Epicuticular Leaf Waxes of *Tilia tomentosa* Moench. and *Tilia* × *europaea* L., Tiliaceae

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Tilia tomentosa Moench., *T.* × *europaea* L., Epicuticular Leaf Wax Composition, β-Amyrin Free and Esterified

Quantity and composition of epicuticular leaf wax of *Tilia tomentosa* Moench. and *T.* × *europaea* L. were examined and showed similar wax compositions. The waxes of these two *Tilia* species contained homologous series of n-alkanes, wax esters, aldehydes, acetates, primary alcohols and fatty acids. In addition to these common epicuticular wax constituents, the triterpenol β-amyrin was found free as well as esterified with long chain fatty acids and in very high amounts with acetic acid in both *Tilia* species.

Introduction

The plant cuticle is an important barrier between plant and environment and evidently forms a protection for the outer plant cells against various external influences. The function of the cuticle is essentially conditioned by its lipid wax layer. An intact epicuticular wax layer is also necessary for healthy growth of plants. Alteration in the surface structure and in the chemical composition of leaf epicuticular waxes show most irreversible damages and may be often caused by environmental factors.

Reports of epicuticular leaf waxes from European deciduous broadleaved trees are very rarely described. Only the wax composition of the leaves and fruits of apple, pear and peach trees are known [1–4].

Wax hydrocarbons were studied from *Juglans regia* [5], *Fagus sylvatica* and *Betula alba* [6]. In this study we want to report the epicuticular leaf waxes of the deciduous broadleaved trees *T. tomentosa* and *T.* × *europaea*. Lime trees, linden or basswoods in general are widespread in the broadleaved forests of the northern temperate zone of Europe and are trees of moderate to large size with alternate long-petioled, ovate-acuminate and serrate leaves. The fragrant flowers emerge from a pedicel which is fused about halfway along a pale green bract [7].

T. tomentosa, the silver lime, has suborbicular-cordate and serrate leaves, dark green and glabrescent above and with dense silvery-white stellate hairs beneath. This lime tree derived from the Balkan peninsula and today found frequently in European cities.

T. × *europaea* called common lime or linden is a natural hybrid between *T. cordata* and *T. platiphyllos,* cultivated from ancient times, and found abundantly in the streets, parks and gardens of Europe. Leaves are obliquely cordate and sharp toothed. They are dull green above and paler beneath, with small tufts of white to brownish hairs in all axils of the veins [7–9].

Materials and Methods

Leaves of *T. tomentosa* and *T.* × *europaea* were harvested in June from trees cultivated in the gardens of the Botanical Institute, University of Cologne. Whole leaves were dipped consecutively into three beakers with 600 ml $CHCl_3$ each for a total of 6 min. The extract was filtered and evaporated to dryness. 500 g fresh leaves from *T. tomentosa* yielded 1.65 g crude wax, which represent 0.3% of the fresh weight or 1.1% of the dry weight. Extraction of *T.* × *europaea* leaves resulted in 1.9 g crude wax, that is 0.4% of fresh weight or 1.3% of dry weight.

The crude wax was redissolved in pentane and fractionated on a silica gel column into three fractions by successive elution with pentane, 2-chloropropane and methanol as described previously [10, 11]. These fractions were separated again by preparative TLC on silica gel precoated plates (Merck 60, Darmstadt) with the following solvents: 1. Toluene (Rf_1) and 2. CH_2Cl_2:EtOAc (24:1) (Rf_2). The isolated compounds were identified by chemical

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reactions, such as methanolysis, ethanolysis, hydrogenation and reduction with $NaBH_4$ as well as by TLC and GC with authentic samples as described recently [11]. GC was carried out with a Hewlett Packard 5710 equipped with FID and an integrator 3380 S. The column used was 20 m glas capillary DUHT OV 101. The temperature was programmed from 160 °C to 340 °C at 4 °C/min.

Yield and composition of the identified compounds are shown in Table I.

The quantitative composition of the lipid wax components are listed in Table II and III.

Table I. Yield and composition of *Tilia tomentosa* and *T.* × *europaea* epicuticular leaf waxes.

