Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 69 (2008) 1197–1207
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Very long chain alkylresorcinols accumulate in the intracuticular wax of rye (*Secale cereale* L.) leaves near the tissue surface

Xiufeng Ji, Reinhard Jetter *

Department of Botany, University of British Columbia, 6270 University Boulevard, Vancouver, Canada V6T 1Z4
Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, Canada V6T 1Z1

Received 10 September 2007; received in revised form 7 December 2007
Available online 29 January 2008

Abstract

Alkylresorcinols (ARs) are bioactive compounds occurring in many members of the Poaceae, likely at or near the surface of various organs. Here, we investigated AR localization within the cuticular wax layers of rye (*Secale cereale*) leaves. The total wax mixture from both sides of the leaves was found to contain primary alcohols (71%), alkyl esters (11%), aldehydes (5%), and small amounts (<3%) of alkanes, steroids, secondary alcohols, fatty acids and unknowns. A homologous series of ARs (3%) was identified by GC–MS and comparison with a synthetic standard of nonadecylresorcinol. The alkyl side chains of the wax ARs contained odd numbers of carbons ranging from C_{19} to C_{27}, with a prevalence of C_{21}, C_{23} and C_{25}. Waxes from both sides of the leaf, analyzed separately in a second experiment, comprised the same compound classes in similar relative amounts and with similar homolog patterns. Finally, the epicuticular and intracuticular wax layers were sampled separately from the abaxial side of the leaf. While ARs accounted for 2% of the intracuticular wax, they were not detectable in the epicuticular wax. The intracuticular wax was also slightly enriched in steroids, whereas the epicuticular layer contained more primary alcohols. All other wax constituents were distributed evenly between both wax layers.

Keywords: Rye; *Secale cereale*; Gramineae; Epicuticular wax; Intracuticular wax; Chain lengths; GC–MS

1. Introduction

1,3-Dihydroxy-5-alkylbenzenes, also known as 5-alkylresorcinols (ARs), are potent bioactive secondary metabolites that have been shown, *e.g.*, to affect bilayer membranes due to their amphiphilic properties, and to interfere with growth regulation of cells through interaction with nucleic acids and enzymes (Kozubek and Tyman, 1999; Prakash Chaturvedula et al., 2002; Kozubek and Demel, 1980). ARs typically occur as homologous series with alkyl chain lengths ranging from C_5 to C_{29} (Kozubek and Tyman, 1999). The alkyl side chains have predominantly odd numbers of carbons, which is significant with regard to AR biogenesis. ARs occur in bacteria, animals, fungi and plants, where they have to date been described in eleven families (Ginkgoaceae, Anacardiaceae, Proteaceae, Myrsinaceae, Primulaceae, Myristicaceae, Fabaceae, Asteraceae, Iridaceae and Poaceae) (Kozubek and Tyman, 1999). They were initially isolated from fruits, seeds and various senescent organs, and only later were they also detected in green leaves and stems.

There is indirect evidence that ARs accumulate at or near the surface of diverse organs, where they exhibit antibacterial and antifungal activities. ARs are present in the outer (aleurone and pericarp) regions of wheat and rye grains, resulting in relatively high AR concentrations in bran and shorts milling fractions as compared to flours (Ross et al., 2003). Extracts of thin fruit peels of *Mangifera indica* (mango), were reported to contain ARs (conferring resistance to *Alternaria alternata*) in concentrations twelve times higher than those of the flesh (Droby and Prusky, 1987). More interestingly, the outer layer of mango flesh accumulated high AR concentrations after peeling, while

* Corresponding author. Tel.: +1 604 822 2477; fax: +1 604 822 6089.
E-mail address: jetter@interchange.ubc.ca (R. Jetter).

0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.12.008

the flesh underneath did not. ARs are also found in the seed covers of *Myristica fragrans* (nutmeg), where they have been reported to confer antibacterial activity against *Staphylococcus aureus* (Orabi et al., 1991). Finally, the fruit shells of *Anacardium occidentale* (cashew nut) are known to accumulate ARs that cause severe dermatitis when the fruit surfaces are brought into contact with human skin (Nogueira Diogenes et al., 1996). Since most occurrences of ARs are in tissues that are exposed at the plant surface, it may be speculated that the respective ARs are produced by epidermal cells and are exposed at the surface to protect the interior tissue. However, direct evidence for the surface accumulation of ARs is scarce.

The surface of primary above-ground plant organs is covered with a cuticle, a protective lipid structure sealing the tissue against the environment. The cuticle serves as the major barrier preventing nonstomatal water loss, helps to protect plant surfaces against pathogens and ultraviolet radiation, and affects plant-insect interactions (Jenks et al., 1994; Holmes and Keiller, 2002; Eigenbrode and Jetter, 2002; Vogg et al., 2004; Fürstner et al., 2005). Plant cuticles consist of cutin, an insoluble polyester lattice typically constituting 40–80% of the cuticle mass, and of soluble cuticular waxes. The latter are complex mixtures of very long chain (VLC) fatty acid derivatives, typically unbranched and fully saturated alcohols, aldehydes, ketones, fatty acids, esters and alkanes (Jetter et al., 2006). Each of these compound classes comprises a homologous series with chain lengths ranging from C_{20} to C_{36} (esters from C_{36} to C_{70}). Depending on the plant species and organ, wax mixtures may also contain cyclic compounds such as triterpenoids and phenylpropanoids (Wen et al., 2006).

Part of the cuticular wax is embedded within the polymer network of cutin, and is designated as intracuticular wax. An additional layer of so-called epicuticular wax is deposited onto the outer surface of the cutin matrix, where it forms the true surface of the tissue (Jeffree, 1986). Both epi- and intracuticular waxes can be extracted together in one step by dipping the intact surface of an organ into organic solvents. Alternatively, both wax layers can be sampled separately by first employing adhesives to strip the epicuticular material, and then organic solvents to extract the remaining intracuticular wax (Jetter et al., 2000). For a number of species, the two wax layers were found to have drastically differing composition, with some constituents of the mixture being enriched in the epi- and others in the intracuticular wax (Jetter et al., 2006). For example, selective analyses of both wax layers on *Taxus baccata* needles showed that relatively polar components tended to accumulate in the intracuticular wax, and that aromatics (phenylpropanoids and phenylbutanoids) were entirely restricted to this layer (Wen et al., 2006).

