Accumulation Patterns of Polyamines and Hydroxycinnamic Acid Conjugates during Microsporogenesis of *Corylus avellana*

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The amounts of free, conjugated (hydroxycinnamic acid amides) and insoluble (poly)amines (putrescine, spermidine, spermine) as well as hydroxycinnamic acid esters (quinic acid esters) were determined during development of male inflorescences of *Corylus avellana* L. The location of these compounds either in sporophytic or gametophytic tissues and the possible correlation of their accumulation with exine formation are discussed.

Introduction

The aliphatic (poly)amines putrescine, spermidine and spermine have been shown to be possibly involved in numerous processes of plant growth and development [1–3]. Conjugates of these amines (amides) with hydroxycinnamic acids (HCAs) have been frequently found in the reproductive organs such as anthers, pollen, ovaries, and seeds [4–9]. HCA Amides are likely to be involved in differentiation of floral structures [10] and pollen fertility [6, 11]. Also, appreciable changes in polyamine levels were observed during *in vitro* anther maturation and gametogenesis [12], pollen tube growth and fertilization [13, 14] as well as post-fertilization development of the embryo [15]. Thus, the metabolism of polyamines and their HCA conjugates may be one of the basic processes of floral differentiation.

The development of the male inflorescences of *Corylus avellana* L. provides a suitable system to study stage-specific accumulations of polyamines and HCA amides. The structure elucidation of caffeoylferuloylspermidine and bisferuloylspermidine from mature pollen grains was recently described [7]. The occurrence of these amides in pollen seems to be characteristic for most members of the Fagaceae and the Betulaceae [8]. Furthermore, the events during microspore and pollen development of Betuloid pollen have been described in detail [16], emphasizing the unique differentiation of the exine. Changes in the levels of polyamines and their HCA amides can easily be correlated with these cytological events, and conclusions about the location of the HCA amides can be drawn confirming results obtained earlier [17].

Materials and Methods

Plant material

Male inflorescence of *Corylus avellana* L. were harvested as indicated in the figures from two trees growing at the botanical garden of the University in Cologne (F.R.G.) during autumn and winter of 1984/85 and 1985/86 and the autumn of 1987. The catkins were weighed immediately after harvest and stored at −20 °C. Developmental stages − sporogenous tis-

Abbreviations: BFS, bis-*E*-feruloylspermidine; Caf-QA, 3-O-*E*-caffeoylquinic acid; CFS, *E*-caffeoyl-(*E*-feruloyl)-spermidine; Coum-QA, 3-O-*E*-(4-coumaroyl)-quinic acid; FS, *E*-feruloylspermidine; HCA, hydroxycinnamic acid; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

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sue, tetrades, young microspores, differentiation of the exine, mature pollen – were determined by light microscopy. Mature pollen grains were collected in the area around Münster (F.R.G.). Male inflorescences of *Alnus glutinosa* (L.) Gaertn. were collected in the area around Cologne at the beginning of September 1987.

Extraction of HCA conjugates

Five catkins or 1 g plant material (three samples per harvest) were frozen with liquid nitrogen and ground in a mortar. HCA Amides were extracted with 80% aq. CH_3OH (×4) and the suspensions allowed to stand for 30 min at room temperature with continuous stirring. After centrifugation, the supernatants were combined, evaporated to dryness (*in vacuo* at 30–35 °C) and the residue redissolved in 10 ml 80% aq. CH_3OH. Extracts were stored at −20 °C until further analysis.

Extraction and benzoylation of free and insoluble polyamines

The extraction procedures followed methods described by Flores and Galston [18] and Dumortier *et al.* [19]. One g plant material (three samples per harvest) was frozen with liquid nitrogen and ground in a prechilled mortar. Free polyamines were extracted with 5% aq. $HClO_4$ while stirring for 1 h at 0 °C. After centrifugation (48,000 × *g*) the supernatants were kept at −20 °C until benzoylation. The pellets and in parallel samples standards containing 1 mM each of putrescine, spermidine and spermine (× HCl; Sigma, Deisenhofen, F.R.G.) were hydrolyzed with 5 ml 4 N HCl for 16 h at 100 °C. After centrifugation the pellets were washed twice with 5 ml H_2O, all supernatants combined and the final volumes determined (storage at −20 °C). Prior to high-performance liquid chromatographic (HPLC) analysis all extracts were benzoylated in triplicate according to Flores and Galston [18].

