Research Article

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The permeation barrier of plant cuticles: uptake of active ingredients is limited by very long-chain aliphatic rather than cyclic wax compounds

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Abstract

BACKGROUND: The barrier to diffusion of organic solutes across the plant cuticle is composed of waxes consisting of very long-chain aliphatic (VLCA) and, to varying degrees, cyclic compounds like pentacyclic triterpenoids. The roles of both fractions in controlling cuticular penetration by organic solutes, e.g. the active ingredients (AI) of pesticides, are unknown to date. We studied the permeability of isolated leaf cuticular membranes from *Garcinia xanthochymus* and *Prunus laurocerasus* for lipophilic azoxystrobin and theobromine as model compounds for hydrophilic AIs.

RESULTS: The wax of *P. laurocerasus* consists of VLCA (12%) and cyclic compounds (88%), whereas VLCAs make up 97% of the wax of *G. xanthochymus*. We show that treating isolated cuticles with methanol almost quantitatively releases the cyclic fraction while leaving the VLCA fraction essentially intact. All VLCAs were subsequently removed using chloroform. In both species, the permeance of the two model compounds did not change significantly after methanol treatment, whereas chloroform extraction had a large effect on organic solute permeability.

CONCLUSION: The VLCA wax fraction makes up the permeability barrier for organic solutes, whereas cyclic compounds even in high amounts have a negligible role. This is of significance when optimizing the foliar uptake of pesticides.
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Keywords: cuticular permeability; active ingredients; very long-chain aliphatic compounds; cyclic compounds; pesticides

1 INTRODUCTION

Understanding the permeation properties of the plant cuticle is vital in ecology and agrochemistry. Because the primary ecological function of the plant cuticle is to prevent desiccation, expanding knowledge of cuticular permeation to water is of great interest.[1] In plant protection, systemic active ingredients (AI) need to diffuse across the cuticle to reach the interior of the plant and their sites of action.[2] The cuticle is the rate-limiting barrier for AI uptake from a deposit. Therefore, understanding the properties of the cuticular permeation barrier to AIs is of major importance. The cuticle consists of a thin continuous layer made up of cutin, polysaccharides and solvent-soluble waxes.[3] The biopolymer cutin is composed of esterified hydroxyl fatty acids of 16- and 18-carbon chain lengths.[4] Cuticular waxes are embedded within the cutin[5] and also occur as epicuticular wax crystals or films deposited on the cuticular surface.[3] Cuticular waxes are composed of very long-chain aliphatic compounds (VLCAs) and cyclic substances such as pentacyclic triterpenoids, sterols and tocopherols.[6,7] VLCAs consist mainly of primary *n*-alcohols, *n*-alkanes, fatty acids and alkyl esters. It is well-documented that cuticular waxes form the major permeation barrier of the cuticle to water[8] and organic solutes.[9,10] As shown by Schönherr, the extraction of cuticular waxes increases cuticular transpiration by up to several orders of magnitude.[8] However, no correlation between water permeability and cuticular thickness or wax coverage has been found.[11,12] In

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S.S. and P.S. carried out the experiments. S.S. wrote the manuscript with support from P.S., K.A., M.B., C.P. and M.R. K.A. and M.R. supervised the project.

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2004, Vogg *et al.*[13] showed that epicuticular waxes in tomato fruit (*Lycopersicon esculentum*) cuticles contributed to the transpiration barrier to only a minor degree, whereas the VLCAs of the intracuticular waxes constituted the primary barrier to water; triterpenoids were less important. For leaves, it has been proposed that intracuticular VLCAs, and for some species epicuticular VLCAs also, account for the majority of cuticular resistance to water transpiration,[7] but direct experimental evidence is lacking to date.

For organic solutes, it has been reported that among different species, the permeance of one compound could vary by up to four orders of magnitude.[9,14] Several studies also showed that cuticular permeability to organic compounds increased by several orders of magnitude after wax extraction.[9,10,15] The contribution of VLCA and cyclic wax components to formation of the barrier against the penetration of organic solutes, including AIs, has not been studied to date. This is surprising because in agrochemistry, it is important to identify the chemical composition and physical state of the cuticular wax fraction(s) determining the rates of foliar AI uptake. Such knowledge would support any effort to optimize the bio-delivery of plant protection agents.

The objective of this study is to provide first insights into the nature of the cuticular diffusion barrier to organic solutes. The approach was to differentiate between the contributions of the VLCA fraction (present in all plant species) and the cyclic compound fraction (present in some species) to the cuticular barrier against the uptake of organic solute. Based on mostly indirect evidence from investigations on the water permeability of cuticles, we propose that VLCAs rather than the cyclic wax fraction constitute the permeation barrier to organic solutes. To test this hypothesis, we devised an experimental approach to identify the permeability of the cuticular barrier to organic solutes using two evergreen species exhibiting different VLCA proportions in their wax. *Prunus laurocerasus* was selected because its leaf cuticular wax contains VLCAs and cyclic compounds, the latter at a very high proportion. To contrast this, *Garcinia xanthochymus* was chosen because its leaf cuticular wax consists almost exclusively of VLCAs. To measure the effect of cyclic compounds and VLCAs on cuticular permeability, a method to selectively extract the cyclic wax fractions of isolated cuticles was developed. Permeation studies were conducted with theobromine and azoxystrobin as polar and apolar model compounds covering the range of lipophilicities of many commercially important pesticide AIs.

