of the soluble phenolic component catechin in previous needles of adult trees altered significantly. In current year's needles, differences were only found in the presence of high SO_2 deposition. In contrast to catechin, picein and total phenolics remained unaffected. The concentration of catechin in the previous year's needles of adult trees correlated positively with needle necroses.

The phenolic components of the cell walls of pine needles were also analysed. The main phenolic compounds were identified as *p*-coumaric acid and ferulic acid. Differences in the levels of *p*-coumaric acid were detected in the needles of adult trees between the three sites, with the highest levels being measured at the site with the lowest pollution (Neuglobsow). However, changes in the *p*-coumaric acid content in young trees were low. No site-related differences were found regarding ferulic acid in adult and young pines. The findings are discussed and compared with data reported in the literature.

Introduction

The enhanced production of phenolic compounds in plants due to both biotic and abiotic stress has already been described (Richter and Wild, 1992). The influence of air pollutants (ozone, sulfur dioxide, fluorine compounds and nitrogen dioxide) on various phenolic compounds in conifers in the field (Richter and Wild, 1992; 1994; Karolewski and Giertych, 1995) and in fumigation experiments (Heller *et al.,* 1990; Jensen and Løkke, 1990; Langebartels *et al.,* 1990; Holopainen *et al.*, 1994; Kainulainen *et al.,* 1995) has already been the target of various investigations. In needles of Norway spruce trees 20–30 and 40–50 years old several phenolics have been examined relating to forest damage (Richter and Wild, 1992; 1994). Membrane damaging agents such as high ozone levels and nutrient imbalances characterising the sites studied were considered as factors for the occurrence of damage symptoms. Field investigations of spruce discovered higher levels of flavonol catechin in the needles of damaged trees compared to unaffected ones. In contrast picein (the β-D-glucopyranoside of *p*-hydroxyacetophenone) was found to decrease in needles of damaged trees, although sharp individual variations prevailed (Richter and Wild, 1992). Unlike older trees, the catechin and picein levels of needles of 3-year-old clonal spruce plants were unaffected by exposure to ozone (Heller *et al.,* 1990). Moreover, spruce needle monoterpenes were hardly affected by air pollutants (Kainulainen *et al.,* 1995). In contrast, needles of Scots pine seedlings exposed to high levels of ozone displayed decreased concentrations of some monoterpenes (Kainulainen *et al.,* 1995). In the case of individual stilbenes, a dose-dependent biochemical response to ozone in primary needles of Scots pine seedlings was observed (Rosemann *et al.,* 1991). The content of individual and total resin acids in pine shoots increased after exposure to ozone or nitrogen dioxide and declined with high doses of sulfur dioxide, although low sulfur dioxide levels had no effect (Kainulainen *et al.,* 1995). The experiments performed by

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Kainulainen *et al.* (1995) also revealed that concentrations of total phenolics in spruce and pine seedlings were not affected by ozone, sulfur dioxide or nitrogen oxides. However, Karolewski and Giertych (1995) found in needles of 9-year-old Scots pines an increase in total phenols and orthodiphenols in an environment polluted by sulfur dioxide and fluorine compounds. There is also some evidence that the levels of total phenolics may fluctuate with nutrient levels (Nerg *et al.*, 1994).

The investigations described above dealt with the whole needle. However, air pollutants' first target of attack is cell walls. Therefore, changes to cell wall-bound compounds ought to be assumed by the impact of harmful substances. Phenolics participate in the formation of cross-linkings in cell wall components, a process which is catalysed by peroxidase (Takahama and Oniki, 1992). This reaction is the last step of enzymic reactions leading to the lignification of cell walls (Elstner and Heupel, 1976). Relatively high concentrations (0.1% of the fresh needle material) of cell wall-bound phenolics were detected in spruce needles, with *p*-coumaric and ferulic acid being the main components (Strack *et al.*, 1987). However, little is known about changes to cell wall–bound phenolics in pine needles under field conditions.

