

Surface Structure and Chemical Composition of Epicuticular Waxes during Leaf Development of *Tilia tomentosa* Moench.

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The very young leaflets of silver lime trees (*Tilia tomentosa*), just unfolding from buds, contained a continuous wax layer without any wax sculptures. The wax on young leaves is quite different in yield and composition than that of mature leaves. After unfolding of leaves a very dynamic biosynthesis of most wax lipids was started. Fifteen days after leaf unfolding the *de novo* biosynthesis of β -amyrenyl acetate and later on of aldehydes could be detected for the first time. The biosynthesis of wax components in silver lime leaves was finished at the end of June and the wax remained nearly constant in amount and composition during the remaining season. At the same time, when β -amyrenyl acetate was found for the first time, wax sculptures were observed in silver lime leaf waxes coming out of the continuous wax layer, exclusively on the upper leaf side. These wax sculptures increased in quantity in the next weeks and resulted in a crystalloid shape of most solitaire quadrangular rodlets. These crystals were remained all over the season and were formed from β -amyrenyl acetate, the dominating main wax component (ca. 49% wax).

Introduction

Epicuticular waxes are found to form a continuous layer, which cover the epidermal cells with their cutin layer, resulting in a nearly impermeable membrane, the cuticula [1]. 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 [2–5]. 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 [6–8]. The micromorphology of epicuticular waxes are also cited helpful for taxonomic and organ specific applications [9–15].

Plant surface waxes consist of homologous series of very long chained lipids and often also of triterpenoids [16, 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* [5]. Leaf waxes of the silver lime tree consist of homologous series of wax lipids and also triterpenoids [19, 20]. The

very young silver lime leaflets just unfolding from buds also contained a continuous wax layer with the components hydrocarbons, wax esters, acetates, alcohols, fatty acids and $\alpha + \beta$ -amyrin, but not aldehydes and β -amyrenyl acetate. This wax is therefore quite different in yield and composition from that of matured leaves. After leaf emerging from buds a very dynamic biosynthesis of wax components was started. During April, May and June wax amount was doubled quantitatively concerning leaf dry weight (0.5% to 1.08% wax/dry weight) or tripled concerning leaf surface area (13 μg to 39 μg wax/cm²). Wax amount per one leaf increased twenty times from 0.208 mg wax in the initial stage to 5.400 mg at the end of June. 15 days after leaf unfolding a *de novo* biosynthesis of β -amyrenyl acetate and later on of aldehydes commenced. From July to November wax amount and composition remained nearly constant [19]. The surface structures of developing silver lime leaves obtained by scanning electron microscopy are discussed and correlated with the chemical composition of these wax layers analyzed at the same stages in the present investigation.

Materials and Methods

Leaves of a silver lime tree (*Tilia tomentosa* Moench.) were harvested from a southern side of a

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free standing tree, more than 25 years old, cultivated in the garden of the Botanical Institute of the University of Cologne. Leaves were collected continuously twice in a week in March, April and May and then once in a week throughout the season up to the end of November. In 1989 the first leaflets just emerging from buds were observed and harvested on 29th March (in 1988 on 12th April, in 1990 on 17th April). The yellow leaves before falling were harvested on 15th November.

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

Silver lime 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*-pentane (hydrocarbons), 2. 2-chloropropane (wax esters, aldehydes, acetates), 3. methanol (alcohols, amyrins, fatty acids) and were analyzed by TLC and GC as described recently by Gölz *et al.* [19, 20]. The patterns of the silver lime leaf wax lipids on 29th March and 30th August (1989) in Fig. 4 were described in peak area percent of the gaschromatograms.