Identification and quantification

This was performed by HPLC. The liquid chromatograph used came from LKB Pharmacia (Freiburg, F.R.G.). Separations were achieved on prepacked MN-Nucleosil C_{18} (5 µm, 250 mm · 4 mm i.d.; Macherey-Nagel, Düren, F.R.G.); elution systems: 1.5% H_3PO_4 and 45% CH_3CN in H_2O at a flow rate of 1 ml · min^{-1} for the benzoylated polyamines and linear gradient within 30 min from solvent A (1.5% H_3PO_4 in H_2O) to 30% solvent B (1.5% H_3PO_4, 20% CH_3COOH and 25% CH_3CN in H_2O) in (A + B) at a flow rate of 1 ml · min^{-1} for the HCA conjugates (detection at 320 nm). Peaks were identified by cochromatography with reference material: *E*-caffeoyl-(*E*-feruloyl)-spermidine (CFS) and bis-*E*-feruloylspermidine (BFS) were isolated from pollen of *C. avellana* [7]. 3-O-*E*-Caffeoylquinic acid (Caf-QA) and 3-O-*E*-(4-coumaroyl)-quinic acid (Coum-QA) came from young male inflorescences of *A. glutinosa* and was isolated and identified as described below. *E*-Feruloylspermidine (FS) was synthesized according to Smith *et al.* [20]. All assignments from extracts were checked by HPLC and thin-layer chromatography (TLC) as documented in Table I. Benzolyated untreated (0.1 mM) and 'hydrolyzed' (1 mM) polyamine standards, authentic HCA spermidines and chlorogenic acid (Serva, Heidelberg, F.R.G.) were used as external standards for quantification.

Isolation and structure elucidation of HCA esters

Young male inflorescences (70 g) of *A. glutinosa* were frozen with liquid nitrogen, ground in a mortar and extracted twice with 80% aq. CH_3OH. The suspensions were allowed to stand for 1 h at room temperature and the combined filtrates concentrated to a minimum volume. The resulting crude syrup was fractionated on a polyamide column equilibrated with H_2O (polyamide SC 6, 70 cm · 4 cm i.d.; Macherey-Nagel) and the following solvents were used: H_2O, 40% aq. CH_3OH, CH_3OH, and 40% $(CH_3)_2CO$ in CH_3OH. The latter eluted the HCA quinic acids which were further separated by TLC on microcrystalline cellulose ('Avicel', Macherey-Nagel) in $CHCl_3$–CH_3COOH (3:2, H_2O saturated). The compounds were eluted with CH_3OH and finally purified by chromatography on a Sephadex LH-20 column with CH_3OH (70 cm · 3 cm i.d.; Pharmacia, Freiburg, F.R.G.).

The identity of Coum-QA and Caf-QA were confirmed from the ^1H and ^{13}C NMR (Bruker AM-300 and WM-400 NMR spectrometers), and FAB-MS (Kratos MS 50 mass spectrometer) data. In each case the number and nature of the aromatic and quinic acid moieties in the molecule were unambiguously determined from identification of the various spin systems in the 1D and 2D ^1H NMR spectra, and from the observation of the appropriate deproton-

ated molecular ions in the negative ion FAB mass spectra. Although substitution at C-3 or C-5 of quinic acid was readily established the differentiation between these was somewhat more difficult initially as both compounds isolated after chromatographic purification showed, in neutral CD_3OD, uncharacteristic coupling constants for this moiety (Table II). Although part of the quinic acid spin systems is second order, due to overlap of H-2A and H-2B, the part involving H-5, H-6A and H-6B is essentially first order. We assume that the compounds are present as salts under these conditions and adopt structures in which the quinic acid moiety is no longer in one favoured conformation.