The occurrence of ARs near the surface of certain plant species and organs, together with the lipophilic character of the AR side chains, raises the question whether the ARs are localized in the cuticular waxes in these systems. Cuticular ARs have been reported only once for seeds of various barley (*Hordeum vulgare*) cultivars (Garcia et al., 1997). Unfortunately, wax analyses have not been carried out for diverse other species known to synthesize ARs. Where wax analyses have been carried out for other AR-containing species, these compounds were not reported for the wax mixtures. For example, rye (*Secale cereale* L.) contains relatively high amounts of ARs (Montsant et al., 2005; Magnucka et al., 2007), but ARs are not mentioned in reports on the cuticular waxes from this species (Streibl et al., 1974; Tulloch and Hoffman, 1974). However, this is not sufficient evidence against the accumulation of cuticular ARs in this species, as the compounds might have been overlooked in the early wax analyses or AR accumulation might be restricted to certain parts of the plants that were not investigated in the wax analyses. The AR reports are referring to rye grains and seedlings, whereas the wax analyses were performed on leaves and straw (i.e. leaves, stems and spikes). It is therefore not clear whether ARs in these instances accumulated partially or even exclusively in this lipophilic compartment, and whether they are targeted to the cuticle to serve biological functions at the tissue surface. It is also not clear how ARs might be partitioned between the epi- and intracuticular wax layers.

The goal of the present investigation was to localize ARs in cuticular wax mixtures of Poaceae. Preliminary experiments had shown that all leaves of rye contain relatively high amounts of ARs, as compared with other species and organs. Here, we performed detailed chemical analyses to address the questions: (1) which VLC compounds are present in the waxes of rye leaves, (2) whether the abaxial and adaxial waxes differ, (3) whether the epicuticular and intracuticular wax layers have different composition, (4) whether ARs exist in the leaf cuticular wax, and (5) whether ARs are restricted to one side of the leaf and/or to either the epi- or intracuticular wax layers.

2. Results and discussion

The goal of the present investigation was to analyze the chemical composition of the cuticular waxes on the adaxial and abaxial sides of *Secale cereale* leaves, with special emphasis on the localization of alkylresorcinols in the epicuticular and intracuticular wax layers. To minimize biological variation, analyses were restricted to well-defined segments of the second leaf. Preliminary results had shown that differentiation and growth of epidermal cells had ceased in the leaf zone 10 cm above the point of emergence, resulting in relatively constant wax mixtures over time. Three sets of experiments were carried out to describe the wax composition with three different levels of spatial resolution. In the first experiment, total wax mixtures were extracted from both sides of the leaves together, in order to acquire overall compositional data comparable with those reported for other grass species. The second and third experiments were aimed at distinguishing the composition

of waxes from both sides of the leaves, and from the epi- and intracuticular wax layers, respectively.

In the initial experiment, the total leaf wax was extracted from the entire exposed leaf surface including both the adaxial and the abaxial sides. The total wax coverage on the leaf was 12.2 ± 1.5 μg/cm^2 (Fig. 1). The mixture contained large amounts of primary alcohols (71%) together with alkyl esters (11%), aldehydes (5%), alkanes (3%), steroids (0.3%), and traces of secondary alcohols as well as fatty acids. The fatty acid, primary alcohol and aldehyde fractions contained homologous series of predominantly even carbon-numbered compounds, with chain lengths ranging between C_{20}–C_{34}, C_{22}–C_{30} and C_{26}–C_{32}, respectively (Table 1). The C_{26} homologs strongly predominated in all three series (Fig. 2), making hexacosanol the single most abundant constituent with 69% (8.7 ± 1.3 μg/cm^2) in the rye leaf total wax mixture. The alkane fraction was found to contain a homologous series ranging from C_{27} to C_{33} (Table 1), with a relatively broad chain length distribution of predominantly odd-numbered compounds and a maximum at C_{31} (Fig. 2). The alkyl esters were identified as a homologous series ranging from C_{40} to C_{52} (even numbers only) with a maximum at C_{44} (Table 2).

Our findings for rye wax are similar to the cuticular wax compositions reported for other Poaceae species. Rice (*Oryza sativa*), wheat (*Triticum aestivum* and *T. durum*), barley (*Hordeum vulgare*) and maize (*Zea mays*) waxes had consistently been found to contain alkanes, primary alcohols, fatty acids and aldehydes similar to those on rye leaves (Tulloch and Hoffman, 1971; Bianchi et al., 1979; Reynhardt and Riederer, 1994; Koch et al., 2005). The cuticular waxes of rye have not been studied in similar detail to date. Wax extracted from rye straw contained alkanes, primary alcohols, fatty acids and esters together with high amounts of β-diketones not found in our study (Streibl et al., 1974). Similar results were reported for wax mixtures extracted from all leaves together (Tulloch and Hoffman, 1974). Overall, the discrepancies between our results and the previous reports cannot be attributed to variability between rye cultivars alone, but are likely to also reflect drastic differences between the wax compositions of various organs of rye. β-diketones are the predominant wax constituents of barley spikes and stems (von Wettstein-Knowles, 1982; Richardson et al., 2007), as well as wheat flag leaves (Tulloch, 1973). By analogy, it seems plausible that the β-diketones previously reported for rye waxes originated from these organs rather than the lower leaves investigated here.

In addition to the typical wax constituents described above, the leaf surface extracts of *S. cereale* were found to contain a number of compounds that had not previously been reported from leaf cuticular waxes. Based on their GC retention behavior and MS characteristics, they were recognized as a homologous series of five compounds differing by $–CH_2—CH_2–$ units. The corresponding TMSi derivatives showed MS fragments of *m*/*z* 73, 268, 281 characteristic for alkylresorcinols (ARs) (Linko et al., 2002; Ross et al., 2003), together with molecular ions

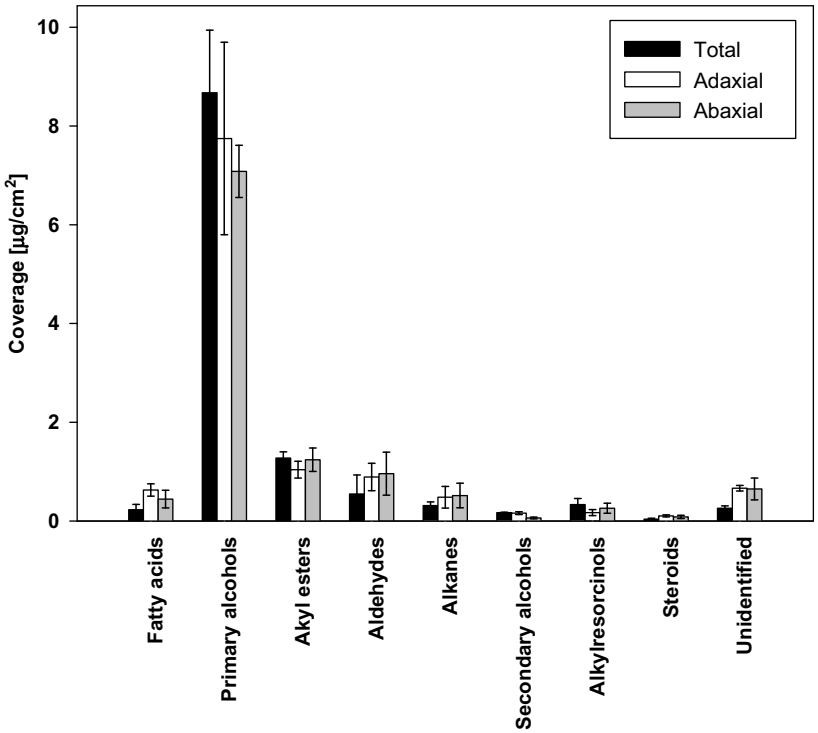


Fig. 1. Compound class composition of cuticular wax mixtures from the second leaf of rye. The coverages of all compound classes are given as mean values ($n = 6$) and SD for the total wax extracted from both sides of the leaf together, and for the wax extracted from the adaxial and abaxial sides separately.

