Annals of Botany 1–8, 2007
doi:10.1093/aob/mcm255, available online at www.aob.oxfordjournals.org

OXFORD JOURNALS
OXFORD UNIVERSITY PRESS

Chemical Composition of the Epicuticular and Intracuticular Wax Layers on Adaxial Sides of *Rosa canina* Leaves

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Received: 25 June 2007 Returned for revision: 7 August 2007 Accepted: 31 August 2007

• *Background and Aims* The waxy cuticle is the first point of contact for many herbivorous and pathogenic organisms on rose plants. Previous studies have reported the average composition of the combined wax extract from both sides of rose leaves. Recently, the compositions of the waxes on the adaxial and abaxial surfaces of *Rosa canina* leaves were determined separately. In this paper, a first report is made on the compositions of the epicuticular and intracuticular wax layers of *Rosa canina* leaves. The methods described enable the determination of which compounds are truly available at the surface for plant–organism interactions.
• *Methods* An adhesive was used to mechanically strip the epicuticular wax from the adaxial leaf surface and the removal was visually confirmed using scanning electron microscopy. After the epicuticular wax had been removed, the intracuticular wax was then isolated using standard chemical extraction. Gas chromatography, flame ionization detection and mass spectrometry were used to identify and quantify compounds in the separated wax mixtures.
• *Key Results* The epicuticular wax contained higher concentrations of alkanes and alkyl esters but lower concentrations of primary alcohols and alkenols when compared to the intracuticular wax. In addition, the average chain lengths of these compound classes were higher in the epicuticular wax. Secondary alcohols were found only in the epicuticular layer while triterpenoids were restricted mainly to the intracuticular wax.
• *Conclusions* A gradient exists between the composition of the epi- and intracuticular wax layers of *Rosa canina* leaves. This gradient may result from polarity differences, in part caused by differences in chain lengths. The outer wax layer accessible to the phyllosphere showed a unique composition of wax compounds. The ecological consequences from such a gradient may now be probed.

Key words: Cuticular wax, *Rosa canina* leaves, surface composition, triterpenoids, alkanes, epicuticular, intracuticular.

INTRODUCTION

The surfaces of leaves, flowers, fruits and non-woody stems are covered with a cuticle made of cutin and waxes (Jetter *et al.*, 2006). Within the cuticular waxes, an intracuticular and an epicuticular layer can be distinguished according to the wax location inside the cutin matrix and exterior to it, respectively (Jeffree, 1986). In many plant species, a relatively thin film of epicuticular material forms a smooth wax surface. In contrast, wax crystals protruding from the film create a microscopically rough surface on other species (Jeffree, 2006).

The primary physiological function of the cuticle, to limit non-stomatal water loss, is thought to be predominantly associated with the intracuticular waxes (Baur, 1998). Epicuticular waxes may also serve physiological functions, including protection against UV light (Reicosky and Hanover, 1978) and moderation of gas exchange through stomatal antechambers of angiosperm needles (Jeffree *et al.*, 1971). Besides, the epicuticular waxes form the true surface of the plant organs and therefore play an important ecological role in the interaction with insects (Müller, 2006) and pathogens (Carver and Gurr, 2006).

The composition of plant cuticular waxes has long been studied using superficial extraction of intact plant material with organic solvents (Jetter *et al.*, 2006). The wax extracts from diverse plant species were reported to consist of homologous series of very-long-chain aliphatics, i.e. fatty acids, aldehydes, primary and secondary alcohols, ketones, and alkanes of chain lengths C_{20}–C_{36}, as well as C_{38}–C_{70} alkyl esters (Jetter *et al.*, 2006). In addition, triterpenoids, tocopherols, or aromatic compounds can be present, in some species only in trace amounts and in others dominating the mixture.

