Phenolic Compounds from Ageing Shoots of *Picea abies*

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Z. Naturforsch. **52c,** 308–312 (1997); received December 19, 1996/January 23, 1997

Picea abies, Ageing, Phenolics, Phenolic Glucosides, Quantification by GC-MS

The influence of ageing on the amount and composition of phenolic compounds and their glucosides was studied in one to five-year-old shoots of *Picea abies*.

The total content of phenolics increased remarkably within the first two years of growth. In this period cinnamic acid derivatives were dominating, but beginning with the end of the first vegetation period a dramatic increase of acetophenones was observed. Obviously at the end of the first vegetation period the cinnamic acid derivatives are subjected to oxidation resulting in an increase of acetophenones.

Introduction

Recently we observed that ageing of plants is connected with the oxidation of unsaturated compounds to epoxides. Thus, sterols are converted to sterol epoxides (Meyer and Spiteller, 1996a) and unsaturated terpenes to terpene epoxides (Meyer and Spiteller, 1996b). Oxidative processes are not restricted to compounds containing double bonds, but may also include phenolics and their glucosides which are one of the major classes of secondary metabolites in spruce. Therefore as a consequence of the ageing process we expected a change in the qualitative and quantitative composition of these compounds.

In the case of spruce shoots it is necessary to distinguish between the period of differentiation into the single components like wood, bark or needles, which occurs in the first year of growth and the ageing of the differentiated shoots. The accumulation pattern of phenolic compounds of spruce shoots during the first vegetation period has been reported previously (Strack *et al.*,1989; Kraus and Spiteller, 1997). This paper deals with changes in the composition of phenolics in spruce shoots between one and five years old.

Abbreviations: MSTFA, N-Methyl, N-trimethylsilyltrifluoroacetamide; GC, gas chromatography; GC-MS, gas chromatography – mass spectrometry; Gluc, glucose.

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Results and Discussion

Spruce shoots, between one and five years old, were harvested in midsummer from one *Picea abies* tree and extracted with methanol. Phenolic compounds in residues of these extracts were isolated by extraction with ethylacetate. Identification of the compounds was achieved by GC and GC-MS after trimethylsilylation with MSTFA. The mass spectra were compared with data known from literature (Kraus and Spiteller, 1990), or spectra of reference compounds. Structures of yet unidentified compounds were elucidated applying Biemann's shift rule (Biemann, 1962) as described previously (Kraus and Spiteller, 1997). Phenolic glucosides were obtained from *n*-butanol extracts. Their aglyca were identified after hydrolysis of the glucosides with β-glucosidase (Deyama *et al.*, 1986) and trimethylsilylation with GC and GC-MS as reported previously (Kraus and Spiteller, 1997). The identified phenolics and their glucosides are listed in Table I.

Quantification was achieved by integrating the total ion current of GC-MS runs of the trimethylsilylated extracts using 3-hydroxy-4-methoxybenzylic alcohol and salicin as internal standards for phenolics and glucosides, respectively.

The results of the quantification of phenolics from young and aged shoots are listed in Table II. The highest content of free phenolics was detected in two-year-old shoots. In the following years the total amount of phenolics decreased and finally reached a remarkably lower level in four to five-year-old shoots than in the young shoots of the first year.