To sum up it may be stated that the levels of phenolic compounds vary considerably between different conifers, and that they are also affected by the age of the trees and the type of pollution. In this study the multiple effects of SO_2 and NO_x on the level of total phenolics, the soluble phenolic components catechin and picein, and the cell wall-bound *p*-coumaric and ferulic acid in the current and the previous year's needles of young and adult pine trees from three differently polluted field sites were investigated. Special attention was devoted to the effect of decreasing SO_2 immissions. The findings of soluble and cell wall-bound phenolics are compared with data from the literature and analysed with respect to whether these parameters constitute suitable biomarkers for the detection of multiple stress.

Material and Methods

Field sites and sampling conditions

Pine needles were obtained from young (20–25 yr old) and adult (40–65 yr old) Scots pine stands (*Pinus sylvestris* L.) growing at three differently polluted areas in eastern Germany. Neulobsow is located in a rural area near Lake Stechlin (Land Brandenburg) about 70 km north of Berlin. Taura (Dahlener Heide, Free State of Saxony) is situated 40 km north-east and downwind from Leipzig. Rösa (Dübener Heide, Land Sachsen-Anhalt) is located 9 km west of Bitterfeld, to the north-east of the industrial district of Halle. The atmospheric concentrations of air pollutants were measured at meteorological stations (Neuglobsow, Melpitz, Pouch) close to the respective sampling sites Neuglobsow, Taura and Rösa. The air pollution situation is described in Table I. It is characterised by site-related differences in annual means of SO_2 and NO_x, whereas ozone levels were found to be similar at all three test sites. In the temporal course a plain decrease in SO_2 pollution was recognizable, while NO_x- and O_3-influence remained nearly unchanged.

At each field site, 5 plots were selected where 15 trees were chosen at random. Sampling of the needles always took place between 10–20 October. One twig was cut from each tree in the sun-crown. First- and second-generation needles were collected for analysis. Mixed samples were prepared by blending equal amounts of needles collected from 15 branches. The mixed needles were

Table I. Yearly mean concentrations of SO_2, NO_x and O_3 in $\mu g\ m^{-3}$ measured at meteorological stations close to the field sites.

Test site	Station of measurement	SO_2				NO_x				O_3			
		1993	1994	1995	1996	1993	1994	1995	1996	1993	1994	1995	1996
Neuglobsow	Neuglobsow	11	7	7	10	10	9	10	11	52	56	51	53
Taura	Melpitz	37	23	19	18	–	14	15	16	40	50	50	44
Rösa	Pouch	61	32	25	21	21	22	21	26	69	64	51	50

Station Neuglobsow and Melpitz of the Umweltbundesamt (distance approx. 5 km), Station Pouch of the Landesamt für Umweltschutz Saxony–Anhalt (distance approx. 3 km).

immediately deep-frozen in liquid nitrogen and stored at −80°C until analysis.

Analysis of soluble phenolic compounds

Soluble phenolics were determined using a modified version of the method described by Richter and Wild (1992). The extracts were filtered (Sartorius membrane filters, regenerated cellulose, 25 mm, 1.2 µm). The subsequent HPLC analysis of soluble phenolics (column: LiChrospher RP-18, 250–4 mm; pre-column: LiChrospher RP-18, 4–4 mm, 5 µm , Merck) was carried out as described by the authors.

Determination of total phenolics in methanolic extracts

The analysis of total phenolics took place following the procedure of Schroer (1992). The residue of evaporated methanolic extracts was dissolved in 2x5 ml hot water and following dilution (1:20) used in the test. After the addition of a sulphanilic acid solution, the sample was mixed, incubated for 30 min at 60 °C in a water bath, and then the reaction was stopped in crushed ice. The absorption of phenolics was measured at 429 nm. The results were plotted in accordance with a tannin standard performed in the same manner.