It has been shown that solvent molecules rapidly enter into the deeper layers of the cuticle and release a mixture of both epi- and intracuticular waxes (Jetter *et al.*, 2000). The resulting extracts reflect the total wax composition, averaging over the entire depth of the cuticle, rather than assessing the composition of the true surface. Only in recent years have methods been developed that allow independent and selective sampling of both the epi- and intracuticular wax layers for chemical analysis (Jetter *et al.*, 2000; Jetter and Schäffer, 2001). They have successfully been employed to study the composition of epicuticular wax crystals, for example on leaves of *Pisum sativum* (Gniwotta *et al.*, 2005) and in the pitcher traps of the carnivorous *Nepenthes* species (Riedel *et al.*, 2003, 2007), as well as the composition of smooth epicuticular wax films, for example on leaves of *Prunus laurocerasus* (Jetter *et al.*, 2000). Hence, it is now possible to describe the

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composition of plant surfaces more accurately and to test which species-characteristic compounds are exposed at the surfaces. The resulting chemical information is necessary to better understand direct contact interactions between plants and herbivores and pathogens landing on the plant cuticle.

Roses are among the plants that are most selected-for by humans, not only for their flowers and fragrance but also probably for the shiny appearance of their foliage. Accordingly, most rose cultivars have leaves covered by smooth epicuticular wax films that are sparsely covered with granules or triangular rodlets (Wissemann, 2000). It seems likely that differences in the compositions of cuticular waxes on the leaves of various rose cultivars cause either smoother or microscopically rougher surfaces, and thus indirectly contribute to the horticultural value of roses.

Roses have been the subjects of several wax composition studies (Baker *et al.*, 1963; Silva Fernandes, 1965; Wollrab, 1968, 1969*a*; Mladenova *et al.*, 1976, 1980; Mladenova and Stoianova-Ivanova, 1977; Jenks *et al.*, 2001). For those reporting leaf wax composition, only the average composition of waxes from both sides of the leaves was given. Only recently did Wissemann *et al.* (2007) for the first time separately analyse the wax composition for the adaxial and abaxial cuticles of *Rosa canina* leaves, a species with triangular rodlets on the abaxial surface. Since none of the previous studies discriminated between epi- and intracuticular wax layers, it still remains to be determined which compounds are available at the surface for plant–organism interactions. We have therefore performed chemical and micromorphological wax analyses on the same rose species, *R. canina*, to address the following questions: (1) which compounds constitute the epicuticular wax layer on the adaxial surface of rose leaves; (2) whether gradients between the epicuticular wax and the underlying intracuticular layer exist; and (3) how much the intra- and epicuticular waxes contribute to the total cuticular wax.

MATERIALS AND METHODS

Plant material

Twigs were harvested in the spring from cultivated plants of the species complex *Rosa canina* L. growing continuously on the campus of the University of Wuerzburg, Germany. Mature leaflets were cut from the twigs using razor blades. Batches of approximately ten leaflets were pooled for extractions and mechanical surface wax removal using either gum arabic or frozen water. Four independent samples were taken with each method or combination of methods except as otherwise noted.

Mechanical wax removal

Circular leaf discs of 12 mm diameter were cut from freshly harvested mature leaves and used without further preparation. For the mechanical removal of epicuticular wax, 10–20 leaf discs were treated with water as a cryo-adhesive, as described by Riedel *et al.* (2003). A defined amount of *n*-tetracosane was added to the extracts as an internal standard. Alternatively, gum arabic was employed as a second adhesive for the selective removal of epicuticular waxes. Prior to the experiment, commercial gum arabic powder (Sigma-Aldrich) was extracted in a Soxhlet apparatus with hot chloroform to remove any soluble lipids and residues. An aqueous solution of gum arabic (1 g mL^{-1}) was applied onto the entire adaxial surface of the leaflets 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 with chloroform at room temperature. An internal standard was added as before. Mechanical wax removal was also repeated on the same leaf surfaces a second and third time with two and one replicates, respectively, in order to determine the completeness of wax removal.

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 exposed surface and filling the cylinder with approximately 1·5 mL of chloroform. The solvent was agitated for 30 s (by pumping with a Pasteur pipette) and removed. When any solvent leaked between the cylinder and leaf disc surface the sample was discarded. Extracts from ten individual leaf discs were pooled for further analysis. Tetracosane was immediately added to all the extracts of cuticular waxes as an internal standard and the solvent removed under reduced pressure. A similar procedure was used to extract intracuticular waxes after the mechanical removal of epicuticular waxes from adaxial surfaces.

