Phenolic Compounds in Needles of Norway Spruce Trees in Relation to Novel Forest Decline. II. Studies on Trees from Two Sites in Middle Western Germany

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The content of several phenolic compounds in needles of 20- to 30-year-old Norway spruce trees (Picea abies) was measured using HPLC. The results of two forestry sites in middle western Germany are reported in this paper. They are part of a research programme on novel forest decline which was carried out in various regions of Germany. Distinct amounts of picein, catechin, piceatannol glucoside, and other phenolic compounds were detected in the studied spruce needles. Additionally, their contents changed in relation to damage. Some compounds, especially catechin, showed increased levels in the needles of the damaged trees compared to the undamaged ones. Here, the values for the undamaged trees of the different sites were similar. Concerning the changes in picein contents, however, there was a great difference between the sites. p-Hydroxyacetophenone was detected in very low amounts only and did not correlate with damage.

These results are compared with earlier findings from another site that shows severe damage. The role of phenolic compounds as indicators of tree damage is discussed.

Introduction

An enhanced production of phenolic compounds and other biologically active substances was repeatedly observed in plants subjected to physiological stress (Howell, 1970; Howell and Kremer, 1973; Friend, 1979; Flint et al., 1985). This was found concerning the influence of both biotic and various abiotic factors. Howell (1974), for example, described that ozone caused changes in the metabolism of phenolics similar to those induced by other factors such as diseases, mechanical stresses, and nutrient deficiencies. Several other authors also summarized in their publications these kinds of phenomena (Tingey et al., 1975; Rubin et al., 1983; Hahlbrock and Scheel, 1987) which were often regarded as a consequence to the loss of cell compartmentalization (Friend, 1976; Matile, 1984; Kemp and Burden, 1986). In this connection phenolic compounds are also studied in forest damage research, where membrane damaging agents like ozone and nutrient imbal-

Abbreviations: d, damaged; DW, dry weight; Ha, Hattgenstein site; pHAP, p-hydroxyacetophenone; PTG, piceatannol glucoside; u, undamaged; Wa, Wallmerod site; ym, yearly mean value.

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found lower levels of picein and higher contents of most other phenolic compounds studied. We now report on results from 20- to 30-year-old spruce trees at two forestry sites in western Germany, one of which shows relatively moderate symptoms of tree damage, while the trees of the other site are apparently healthy. The goal of the present study was to find out if changes in the content of phenolic compounds follow a general response pattern at different sites which is necessary for their evaluation as biochemical parameters characterizing tree damage.

The studies presented here are part of a project dealing with the physiological, biochemical, and histological characterization of spruce needles from open-air habitats in order to find diagnostic parameters for spruce tree damage (Benner and Wild, 1988; Wild et al., 1990; Hasemann and Wild, 1990; Tenter and Wild, 1991; Richter and Wild, 1992; Yang et al., 1993; Schmieden et al., 1992).

Materials and Methods

Description of the sites

Hattgenstein

This area is part of the Hunsrück mountains in western Rhineland-Palatinate (Forestry Office Idar-Oberstein, Forest District Hattgenstein, Division 257 b). It is located at approximately 650 m above sea level showing a slight inclination towards the southeast. The soil of the studied plantation is a podsolated acidic brown-earth which is covered by raw humus and set on quartzite. Its pH value ranges between 2.7 and 3.5 in the upper and between 3.6 and 3.8 in the lower layers. The soil is short of nutrients (Sabel, 1991).

At this site ozone is the main air pollutant on account of high levels that often rise to half-hourly mean values of 200–300 μg m⁻³. Maximal monthly mean values amounted to 140 μg m⁻³ in 1985 and 1986, and 120 μg m⁻³ in 1989. The immision and climatic data were registered at the station Leisel which is situated at a distance of 6 km from the experimental site at about the same altitude (ZIMEN, Central Network for the Measurement of Immissions in Rhineland-Palatinate, 1985–1989).

