Surface Structures and Chemical Composition of Epicuticular Waxes during Leaf Development of *Fagus sylvatica* L.

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Dedicated to Professor Ludwig Bergmann on the occasion of his 65th birthday

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The surface structures of beech (*Fagus sylvatica*) leaf waxes were studied by SEM and correlated with chemical compositions of the extracted wax lipids during one vegetation period. The very young leaflets just unfolding from buds contained already a wax layer without any wax sculptures or crystalloids. This wax layer is quite different in yield and composition to that of mature leaves. With the unfolding of beech leaves, a dynamic biosynthesis of several wax lipids was started, but the biosynthesis of wax esters was not continued further.

Ten days after leaf unfolding the *de novo* biosynthesis of aldehydes could be detected for the first time. Aldehyde amount increased rapidly to about 13% of the wax. The predominant individual wax lipids synthesized were aldehydes, alcohols and fatty acids with C_{28} and hydrocarbons with C_{27} chain lengths, respectively. The biosynthesis of wax lipids in beech leaves was completed at the end of May and remained nearly constant in quantity and composition during the remaining season.

At the same time when aldehydes were found for the first time, wax sculptures were observed on beech leaf waxes coming out of the continuous wax layer, exclusively on the upper leaf side. These wax sculptures increased in size and quantity in the following time and were present on the upper leaf side all over the season, only some wax sculptures show a trend to crystalline forms.

Introduction

All aerial organs of higher plants are covered by a thin continuous wax layer on the surface of the cuticle [1, 2]. Wax layers are a protective and a transpiration barrier of the epidermal plant cells, and are responsible for the controlled transpiration and gas exchange through the stomata [3–6]. On mature leaves the continuous wax layers are often superimposed with wax sculptures or wax crystals. On deciduous broadleaf trees, the lower and the upper leaf sides show most different surface structures [7–9]. The micromorphology of epicuticular waxes are also cited as being helpful for taxonomic and organ-specific applications [10–16].

Plant surface waxes consist of homologous series of very long chained lipids and often also of triterpenoids [2, 17]. The composition of these waxes change during leaf development and growth as shown recently for *Fagus sylvatica* [18], *Tilia tomentosa* [19] and *Citrus aurantium* [6]. Leaf waxes of the beech tree consist of homologous series of only wax lipids [18, 20].

The very young beech leaves, just unfolding from buds were already covered with a wax layer, consisting only of homologous series of wax lipids without any triterpenoids. These waxes from young unfolding leaves showed a quite different lipid composition to that of mature leaves [18, 20]. The folded leaves in buds contained hydrocarbons, wax esters, benzyl acyl esters, alcohols and fatty acids from the beginning, but no aldehydes. Aldehydes were identified only after ten days of leaf unfolding. The biosynthesis of wax lipids was very dynamic in the first five weeks after leaf emerging. During this time the wax lipids were doubled quantitatively concerning leaf dry weight (0.36% to 0.75%) or leaf surface area (8 to 18 µg wax per cm^2) but were found to ten times for one leaf (100 µg to 961 µg wax per leaf). During May lipids with chain length C_{28} were synthesized predominantly resulting as main components for

Abbreviations: SEM, scanning electron microscopy; GC, gas chromatography; CC, column chromatography; TLC, thin layer chromatography.

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aldehydes, alcohols and fatty acids and C_{27} for hydrocarbons, respectively. Therefore a rapid change in chain lengths composition was observed. In contrast, wax esters and benzyl acyl esters were no more synthesized after unfolding of beech leaves. After May, wax lipids remained nearly constant in quantity and composition during the remaining season [18, 21].

During leaf development and one vegetation period the surface structures of *Fagus sylvatica* were studied continuously by scanning electron microscopy in the present investigation. These results were correlated with the chemical composition of the leaf wax layers, analyzed at the same stages, in order to study the variations of surface structures and their chemical implications.

Materials and Methods

Leaf harvesting

Leaves of a beech tree (*Fagus sylvatica* L. *cv. pendula*) were harvested from the southern west side of a free standing tree, more than 50 years old, cultivated in the garden of the Botanical Institute of the University of Cologne. Leaves were collected continuously twice in a week in April and May and then once in a week throughout the season up to the end of November. In 1989 as well as 1990 the first leaflets unfolding from buds were observed on 24th April. The yellow leaves about to fall were harvested on 19th November.

