

Developmental and Seasonal Variations in the Epicuticular Waxes of Beech Leaves (*Fagus sylvatica* L.)

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Dedicated to Professor Wilhelm Menke on the occasion of his 80th birthday

Fagus sylvatica, Fagaceae, Developmental Factors, Seasonal Variations, Epicuticular Waxes

The leaf epicuticular waxes of beech trees (*Fagus sylvatica* L.) were analyzed continuously all over one vegetation period with preparations every week from April 24 to November 15. The folded leaves in buds contained hydrocarbons, wax esters, benzyl acyl esters, alcohols and fatty acids from the beginning, but not aldehydes. Aldehydes were identified only after 10 days of leaf unfolding. The biosynthesis of wax lipids was rapid in the first three to five weeks till May 30. During this time the wax lipids were doubled quantitatively and the chain length specificity has also changed in all wax lipid classes. From June to October the composition of the wax lipid classes and also the chain length specificity remained constant with the exception of fatty acids. The biosynthesis of wax lipids was found to be very dynamic in the first five weeks and correlated with the leaf development and growth.

Introduction

The surface wax layer represents the interface between land plants and their aerial environment. The phylogenetical development of land plants was essentially conditioned with the biosynthesis of these wax lipids [1]. Wax lipids are found to form a continuous wax layer covering the cutin layers [2, 3]. This results in a nearly impermeable membrane [4], the cuticle. The waxes consist of homologous series of long chain wax lipids and often triterpenoids [5]. The function and physiological role of epicuticular waxes is essential for the healthy growth of plants. The wax layer is responsible for the controlled transpiration and the gas exchange through the stomata. These processes collapse immediately when the waxes washed out. This layer is furthermore a protection for the outer plant cells against environmental factors such as climate, mechanical damages [5] and pathogenic infections [6]. The chemical composition of these surface waxes were found to be organ and species specific [7–11] and to vary with light [12] and age. Several authors reported the seasonal variations of epicuticular waxes in the developing leaves of *Brassica* [13], wheat [14], *Sorghum* [15, 16], maize

[17], blueberry [18], peach [19], box [20] and the leaves and fruits of banana [21] and orange [22] and tomato fruits [23]. *Fagus sylvatica*, popularly known as Common or European Beech, is an important forest tree in Central and South Europe [24]. Recently we have reported the organ specific composition of epicuticular waxes of mature beech leaves and seeds [8]. The beech leaf waxes contained only homologous series of wax lipids without any triterpenoids. In the present investigation we report the developmental and seasonal variations of surface waxes from beech leaves. The knowledge of changes in leaf wax characteristics during the whole season might be important to interpret the plant response to environmental factors.

Materials and Methods

Leaves of beech (*Fagus sylvatica* cv. *pendula*) were harvested from a tree cultivated in the Garden of the Botanical Institute, University of Cologne. The leaf samples were collected from 24th April 1989 to 15th November 1989. Three to five branches in a length of about 1 m were harvested continuously twice in a week for the first three weeks and then once in a week throughout the season from a southern-western side of a free standing tree. In addition, sun and shade leaves were harvested thrice in the month of September to

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compare the results. During the final stages of the season in October/November faded brown leaves were also studied separately. The areas of the representative leaves were measured using a planimeter Kontron MOP-AM 02. Dry weight of the leaves was determined by heating them in an oven at 110 °C for 3 h. Surface waxes were extracted twice from leaves (about 50 g) by immersing them for 2 min in chloroform. The waxes were fractionated on a silica gel column and checked by TLC (solvent: toluene). The identification and quantification of the waxes was done as described previously [8]. The means and standard deviations in the Tables and Figures resulted from 4 preparations in one month. GC was carried out with a Hewlett Packard 5710 unit fitted with a FID and an integrator 3380 A on a capillary column OV 1 (10 m). The column temperature was programmed from 140 °C to 340 °C as required.