From a population of 25- to 30-year-old Norway spruce trees (Picea abies (L.) Karst.), height ca. 10 m, ten trees were chosen in the spring. The apparently healthy trees (damage class 0-1) were standing in close vicinity of the damaged trees (damage class 1–2; for classification parameters see Forest Damage Survey (Waldzustandsbericht 1989). The damage symptoms comprise needle loss and yellowing mainly on the light-exposed parts of needles. Older needles were generally more affected than younger ones. Needle samples of single trees were taken on four dates during the vegetation period in 1989.

Wallmerod

The area is situated in the Westerwald mountains at an altitude of 495 m above sea level on an almost even plateau (Forestry Office Wallmerod, Forest District Höhn, Division 1 C). Slightly stony, loess loam-rich brown-earth constitutes the soil, that shows a good supply of nutrients. No signs of enhanced needle loss nor yellowing of needles were observed on the Norway spruce trees of this site. Five 20-year-old trees (damage class 0) were selected for the following measurements. Needles were combined to bulk samples for each generation since the results of a number of previous years demonstrated on various parameters a pronounced physiological homogeneity of the needles originating from different trees (Wild et al., 1990).

Plant material and sampling

During the growth period 1989 spruce needles were harvested at the two forestry sites. For that, south to south-west exposed branches were taken from each sixth to eighth whorl of the spruce trees. After separating the different needle age classes, the twig-segments were immediately frozen in liquid nitrogen and the needles were stripped off. The latter were kept in liquid nitrogen until storage at −80 °C. The second needle age class (needles sprouted in the previous year), which was always still completely green, was used for the experiments. For each date, harvesting at Hattgenstein and Wallmerod was carried out on subsequent days.

Extraction of phenolic compounds

0.5 g of deep-frozen needles together with 100 μl internal standard (gallic acid, 45 mM in 50% meth-
anol) were homogenized in 3.5 ml 80% aqueous methanol, that was chilled to -20 °C before (Ultra Turrax T25, Janke & Kunkel). After centrifugation of the homogenate the pellet was washed with 1.5 ml chilled 80% methanol. The combined supernatants were adapted to room temperature and afterwards the volume was made up to 5 ml to compensate evaporation of methanol. In order to remove remaining particles the extract was filtered (Sartorius membrane filters, regenerated cellulose, 13 mm, 0.2 μm). 1 ml of the filtrate was purified from lipids by solid phase extraction (SEP-PAK C18 Cartridges, Waters/Millipore).

For following HPLC analysis 1 ml of natrium acetate buffer (0.5% glacial acetic acid plus NaOH), pH 3.3, was added. The extracts were directly analyzed or stored at -20 °C. From each needle sample two (single trees) or three (bulk samples) parallel extracts were prepared.

High performance liquid chromatography

The extracted phenolic compounds were separated and quantitatively determined by reversed phase HPLC (HPLC two-pump system, LKB; Nucleosil C18 column, 125 mm x 8 mm x 4 mm, 5 μm, 100 Å, Macherey-Nagel; pre-column, LiChrospher RP-18, 5 μm, LiChroCart 4-4, Merck). Elution was performed at room temperature with a flow rate of 1 ml min−1. The mobile phase consisted of 0.5% natrium acetate buffer, pH 3.3 (eluent A) and methanol (LiChrosolv Gradient grade, Merck; eluent B). The separation was achieved by a four-step gradient running from 17% to 80% eluent B according to Richter and Wild (1992). The phenolic compounds were detected through absorption of light at a wavelength of 275 nm (Shimadzu, SPD-6 AV). Each needle extract was analyzed twice.

For more details concerning the identification of the respective phenolic compounds see Richter and Wild (1992).

Results

Chromatogrammes of methanol-soluble phenolic compounds from spruce needles of the Wallmerod and Hattgenstein sites can be seen in Fig. 1. Both needle samples derive from undamaged trees. Distinct amounts of picein, catechin, piceatannol glucoside, and two still unidentified compounds (see Richter and Wild, 1992) and lower levels of epicatechin and pHAP were found. The levels of the studied phenolic compounds do not show distinct seasonal variations in the spruce needles of the considered sites, as far as the different harvest dates are regarded. Only the values for the damaged trees at the Hattgenstein site tend to increase during summertime (Fig. 2–6, Table I).