Scanning electron microscopy

Fresh and air-dried leaves were prepared for SEM by sputtering with gold using an Emscope sputter coater and examined in a Hitachi S-405 A scanning electron microscope or a Cambridge Stereo Scan 200.

Wax analysis

Beech leaf waxes were extracted with $CHCl_3$. The wax extracts were fractionated on a silica gel column in three fractions, using the solvents: 1. *n*-

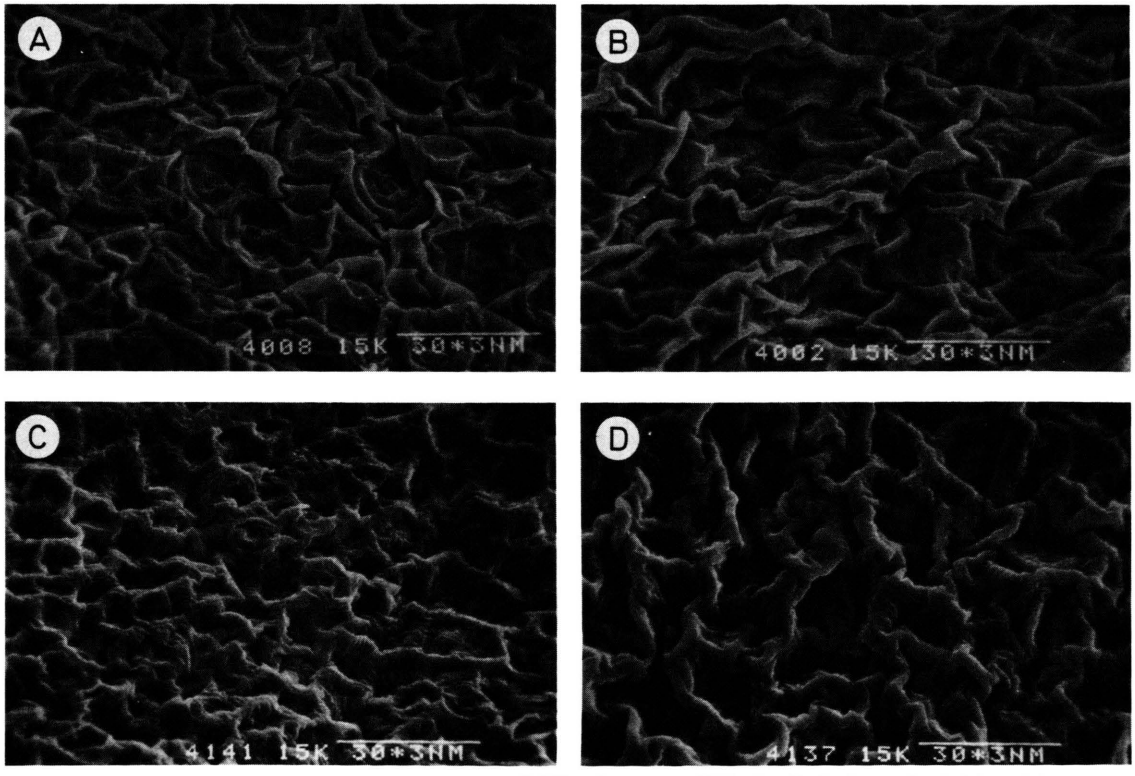

Fig. 1. *F. sylvatica* leaf surface from very young unfolding leaves on 27th April. A: lower leaf side, with a continuous wax layer; B: upper leaf side, with a continuous wax layer; C: lower leaf side, washed with chloroform; D: upper leaf side, washed with chloroform. Bar = 30 μm.

pentane (hydrocarbons), 2. 2-chloropropane (wax esters, benzyl acyl esters and aldehydes), 3. methanol (alcohols and fatty acids), and were analyzed by GC as described recently by Prasad and Gülz [18, 20].

The patterns of the beech leaf wax lipids on 24th April and 15th August in Fig. 4 were described in peak area percent of the gas chromatograms.

Results

Leaf surface structure

The study of surface lipids with SEM implies very careful preparation methods and excludes all fixation preparations that could dissolve the wax lipids or destroy the original structures. Most usual are the sputtering of fresh and carefully air-dried leaves with gold [10].