Table 1
Very long chain compounds identified in rye leaf waxes

Compound class	Chain length															
	C_{19}	C_{20}	C_{21}	C_{22}	C_{23}	C_{24}	C_{25}	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	C_{34}
Fatty acids		To		To		To		**To**		To		To		To		To
		Ad		Ad		Ad		**Ad**		Ad		Ad		Ad		Ad
		Ab		Ab		Ab		**Ab**		Ab		Ab		Ab		Ab
		Ep In		Ep In		Ep In		**Ep In**		Ep In						
Primary alcohols				To		To	To	**To**	To	To		To				To
				Ad		Ad	Ad	**Ad**	Ad	Ad		Ad				Ad
				Ab		Ab	Ab	**Ab**	Ab	Ab		Ab				Ab
				Ep In		Ep In	Ep In	**Ep In**	Ep	Ep In	Ep	Ep In				
Aldehydes								**To**		To		To		To		
								Ad		Ad		Ad		Ad		
								Ab		Ab		Ab		Ab		
								Ep In		Ep In						
Alkanes									To		To	To	**To**	To	To	
									Ad		Ad	Ad	**Ad**	Ad	Ad	
									Ab		Ab	Ab	**Ab**		Ab	
								Ep In	Ep In		Ep In	Ep In	**Ep In**	Ep In	Ep In	
Secondary alcohols (OH at C_{14}–C_{17})															**To**	
															Ad Ab	
															Ep In	
Alkylresorcinols	To		To		**To**		To		To							
	Ad		Ad		**Ad**		Ad		Ad							
	Ab		Ab		**Ab**		Ab		Ab							
	In		In		**In**		In		In							

Wax mixtures were sampled by extracting both sides of the leaf together (To), by extracting the adaxial (Ad) or the abaxial (Ab) sides selectively, or by removing the epicuticular (Ep) and the intracuticular (In) wax layers consecutively from the abaxial side. Predominant homologs are highlighted in bold face.

$[C_6H_3(OTMSi)_2(CH_2)_nCH_3]^+$ and corresponding fragments $[M–15]^+$ indicating the loss of a methyl group. To verify whether these wax constituents were indeed ARs, one representative of the homologous series was synthesized. This standard of 1,3-dihydroxy-5-nonadecylbenzene (**1**; AR 19:0) (Fig. 3) was found to have identical mass spectral characteristics as the wax compounds. Furthermore, the standard co-eluted with one of the rye wax constituents under the GC conditions used. This finding not only confirms the exact alkyl chain length, but also the isomer configuration of the wax ARs. It had previously been shown that various isomers of comparable phenolics, including variation in both side-chain and ring-positional configuration, have sufficiently different physical properties to be separated by GC (Fritz and Moore, 1987). Thus, even though individual AR isomers may not be distinguished by MS alone, they can usually be identified by GC–MS co-injection experiments. GC–MS comparison with the authentic standard therefore unambiguously established the structure of one rye wax constituent as AR 19:0 (**1**).

Based on the equal distances between GC peaks, all the other rye wax constituents with identical MS characteristics were identified as homologous ARs with alkyl chain lengths C_{19} to C_{27} (**1**–**5**) (Table 1, Fig. 3). The series of ARs was dominated by homologs with odd-numbered alkyl side chains, mainly by ARs 21:0 (**2**), 23:0 (**3**) and 25:0 (**4**) (Fig. 4). Overall, the AR fraction contributed 0.3 ± 0.1 μg/cm^2 to the wax coverage on the leaves, accounting for approximately 3% of the total wax mixture. After identification of the ARs, only 2% of the wax mixture remained unidentified.

To test whether ARs are restricted to the cuticular wax of rye leaves or whether similar AR homologs also occur in the interior of the organ, surface waxes were first removed and then standard protocols for analysis of internal ARs were applied. ARs were quantified against an internal standard of synthetic AR13:0 (**6**) using the intensity of the characteristic MS fragment (*m/z* 268). Internal AR amounts were found to be 9 μg/g fresh weight, as compared to 135 μg/g of cuticular ARs. Cuticular ARs thus accounted for approximately 94% of the total AR contents of the leaf.

In previously published analyses of rye waxes, ARs were not reported (Streibl et al., 1974; Tulloch and Hoffman, 1974). However, ARs had been detected in total lipid extracts from various organs of *S. cereale*. Rye grains were found to contain 559 μg/g dry weight of AR with alkyl chain lengths ranging from C_{17} to C_{25} (odd numbers only) and a strong predominance of ARs 17:0, 19:0 and 21:0 (Montsant et al., 2005). In rye seedlings, Magnucka et al. (2007) detected 3.1 μg/g dry weight of ARs 15:0 to 25:0, with AR 17:0, AR 19:0 and AR 23:0 as the main homologs. Unfortunately, it is not clear whether these ARs were located in the cuticular waxes of respective organs, or