0939–5075/97/0500–0308 $ 06.00

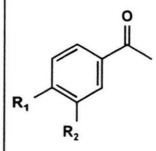
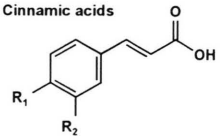
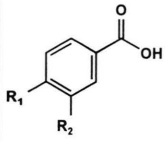
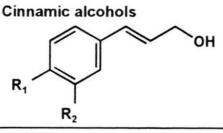
Compound		No.	Compound		No.
Acetophenones	R_1 = OH, R_2 = H	1	**Benzaldehydes**	R_1 = OH, R_2 = H	8
	R_1 = OH, R_2 = OCH_3	2		R_1 = OH, R_2 = OCH_3	9
	R_1 = R_2 = OH	3		R_1 = R_2 = H	10
	R_1 = OGluc, R_2 = H	1a	**Cinnamic acids**	R_1 = OH, R_2 = H	11
	R_1 = OGluc, R_2 = OCH_3	2a		R_1 = OH, R_2 = OCH_3	12
	R_1 = OGluc, R_2 = OH	3a		R_1 = OGluc, R_2 = H	11a
	R_1 = OH, R_2 = OGluc	3b		R_1 = OGluc, R_2 = OCH_3	12a
Benzoic acids				R_1 = OGluc, R_2 = OH	13a
	R_1 = R_2 = H	4	**Cinnamic alcohols**		
	R_1 = OH, R_2 = H	5		R_1 = OGluc, R_2 = H	14a
	R_1 = OH, R_2 = OCH_3	6		R_1 = OGluc, R_2 = OCH_3	15a
	R_1 = R_2 = OH	7			
	R_1 = OGluc, R_2 = H	5a			
	R_1 = OGluc, R_2 = OCH_3	6a			
	R_1 = OGluc, R_2 = OH	7a			

Table I. Identified phenolics and phenolic glucosides from shoots of *Picea abies*.

The accumulation pattern of single compounds differed: The amount of derivatives of acetophenones, benzoic acids and benzaldehydes increased over the first two years of growth and reached a maximum in the third year. Then it dropped again.

In contrast the highest amount of unsubstituted cinnamic acid (**10**) was detected in one-year-old shoots. The amount of p-coumaric acid (**11**), biogenetically derived from (**10**) (Harborne, 1980), was maximal in two-year-old shoots. The content of ferulic acid (**12**), its precursor is (**11**), reached the maximum value in the third year.

Table II. Amount of phenolics (0.01 μmol/g dry weight) in ageing spruce shoots.

Compound No.	Age of shoots (years) 1	2	3	4	5
Acetophenones					
1	63	455	646	202	135
2	1	2	2	1	1
3	1	4	3	1	1
Benzoic acids					
4	1	2	6	3	2
5	3	8	12	5	4
6	3	4	6	2	2
7	1	13	13	10	5
Benzaldehydes					
8	2	4	5	5	3
9	2	3	4	3	2
Cinnamic acids					
10	42	20	20	3	2
11	1083	2258	1017	187	127
12	106	121	132	27	22
Total	**1308**	**2894**	**1866**	**449**	**306**

Following conclusions are drawn from these observations: During the first year of growth mainly derivatives of cinnamic acids were formed (Fig. 1). Other phenolics were generated at the end of the first vegetation period (Kraus and Spiteller, 1997; Slimestad and Hostettmann, 1996) and in the following years by transformation of cinnamic acids to higher oxidized compounds. Beginning from the second year the content of total cinnamic acids decreased and that of acetophenones increased. Thus, a typical change in the percentual composition of the phenolic fraction was observed as shown in Fig. 2.

The considerable drop of the total amount of phenolics in four and five-year old shoots may be caused by further oxidation and polymerisation of simple phenolics to higher molecular compounds

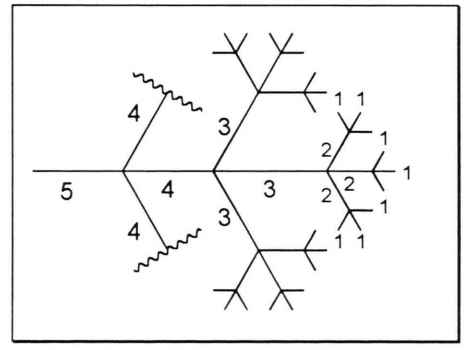


Fig. 1. Illustration of the determination of the age of spruce shoots in a spruce twig. The numbers indicate the age of the shoots in years.