Results

The very young silver lime leaflets were rolled in buds. Beginning with the first emergence of buds these leaflets were analyzed continuously for their epicuticular wax composition [19] as well as their surface structures by scanning electron microscopy. SEM-pictures from the lower (abaxial) and the upper (adaxial) leaf sides of just unfolding leaves were recorded first on 29th March in 1989 (on 12th April in 1988, on 17th April in 1990) and then continuously all over the season. The folded leaves, just emerging from buds were already covered with a thin continuous wax layer (Fig. 1). The lower leaf side (abaxial) contained numerous ordinary trichomes in form of most eight cellular stellate hairs in a very dense arrangement (Fig. 1, A). Therefore it is very difficult to get a SEM-picture of the lower leaf surface at this early stage. On matured leaves, however, when the hairs were not so densely arranged because of the differential cell enlargement, it was possible to get a picture of the background. Always a continuous wax layer was observed without any wax sculptures on this leaf

side. The upper leaf side (adaxial) contains only solitary stellate hairs (Fig. 1, C) and additionally solitary glandular trichomes (Fig. 1, D). The cell walls of trichomes are commonly of cellulose and are covered with a cuticula and also waxes [21–23]. At this early developmental stage a continuous wax layer without any wax sculptures was found (Fig. 1, B, C, D). The epidermal cells were small and not yet filled with water and cell substances. On the upper leaf side also several eight cellular scars in form of “channels” could be seen (Fig. 1, C). The walls of these scars were also covered with wax.

Fifteen days after leaf unfolding a distinct variation in the surface wax structures of the upper leaf side was observed with wax sculptures coming out of the wax layers. Many crystal-germs appeared in the wax motherlye (Fig. 2, A). These wax sculptures were observed first on 13th April in 1989 (on 27th April in 1988, on 30th April in 1990). Again after 10 to 14 days definite wax crystalloids in form of most solitary quadrangular rodlets were seen to be dispersed over the leaf surface (Fig. 2, D, B). Clusters of rodlets were arranged over the scars (Fig. 2, C).

In Fig. 3 the same wax crystalloids are figured in greater magnification. A and B represent shapes of solitary most quadrangular rodlets erecting from the wax layer with a pointing at the basis. But not all wax sculptures were developed to a wax crystalloid. Above the scars were observed clusters of quadrangular rodlets (C + D) with variations in shape and largeness. The wax layers on the lower as well the upper leaf sides were washed out with CHCl_3 resulting in a precise drawing of epidermal cells, walls and hairs. Some glandular trichomes seem to be cut off by this treatment leaving back the eight cellular scars.

The composition of surface waxes of silver lime leaves were continuously analysed at the same stages to the SEM pictures during one vegetation period and presented recently by Gölz *et al.* [19]. The wax extracts from very young, just emerging leaflets on 29th March 1989 contained of homologous series of wax lipids composed of hydrocarbons, wax esters, acetates, alcohols, fatty acids, $\alpha + \beta$ -amyrin and their esters with long chain fatty acids of the latter substances. But aldehydes and β -amyrenyl acetate were completely absent at that early developmental stage. Fig. 4 demonstrates

this leaf wax composition on 29th March, chain lengths and distribution patterns are listed. In the wax of this very young leaflets, the dominating lipid class was wax esters (43%) followed by alcohols (24%), acetates (15%), fatty acids (7%), amyrins (6%) and hydrocarbons (5%). In this early stage, α -amyrin was predominated than β -amyrin. The wax composition resulted in a wax layer of more fluid consistency without any wax sculptures as shown in Fig. 1. This wax consistency is able to continue the unfolding movements in the initial stage and also the differential cell enlargements and growth of leaves in the following stages. After leaf unfolding a dynamic biosynthesis of epicutic-

lar waxes was observed from April to June. Wax content doubled or tripled during that time. Fifteen days after leaf unfolding the *de novo* biosynthesis of β -amyrinyl acetate and later on of aldehydes commenced. During leaf development β -amyrinyl acetate became the dominating epicuticular wax component comprising 49% of total wax (Fig. 4). An increasing trend was also found for the synthesis of hydrocarbons, aldehydes, alcohols, fatty acids and β -amyrin. During that time chain lengths specificity changed for hydrocarbons and fatty acids, β -amyrin became predominant to α -amyrin. The synthesis of wax esters and acetates, however, was almost stopped after leaf unfolding.

