Chemical composition of the epicuticular and intracuticular wax layers on the adaxial side of *Ligustrum vulgare* leaves

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Summary

• Previous research has shown that cuticular triterpenoids are exclusively found in the intracuticular wax layer of *Prunus laurocerasus*. To investigate whether this partitioning was species-specific, the intra- and epicuticular waxes were identified and quantified for the glossy leaves of *Ligustrum vulgare*, an unrelated shrub with similar wax morphology.

• Epicuticular wax was mechanically stripped from the adaxial leaf surface using the adhesive gum arabic. Subsequently, the organic solvent chloroform was used to extract the intracuticular wax from within the cutin matrix. The isolated waxes were quantified using gas chromatography with flame ionization detection and identified by mass spectrometry. The results were visually confirmed by scanning electron microscopy.

• The outer wax layer consisted entirely of homologous series of very-long-chain aliphatic compound classes. By contrast, the inner wax layer was dominated (80%) by two cyclic triterpenoids, ursolic and oleanolic acid.

• The accumulation of triterpenoids in the intracuticular leaf wax of a second, unrelated species suggests that this localization may be a more general phenomenon in smooth cuticles lacking epicuticular wax crystals. The mechanism and possible ecological or physiological reasons for this separation are currently being investigated.

Key words: cuticular wax, leaf surface, plant cuticles, privet (*Ligustrum vulgare*), triterpenoids.

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Introduction

The nonwoody surfaces of plants are separated from the surrounding atmosphere by the cuticle, a layer consisting of cutin and wax (Jetter *et al.*, 2006). This wax is both impregnated in (intracuticular) and exterior to (epicuticular) the cutin biopolymer (Jeffree, 1986). Epicuticular wax may exist as a smooth film in some species, typically rendering their surfaces glossy, or it may be textured by protruding wax crystals in other species (Jeffree, 2006). The wax forms a barrier to deleterious water loss, which is the primary function of the cuticle (Baur, 1998). Other secondary functions have been suggested, such as ultraviolet (UV) light reflection (Reicosky & Hanover, 1978). Also, as the epicuticular wax is the first physical barrier encountered by external organisms, the wax likely plays a role in plant–insect (Müller, 2006) and plant–pathogen interactions (Carver & Gurr, 2006).

Plant cuticular waxes represent complex mixtures of very-long-chain aliphatics and cyclic compounds (Jetter *et al.*, 2006). The aliphatics include fatty acids, aldehydes, primary

and secondary alcohols, ketones, and alkanes, with chain lengths ranging from C_{20} to C_{36} in homologous series. Alkyl esters from C_{38} to C_{70} may also be present. In addition to these straight-chain compounds, cyclic compounds such as triterpenoids, tocopherols, and aromatic compounds may be found in either large or small quantities, depending on the species.

The cuticular waxes of plants have traditionally been considered to be a homogenous mixture, mainly because organic solvents indiscriminately extract both epi- and intracuticular wax (Jetter *et al.*, 2000). However, recently developed methods for physically removing the epicuticular wax before extraction with solvents permit the composition of each layer to be analyzed independently (Jetter *et al.*, 2000; Jetter & Schäffer, 2001). Compounds present in the outer or inner wax region of different plant species can now be identified.

Initial studies on the glossy-leafed *Prunus laurocerasus* reported that the epicuticular wax was composed exclusively of aliphatic compounds, while the intracuticular wax contained high percentages of two cyclic triterpenoids (Jetter *et al.*, 2000). Triterpenoids are also known to form surface wax crystals on other species such as *Ricinus communis* (Guhling *et al.*, 2006) and *Macaranga* spp. (Markstädter *et al.*, 2000). However, for glossy-leafed plants, it remains unknown whether or not the localization of triterpenoids in the intracuticular wax of *P. laurocerasus* (Rosaceae) is a species-specific finding. To further explore this issue, the following questions were addressed for the unrelated evergreen shrub *Ligustrum vulgare* (Oleaceae) which also has glossy leaves devoid of epicuticular wax crystals. (1) What is the quantity and composition of the cuticular wax? (2) Is this composition homogenous? (3) If not, how does the epicuticular wax film differ from the intracuticular wax? (4) How much does each layer contribute to the overall wax?