Analysis of cell wall-bound phenolics

To prepare the cell wall fractions, hydrolysis and the HPLC determination of hydroxycinnamic acids using a modified version of the method described by Strack *et al.* (1988) were performed. About 500 mg frozen plant material was powdered and stirred twice for 1 min with 10 ml 80% aqueous methanol. After centrifugation the pellet was washed with 30 ml methanol, 30 ml water, 30 ml acetone and 30 ml ethyl ether before air drying. The dry residue was regarded as the cell wall preparation (c.w.). 30 mg of the preparation was hydrolysed with 2 ml 1 N sodium methylate at 80 °C for 2 h, followed by 1 h at ambient temperature. 1 ml of the mixture was acidified with 100 µl phosphoric acid and centrifuged. An aliquot of 20 µl of the hydrolysed phenolic compounds was separated using HPLC (column: Nucleosil C_{18}, 250–3 mm, 5 µm, Macherey-Nagel, Düren, Germany). The solvent system used was a gradient of A (1.5% phosphoric acid) and B (acetonitrile/water 8/2 v/v). A linear gradient was applied from 0% B in A to 100%B over 30 min. The solvent flow rate was 1 ml/min. Hydroxycinnamic acids were detected at 310 nm.

Needle necroses (damage index)

Needle necroses for the first and second age classes were described by judging needle colour (chloroses) and visible damage (necroses) according to the following key factor, which was stated random by the authors with: 1=green needles, no signs of chlorophyll loss in the needle tip, 1.5= green needles with partially chlorotic tips, 2=all needles with chlorotic tips, 2.5=some needles with 1–5 mm necrotic tips, 3=all needles with 1–5 mm necrotic tips, 3.5=some needles with 5–10 mm necrotic tips, 4=all needles with 5–10 mm necrotic tips. The index of needle necroses making up each class was summed so that mean values are based on totals of 15 twigs per plot.

Statistics

At least 3 aliquots of individual needle sample were measured. Tables were used to calculate site means (n=5 ± standard error). The Mann Whitney U-Test was used for testing pairwise differences between sites. Data which were not different at $P<0.05$ are marked with the same letter in the table; significant differences are indicated by different letters.

Results

The chromatogram of soluble phenolic substances from pine needles has a very complex pattern (Fig. 1 A). In needles of *Pinus sylvestris* L. the levels of total phenolics vary between 9.94 and 61.63 mg g^{-1} d.w. The concentrations of the phenolic compounds studied were in the range of 0.1% (picein) and 4.0% (catechin) of the total phenolics. The peaks were identified by adding a mixture of reference compounds to the sample solution before injection. At the beginning of the investigations into soluble phenolic compounds, we also measured *p*-hydroxyacetophenone (*p*-HAP; the aglycon of picein) and epicatechin (the structural isomer of catechin). However, the levels of *p*-HAP and epicatechin found (0.03% and 0.06%

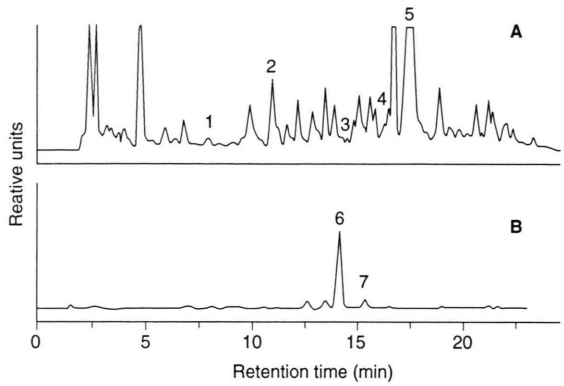

Fig. 1. Chromatogram of soluble phenolics (A) and chromatogram of cell wall-bound phenolic compounds (B) from current year's pine needles of the test site Rösa. 1 picein, 2 catechin, 3 epicatechin, 4 *p*-hydroxyacetophenone, 5 unidentified compound, 6 *p*-coumaric acid, 7 ferulic acid.

of the total phenolics content respectively) were too small to be worth evaluating as the phenolic substances with the highest peaks in the chromatogram could not be identified under these circumstances. Moreover, comparison of the peak height of unidentified compounds in needles from the highly polluted site at Rösa and the far less polluted site at Neuglobsow exhibited no visible differences.