Chemical analysis

Prior to GC analysis, chloroform was evaporated from the samples under a gentle stream of N_2 while heating to 40 °C. Then the wax mixtures were treated with bis-*N,N*-(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma-Aldrich) in pyridine (30 min at 70 °C) in order to transform all hydroxyl-containing compounds into the corresponding trimethylsilyl (TMSi) derivatives. The qualitative composition was studied with a capillary GC (6890 N, Agilent, Avondale, PA; column 30 m HP-1, 0·32 mm i.d., df = 0·1 μm, Agilent), with He carrier gas inlet pressure programmed for constant flow of 1·4 mL min^{-1}, and a mass spectrometric detector (5973 N, Agilent). GC was carried out with temperature-programmed on-column injection at 50 °C, oven 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 a capillary GC with a flame ionization detector under the same GC conditions as above, but with H_2 carrier gas inlet pressure regulated for constant flow of 2 mL min^{-1}. Single compounds were quantified

against the internal standard by automatically integrating peak areas.

Scanning electron microscopy

Samples consisting of untreated and treated leaf discs (see above) were air-dried overnight before mounting on stubs using double-sided adhesive tape. Samples were then coated with 5 nm of Au using a Cressington Sputter Coater 208HR (Ted Pella, Inc., Redding, California, USA) and investigated with a Hitachi S4700 field emission SEM (Nissei Sangyo America, Ltd., Pleasanton, California, USA) using a 1 kV accelerating voltage and a 12 mm working distance.

RESULTS

A first experiment analysed the total wax mixture from the adaxial side of rose leaves by gas chromatography and mass spectrometry in order to confirm the results of Wissemann *et al.* (2007) and provide a base-line for further analysis. To sample both epi- and intracuticular wax layers together, the intact tissue had to be extracted exhaustively. Protocols for the selective extraction of wax from one leaf side have been described in recent years and were since used to investigate a small number of broad-leaf plant species (Jetter *et al.*, 2000; Riedel *et al.*, 2003; Gniwotta *et al.*, 2005; Guhling *et al.*, 2005). The same procedures, holding the organic solvent in glass cylinders pressed onto the intact tissue, were employed here to extract the total wax mixture from the adaxial side of fresh rose leaf discs. In preliminary experiments various conditions for extraction were tested (data not shown). It was found that application of chloroform at room temperature for 2×30 s resulted in exhaustive extraction of soluble cuticular lipids. The combined extracts were found to contain 28 ± 2 μg cm^{-2} of wax (Fig. 1).

Seven compound classes were identified in the total wax from the adaxial sides of *R. canina* leaves (Fig. 2). The mixture was dominated by alkanes (36 %) and primary alcohols (23 %), followed by alkyl esters (11 %) and triterpenoids (7 %). Secondary alcohols, alkenols (unsaturated primary alcohols) and benzyl esters were all detected as minor components (1–2 %), leaving only 14 % of the wax mixture unidentified.

Within the class of triterpenoids, twelve compounds were identified (Fig. 3). Lupeol was the most abundant single triterpenoid constituent (3%). α-amyrin and β-amyrin were detected together with their palmitate and stearate esters, as well as the corresponding triterpenoid acids oleanolic acid, ursolic acid and hederagenin. In all other compound classes, homologous series of aliphatics were identified with chain-length distributions typical for cuticular wax mixtures. Free primary alcohols with chain lengths ranging from C_{22} to C_{32} were detected, with a strong predominance of even-numbered homologues and a maximum at C_{26}. Similar chain-length distributions were also found for the alcohol moieties of alkyl esters and the free unsaturated alcohols. The alkyl esters were formed by fatty acid moieties ranging from C_{12} to C_{22}, while the benzyl esters contained mainly C_{32}–C_{36} acids. Both classes of esters showed a strong predominance of even-numbered acyl homologues. In contrast, alkanes and secondary alcohols were the only classes of compounds in which odd numbers of carbons were found to prevail, with only trace amounts of even-numbered homologues being present.