Addition of a small amount of DCl to the methanolic solution of Coum-QA caused significant changes for the shifts of C-7 to give the more characteristic shift of the carboxylic acid group and smaller shifts for the rest of the molecule (Table III). More significantly although the ^1H shifts showed only small changes the coupling constants were now characteristic of a fixed quinic acid ring conformation with the C-3 substituent in an axial position (Table II).

The close similarity of the ^{13}C shifts of the quinic acid moiety in both compounds (Table III) confirms that both have the same substitution patterns. Small amounts of the corresponding *Z*-isomer were present and presumably arise from the isolation procedure. Only the data for the *E*-isomer are reported.

Results

Microspore and pollen development

The male inflorescences of *C. avellana* develop in early August, although anthers with sporogenic tissue (pollen mother cells) are not observed before late August or early September. Subsequently the young microspores are released and development of the pollen wall, mainly of the exine layers, proceeds. Dormancy lasts from November until February or early March. At the beginning of dormancy the average weight of a single catkin has increased from about 30 mg in early August to 200–300 mg in late October or November. No further significant changes in weight were observed until deshiscence of the anthers.

Accumulation patterns of the metabolites

The stage-specific levels of the free, HCA-conjugated (amides) and insoluble polyamines, as well as the HCA esters and a major flavonoid (quercetin 3-glucosylgalactoside), are shown in Fig. 1 and 2. Table I summarizes some chromatographic data of the HCA conjugates (amides and esters).

The levels of free polyamines range from less than 10 nmol (spermine) to about 60 nmol (spermidine) per inflorescence. With the exception of a spermidine peak in late November, which is preceded by a peak of the HCA spermidines in early November and followed by a peak in the insoluble polyamine fraction in early December, no further major changes in the amounts of free polyamines was observed. Only traces of insoluble polyamines were detectable to the end of September. Accumulation of insoluble spermidine and putrescine was found to occur after the release of the young microspores. In 1985, as well as in 1987, the amount of accumulated spermidine exceeds that of putrescine. The level of insoluble putrescine shows no major changes after the release of young microspores.

Analysis of the HCA conjugates by HPLC revealed complex patterns of phenolics including flavonoids (details not documented), *e.g.* the major quercetin 3-glucogalactoside [21]. Fig. 2 shows the

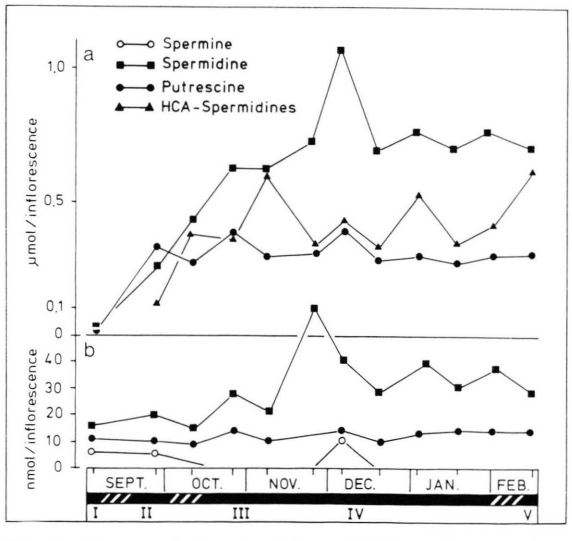

Fig. 1. Changes in levels of free, HCA-conjugated (total of amides) and insoluble spermidine as well as free and insoluble putrescine and spermine during development of male inflorescences of *Corylus avellana* in 1985/1986. (a) Insoluble and conjugated polyamines; (b) free polyamines; I: pollen mother cells, II: tetrad stage, III: microspores, IV: dormancy, V: mature pollen. Note differently scaled ordinates.