	T. tomentosa		*T.* × *europaea*		TLC	
	[mg]	% wax	[mg]	% wax	Rf_1	Rf_2
Wax lipids						
Hydrocarbons	65	4.0	80	4.2	0.70	
Wax esters	207	12.5	266	14.0	0.64	
Aldehydes $C_{22}-C_{34}$	+	+	8	0.4	0.45	0.77
Acetates $C_{22}-C_{34}$	12	0.7	125	6.6	0.35	0.71
Alcohols	220	13.3	227	11.9	0.06	0.30
Fatty acids	42	2.5	43	2.3	0.02	
Triterpenoids						
β-Amyrin esters	186	11.3	124	6.5	0.70	
β-Amyrin acetates	566	34.3	648	34.1	0.37	0.71
β-Amyrin	167	10.1	54	2.8	0.06	0.30
β-Sitosterol	9	0.5	16	0.9	0.03	0.20
Unidentified	18	1.1	20	1.1		
Lost on column	158	9.6	289	15.2		
	1650	99.9	1900	100		

+ = traces

Table II. Quantitative composition of wax lipids from *T. tomentosa* leaves.

No. of C-atoms	*n*-Alkanes	Alcohols free	Alcohols esterified	Fatty acids free	Fatty acids esterified	No. of C-atoms	Wax esters
14				+	+		
16				30.9	70.2	38	+
18:2				2.4		39	
18:1				5.7		40	13.5
18:0				11.3	1.8	41	1.0
19						42	48.0
20		+		+	+	43	1.0
21	0.3					44	24.0
22	0.6	0.7	1.7	+	1.8	45	+
23	9.3	+			+	46	5.3
24	2.5	29.6	16.2	40.0	15.9	47	+
25	7.1	+			+	48	4.3
26	0.6	47.6	52.6	9.8	3.6	49	+
27	6.2	+			+	50	2.6
28	1.7	18.0	24.1	+	2.0	51	+
29	34.8	+			+	52	0.5
30	2.0	3.2	4.4	+	1.4	53	+
31	30.2	+			+	54	+
32	1.3	0.9	1.0	+	1.1		
33	2.1				+		
34	+				2.2		
35	+						

Table III. Quantitative composition of wax lipids from *T.* × *europaea* leaves.

No. of C-atoms	*n*-Alkanes	Alcohols free	Alcohols esterified	Fatty acids free	Fatty acids esterified	No. of C-atoms	Wax esters
14				+	+		
16				22.9	82.2	38	+
18:2				2.6		39	+
18:1				2.6		40	20.9
18:0				4.6	1.1	41	1.2
19						42	52.9
20				+	+	43	+
21	0.3					44	18.7
22	0.7	1.1	+	10.1	+	45	+
23	10.3	+		+	+	46	3.1
24	2.4	28.7	18.1	35.7	8.4	47	+
25	7.8	+		4.5	+	48	3.1
26	0.6	48.2	58.7	12.6	3.3	49	+
27	6.0	+		+	+	50	+
28	1.1	17.7	20.5	2.0	2.4	51	+
29	34.2	+		+	+	52	+
30	1.5	2.5	2.7	2.6	1.4	53	+
31	30.3	+				54	+
32	1.1	1.9	+	+	1.2		
33	2.8						
34	+						
35	+						

Results and Discussion

Epicuticular waxes of *T. tomentosa* leaves were extracted in amounts of 1.1% of dry weight and of *T.* × *europaea* in 1.3%. These waxes represent a normal yield from leaves without other secreted compounds. They were found to contain a complex mixture of different components. The presence of common long chained and mostly saturated lipid components was detected in homologous series of alkanes, wax esters, aldehydes, acetates, primary alcohols and fatty acids. These substances yielded about 33.0% and 39.4% of the crude wax and resulted from the lipid metabolism. In addition a number of triterpenoids were found which originated from the isoprene metabolism. These latter substances yielded about 57.2% and 44.3% of the crude waxes of both *Tilia* species.

Common lipid components

Hydrocarbons (4.0% and 4.2%) were fractionated with pentane on a silica gel column (fraction 1). Most hydrocarbons were found to consist of n-alkanes (about 99%) with chain lengths ranging from C_{21} to C_{35}. The components of greater concentrations were nonacosane and hentriacontane (see Table II and III). Traces of alkenes, about 1% of the hydrocarbons could be identified by TLC on silica gel plates which were impregnated with $AgNO_3$ and by hydrogenation with Pd-catalysator [12].