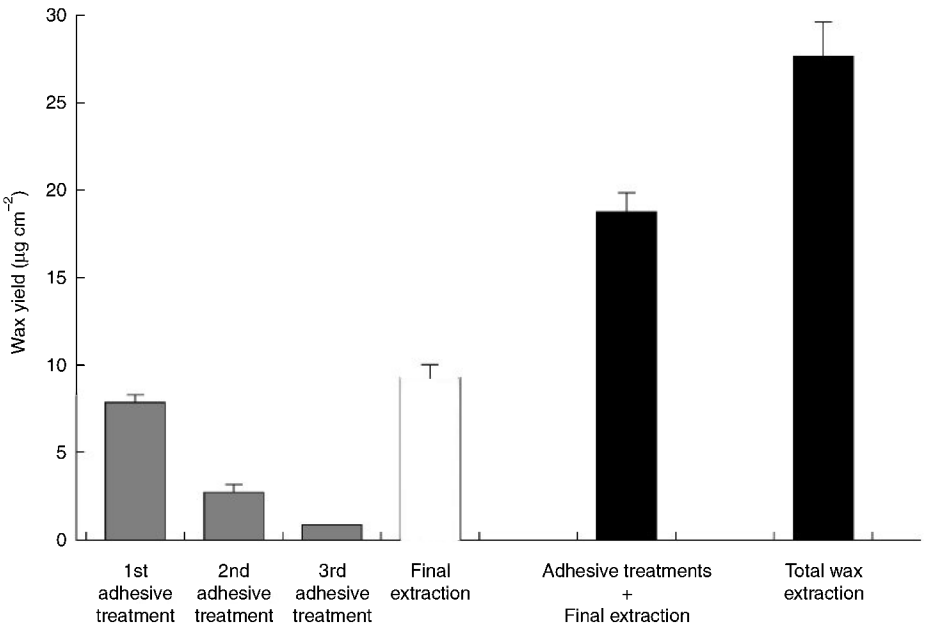

FIG. 1. Wax yields (μg cm^{-2}) from *Rosa canina* leaves sampled by a combination of mechanical and extractive methods. Three consecutive treatments with water as a cryo-adhesive were employed to remove the epicuticular wax layer, and a following extraction with chloroform was employed to remove the intracuticular wax layer. In an independent experiment, the bulk cuticular wax (total extraction) was sampled directly using chloroform. Averages of four independent samples (except for second and third adhesive treatments) are given with s.e.