Results

The composition of beech leaf epicuticular waxes has been reported recently [8] from the matured leaves harvested in summer (July, September 1988). The wax contained only homologous series of long chain wax lipids. In the present investigation we have studied the developmental and seasonal factors concerning the surface wax composition. The leaf waxes were therefore analyzed every week continuously during the whole season from

April 24 to November 15, 1989. In the initial stages of leaf development the waxes were isolated twice in a week for a period of three weeks with the intention to get more information on leaf development and wax biosynthesis in this very active biosynthetic phase. The very young leaves unfolding from the buds appeared on April 24, 1989. The young leaves in the buds were folded like a fan and found to contain surface wax from the beginning of their unfolding phase of about 0.3% wax per dry weight with a layer of about 8 µg wax per cm² surface area. This early wax layer consisted of homologous series of hydrocarbons, wax esters, benzyl acyl esters, alcohols and fatty acids. At the beginning, aldehydes were completely absent in the waxes. They were found for the first time of about 1% of waxes in the preparation of May 5. From that day, a rapid biosynthesis of aldehydes was observed. The rapid wax lipid biosynthesis in beech leaves is documented in Tables I and II and Fig. 1–6. Table I summarized the epicuticular wax composition at the beginning of leaf development and mean values of mature leaves from June to November.

Developmental and seasonal factors

The very young folded leaves showed a dry weight of about 29% in April. Little increase was observed in the next month, but the dry weight raised to 43% in early June during the leaf development. The dry weight was almost constant in

Table I. Yields and composition of surface waxes of beech leaves at date 24. 4. 1989, mean values from June to November 1989 (n = 25) and shade leaves in September (lipids in % dry wt).

| | 24.4 | 5.5 | $\bar{x}_{25} \pm s$ (June–November) | | Shade leaves September |
|-------------------------------------|-------|-------|---|--------|---------------------------|
| Dry weight [%] | 29 | | 43 | ± 3 | 37 |
| Leaf area [cm ²] | 5 | | 28 | ± 3 | 30 |
| % Wax/dry wt | 0.36 | | 0.75 | ± 0.12 | 0.90 |
| µg Wax/cm ² leaf surface | 8 | | 18 | ± 4 | 14 |
| µg Wax/one leaf | 100 | | 962 | ± 212 | 750 |
| Hydrocarbons | 0.030 | | 0.111 ± | 0.028 | 0.130 |
| Wax esters | 0.085 | | 0.095 ± | 0.030 | 0.094 |
| Benzyl acyl esters | 0.005 | | 0.005 ± | 0.002 | 0.003 |
| Aldehydes | – | 0.001 | 0.101 ± | 0.020 | 0.147 |
| Alcohols | 0.190 | | 0.309 ± | 0.074 | 0.358 |
| Fatty acids | 0.042 | | 0.132 ± | 0.033 | 0.200 |
| Wax | 0.358 | | 0.780 ± | 0.015 | 0.932 |

the following months but decreased in November (Fig. 1 A).

The folded leaves in the buds had a leaf area of about 5 cm² at the initial stage. The leaves grew up to six times (30 cm²) by May and remained constant during the season (Fig. 1 B). The leaf growth was finished by May 16 when the largest leaf area

was reached but the synthesis of biomass continued up to May 30.

Surface waxes were present in the folded leaves of buds of about 0.3% of dry weight. The wax amount was increased to 1% in the next three weeks till May 16, decreased in the following two months to about 0.75% and remained constant over the remainder of the season (Fig. 1 C).

A continuous wax layer of 8 µg wax per cm² leaf surface was found in the initial days. The surface concentration of leaf was increased in the next 5 weeks (May 30) to about 20 µg per cm² leaf surface and was constant till August. Lower values (about 14 µg/cm²) were found in the following months (Fig. 1 D). Wax amounts correlated to one leaf resulted in a very low value of about 100 µg wax per leaf for the first preparation by April 29. Within the next five weeks a very rapid increase was observed up to 1200 µg wax per leaf. After that time a slow and continuous decrease to about 800 µg wax per leaf was found (Fig. 1 E). The seasonal patterns for wax amounts indicate a very active biosynthesis of wax components in the first three weeks. In the following time the waxes remained nearly constant.