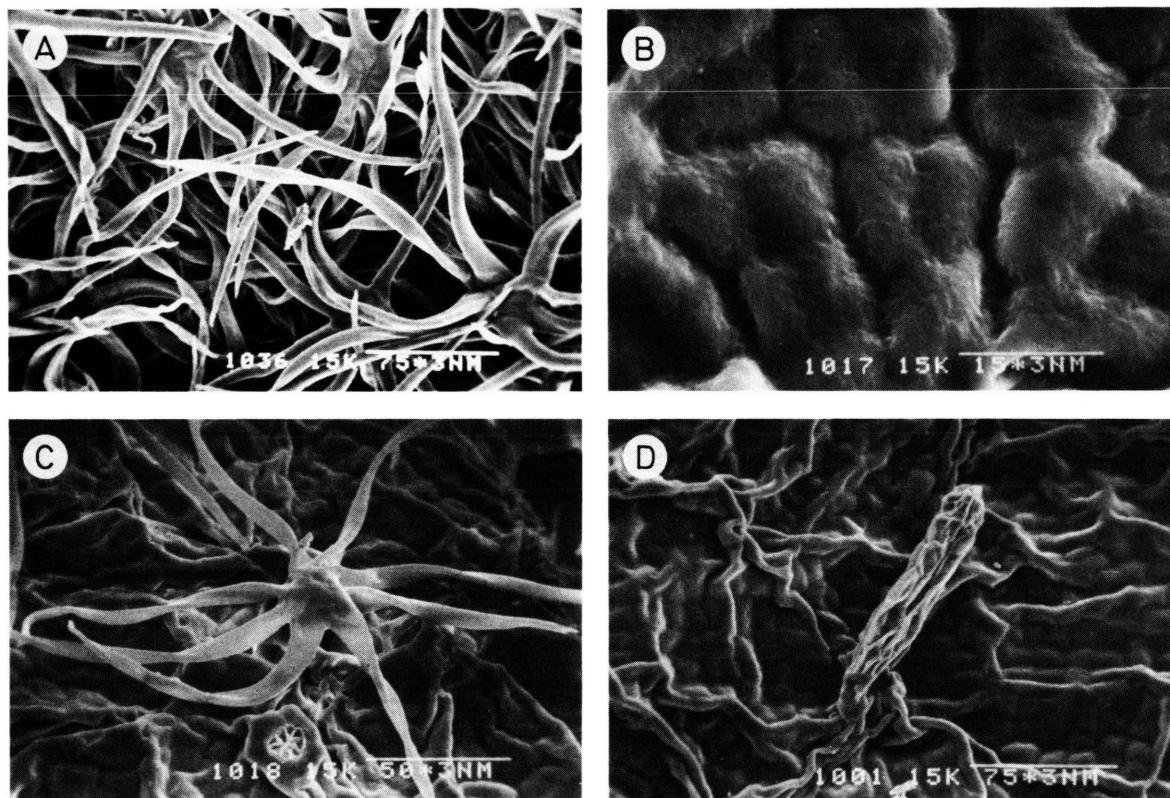


Fig. 1. *Tilia tomentosa* leaf surface structures of leaflets just emerging from buds

A lower leaf side with dense arrangement of stellate hairs. Bar = 75 μ m;

B upper leaf side with a continuous wax layer. Bar = 15 μ m;

C upper leaf side with a continuous wax layer, a solitary stellate hair and an open scar. Bar = 50 μ m;

D upper leaf side with a continuous wax layer and one solitary glandular trichome. Bar = 75 μ m.

From July to November the wax amount and composition remained nearly constant. The composition and distribution patterns on 30th August are representative for wax layers in that time and are figured therefore in Fig. 4. From July to November β -amyrenyl acetate was dominated with 49% of total wax. The other wax components followed with distinct lower values for alcohols (18%), fatty acids (10%), amyrins (7%), hydrocarbons (6%), wax esters (4%), aldehydes (3%) and acetates (3%). Silver lime leaf waxes of this composition exhibited a surface structure consisted of numerous single angular rodlets all over the upper leaf side.