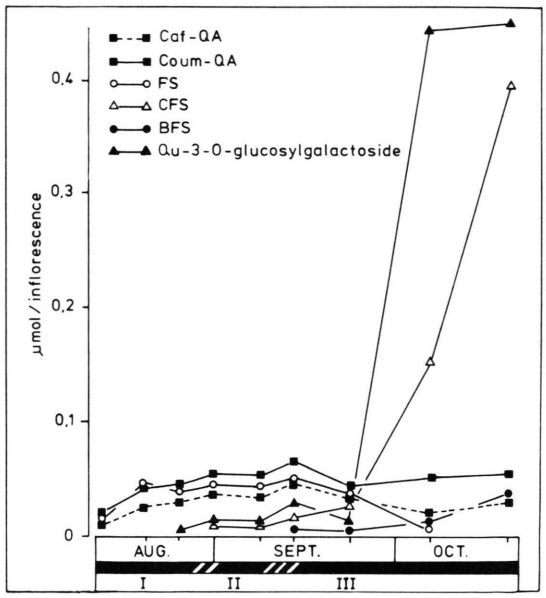

Fig. 2. Changes in levels of HCA conjugates (amides, esters) and quercetin 3-glucosylgalactoside during early development of male inflorescence of *Corylus avellana* in autumn 1987. BFS = bis-*E*-feruloylspermidine; Caf-QA = 3-O-caffeoylquinic acid; CFS = *E*-caffeoyl-(*E*-feruloyl)-spermidine; Coum-QA = 3-O-(4-coumaroyl)-quinic acid; FS = *E*-feruloylspermidine; Qu = quercetin. I: pollen mother cells, II: tetrad stage, III: young microspores.

quantitative changes of these compounds. The mature pollen grains contain only the amides CFS and BFS and the quercetin glycoside as the major flavonoid. The other HCA conjugates, the amide FS and the esters Caf-QA and Coum-QA, seem to occur in other parts of the inflorescence and/or the young microspores. Caf-QA and Coum-QA are present in all stages of development and their concentrations ranged from 25–75 nmol per inflorescence. FS accumulated slightly during initial growth of male catkins in August and significantly declined during exine formation in October. At the same time CFS and, to a lesser extent, BFS were formed.

Discussion

The results of the microscopic examination of anthers agree with a detailed study on microspore and pollen differentiation in male inflorescence of *Betula verrucosa* [16]. It can be assumed that events during exine formation follow the same pattern in catkins of *C. avellana,* since the appearance of microspores and pollen grains are almost identical within these two genera [22]. The juvenile exine is characterized by a period of polymerization of sporopollenine leading to the mature exine [23]. Some changes in the biochemistry of polyamines and phenolics can be correlated with events during microspore and pollen differentiation of *C. avellana.*

The levels of free polyamines were not found to correlate with differentiation of the male inflorescences. If free polyamine are involved in plant growth regulation, they might be effective in the picomole range. This has also been suggested in a recent study of seedling development [24]. Also, the levels of the two HCA quinic acids do not seem to correlate with the accumulation of the HCA spermidines. Chlorogenic acid has been shown to be a possible acyl donor in the biosynthesis of HCA conjugates [25–27]. No evidence for such a transacyla-

Table I. Chromatographic data of the hydroxycinnamic acid conjugates (amides, esters) from male inflorescence of *Corylus avellana.*

Compound	TLC R_f (×100) S1	S2	Appearance UV/UV + NH_3	Ninhydrin	HPLC R_t, min
Caf-QA	15	45	bl/gr	–	3.4
Coum-QA	29	65	abs/d-bl	–	4.9
FS	44	29	bl/bl	violet	4.0
CFS	45	56	bl/gr-bl	–	12.9
BFS	68	66	bl/gr-bl	–	16.3

TLC ('Avicel'): S1 = $CHCl_3$–CH_3COOH–H_2O (3:2, H_2O saturated); S2 = *n*-butanol–CH_3COOH–H_2O (6:1:2); appearance: absorbing (abs) or fluorescent under UV (350 nm) with and without NH_3 (d-bl = dark blue; bl = blue; gr = green; gr-bl = green-blue).

Table II. ^{1}H NMR chemical shifts (ppm) and coupling constants (Hz) of the hydroxycinnamic acid-quinic acid esters.

