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PHENYLPROPANOID PATTERNS IN FAGALES POLLEN AND THEIR PHYLOGENETIC RELEVANCE

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Key Word Index—*Alnus*; *Betula*; *Corylus*; Betulaceae; Fagaceae; Hamamelididae; hydroxycinnamic acid amides; polyamines; spermidine; flavonoids; quercetin 3-*O*-glucosylgalactoside; chemosystematics.

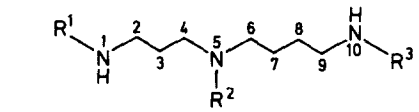
Abstract—The occurrence of hydroxycinnamic acid-spermidine amides and quercetin 3-*O*-glucosylgalactoside in pollen of Fagales and some other taxa of the Hamamelididae (*sensu* Takhtajan and Cronquist) has been investigated. The genera *Alnus*, *Betula*, *Corylus*, and *Quercus* show characteristic patterns of N^1,N^{10}-di-(*E*)-feruloylspermidine, N^5,N^{10}-di-(*E*)-feruloylspermidine, N^1,N^{10}-(*E*)-caffeoyl-(*E*)-feruloylspermidine and several *N*,*N*-di-*p*-coumaroylspermidines. The occurrence of these amides—together with some other unidentified hydroxycinnamic acid conjugates and quercetin 3-*O*-glucosylgalactoside—in extracts of the pollen grains support suggested relationships among Fagales, Juglandales, and Myricales.

INTRODUCTION

The systematic position of the Fagales as one of the derived orders of the Hamamelididae is well accepted in recent systematic treatments [1–4]. However, the relationships among Fagales, Juglandales, Myricales, and other 'Amentiferous' orders, as well as the taxonomic subdivision of the Fagales into families and subfamilies are still debated. Most authors [1, 2, 4] emphasize the close relationship between Fagales, Juglandales, Myricales, and Hamamelidales which has been additionally confirmed by some recent serosystematic studies [5–7]. Thorne [3] does not place the Juglandales and Myricales in the superorder Hamamelidiflorae, but in the Rutiflorae. Urticales are frequently classified in the Malviflorae [3, 4].

Fagales is traditionally divided into Betulaceae and Fagaceae, but Cronquist [2] includes Balanopaceae and Nixon [8] proposes separating the Nothofagaceae from the Fagaceae. While Betuloideae and Coryloideae are acknowledged as distinct subfamilies of Betulaceae [1, 2, 9], the taxonomic treatment of subgroups in the Fagaceae is still controversial [1, 2, 8]. The exine ornamentation of Fagaceae pollen, however, was shown to give a good diagnostic feature allowing discrimination among three subfamilies of Fagaceae: Castanoideae, Fagoideae, and Quercoideae [10]. Phytochemical and biochemical data may contribute useful characteristics for the systematic investigation of Fagales and related orders.

The accumulation of hydroxycinnamic acid (HCA) amides in the reproductive organs of higher plants has been reported from a large number of families [11]. Some of these compounds are suggested to be markers of pollen fertility [12] and may also protect pollen grains from damaging UV light and microbial infection [13]. N^1,N^{10}-(*E*)-Caffeoyl-(*E*)-feruloylspermidine and N^1,N^{10}-di-(*E*)-feruloylspermidine (Fig. 1, CI and CII) were recently identified from pollen of *Corylus avellana* L. [14] and were



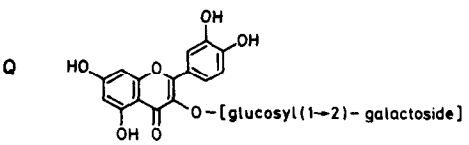
P $R^1=R^2=p$-Coumaroyl; $R^3=H$

AI, AII { $R^1=R^2=p$-Coumaroyl; $R^3=H$ or; $R^1=R^3=p$-Coumaroyl; $R^2=H$ or; $R^2=R^3=p$-Coumaroyl; $R^1=H$

B $R^2=R^3=$Feruloyl; $R^1=H$

CI $R^1=$Caffeoyl; $R^3=$Feruloyl; $R^2=H$ or $R^1=$Feruloyl; $R^3=$Caffeoyl; $R^2=H$

CII $R^1=R^3=$Feruloyl; $R^2=H$



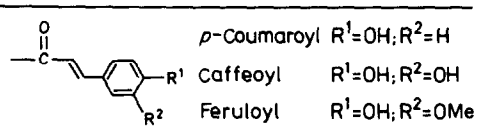


Fig. 1. Scheme of the structures of the hydroxycinnamic acid-spermidine amides and quercetin 3-*O*-glucosylgalactoside from Fagales pollen.