As can be seen in Table II, young pine stands (20–25 yr old) showed no reaction in catechin level depending on air pollution. In adult trees (40–65 yr old) the catechin content of the current year's needles exhibited site-related differences in 1993, with the lowest level being in Taura, whereas in 1995 and 1996 no differences were found. In the previous year's needles, clear differences were observed concerning catechin concentration between the three sites in 1995 and 1996. In the year 1992 a pollution-related increase was only ascertained in Rösa. After the catechin content had increased 1.4- to 2.1-fold in the previous year's needles from 1992 to 1995, a significant change was only established between 1992 and 1996 in Taura. Compared to the first needle age class the catechin level of the second needle age class was significantly enhanced in the years 1995 and 1996. In current year's needles the catechin levels increased from 1993 to 1996 in Neuglobsow and Taura. In both needle age classes the catechin levels were subject to annual variations at all three sites.

In the picein content of the pine needles (Table II), site-dependent differences were only found in 1992 for the second needle age class of adult trees. Just as in the case of catechin, the picein level of the previous year's needles was significantly higher than in the current year's needles (1995 and 1996). In contrast to catechin, the picein level of the first needle age class decreased from 1993 to 1996, but remained unaffected in the second needle age class from 1992 to 1996 with the exception of Taura.

The total phenolic content (Table II) essentially displayed no site-dependent variations in needles of young and adult trees. However, in previous year's needles the contents were significantly higher than in current year's needles. Over the course of time (1992–96 and 1994–96) there were significant decreases in both the first and the second needle age.

We were interested in examining the harm caused by air pollutants at the level of phenolic compounds in visually necrotic and green needle tissues. Therefore the phenolic levels in 8 segments of the previous year's needles (n=500 to 600) with tip necroses harvested in October 1996 from the test site in Rösa were studied. As can be seen in Table III, in the first mostly necrotic segment (0–5 mm) the amounts of catechin and total phenolics were about 1.5-times higher than in the following segments. Picein exhibited a small increase in the second segment (5–15 mm). In the other segments there were similar levels with regard to both catechin and total phenolics.

Study of the cell walls revealed considerable differences compared to the needle as a whole. Cell walls were found to contain a very much lower diversity of phenolics than soluble extracts of total needles. The chromatogram of phenolic compounds from the test site Rösa (Fig. 1 B) showed *p*-coumaric acid to be the main component of the cell wall-bound phenolics in Scots pine needles at 11.5–14.7 μmol g^{-1} c.w., compared to 1.8–2.2 μmol g^{-1} c.w. ferulic acid. The values given in Table IV demonstrate that p-coumaric acid levels in the needles of young stands exhibit only low site differences, but are much more site-dependent in adult stands. Just as in the case of total phenolics (1994) and catechin (1993), the lowest concen-

Table II. Contents of catechin, picein and total phenolics (mean values ± standard errors) in extracts of current and previous year's needles from adult (40–65 yr old) and young (20–25 yr old) pine stands. Significance was determined according to the Mann Whitney U–test. Different small letters (a, b, c) within the rows indicate significant differences between the sites and different large letters (A, B) within the columns indicate significant temporal differences at $P<0.05$ (reference year 1992,1993 or 1994).