2 MATERIALS AND METHODS

2.1 Chemicals

For isolation of cuticular membranes, cellulase (NCBE, University of Reading, Reading, UK), pectinase (Trenolin Super DF, ERBSLÖH, Geisenheim, Germany), citric acid monohydrate (AppliChem, Darmstadt, Germany) and sodium azide (Sigma-Aldrich, Steinheim, Germany) were used.

Wax analysis was carried out using chloroform and methanol (Roth, Karlsruhe, Germany; purity > 99.8%) for the extraction of cuticular membranes. The internal standard *n*-tetracosane was from Sigma-Aldrich. Derivatization was done using *N*,*O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA; Macherey-Nagel, Düren, Germany) and pyridine (Roth).

Permeation experiments were conducted using the lipophilic fungicide azoxystrobin (methyl (2*E*)-2-(2-{[6-(2-cyanophenoxy) pyrimidin-4-yl]oxy} phenyl)-3-methoxyacrylate) from Syngenta Crop Protection AG (Münchwilen, Switzerland; 97% purity, log $K_{O/W}$ 2.5[16]). The alkaloid theobromine (3,7-dimethylpurine-2,6-dione, log $K_{O/W}$ -0.78[17]) was from Sigma-Aldrich (purity > 99%). The chemical structures of azoxystrobin and theobromine are given in Fig. S1. High-purity water was used for permeation experiments (Millipore Milli-Q Gradient Water Purification System, Millipore Sigma, Burlington, MA, USA). Water, methanol, acetonitrile and formic acid (99.9% pure) for UPLC-MS were from Biosolve BV (Valkenswaard, The Netherlands).

2.2 Plant materials

Fully expanded leaves of *P. laurocerasus* cv. Herbergii (laurel cherry) and *G. xanthochymus* (Mysore Gamboge) were harvested from the botanical garden of the University of Würzburg, Germany. Cuticular membranes (CM) of the non-stomatous, adaxial side were isolated enzymatically using the method described by Schönherr and Riederer.[18] For enzymatic isolation, a solution of 1% (v/v) cellulase, 1% (v/v) pectinase and 1 mM sodium azide in 20 mM citric buffer was used. After isolation, CM were stored in demineralized water at room temperature until further use.

2.3 Wax extraction

Dewaxed cuticles (MX) were obtained by extracting CM twice with chloroform (5 mL) for 30 min. Extracting CM with methanol (5 mL) for 20 h was done to obtain membranes without triterpenoids. These membranes are referred to as M. To check whether extraction of triterpenoids was successful, M were also treated subsequently with chloroform. The differently treated membranes were stored in demineralized water at room temperature until further use. To study the extraction behaviour of a *P. laurocerasus* leaf with methanol in comparison with an isolated cuticle, the adaxial side of the leaf was treated with methanol for 30 s. Wax extracts were evaporated under a flow of nitrogen and the composition was analysed as follows.

2.4 Wax analysis

An internal standard solution was prepared by dissolving *n*-tetracosane in chloroform and added to the wax extracts. Solvent was evaporated using a flow of nitrogen. Derivatization with 10 µL BSTFA and 10 µL pyridine was carried out at 70 °C for 30 min using a heating module (Pierce Reacti-Therm, Pierce Chemical, Dallas, TX, USA). The mixture was redissolved in chloroform and used for gas chromatography (GC).

GC-mass spectrometry (GC-MS; 6890 N, GCSystem; Agilent Technologies, Santa Clara, CA, USA) was used to identify wax compounds. Helium was used as a carrier gas. A MS detector (*m/z* 50–750, MSD 5977A, Agilent Technologies) was applied. On-column injection with a capillary column (30 m × 0.32 mm, DB-1, 0.1 µm film: J&W Scientific, Agilent Technologies) was used. The sample was injected at 50 °C and the temperature was held at 50 °C for 2 min. The temperature was then increased to 200 °C at a heating rate of 40 °C min^{-1}, and held at 200 °C for 2 min. The temperature was then increased to 320 °C (heating rate: 3 °C min^{-1}) and held for 30 min. Identification was undertaken using Wiley 10th/NIST 2014 mass spectral library (W10N14; John Wiley & Sons, Hoboken, NJ, USA), reference specimen or spectra interpretation.

Quantification was undertaken using GC flame ionization detection (GC-FID, 6850N, GCSystem; Agilent Technologies). Similar GC conditions as before were used to separate compounds, except that hydrogen gas was used as the carrier gas.