Materials and Methods

Plant material

Branches of *Ligustrum vulgare* L. (privet) were collected from cultivated plants growing on the campus of the University of Wuerzburg, Wuerzburg, Germany. Approximately 10 mature leaves were excised from the branch using a razor blade and pooled for each treatment. Five independent replicates were analyzed per treatment.

Mechanical wax removal

Gum arabic was employed as an adhesive for the selective removal of epicuticular waxes. Before the experiment, commercial gum arabic powder (Sigma-Aldrich, Oakville, Canada) was extracted in a Soxhlet apparatus with hot chloroform to remove any soluble lipids and residues. An aqueous solution of the adhesive (1 g ml^{-1}) was applied onto the entire adaxial surface of the leaves using a small paintbrush. After 30 min, the solution was dry and a thin polymer film could be peeled off in pieces which were collected and extracted overnight with chloroform at room temperature. A defined amount of *n*-tetracosane was added to the extracts as an internal standard. The treated surface area was subsequently measured digitally by scanning photocopies of the leaves.

Wax extraction

Total wax extraction from the adaxial surface was achieved by placing the intact leaf onto a flexible rubber mat, gently pressing a glass cylinder, 10 mm in diameter, onto the center of the exposed leaf surface and filling the cylinder with approx. 1.5 ml of chloroform (Jetter *et al.*, 2000). The solvent was agitated for 30 s (by pumping with a Pasteur pipette) and removed. When any solvent leaked between cylinder and leaf surface, the sample was discarded. Tetracosane was immediately added to all the extracts of cuticular waxes as an internal standard and the solvent was removed under reduced pressure. A similar procedure was used to extract intracuticular waxes after mechanical removal of epicuticular waxes from adaxial surfaces.

Chemical analysis

Before gas chromatography (GC) analysis, chloroform was evaporated from the samples under a gentle stream of nitrogen (N_2) while heating to 50°C. Then the wax mixtures were treated with bis-*N,N*-(trimethylsilyl)trifluoroacetamide (BSTFA; Sigma-Aldrich) in pyridine (30 min at 70°C) to transform all hydroxyl-containing compounds into the corresponding trimethylsilyl (TMSi) derivatives. The qualitative composition was studied with capillary GC (5890N; Agilent, Avondale, PA, USA; column 30 m HP-1, 0.32 mm inner diameter, film thickness = 0.1 μm; Agilent) with helium (He) carrier gas inlet pressure programmed for constant flow of 1.4 ml min^{-1} and mass spectrometric detector (5973N; Agilent). GC was carried out with temperature-programmed injection at 50°C, with the oven temperature held for 2 min at 50°C, raised by 40°C min^{-1} to 200°C, held for 2 min at 200°C, raised by 3°C min^{-1} to 320°C and held for 30 min at 320°C. Individual wax components were identified by comparison of their mass spectra with those of authentic standards and literature data. The quantitative composition of the mixtures was studied using capillary GC with flame ionization detector under the same GC conditions as above, but with hydrogen (H_2) carrier gas inlet pressure regulated for a constant flow of 2 ml min^{-1}. Single compounds were quantified against the internal standard by automatically integrating peak areas.

Scanning electronic microscopy

Samples consisting of untreated leaves and treated leaves (as described in the previous sections) were mounted on stubs