Fraction 2 contained wax esters, aldehydes and acetates. This fraction was separated again by TLC with the solvent toluene. Wax esters (Rf_1: 0.64) were present in homologous series ranging from C_{38} to C_{54} in amounts representing 12.5% and 14.0% of the waxes. The wax ester C_{42} was dominating, existing primarily of the alcohol C_{26} and the fatty acid C_{16} as shown by their ethanolysis products. The individual GC ester peaks presented wax esters of the same total carbon number, but containing mostly mixtures of isomeric esters. This supposition is based on the correlation between the composition of wax ester peaks and their saponification products and literature data [13, 14].

Ethanolysis of the wax esters yielded primary alcohols ranging from C_{22} to C_{32} with hexacosanol as the main component and fatty acids in form of fatty acid ethyl esters (FAEE) ranging from C_{14} to C_{32}. Palmitic acid was the dominating fatty acid. All fatty acids from the wax esters were found to be saturated (see Table II and III).

Aldehydes (Rf_1: 0.45) were present in fraction 2 in a small amount (about 1% of the wax) ranging from C_{22} to C_{34}. They were identified by reduction with $NaBH_4$ to the corresponding primary alcohols (Rf_1: 0.06).

Acetates (Rf_1: 0.35) were also isolated by preparative TLC from fraction 2 and could be identified by saponification with HCl/MeOH yielding the corresponding primary alcohols (Rf_1: 0.06) which ranged from C_{22} to C_{34}. The main component was hexacosanol.

Fraction 3 contained alcohols and fatty acids. This fraction was esterified with HCl/MeOH in order to obtain fatty acid methyl esters (FAME). The methyl esters were separated from alcohols with silica gel column chromatography. Elution with 2-chloropropane yielded FAME, and with MeOH were eluted alcohols [11]. The GC analysis of the FAME (2.5% and 2.3% of the wax) showed numerous compounds ranging from C_{14} to C_{34} with none of the fatty acids dominating. Weak maxima are shown at C_{16} and C_{24}. Unsaturated fatty acids were found only in small amounts with chain length C_{18} (see Tables II and III).

Primary alcohols were present in high amounts of 13.3% and 11.9% of the waxes with chain length ranging from C_{18} to C_{34}. Tetracosanol and hexacosanol were the dominating primary alcohols (see Tables II and III).

The two *Tilia* species studied contain the same lipid components with similar distribution patterns and identical distinct maxima in each homologous series.

Triterpenoids

The common lipid wax components just described were accompanied with one triterpenol and its esters. In the alcohol fraction of this waxes β-amyrin was found. This triterpenol was identified by TLC (Rf_2: 0.33), a positive carbazole colour reaction, GC and comparison with an authentic sample [11, 12]. Near after the β-amyrin peak another small peak in the GC was detected which could be separated by preparative TLC, solvent CH_2Cl_2: EtOAc (24:1) and identified as β-sitosterol (Rf_2: 0.20) in trace amounts. This sterol showed a specific carbazole colour reaction and the same Rf- and RT-values as authentic β-sitosterol [12].

Accompanied with the primary alcohol acetates β-amyrin acetate was found in the very high concentrations of 34.3% and 34.1% of the waxes. This acetate was also methanolyzed with HCl/MeOH and yielded the alcohol β-amyrin. β-Amyrin acetate and the corresponding β-amyrin were identified by TLC and GC and comparison with authentic samples.

Wax esters of long chained fatty acids and alcohols contained also β-amyrin esters in remarkable amounts of 11.3% and 6.5% of the waxes. These β-amyrin esters (Rf_1: 0.7) could be separated from wax esters (Rf_1: 0.64) by preparative TLC on silica gel plates, solvent toluene. The ethanolysis of the isolated triterpenol esters yielded β-amyrin and the very long chained fatty acids ranging from C_{22} to C_{32}.

T. tomentosa and *T.* × *europaea* contained the same triterpenoids β-amyrin, β-amyrin acetate and β-amyrin fatty acid esters. The two species differ in the amounts of β-amyrin and β-amyrin fatty acid esters (see Table 1), but the triterpenol derivative β-amyrin acetate was found to be the dominating major component of the epicuticular waxes in both *Tilia* species.

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