Beech leaflets in buds are folded like a fan. With the first emergence of these leaflets the experiments were started. SEM pictures from the lower (abaxial) and the upper (adaxial) beech leaf sides were taken from 24th April continuously all over the season. The variations of leaf surface structures were studied in this manner during leaf development and vegetation period. The folded leaves just emerging from buds contained already a wax layer on the lower as well as on the upper leaf sides, as demonstrated in Fig. 1 A and B in preparations of very young leaves on 27th April. On both leaf sides the epidermal cells are covered with a wax layer without any wax sculptures or crystalloids. Young stomata could be seen on the lower leaf side, but no trichomes are on both leaf sides. These wax layers could be washed out by dipping the leaves in $CHCl_3$ for a short time up to 1 min. By this treatment all epicuticular waxes were washed out, but no lipids from the inner part of the epidermal cells were extracted as checked by chemical analysis, and the cutin layer was not destroyed by this manner. The extracted epicuticular waxes from these very young leaves just emerging

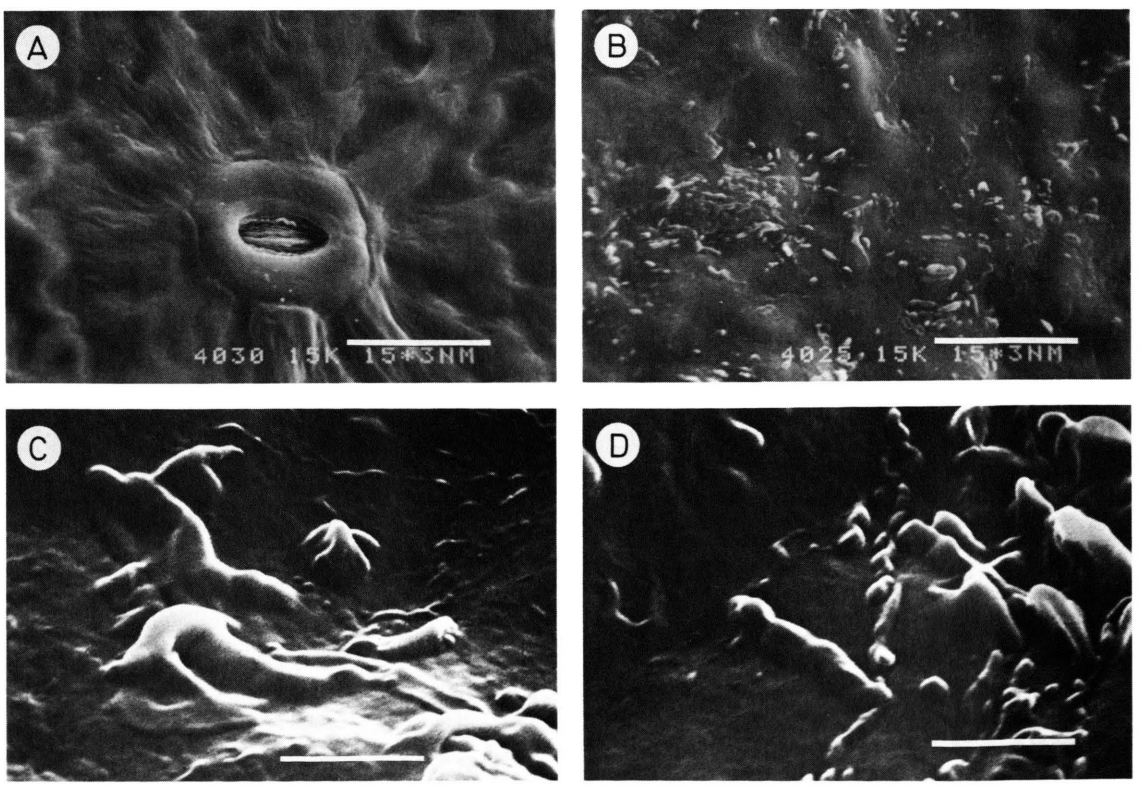

Fig. 2. *F. sylvatica* leaf surface from unfolded leaves on 4th May. A: lower leaf side with a continuous wax layer without any wax sculptures; B: upper leaf side with a continuous wax layer and additionally wax sculptures. Bar = 15 μm. C and D: different wax sculptures from the upper leaf side. Bar = 2 μm.

from buds were analyzed by CC, TLC and GC. The composition of the epicuticular waxes of this early stage is listed in Fig. 4.