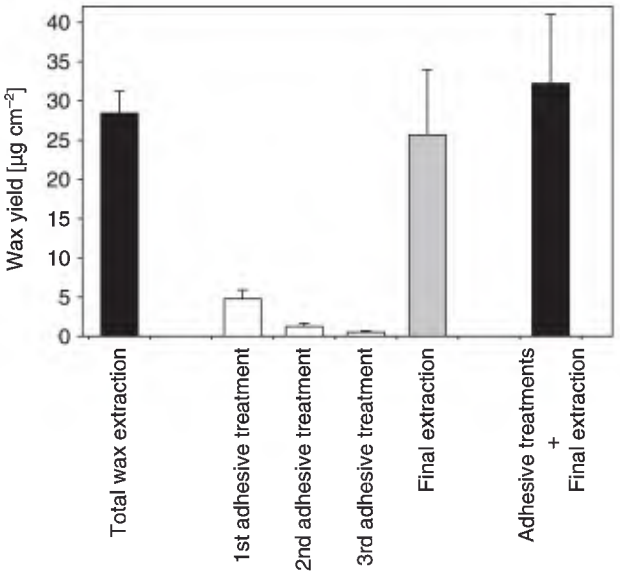

Fig. 1 Wax yields from adaxial sides of *Ligustrum vulgare* leaves sampled using a combination of mechanical and extractive methods. In a first experiment, the total cuticular wax was sampled directly using chloroform. In an independent second experiment, three consecutive treatments with the adhesive gum arabic were employed to remove the epicuticular wax layer, followed by extraction with chloroform to remove the intracuticular wax layer. Wax yields are given as mean values (± standard deviation; $n = 5$).

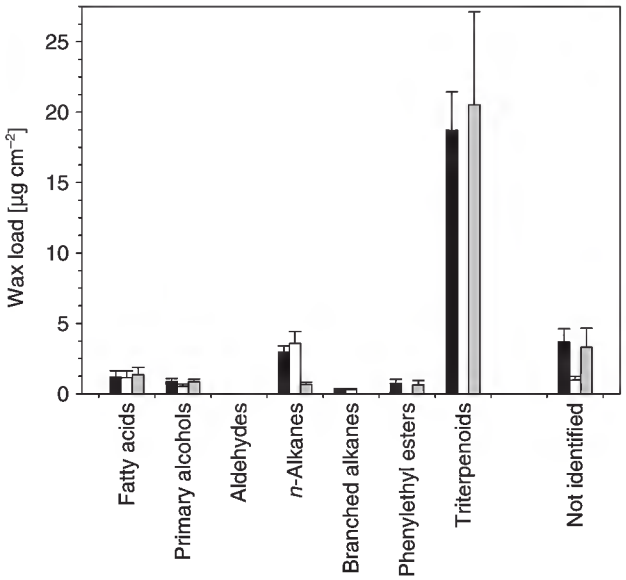

Fig. 2 Composition by compound class of the total wax mixture (black bars) and the epicuticular (white bars) and intracuticular (gray bars) wax layers on the adaxial leaf surfaces of *Ligustrum vulgare*. Amounts of the different compound classes are given as mean values (± standard deviation; $n = 5$).

using double-sided adhesive tape and allowed to air-dry overnight. Samples were then coated with 5 nm of gold (Au) in a Cressington Sputter Coater 208 H (Ted Pella, Inc., Redding, CA, USA) before viewing under a Hitachi S4700 field emission scanning electron microscope (SEM; Nissei Sangyo America, Ltd, Pleasanton, CA, USA) at a 1-kV accelerating voltage and a 12-mm working distance. The order of magnitude of epicuticular film thickness was approximated from the height difference between adjacent untreated and gum arabic-treated surfaces.

Results

The cuticular wax components of the adaxial side of privet leaves were identified and quantified by GC and mass spectrometry. Preliminary experiments showed that two applications of room-temperature chloroform for 30-s durations exhaustively extracted the total cuticular wax from privet leaves. With the chloroform restricted to the adaxial surface by a glass cylinder, these selective extractions produced a total of 28 ± 3 (mean ± standard deviation) μg cm^{-2} of wax from the adaxial surface (Fig. 1). The wax mixture contained six identifiable compound classes (Fig. 2). Triterpenoids were most abundant (66%), and were accompanied by smaller amounts of *n*-alkanes (11%), fatty acids (4%), primary alcohols (3%), branched alkanes (1%), and aldehydes (< 1%). For the total wax mixture, 13% could not be identified.

The triterpenoid class consisted of only two compounds, ursolic and oleanolic acid (Fig. 3). The remaining very-long-chain compound classes were present as homologous series. The *n*-alkanes ranged from C_{25} to C_{35}, with tritriacontane (C_{33}) as the dominant homolog. Similarly, the *iso*-alkanes were also dominated by odd-numbered carbon numbers (C_{29} and C_{31}), but with even-numbered chain lengths (C_{28} and C_{30}, respectively). Conversely, *anteiso*-alkanes had even carbon numbers (C_{32} and C_{34}), as did the other compound classes. Fatty acids and primary alcohols ranged from C_{20} and C_{22} to C_{34}, respectively, peaking at C_{32}. Tetratriacontanal (C_{34}) was the only aldehyde detected.