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localized in the structures of the exine [15]. The related compounds N^5,N^{10}-di-(*E*)-feruloylspermidine (Fig. 1, **B**) and *N,N*-disubstituted *p*-coumaroylspermidines (Fig. 1, **P, AI, AII**) have been isolated from pollen of *Alnus glutinosa* (L.) Gaertn., *Betula verrucosa* Ehrh., and *Pterocarya fraxinifolia* (Lam.) Spach [16].

The occurrence of flavonoids has frequently been investigated in chemosystematic studies. While pollen flavonol aglyca are usually simple structures such as kaempferol and quercetin [17], their glycosylation patterns mostly are more complex than those found in the vegetative plant parts [18, 19]. Quercetin 3-*O*-glucosylgalactoside has been identified as the major flavonol in pollen of *Corylus avellana* L. [20] and evidently is located on the surface of the exine [21].

In the present study pollen from 67 species was investigated for the occurrence of quercetin 3-*O*-glucosylgalactoside and the HCA-spermidine amides which were found to be restricted to a few orders. The location of these compounds in the structures of the pollen exine has been deduced from their characteristic extraction behaviour using a successive procedure to remove flavonoids first and subsequently the HCA amides [14]. The extracts were analysed by thin-layer chromatography (TLC) including spray reagents and by high-performance liquid chromatography (HPLC).

RESULTS AND DISCUSSION

Figure 1 shows the structures of the analysed phenylpropanoids (HCA-spermidine amides and quercetin 3-*O*-glucosylgalactoside) which were recently identified [14, 16, 20]. Some of their chromatographic data and their occurrence in pollen (or spores) from 67 species are summarized in Table 1 and 2, respectively. The aqueous extraction procedure of pollen material (extract I, see experimental) yielded about 90% of the total flavonoids and almost none of the HCA amides. The latter are extractable by subsequent repeated methanolic treatments (6 times, 1 hr each). The first three extractions combined (extract II) gave about 60% of the total HCA-amide content which was calculated by quantitative HPLC (see Table 2). The sixth fraction (extract III) was also analysed for the presence of the HCA amides (Fig. 2). The unusual extraction behaviour of these conjugates suggests that they are integrated in the structures of the pollen exine, although they are not covalently attached to the sporopollenin [14, 16]. The examination of this sixth fraction of the methanolic extract showed that, for all but two samples, the HCA amides detected in extract II (first three combined methanolic extractions; see Table 2) might have an analogous location in the exine. The HCA amides and quercetin 3-*O*-glucosylgalactoside were identified in unknown extracts by cochromatography with authentic samples from *Alnus glutinosa* (**AI, AII**), *Betula verrucosa* (**B**), and *Corylus avellana* (**CI, CII**, see Fig. 1 and Table 1). **P** from *Pterocarya fraxinifolia* (N^1,N^5-di-*p*-coumaroylspermidine), however, could not be chromatographically distinguished from **AI** from *Alnus glutinosa* (N^1,N^5-,N^5,N^{10}-or N^1,N^{10}-di-*p*-coumaroylspermidine). Figure 2 shows four selected diagrams from the HPLC analyses of the HCA-spermidine amides (extract III).