NMR data	Caf-QA CD_3OD	Coum-QA CD_3OD	Coum-QA CD_3OD + DCl
Shifts			
H-2a	2.165–2.040	2.165–2.043	2.214–2.155
H-2e			2.253
H-3	5.435	5.437	5.400
H-4	3.814	3.816	3.687
H-5	4.059	4.063	4.204
H-6a	1.962	1.963	2.003
H-6e	2.165–2.040	2.110	2.214–2.155
H-2′	7.088	7.498	7.497
H-3′	–	6.843	6.849
H-5′	6.813	6.843	6.849
H-6′	6.985	7.498	7.497
H-7′	7.626	7.690	7.697
H-8′	6.352	6.413	6.411
Couplings			
(2a–2e)	–	–	(–) 14.7
(3–2a)	7.7[a]	7.6[a]	4.4
(3–2e)	4.9[a]	4.8[a]	3.6
(3–4)	3.1	3.0	3.5
(4–5)	6.5	6.5	8.6
(5–6a)	6.5	6.6	9.8
(5–6e)	4.5	4.6	4.3
(6a–6e)	(–) 14.1	(–) 13.8	(–) 13.6
(2′–6′)	2.1	(2′–3′) 8.7	8.7
(5′–6′)	8.2	+(2′–5′)	
(7′–8′)	15.9	16.0	16.0

[a] These are splittings observed on H-3 which is part of a second spin system as H-2a and H-2e overlap. No attempt was made to perform an interactive spectral analysis as the latter signals overlapped with H-6e and the situation was made more complex by the presence of small amounts of *Z*-isomers.

Table III. ^{13}C NMR chemical shifts (ppm) of the hydroxycinnamic acid-quinic acid esters.

NMR data	Caf-QA CD_3OD	Coum-QA CD_3OD	Coum-QA CD_3OD + DCl
C-1	75.82	75.80	75.39
C-2	37.39	37.37	36.43
C-3	72.60	72.59	72.66
C-4	73.63	73.61	74.01
C-5	69.94	69.91	68.61
C-6	39.41	39.39	40.92
C-7	181.53	181.57	176.54
C-1′	128.18	127.54	127.44
C-2′	115.35	131.02	131.06
C-3′	146.75[a]	116.84	116.84
C-4′	149.35[a]	161.06	161.10
C-5′	116.58	116.84	116.84
C-6′	122.79	131.02	131.06
C-7′	146.70	146.35	146.48
C-8′	116.01	115.99	115.89
C-9′	168.82	168.82	168.93

[a] Assignments interchangeable.

tion could be found in the male catkins. It is likely that formation of these amides proceeds *via* the HCA-CoA thioesters as recently found for HCA putrescines in tobacco cell cultures [28]. The stage-specific occurrence of FS during early microsporogenesis might be due to its conversion to CFS and BFS, rapidly accumulating during exine formation and differentiation.

Free and insoluble polyamines were detected in whole inflorescences. A significant portion of insoluble putrescine and spermidine could not be recovered in methanolic extractions as HCA amides. They could be bound either to the pollen grains or to other structures of the inflorescence such as cell walls [19, 29, 30]. The HCA quinic acids and FS were not found in pollen grains. They obviously accumulate in the sporophytic part of the inflorescence. Chlorogenic acid (5-O-caffeoylquinic acid) has been found

in leaves of members of the Betulaceae [31]. Mature pollen grains of *C. avellana* are known to contain the two HCA amides CFS and BFS. These compounds could only be removed quantitatively from the pollen material with organic solvents and continuous, repeated extractions for up to 140 days. It has been suggested that they occur on the outside of the exine as well as inclusions of the exine cavities [17]. This view agrees with the present developmental studies: Accumulation starts with the release of microspores from the tetrades and proceeds for several weeks until dormancy (Fig. 1). This corresponds to the phases of initiation of exine formation and differentiation. It can be assumed that during exine development a considerable portion of the HCA spermidines are incorporated into its complex structures without being covalently attached to sporopollenine (for ultrastructural analyses of the exine of *Betula* pollen grains see ref. [23, 32]).

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