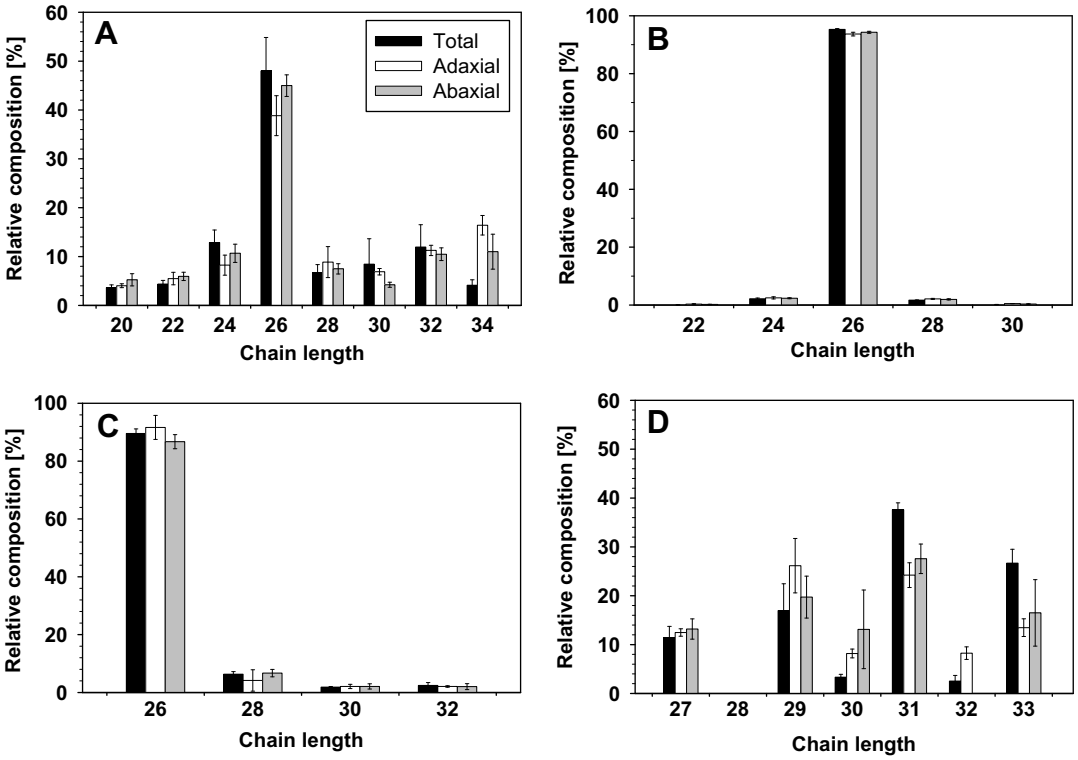


Fig. 2. Chain length distributions of individual wax components in the total, adaxial and abaxial wax mixtures on the second leaf of rye. Percentages of individual homologs within the series of (A) fatty acids, (B) primary alcohols, (C) aldehydes and (D) alkanes are shown as means ($n = 6$) with SD.

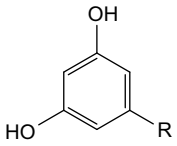
Table 2
Homolog and isomer composition of very long chain alkyl esters identified in rye leaf waxes

Ester chain length	Chain lengths of esterified acids						
	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}	C_{26}
C_{40}	To Ad Ab Ep In	To Ad Ab Ep In	**To** **Ad Ab** **Ep In**	To Ad Ab Ep In			
C_{42}			**To** **Ad Ab** **Ep In**	To Ad Ab Ep In			
C_{44}		To Ad Ab Ep In	**To** **Ad Ab** **Ep In**				
C_{46}				**To** **Ad Ab** **Ep In**	To Ad Ab Ep In		
C_{48}					**To** **Ad Ab** **Ep In**	To Ad Ab Ep In	To Ad Ab Ep In

Wax mixtures were sampled by extracting both sides of the leaf together (To), by extracting the adaxial (Ad) or the abaxial (Ab) sides selectively, or by removing the epicuticular (Ep) and the intracuticular (In) wax layers consecutively from the abaxial side. Predominant acyl homologs are highlighted in bold face.

whether they had accumulated partially or exclusively in the interior tissues. Future AR analyses of these organs should distinguish between these possibilities. Interestingly, the AR mixtures from rye grains and seedlings both had chain length ranges starting and ending with lower homologs than the leaf cuticular ARs. The homologous series of leaf ARs was also found to peak at longer chain lengths than those of the other organs.

The identification of ARs in the total wax from rye leaves raised the question whether these compounds are present in both the adaxial and abaxial wax mixtures. A second experiment was consequently designed to separately



R*	Name	Number in text	Abbreviation
$C_{19}H_{39}$	5-Nonadecylresorcinol	**1**	AR19:0
$C_{21}H_{43}$	5-Heneicosylresorcinol	**2**	AR21:0
$C_{23}H_{47}$	5-Tricosylresorcinol	**3**	AR23:0
$C_{25}H_{51}$	5-Pentacosylresorcinol	**4**	AR25:0
$C_{27}H_{55}$	5-Heptacosylresorcinol	**5**	AR27:0
$C_{13}H_{27}$	5-Tridecylresorcinol	**6**	AR13:0

* R are *n*-alkyl chains.

Fig. 3. Structures of alkylresorcinols identified in rye leaf wax (**1**–**5**) and synthesized as standards for structure elucidation (**1**) as well as quantification (**6**).

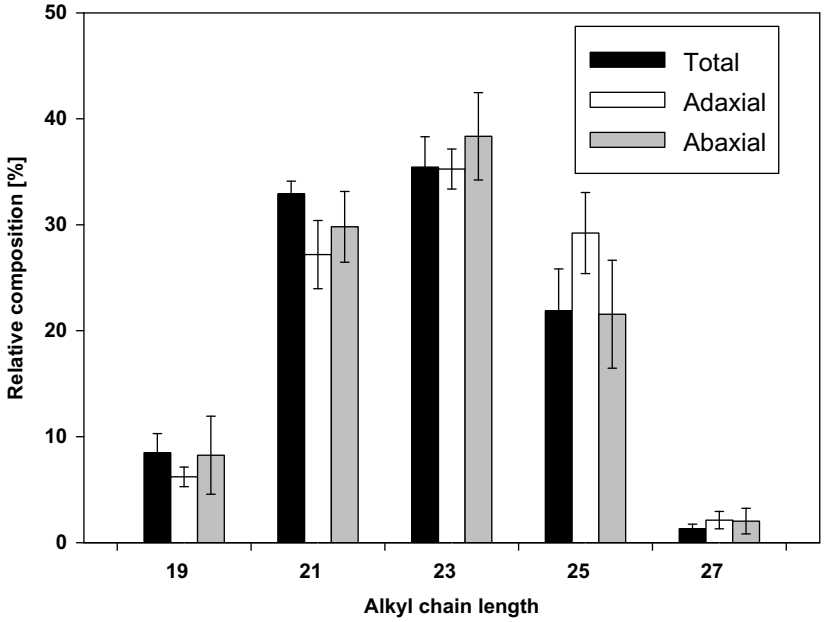


Fig. 4. Chain length distributions of alkylresorcinols in the total, adaxial and abaxial wax mixtures on the second leaf of rye. Percentages of individual homologs within the fraction are shown as means ($n = 6$) and SD.

analyze the waxes from both sides of the *S. cereale* leaves. To this end, a method was used that had previously been established for the separate extraction of waxes from both sides of gymnosperm needles (Wen et al., 2006). It relies on gentle brushing of one leaf surface with $CHCl_3$–soaked fabric glass and collecting the organic wax solutions for GC–MS analyses. Preliminary experiments, in which the rye leaf surfaces were brushed 20 times, yielded only approximately two thirds of the total wax amounts present. After brushing for more than 60 times the extracts started to appear green, indicating contamination by chlorophyll and possibly also other lipids from internal tissues. Therefore, a standard protocol limited to 60 times brushing was used to extract the surface waxes from both sides of rye leaves. It yielded 12.1 ± 2.5 μg/cm^2 and 11.5 ± 2.0 μg/cm^2 from the adaxial and abaxial surfaces, respectively. These findings correspond to an overall average wax coverage of 11.8 ± 3.2 μg/cm^2, which is not significantly different from the total wax load found in our first experiment. It can be concluded that brushing of both sides of rye leaves with fabric glass allowed the separate and exhaustive sampling of adaxial and abaxial cuticular waxes.