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2.5 UHPLC–MS analysis

Ultra high-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS; ACQUITY HcClass UHPLC high class system with QDa detector, Waters, Eschborn, Germany) was used to identify and quantify the organic solutes. A sample volume of 2 µL was injected into a Luna Omega C_{18} polar column (particle size 1.6 µm, 50 mm × 2.1 mm; Phenomenex, Aschaffenburg, Germany). The column was equilibrated to 35 °C. The autosampler (Acquity Flow Through Needle, Waters) was held at 25 °C. The assay had an initial gradient of 97% formic acid (0.1%, v/v) and 3% acetonitril. The flow rate was set to 0.5 mL min^{-1}. After 1.2 min, the amount of formic acid was reduced linearly to 10% and acetonitrile was increased to 90%. This was held until 1.5 min. The initial gradient was reached again after 2.2 min. Column equilibration using the initial gradient was done until 2.8 min. Prefilter (Krud-Katcher ULTRA HPLC in-line filter 0.5 µm depth filter × 0.004in ID, Phenomenex) and pre-column (Security Guard ULTRA cartridges, UHPLC fully porous C_{18} and SecurityGuard ULTRA holder, Phenomenex) were used.

The QDa detector with electrospray ionization (ESI) was operated in positive mode. Ions of mass 404 (azoxystrobin) and 181 (theobromine) were detected in single ion recording mode. The sampling frequency was set to 15 Hz. The ESI probe temperature was set to 600 °C. Capillary voltage was adjusted to 0.8 kV. Cone voltage was set to 20 V. Calibration in the range of 0.001 to 1.0 µg mL^{-1} was done using standard solutions of azoxystrobin and theobromine in purified water. Coefficients of determination were > 0.997.

2.6 Transport experiments

Permeation experiments were performed using two half-cells made of stainless steel with a sampling port, mounting the membrane between the donor and receiver compartments. The physiological outer sides of the membranes were orientated towards the donor compartment. Teflon paste (Roth) was used to seal the interface between the membrane and cells. The cells were sealed with adhesive tape (Beiersdorf, Hamburg, Germany).

One millilitre of an aqueous solution of theobromine (100 µg mL^{-1}) was applied to the donor compartment. Subsequently, the receiver compartment was filled with 1 mL of high-purity water. After adding solution and water to the compartments, the sampling ports were sealed with adhesive tape (Beiersdorf). The chambers were put on a Rotamax 120 (Heidolph Instruments, Schwabach, Germany) at a rotational speed of 50 rpm. The temperature was held at 25 ± 0.1 °C using an incubator (IPP110, Memmert, Schwabach, Germany). At time intervals of 2–24 h, 30 µL aliquots were taken from the receiver compartment and replaced by 30 µL purified water. Aliquots were analysed by UHPLC-MS.

Because of the low water solubility of axozystrobin, the following procedure was performed: 100 µL of a solution of azoxystrobin in acetonitrile (10 000 µg mL^{-1}) was added to each donor cell. The solvent was evaporated under nitrogen gas. The membrane was mounted between the donor and receiver cell as described previously. After sealing the chamber with adhesive tape (Beiersdorf), 1 mL of high-purity water was applied to each cell. This was done to obtain a constantly saturated azoxystrobin solution in the donor compartment. The concentration of azoxystrobin in the donor solution was assumed to be steady, because new azoxystrobin could dissolve into the donor from solid azoxystrobin after permeating the membrane into the receiver compartment.

The permeances of theobromine and azoxystrobin were determined for CM, M and MX.

Plotting the amount M (g) of a compound that permeated the membrane as a function of time t (s), the flow rate F (µg s^{-1}) represents the slope and can be determined by

$$F = \frac{\Delta M}{\Delta t} \quad (1)$$

Using the concentration difference Δc between the donor and receiver compartment, F (µg s^{-1}) and the membrane area A (m^2), the permeance P (m s^{-1}) of a solute can be calculated by:[19]

$$P = \frac{F}{A \cdot \Delta c}. \quad (2)$$

For azoxystrobin, Δc was calcuclated using the concentration difference between the saturated donor compartment and the receiver compartment (c of receiver was nearly zero). Therefore, three separate donor cells with saturated azoxystrobin solution were prepared, as described previously. A 100 µL aliquot was removed from the upper solution of the donor compartment, filtered and diluted. Concentration was determined by UHPLC–MS. Exemplary permeation of azoxystrobin and theobromine is given in Fig. S2.

2.7 Statistics

Statistical analysis was carried out using OriginPro 2019 (64-bit, 9.6.0.172). Outliers were removed in accordance with the interquartile range method. Permeance data did not show normality according to the Shapiro–Wilk test ($P < 0.05$). Lognormal transformation of the permeance did not result in normality. Therefore, non-parametric statistical analysis was used to examine the permeance data without transformation. Kruskal–Wallis analysis of variance (ANOVA) with post-hoc Dunn's test ($P < 0.05$) was selected to detect significant differences between CM, M and MX of one compound and species. Owing to non-normality, median values (25th and 75th percentile) instead of mean values (standard deviation) were used in this study and to calculate the effect of wax extraction. Normality was given for wax data. A t-test was conducted to detect statistically significant differences between pure chloroform extract and combined methanol and chloroform extract.