The changes in the individual wax lipid classes during the season were also studied in detail. They are figured as total and individual components. All values in the figures are described per dry weight (Fig. 2–6). The lipid classes with respect to % per surface wax are summarized in Table II.

Hydrocarbons

Hydrocarbons were found in a homologous series ranging from C₂₁ to C₃₁ all over the season. In the first days of leaf development hydrocarbons were present about 8% of waxes. This amount increased very rapidly within four weeks to about 16% and decreased in the following months to nearly a constant value (Table II). These changes were resulted primarily by the biosynthesis of the hydrocarbon C₂₇. Heptacosane was the main component in the hydrocarbon fraction and showed the same seasonal pattern as that of total hydrocarbons. C₂₅ and C₂₉ were found in lower concentrations, nearly constant amounts all over the season. The other alkanes were found in very small amounts (Fig. 2 A and B).

The presence of unsaturated hydrocarbons with carbon number 27 and 29 in the first 8 days of leaf

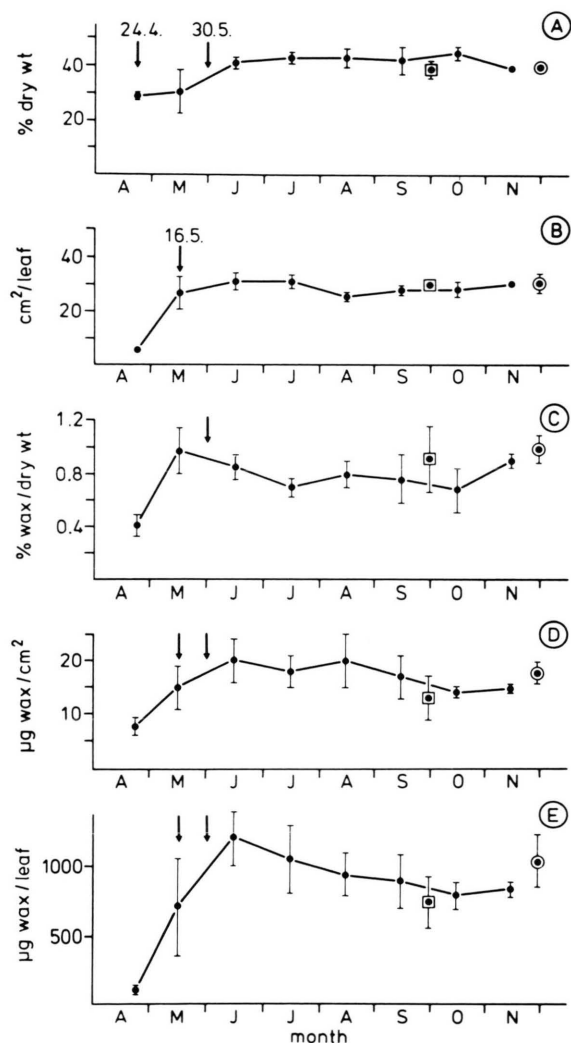


Fig. 1. Seasonal variations and developmental factors in the surface waxes of beech leaves
 A dry weight of the leaves in %;
 B leaf area in cm²;
 C % surface wax per dry weight;
 D µg wax per cm² leaf surface;
 E µg wax per one leaf
 □ = shade leaves, ○ = faded leaves.