All the compound classes previously identified in the total wax mixture from entire rye leaves (see above) were also detected in the separate extracts from both the adaxial and abaxial sides (Fig. 1). There were no significant differences between the compound class percentages on both sides of the leaves, and between the homolog patterns

within these classes (Tables 1 and 2, Fig. 2). ARs were found to have approximately equal coverages of 0.2 ± 0.1 μg/cm^2 on the abaxial side of the leaf (accounting for 1.4 ± 0.4% of the wax mixture) and of 0.3 ± 0.1 μg/cm^2 on the abaxial side (accounting for 2.2 ± 0.5% of the wax). Similarly, the chain length distributions in the AR fractions from both sides of the leaves were found to match closely (Table 1, Fig. 4).

Overall, our results show that rye leaves are covered by adaxial and abaxial cuticles with relatively similar wax composition. They are thus an example where the two surfaces of the same organ, even though they have physiologically and ecologically different significance, share the same overall makeup. Separate analyses of waxes from adaxial and abaxial leaf sides had previously been performed for relatively few other species, and in many cases marked differences in composition had been reported. For example, the adaxial wax of *Pisum sativum* was found to be dominated by primary alcohols, whereas the corresponding abaxial wax contained large amounts of alkanes (Holloway et al., 1977; Eigenbrode et al., 1998; Gniwotta et al., 2005).

A third experiment was carried out to investigate whether compositional differences existed between the intracuticular and the epicuticular wax layers on rye leaves. In particular, this was to address the question whether ARs are localized in the inner wax layer or exposed at the very surface, or whether they occur throughout the cuticular wax. Since no pronounced differences had been found between the compositions of the adaxial and abaxial waxes, this last experiment focused only on the latter side of the rye leaf.

Gum arabic was used as an adhesive for the mechanical sampling of the surface wax layer, and could be applied to the same leaf four times without damaging the abaxial surface. The consecutive glue treatments yielded slightly decreasing wax amounts of 2.3 ± 1.0, 2.3 ± 0.5, 2.2 ± 0.5, and 1.7 ± 0.4 μg/cm^2. Statistical analyses showed no significant difference between the cumulative wax yields after the 3rd and 4th treatments (ANOVA, $N = 6$, $P = 0.066$), indicating that the four successive treatments together had exhaustively removed the mechanically accessible wax and that a fifth gum arabic treatment would not have yielded more material. Similar to previous studies, it can be concluded that the wax sampled in this way had been located outside the mechanically resistant cutin matrix, and hence comprised the complete epicuticular wax layer. When the leaf surface was, after four treatments with gum arabic, finally extracted with $CHCl_3$, substantial quantities of wax could be retrieved. The corresponding wax yield after the final chloroform extraction (6.0 ± 2.0 μg/cm^2) differed significantly from that of the 4th adhesive treatment (ANOVA, $N = 6$, $P < 0.0005$), indicating that extraction yielded wax from a distinct layer inside the cuticle. Respective wax samples must therefore be interpreted as intracuticular wax. The overall wax yield of all steps in this experiment (epicuticular plus intracuticular wax) closely matched the yield found for direct extraction of the same surface in the previous experiment (ANOVA, $N = 6$, $P = 0.160$).

The intracuticular and epicuticular wax layers shared very similar qualitative and quantitative compositions, both in terms of compound classes and chain length patterns within fractions (Tables 1 and 2, Figs. 5 and 6). As

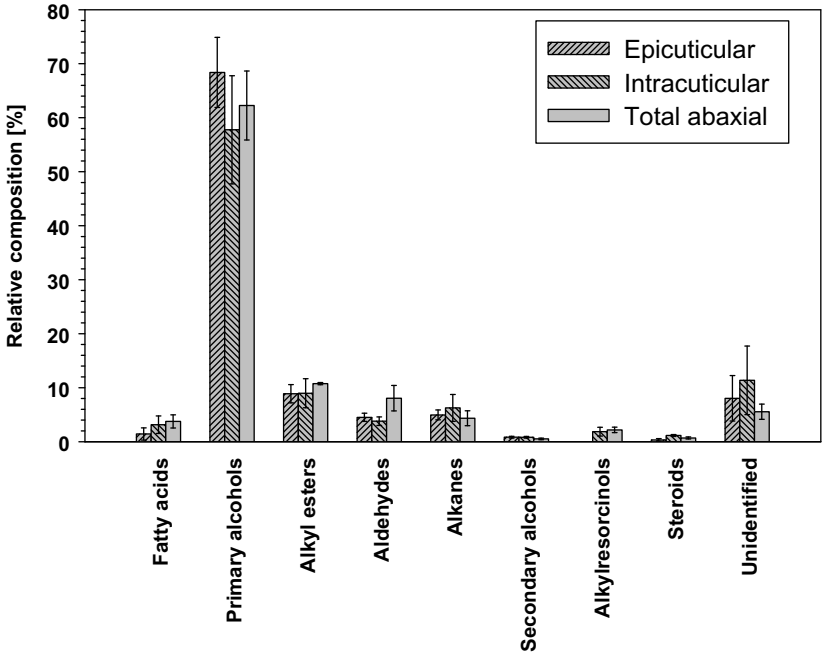


Fig. 5. Relative amounts of wax compound classes within the epicuticular, intracuticular, and total abaxial wax mixtures on the second leaf of rye. The results from the four gum arabic treatments were taken together to show the composition of the epicuticular waxes, whereas the final extraction results represent the composition of the intracuticular waxes.

two exceptions to this overall homogeneous wax composition, primary alcohols were found at higher percentage in the epicuticular wax than in the intracuticular layer, while steroids showed the opposite distribution. The latter result confirmed previous findings for other species, where cyclic components also established gradients going from higher