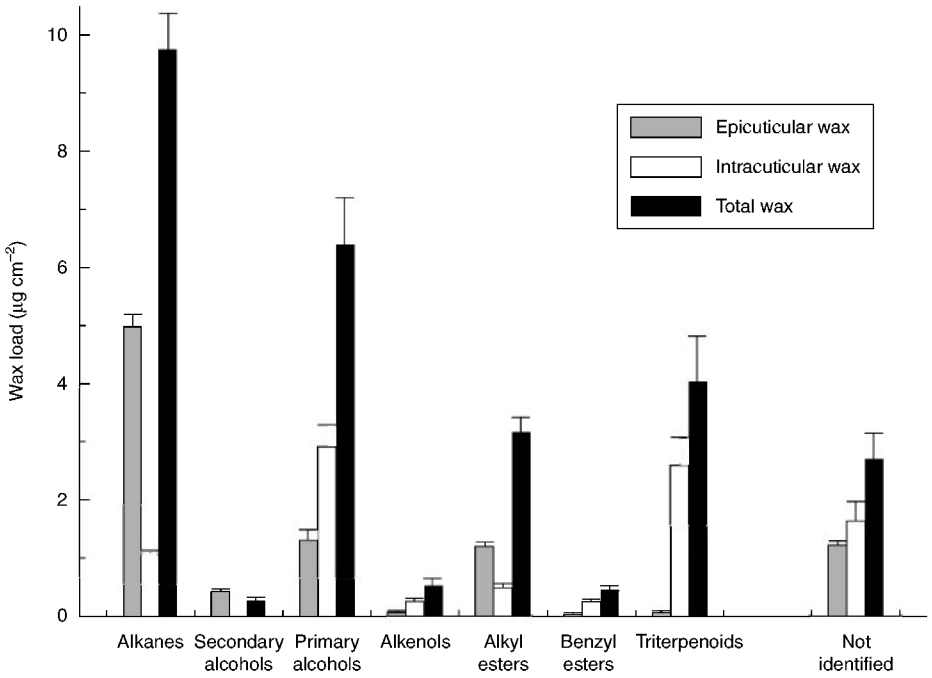

FIG. 2. Composition (μg cm^{-2}) of different compound classes found in the total wax mixture and the epicuticular and intracuticular wax layers on the adaxial surfaces of *Rosa canina* leaves. Averages of four independent samples (generated using a cryo-adhesive followed by chloroform extraction) are given with s.e.

A second experiment was designed to assess the composition of the epicuticular wax film covering the adaxial surface of *R. canina* leaves, and to compare it with the underlying intracuticular wax layer. To this end, both wax layers had to be sampled separately with high selectivity. In similar studies on other plant species it had been shown that the necessary spatial resolution could be achieved by a combination of sampling methods (Jetter and Schäffer, 2001; Vogg *et al.*, 2004; Gniwotta *et al.*, 2005; Guhling *et al.*, 2005; Wen *et al.*, 2006). As organic solvents such as chloroform mobilize a mixture of epi- and intracuticular waxes (Jetter *et al.*, 2000), extraction protocols do not have the necessary selectivity to probe both layers separately. Instead, the epicuticular wax must be stripped off the surface by employing adhesives that are free of organic solvents. After exhaustive removal of epicuticular wax, the remaining intracuticular wax can be extracted.

Preliminary experiments had shown that both gum arabic and frozen water could be used as adhesives, enabling the reproducible removal of surface wax from *R. canina* leaves (data not shown). When the adaxial surface was treated repeatedly, the wax yields decreased drastically and were approaching zero after the third adhesive application (Fig. 1). In sharp contrast, subsequent extraction of the adaxial surface with chloroform yielded relatively high amounts of wax. These results, taken together, showed that the adhesive stripping was selective for waxes located outside the mechanically resistant cutin matrix, and hence for the epicuticular wax layer. Repeated application of frozen water ensured the exhaustive removal of epicuticular material and, consequently, the remaining material released in the final extraction step must be interpreted as intracuticular wax.

The adaxial surface of *Rosa canina* leaves, when viewed by scanning electron microscopy, had an overall flat appearance and entirely lacked stomata. At higher magnifications, an epicuticular film textured with wax granules could be observed (Fig. 4). Similar surface micromorphologies for select *Rosa* spp. have been reported in a previous study (Wissemann, 2000), with only small differences probably resulting from variations between the adaxial versus abaxial surfaces. The removal of epicuticular wax by adhesives was also confirmed using SEM. A single application of gum arabic removed the majority of the epicuticular wax film from the surface while leaving the adjacent untreated zone untouched (Fig. 4). The border between treated and untreated areas was clearly delineated by an irregularly curved continuous line. After three consecutive applications of gum arabic, no visible traces of epicuticular wax remained (data not shown). This is in good accordance with the decreasing yields quantified by gas chromatography. Viewing the leaf surface after treatment with chloroform revealed a surface lacking any discernable cuticular wax (data not shown).

Adding the yields of consecutive adhesive treatments with frozen water, the adaxial leaf surface of *R. canina* was found to be covered with 9 ± 1 μg cm^{-2} of epicuticular wax, while intracuticular wax amounted to another 9 ± 1 μg cm^{-2} (Fig. 1). Similar results were found for consecutive treatments with gum arabic (data not shown). The total wax load, found in this experiment in both layers together, was very similar to the value for the total wax extraction in the previous experiment. Both experiments thus mutually confirmed each other.