Year	Neuglobsow	Taura	Rösa
	Catechin (μg g^{-1} d.w.)		
Adult stands / Current year's needles			
1993	539.16 ± 12.67 a; A	403.08 ± 22.48 b; A	930.37 ± 76.64 c; A
1995	841.38 ± 82.20 a; B	822.27 ± 51.15 a; B	1423.25 ± 276.70 a; A
1996	704.03 ± 41.55 a; B	643.71 ± 28.06 a; B	743.36 ± 53.49 a; A
Adult stands / Previous year's needles			
1992	915.57 ± 27.06 a; A	871.54 ± 49.68 a; A	1381.48 ± 90.33 b; A
1995	1246.55 ± 109.74 a; B	1832.34 ± 23.99 b; B	2592.19 ± 98.21 c; B
1996	872.93 ± 30.05 a; A	1359.61 ± 90.45 b; B	1139.22 ± 43.51 b; A
Young stands / Current year's needles			
1993	517.21 ± 68.60 a	603.08 ± 77.12 a	560.79 ± 40.58 a
	Picein (μg g^{-1} d.w.)		
Adult stands / Current year's needles			
1993	33.94 ± 2.01 a; A	28.51 ± 2.43 a; A	34.74 ± 1.24 a; A
1995	10.66 ± 1.47 a; B	7.22 ± 1.02 a; B	10.58 ± 2.20 a; B
1996	7.23 ± 0.65 a; B	7.05 ± 0.28 a; B	5.20 ± 0.41 b; B
Adult stands / Previous year's needles			
1992	23.60 ± 1.50 a; A	14.46 ± 1.08 b; A	15.54 ± 0.54 b; A
1995	19.27 ± 2.86 a; A	16.11 ± 3.05 a; A	17.05 ± 1.95 a; A
1996	20.34 ± 1.23 a; A	17.98 ± 0.70 a,b; B	17.14 ± 0.87 b; A
Young stands / Current year's needles			
1993	32.41± 2.54 a	34.24 ± 1.59 a	30.02 ± 2.03 a
	Total phenolics (mg g^{-1} d.w.)		
Adult stands / Current year's needles			
1994	21.54 ± 1.22 a,b; A	17.80 ± 1.03 a; A	22.56 ± 1.62 b; A
1995	31.84 ± 3.99 a; A	30.69 ± 2.89 a; B	27.67 ± 3.11 a; A
1996	9.94 ± 0.80 a; B	10.17 ± 0.51 a; B	12.23 ± 1.75 a; B
Adult stands / Previous year's needles			
1992	51.75 ± 3.00 a; A	61.63 ± 2.54 b; A	59.21 ± 1.99 a,b; A
1995	49.91 ± 1.03 a; A	35.19 ± 1.64 b; B	44.17 ± 2.55 a; B
1996	29.51 ± 1.45 a,b; B	26.64 ± 1.48 a; B	31.54 ± 1.85 b; B
Young stands / Current year's needles			
1994	21.38 ± 0.91 a	19.13 ± 2.09 a	18.57 ± 3.02 a

tration of *p*-coumaric acid was found in Taura; the highest levels were measured at the Neuglobsow site with the lowest pollution. Data of ferulic acid (Table IV) showed no site-related differences in the needle levels of young and adult trees.

Discussion

The pattern of soluble phenolic compounds in extracts of pine and spruce needles exhibits considerable differences. The levels of catechin (1.39–8.93 μmol g^{-1} d.w.) and picein (0.02–0.12 μmol g^{-1} d.w.) found in pine needles during this study are much lower than the concentrations measured in spruce needles. Heller *et al.* (1990) reported 13.4–32 μmol g^{-1} f.w. catechin and 15.9–47.7 μmol g^{-1} f.w. picein for the first needle age class. Richter and Wild (1992) found 55–100 μmol g^{-1} d.w. catechin and 50–80 μmol g^{-1} d.w. picein in the second needle age class. According to Strack (1988), picein is the main soluble phenolic component in spruce needles, whereas its aglycon *p*-hydroxyacetophenone only appears as a side-component. The flavonoid compounds catechin and epicatechin behave similarly: catechin is also found to

Table III. Contents of catechin, picein (μg g^{-1} d.w.) and total phenolics (mg g^{-1} d.w.) in different needle segments (mm) of a mixed sample from 500 to 600 previous year's needles from adult (40–65yr old) pine stands of the test site Rösa harvested in October 1996.

Needle segment	Catechin	Picein	Total phenolics
0–5	2243.40	18.30	47.59
5–15	1370.70	22.77	32.46
15–25	1588.37	20.00	27.31
25–35	1493.03	18.47	25.26
35–45	1329.63	19.02	25.77
45–55	1378.38	18.22	24.28
55–65	1399.94	18.67	20.01
>65	1502.37	18.68	24.98

Table IV. Contents (mg g^{-1} c.w.) of cell wall–bound phenolics p–coumaric and ferulic acid (mean values ± standard errors) of current year's needles from adult (40–65 yr old) and young (20–25 yr old) pine stands harvested in October 1994. Significance was determined according to the Mann Whitney U–test. Different letters within the columns indicate significant differences between the sites at $P<0.05$.