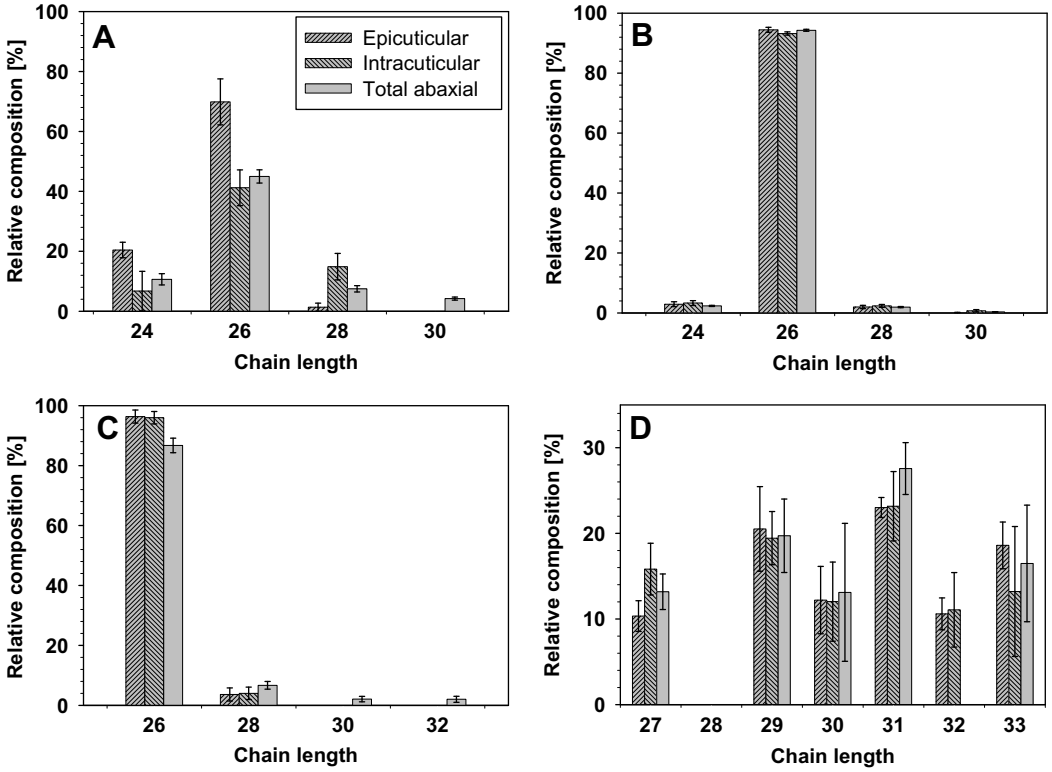


Fig. 6. Chain length distributions of individual wax components in the epicuticular, intracuticular, and total abaxial wax mixtures on the second leaf of rye. Percentages of individual homologs within the series of (A) fatty acids, (B) primary alcohols, (C) aldehydes and (D) alkanes are shown as means ($n = 6$) with SD.

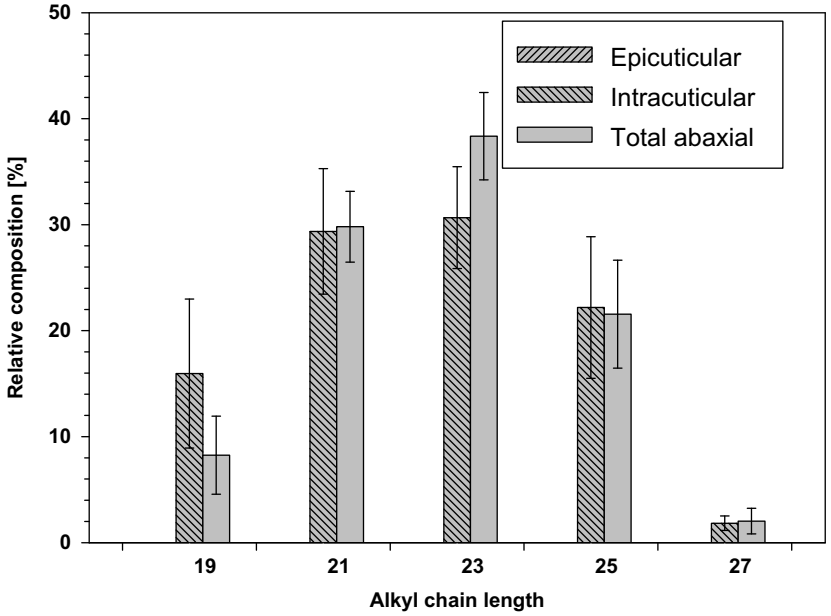


Fig. 7. Chain length distributions of alkylresorcinols in the epicuticular, intracuticular, and total abaxial wax mixtures on the second leaf of rye. Percentages of individual homologs within the fraction are shown as means ($n = 6$) with SD.

concentrations inside the cuticle to lower concentrations at the surface (Wen et al., 2006).

Finally, the most drastic gradient between the epicuticular and intracuticular waxes of rye leaves was found for the ARs. These compounds could not be detected in the epicuticular wax, but were instead restricted entirely to the intracuticular layer of wax (Fig. 5). This layer was found to contain 0.2 ± 0.1 μg/cm^2 of ARs, accounting for 2.0 ± 0.8% of the intracuticular wax mixture. The AR fraction was dominated by the homolog with C_{23} alkyl side chain (Fig. 7). Overall, the concentration and chain length patterns of ARs in the intracuticular wax samples largely confirmed our previous findings for the overall wax mixture from the abaxial side of the rye leaf. This result further underlines that all the ARs present in the rye leaf cuticle are largely restricted to the inner wax layer. They reach their highest concentration slightly underneath the surface and may be exposed at the true surface only in very small amounts due to wax diffusion.

3. Conclusions

In summary, we have detected ARs with alkyl chain lengths from C_{19} to C_{27} in the leaf cuticular waxes of rye. The rye leaf ARs were found to be largely restricted to the cuticle, with relatively low concentrations detected in the internal lipids of this organ. ARs accumulated in similar amounts in the cuticular waxes of both sides of the leaves. While they were found at relatively high concentrations in the intracuticular layers, they were absent from the epicuticular wax. This finding implies that the rye leaf ARs are located very close to the surfaces of the leaves, but are covered by a thin layer of epicuticular wax. It can be concluded that the major AR amounts are not exposed at the very surface of the tissue, and are not available for direct contact with microorganisms and insects that land on the leaf surface.

4. Experimental

4.1. Plant growth

Rye (*Secale cereale* L. cv. Esprit) seeds were purchased from Capers, Vancouver and germinated directly in soil. Plants were grown in several batches in a greenhouse at the University of British Columbia (23 °C, 14 h light at approximately 120 μmol m^{-2} s^{-1}). Before the third leaves had reached approximately half of their final length (typically three weeks after planting), the second leaves were harvested and prepared for wax analysis. To this end, a 5 cm-long segment was cut out of the second leaf 10 cm away from the point of emergence from the sheath of the first leaf. Wax was sampled from the segment using one of three methods (see below), and six independent parallels were analyzed for each method. Sampled surface areas were calculated based on leaf widths and segment length.

4.2. Wax extraction

The total wax mixture was extracted by immersing the whole leaf segments twice for 30 s into $CHCl_3$ containing *n*-tetracosane as an internal standard. The resulting solutions were concentrated, transferred into small vials, taken to dryness under a gentle stream of N_2, and stored at 4 °C. For selective extraction of either adaxial or abaxial wax, respective sides of the rye leaf segments were brushed approximately 60 times with fabric glass soaked with $CHCl_3$. Before use, the fabric glass was cleaned by thorough extraction in $CHCl_3$ using a Soxhlet apparatus. Tetracosane was added as an internal standard and the wax extracts were stored at 4 °C.