Table II. Composition of surface wax lipids of beech leaves during a vegetation period (in % wax, $\bar{x} \pm s$; s. l. = shade leaves).

| | Hydrocarbons | Wax esters | Benzyl acyl esters | Aldehydes | Alcohols | Fatty acids |
|---------------------|----------------|----------------|--------------------|----------------|----------------|----------------|
| <i>Green leaves</i> | | | | | | |
| April | 8.1 \pm 2.2 | 26.3 \pm 3.0 | 2.3 \pm 0.7 | | 46.8 \pm 3.0 | 8.9 \pm 2.3 |
| May | 15.3 \pm 1.8 | 15.7 \pm 4.4 | 2.5 \pm 2.0 | 9.6 \pm 7.8 | 45.1 \pm 4.9 | 8.0 \pm 2.5 |
| June | 14.3 \pm 2.1 | 11.3 \pm 1.5 | 0.6 \pm 0.1 | 14.8 \pm 1.4 | 41.4 \pm 1.2 | 14.6 \pm 5.0 |
| July | 13.9 \pm 0.8 | 10.6 \pm 1.8 | 0.5 \pm 0.1 | 14.6 \pm 2.3 | 40.4 \pm 2.5 | 17.7 \pm 2.7 |
| August | 12.5 \pm 2.3 | 9.8 \pm 1.5 | 0.3 \pm 0.1 | 12.7 \pm 1.5 | 40.8 \pm 3.0 | 20.1 \pm 2.4 |
| September | 13.5 \pm 3.1 | 10.3 \pm 0.9 | 0.2 \pm 0.1 | 12.8 \pm 2.4 | 34.4 \pm 5.2 | 22.2 \pm 1.7 |
| September s. l. | 13.5 \pm 3.6 | 9.1 \pm 2.0 | 0.2 \pm 0.1 | 14.8 \pm 1.7 | 39.1 \pm 3.0 | 19.9 \pm 1.3 |
| October | 12.4 \pm 4.2 | 11.8 \pm 0.4 | 0.3 \pm 0.1 | 12.2 \pm 0.9 | 33.1 \pm 2.0 | 23.3 \pm 0.5 |
| November | 12.6 \pm 2.0 | 11.4 \pm 2.3 | 0.3 \pm 0.1 | 12.6 \pm 0.5 | 41.9 \pm 0.7 | 20.0 \pm 1.4 |
| <i>Faded leaves</i> | | | | | | |
| October | 10.4 \pm 2.1 | 11.7 \pm 1.2 | 0.2 \pm 0.1 | 10.5 \pm 0.5 | 36.5 \pm 1.7 | 29.3 \pm 2.2 |
| November | 9.9 \pm 1.0 | 11.6 \pm 1.6 | 0.2 \pm 0.1 | 11.7 \pm 4.1 | 42.0 \pm 5.2 | 29.5 \pm 8.2 |

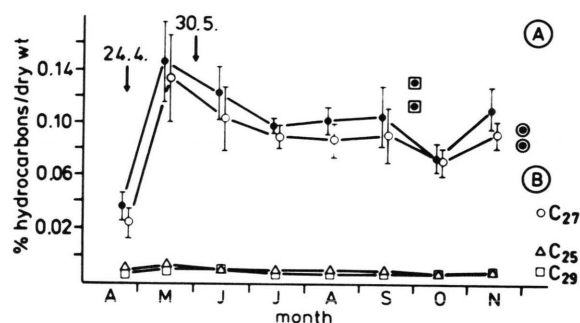


Fig. 2. Seasonal variations of hydrocarbons in beech leaf waxes

A total hydrocarbons;
 B individual components
 □ = shade leaves, ○ = faded leaves.

development in high amounts of about 21 to 38% of the hydrocarbon fraction was a remarkable observation. In preparations after May 2, these unsaturated hydrocarbons could not be identified again. The unsaturated compounds may be characteristic for the waxes in folded leaves in the buds and may be destroyed or polymerized by a radical mechanism in the presence of light and air after the unfolding of leaves.

Wax esters

In the initial stage of leaf development the wax esters were found in high amounts of 27% of the

waxes. The content of wax esters decreased in the following time to about 12%. The wax esters per dry weight showed a maximum in May and then decreased in June and remained constant till the end of the season (Table II).