Discussion

Fifteen days after leaf unfolding from buds β -amyrenyl acetate was observed for the first time in surface waxes from silver lime leaves. At the same time the formation of wax sculptures on only the upper leaf side was also noticed. The beginning of the biosynthesis of β -amyrenyl acetate may be the initiative step for forming these crystal-germs. In the following days many of these germs developed to crystalline in form of solitary quadrangular rodlets and spreaded all over the matured upper leaf surface and may be composed of only β -amyrenyl acetate, the dominating main wax component (49% of total wax) of mature silver lime

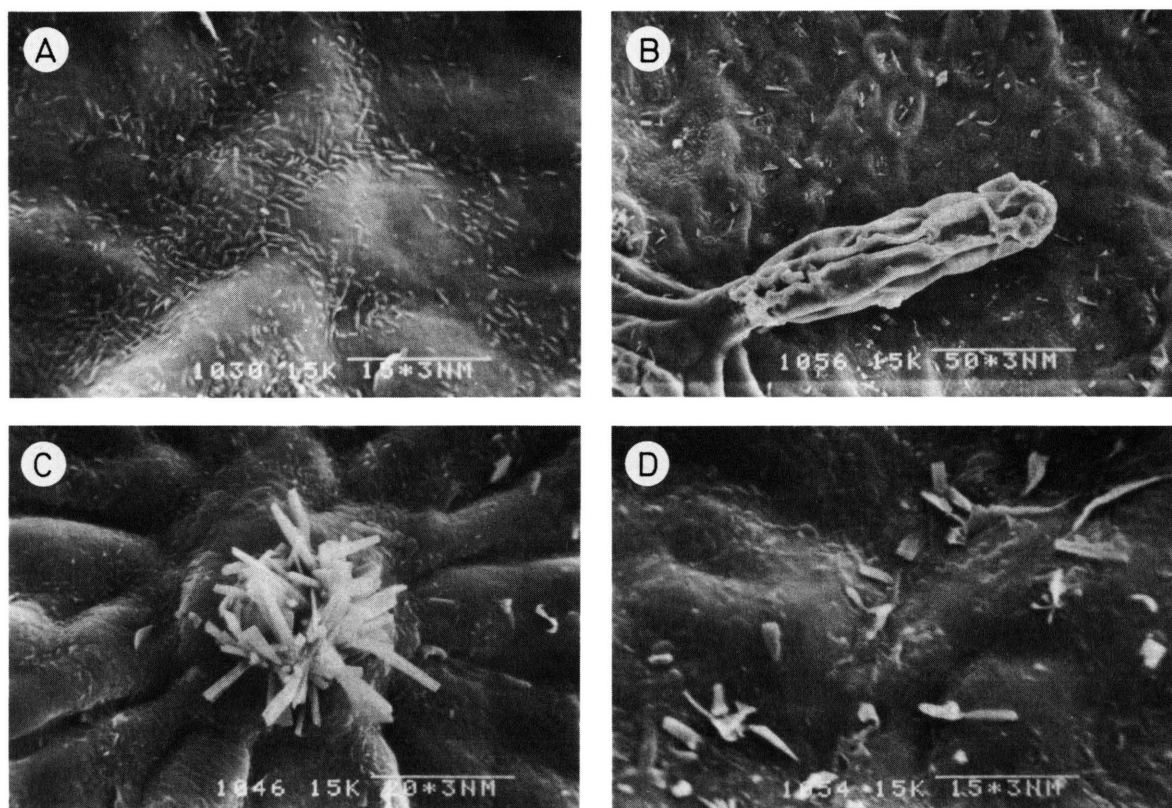


Fig. 2. *Tilia tomentosa* upper leaf surface structures of unfolding leaves

- A upper leaf side with a continuous wax layer and first wax sculptures, 15 days after leaf unfolding. Bar = 15 μ m;
- B upper leaf side with a continuous wax layer, single wax crystals and one glandular trichome, 30 days after leaf unfolding. Bar = 50 μ m;
- C upper leaf side with a cluster of quadrangular rodlets over a scar, 30 days after leaf unfolding. Bar = 20 μ m;
- D upper leaf side with a continuous wax layer, wax sculptures and most solitary wax crystals in the shape of quadrangular rodlets, 30 days after leaf unfolding. Bar = 15 μ m.