3 RESULTS

3.1 Wax extraction

The adaxial leaf cuticular wax of *G. xanthochymus* consisted mainly of VLCAs (97% of total wax, Fig. 1). VLCA chain lengths ranged between C_{29} and C_{36}. *n*-Alkanes made up the major fraction of VLCAs (77%), followed by 23% alcohols (Table 1). Tritriacontane and hentriacontane were the predominant compounds found in alkanes. The primary alcohols in *G. xanthochymus* wax were composed mainly of 1-tetratriacontanol (10%). Cyclic compounds were a minor fraction in wax of *G. xanthochymus* yielding 0.15 ± 0.02 µg cm^{-2}. They amounted to 3% of the total wax and consisted of sterols, tocopherols and triterpenoids. VLCAs yielded 6.00 ± 0.03 µg cm^{-2} (Table 1).

Triterpenoids made up the major fraction of the adaxial leaf cuticular wax of *P. laurocerasus*, where the cyclic fraction yielded 53.27 ± 2.79 µg cm^{-2} (Fig. 1, Table 1). Ursolic acid, as the major triterpenoid, amounted to 75% of the total wax. VLCAs yielded 12% of the total wax (7.49 ± 0.92 µg cm^{-2}) with chain lengths ranging from C_{20} to C_{50}. *n*-Alkanes comprised 67% of VLCAs, consisting mainly of nonacosane and hentriacontane. Only 4%

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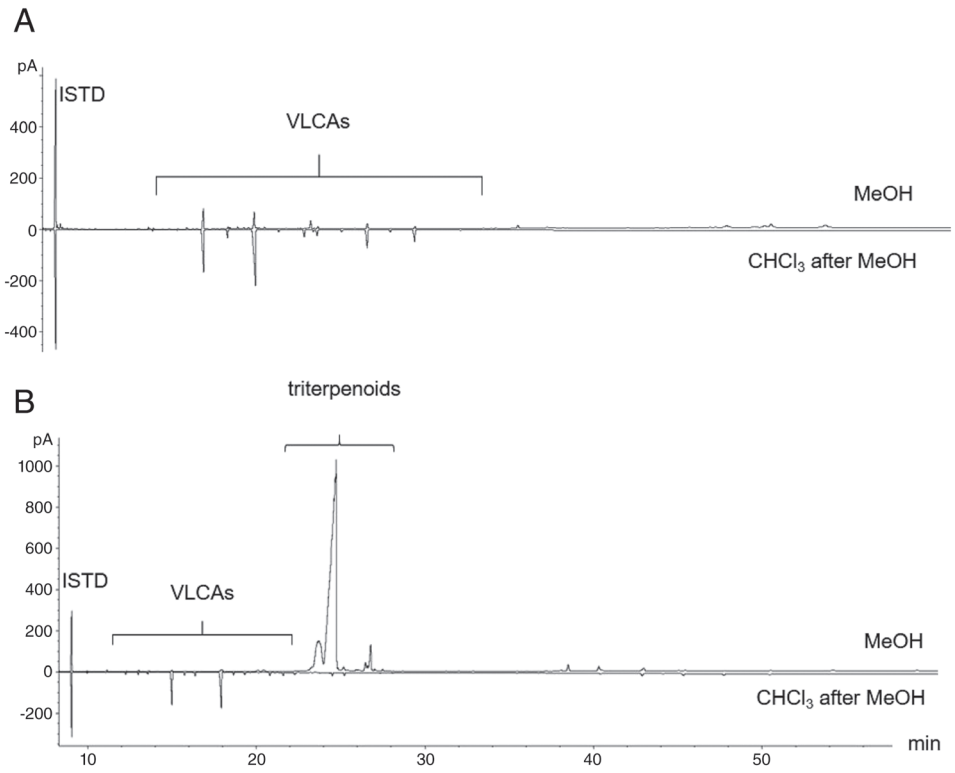

Figure 1. Gas chromatography flame ionization detection chromatograms of the methanol extract (MeOH) and subsequent chloroform extract ($CHCl_3$ after MeOH) of isolated membranes of *Garcinia xanthochymus* (A) and *Prunus laurocerasus* (B) showing differences in intensities of very long-chain aliphatic compounds (VLCAs) and triterpenoids. ISTD, internal standard.

Table 1. Composition of the cuticular waxes of *Garcinia xanthochymus* and *Prunus laurocerasus* determined by combined extracts of methanol and subsequent chloroform treatment

Compound classes	*Garcinia xanthochymus*	*Prunus laurocerasus*
Fatty acids	N.D.	0.17 ± 0.10
Primary alcohols	1.39 ± 0.01	1.33 ± 0.41
n-alkanes	4.60 ± 0.04	4.99 ± 0.17
Alkyl esters	N.D.	0.97 ± 0.003
Methyl ester	N.D.	0.02 ± 0.003
Sterols	0.04 ± 0.002	N.D.
Tocopherols	0.04 ± 0.02	0.011 ± 0.011
Triterpenoids	0.08 ± 0.003	53.26 ± 3.21
Not identified	0.77 ± 0.05	4.11 ± 1.01
Total	6.15 ± 0.05	60.76 ± 2.74

Data are given as means ± standard deviations (μg cm^{-2}; $n = 4$).
N.D., not detected.

of total wax was composed of primary alcohols, alkyl esters and fatty acids. Total adaxial cuticular wax coverage of *G. xanthochymus* amounted to 6.2 ± 0.05 μg cm^{-2}. It was 10-fold lower than the total adaxial cuticular wax coverage of *P. laurocerasus* (60.76 ± 2.74 μg cm^{-2}).VLCA coverage of *P. laurocerasus* leaf wax yielded 7.5 ± 0.9 μg cm^{-2}.