In Fig. 1 C and D can be seen very young epidermal cells with their cutin layer after washing out the waxes with $CHCl_3$. All epidermal cells of the mature leaves may be determined in these young leaflets. During the following time in April and May a different epidermal cell enlargement could be observed.

Ten days after leaf unfolding a variation in wax structures of the upper leaf side was observed, but not on the lower side (Fig. 2A and B). According to Fig. 2C and D wax sculptures are coming out from the continuous wax layer of the upper leaf side without crystalline structures at this developmental stage.

In the following four weeks in May, the wax sculptures increased in size and quantity and were nearly constant in the following months. Fig. 3 shows these wax layers from a mature leaf at the end of June. On the lower leaf side no wax sculptures are seen, but numerous wax sculptures are present on the upper leaf side. Only some wax sculptures show a trend for crystallization (Fig. 3C and D).

Again, the wax layers from mature beech leaves were washed out with $CHCl_3$ resulting in a precise drawing of epidermal cells and walls. The morphologically intact cutin layers above the epidermal cells could be seen in a clear structure on its SEM pictures. But this cutin layer is no more a transpiration or protective barrier after removal of the surface wax layer [3–6, 22]. Water and gaseous substances from the inner part of the cells diffuse through the cell walls and also the cutin. The washed beech leaves reached their dry weight within 90 min at room temperature. The essential presence of epicuticular waxes for the normal function of the cuticula could be demonstrated by this simple experiment.

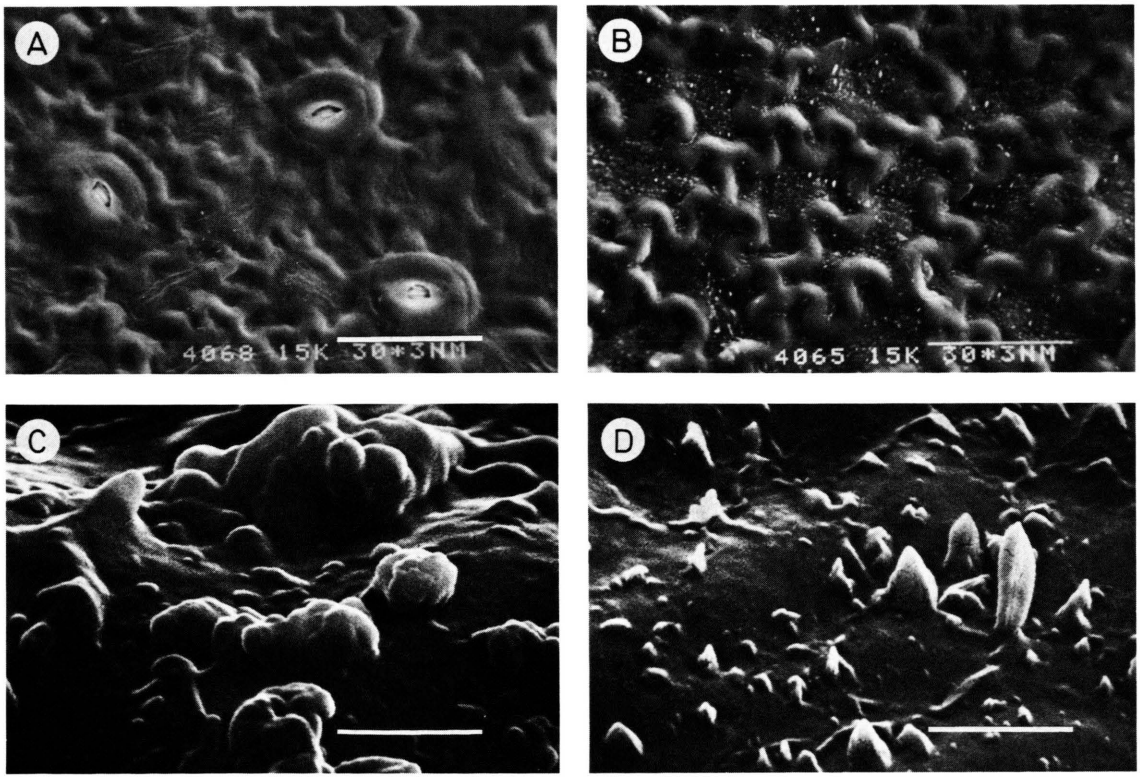

Fig. 3. *F. sylvatica* leaf surface from matured leaves at the end of June. A: lower leaf side with a continuous wax layer without any wax sculptures; B: upper leaf side with the additional wax sculptures. Bar = 30 μm. C and D: wax sculptures from the upper leaf side with a trend to crystallization. Bar = 1.6 μm.