Test site	*p*–Coumaric acid	Ferulic acid
	Adult stands	
Neuglobsow	2.42 ± 0.06 a	0.42 ± 0.01 a
Taura	1.89 ± 0.03 b	0.35 ± 0.01 b
Rösa	2.13 ± 0.04 c	0.36 ± 0.02 a,b
	Young stands	
Neuglobsow	2.38 ± 0.08 a	0.42 ± 0.02 a
Taura	2.10 ± 0.06 b	0.38 ± 0.02 a
Rösa	2.18 ± 0.07 a,b	0.38 ± 0.02 a

be the main component, whereas the quantities of epicatechin detected are much smaller.

In pine needles neither catechin nor picein constitute main components among the soluble phenolic compounds. The quantities of *p*-hydroxyacetophenone and epicatechin are insignificant. These differences in the secondary substance metabolism of different conifers have not yet been explained.

In addition to the total phenol content of soluble phenols, we also focused on catechin and picein as they have proved to be good indication parameters in field investigations. The goal of the present work was to study the multiple effects of SO_2 and NO_x on the levels of selected phenols in pine needles, with the effects of decreasing SO_2 immissions over time being of particular interest. The findings showed that of the soluble phenolics only catechin and of the cell wall-bound compounds only *p*-coumaric acid displayed site-related changes. Moreover, only the adult stands (but not the young trees) showed a reaction in terms of phenol level, which concerning the soluble phenolics is restricted to the second needle age class. In the first needle age class, the phenolics are subject to sharp metabolic changes (Dittrich *et al.*, 1989), which is why the second needle age class is often preferred when examining phenolic substances (Richter and Wild, 1994).

In the adult pine stands investigated, the catechin level in the previous year's needles was significantly higher than in the current year's needles. This phenomenon was also found to apply to picein and total phenolic contents. These results coincide with the findings of papers (Hatcher, 1990; Heller *et al.*, 1990; Kainulainen *et al.*, 1994; Nerg *et al.*, 1994) in which lower phenol concentrations were detected in younger conifer needles than in older ones.

The investigations described in the literature (Kainulainen *et al.*, 1995; Karolewski and Giertych, 1995) lead us to suppose that SO_2 and NO_x affect the phenolic levels of pine needles. By contrast, our study indicates that the changes in the levels of soluble phenolics are not caused by immissions effects. Although catechin shows site-related differences depending on SO_2 pollution and picein and total phenol decrease over time, there is no connection between differences in the three sites among the individual phenolic components and changes over time. Similarly, no correlation was detected between the phenolics investigated and the sulfur and nitrogen components occurring depending on the immissions situation.

By contrast it has been reported in a number of studies that nutrient availability and in particular the nitrogen level affects the levels of secondary metabolites in plants, with the concentrations of mineral nutrients (Balsberg Påhlsson, 1989) and nitrogen (Haukioja *et al.*, 1985; Dustin and Cooper-Driver, 1992; Nerg *et al.*, 1994) negatively correlating with the phenolic level. However, when considering the entire test area during all the years of investigation, we only found minor impact on the part of nitrogen on the catechin and total phenolic levels in the pine needles. In the second needle age class, the phenolic levels tended to rise as nitrogen increased, although firm statistical findings were not produced.

The effect of nitrogen on various phenolic compounds is unclear. For example in the case of terpene, reductions, no change and increases have all been reported under the influence of nitrogen (Nerg *et al.,* 1994). A lack of nutrients (N, P, etc.) combined with unrestricted light and photosynthesis result in a low level of nitrogen in the leaves of the plants and the accumulation of secondary carbon-based metabolites such as phenolics. However, if nutrient availability increases but photosynthesis is limited, nutrients will accumulate and there will be a lack of carbon skeletons, and under these circumstances the plants will reduce the levels of carbon-based secondary substances (Haukioja *et al.,* 1985; Dustin and Cooper-Driver, 1992). A decrease in phenol concentrations in the pine needles investigated was not detected with rising nitrogen content. Changes to photosynthesis cannot be the reason for alterations to the phenol levels either since photosynthesis in the three test areas did not differ with respect to the effect of immissions (Dudel *et al.,* 1995). It must therefore be assumed that changes in the phenol budget are caused not by one single parameter but rather the complex interplay between different metabolic processes.