Methanol extraction of *G. xanthochymus* cuticles resulted in the quantitative extraction of cyclic compounds (Fig. 2). The subsequent chloroform extract consisted exclusively of VLCAs; 15% of VLCAs were extracted using methanol, comprising hentriacontane, tritriacontane and 1-dotriacontanol. Extracting waxes using methanol followed by chloroform resulted in total wax coverages similar to those found in wax extracts using chloroform alone. No significant differences in total wax coverage between these two extraction methods were found. Looking at the selective extraction of CM of *P. laurocerasus*, the results were as described above for *G. xanthochymus*. Methanol extracted the cyclic compounds completely (Fig. 2); 10% of VLCAs were extracted using methanol. These VLCAs consisted mainly of heptacosane, 1-tetracosanol and 1-hexacosanol. The remainder of the VLCAs were found in the subsequent chloroform extract. Treating the adaxial sides of intact leaves of *P. laurocerasus* with methanol for 30 s did not remove any triterpenoids, in contrast to the quantitative removal when extracting isolated cuticles with methanol (Fig. 3).

3.2 Cuticular permeabilities

The permeance of theobromine and azoxystrobin measured for the two species *G. xanthochymus* and *P. laurocerasus* with isolated cuticular membranes (CM), methanol-extracted membranes (M) and chloroform-extracted membranes (MX) ranged from 0.6 (0.4–1.0, 25th to 75th percentile) × 10^{-11} m s^{-1} to 1.5 (0.2–2.6) × 10^{-9} m s^{-1} (Fig. 4, Table S1). The lowest permeance was observed for theobromine with CM of *P. laurocerasus*, and the highest for theobromine with MX of *G. xanthochymus*. The permeance of theobromine with CM of *G. xanthochymus* was 13.9 (1.1–32.0) × 10^{-11} m s^{-1}, although the same compound showed a permeance of 8.3 (3.4–23.0) × 10^{-12} m s^{-1} with CM of *P. laurocerasus*. Fully dewaxing the membranes using chloroform resulted in a wax extraction effect (P_{MX} P_{CM} $^{-1}$) of 11 for *G. xanthochymus*, whereas *P. laurocerasus* showed 19-fold higher permeances with MX in comparison with CM (Table S1). *G. xanthochymus* showed a permeance

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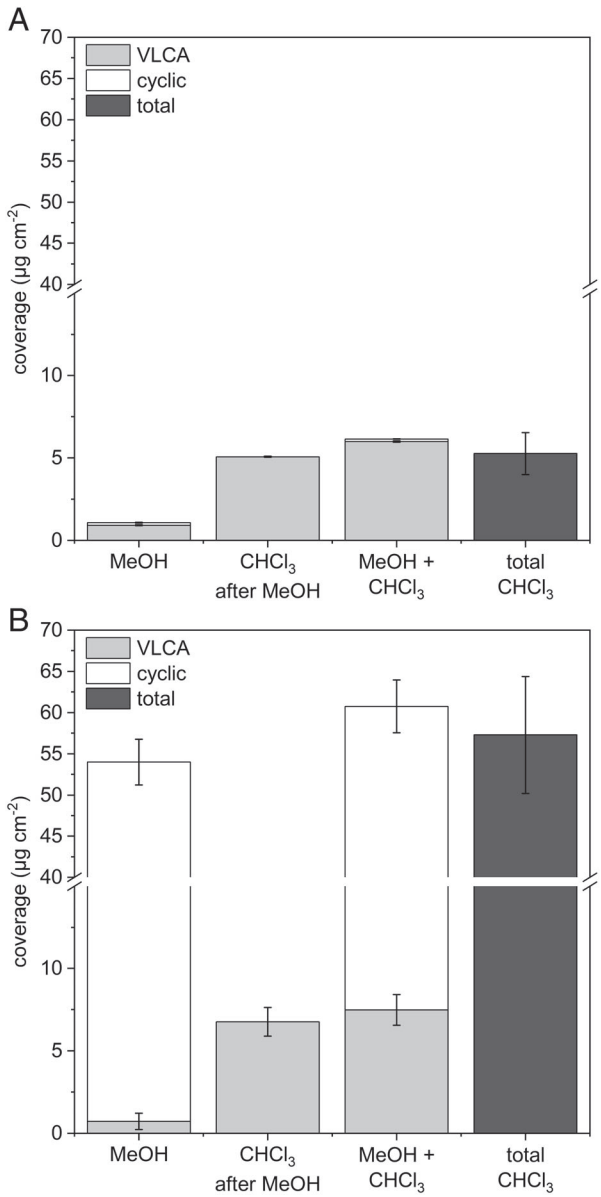