As seen in Table 2, the accumulation of the analysed phenylpropanoids in pollen is restricted to the subclass Hamamelididae *sensu* Takhtajan and Cronquist [1, 2]. Traces of some of these compounds, however, could be detected in members of other taxa. The quercetin 3-*O*-glucosylgalactoside (**Q**) was the main flavonoid of pollen from the Betulaceae and was found in all but six of the 41 species of Hamamelididae examined. *Quercus* pollen accumulates four or five flavonoids (not identified) including quercetin 3-*O*-glucosylgalactoside. Traces of this compound could be detected in pollen from *Ulmus laevis* Pall. The Ulmaceae and Fagaceae both show appreciable amounts of an unidentified kaempferol conjugate that also occurs in pollen of *Corylus avellana* [14] and most other Betulaceae. Thus the flavonoid patterns of the Ulmaceae (Urticales) investigated here show similarities to those of some Fagales. Suggested relationships between Fagales, Juglandales, Myricales, and Hamamelidales are supported by the occurrence of quercetin 3-*O*-glucosylgalactoside in pollen of *Liquidambar styraciflua* L., *Platanus* × *hybrida* Brot., *Myrica gale* L., *Pterocarya fraxinifolia* (Lam.) Spach., *Juglans nigra* L., and *J. regia* L.

Accumulation of the HCA-spermidine amides in the pollen exine seems to be restricted to the orders Fagales, Juglandales, and Myricales. Within the Betulaceae the pattern of distribution of the compounds correlates within genera and subfamilies. N^1,N^{10}-caffeoylferuloylspermidine (**CI**) and N^1,N^{10}-diferuloylspermidine (**CII**)

Table 1. Summary of the chromatographic data of quercetin 3-*O*-glucosylgalactoside (**Q**) and hydroxycinnamic acid-spermidine amides (see Table 2)

Compound	TLC ($R_f \times 100$)			Appearance on TLC			HPLC
	S1	S2	S3	UV/UV + NH_3	Nitroan.	Dragen.	(R_t, min)
Q	11	39	5	abs/yellow	ochre	–	6.0
P/AI	62	51	72	abs/d-blue	yellow	red brown	12.3
AII	88	63	79	abs/d-blue	yellow	l-red brown	13.9
B	83	67	83	blue/g-blue	ochre	red brown	16.3
CI	62	51	72	blue/g-blue	ochre	l-red brown	10.7
CII	88	63	79	blue/g-blue	ochre	l-red brown	15.9

TLC: S1 = $CHCl_3$–HOAc–H_2O (3:2, H_2O satd; microcrystalline cellulose, Macherey–Nagel, Düren, F.R.G.); S2 = *n*-BuOH–HOAc–H_2O (6:1:2; microcrystalline cellulose); S3 = H_2O–MeCOEt–MeOH–acetylacetone (13:3:3:1; polyamide DC-6, Macherey–Nagel, Düren, F.R.G.).

Appearance: absorbing (abs) or fluorescent under UV with and without NH_3 vapour: d = dark; g = green; spray reagents (appearance under daylight): nitroan. = 4-nitroaniline; Dragen. = Dragendorff III; l = light.

Table 2. Survey of pollen and spores for hydroxycinnamic acid-spermidine amides and flavonolglycosides (HPLC and TLC of extract I and II, see Experimental)