In order to discriminate between epicuticular and intracuticular waxes, gum arabic was used as an adhesive for mechanical removal of the outer layer and then solvent extraction was employed to sample the inner wax layer. Prior to use, gum arabic powder (Sigma–Aldrich) was thoroughly delipidated with $CHCl_3$ in a Soxhlet apparatus, dried and dissolved in distilled water (1 g ml^{-1}). The glue solution was spread onto the abaxial side of the leaf segment and air-dried for 40 min. The hardened adhesive film was stripped off, broken into pieces and extracted with $CHCl_3$ containing tetracosane as internal standard. The process was repeated three more times on the same side of the leaf segment and the resulting wax solutions were dried and stored at 4 °C. After the adhesive treatments, the same side of the leaf segment was extracted by brushing 20 times with $CHCl_3$-soaked fabric glass to sample the intracuticular wax.

4.3. 5-n-Tridecylresorcinol (*6*, AR13:0)

Synthesis of AR13:0 (**6**) was carried out as described by Fürstner and Seidel (1997).

4.4. 5-n-Nonadecylresorcinol (*1*, AR19:0)

AR19:0 (**1**) was synthesized using similar methods as described by Fürstner and Seidel (1997): a solution of triflic anhydride (2.1 g, 7.4 mmol, Sigma–Aldrich) in CH_2Cl_2 (9 ml) was slowly added to a solution of 3,5-dimethoxyphenol (**7**) (1.5 g, 9.8 mmol, Sigma–Aldrich) and 2,6-lutidine (1.6 ml, 13.7 mmol, Sigma–Aldrich) in CH_2Cl_2 (48 ml) at −10 °C. The solution was brought to 0 °C and stirred for 2 h before adding H_2O (5 ml). The organic layer was removed and dried with Na_2SO_4. After removing the solvent under vacuum, the crude product was purified by flash CC on packed silica gel with hexane:CH_2Cl_2 (1:1). After removing the solvents and drying overnight, 3,5-dimethoxyphenol triflate (**8**) was obtained as a yellow syrup with 90% yield. 1-tridecene (**9**) (474 mg, 0.7 ml, 2.6 mmol, Sigma–Aldrich) and 9-borabicyclo[3.3.1]nonane (9-BBN) (5.2 ml, 2.6 mmol, Sigma–Aldrich) in THF (60 ml) were stirred for 2 h under N_2 at room temperature. Then NaOMe (0.17 g, 3 mmol), triflate (**8**) (0.65 g, 2.3 mmol)

and $PdCl_2$ (dppf) (56 mg, 0.07 mmol, Sigma–Aldrich) were added, the mixture was heated until reflux began, this being maintained for 1 h, the solvent was next removed and CH_2Cl_2 (10 ml) was added. Insoluble residues were removed by passage of the CH_2Cl_2 solution through a short column of silica. After evaporation of the solvent, the crude product was purified by flash CC with hexane/EtOAc (15:1) as eluent, to produce 1,3-dimethoxy-5-nonadecylbenzene (**10**) as a colorless solid (54% yield). A mixture of **10** (0.5 g, 1.2 mmol) and 9-iodo-9-BBN (0.41 ml, 2.52 mmol, Sigma–Aldrich) in hexane (25 ml) was stirred for 3 h at room temperature. Then the solvent was removed under vacuum and the residue dissolved in Et_2O (15 ml). Ethanolamine (0.14 ml, 2.2 mmol, Sigma–Aldrich) in THF (1 ml) was added to precipitate the 9-BBN ethanolamine adduct, the mixture was stirred for 3 h, and the precipitate was filtered off. The filtrate was taken to dryness, and the crude product was purified by flash column chromatography using hexane/EtOAc (2:1) as eluent, giving analytically pure **1** (61% yield).

4.5. Extraction of ARs from internal tissue of rye leaves

Analysis of ARs in the internal tissue of rye leaf was based on the method described by Deszcz and Kozubek (2000). After surface wax removal, rye leaves were homogenized in liquid N_2 and extracted with $CHCl_3$:MeOH (2:1, v/v) containing synthetic 1,3-dihydroxy-5-tridecylbezene (**6**, AR13:0) as internal standard. The extract was filtered and separated on TLC (silica) with $CHCl_3$:EtOAc (85:15). Bands were scraped off and extracted with $CHCl_3$, the solvent was removed and the resulting samples stored at 4 °C for analysis.

4.6. Chemical analysis

Wax extracts were subjected to reaction with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA; 10 μl, Sigma–Aldrich) in pyridine (10 μl) at 70 °C for 30 min to transform all hydroxyl-containing compounds into the corresponding trimethylsilyl (TMSi) derivatives (Deas et al., 1974). The resulting solutions were diluted with $CHCl_3$ (100 μl) prior to analysis by GC–MS and GC–FID. The qualitative composition of the wax mixtures was studied using capillary GC (5890 N, Agilent, Avondale, PA; column 30 m HP-1, 0.32 mm i.d., Agilent) with He carrier gas inlet pressure programmed for constant flow of 1.4 ml min^{-1} and MS 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 to 200 °C, held for 2 min at 200 °C, raised by 3 °C min to 320 °C and held for 30 min at 320 °C. Individual compounds were identified by comparison of characteristic fragments with those of authentic standards and literature data. Quantitative analysis was carried out using GC–FID under the same GC conditions as above, but with H_2 carrier gas inlet pressure regulated for constant flow of 2 ml min^{-1}. Individual compounds were quantified against the internal standard by automatically integrating peak areas. All quantitative data are given as means of six parallel experiments and standard deviations. Statistical analyses were performed with SPSS 13.0 (SPSS, USA).

Acknowledgements

This work has been supported by the Natural Sciences and Engineering Research Council (Canada), the Canada Research Chairs Program, and the Canadian Foundation for Innovation.

References

Bianchi, G., Lupotto, E., Russo, S., 1979. Composition of epicuticular wax of rice, *Oryza sativa*. Experientia 35, 1417.

Deas, A.H.B., Baker, E.A., Holloway, P.J., 1974. Identification of 16-hydroxyoxodecanoic acid monomers in plant cutins. Phytochemistry 13, 1901–1905.

Deszcz, L., Kozubek, A., 2000. Higher cardol homologs (5-alkylresorcinols) in rye seedlings. Biochim. Biophys. Acta 1483, 241–250.

Droby, S., Prusky, D., 1987. Induction of antifungal resorcinols in flesh of unripe mango fruits and its relation to latent infection by *Alternaria alternata*. Physiol. Mol. Plant Pathol. 30, 285–292.

Eigenbrode, S.D., Jetter, R., 2002. Attachment to plant surface waxes by an insect predator. Integr. Comp. Biol. 42, 1091–1099.

Eigenbrode, S.D., White, C., Rohde, M., Simon, C.J., 1998. Epicuticular wax phenotype of the well mutation and its effect on pea aphid populations in the greenhouse and in the field. Pisum Genet. 20, 13–17.

Fritz, J.O., Moore, K.J., 1987. Separation and quantification of lignin-derived phenolic monomers using high-resolution gas chromatography. J. Agric. Food Chem. 35, 710–713.

Fürstner, A., Seidel, G., 1997. Shortcut syntheses of naturally occurring 5-alkylresorcinols with DNA-cleaving properties. J. Org. Chem. 62, 2332–2336.