Consequently other biochemical parameters (Schulz *et al.,* 1996) listed in Table V were included in the investigations and subjected to multivariate data evaluation using factor analysis (Berg *et al.,* 1995; Soares *et al.,* 1995). The composition of the individual complexes of characteristics (Table VI) reveals that the complex of characteristics of the 2nd factor expresses nitrogen effects, while the complex of characteristics of the 3rd factor expresses sulfur effects. The complex of characteristics of the 1st factor indicates that there is a connection between phenolics, the mineral nutrient magnesium and the necroses. In the adult pine stands investigated, needle necroses occur to a greater extent in the previous year's needles, while the catechin level in the entire needle extract also rises. In fact a positive correlation exists between the catechin level and the necroses (Fig. 2). It ought to be assumed that the rise observed in the catechin level is caused by defence reactions, an assumption which is backed up by findings reported in the literature (Karolewski, 1990; Giertych and Karolewski, 1993). The defence mechanisms are primarily linked to an intensified lignification process, resulting in the healthy tissue being separated from the necrotic tissue (Karolewski and Giertych, 1994), with various changes occurring in the metabolism of the phenolics depending on the degree of damage (Karolewski and Daszkiewicz, 1988). In addition it could also be concluded that the defence mechanism only takes effect after long pollutant exposure and correspondingly severe damage. A positive correlation between the catechin level and the degree of damage was also observed by Richter *et al.* (1996) in the second needle age class in adult spruce stands (40–60 yr). Moreover, the results of our investiga-

Table V. Contents of different biochemical parameters (mean values ± standard errors) in extracts of current year's needles from adult (40–65 yr old) pine stands in 1993 (SCHULZ *et al.,* 1996). Significance was determined according to the Mann Whitney U–test. Different small letters (a, b, c) within the rows indicate significant differences between the sites at $P<0.05$.

Parameter	Neuglobsow	Taura	Rösa
Magnesium (mg g^{-1} d.w.)	0.81 ± 0.03 a	0.87 ± 0.04 a	0.91 ± 0.03 a
Non-protein nitrogen (mg g^{-1} d.w.)	3.00 ± 0.07 a	3.56 ± 0.16 b	6.90 ± 1.09 c
Sulfate (mg g^{-1} d.w.)	0.72 ± 0.02 a	1.38 ± 0.02 b	1.47 ± 0.04 b
Sulfur (mg g^{-1} d.w.)	1.25 ± 0.05 a	1.67 ± 0.05 b	1.85 ± 0.06 b
Glucose (mg g^{-1} d.w.)	4.22 ± 0.28 a	3.21 ± 0.35 b	2.37 ± 0.31 b
Glutamine (µg g^{-1} d.w.)	104 ± 13 a	272 ± 29 b	558 ± 22 c
Glutathione (µg g^{-1} d.w.)	314 ± 12 a	469 ± 12 b	441 ± 24 b
PEP-Carboxylase (Unit g^{-1} d.w.)	0.59 ± 0.03 a	1.55 ± 0.41 b	1.15 ± 0.12 b
Necroses	1.62 ± 0.09 a,b	1.52 ± 0.02 a	1.81 ± 0.07 b

Table VI. Factor loadings of the rotated matrix for 11 needle characteristics.