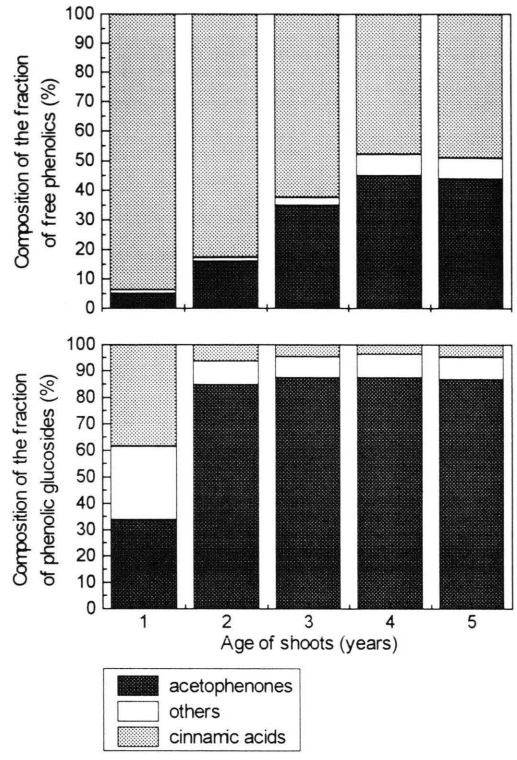


Fig. 2. Percentual composition of the fractions of free phenolics and phenolic glucosides.

that escape GC-MS investigations. A similar oxidative polymerisation of phenolics occurs after action of several stressing factors on plants (Rhodes and Wooltorton, 1978).

Evidently the total content of phenolic glucosides of young and aged spruce shoots diverged from the pattern observed for their free aglyca (Table III). A remarkably small amount of phenolic glucosides was detected in one-year-old shoots. The total content, representing also the accumulation pattern for most single compounds, reached a maximum in three-year-old shoots. Still considerable amounts were detected in two- and four-year-old shoots. In the fifth year of growth the content of phenolic glucosides dropped, although slower than observed for free phenolics. Apparently this comparatively small decrease is caused by the increasing hardness of the ageing plant material which renders the complete extraction of polar compounds difficult. Generally the deviations in the content of phenolic glucosides of ageing shoots

Table III. Amount of phenolic glucosides (0.1 μmol/g dry weight) in ageing spruce shoots.

Compound No.	Age of shoots (years) 1	2	3	4	5
Acetophenones					
1a	95	2328	2805	2407	1425
2a	tr.*	2	2	1	1
3a+b	2	79	48	39	19
Benzoic acids					
5a	16	83	109	100	55
6a	3	7	11	10	5
7a	1	6	10	10	6
Cinnamic acids					
11a	16	85	68	62	53
12a	17	14	14	12	14
13a	78	83	72	29	14
Cinnamic alcohols					
14a	34	21	15	8	2
15a	13	82	78	88	45
Total	**275**	**2790**	**3232**	**2766**	**1639**

* = Traces, less than 0.1 μmol/g dry weight.

were smaller than those in the content of free phenolics.

The formation of phenolic glucosides obviously occurs at the end of the first vegetation period. As the experiments presented were carried out in midsummer the glucosylation was not terminated yet and the content of phenolic glucosides in one-year old shoots was therefore very low. Once glucosylated, however, only minor changes are observed, concerning the amount of single compounds as well the percentual composition of the fraction of phenolic glucosides.

Figure 2 illustrates that in the first year of growth the composition of the fraction of phenolic glucosides was rather heterogeneous. In elder shoots about 85% of the total phenolic glucosides were derivatives of acetophenones.

Summing up ageing of spruce shoots is expressed by a strong increase in the content of acetophenone derivatives in the fraction of free phenolics, whereas young shoots are rich in precursor molecules, cinnamic acids. In the fraction of phenolic glucosides these changes are negligible after the period of differentiation.

Material and Methods

Gas-chromatography/mass spectrometry

GC-MS measurements were performed on a DB-1 fused silica capillary column (length: 30 m;

inner diameter: 0.32 mm; film thickness: 0.1 µm; carrier gas: H_2 2 ml/min; temperature prog.: 80–280° at 3°/min). The GC was coupled to a double focusing mass spectrometer running under EI conditions at 70 eV.