The epicuticular wax on the adaxial side of rose leaves contained alkanes (52 %), primary alcohols (18 %), alkyl

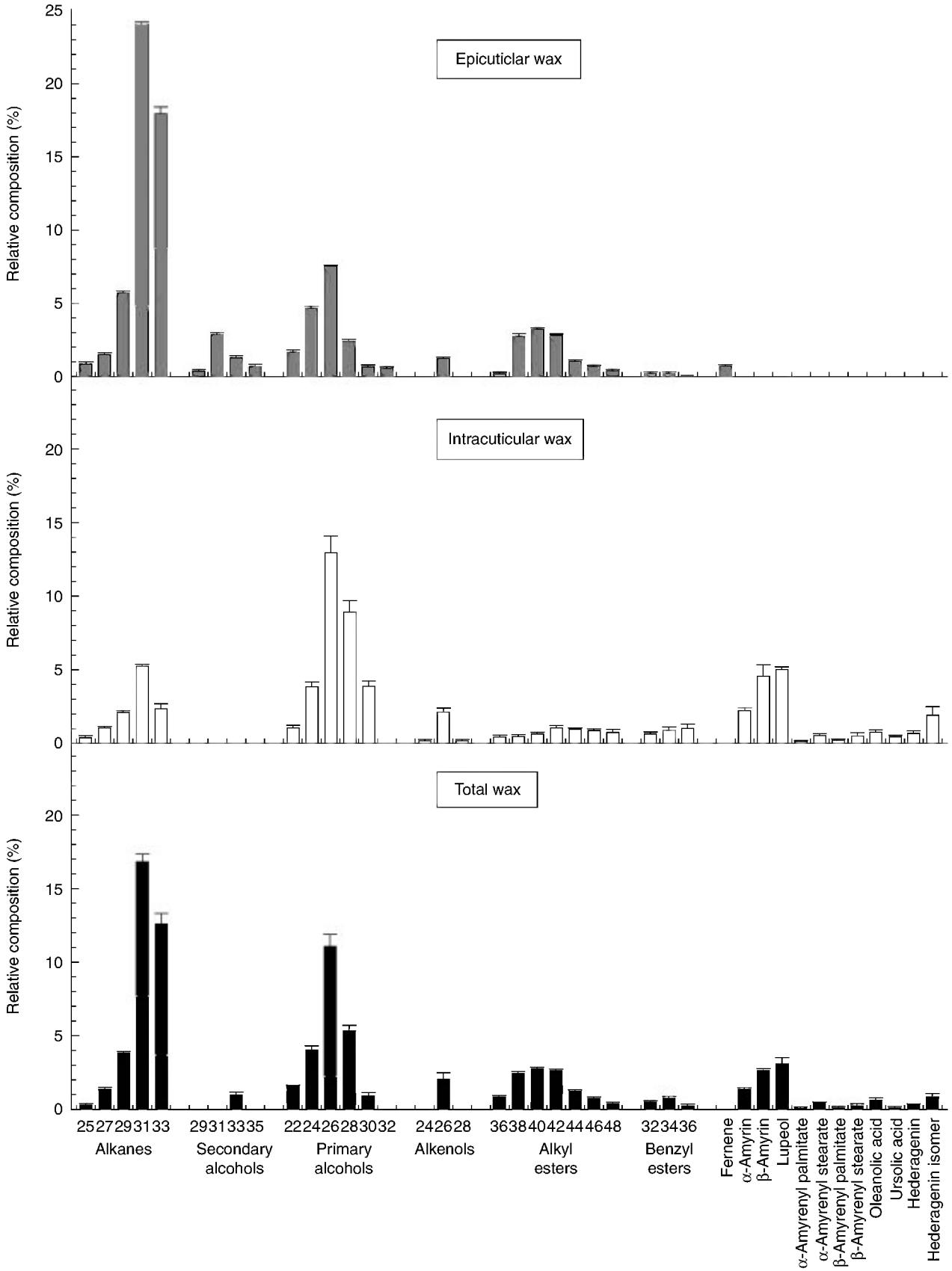

FIG. 3. Composition of the total wax mixture and the epicuticular and intracuticular wax layers on adaxial surfaces of *Rosa canina* leaves. Relative quantities (%) of individual compounds are listed according to chain lengths in the homologous series of aliphatic compound classes, or as triterpenoid isomers and derivatives. Averages of four independent samples (generated using a cryo-adhesive followed by chloroform extraction) are given with s.e.