Figure 2. Wax fractions of methanol (MeOH), subsequent chloroform extract ($CHCl_3$ after MeOH), combined wax fractions (MeOH + $CHCl_3$), and total wax amount of pure chloroform extract ($CHCl_3$) of adaxial leaf cuticles of *Garcinia xanthochymus* (A) and *Prunus laurocerasus* (B) grouped by very long-chain aliphatic compounds (VLCA), cyclic compounds (cyclic) and total wax amount (total).

of theobromine with MX of 1.5 $(0.2–2.6)\times10^{-9}$ m s^{-1}, whereas the permeance of *P. laurocerasus* was 1.5 $(1.2–1.9)\times10^{-10}$ m s^{-1}. Permeances with membranes of *P. laurocerasus* using azoxystrobin were higher in comparison with the same membranes using theobromine (Mann–Whitney rank sum test, $P<0.05$). The permeance with MX yielded 1.2 $(0.9–1.6)\times10^{-9}$ m s^{-1} and was 37-fold higher than the permeance with CM (Fig. 4, Kruskal–Wallis ANOVA with Dunn's test, $P<0.05$). The effect of wax extraction was greater using membranes of *P. laurocerasus* (19 theobromine, 37 azoxystrobin) than using *G. xanthochymus* (11 theobromine, 4 azoxystrobin). Removing the cyclic wax fraction using methanol did not alter the permeance of theobromine and azoxystrobin in comparison with CM for either species (Fig. 4). No statistically significant difference was found between CM and M. Fully dewaxing the membranes resulted in a statistically significant increase in the permeance with MX in comparison with CM and M (Kruskal–Wallis ANOVA with post-hoc Dunn's test, $P<0.05$).

4 DISCUSSION AND CONCLUSION

The aim of this study was to examine the contribution of VLCAs and cyclic wax compounds to the cuticular permeation barrier for organic solutes. We hypothesized that this barrier consists of VLCAs rather than the cyclic wax fraction and tested this hypothesis experimentally by applying a selective wax extraction method to isolated cuticular membranes.

4.1 Wax extraction

Two evergreen species (*G. xanthochymus* and *P. laurocerasus*) were selected for this study because of fundamental differences in composition of their cuticular wax. The cuticular wax of *G. xanthochymus* contains only a small fraction of cyclic components (3%), whereas that of *P. laurocerasus* consists of 88% cyclic triterpenoids (Table 1). GC-MS analyses confirmed that using methanol, an almost complete selective extraction of the cyclic wax fraction can be achieved (Figs 1 and 2), whereas VLCAs remained almost entirely in the cuticular membrane. This wax fraction could be removed by subsequent treatment with chloroform. Combining both extracts resulted in a total wax composition equivalent to that obtained by extracting whole cuticles with chloroform.

The waxes associated with the plant cuticle impregnate the cutin matrix (intracuticular waxes) and are deposited on the outer surface as a thin film (epicuticular waxes), sometimes together with epicuticular wax crystals.[20] Previous studies have demonstrated that the epicuticular wax of the plant species investigated consisted of VLCAs, whereas the cyclic fraction was located exclusively in the interior of the cutin matrix.[7,21,22] Treating the intact adaxial surface of leaves of *P. laurocerasus* with methanol did not release any triterpenoids, proving that the cyclic compounds are located in the inner layers of the cuticle and are effectively shielded from the extractive action of methanol by a superficial layer of VLCAs (Fig. 3). Using enzymatically isolated plant cuticles, however, overcomes this obstacle, as methanol can reach the intracuticular cyclic deposits by entering the cuticle from its inner, not VLCA-coated side. Supporting this notion of differential solubility, a recent study showed that only small amounts of the main epicuticular wax compound 1-octacosanol could be extracted from wheat leaf surfaces using methanol.[23] By contrast, chloroform could quantitatively extract 1-octacosanol after 3 min. The authors state that polar solvents like methanol or isopropanol solubilize long-chain aliphatic primary alcohols to a very low degree, as also found by Hoerr *et al.*[24] Because VLCA alkanes are even less polar than their corresponding alcohols, they can be assumed to be less soluble in methanol. This was confirmed by experiments using shorter chain compounds showing a higher solubility in methanol, e.g. for 1-decanol (>1310 g 100 mL^{-1})[24] and *n*-decane (8.1 g 100 mL^{-1}) at 20 °C.[25] To our knowledge, for higher alcohols and alkanes above 18 carbon atoms, solubility data in methanol have not yet been published. However, it is clear that a higher hydrocarbon chain length will drastically decrease solubility, as seen with alcohols in the range C_{10} (18.8 g 100 mL^{-1}) to C_{18} (0.3 g 100 mL^{-1}).[24] By contrast, higher solubility in methanol was reported for cyclic triterpenoids like ursolic acid (0.76 g 100 mL^{-1}), the main compound of the cyclic wax fraction of *P. laurocerasus*.[26]