Scanning electron microscopy revealed that the adaxial surface of privet leaves contained only irregularly margined, flat, epidermal pavement cells (no guard cells or trichomes; data not shown). The surface was minutely granulated but lacked distinguishable surface crystals (Fig. 4a). Overall, the surface appeared to be covered by a relatively smooth film of wax. A single application of gum arabic produced a very smooth surface with a curvilinear line dividing the leaf between native and gum arabic-treated regions. Although a single application of gum arabic removed most of the epicuticular wax, small patches of epicuticular wax remained. However, three consecutive applications of gum arabic essentially removed all of the epicuticular wax film (Fig. 4b).

Preliminary experiments showed that the adhesive gum arabic reproducibly removed the surface wax from privet leaves (data not shown). When the adaxial surface was treated repeatedly, the wax yields decreased and were approaching

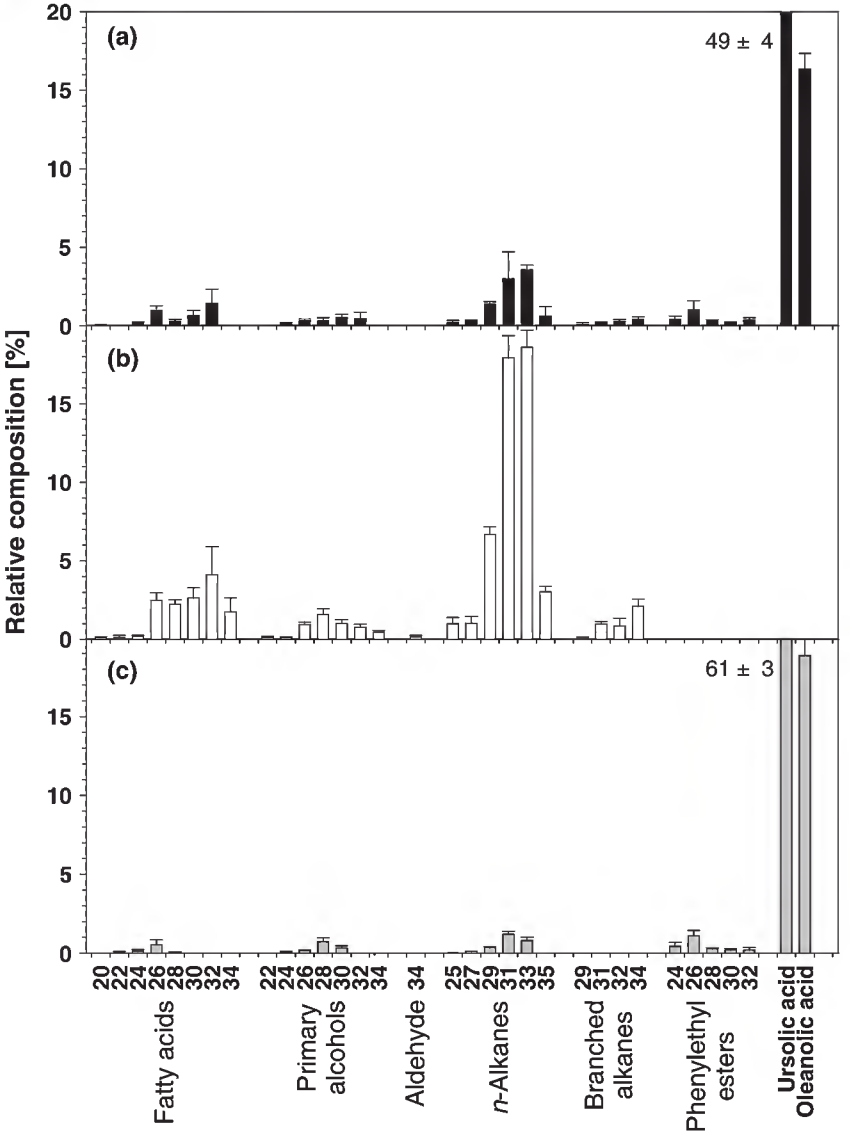

Fig. 3 Composition of (a) the total wax mixture and the (b) epicuticular and (c) intracuticular wax layers on adaxial leaf surfaces of *Ligustrum vulgare*. Compounds are listed according to chain lengths in the homologous series of aliphatic compound classes, or as triterpenoid isomers. Relative quantities of the individual compounds are given as mean values (± standard deviation; $n = 5$).

zero after the third adhesive application (Fig. 1). The wax yield of three consecutive gum arabic treatments on the adaxial leaf surface of privet added to 6.6 ± 1.6 μg cm^{-2}. This epicuticular wax consisted of alkanes (55%), branched alkanes (5%), fatty acids (18%), primary alcohols (8%), and aldehydes (< 1%; Fig. 2). Notably absent were the triterpenoids. The chain length distributions matched those found in the total wax mixture (see above). Hentriacontane (C_{31}) and tritriacontane (C_{33}) were the most abundant compounds and were present at nearly equivalent amounts (Fig. 3). Together they comprised nearly 36% of the epicuticular wax.