The content of catechin, epicatechin, piceatannol glucoside, and the two unknown phenolic compounds, which are eluted from the column after 5.5 and 9.1 min, shows elevated levels for the damaged trees compared to the undamaged ones on the single harvest dates (Fig. 2–4, Table I). This increase is significant for catechin and "peak 9.1'" in summer and for catechin it is also significant for autumn (Fig. 2 and 4). Yearly mean values of the concentrations of catechin, PTG, and of the two still unidentified compounds show significant increases in the needles in relation to damage. Comparable concentrations of all these compounds were found in the undamaged trees at the Hattgenstein and Wallmerod sites. Sometimes a tendency to even lower values can be observed at the

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Sample</th>
<th>pHAP (μmol g⁻¹ DW)</th>
<th>Epi-catechin (μmol g⁻¹ DW)</th>
<th>Peak 5.5' (μmol g⁻¹ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>03-May-89</td>
<td>Wa</td>
<td>3.6</td>
<td>18.8</td>
<td>7.0</td>
</tr>
<tr>
<td>02-May-89</td>
<td>Ha u</td>
<td>4.4 (4.3)</td>
<td>20.7 (6.6)</td>
<td>6.4 (1.5)</td>
</tr>
<tr>
<td></td>
<td>Ha d</td>
<td>3.6 (2.3)</td>
<td>28.1 (3.8)</td>
<td>7.2 (0.8)</td>
</tr>
<tr>
<td>19-Jun-89</td>
<td>Wa</td>
<td>2.0</td>
<td>22.2</td>
<td>7.3</td>
</tr>
<tr>
<td>20-Jun-89</td>
<td>Ha u</td>
<td>1.8 (1.0)</td>
<td>24.1 (4.1)</td>
<td>6.8 (1.7)</td>
</tr>
<tr>
<td></td>
<td>Ha d</td>
<td>3.3 (1.3)</td>
<td>28.8 (1.7)</td>
<td>7.9 (1.6)</td>
</tr>
<tr>
<td>14-Aug-89</td>
<td>Wa</td>
<td>1.5</td>
<td>24.3</td>
<td>9.4</td>
</tr>
<tr>
<td>15-Aug-89</td>
<td>Ha u</td>
<td>3.7 (3.1)</td>
<td>27.9 (6.6)</td>
<td>9.0 (2.1)</td>
</tr>
<tr>
<td></td>
<td>Ha d</td>
<td>3.7 (2.9)</td>
<td>29.7 (9.4)</td>
<td>10.5 (1.3)</td>
</tr>
<tr>
<td>09-Nov-89</td>
<td>Wa</td>
<td>3.6</td>
<td>24.5</td>
<td>9.4</td>
</tr>
<tr>
<td>10-Nov-89</td>
<td>Ha u</td>
<td>2.1 (1.1)</td>
<td>27.4 (4.1)</td>
<td>9.0 (1.6)</td>
</tr>
<tr>
<td></td>
<td>Ha d</td>
<td>2.4 (0.9)</td>
<td>30.1 (5.5)</td>
<td>9.5 (1.2)</td>
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<tr>
<td>yearly</td>
<td>Wa</td>
<td>2.7 (1.1)</td>
<td>22.4 (2.7)</td>
<td>8.3 (1.3)</td>
</tr>
<tr>
<td>mean value</td>
<td>Ha u</td>
<td>2.9 (1.2)</td>
<td>25.8 (4.4)</td>
<td>7.8 (1.4)</td>
</tr>
<tr>
<td></td>
<td>Ha d</td>
<td>3.3 (0.6)</td>
<td>29.9 (1.9)</td>
<td>8.8 (1.5)*</td>
</tr>
</tbody>
</table>

* p < 0.05 for the difference between Ha u and Ha d.
The picein content in the needles of damaged spruce trees from the Hattgenstein site is two to three times higher than that of the undamaged trees from the same site (Fig. 5). This difference is significant for all harvest dates and the annual mean value though there are considerable variations between the individual trees. The needles of the healthy trees at the Wallmerod site, however, contain similar amounts of picein compared to the needles of the damaged trees at Hattgenstein, and their yearly mean values are almost identical.