Fürstner, R., Barthlott, W., Neinhuis, C., Walzel, P., 2005. Wetting and self-cleaning properties of artificial super hydrophobic surfaces. Langmuir 21, 956–961.

Garcia, S., Garcia, C., Heinzen, H., Moyna, P., 1997. Chemical basis of the resistance of barley seeds to pathogenic fungi. Phytochemistry 44, 415–418.

Gniwotta, F., Vogg, G., Gartmann, V., Carver, T.L.W., Riederer, M., Jetter, R., 2005. What do microbes encounter at the plant surface? Chemical composition of pea leaf cuticular waxes. Plant Physiol. 139, 519–530.

Holloway, P.J., Hunt, G.M., Baker, E.A., Macey, M.J.K., 1977. Chemical composition and ultrastructure of the epicuticular wax in four mutants of *Pisum sativum* (L). Chem. Phys. Lipid 20, 141–155.

Holmes, M.G., Keiller, D.R., 2002. Effects of pubescence and waxes on the reflectance of leaves in the ultraviolet and photosynthetic wavebands: a comparison of a range of species. Plant, Cell Environ. 25, 85–93.

Jeffree, C.E., 1986. The cuticle, epicuticular waxes and trichomes of plants, with reference to their structure, functions and evolution. In: Juniper, B., Southwood, R. (Eds.), Insects and The Plant Surface. E. Arnold, London, pp. 23–135.

Jenks, M.A., Joly, R.J., Peters, P.J., Rich, P.J., Axtell, J.D., Ashworth, E.N., 1994. Chemically induced cuticle mutation affecting epidermal conductance to water vapor and disease susceptibility in *Sorghum bicolor* (L). Moench. Plant Physiol. 105, 1239–1245.

Jetter, R., Kunst, L., Samuels, A.L., 2006. Composition of plant cuticular waxes. In: Riederer, M., Müller, C. (Eds.), Biology of the Plant Cuticle. Blackwell, Oxford.

Jetter, R., Schäffer, S., Riederer, M., 2000. Leaf cuticular waxes are arranged in chemically and mechanically distinct layers: evidence from *Prunus laurocerasus* L. Plant, Cell Environ. 23, 619–628.

Koch, K., Barthlott, W., Koch, S., Hommes, A., Wandelt, K., Mamdouh, W., De-Feyter, S., Broekmann, P., 2005. Structural analysis of wheat wax (*Triticum aestivum*, c.v. '*Naturastar*' L.): From the molecular level to three dimensional crystals. Planta 223, 258–270.

Kozubek, A., Demel, R.A., 1980. Permeability changes of erythrocytes and liposomes by 5-(*n*-alk(en)yl)resorcinols from rye. Biochim. Biophys. Acta 603, 220–227.

Kozubek, A., Tyman, J.H.P., 1999. Resorcinol lipids, the natural non-isoprenoid phenolic amphiphiles and their biological activity. Chem. Rev. 99, 1–25.

Linko, A.-M., Parikka, K., Wähälä, K., Adelrcreutz, H., 2002. Gas chromatographic–mass spectrometric method for the determination of alkylresorcinols in human plasma. Anal. Biochem. 308, 307–313.

Magnucka, E.G., Suzuki, Y., Pietr, S.J., Kozubek, A., Zarnowski, R., 2007. Action of benzimidazole fungicides on resorcinolic lipid metabolism in rye seedlings depends on thermal and light growth conditions. Pestic. Biochem. Physiol. 88, 219–225.

Montsant, A., Zarka, A., Boussiba, S., 2005. Presence of a nonhydrolyzable biopolymer in the cell wall of vegetative cells and astaxanthin-rich cysts of *Haematococcus pluvialis* (Chlorophyceae). Marine Biotechnol. 3, 515–521.

Nogueira Diogenes, M.J., De Morais, S.M., Ferreira Carvalho, F., 1996. Contact dermatitis among cashew nut workers. Contact Dermat. 35, 114–115.

Orabi, K.Y., Mossa, J.S., Ej-Feraly, F.S., 1991. Isolation and characterization of two antimicrobial agents from mace (*Myristica fragrans*). J. Nat. Prod. 54, 856–859.

Prakash Chaturvedula, V.S., Schilling, J.K., Miller, J.S., Andriantsiferana, R., Rasamison, V.E., Kingston, D.G.I., 2002. New cytotoxic bis-5-alkylresorcinol derivatives from the leaves of *Oncostemon bojerianum* from the Madagascar rainforest. J. Nat. Prod. 65, 1627–1632.

Reynhardt, E.C., Riederer, M., 1994. Structures and molecular dynamics of plant waxes II. Cuticular waxes from leaves of *Fagus sylvatica* L. and *Hordeum vulgare* L. Eur. Biophys. J. 23, 59–70.

Richardson, A., Wojciechowski, T., Franke, R., Schreiber, L., Kerstiens, G., Jarvis, M., Fricke, W., 2007. Cuticular permeance in relation to wax and cutin development along the growing barley (*Hordeum vulgare*) leaf. Planta 225, 1471–1481.

Ross, A.B., Shepherd, M.J., Schüpphaus, M., Sinclair, V., Alfaro, B., Kamal-Eldin, A., Aman, P., 2003. Alkyl resorcinols in cereals and cereal products. J. Agric. Food Chem. 51, 4111–4118.

Streibl, M., Konecny, K., Trka, A., Ubik, K., Pazlar, M., 1974. Composition of the wax of rye straw. Coll. Czech 39, 475–479.

Tulloch, A.P., 1973. Composition of leaf surface waxes of *Triticum* species: variation with age and tissue. Phytochemistry 12, 2225–2232.

Tulloch, A.P., Hoffman, L.L., 1971. Leaf wax of durum wheat. Phytochemistry 10, 871–876.

Tulloch, A.P., Hoffmann, L.L., 1974. Epicuticular waxes of *Secale cereale* and *Triticale hexaploide* leaves. Phytochemistry 13, 2535–2540.

Vogg, G., Fischer, S., Leide, J., Emmanuel, E., Jetter, R., Levy, A.A., Riederer, M., 2004. Tomato fruit cuticular waxes and their effects on transpiration barrier properties: functional characterization of a mutant deficient in a very-long-chain fatty acid β-ketoacyl-CoA synthase. J. Exp. Bot. 55, 1401–1410.

von Wettstein-Knowles, P., 1982. Biosynthesis of epicuticular lipids as analysed with the aid of gene mutations in barley. In: Wintermans, J.F.G.M., Kuiper, P.J.C. (Eds.), Biochemistry and Metabolism of Plant Lipids. Elsevier, pp. 69–78.

Wen, M., Buschhaus, C., Jetter, R., 2006. Nanotubules on plant surfaces: chemical composition of epicuticular wax crystals on needles of *Taxus baccata* L. Phytochemistry 67, 1808–1817.