Solvent extraction of the adaxial leaf surface after the gum arabic treatments yielded 25.6 ± 8.3 μg cm^{-2} (Fig. 1). The intracuticular wax was strongly dominated by triterpenoids (80%). Small quantities of alkanes (3%), fatty acids (1%), and alcohols (1%) were also present (Fig. 2). Branched alkanes and aldehydes were not detectable. The aliphatics tended to have shorter chain lengths than in the total cuticular wax, with maxima for hentriacontane (C_{31}), hexacosanoic acid (C_{26}), and octacosanol (C_{28}; Fig. 3). The quantities of the two triterpenoids relative to each other resembled those found in the total cuticular wax.

Discussion

The compounds identified and quantified in the adaxial wax of *L. vulgare* leaves have all been reported in plant waxes before. The very-long-chain aliphatics were typical of the general suite of straight chain compound classes and chain lengths found in the waxes of many other species. Also, the

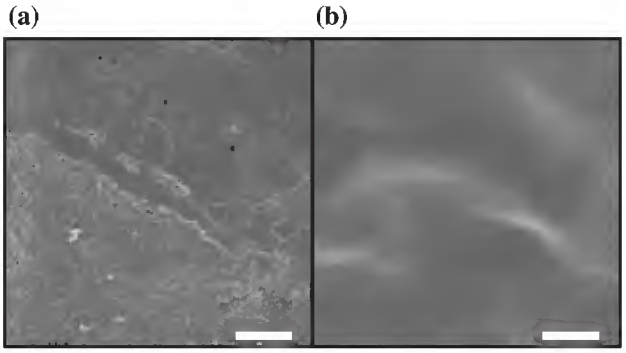

Fig. 4 Scanning electron micrographs of *Ligustrum vulgare* adaxial leaf surfaces before and after treatment with gum arabic. (a) The adaxial surface after a single treatment with gum arabic (top right) is clearly delineated from the epicuticular wax film-covered native surface (bottom left). (b) Three consecutive treatments with gum arabic produce a smooth adaxial surface. Bars, 1 μm.

two triterpenoids present were the same as those found in *P. laurocerasus* (Jetter *et al.*, 2000).

Although the above analysis of the total soluble lipids allows the identification of all compounds present, it does not reveal possible partitioning of compounds within the cuticle. Is the wax composition homogenous for privet leaves or do compounds tend to partition along an inside-to-outside gradient? To answer this question, the epicuticular wax film overlying the adaxial surface was analyzed separately from the intracuticular wax.