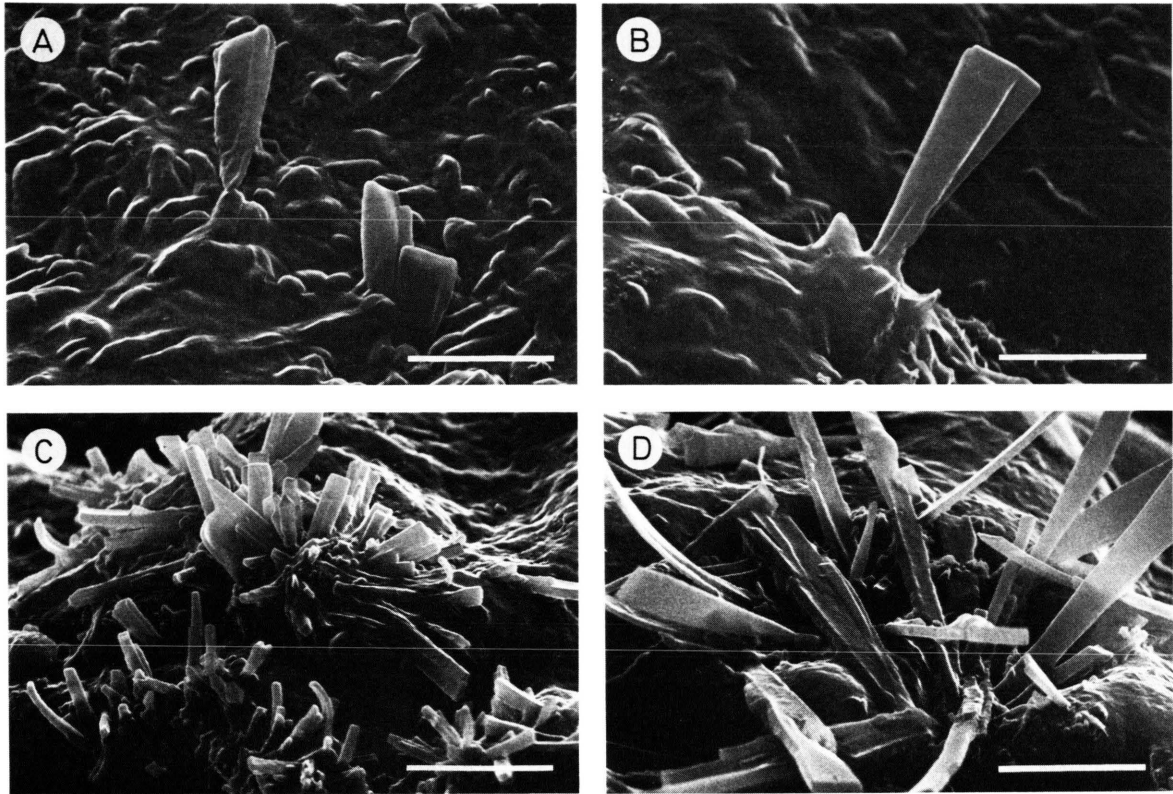


Fig. 3. *Tilia tomentosa* upper leaf side, crystalloid wax structures of mature leaves.
A + B solitary wax crystals in shapes of quadrangular rodlets. Bar = 3 μ m;
C + D clusters of wax crystals in shapes of quadrangular rodlets. Bar = 5 μ m.