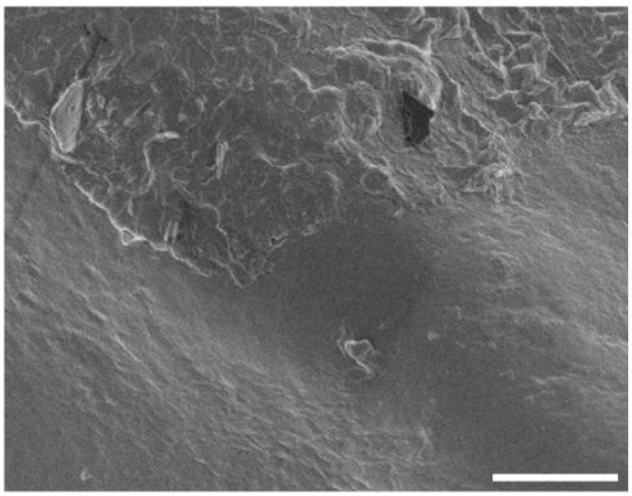

FIG. 4. Scanning electron micrograph of the adaxial leaf surface of *Rosa canina* with and without a single application of adhesive. The untreated, native adaxial surface (top) shows the epicuticular film with embedded wax crystals. It is delineated from the treated surface (bottom), where a single application of gum arabic left the surface visibly devoid of nearly all of the epicuticular wax film. Scale bar = 2 μm.

esters (12 %), secondary alcohols (5 %) and small amounts of alkenols, benzyl esters, and triterpenoids (approximately 1% each; Fig. 2). The chain-length distributions of the aliphatic compound classes were very similar to those in the total wax mixture (see above), except for a decrease in C_{28} alcohol and a corresponding increase for C_{24} alcohol in the epicuticular wax (Fig. 3). In addition, an increased range of secondary alcohols could be observed in the epicuticular wax mixture, because these compounds were no longer obscured by triterpenoids as in the total wax mixture. Hentriacontan-12-ol (C_{31}) was the most abundant secondary alcohol, identified together with nonacosan-10-ol (C_{29}), tritriacontan-12-ol (C_{33}) and pentatriacontan-12-ol (C_{35}). The triterpenoids lupeol, α-amyrin and β-amyrin, as well as their derivatives could not be detected in the epicuticular wax mixture.

The intracuticular wax layer on the adaxial side of rose leaves comprised similar compound classes as the adjacent epicuticular layer, with the exception of secondary alcohols (Fig. 2). Alkanes (12 %) and alkyl esters (5 %) were present at lower concentrations in the intracuticular wax than in the epicuticular film. In contrast, the intracuticular layer contained relatively high amounts of primary alcohols (31 %), alkenols (2 %) and triterpenoids (13 %). The homologue distribution of aliphatics and the percentages of individual triterpenoids (Fig. 3) both resembled the composition described for the total cuticular wax (see above).

DISCUSSION

All the compound classes and homologues identified here have been reported in plant waxes before. Other rose species have been found to have similar qualitative compositions of total leaf wax (Baker *et al.*, 1963; Silva Fernandes, 1965; Wollrab, 1968, 1969*a*; Mladenova *et al.*, 1976, 1980; Mladevnova and Stoianova-Ivanova, 1977; Jenks *et al.*, 2001). Wissemann *et al.* (2007) reported very similar amounts for most of the compound classes in the adaxial wax of *Rosa canina* leaves, with the additional occurrence of small quantities of aldehydes, fatty acids, coumarates and phenylethyl esters. Most importantly, our extraction conditions yielded higher amounts of triterpenoids than in the previous study. Similar wax compositions have also been reported for the leaves of other Rosaceae, including *Malus domestica* (Silva Fernandes *et al.*, 1964), *Pyrus communis* (Wollrab, 1967), *Prunus laurocerasus* (Jetter *et al.*, 2000), *Rubus fruticosa* (Wollrab, 1969*b*), *Dryas octopetala* (Lütz and Gülz, 1985) and three *Fragaria* species (Baker and Hunt, 1979).