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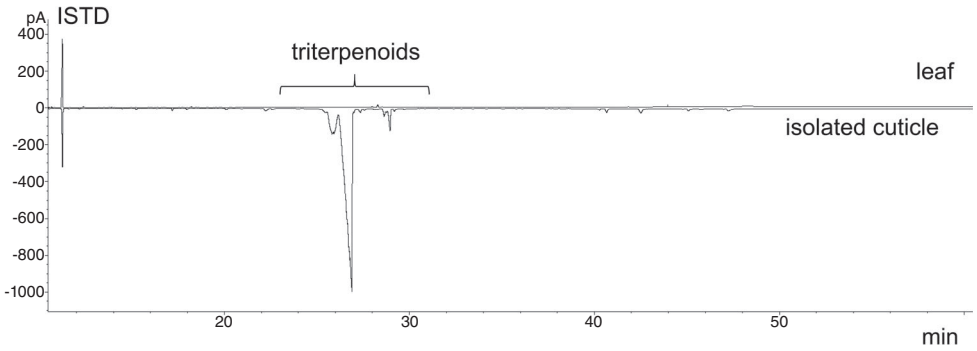

Figure 3. Gas chromatography flame ionization detection chromatograms of methanol extracts of an isolated cuticle and the adaxial side of an intact leaf of *Prunus laurocerasus*. ISTD, internal standard.

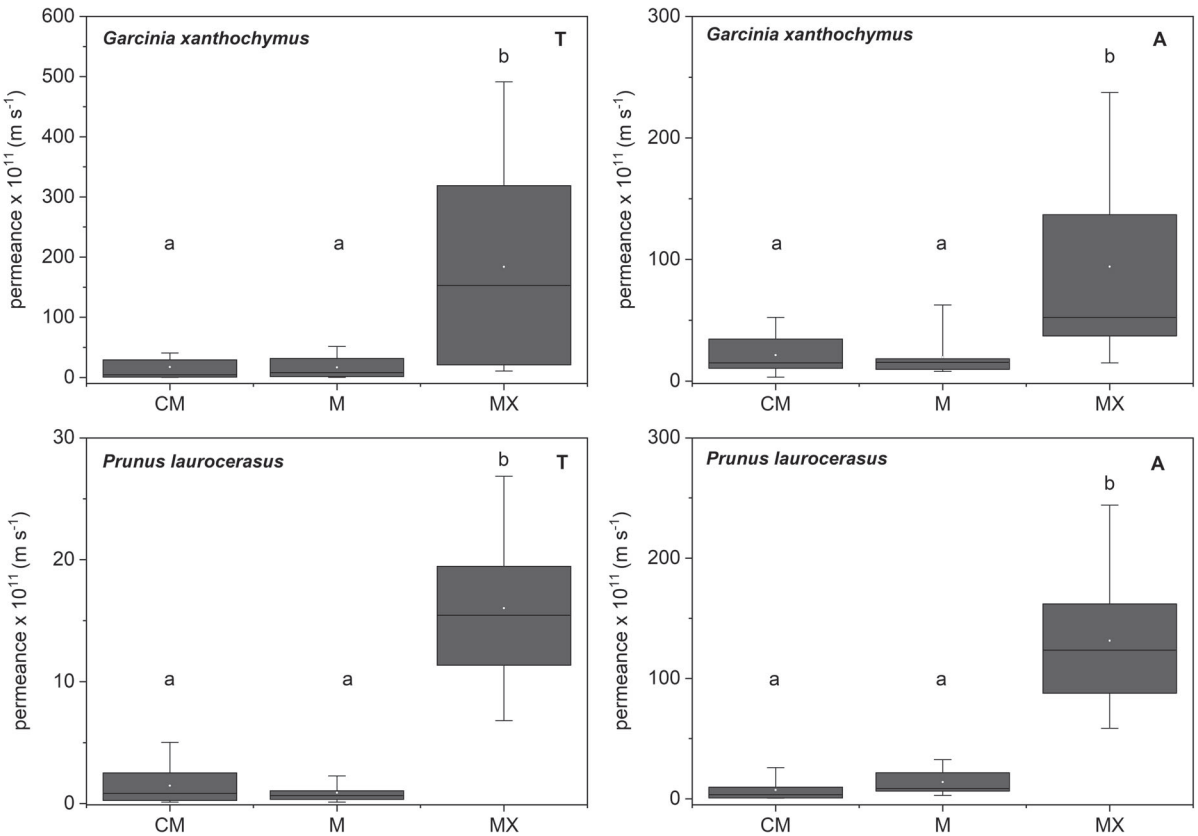

Figure 4. Permeances of theobromine (T) and azoxystrobin (A) of isolated cuticular membranes (CM), methanol-treated membranes (M) and chloroform-treated membranes (MX) of *Garcinia xanthochymus* and *Prunus laurocerasus*. Box represents 25th and 75th percentiles. Horizontal lines within boxes represent the median. Whiskers indicate the 10th and 90th percentiles. Different letters above the box indicate significant difference (Kruskal–Wallis –ANOVA with Dunn's test, $8 \leq n \leq 30$; $P < 0.05$).