*p*-Hydroxyacetophenone, the aglycone of picein, was found in only very low amounts, and differences between individual trees are considerable. No damage-related changes were observed (Table I).

The sum of all detected phenolic compounds – except picein – (Fig. 6) always shows distinct and significant increases in the needles of the damaged trees compared to those of the undamaged trees at the Hattgenstein as well as at the Wallmerod site. It has to be considered that this sum also includes compounds that do not distinctly react to damage.
Fig. 2. Contents of catechin in spruce needles of the second age class from the Wallmerod and Hattgenstein sites. Values were obtained from bulk samples for Wallmerod and mean values from five trees for Hattgenstein on various harvest dates in 1989 (ym = yearly mean value). Standard deviations are given as bars. Significance values were determined according to Student's t-test for differences at Hattgenstein on the individual harvest dates: June and August: $p = 0.04$; November: $p = 0.09$ (difference for May is not significant); ym 1989: $p = 0.03$. □ Wallmerod undamaged trees, □ Hattgenstein undamaged trees, ■ Hattgenstein damaged trees.

Fig. 3. Contents of PTG in spruce needles from the Wallmerod and Hattgenstein sites. Significance value for ym 1989: $p = 0.02$ (Ha). For details see Fig. 1.

Fig. 4. Contents of a phenolic compound with $t_R = 9.1$ min (probably o-coumaric acid glucoside) in spruce needles from the Wallmerod and Hattgenstein sites. Values are calculated as gallic acid equivalents. Significance values for Hattgenstein on the individual harvest dates: $p = 0.04$ (June) and $p = 0.08$ (August); ym 1989: $p = 0.01$. For details see Fig. 1.

Fig. 5. Contents of picein in spruce needles from the Wallmerod and Hattgenstein sites. Significance values for Hattgenstein on the individual harvest dates: $p = 0.02–0.03$; ym 1989: $p = 3.5 \times 10^{-6}$. For details see Fig. 1.

Discussion

In continuation of our studies described in part I of this paper (Richter and Wild, 1992), we examined phenolic compounds in needles of young spruce trees from two other sites. The results of the analysis concerning the Wallmerod and Hattgenstein sites showed an occurrence and a distribution of phenolic compounds quite similar to those of the Freudenstadt site. Furthermore, the data show that the method which was developed earlier (Richter and Wild, 1992) is very suitable to analyze single main phenolic compounds in spruce needles from different spruce tree sites.

For comparative studies on damaged as well as on undamaged trees it is important to choose the second needle age class since current year’s needles show pronounced developmental changes in the composition of phenolic compounds (Dittrich et al., 1989). At the sites in question as well as at the Freudenstadt site, the levels of phe-
nolic compounds remained rather constant in the studied needles of the undamaged trees during the growth period (see also Yee-Meiler, 1974; Osswald and Benz, 1989). Thus, seasonal variations do not interfere with damaged-related differences.

At the Hattgenstein site the contents of a number of phenolic compounds – particularly catechin – were increased in relation to damage. These changes can be seen on single harvest dates and are even more pronounced in the yearly mean values. Kicinski et al. (1988) also reported an increase in catechin contents in relation to damage in fumigated young spruce trees. The results for PTG on the harvest dates are affected by large individual variations among the trees. This may be explained by the fact that the trees at the Hattgenstein site are of heterogeneous provenance. Solhaug (1990) found that contents of stilbene glucosides vary not only in different Picea species but also with provenance. Catechin and other phenolics occur in relatively equal amounts in the trees studied here. Values for these compounds in undamaged trees are similar at both the Hattgenstein and Wallmerod sites.