Characteristics	Factor 1	Factor 2	Factor 3
Total phenolics	0.88322	0.01487	−0.02668
Magnesium	−0.85659	−0.08116	−0.00482
Necroses	0.78157	0.39470	−0.00168
Catechin	0.68177	0.40974	0.31214
Non-protein nitrogen	0.13260	0.91689	0.07065
PEP-Carboxylase	0.27234	0.84083	0.25678
Glutamine	0.24986	0.79412	0.43686
Glucose	0.50280	−0.58246	0.44955
Glutathione	−0.18550	0.04909	0.91271
Sulfate	0.06038	0.14452	0.88645
Sulfur	0.28711	0.34425	0.85212

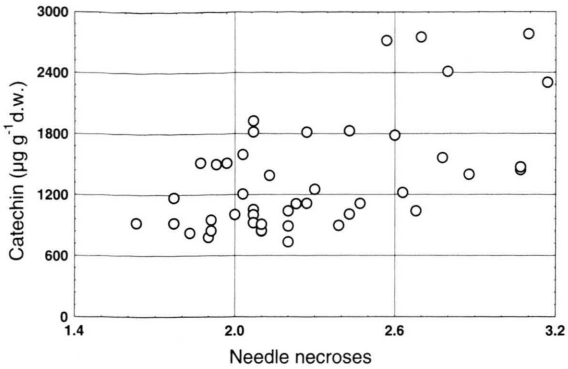

Fig. 2. Correlation between the catechin content and the state of necroses in previous year's needles of adult pine stands (40–65 yr old) at the three field sites. For determination of needle necroses see Materials and Methods.

tions demonstrate that no correlation exists between the catechin level and damage symptoms in young pines (20–25 yr).

Tip necroses are plain in the second needle age class at the test site in Rösa. When examining the individual needle segments, significantly higher total phenol and catechin levels were found in the first 5 millimetres (i.e. the visually damaged part of the needles) than the following visually undamaged segments (Table III). It can merely be supposed that the higher phenol level in the tips of the needles is caused by the zone between the necrotic and undamaged tissue. In contrast to Karolewski (1990), who was able to separate the intermediate zone in young pine seedlings as a dark band, this is not possible in the needles of adult pine stands.

What actually happens in the cell walls under the impact of pollutants? Although *p*-coumaric acid significantly decreases in the more heavily polluted areas (Table IV), the differences in level are only minor. A clear relationship between the immissions situation and the cell wall-bound phenolics was not found. One possible explanation for the decrease in p-coumaric acid in the cell walls of the more heavily polluted needles could be the increasing requirement for phenol acid as a preliminary stage for lignin biosynthesis. The involvement of *p*-coumaric acid in lignification processes has been described (Maillard and Berset, 1995). The polymerisation of lignin in the cell wall takes place after the oxidation of hydroxycinnamic acid alcohol to phenoxy radicals. The half-life of these free radicals is reached just before they react with each other to form lignin and create bonds between lignin and the polysaccharides of the cell walls (Bolwell, 1988). Rosemann *et al.* (1991) arrived at similar conclusions concerning the decreasing level of stilbenes in pine needles. They supposed that the decrease is due to metabolisation such as glycosylation or oxidation.

Fluctuations in the phenolic level which occur over time in particular with respect to catechin (current and previous year's needles) and total phenol (current year's needles) have already been described for conifers (Hatcher, 1990). They can probably be explained by the non-linear nature of phenolic reactions (Giertych and Karolewski, 1993). A rise due to the activation of the biosynthesis of the phenolics is followed by a decrease owing to oxidation and polymerisation, which in turn is followed by an increase.

In our areas of investigation, the levels of phenolic compounds depending on immissions pollution are not directly affected by either nitrogen or sulfur. Dose-effect relations were not detected at the individual test areas. By contrast, a causal link was ascertained between the necroses caused by air pollution and catechin. The present findings permit the conclusion that total phenol content, picein and ferulic acid in pine needles show no noteworthy reaction to airborne pollutants and are thus unsuitable for detecting effects. In spruces, too, picein can be disregarded as a parameter for the diagnosis of damage owing to level fluctuations (Richter and Wild, 1994). Catechin and *p*-coumaric acid in pine needles react to multiple stress, although in the case of catechin the age of the trees (40–65 yr) and of the needles is of decisive importance. However, these two phenolics make unsuitable early indication parameters as they only exhibit reactions following severe damage.

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