Taxon	Source	Compound						
		Q	K	P/AI	AII	B	CI	CII
Pteridophyta								
Equisetales								
Equisetum arvense L.	BK	—	—	—	—	—	(+)	—
Filicales								
Adiantum raddianum K. B. Presl	BK	—	—	—	—	—	(+)	—
Asplenium adiantum-nigrum L.	BK	—	—	—	—	—	—	—
Doodia media R. Br.	BK	—	—	—	—	—	—	—
Cibotium schiedei Schlentend. et Cham.	BK	—	—	—	—	—	—	—
Spermatophyta								
Coniferophytina								
Pinales								
Pinus mugo Turra	M	—	—	—	—	—	—	—
Magnoliophytina								
Dicotyledoneae								
Ranunculales								
Thalictrum minus L. var. *jacquiniana*	BK	—	—	—	—	—	—	—
Papaverales								
Papaver rhoeas L.	BK	—	—	—	(+)	—	—	—
Hamamelidales								
Liquidambar styraciflua L.	AU	○	—	—	—	—	—	—
Platanus × *hybrida* Brot.	BK	●	○	—	—	—	—	—
Urticales								
Cannabis sativa L.	W	—	—	—	—	—	—	—
Ulmus glabra Huds. emend. Moss.	W	—	●	—	—	—	—	—
U. laevi Pall.	BD	(+)	●	—	—	—	—	—
Zelkova serrata (Thunb.) Mak.	W	—	○	—	—	—	—	—
Fagales								
Fagaceae (incl. Nothofagaceae)								
Fagoideae								
Fagus sylvatica L.	W	—	+	—	—	—	—	—
Nothofagus antartica (G. Forst.) Oerst.	W	—	(+)	—	—	—	—	—
Quercoideae								
Quercus cerris L.	W	(+)	—	—	(+)	—	—	—
Q. macrocarpa Michx.	AU	○	(+)	+	○	—	—	—
Q. robur L.	W	◑	○	—	○	—	—	—
Q. rubra L.	W	○	+	+	+	—	—	—
Q. shumardii Buckl.	AU	◑	+	○	○	—	—	—
Q. sinuata Walt.	AU	◑	(+)	+	—	—	—	—
Q. stellata Wangenh.	AU	●	+	◑	—	●	—	—
Q. texana Buckl.	AU	◑	(+)	◑*	○*	—	—	—
Q. turbinella Greene	PNF	○	(+)	○	—	—	—	—
Q. virginiana Mill.	AU	○	(+)	+	+	—	—	—
Betulaceae								
Betuloideae								
Alnus cordifolia Tenore	W	●	—	◑	◑	—	—	—
A. glutinosa (L.) Gaertn.	W	●	—	◑	○	—	—	—
A. laciniata Ehrh.	W	●	—	◑	◑	—	—	—
A. viridis (Chaix.) DC	W	●	(+)	◑	◑	—	—	—
Betula albosinensis Burk. var. *septentrionalis*	BD	●	(+)	—	—	○	—	—
B. alleghaniensis Britt.	BD	●	—	—	—	●	—	—
B. humilis Schrank	BD	●	—	—	—	○*	—	—
B. jacquemontiana Spach.	BD	●	—	—	—	●	—	—
B. medwediewii Regel	BD	○	—	○	+	—	—	—
B. neo-alaskana Sarg.	BD	●	—	—	—	●	—	—
B. nigra L.	BD	●	(+)	—	—	◑	—	—
B. papyrifera Marsh.	BD	●	—	—	—	◑	—	—
B. verrucosa Ehrh.	W	●	—	—	—	◑	—	○
Coryloideae								

Table 2. *Continued*

Taxon	Source	Compound						
		Q	K	P/AI	AII	B	CI	CII
Carpinus betulus L.	W	●	—	—	—	—	◑	○
C. japonica Blume	BD	●	(+)	○	○	—	○	○
Corylus avellana L.	W	●	○	—	—	—	◑	○
C. colurnoides C. Schn.	W	●	—	—	—	—	◑	○
Ostrya carpinifolia Scop.	W	●	○	—	—	—	●	○
Myricales								
Myrica gale L.	W	●	+	—	○	—	—	—
Juglandales								
Carya illinoensis (Wangenh.) K. Koch	AU	—	—	—	—	—	—	—
Juglans nigra L.	BK	●	○	●	○	—	—	—
J. regia L.	BK	●	—	●	+	—	—	—
Pterocarya fraxinifolia (Lam.) Spach.	W/BD	○	—	●	—	—	—	—
Caryophyllales								
Lampranthus multiseratius N. E. Brown	BK	—	—	—	—	—	(+)	—
Epiphyllum Hybr.	BK	—	—	—	—	—	—	—
Polygonales								
Rumex acetosella L.	W	—	—	—	—	—	—	—
Salicales								
Populus tremula L.	W	—	—	—	—	—	—	—
Malvales								
Lavatera spec.	BK	—	—	—	—	—	—	—
Abutilon pictum (Gill. ex Hock. et Arn.) Walp.	BK	—	—	—	—	—	(+)	—
Rutales								
Pistacia mexicana Swingle	AU	—	+	—	—	—	—	—
Geraniales								
Geranium pratense L.	BK	—	—	—	—	—	—	—
Rhamnales								
Ziziphus jujuba Mill.	AU	(+)	—	—	—	—	—	—
Scrophulariales								
Digitalis purpurea L.	BK	—	—	—	—	—	—	—
Campanulales								
Campanula persicifolia L.	BK	—	—	—	—	—	—	—
Monocotyledoneae								
Liliales								
Lilium pumilum Del.	BK	—	—	—	—	—	—	—
Tulipa gesneriana L.	BK	—	—	—	—	—	—	—
Narcissus poeticus L. (anthers)	BK	—	—	—	—	—	(+)	—
Cyperales								
Carex montana L. (anthers)	BK	—	—	—	—	—	(+)	—
Eriophorum angustifolium Honck.	W	—	—	—	—	—	—	—
Typhales								
Typha angustifolia L.	W	—	—	—	—	—	—	—
T. latifolia L.	OI	—	—	—	—	—	—	—