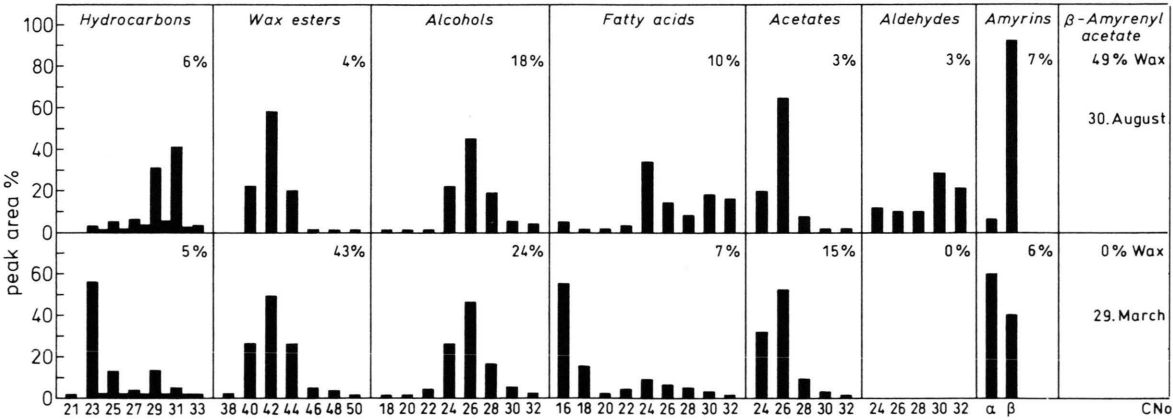


Fig. 4. Composition and distribution patterns of epicuticular waxes of *Tilia tomentosa* leaves on 29th March and 30th August.

leaves. In this case a very definite correlation could be observed with the beginning of biosynthesis and crystal development of one wax component, the β -amyrenyl acetate.

Most deciduous broadleaf trees have different surface structures on the lower as well the upper leaf side as shown recently for *Quercus robur*, *Acer pseudoplatanus*, *Juglans regia* [7], *Fagus sylvatica* [8] and also for *Tilia tomentosa* in the present investigation. The silver lime leaves have primarily a quite different morphology concerning trichomes. The lower leaf sides showed very dense arrangements of stellate hairs and stomata. On the upper leaf side solitary stellate hairs with solitary glandular trichomes were found. Furthermore the lower leaf side was covered with a continuous wax layer without any wax sculptures and crystalloids. In contrast, on the upper leaf side wax sculptures and also wax crystals were formed in shapes of quadrangular rodlets during leaf development. Therefore, in the lower and upper epidermal cells may be located different biosynthesis pathways for the individual wax components. β -Amyrenyl acetate may be synthesized predominantly in the upper epidermal cells.

In addition to the present study on *Tilia tomentosa* [19, 20] recently the chemical composition and the surface structures of leaf waxes were correlated with leaf development for another two deciduous broadleaf trees, *F. sylvatica* [8, 18] and *Q. robur* [Gülz *et al.*, unpublished]. These studies revealed many factors vary for different plants like the moment for leaf emerging, duration of leaf development, wax composition, wax surface structures,

wax crystals and leaf morphology. But several general observations were also found. The folded leaflets in buds also contained a continuous wax layer without any wax sculptures or crystalloids and are quite different in yield and composition to that of mature leaf waxes.

After leaf unfolding a dynamic biosynthesis of wax components was started. Especially wax lipids with very long chain lengths were synthesized after leaf unfolding. However, the synthesis of wax esters was stopped.

About 10 to 15 days after leaf unfolding the *de novo* biosynthesis of distinct wax substances was started, aldehydes on *F. sylvatica* and *Q. robur* leaves and β -amyrenyl acetate on *T. tomentosa* leaves. At the same time variations in the leaf surface structures were also observed. Wax sculptures and crystalloid structures were formed out and superimposed on the continuous wax layers.

After 5 to 12 weeks the wax biosynthesis was completed and wax amount, composition, and surface structures remained nearly constant over the remaining season.

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