Gas-chromatography

The column conditions for analytical GC were the same as for GC-MS. Detector: FID; injector temperature: 270°; detector temperature: 290°; split ratio 1:30. Retention indices (R_I) were calculated according to Kováts (1958) with *n*-alkanes C_{10}-C_{36} as reference compounds.

Chemicals

MSTFA was from Machery und Nagel (Düren, FRG); β-glucosidase was bought from Sigma (Deisenhofen, FRG). Solvents were distillated before use. All other chemicals were of analytical grade.

Trimethylsilylation

0.3 mg of dried sample were dissolved in 10 µl THF (purified and dried) and 20 µl MSTFA added. The mixture was allowed to stand at room temperature for 12 h. 1 µl of the mixture was then subjected to GC and GC-MS.

Plant material

Spruce shoots were harvested by mid of June 1995 from one 20-year-old *Picea abies abies* tree, grown in a garden in Bayreuth, Germany. Shoots including needles were taken from a twig. The age of the shoots was determined as shown in Fig. 1. Between 8 and 14 shoots (corresponding 10 g fresh weight) of the same age were combined and extracted. The experiments were repeated at least 3 times.

Isolation of phenolics

Shoots (10 g fresh weight) were homogenised in 100 ml CH_3OH with a commercial Ultra Turrax at room temperature for three minutes. The solution was allowed to stand for 4 h. Then solid material was removed by centrifugation. The residue was reextracted twice with 30 ml CH_3OH/H_2O 1:1 (v/v). The combined supernatants were evaporated to dryness, resuspended in 60 ml CH_3OH/H_2O 9:1 (v/v) and extracted with 2 x 25 ml cyclohexane to remove non polar compounds. The residue of the CH_3OH/H_2O layer, obtained by evaporation in vacuum, was resuspended with 60 ml H_2O and extracted with 3 x 30 ml ethylacetate. After removal of the solvent in vacuum the residue was dissolved in 80 ml ethanol and extracted with 3 x 25 ml diluted KOH (2%, aqueous). The combined KOH layers were immediately acidified with 2N HCl (pH = 4) and reextracted with 3 x 20 ml ethylacetate. The organic layers were washed with 2 x 10 ml H_2O, dried over Na_2SO_4 and concentrated in vacuum. Phenolics were identified and quantified after trimethylsilylation by GC-MS.

Isolation of glycosides

The remaining aqeous layer obtained after extraction with cyclohexane and ethylacetate was extracted with 3 x 15 ml *n*-butanol. The organic layers were washed with 10 ml H_2O and concentrated in vacuum.

Enzymatic hydrolysis of glucosides (Deyama et al., 1986)

10 mg of the residue of the glucoside fraction were dissolved in 15 ml acetate buffer (0.1 M; pH = 5.0). Then about 5 mg ß-glucosidase (Sigma, Deisenhofen, FRG) were added. The mixture was incubated overnight at 37° and extracted with 3 x 10 ml ethylacetate. The organic layers were washed, dried over Na_2SO_4 and concentrated in vacuum.

Quantification of phenolics

The absolute content of the phenolics and glucosides in shoots was determined from total ion current of GC-MS measurements of the trimethylsilylated derivatives of ethylacetate and hydrolysed n-butanol extracts, respectively. 4-Methoxy-3-hydroxybenzylic alcohol (R_I = 1625) and salicin (aglycon: R_I = 1430) were added as internal standards to the plant extracts immediately after homogenisation. Peak areas were integrated. Quantifications were performed at least 3 times to ensure reproducibility. The deviations were within a range of 15 %.

Data from mass spectra after trimethylsilylation: see Kraus and Spiteller (1997).

Acknowledgements

We thank the Fonds der Chemischen Industrie for financial support and Mr. M. Glaeßner for running the mass spectra.

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