Epicuticular Waxes of Seed Coats from Species of the Genus *Cistus* L. (Cistaceae)

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Epicuticular waxes were extracted with hexane from seed coats of 16 species from the genus *Cistus* (Cistaceae) in amounts corresponding to an average of 0.07% per dry weight. All cuticular waxes obtained from the various *Cistus* species consisted of hydrocarbons, wax esters, triterpenol esters, aldehydes, primary alcohols and free fatty acids. These compounds were present as homologues in changing patterns. In addition two unidentified acids and two unknown alcohols were found. Epicuticular waxes from seed coats differed qualitatively and quantitatively from those of *Cistus* leaves and petals and confirmed the existence of organ specific compositions of epicuticular waxes. The wax components secreted in this composition result in a cover which in scanning electron microscopic pictures resembles a smooth layer of solidified fluid lacking any crystalline structure.

Introduction

All organs of higher plants are regularly protected by a thin epicuticular wax layer against their environment air. The wax layer consists usually of a complex mixture of long chain and lipophilic components which cause nearly complete impermeability of this natural plant surface. The regulation of water transpiration and gas metabolism through the stomata are a consequence of this hydrophobic and nearly impermeable outer membrane. The wax layer is furthermore a barrier of aerial plant cells against the attack by solid, liquid or gas air pollutions as well as bacteria, viruses, fungi and insects etc. Therefore, the structure of plant surfaces and the composition of epicuticular waxes have been studied in detail from leaves, stems, petals and fruits of many plants [1–3]. But only a few data are known about single components of seeds or seed coats [4–7]. Extensive results on waxes of seed coats from *Jojoba* were published recently [8–9]. *Cistus* species have been investigated in detail regarding epicuticular waxes from leaves and petals [10–13] as well as another Cistaceae such as *Halimium halimifolium* [14]. In continuation of these investigations epicuticular waxes were now isolated and analysed from seed coats of 16 *Cistus* species.

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Materials and Methods

Cistus seeds for the following studies are the same as described by Krollmann and Gülz [15]. All known native *Cistus* species [16] were analysed with the two exceptions of *C. osbeckiaefolius* and *C. varius*. Batches of 5 g of whole seeds were extracted by refluxing with 25 ml hexane for three times, altogether for 30 min. The cuticular waxes were fractionated on silica gel columns (Kieselgel 60, Merck). Hydrocarbons were eluted with pentane, wax esters, triterpenol esters and aldehydes with 2-chloropropane and free fatty acids and alcohols with methanol. Alkenes were separated from alkanes by TLC in isooctane as developing solvent on silica gel plates impregnated with $AgNO_3$ (0.6 g $AgNO_3$/10 ml acetonitrile).

Wax esters (R_f value 0.65) and aldehydes (R_f value 0.42) were separated by preparative TLC with benzene. Aldehydes were identified by reduction to primary alcohols with $NaBH_4$ in dioxane. Wax esters were transacylated with 5% HCl in ethanol, the saponification products were isolated by preparative TLC. Free fatty acids were esterified with diazomethane and then separated from alcohols by TLC with benzene. Acetylated alcohols were separated by TLC with petroleum : ether : acetic acid (80 : 20 : 1) for developing solvent.

Gas-liquid-chromatography:

Hewlett-Packard model 5710 A with FID and integrator 3380 S.

25 m glass capillary column DUHT-OV-101 for hydrocarbons, wax esters, aldehydes and alcohols.
10 m glass capillary column FFAP for fatty acid methyl esters, ethyl esters and alcohol acetates.

Results and Discussion

Whole *Cistus* seeds were extracted with hot hexane for three times. Further extractions did not increase the amounts of cuticular waxes. This fact as well as the quantity and composition of the isolated waxes, which differed from the lipids extracted from homogenized seeds [15], indicated the presence of epicuticular waxes on seed coats. Thus it may be concluded, that only waxes from seed coats were extracted by this method and not lipids from other parts of the seeds.

From 5 g of *Cistus* seeds epicuticular waxes could be isolated in amounts of 1 mg to 5 mg with an average of 3 mg or 0.07% of seed dry weight. Only from *C. parviflorus* waxes were extracted in higher amounts of 0.20%.

All epicuticular waxes from seed coats of 16 *Cistus* species contained homologues of alkanes, alkenes, wax esters, triterpenol esters, aldehydes, free fatty acids and primary alcohols.

Seeds of *C. ladanifer* were present in greater amounts. Parallel preparations and analyses were carried out in this species resulting in representative means, which are listed in Tables I and II.