The individual wax esters were present in homologous series from C_{36} to C_{54} , without any predominant component. During the season the individual components changed in their relative amounts, with a trend of increase for C_{40} , C_{42} , C_{44} , and decrease for C_{50} and C_{52} . C_{42} and C_{44} wax esters were found each in concentrations of more than 20% of the esters (Fig. 3A and B).

Benzyl acyl esters

In addition to the esters of long chain alcohols and fatty acids, beech leaf waxes were found to contain another series of esters resulting from an aromatic alcohol (benzyl alcohol) and long chain fatty acids with chain lengths ranging from C_{22} to C_{28} .

Benzyl acyl esters were detected as 2.3% of waxes in the first preparation. They decreased to 0.5% by July and then remained constant over the season (Table II; Fig. 3C). The benzyl acyl esters C_{29} and C_{31} were predominant in the first two weeks. Then the C_{35} ester dominated with about 65% of the benzyl acyl esters throughout the season.

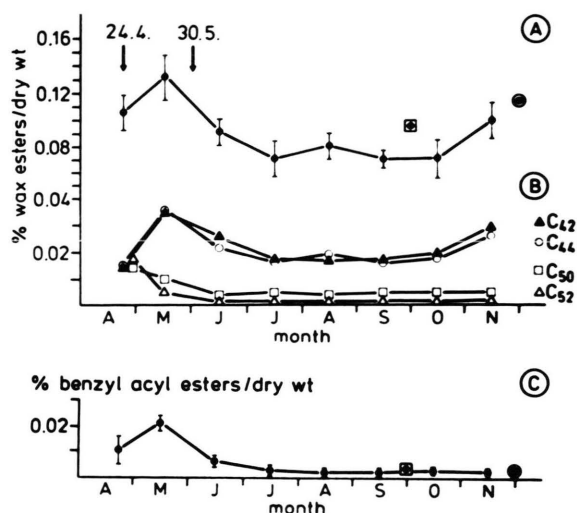


Fig. 3. Seasonal variations of wax esters and benzyl acyl esters in beech leaf waxes

A total wax esters;
 B individual components;
 C total benzyl acyl esters
 □ = shade leaves, ○ = faded leaves.

Aldehydes

Aldehydes were completely absent in the first two weeks of leaf development. On May 5, aldehydes were found for the first time of about 1% of the surface waxes. Within 10 days the aldehyde content was increased to 16% in a rapid way and then a light decreasing trend was observed during the remaining season (Table II). Aldehydes were found in a homologous series of C₂₂ to C₃₀. The predominant aldehyde has the chain length C₂₈ from the beginning of their biosynthesis and reached 85–90% of the aldehydes after two weeks. The aldehyde C₂₆ was found with 32% in the early stage, but it fell down to 5% within two weeks and was constant over the season (Fig. 4 A and B).

Alcohols

Primary alcohols were the main wax components in the surface wax of beech leaves throughout the season. At the initial stage of leaf development they were found with 47% of wax, but fell down to about 40% in the following months (Table II). The alcohols were present in homologous series ranging from C₁₆ to C₃₂. Alcohol C₂₈ was

dominating with about 50% followed by C₂₀ with about 20% in mature leaves. In the initial stage C₂₄ and C₂₂ were the main alcohols but only for two weeks, then C₂₈ increased very rapidly (Fig. 5 A and B).

Fatty acids

In the initial stage of leaf development the fatty acids represented about 10% of the waxes. In the following period they increased continuously all over the season (Table II). This pattern was determined by octacosanoic acid (Fig. 6 A and B). Fatty acids were found in a series of homologues ranging from C₁₄ to C₃₂. After May, fatty acid C₂₈ dominated with continuously increasing amounts from 10% to 60%. The other fatty acids were present each in amounts of about 20% in the initial stage and decreased to less than 10% during the following months. The seasonal pattern of fatty acids was entirely different from the other lipids with a continuously increasing trend over the season.