Q = Quercetin 3-*O*-glucosylgalactoside; **K** = unidentified kaempferol conjugate; **P** = N^1,N^5-di-*p*-coumaroylspermidine; **AI/AII** = N^1,N^5-, N^5,N^{10}- or N^1,N^{10} di-*p*-coumaroylspermidine [16], **P** and **AI** could not be distinguished by chromatographic analyses; **B** = N^5,N^{10}-diferuloylspermidine; **CI** = N^1,N^{10}-caffeoylferuloylspermidine; **CII** = N^1,N^{10}-diferuloylspermidine (for structures see Fig. 1).

AU = Area at Austin, U.S.A. (spring/summer 1986); BD = Botanic Garden Dortmund, F.R.G. (spring 1986, 1987); BK = field at Botany Department Universität, Köln (spring/summer 1985, 1987); M = Area at Mönchengladbach, F.R.G. (spring 1985); OI = area at Ostia, Italy (June 1985); PNF = Prescott National Forest, Arizona, U.S.A. (May 1986); W = collection Wiermann.

— = Not detected; (+) = traces, only detected with HPLC; + = 40–100 nmol/100 mg pollen; ○ = 100 nmol–1 μmol/100 mg pollen; ◐ = 1–2 μmol/100 mg pollen; ● = 2 μmol/100 mg pollen, * = not detected in extract III (< 10 mg pollen).

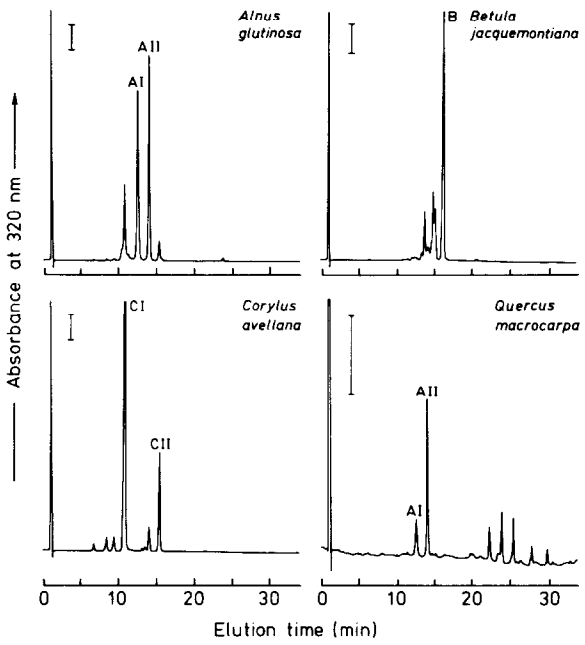

Fig. 2. Selected diagrams from HPLC analyses of hydroxycinnamic acid–spermidine amides (extract III; see 'experimental') from Fagales pollen. For peak identification see Fig. 1 (**AI** could not be chromatographically distinguished from **P**). Bar = 0.01 absorbance units. Separations were achieved on a Nucleosil C-18 column (3 μm, 100 × 4 mm i.d.; Macherey–Nagel, Düren, F.R.G.) at a flow rate of 1 ml/min with a two-step linear gradient: in 20 min from 30% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O) to 60% solvent B in A and subsequently in 13 min to 100% solvent B.