Repeated application of gum arabic to the adaxial surface of privet leaves resulted in continually decreasing yields. This demonstrated the selective removal by gum arabic of surface wax up to the mechanically resistant cutin matrix. However, the subsequent extraction with chloroform yielded a nearly 50-fold increase in extracted wax as compared with the third gum arabic treatment. Thus, the mechanically removed wax must be interpreted as epicuticular wax while the remaining wax subsequently extracted with solvent must be intracuticular wax. Moreover, because the combined yields of epicuticular plus intracuticular wax (sum: 32 ± 9 μg cm^{-2}) were confirmed by a second independent experiment using total wax extraction (28 ± 3 μg cm^{-2}), the selective extractions of epi- and intracuticular wax were also exhaustive. These results for the total wax loads on the adaxial sides of *L. vulgare* leaves also match the wax quantity found on the adaxial side of *P. laurocerasus* leaves (28 μg cm^{-2}; Jetter *et al.*, 2000).

Scanning electron microscopy images visibly supported these chemical data. Only small patches of epicuticular wax were remaining after a single application of gum arabic, and were no longer visible after three consecutive applications. These findings parallel the diminishing quantities of wax found by chemical analysis of consecutive gum arabic applications. Using the quantity of mechanically removed wax (6.6 ± 1.6 μg cm^{-2}) and an approximate density of $0.8–1.0 \times 10^6$ g m^{-3} for very-long-chain aliphatics (Le Roux, 1969), the epicuticular film thickness was expected to be 65–80 nm. This matches the magnitude of film thickness observed by SEM.

The relative partitioning of wax compounds on the adaxial side of privet leaves can now be accurately described. The identified compounds in the outer layer were exclusively very-long-chain aliphatics, with the majority of compounds being alkanes. No triterpenoids were detectable. In contrast, the intracuticular wax was composed almost entirely of two triterpenoids, ursolic acid and its isomer oleanolic acid, which were present in a 3 : 1 ratio, respectively. Less than 5% of this inner wax layer was aliphatic in nature. This quantity of aliphatics is within the error margin of complete epicuticular wax extraction.

The aliphatic constituents of the epi- and intracuticular waxes differed in their relative amounts. Branched alkanes and aldehydes were detectable only in the epicuticular wax, although even in the outer layer these were present at very low quantities. More strikingly, the intracuticular aliphatics all contained shorter chain lengths for compound class maxima and shorter homolog ranges compared with the epicuticular wax. Because compounds with shorter chain lengths are slightly more polar than longer compounds within the same compound class, the intracuticular aliphatics contained a higher proportion of polar constituents. This same pattern of shorter intracuticular and longer epicuticular aliphatic compounds has been found in the leaf and needle waxes of *Rubus fruticosus* and *Taxus baccata*, respectively (Haas & Rentschler, 1984; Wen *et al.*, 2006). The present work further supports the hypothesis that a gradient occurs between outer unpolar and inner less unpolar wax.

Prunus laurocerasus cuticles, which have the same two triterpenoids as privet, displayed the same intracuticular partitioning of the triterpenoids. Also, similar differences between epi- and intracuticular wax, albeit with different triterpenoids, have been reported in other species, including the leaves of *Macaranga tanarius* (Guhling *et al.*, 2005), the stems of *Ricinus communis* (Guhling *et al.*, 2006) and the fruit of *Lycopersicon esculentum* (Vogg *et al.*, 2004). Although some triterpenoids have been shown to produce surface crystals, no cases have been reported where triterpenoids are present in a smooth epicuticular wax film. This clearly suggests that, in general, for species with glossy cuticles, the triterpenoids tend to be located exclusively in the intracuticular wax.

The mechanism for establishing such a gradient remains to be determined. It is possible that the physicochemical properties of triterpenoids, including the size and polarity of the molecules, may hinder their outward movement. Alternatively, they may interact with other intracuticular components such as cutin or cellulose microfibrils that extend from the epidermal cell wall. Moreover, it has been suggested that physiological or ecological functions may be linked to such partitioning (Jetter *et al.*, 2000), but they also remain to be investigated.

In conclusion, the identified composition of the outer adaxial surface film on privet leaves was comprised entirely of very-long-chain aliphatics. Conversely, the vast majority of the intracuticular wax consisted of two triterpenoids. This very closely matches the transversal gradients of compounds in the leaf cuticle of the unrelated species *P. laurocerasus*, and thus suggests that the pattern may be generalized across many species that contain triterpenoids in smooth plant cuticles.

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