Shade leaves

In September shade leaves were also analyzed in three preparations and compared with sun leaves for the seasonal studies in this month. The shade leaves have nearly the same leaf area but the values for dry weight were lower, 37% instead of 42%. Sun leaves are thicker than shade leaves [25, 26]. The wax amounts showed also different values. Shade leaves contained more wax per dry weight,

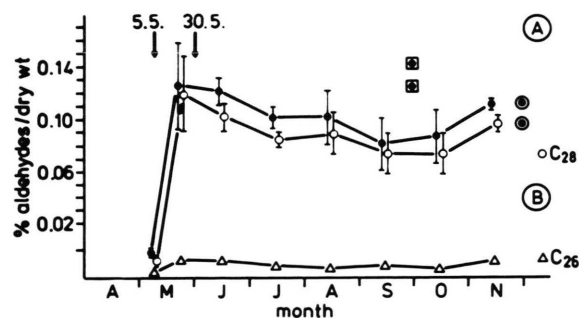


Fig. 4. Seasonal variations of aldehydes in beech leaf waxes

A total aldehydes;
 B individual components
 □ = shade leaves, ○ = faded leaves.

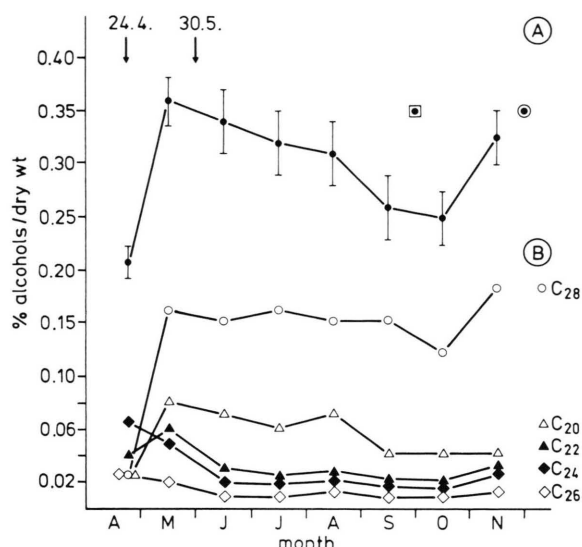


Fig. 5. Seasonal variations of alcohols in beech leaf waxes

A total alcohols;

B individual components

□ = shade leaves, ○ = faded leaves.

0.90% instead of 0.75%. The surface area had nearly the same wax layer, 14 μg wax/ cm^2 instead of 13 μg wax/ cm^2 (Fig. 1A–C). The wax and the lipid components showed the same composition with only little differences in amounts. All lipids have higher values per dry weight for shade leaves because of the lower dry weight. The distribution

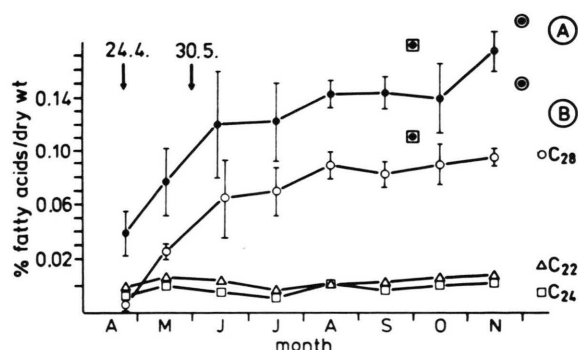


Fig. 6. Seasonal variations of fatty acids in beech leaf waxes

A total fatty acids;

B individual components

□ = shade leaves, ○ = faded leaves.

patterns of the homologue lipids are the same as for sun leaves regarding the standard deviations (Table I and II).

Faded leaves

The faded brown leaves in October and November have nearly the same dry weight as that of green leaves collected in November. They showed lower values against those for June to October. At this time organic substances were transported from leaves into branches and stems of trees. The biomass of leaves is therefore reduced at the end of the vegetation period (Fig. 1A–D). The wax lipids showed nearly the same composition as green leaves. The alcohols and fatty acids showed higher % values per wax, the values for aldehydes and hydrocarbons are lower (Table II). The distribution patterns of the homologue lipids are comparable with those of green leaves collected in October/November.