Chemical wax analysis

Parallel to the SEM preparations, the surface waxes of beech leaves were analyzed during a vegetation period and presented recently by Prasad and Gülz [18]. The CC, TLC and GC analysis of the extracted surface waxes from very young leaves, just unfolding from buds on 24th April resulted in the presence of only homologous series of wax lipids which contained of hydrocarbons, wax esters, benzyl acyl esters, alcohols and fatty acids but no aldehydes. Leaf wax composition and the distribution patterns of the individual lipid classes are demonstrated in Fig. 4.

In the wax of very young leaves at 24th April alcohols (50%) were dominating followed by wax esters (28%), fatty acids (10%) and hydrocarbons (9%). The distribution patterns of all these lipids showed no significant dominating chain length. Most lipid chain lengths were present in almost similar concentrations with a predominance of more short chain lenghts. So this wax lipid composition resulted in a continuous wax layer of more fluid consistancy as shown in Fig. 1.

After leaf unfolding a dynamic biosynthesis of several wax lipids was observed resulting in a rapid doubling of wax amounts per dry weight or µg wax per leaf surface area. Predominantly lipids with chain length C_{28} for aldehydes, alcohols and fatty acids and C_{27} for hydrocarbons were synthesized. These chain lengths became the main components of the individual lipid classes. Thus the chain length specificity changed in all wax lipid classes during May. However, the synthesis of wax esters and benzyl acyl esters was almost stopped after the unfolding of leaves. Aldehydes were detected in beech leaf waxes for the first time 10 days after leaf unfolding (4th May) and increased very rapid in May from 0 to 13% of total wax.

The biosynthesis of wax lipids was completed at the end of May. The wax lipids remained nearly constant in amount and composition during the following months. Wax composition on 15th August in Fig. 4 is representative for June to October. At that time also alcohols (42%) were dominating followed by fatty acids (21%), aldehydes (13%), hydrocarbons (13%) and wax esters (10%). Beech leaf waxes of this composition resulted in surface wax sculptures but only on the upper leaf side.

Discussion

Aldehydes and wax sculptures

The first detection of aldehydes in beech leaf waxes was found at the same time when wax sculptures on the upper leaf side were observed on 4th May, ten days after leaves emerging from buds. Thus the aldehyde biosynthesis may be the initiative step for forming these wax sculptures. But it may be not definitive that these sculptures consist