4.2 Cuticular permeability

In both species studied, dewaxing the isolated cuticles using chloroform increased the permeance for theobromine by factors of 19 in *P. laurocerasus* and 11 in *G. xanthochymus*, whereas the permeance for azoxystrobin was increased by factors of 37 (*P. laurocerasus*) and 4 (*G. xanthochymus*). Increased permeances after chloroform extraction confirmed the well-established fact that waxes form the major cuticular permeation barrier to organic solutes.[9,10,15] The present study, shows that the VLCA and cyclic wax fractions contribute differently to the barrier against the diffusion of organic solutes across the cuticle. Treatment with methanol did not significantly increase the permeability of isolated cuticles from *P. laurocerasus* and *G. xanthochymus* for the two solutes (Kruskal–Wallis ANOVA with post-hoc Dunn's test, $8 \leq n \leq 30$; $P < 0.05$). This was true even in the case of *P. laurocerasus* where methanol treatment released cyclic compounds making up > 80% of the total wax. In both species and for both solutes, only full dewaxing of the cuticles with chloroform significantly increased the permeability. These results provide strong experimental evidence that the VLCA fraction of the cuticular wax constitutes the permeation barrier to organic solutes, whereas cyclic compounds when present have no impact on permeability.

To the authors' knowledge, similar studies testing the effects of VLCA and cyclic wax fractions on organic solute permeability of cuticles have not been reported to date. The results here, however, agree with the finding by Jetter and Riederer that in eight

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species, the fatty acyl wax fraction was associated with cuticular water permeability.[7] Another study proposed the hypothesis that cyclic compounds in tomato fruit cuticles do not constitute the permeation barrier to water.[13]

For the present study, lipophilic azoxystrobin and hydrophilic theobromine were chosen as model compounds that cover the range of lipophilicities exhibited by the active ingredients of common pesticides. It is assumed that lipophilic solutes cross the cuticle by diffusing through the cuticular waxes to reach the interior of the leaf.[9,10] They are thought to diffuse in the lipophilic waxes associated with the cuticle,[27] whereas uncharged hydrophilic solutes are thought to take a different pathway.[15,28] Thus, separate pathways across the cuticular barrier, one lipophilic and one hydrophilic, are taken by solutes depending on their lipophilicity. The lipophilic pathway is thought to lead across deposits of waxes like VLCAs,[29] whereas the polar pathway has been associated with hydrated functional groups in the cuticular network leading to an aqueous continuum. Among others, this might be water-filled polysaccharide strains extending from the epidermal cell wall partially to the outer surface of the cuticle.[30,31] The lipophilic pathway is depicted as waxes consisting of crystalline and amorphous fractions.[29,32,33] Lipophilic solutes are assumed to diffuse through amorphous parts while the crystalline fraction is inaccessible.[34] Therefore, the diffusion path length may be much longer than expected by the thickness of the barrier.[35] Extracting these chrystalline blockages leads to a significant shortening of the pathway, which is reflected by a considerable increase in the permeability for lipophilic solutes.

With a log K_{OW} of −0.78, theobromine is a hydrophilic compound and in contrast to azoxystrobin (log K_{OW} = 2.5) diffuses across the cuticle *via* the hydrophilic pathway. Although polar solutes take a different pathway from lipophilic ones, removal of waxes from the cuticles studied here also increased the overall permeability for hydrophilic theobromine. These apparently contradictory findings can be reconciled by assuming that in the native cuticle, wax blocks some hydrophilic pathways before they reach the cuticular surface. These blocks are removed by wax extraction, increasing the availability of polar pathways across the cuticle.[15,36]

This study indicates that cyclic wax compounds do not contribute to the diffusion barrier for hydrophilic or lipophilic compounds. Therefore, the question arises, which selective advantages can be attributed to the, in many species significant, fraction of cyclic compounds, especially triterpenoids, present in the inner matrix of the cuticle? Tsubaki *et al.* showed experimentally that the cuticular triterpenoids of the persimmon fruit act as nano-fillers substantially increasing the mechanical stability of the cuticle.[37] This argument was further taken to explain the high thermal stability in terms of water permeability of the leaf cuticle of the desert plant *Rhazya stricta*.[38] It was hypothesized that cyclic compounds inhibit the thermal expansion of cutin and, thus, even at high temperatures, prevent mechanical damage to the aliphatic wax barrier. Also, triterpenoids are known to have antioxidant[39] and antimicrobial functions.[40–42]

In conclusion, this study showed experimentally that cyclic cuticular wax components, even when present in high amounts, do not contribute to the barrier against the diffusion of polar and apolar organic solutes across the plant cuticle. This is not only of fundamental importance for a better understanding of cuticular barrier functions, but is also of appreciable significance for applied aspects in agrochemistry. It is now clear that the target for improving foliar uptake by active ingredients *per se* or its enhancement by formulation additives is exclusively the barrier made up by VLCA compounds. This is of significance for rationally optimizing pesticide applications under ecological and economic constraints.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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