Discussion

Most of the wax lipid classes of beech leaves, namely hydrocarbons, wax esters, benzyl acyl esters, aldehydes and alcohols showed a similar seasonal behaviour. They exhibited an increasing phase till May, with maxima at this time. They declined in June and were nearly constant over the following months. The maxima in most of the illustrated graphics were conditioned by a very active biosynthesis of these lipids in May. During this month the leaves showed a relatively low dry weight. The leaf area extended and the leaf development was completed till May 16, but the biosynthesis of other organic substances for cell wall materials and storage substances may be forced and resulted in increasing values for dry weight, till May 30. After that time dry weight and leaf area values are nearly constant all over the year with the exception of late October/November. The maxima in the seasonal graphs therefore resulted when the wax lipids were correlated with dry weight. But the maxima in May were not observed when the lipid classes were correlated in % of total waxes (Table II).

The graphs of the wax lipid patterns (Fig. 2–6) indicated a very dynamic phase for biosynthesis of leaf wax lipids in the first three weeks after unfold-

ing. The enzymes for these reactions might be inactivated after that time with the exception of those for the biosynthesis of very long chained fatty acids, which increased over the whole season. Additionally the values for dry weight and leaf area (Fig. 1 A and B) showed that leaf development was completed by May 30. Leaf development and biosynthesis of most wax lipids were terminated parallel during that time.

The decreasing parts of the graphs for μg wax per cm^2 leaf surface and per one leaf (Fig. 1 D and E) at the end of the season may be caused by external environmental factors such as light, temperature, air pressure, rain, wind, dust etc. Similar seasonal decreasing values were found on wax studies about *Cistus laurifolius* [27] and *Buxus sempervirens* [20]. Sublimation of lipids and mechanical rubbing or washing might be the cause for these observations. The transformation of organic substances and their transport in the stems just before the leaves faded and fall down may be the reason for dry weight reduction in late October and November.

Except aldehydes all the wax lipids are present in the surface wax of folded leaves within the buds. Only aldehyde synthesis starts after unfolding of the leaves. This observation indicates an individual activation process for the biosynthetic enzymes for aldehydes and confirms again a quite different pathway for aldehydes than those for alcohols [28]. In the first five weeks of leaf development very dynamic processes were observed, including rapid changes in chain lengths composition concerning the single lipid classes and also a saturation of hydrocarbons. After the unfolding of leaves from the buds predominantly lipids with the chain length C_{28} were synthesized resulting as main components for fatty acids, aldehydes, alcohols and C_{27} for hydrocarbons (Fig. 2 B–6 B). This observation indicates a strong chain length specificity in the biosynthesis of wax lipids during the grow-

ing of leaves, a characteristic observation for beech leaves. During the first three weeks after unfolding of leaves hydrocarbons, aldehydes, alcohols and fatty acids were synthesized with very increasing amounts. Wax esters and benzyl acyl esters showed no significant increase during this time and the following vegetation period (Table I and II; Fig. 3).

The very long chain fatty acids are the biosynthetic precursors for all other wax lipids [29, 30]. During the rapid biosynthesis of all wax lipids in the first three weeks, the amount of fatty acids was comparatively low but increased after the deactivation of those enzymes used for biosynthesis of hydrocarbons, wax esters, aldehydes and alcohols.

The presence of unsaturated hydrocarbons and a dominating of wax lipids with shorter chain lengths in the waxes of young, folded leaves conditioned the surface wax layer with more liquid consistency. So this wax consistency was able to continue the unfolding movements of the leaves in the initial stage and also the growing process of the leaves in the following stages. But on the other hand, unfolded leaves during the first three weeks, are very susceptible to environmental factors because of their chemical wax composition, their wax consistency and their thin wax layer, too. The rapid synthesis of only saturated and very long chained wax lipids within three weeks resulted in a wax layer of more solid consistency. Waxes with a solid consistency are a better protection for leaves against environmental factors.

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