Hydrocarbons

Hydrocarbons were present in a complex mixture of mostly odd-carbon-numbered homologues with chain lengths ranging from C_{17} to C_{35}. Homologous series of *n*-alkanes with similar, not very steep alkane patterns were dominating and the main component was always *n*-nonacosane (32.0% ± 10.2%). In *C. salvifolius* this main component accounted to 56.0%. As a representative example the

Table I. Composition and yield of epicuticular waxes from *C. ladanifer* seed coats.

	[mg]	[% dry wt]
fresh weight	10 000	90.6
waxes	7.0	0.08
alkanes	1.4	0.016
alkenes	0.1	0.001
wax esters triterpenol esters	0.3	0.003
aldehydes	traces	+
fatty acids	1.9	0.021
2 unidentified acids	1.4	0.016
alcohols	0.1	0.001
2 unidentified alcohols	1.6	0.018

Table II. Composition (peak area %) of epicuticular waxes from *C. ladanifer* seed coats.

No. of C-atomes	Alkanes	Wax esters	Saponification		Free acids	Free alcohols
			acids	alcohols		
14			+	+	3.2	
16			41.1	1.8	61.0	
16 : 1			+		+	
17	+		1.6	+	+	
18	0.4		9.0	4.4	9.6	
18 : 1			1.2		10.5	
18 : 2					8.0	
18 : 3					+	
19	1.1		0.2	1.0	+	
20	1.5		27.6	8.9	4.5	
20 : 1			0.5			
21	2.7		1.6	1.0		
22	1.3		10.5	19.1	1.7	+
22 : 1			0.7		1.5	
23	6.3		0.5	2.2		
24	1.6		5.5	29.6	+	10.6
24 : 1			+			
25	8.5		+	4.7		3.1
26	1.4		+	20.4		74.8
27	19.9			1.7		1.0
28	5.4			5.2		10.6
29	35.3			+		
30	1.8			+		+
31	8.6					
32	+					
33	+					
34	+					
35	+					
36		1.2				
37		+				
38		5.4				
39		1.6				
40		17.7				
41		4.6				
42		27.2				
43		4.2				
44		19.8				
45		3.1				
46		10.3				
47		1.6				
48		3.3				
49		+				
50		+				
	alkenes 4.2%	triterpenol esters +	triterpenols +			

+ = < 0.1%

composition of alkanes from *C. ladanifer* is listed in Table II. From all 16 *Cistus* species the following alkane components were found (means and standard deviations in brackets): C_{23} (6.4% ± 2.7%), C_{25} (11.0% ± 7.0%), C_{27} (20.3% ± 4.2%), C_{29} (32.0% ± 10.2%), C_{31} (4.6% ± 1.1%).

All hydrocarbon fractions contained also alkenes in amounts from 1% to 10%. An extreme value of 18% was found in *C. parviflorus.* Alkenes were identified as members of homologous series from C_{23} to C_{35}, with C_{31} as the main component. IR-Spectra indicated double bonds in cis-configuration. In analogy to alkenes from *Cistus* petals, these alkenes may be mixtures of isomeric monoenes [11]. Remarkable are unusually high amounts of even-numbered hydrocarbons, 13.4% for *C. ladanifer.*

Wax esters

Wax ester fractions, eluted with 2-chloropropane from silica gel columns, contained esters of long-chain fatty acids with long-chain alcohols or triterpenols and a small proportion of free aldehydes. Wax esters were found in all *Cistus* species predominantly as straight chain even-carbon-numbered homologues with chain lengths from C_{36} to C_{50}. They show similarly flat patterns with main components C_{42} or C_{40} for *C. albanicus* and *C. psilosepalus.* The composition of wax esters from *C. ladanifer* is listed in Table II.

From all 16 *Cistus* species the following wax esters were found (means and standard deviations in brackets): C_{38} (10.2% ± 5.1%), C_{40} (21.5% ± 7.3%), C_{42} (23% ± 3.4%), C_{44} (17.0% ± 4.8%) and C_{46} (10.1% ± 4.9%).

All wax ester fractions contained in addition triterpenol esters in amounts of usually less than 10%. Extremely high is the proportion of these esters in *C. palhinhae* (36%). The identification of these triterpenols is described together with the alcohols in the following section.