Picein shows pronounced increases in relation to damage at the Hattgenstein site, but levels for the healthy trees at the Wallmerod are similar to those of the damaged trees from the former site. Additionally, variations among individual trees are very large as it was found for the Freudenstadt site (Richter and Wild, 1992). As to pHAP, we found it again in only very low amounts at all considered sites (Osswald and Benz, 1989; Heller et al., 1990). In our studies it shows no damage-related trends. Picein and pHAP have frequently been studied in forest decline research. Many authors found a reduction of picein related to severe damage whereas others could not observe any differences at all (Grill et al., 1975; Hoque, 1984; Kicinski et al., 1988; Strack, 1988; Osswald and Elstner, 1989; Heller et al., 1990; Jensen and Løkke, 1990; Richter and Wild, 1992). The values of Osswald and Benz (1989), however, indicate an increase of picein in damaged trees. Damage-related differences in levels of phenolic compounds can be better interpreted if the results of Wallmerod and Hattgenstein are compared with those of the Freudenstadt site. This summarizing arrangement of results (Fig. 7) demonstrates that the sites studied show opposite trends in damage-related differences of picein contents in spruce needles (Fig. 7b) thus explaining the different results published in literature. We conclude that picein and pHAP can be ruled out as parameters for damage diagnosis.

For the levels of catechin, epicatechin, PTG, and the two still unidentified compounds that are probably p-coumaroyl quinic acid and o-coumaric acid glucoside (Kicinski and Kettrup, 1987; Richter and Wild, 1992) the comparison of all studied sites (Fig. 7a, c–e) demonstrates a clear damage-related increase. Catechin, epicatechin, and “peak 9.1” show the highest values for the damaged trees at the Freudenstadt site, which belong to the highest damage class of all studied trees. The comparison further shows that absolute quantities of these compounds from undamaged trees are equal on all studied sites. Obviously, increasing degrees of damage correlate with rising levels of certain phenolic compounds, which is apparently independent from the site. So the observation that damage-related increases at Hattgenstein are not always significant can be explained by the fact that differences between the degrees of damage of the trees at the Hattgenstein site are small whereas they are great at the Freudenstadt site. Thus, for diagnostic studies on trees of low damage classes a larger number of samples is necessary for the statistical evaluation of results.
If the contents of all studied phenolic compounds – except picein – are summarized, the already described damage-related increases become even more evident although compounds that did not significantly increase in the needles of the damaged trees of the Hattgenstein site (but did so at the Freudenstadt site) are included. Several authors (Yee-Meiler, 1974, 1978; Grill et al., 1975; Kicinski et al., 1988) report on damage-related increases in total phenols in spruce needles. Most of them used colorimetrical tests that do not apply to picein and pHAP. In our studies at all three sites, a rise in the contents of “total phenols” with increasing damage class was observed. Thus, comparable absolute amounts of certain single and “total” phenolic compounds from spruce needles of the different sites and their damage-related increases make
these compounds seem to be suitable parameters for damage diagnosis.

The stress factors that characterize the studied sites are ozone and nutrient deficiencies, especially magnesium deficiency at the Freudenstadt site. Although there are generally no seasonal variations, the levels of phenolic compounds in the needles of the damaged trees seem to rise most during summertime when the emissions of ozone are highest. Generally, the observed damage-related increases can be explained by the membrane-damaging action of ozone on trees weakened by nutrient deficiencies (Howell, 1974). This accumulation of phenolic compounds may play a role in the further protection of the damaged trees against pathogens and predators (Swain, 1977; Rhodes, 1985).

At all of the considered sites, several other physiological and histological parameters were studied in relation to novel forest decline within the same project. They generally confirm the results found for phenolic compounds, and all of them demonstrate a shift in the metabolism of the considered trees towards various protective mechanisms (Wild et al., 1990; Wild et al., 1994, in press). The next step is the screening of several parameters including phenolic compounds over a large number of only slightly damaged trees in different areas of Germany.

Acknowledgements

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