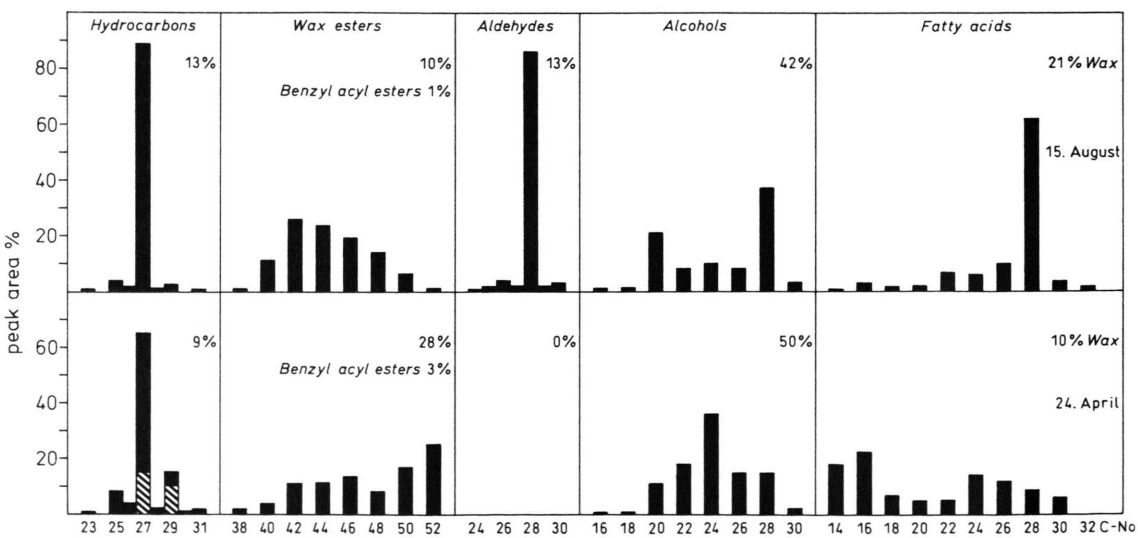

Fig. 4. Composition and distribution patterns of epicuticular waxes from *F. sylvatica* leaves on 24th April and 15th August.

of aldehydes. The amount of aldehydes is not very high at that time and other lipids were also synthesized very rapidly especially with one very long chain length C_{28}. The main lipid class in beech leaf wax consists of alcohols with more than 40% of total wax.

All identified individual lipids (saturated) of epicuticular waxes are solid and crystalline in pure form at room temperature. But all wax lipid classes are present in form of homologous series. Therefore these homologues are solved in each other with the consequence of a melting point depression of the individual substances. This melting point depression will be forced with the presence of six different lipid classes in form of homologous series as found in beech leaf waxes. Therefore these surface layers have mostly a more fluid consistancy in form of a solidified melt and are named wax.

Wax crystalloids are found on leaves mainly when one lipid class is present in high concentration (*ca.* 40%) and additionally with one dominating main component (*ca.* 80%). In beech leaf waxes primary alcohols were analyzed with about 42% wax for mature leaves. But the distribution pattern of the alcohols is not very steep. Alcohol C_{20} is present with 20%, alcohol C_{28} with 40% in the alcohol fraction. In contrast, the aldehydes show a very steep distribution pattern with one dominating aldehyde C_{28} with more than 80%, but the aldehydes are present only in a concentration of 13% wax [18]. Therefore on beech leaves are found a continuous wax layer with only wax sculptures, only some sculptures show a trend for crystallization.

A continuous wax layer superimposed with crystalloids was found on mature leaves of *Tilia tomentosa* [9]. Numerous crystals in shape of quadrangular rodlets are coming out of the wax mother liquor and contained of β-amyrenyl acetate. This substance is present in the high concentration of about 49% of *Tilia* leaf wax [9, 19].

Lower and upper leaf sides

Different wax surface structures on the lower and upper leaf side are found often for mature leaves of deciduous broadleaf trees as shown recently for *Quercus robur, Acer pseudoplatanus* and *Juglans regia* [8]. Different surface structures were also observed on leaves of *Tilia tomentosa* [9]. The different surface structures on the upper and lower leaf side for *F. sylvatica* is therefore not unusual. The formation of wax sculptures only on the upper leaf side of beech leaves was supported with the separate analysis of wax extracts from the lower as well as the upper leaf side. From the upper leaf side more wax (25%) was extracted. The content of aldehydes is also much more higher (4 times) and the main components with chain length C_{28} were also present in higher concentrations in the wax of the upper leaf side.

Common developmental factors

The chemical composition and the surface structure of leaf waxes were correlated during leaf development of three deciduous broadleaf trees, *F. sylvatica* [18], *T. tomentosa* [9, 19] and *Q. robur* (Gülz *et al.*, unpublished) and some general observations resulted from these studies.

The folded leaflets in the buds contained a wax layer without any crystalloid structures and showed quite differences in wax amount and composition to those of mature leaves. After leaf unfolding a dynamic biosynthesis of wax lipids has been started and especially wax lipids with very long chain lengths were synthesized and in all cases the synthesis of wax esters was stopped. About 10 days after leaf unfolding the *de novo* synthesis of aldehydes for *F. sylvativa* and *Q. robur,* and after 15 days of β-amyrenyl acetate for *T. tomentosa* was started. At the same time (after 10 to 15 days) variations in the surface structures of leaf waxes were also observed. Wax sculptures and crystalloid structures were formed out on the continuous wax layer. Lower and upper leaf sides developed different surface structures. After 5 to 12 weeks the wax lipid biosynthesis was completed in leaves and wax amount, composition and surface structure remained nearly constant over the remaining season. Nevertheless, many individual variations were found for epicuticular wax biosynthesis, wax composition and surface structure for the leaves of deciduous broadleaf trees.

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