Based on the present results, the chemical composition of the outermost wax layer on adaxial sides of *R. canina* leaves can now be described very accurately. Some of the components found at high levels in the bulk wax mixture were also present at high concentration near the surface, including the alkanes and alkyl esters. At the same time, some characteristic components, for example the secondary alcohols, were accumulating mainly in the epicuticular wax layer.

A comparison between the composition of epicuticular and intracuticular wax from the adaxial side of rose leaves revealed a number of differences. Most notably, the triterpenoids were found at low concentrations or missing in the epicuticular wax, whereas they accumulated to relatively high concentrations in the intracuticular wax. Similar gradients of cuticular triterpenoids have been reported for the leaves of the closely related Rosaceae species *Prunus laurocerasus* (Jetter *et al.*, 2000), albeit for a different triterpenoid mixture dominated by oleanolic and ursolic acids. Cuticular triterpenoids have also been found to accumulate exclusively in the intracuticular wax of a number of other plant species, including the leaves of *Macaranga tanarius* (Guhling *et al.*, 2005) and *Ligustrum vulgare* (Buschhaus *et al.*, 2007), the stems of *Ricinus communis* (Guhling *et al.*, 2006) and the fruit of *Solanum lycopersicum* (Vogg *et al.*, 2004).

The epicuticular wax and the intracuticular layer of rose leaves further differed in the relative amounts of aliphatic compounds, with high amounts of alkanes and alkyl esters present mainly in the outer layer, and primary alcohols accumulating in the inner compartment. Similar gradients had been found for the very-long-chain components of other plant waxes (Gniwotta *et al.*, 2005; Guhling *et al.*, 2005), including the gymnosperm *Taxus baccata*. Although the epicuticular wax of this species contained relatively high amounts of fatty acids and primary alcohols, the corresponding intracuticular wax was found to have higher percentages of shorter-chain-length homologues in both compound classes (Wen *et al.*, 2006). This finding was interpreted in terms of the polarity of wax constituents: within a given compound class, representatives with shorter chain length are slightly more polar than the higher homologues. Earlier work had also suggested that the intracuticular wax of blackberry leaves (*Rubus fruticosus*) contained larger amounts of polar components, e.g. relatively short-chain fatty acids and free alcohols, than the corresponding epicuticular wax (Haas and Rentschler, 1984). Taking all

these results together, our present data lend further support to the hypothesis that the more polar wax components tend to accumulate in the intracuticular compartment.

It is well established that many insect species use cuticular wax cues, frequently in combination with other plant characteristics, for host recognition (Müller and Riederer, 2005; Heisswolf *et al.*, 2007). However, it remains unknown whether any of the many insect species that associate with the horticulturally and economically important *Rosa* genus (Bruun, 2006) respond to leaf-surface compositions. To begin to address this issue, the compounds truly present on the surface of the plant cuticle need to be identified. The chemical data reported here thus provide a foundation for further studies into the mechanisms of rose–insect interactions.

In conclusion, we found that the adaxial surface of *R. canina* leaves had a characteristic wax composition rich in certain aliphatic compounds. Secondary alcohols were found only in the part of the wax mixture exposed at the surface, while triterpenoids accumulated to high concentrations only in the intracuticular wax. Thus, drastic compositional differences exist between the two wax layers on rose leaves. This analysis provides a foundation for further research into interactions between rose surfaces and insect herbivores or pathogenic micro-organisms.

ACKNOWLEDGEMENTS

Funding for this work was provided by Deutsche Forschungsgesellschaft; Natural Sciences and Engineering Research Council; Canada Research Chairs Program; and Canadian Foundation for Innovation. We acknowledge technical help by Stephanie Full, Stephan Knapek, Lisa Brumm, and the University of British Columbia BioImaging Facility staff. We also thank anonymous reviewers for their comments.

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