were found in all the five investigated members of the subfamily Coryloideae: *Corylus avellana*, *C. colurnoides* C. Schn., *Carpinus betulus* L., *C. japonica* Blume, and *Ostrya carpinifolia* L. Two *N*,*N*-di-*p*-coumaroylspermidines (**AI**, **AII**) were found in pollen of *Alnus* species, while the major HCA amide in *Betula* pollen was N^5,N^{10}-diferuloylspermidine (**B**). These results are in good agreement with a serosystematic study on pollen proteins of Betulaceae [9]. The subfamilies Coryloideae and Betuloideae are distinguished by the occurrence of the N^5,N^{10}-substituted spermidines in the Betuloideae and mainly the N^1,N^{10}-substituted type in the Coryloideae. Within the Fagaceae all investigated species of the subfamily Quercoideae contain at least one of the three dicoumaroylspermidines. But it is noteworthy that the *Quercus* species examined show in addition a pattern of less polar HCA derivatives which seems to be characteristic for this genus (Fig. 2). Pollen from *Fagus sylvatica* L. and *Nothofagus antarctica* (G. Forst.) Oerst. (Fagoideae) also contain unidentified HCA conjugates, even in extract III. Samples of the subfamily Castanoideae were not available for the current investigation.

It is interesting to note that species of the Juglandales and Myricales also contain HCA amides. N^1,N^5-dicoumaroylspermidine (**P**) could be identified from pollen of *Pterocarya fraxinifolia* [16]. *Myrica gale*, *Juglans nigra*, and *J. regia*. also contain appreciable amounts of a chromatographically identical di-*p*-coumaroylspermidine probably with N^5-substitution in the pollen grains. This again affirms a close relationship of these orders to the Fagales. None of the studied phenylpropanoids could be found in *Pistacia mexicana* Swingle pollen, which is in agreement with most taxonomic treatments.

Evidently the phenylpropanoid patterns (flavonol glycosides and HCA amides) in pollen form significant chemosystematic characteristics at the generic and subfamily levels of Betulaceae and probably also Fagaceae. Similar phytochemical patterns do occur in related Hamamelidian orders, but their systematic significance has not yet been demonstrated.

EXPERIMENTAL

Plant material. Pollen (or spore) material was collected in spring and summer of 1985, 1986, and 1987 from various localities (Table 2), freeze- or air-dried and stored at −20°.

Preparation of extracts. Three extracts (I-III) were obtained from all samples and used for chromatographic analyses. Usually the extractions were performed with 100 mg pollen (or spores). Each sample was extracted first 3 times with 2 ml H_2O (1 hr each at room temp.). After centrifugation the supernatants were combined and used for determination of flavonoids (extract I). Then the pollen (or spores) were extracted × 6 with 2 ml 80% aq. MeOH (1 hr each at room temp.). After centrifugation the supernatants of the first three extractions, containing 60% of total HCA amide content, were combined (extract II) and used for quantitative determination of the HCA amides. In addition the 6th fraction (extract III) was also analysed. The standard extraction procedure was established with pollen from *Corylus avellana* for 100 hr to show (i) that there are almost no HCA amides in the H_2O fraction and (ii) that only the HCA amides investigated here occur in extract III.

Chromatographic identification and quantification of phenylpropanoids. Extracts I, II, and III were analysed by HPLC, extracts II and III by TLC—and in some cases also extract I—were used for TLC flavonoid identification (see Table 1). The plates were sprayed with Dragendorff III reagent and 4-nitroaniline [nos 89 and 178 in ref. 22]. HPLC analysis was carried out with a two-pump system (LKB Instrument, Gräfelfing, F. R. G.) connected with an UV/Vis variable wavelength detector and an autosampler (Spark, Emmen, Netherlands), injecting 20 μl. Chromatographic conditions are described in Fig. 2. Quantitative calculations were obtained by a Shimadzu (Kyoto, Japan) Data Processor Chromatopac C-R3A. Rutin, nicotiflorin (Roth, Karlsruhe, F. R. G.), and quercetin 3-*O*-glucosylgalactoside (*Corylus avellana* pollen) [20] were used as standards for flavonol investigation. The HCA amides [14, 16] were quantified using a $\log\varepsilon$ ($cm^{-1} \times mol^{-1}$) at λ_{max}^{MeOH} of 4.33 (codonocarpine) [23].

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