Saponification products

Saponification of cuticular wax esters from *Cistus* seed coats yielded mainly saturated long chained fatty acids and alcohols with chain lengths from C_{12} to C_{28}. Only very small amounts of monounsaturated acids, altogether up to 2.5%, were found. Major components in the fatty acid fraction were palmitic acid and arachidic acid, in the alcohol fraction tetracosanol, hexacosanol and docosanol. In addition the triterpenols could be identified by TLC and GLC with authentic samples as α-amyrine and β-amyrine. A third triterpenol could not be identified.

The composition of saponification fatty acids and alcohols from seed coats of *C. ladanifer* is listed in Table II.

Aldehydes

Aldehydes were analysed in all *Cistus* species. They were present in about 1% of the 2-chloropropane fraction. Only in *C. albanicus* these substances were found in higher amounts up to 10%. Aldehydes from seed coats were found to represent a homologous series with chain lengths ranging from C_{16} to C_{28}, displaying a very steep pattern with the main component hexacosanal (85%).

Free fatty acids and alcohols

The largest quantity (63%) of the extracted epicuticular waxes was eluted with methanol. This fraction contained not only normal fatty acids and primary alcohols, but also unidentified acids and two unknown alcohols. After esterification, the fraction was separated by TLC resulting in two spots with R_f-values 0.4 and 0.06. The spot with R_f-value 0.4 contained fatty acid homologues with chain lengths from C_{14} to C_{24} with a predominance of saturated fatty acids and palmitic acid as main component (61%) (Table II). In addition two not identified acids were found in great amounts, 42% of the acids. In GLC these substances emerge shortly after the saturated fatty acid C_{20}.

The spot with R_f-value 0.06 contained small amounts of primary alcohols with chain lengths from C_{22} to C_{30} (Table II) and two not identified alcohols. They could be separated from the primary alcohols after acetylation by TLC. In GLC the peaks for these unknown alcohols have a position between the saturated acids C_{22} and C_{24}.

Surface layer

Epicuticular waxes from seed coats of all *Cistus* species are characterized by flat distribution patterns for all wax substances and the presence of homologues with relative short chains.

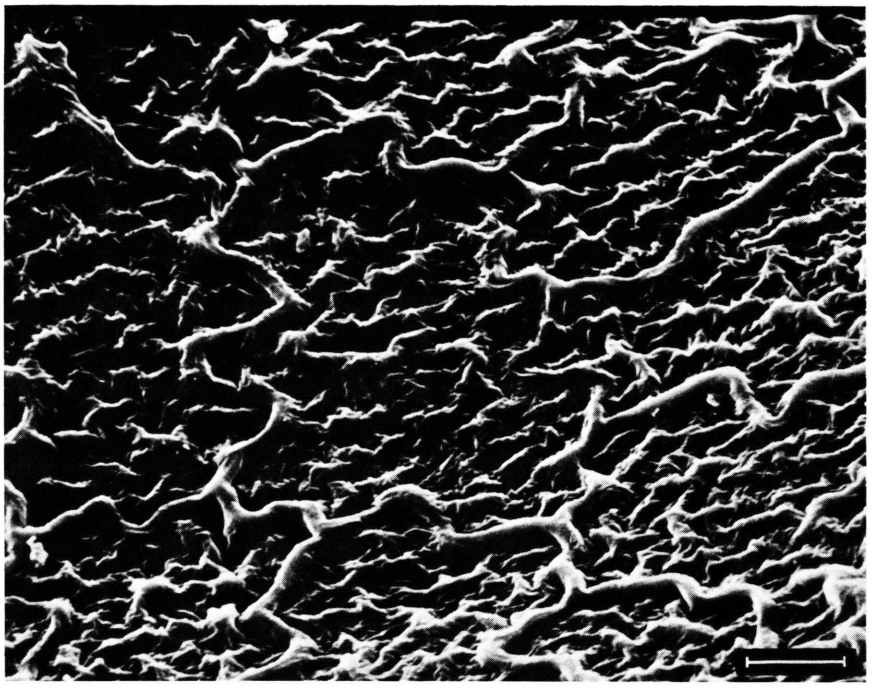

Fig. 1. Scanning electron micrograph of the surface of *C. ladanifer* seed coat; bar = 10 µm.

The wax components secreted in this composition result in a cover which in scanning electron microscopic pictures resembles a smooth layer of solidified fluid without any crystalline structure on the surface of *Cistus* seed coats.

The same epicuticular wax components are present on all *Cistus* seeds, but they differ quantitatively and in their distribution patterns. Furthermore, the quantitative and in some cases also the qualitative composition of epicuticular waxes from seed coats are quite different from those of *Cistus* leaves and petals [10, 13] and confirm the existence of organ specific compositions of epicuticular wax layers in this genus.

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